MECHANISMS INVOLVED IN LYSIS OF LUTEAL TISSUE IN THE MARE

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THESIS SUBMITTED
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TABLE OF CONTENTS

Acknowledgements	iii
List of Figures	iv
List of Tables	vi
List of Plates	viii
SUMMARY	1
CHAPTER ONE	3
GENERAL INTRODUCTION	3
1.1 Introduction	3
1.2 Origins of the corpus luteum	3
1.3 Ovulation	5
1.4 Formation of the corpus luteum	5
1.5 Life-span of the corpus luteum	8
1.6 Prostaglandins: Chemistry, synthesis and metabolism	8
1.7 Stimuli for prostaglandin release	11
1.8 Patterns of prostaglandin release	14
1.9 Transportation of prostaglandin to the site of action	14
1.10 Mode of action of prostaglandins at the corpus luteum	15
1.11 Prolonged dioestrus	17
1.12 Aims of the project	17
CHAPTER TWO	19
2.1 INTRODUCTION	19
2.2 MATERIALS AND METHODS	20
2.2.1 Mares	20
2.2.2 Behavioural studies	20
2.2.3 Palpation of the genital tract	20
2.2.4 Identification of the day of ovulation	20
2.2.5 Examination of the cervix	21
2.2.6 Blood sampling regimes	21
2.2.7 Plasma progesterone estimations	21
2.3 RESULTS	22
2.4 DISCUSSION	30
CHAPTER THREE	33
3.1 INTRODUCTION	33
3.2 MATERIALS AND METHODS	34
3.2.1 Mares	34
3.2.2 Blood sampling regimes	34
3.2.3 Uterine biopsy technique	36
3.2.4 Teasing	37
3.2.5 Palpation of the genital tract *per rectum*	37
3.2.6 Plasma progesterone estimations	37
3.2.7 Estimation of 15 keto 13,14 dihydroprostaglandin F-2α PGFM	37
3.2.8 Prostaglandin F-2α (PGF-2α) estimation	48

TABLE OF CONTENTS (Continued)

3.3 RESULTS	55
3.4 DISCUSSION	85
CHAPTER FOUR	94
4.1 INTRODUCTION	94
4.2 MATERIALS AND METHODS	95
4.2.1 Source of luteal and endometrial tissue	95
4.2.2 Preservation of tissue	95
4.2.3 Fresh and frozen tissue samples	95
4.2.4 Determination of the stage of the oestrous cycle	95
4.2.5 Tissue culture methods	97
4.2.6 Prostaglandin F-2α (PGF-2α) estimation	98
4.2.7 Statistical analysis	98
4.2.8 Tissue progesterone estimation	98
4.2.9 Identification of viable cells	99
4.3 RESULTS	100
4.4 DISCUSSION	144
CHAPTER FIVE GENERAL DISCUSSION	155
BIBLIOGRAPHY	160

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LIST OF FIGURES

Figure 1:1 10

Figure 2:1 Behavioural, cervical, ovarian and plasma progesterone changes in Mare 1 during one inter-oestrous period 23

Figure 2:2 Behavioural, cervical, ovarian and progesterone changes in Mare 2 during one inter-oestrous period 25

Figure 2:3 Behavioural, cervical, ovarian and plasma progesterone changes in Mare 3 during one inter-oestrous period 27

Figure 2:4 Behavioural, cervical, ovarian and plasma progesterone changes in Mare 4 during one inter-oestrous period 29

Figure 3:1 PGFM standard curve in plasma 1st assay method 41

Figure 3:2 PGFM antibody dilution curve Steranti antiserum 44

Figure 3:3 PGFM standard curve in PBS buffer and parallelism curve 47

Figure 3:4 PGF-2α antibody dilution curve 50

Figure 3:5 PGFM antibody dilution curve (Steranti antiserum) PGF-2α standard curve and parallelism curve 54

Figure 3:6 Circulating plasma PGFM and progesterone concentrations in daily blood samples from mare 1 during one inter-oestrous period 59

Figure 3:7 Circulating plasma PGFM and progesterone concentrations in daily blood samples from mare 2 duing one inter-oestrous period 61

Figure 3:8 Circulating plasma PGFM and progesterone concentrations in hourly blood samples from mare 3 at the expected time of luteolysis 63

Figure 3:9 Circulating plasma PGFm and progesterone concentrations in hourly blood samples from mare 4 at the expected time of luteolysis 65

Figure 3:10 Circulating plasma and progesterone concentrations in 3 hourly blood samples taken from mare 5 over a three day period at the expected time of luteolysis 67

LIST OF FIGURES (Continued)

Figure 3:11 Circulating plasma PGFM and progesterone concentrations in hourly blood samples taken from mare 6 over a three day period at the expected time of luteolysis 69

Figure 3:12 Circulating plasma PGFM and progesterone concentrations in hourly blood samples from mare 7 over a three day period at the expected time of luteolysis 71

Figure 3:13 Circulating plasma PGFM and progesterone concentrations in hourly blood samples from mare 8 over a three day period at the expected time of luteolysis 73

Figure 3:14 Circulating plasma PGFM and progesterone concentrations in daily blood samples from mare 9 in prolonged dioestrous 75

Figure 3:15 Circulating plasma PGFM and progesterone concentrations in hourly blood samples taken from mare 10 at the expected time of luteolysis 77

Figure 3:16 Circulating plasma PGFM and progesterone concentrations in hourly blood samples taken from mare 11 in prolonged dioestrus over a three day period 79

Figure 3:17 PGFM and progesterone concentrations in timed plasma samples taken before and after a uterine biopsy from mare 11 80

Figure 3:18 Plasma PGFM, PGF-2α and progesterone concentrations in timed blood samples from mare 13 before and after an injection of 'lutalyse' 82

Figure 3:19 Plasma PGFM, PGF-2α and progesterone concentrations in timed blood samples from mare 12 before and after an injection of 'lutalyse' 84

LIST OF TABLES

Table 2:1 Mares 20

Table 3:1 Mares 34

Table 3:2 Comparison of PGFM assay methods 1 and 2 56

Table 3:3 Effects of varying incubation time on percentage binding 57

Table 4:1 Comparisons of PGF-2α production from fresh and frozen sections of luteal and endometrial tissue 101

Table 4:2 PGF-2α production by luteal tissue samples taken from mares at different stages of the oestrous cycle 103

Table 4:3 PGF-2α production by luteal cells in single cell suspension from mares at different stages of the oestrous cycle 105

Table 4:4 Production of progesterone by luteal cells in single cell suspension from mares at different stages of the oestrous cycle 107

Table 4:5 Effects of aspirin on PGF-2α production by luteal cells in single cell suspension from mares at different stages of the oestrous cycle 109

Table 4:6 PGF-2α by endometrial explant tissue samples from mares at different stages of the oestrous cycle 111

Table 4:7 PGF-2α production by endometrial cells from different areas of the uterus from mares at different stages of the oestrous cycle 113

Table 4:8 PGF-2α production by endometrial cells in single cell suspension from mares at different stages of the oestrous cycle 115

Table 4:9 Effects of aspirin on PGF-2α production by endometrial cells in single cell suspension from mares at different stages of the oestrous cycle 117

Table 4:10 Comparison of PGF-2α production by explant tissue samples from luteal and endometrial tissues 119

LIST OF TABLES (Continued)

Table 4:11 Comparison of PGF-2α production by single cell suspension cultures of luteal and endometrial tissues at different stages of the oestrous cycle 121

Table 4:12 Effects of oxytocin on PGF-2α production by luteal tissue explants from mares at different stages of the oestrous cycle 123

Table 4:13 Effects of oxytocin on PGF-2α production by luteal cells in single cell suspension from mares at different stages of the oestrous cycle 125

Table 4:14 Effects of oxytocin on PGF-2α production by endometrial tissue explants from mares at different stages of the oestrous cycle 127

Table 4:15 Effects of oxytocin on PGF-2α production by endometrial cells in single cell suspension cultures from mares at different stages of the oestrous cycle 129

Table 4:16 Effects of freezing/storage on PGF-2α production by endometrial and luteal tissues 131

LIST OF PLATES

Plate 4:1 Gross examination Early luteal phase 133

Plate 4:2 and 4:3 Histological examination Early luteal phase 134

Plate 4:4 Gross examination Mid luteal phase 136

Plate 4:5 and 4:6 Histological examination Mid luteal phase 137

Plate 4:7 Gross examination Late luteal phase 139

Plate 4:8 and 4:9 Histological examination Late luteal phase 140

Plate 4:10 Gross examination Follicular phase 142

Plate 4:11 and 4:12 Histological examination Follicular phase 143

1 SUMMARY

This thesis set out to investigate the role of Prostaglandin F-2α (PGF-2α) in luteolysis in the mare and to determine any alterations in PGF-2α production between normal cycling mares and mares in prolonged dioestrus.

As luteolysis is not overtly expressed, a method for determining the expected time of luteolsysis had to be established. Four mares were examined by 'teasing' with a stallion, examination of the reproductive tract *per rectum* and plasma progesterone estimations. These investigations allowed expertise to be gained in determining the day of ovulation which was counted as Day 0. Thirteen days were counted forward to determine the expected time of luteolysis.

In an attempt to establish the PGF-2α pattern of release at luteolysis the metabolite 13,14 dihydro 15 keto PGF-2α (PGFM) was measured in the plasma as an accurate marker for PGF-2α release. A PGFM radio-immunassay was therefore modified and eleven mares were blood sampled and the plasma was assayed for PGFM concentrations. The mares were bled under different blood sampling regimes and an overall pattern of PGFM release became apparent. At Day 14 post-ovulation a series of pulses was observed in the plasma which increased in frequency until they appeared to be terminated by the largest PGFM pulse. This pattern appeared to be crucial to the outcome of luteolysis. Three out of the eleven mares did not return to standing oestrus despite demonstrating raised PGFM concentrations in the circulating plasma. The pattern of release in these mares appeared altered compared to the normal cycling mares. Also the overall concentrations released appeared reduced and these two factors may have resulted in the mares remaining in a state of prolonged dioestrus. Two of the mares in prolonged dioestrus had uterine biopsies taken and both returned to standing oestrus within five days of the biopsy. No significant pulses of PGFM were picked up in the plasma during and for several hours after uterine biopsy but this could have been due to inadequate blood-sampling regimes in the mares involved. As the mares in prolonged dioestrus appeared to be capable of a degree of prostaglandin release, it was queried whether a secondary source of PGF-2α was present in the mare, which was not being stimulated in the mares in prolonged dioestrus.

The ovary seemed the obvious place for a secondary source of PGF-2α, in particular the corpus luteum (CL), therefore a tissue culture method and a PGF-2α radio-immunassay were established. Sections of luteal tissue and, as a comparison, endometrial tissue were collected and frozen in liquid nitrogen before culturing *in vitro* to determine PGF-2α production by the tissues at the different stages of the oestrous cycle. However as no reproductive history of the mares was known, histology of the luteal tissue was used to group the mares into early, mid, late luteal and follicular phases.

The luteal tissue appeared to be capable of PGF-2α at all stages of the oestrous cycle but it was during the late luteal phase, around the expected time of luteolysis that the most significant increase in PGF-2α production by the tissue was observed. This increase was thought to be involved in luteolysis, as no decrease in plasma or tissue progesterone concentration was observed. It appeared therefore that the mare had a secondary source of PGF-2α release in the CL which in some way supplemented the PGF-2α from the uterine endometrium. Any breakdown in communication between the two areas may lead to the mare not returning to standing oestrus and entering a state of prolonged dioestrus.

CHAPTER ONE
GENERAL INTRODUCTION

1.1 Introduction

The oestrous cycle of the mare can be divided into the follicular or standing oestrous phase, during which time the mare will accept mating, and the luteal or dioestrous phase, when the mare rejects the stallion. During the luteal phase of the oestrous cycle a temporary endocrine gland, the corpus luteum (CL) formed from the remnants of the ovulated follicle, is present in the ovary. This gland secretes the steroid hormone progesterone, which is essential for the establishment and maintenance of pregnancy, by maintaining a suitable uterine environment for the growth and development of the conceptus.

1.2 Origins of the corpus luteum

In the mare as in all the domestic species, corpus luteum formation occurs after ovulation of the mature follicle (Ginther, 1979). The follicle that is destined to ovulate grows and develops rapidly during standing oestrus, reaching a size of 60-70mm in Thoroughbred mares at ovulation (Ginther, 1979). Throughout this period of growth and maturation, the developing follicles secrete increasing concentrations of the steroid hormone oestradiol, principally oestradiol β, which enters the circulation and brings about 'switching on' of the behavioural centres and the mares acceptance of the stallion (Noden, Oxender and Hafs, 1975). Both granulosa and theca cells are involved in the production of oestradiol β in maturing follicles.

During the early stages of follicular growth the theca interna layer undergoes marked capillary invasion and increased androgen production, while the granulosa cell layer also undergoes growth and development and is concerned with the aromatisation of androgens to oestrogens. The more vascular follicles are usually found to have the highest steroid content (Younglai, 1971).

The follicular changes are initiated by Follicle Stimulating Hormone (FSH), a gonadotrophin which is released in a bimodal surge pattern from the anterior pituitary (Evans and Irvine, 1975). One of the two FSH surges is reported to occur during dioestrus, 10-13 days prior to ovulation in all mares, despite the extreme variability of oestrous cycle length in the mares. This mid-dioestrous increase in

FSH is thought to be responsible for the development of the follicle destined to ovulate during the following standing oestrus. A controlling mechanism of FSH synthesis and release from the anterior pituitary in the mare has now been established. This is thought to be the compound inhibin, produced by the granulosal cells of the follicle and found in the follicular fluid. Inhibin, in conjuction with oestradiol, acts by negative feedback directly on the anterior pituitary to suppress FSH production (Franchimont *et al*, 1979). Due to the stimulate actions of FSH an average of 3.5 follicles attain a size of 20-25mm prior to standing oestrus in the mare (Irvine, 1981). However usually only one of these follicles will mature fully and ovulate while the others will become atretic. Reports suggest that the follicle destined to ovulate is selected very early in standing oestrus and indeed is probably selected prior to overt behavioural oestrus (Irvine, 1981).

The factors controlling the destiny of the developing follicles are very poorly understood but recently it has been reported that the follicle with the highest concentrations of oestrogen is the one which ovulates.

Subsequent growth and maturation of the follicle, after this pre-ovulatory growth, is luteinising hormone-dependent and occurs relatively rapidly.

In most of the domestic species other than the mare, luteinising hormone (LH) is released in a pulsatile pattern with a sudden, large, short-lived surge occurring just prior to ovulation (Baird, Swanston and Scaramuzzi, 1976). However in the mare the pattern of LH secretion is very different.

In this species LH concentrations do not reach a sudden peak around ovulation but show a gradual increase in the circulating concentrations during late dioestrus, reaching a peak about 18-24 hours after ovulation, after which there is a gradual decrease in the circulating concentrations to baseline during early dioestrus (Hughes *et al*, 1980; Urwin and Allen, 1983).

In the mare LH release appears to be episodic in nature but the amplitude and frequency of the pulses varies greatly between mares and within mares during different stages of the oestrous cycle. No uniformity in the pattern of this pulsatile release is apparent within a group of mares, however this episodic release of LH is superimposed onto an increasing baseline concentration in all mares such that the overall concentration of LH is increasing markedly towards ovulation (Evans *et al*, 1979).

Unlike the other domestic species the half-life of LH in the plasma of the mare is very long (about 1.5-1.8 days) and this slow metabolism is reflected by the prolonged elevated LH concentrations demonstrated in the circulating plasma (Geshwind *et al*, 1975). The mare therefore has significantly elevated LH for almost one half of her 21 day cycle. The reason for this pattern of LH release in the mare has not been elucidated. However these prolonged high levels of LH appear to be responsible for the long standing oestrous period of the mare (5-6 days) and in addition may explain why double ovulations, with the second occurring as late as 4 days after the first, are frequently reported in the mare (Stabenfeldt, Hughes and Evans, 1972).

1.3 Ovulation

During their development the follicles grow close to, and protrude from, the ovarian surface until prior to ovulation when migration to the hilus, or ovulatory fossa, of the ovary occurs (Witherspoon and Talbot, 1970). This hilus, unlike the rest of the ovarian surface, is not surrounded by peritoneum and is in close apposition to the infundibulum. Thus, in the mare, ovulation is only able to occur from this site.

Although the exact events involved in the ultimate release of the ovum have not been fully established there is an increasing body of evidence to suggest that prostaglandins are closely involved in the ovulatory process (Armstrong, 1986). Significant concentrations of prostaglandin F-2α (PGF-2α) and prostaglandin E-2 (PGE-2) are present in the follicular fluid prior to ovulation and the inflammatory changes evident in the membrane of the follicle appear to be mediated by PGF-2α and PGE-2. LH stimulation of the ovulatory follicle leads to an increase in the intracellular concentrations of the enzymes involved in prostaglandin synthesis. PGF-2α and PGE-2 also stimulate the release of plasminogen activator substance, responsible for the conversion of plasminogen to plasmin, which is involved in the breakdown of the follicle wall at ovulation (Poyser, 1981). Contractions of the ovarian tissue around the ovulatory follicle assisting in the expulsion of the ovum also occur and are thought to be mediated by the prostaglandins.

1.4 Formation of the corpus luteum

Immediately after ovulation the follicle wall collapses, in a highly folded state, into the space left by the escaping ovum and the space gradually fills with blood to

form the corpus haemorrhagicum (Van Nierkerk, 1975). This structure can be palpated *per rectum* as a large soft depression in the ovarian substance, often representing about 70% of the size of the original follicle (Ginther, 1979). The corpus haemorrhagicum gradually undergoes luteinisation of the granulosa cells with subsequent hypertrophy and hyperplasia of the cells to produce the fully functional corpus luteum (CL). By Day 3 post-ovulation luteinisation of the granulosa cells is complete and by Day 4 post-ovulation the CL has reached its maximum size (Van Nierkerk, 1975).

Recently several authors have proposed a two cell model for the structure of the CL in the ewe and the cow (Fitz *et al*, 1982: Fitz *et al*, 1984: Schwall *et al*, 1986). The two cells, termed large and small cells based on the variation in their size, appear to be physiologically distinct although capable of interaction. Schwall *et al* (1986) reported PGF-2α receptor sites only on the cell membrane of the large cells. As both cells are capable of progesterone production they must both be lysed at luteolysis to reduce the circulating plasma progesterone to baseline.

As the CL matures it gradually organises to become brown and more fibrous with a gradual reduction in size. Subsequently, due to ageing, the colour alters from brown to yellow/orange as the amount of pigment increases. Lysis of the CL occurs around Day 14 post-ovulation in the mare with plasma progesterone reaching baseline prior to standing oestrus (Neely *et al*, 1979). The biochemical changes observed in the CL during luteolysis appear to be separate from the structural changes observed as the CL ages. The biochemical changes are referred to as functional luteolysis and the structural changes as structural luteolysis (Horton and Poyser, 1976). It seems likely that the mare resembles the ewe in this aspect of reproduction in that the functional luteolytic changes precede the structural changes but both are mediated by PGF-2α. PGF-2α has been reported to increase the release of lysosmal enzymes in the luteal cells of the rat and these enzymes may be responsible for the structural changes observed in the luteal cells (Poyser, 1981).

The CL is responsible for the secretion of the steroid hormone progesterone which is synthesised from the precursor cholesterol by the granulosa cells (Ginther, 1979). Within 24 hours of ovulation some of the luteinised granulosa cells are actively synthesising and secreting progesterone (Vans Rensburg and Van Nierkerk, 1968: Van Nierkerk, 1975). Progesterone concentration increases rapidly in the plasma during this time to reach a plateau by Day 6 post-ovulation (Palmer, 1978).

The ratio of increased progesterone concentration to decreased oestrogens is thought to be responsible for 'switching off' overt oestrous behaviour and the mare's rejection of the stallion (Noden, Oxender and Hafs, 1975). The luteal cells continue to secrete progesterone until Day 14 of the oestrous cycle when, if pregnancy is not established, lysis of the CL takes place and progesterone secretion is terminated (Oxender *et al*, 1974).

The gonadotrophin LH and possibly FSH appear to be vital for the growth, development and function of the CL of the mare. Pineda *et al* (1973) demonstrated that if antisera to LH was injected into mares in early dioestrus the CL did not develop normally but showed signs of early degeneration with no increase in the circulating plasma progesterone. However the antisera may have had some FSH activity as well as LH, as it consisted of a crude pituitary extract and therefore FSH, along with LH, may have been responsible for the effects observed. However in the mare, as in many of the domestic species, receptors for LH but not FSH have been identified on the cell surface of the luteal cells (Kimball and Wynegarden, 1977). Stewart and Allen (1979) reported that in the mare the LH receptors have a higher specificity for LH than the LH receptors of the other domestic species. The binding of LH to the receptors on the luteal cell membranes leads to an intracellular increase in cyclic adenosine monophosphate (cAMP) synthesised from adenosine triphosphate (ATP) (Richardson, 1987). This reaction is catalyzed by the enzyme adenylate cyclase bound to the cell membrane and triggered by the binding of LH to the LH receptor site. Cyclic AMP is a vital co-factor in the synthetic pathway of progesterone and an increase in the concentration of cAMP leads to an increased synthesis and release of progesterone from the luteal cells. However some authors report that progesterone synthesis and release by the CL is not solely dependent on the stimulatory action of LH and that the CL is capable of autonomous secretion of progesterone, for at least part of the oestrous cycle in most of the domestic species (Rothchild, 198l). Progesterone therefore apparently acts as a positive stimulus for the continued synthesis of progesterone. Indeed reports show that the CL of the ewe, guinea pig and pig can grow and secrete progesterone after hypophysectomy for almost the whole luteal phase (Rothchild, 198l). PGE-2 has also been suggested as a luteotrophic agent mimicking the effects of LH at its receptor site (Massicote *et al*, 1984). However little is known of the circulating plasma PGE-2 concentrations throughout the oestrous cycle in the mare. As previously discussed, a wave of FSH

is recorded during dioestrus in the mare which appears to stimulate a mid-dioestrous wave of follicles. However the fact that this wave of FSH may have a role to play in the stimulation of progesterone secretion cannot be ruled out, particularly in the mare in which so little is known of the stimulating and controlling mechanisms of the CL.

1.5 Life-span of the corpus luteum

In cycling mares with an interoestrous period of 15-17 days, lysis of the CL occurs around Day 14 post-ovulation (Neely *et al*, 1979). This results in a decline in circulating plasma progesterone to baseline and is considered to be mediated by the luteolysin PGF-2α, synthesised and released from the uterine endometrium of the mare (Neely *et al*, 1979). The decline in the circulating plasma progesterone occurs over the following 36-48 hours. The importance of the uterus in luteolysis in the mare was first reported by Ginther and First (1971) who observed that hysterectomy in the mare significantly prolonged the lifespan of the CL. PGF-2α has been positively identified as the luteolysin in the ewe, the species in which most of the work in this field has concentrated (McCracken *et al*, 1972: Goding, 1974). In the mare, as in the ewe, there is an increase in the circulating plasma PGF-2α around the time of luteolysis. In addition exogenous prostaglandin administered during dioestrus effects a return to standing oestrus within 2-5 days in the mare (Allen and Rowson, 1973: Allen and Cooper, 1975: Kenney *et al*, 1975). Therefore workers in the field hypothesized that, as in the ewe, PGF-2α was the luteolysin in the mare (Douglas and Ginther, 1976).

1.6 Prostaglandins: Chemistry, synthesis and metabolism.

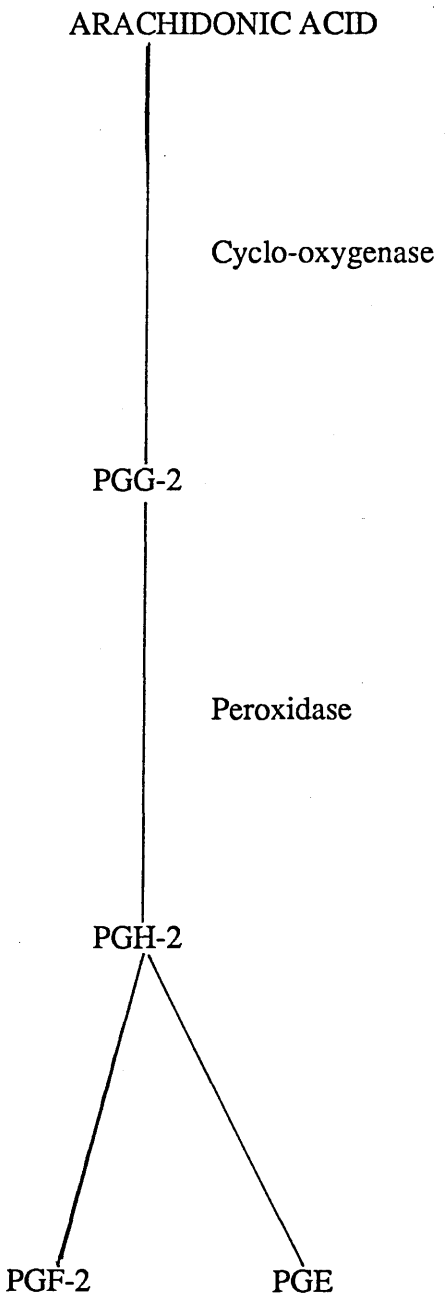
As far as reproduction is concerned the most important prostaglandins are PGE-2 and PGF-2α. The exact role of PGE-2 has not been fully established but it appears to have luteotrophic properties and may be involved in the maternal recognition of pregnancy, acting to combat the luteolytic actions of PGF-2α (Richardson, 1986). The role of PGF-2α has been more fully investigated and although not fully understood, this compound does appear to be directly involved in luteolysis.

Both PGE-2 and PGF-2α are 20 carbon long-chain fatty acids with double bonds at C5 and C13 and a 15(S) hydroxyl group which seems to be important for their biological activity. Their differences arise in the structure of the functional groups of the cyclo-pentane ring, PGF-2α differing from PGE-2 only in the pres-

ence of a hydroxyl group attached to the cyclo-pentane ring in place of an oxo group (Poyser, 1981).

The prostaglandins PGF-2α and PGE-2 are synthesised from the common precursor arachidonic acid (Figure 1:1, Lands, 1979). Arachidonic acid (AA) is stored in esterified form as arachidonate in the phospholipid fraction of the cell membrane. A variety of stimuli are able to release AA from the membrane bound glycerophospholipid, mainly through hydrolysis by phospholipase A (PLA-2) and phospholipase C (PLC: Vogt, 1978). However there appears to be different endogenous pools of AA present in the cell membrane. PLA-2 appears to be responsible for release of AA destined to be the precursor of PGF-2α while PLC releases AA for PGE-2 synthesis (Poyser, 1987). The concentration of free AA available within the cell is normally extremely low, unless the cell is stimulated, therefore the release of free AA is considered to be the rate-limiting step in the biosynthesis of prostaglandins (Leaver and Poyser, 1981). Around Day 14 post-ovulation in the mare there is an increased release of AA from the endometrial cell membrane due to increased PLA-2 activity. This leads to increased PGF-2α synthesis due to the actions of complex microsomal enzymes, the first of which is fatty acid cyclo-oxygenase (Lands, 1979). Cyclo-oxygenase catalyses the oxygenation of AA to the endoperoxide PGG-2 and is readily inhibited by the non-steroidal anti-inflammatory drugs (NSAID), such as aspirin and indomethacin (Vane, 1971: Poyser, 1985). PGG-2 is rapidly converted into the peroxide PGH-2 by the action of a peroxidase enzyme. These intermediates are biologically very potent, being strong inducers of platelet aggregation, vasoconstriction and contraction of respiratory smooth muscle among other reactions. However they are transitory compounds with half-lives of about 8 seconds and so their actions tend to be exerted in close proximity to their tissues of origin (Poyser, 1981). The endoperoxides are rapidly converted into a number of different compounds, the classical primary prostaglandins, PGF-2α, PGE-2 and PGD-2 among others, by the specific actions of an appropriate enzyme (Poyser, 1984a). PGF-2α can be synthesised in three different ways from the endoperoxide PGH-2. Firstly PGH-2 can be reduced to produce PGF-2α. This reaction is thought to be enzymatically controlled (Poyser, 1984a). The second pathway is the conversion of PGE-2 into PGF-2α by the actions of the enzyme 9-keto-reductase. The third method is the reduction of the 11-oxo group of PGD-2.

Figure 1:1


ARACHIDONIC ACID
Cyclo-oxygenase
PGG-2
Peroxidase
PGH-2
PGF-2
PGE

Although the prostaglandins have a high biological potency they are rapidly metabolised to essentially inactive compounds. The organs mainly responsible for prostaglandin metabolism are the lungs (Piper, Vane and Wyllie, 1970), although to a lesser extent metabolism also takes place in the liver and kidneys (Samuelsson *et al*, 1975). The first metabolic attack by 15 hydroxyl prostanoate dehydrogenase converts the 15 hydroxyl group to an oxo group very rapidly. This is then followed by reduction of the C3 double bond by a reductase enzyme, further reducing the potency to produce 15 keto-13,14 dihydro prostaglandin F-2α (PGFM). This metabolite has a much longer half-life than the parent compound and so PGFM is more readily measured in the plasma. Assays on the plasma of the domestic species therefore record PGFM concentrations as an indicator of the circulating plasma prostaglandin concentrations (Granstrom and Kumlin, 1987).

The major final degradation products are excreted in the urine after extensive oxidisation in the liver to the tetranor metabolites. These compounds have an even longer half-life than the 15 keto-13,14 dihydroprostaglandin metabolite, and have therefore been measured by some authors to gain further information about prostaglandin concentrations in the circulating plasma (Kindahl *et al*, 1984). However both plasma and urine analysis of PGF-2α metabolites gives no indication of site of synthesis and release of the parent compound.

The prostaglandins PGF-2α and PGE-2 can have another metabolic fate. The presence of a prostaglandin-9-oxo reductase, which is capable of catalysing the reduction of the 9-oxo group at C9 in PGE-2 to form PGF-2 compounds is found in many tissues including the ovary (Watson *et al*, 1979). The reverse reaction in which the formation of PGE compounds from PGF-2 by the enzyme prostaglandin 9-hydroxyldehydrogenase has also been reported. Although these reactions may occur more frequently in some species than in others, they may be a vital pathway in the alteration of the proportion of the two compounds (Oliw, Granstrom and Anggard, 1981).

1.7 Stimuli for prostaglandin release

It is apparent that no single stimulatory factor is involved in the stimulation of prostaglandin synthesis but a complicated interaction of many separate factors.

Several authors report that oestrogens play an important role in the stimulation of PGF-2α production from the endometrial cells and indeed, in most of the domestic species, an increase in the circulating oestrogen concentration is observed prior to, or during, the luteolytic increase in PGF-2α (Poyser, 1981). However exogenous oestrogens only stimulate PGF-2α production from the uterus if the uterus has had previous exposure to progesterone for a period of at least 10 days (Blatchley and Poyser, 1974: McCracken *et al*, 1981). In the mare the dioestrous wave of follicles, releasing increasing concentrations of oestrogens, may be involved in stimulating an increase in PGF-2α synthesis and release from the progesterone-primed uterus. Work done to date on the guinea pig suggests that oestrogens are able to passage into the nucleus of the endometrial cell and act on the DNA surface to stimulate DNA synthesis (Horton and Poyser, 1976). RNA then increases the synthesis of the enzymes involved in PGF-2α synthesis. Oestrogens are also capable of increasing the intracellular calcium concentration responsible for triggering the PLA-2 enzyme. This enzyme is optimally active in the guinea pig uterus at a concentration of 7mM Ca^2 (Downing and Poyser, 1983). An increased intracellular calcium concentration will therefore lead to activation of the enzymes and the release of AA from the cell membrane. This effect, coupled with the intracellular increase in PGF-2α synthetic enzymes, may be an important aspect of the oestrogen stimulation of prostaglandin production.

Although it appears essential that the uterus is first primed with progesterone before oestrogen can stimulate the synthesis of prostaglandin, the exact role of progesterone has not been clarified. Sar and Stumpf (1974) showed that progesterone enters the nucleus of the cell in a similar manner to that of oestrogen, but the nuclear response to the presence of progesterone remains unclear. Poyser (1981) suggests that progesterone is involved in the release of AA from its bound source in the cell membrane.

However oestrogens acting on a progesterone-primed uterus are not the only stimulatory factors involved in the release of PGF-2α from the uterus. As early as 1959, Armstrong and Hansell recognized that exogenous oxytocin, infused into cycling cows during dioestrus, resulted in an early return to standing oestrus. Recent evidence in the ewe suggests that the luteolytic effect of oxytocin is mediated via the release of prostaglandins from the uterus and many authors now believe that oxytocin is intimately involved in the process of luteolysis in several

species (Flint and Sheldrick, 1983: Sheldrick, Mitchall and Flint, 1980 :McCracken *et al*, 1981: Cooke and Homiedia, 1982 and 1983: Mitchell *et al*, 1982: Mitchell, Flint and Turnball, 1975). However the evidence for the involvement of oxytocin in the oestrous cycle of the mare is a confused and contradictory one. Neely *et al*, (1979b) were unable to induce precocious oestrus in mares with the administration of exogenous oxytocin, although Goff *et al* (1987) reported increased plasma prostaglandin in response to exogenous oxytocin. Tetzke *et al* (1987), using an intensive programme of blood collections, reported that the circulating concentration of oxytocin fluctuated markedly throughout the oestrous cycle of the mare. The concentrations increased during mid to late dioestrus and declined during oestrus. This pattern resembles that reported in the ewe, although actual concentrations of oxytocin are higher in the mare than in the ewe. Burns *et al* (1981) also reported that the concentrations of oxytocin fluctuated throughout the oestrous cycle of the mare but they reported that the concentrations reached a peak during oestrus and early dioestrus with baseline concentrations throughout mid to late dioestrus and no increase at the time of luteolysis.

In the ewe however the role of oxytocin is clearer and in a series of experiments Flint and Sheldrick were able to demonstrate that plasma oxytocin fluctuated throughout the oestrous cycle of the ewe, rising during mid dioestrus but declining during prostaglandin mediated luteolysis (Sheldrick and Flint, 1981: Flint and Sheldrick, 1983).

In the ewe and the cow the source of oxytocin appears to be the ovary and a precursor molecule for oxytocin has been identified in the cells of the bovine CL (Wathes *et al*, 1983). In these species oxytocin is thought to attach to receptor sites in the endometrium specific for oxytocin, thus stimulating the release of prostaglandin (Roberts *et al*, 1976: Sheldrick and Flint, 1985). McCracken *et al*, (1981) suggest that oestrogens act on the progesterone-primed uterus not only to stimulate prostaglandin synthesising enzymes but also to increase the concentration of oxytocin receptors on the endometrial cell surface and to increase the binding of free oxytocin to these receptor sites. The coupling of oxytocin to the endometrial receptor sites leads to an increase in the synthesis and release of PGF-2α from the cells. However Poyser (1984b) reported that the release of PGF-2α occurred from Day 12 post-ovulation in the ewe, 2 days before the endometrium became sensitive to the

effects of oxytocin, thus suggesting that oxytocin is not initially involved in the release of PGF-2α

1.8 Patterns of prostaglandin release

Once stimulated, prostaglandin synthesis and secretion proceed rapidly. The endometrial cell does not store prostaglandin but secretes it immediately (Piper and Vane, 1971). In the mare Stabenfeldt *et al* (1981) reported that around Day 14 post-ovulation, PGF-2α was released into the plasma as a series of short-lived surges or pulses. The pulses of PGF-2α were released over a timespan of 36-48 hours and apparently resulted in a sequential drop in plasma progesterone to baseline. The mares were observed to return to standing oestrus within 6-7 days of the first release of PGF-2α. A pulsatile pattern of PGF-2α release has been identified in all the domestic species which depend on the uterus as the major source of PGF-2α (Horton and Poyser, 1976: Poyser, 1981). Although the controlling mechanism of this pulsatile release of PGF-2α remains unclear, many authors suggest that oxytocin is the 'pulse generator' for prostaglandin release (Flint and Sheldrick, 1983: McCracken *et al*, 1981). Flint and Sheldrick (1983) reported that the CL will release oxytocin in a pulse representing the total amount of oxytocin synthesised by the cells at any one time. Oxytocin will attach to the receptor sites on the cell membranes of the endometrial cells and stimulate PGF-2α release in a pulsatile pattern. McCracken *et al*, (1981) suggested that the episodic release of PGF-2α was due to down-regulation of the oxytocin receptor sites in the endometrium which took some time to recover and as a result no PGF-2α was released during the recovery period. Poyser (1984b) however reported that the pulsatile release of PGF-2α occurred independently of the hormonal status of the animal and that the pattern of prostaglandin release was an intrinsic property of the uterus and did not depend on hormonal stimuli. Due to destruction of cyclo-oxygenase during PGF-2α synthesis new 'batches' of cyclo-oxygenase have to be produced thus leading to delays in PGF-2α synthesis and a pulsatile pattern of release (Poyser, 1981).

1.9 Transportation of prostaglandin to the site of action

Once the prostaglandins have been synthesised in the uterus they must travel to their site of action in the ovary. In ruminants prostaglandins travel to the ovary via the counter-current exchange system (McCracken *et al*, 1973: Einer-Jensen and McCracken, 1981: Ginther, 1974). This system is a complicated plexus involving

the uterine vein and the ovarian artery, such that the walls of the vein and the artery are in very close apposition. Due to the varying tensions between the uterine vein and ovarian artery prostaglandins are able to pass directly from one to the other. Thus a very concentrated surge of PGF-2α in its unmetabolised form is able to reach the ovary of the ruminant without first passing through the lungs. The importance of this local exchange of substances in the oestrous cycle of the ewe is readily demonstrated when separation of the ovarian artery and uterine vein is carried out, as it leads to persistence of the CL beyond the normal interoestrous period, despite increased plasma PGF-2α concentrations (Barrett *et al*, 1971:Abdel Rahim *et al*, 1984). However in the mare the uterine vein and ovarian artery are not closely apposed and no exchange of substances is possible between them (Ginther, 1974a). The absence of this local transfer system is easily demonstrated by partial hysterectomy of the ipsilateral horn (Ginther and First, 1971). In the mare, unlike the ruminant, this had no effect on the lifespan of the CL which was lysed as the concentrations of PGF-2α increased in the plasma. This would suggest that the prostaglandin may travel to the ovary via a systemic rather than a local pathway.

1.10 Mode of action of prostaglandins at the corpus luteum

Various theories have been suggested as to the mechanisms involved in the PGF-2α mediated lysis of the CL. In 1970 Pharriss suggested that vasoconstriction mediated by PGF-2α may cause death of the CL due to a lack of nutrients reaching the ovary. More recent reports suggest that there is little alteration to the overall ovarian blood flow during luteolysis, although luteal blood flow appears to be selectively reduced. However this reduction in luteal blood flow occurs after progesterone has begun to decline (Horton and Poyser, 1976: Einer-Jensen and McCracken, 1977). It is not apparent therefore if the reduction in ovarian blood flow is a cause or an effect of luteolysis.

Labhetswar (1973) suggested that cessation of luteal function resulted from altered gonadotrophin secretion by the anterior pituitary. This work suggested that PGF-2α inhibited gonadotrophin hormone secretion at the pituitary. However Jochle *et al* (1987) reported that if circulating progesterone concentrations are low then an increase in circulating plasma LH and FSH is observed in response to PGF-2α administration in the mare. In addition Poyser (1981) reported that PGF-2α is a vital trigger for the release of gonadotrophin from the hypothalamus. This would suggest that PGF-2α will stimulate, not inhibit gonadotrophin secretion

at the pituitary. Behrman (1979), and Strauss and Stambaugh (1974) suggested an anti-gonadotrophic effect at the CL with a direct effect on the progesterone secretory function. This theory has been strongly favoured by many authors. Vernon *et al* (1979) demonstrated the existence of definite PGF-2α receptor sites on the luteal cell membranes of the mare. Kimball and Lauderdale (1975) also reported the existence of separate PGF-2α, PGE-2 and LH receptor sites on the surface membrane of bovine luteal cells. This suggested that PGF-2α acted independently of the LH receptor sites and not by a process of competitive binding at the LH receptor sites.

PGF-2α is thought to react with its receptors on the luteal cell membrane and uncouple the adenylate cyclase:cAMP system, by which LH stimulates the progesterone synthetic pathway (Horton and Poyser, 1976). This prevents the LH dependent cAMP accumulation within the cell, possibly by increasing intracellular calcium concentrations which inhibit the activation of adenylate cyclase and the intracellular increase in cAMP. The reduction in intracellular cAMP leads to deactivation of cholesterol esterase enzymes which are vital for progesterone synthesis. PGF-2α also blocks the effects of dibutryl cyclic AMP (Jordan, 1981), a compound capable of stimulating the progesterone synthetic pathway but at a step further along the pathway from the cAMP site. This would suggest that PGF-2α is also capable of inhibiting the synthetic pathway at sites other than the adenylate cyclase-cAMP site.

After the initial PGF-2α release, progesterone begins to decline to baseline. Roser *et al* (1982) reported a significant decline in luteal and plasma progesterone concentrations in the mare three hours after treatment with exogenous PGF-2α. Stabenfeldt *et al* (1981) demonstrated that PGF-2α release preceded the progesterone decline by 3-5 hours in the plasma of cycling mares.

The reaction of the luteal cells to the effects of PGF-2α is not consistent throughout the lifespan of the CL. In the mare exogenous PGF-2α will not lyse the CL until at least Day 4-5 post-ovulation (Allen and Rowson, 1973) and endogenous PGF-2α released from the uterus by saline infusion or biopsy does not lead to lysis of the CL during early dioestrus (Stabenfeldt *et al*, 1981). Indeed a stimulatory effect of PGF-2α on progesterone secretion has been observed in dispersed cultured monkey (Stouffer *et al*, 1979), human (Richardson, 1987) and bovine luteal cells (Benhaim *et al*, 1981) during the mid luteal phase of the oestrous cycle. However

these results have not been corroborated by *in vivo* work. The reason for the refractoriness of the luteal cells during the developmental stages of the cycle has not been clarified. Although the number of PGF-2α receptors in the CL of the mare was found to vary throughout the oestrous cycle no pattern to the changes was evident (Kimball and Wyndegarden, 1977). Increased binding of PGF-2α to its receptor site was reported in the bovine during the later stages of the oestrous cycle (Rao *et al*, 1979). Schwall *et al* (1986) suggested that the large luteal cells which contain the PGF-2α receptor sites do not develop until several days after the CL has begun functioning and therefore PGF-2α was not able to bind to the cell membrane and exert its effect on progesterone production.

1.11 Prolonged dioestrus

The mare frequently demonstrates a breakdown in the events of luteolysis in which lysis of the CL does not occur at the expected time (Stabenfledt *et al*, 1976). The CL will persist in the ovary leading to a state of prolonged dioestrus which, if untreated, can last up to 90-100 days. Although prolonged dioestrus has been recognized in the other species the incidence is very low, 2% in ruminants (Bulman and Lamming, 1977) compared to between 20-30% in the mare (Stabenfeldt *et al*, 1981: Ginther, 1979). This condition obviously leads to considerable economic loss in breeding mares although it can be treated with exogenous PGF-2α, which will usually return the mare to standing oestrus within 2-5 days (Allen and Cooper, 1975: Allen *et al*, 1974).

1.12 Aims of the project

The action of PGF-2α at the CL during luteolysis in the domestic species appears to be a complicated interaction of events, with no one event of greater importance than the others. Luteolysis in the mare has not been sufficiently investigated and there are many gaps in our understanding of this event in the mare. The mare is anatomically and physiologically different from the ruminants in many areas of reproduction and therefore the findings reported in these animals cannot be extended to the mare. As a result a great deal of investigative work is required to fully understand the reproductive cycle of the mare and the events of luteolysis, which differ so markedly from that of the ewe and the cow. The purpose of this project was therefore to try to assess more accurately the events of luteolysis with

respect to PGF-2α synthesis, its release from the endometrial and luteal tissue and the role that it plays in the events of luteolysis in the mare.

2 CHAPTER TWO
BEHAVIOURAL AND CLINICAL STUDIES OF FOUR NORMAL CYCLING MARES.

2.1 INTRODUCTION

In the mare many studies have already been undertaken into the behavioural, clinical and hormonal changes that occur during the oestrous cycle. However to date no obvious detectable changes in any of these parameters have been found associated with lysis of the functional corpus luteum.

The aims of the work outlined in this chapter were firstly to gain sufficient expertise in the examination of mares using the techniques of teasing by stallion, blood sampling for plasma progesterone analysis and examination of the reproductive tract *per rectum*, such that the day of ovulation could be accurately assessed. Secondly to try to determine if any correlation existed between the fluctuations in the width, tone and palpability of the cervix and plasma progesterone concentrations during one oestrous cycle. The reason for these studies was to attempt to identify an accurate method for determining when luteolysis was occurring in the oestrous cycle of the mare.

2.2 MATERIALS AND METHODS

2.2.1 Mares

Four mares were used in the study. Details of which are outlined in Table 2:1:

Table 2.1

MARE	BREED	AGE	PREVIOUS FOALS
Mare 1	Highland X	12	1
Mare 2	New Forest	9	3
Mare 3	Welsh	11	2
Mare 4	Hunter	13	unknown

2.2.2 Behavioural studies

Teasing was carried out daily throughout a complete oestrous cycle in each of the mares. Each mare was teased individually, for at least 5 minutes, in the presence of an eight year old Welsh pony stallion. Results were recorded using the method of Munro *et al*, (1979), with modifications. The modifications involved giving the mare a +/-1 or +/-2 score overall depending on whether the individual behavioural responses were exhibited occasionally or continuously, in response to the stallion.

2.2.3 Palpation of the genital tract

Palpation of the genital tract *per rectum* was carried out daily during the oestrous cycle of each mare. The mare was restrained in wooden stocks. Ovaries, uterus and cervix were palpated. Parameters recorded were: ovarian size and consistency; size position and consistency of the largest follicle present on the ovaries; cervical size, consistency and state.

2.2.4 Identification of the day of ovulation

The main criterion for determining the day of ovulation was softening of the surface of a large mature follicle, followed by loss of this follicular structure and a marked reduction in ovarian size. In some of the mares in this group a painful response was elicited, on the day of ovulation and in some cases for several days after, on palpation of the ovary in which ovulation had occurred. The

timespan and the degree of response varied between the mares and note was taken of the degree of response and the length of time it was elicited.

2.2.5 Examination of the cervix

A measurement of the width of the cervix was obtained by the use of a Rectal measuring device (Physics department, University of Exeter). This machine consisted of two finger probes, linked to a read-out dial. These probes were lubricated well and one was place on the thumb, the other on the second finger. They were positioned over the cervix *per rectum* and the different measurements in millimeters were recorded. During examination of the cervix observations were also made on the consistency and state of the cervix. The scoring system outlined below was used to score each cervix:

0 Very wide flaccid cervix, whose borders could not be accurately determined. Width 40-50mm

\+ Loss of tension but still formed. Width 30-39mm

++ Closed cervix but little apparent tone. Width 25-29mm

+++ Tightly closed presenting as an obvious ridge. Width < 25mm

Measurements were taken daily throughout one oestrous cycle in the mares.

2.2.6 Blood sampling regimes

Blood samples were collected daily throughout one oestrous cycle by veni-puncture from the jugular vein. A 19G 1 1/2" needle (Terumo) and heparinised monovets (Sarstedt) were used. Immediately after collection the samples were centrifuged (20 mins, 4°*C*, 2000g) decanted into labelled plastic bottles and stored at -20°*C* until assayed for progesterone concentrations.

2.2.7 Plasma progesterone estimations

Plasma progesterone concentrations were estimated using an established radio-immunassay (RIA) procedure as described by Munro *et al* (1979). The intra-assay and inter-assay coefficients were calculated as 13%. The limit of sensitivity was 0.5 ng/ml.

2.3 RESULTS

Figure 2:1 Mare 1

Behavioural, cervical, ovarian and plasma progesterone changes in Mare 1 during one inter-oestrous period.

Ovulation was recorded in this mare 24 hours before the end of overt oestrus when progesterone was basal. A marked painful response was elicited on the day of and the day after ovulation in this mare. Throughout the standing oestrous period the borders of the cervix could still be readily defined *per rectum* although some loss of tone was apparent. Complete relaxation of the cervix was observed 5 days after ovulation when progesterone concentrations were already increasing.

Figure 2: 1

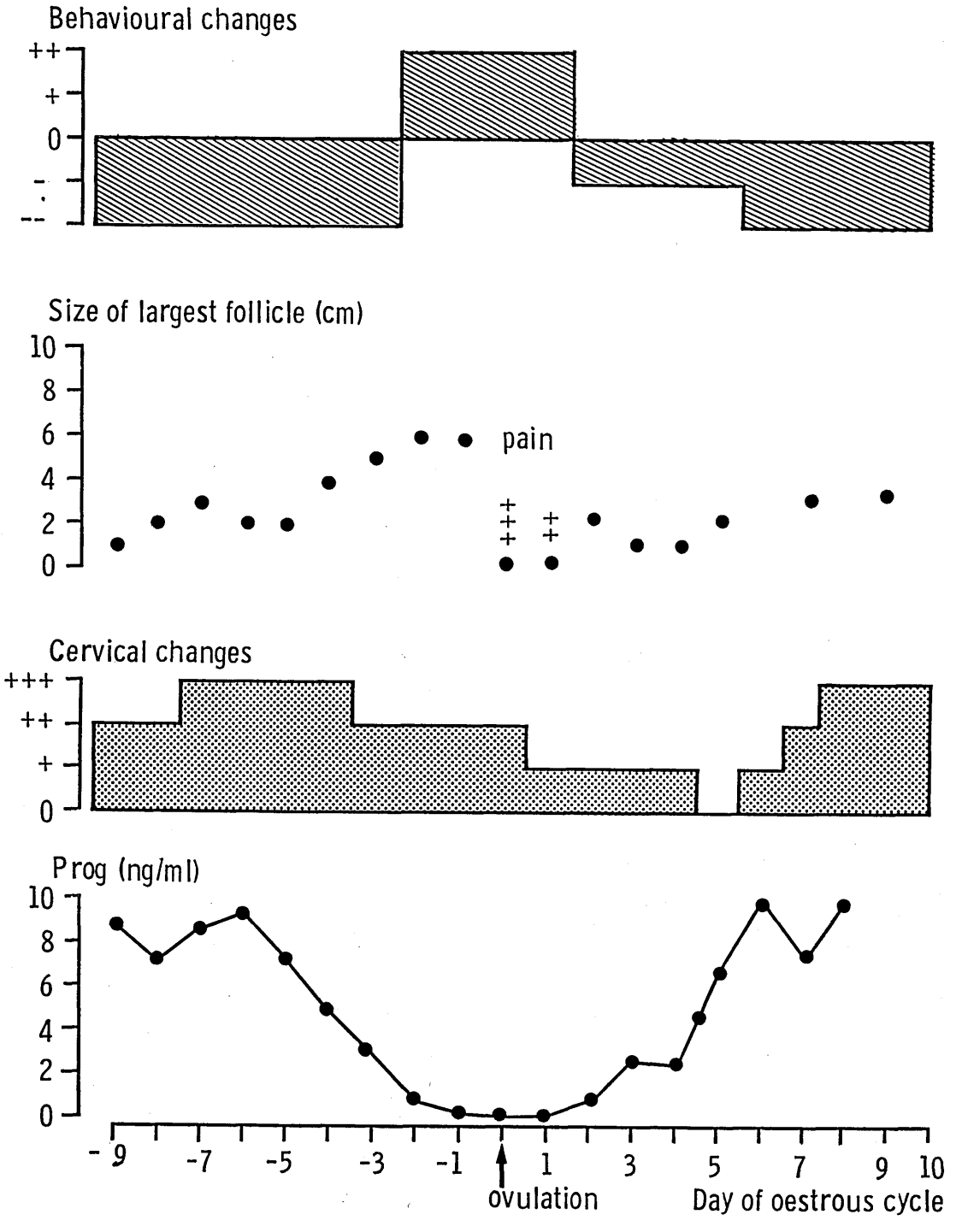

Behavioural changes
++
+
0
-
--
Size of largest follicle (cm)
10
8
6
4
2
0
pain
Cervical changes
+++
++
+
0
Prog (ng/ml)
10
8
6
4
2
0
-9
-7
-5
-3
-1
1
3
5
7
9
10
ovulation
Day of oestrous cycle

Figure 2:2 Mare 2

Behavioural, cervical, ovarian and plasma progesterone changes in Mare 2 during one inter-oestrous period.

Ovulation occurred in this mare 48 hours before the end of overt oestrus. A painful response was elicited on the day of ovulation only. A gradual transition from behavioural dioestrus to standing oestrus was observed. The cervix gradually relaxed 7 days before ovulation to become soft and dilated 2 days prior to ovulation. These changes were associated with low progesterone concentrations.

Figure 2: 2

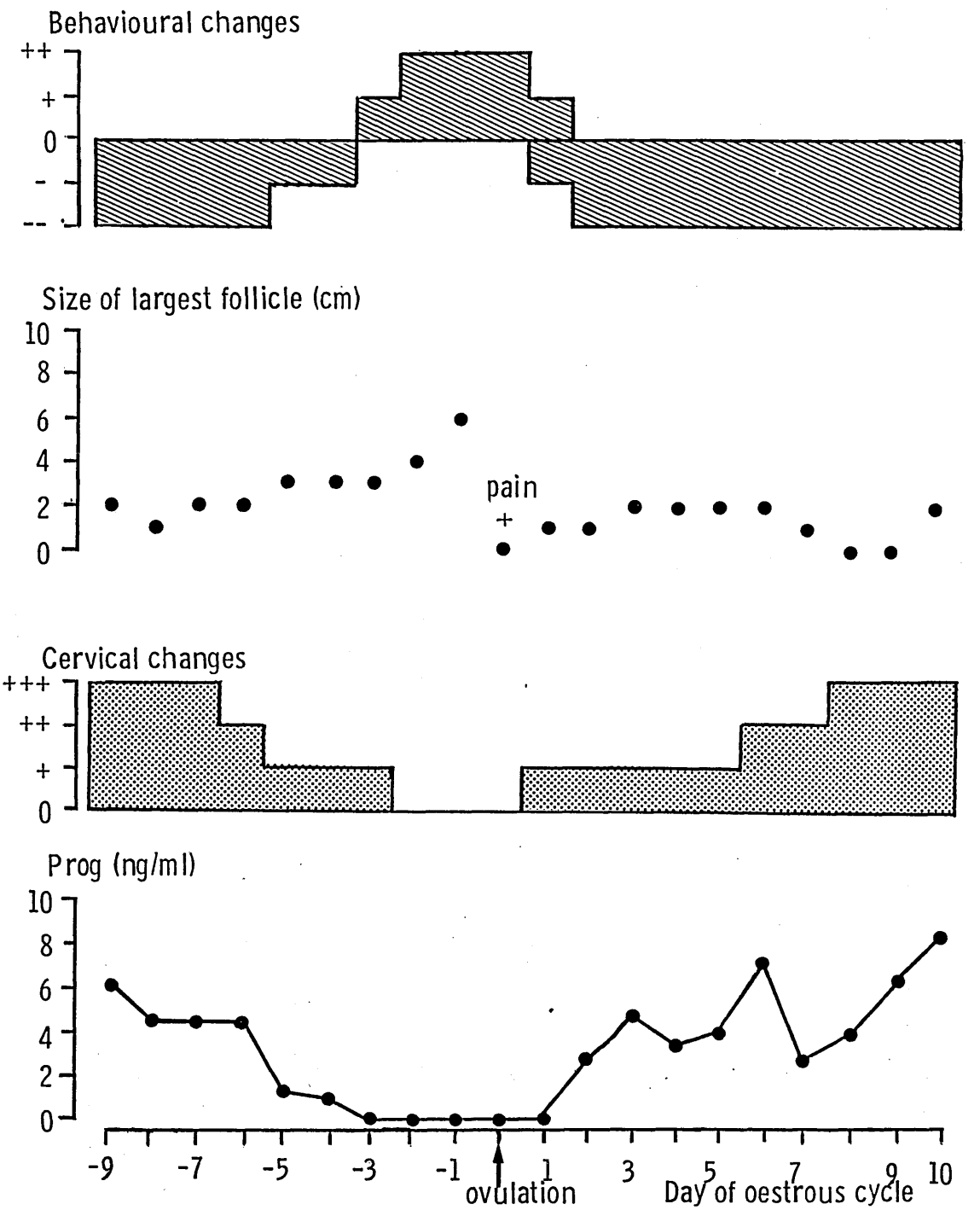

Behavioural changes
++
+
0
-
--
Size of largest follicle (cm)
10
8
6
4
2
0
pain
Cervical changes
+++
++
+
0
Prog (ng/ml)
10
8
6
4
2
0
-9
-7
-5
-3
-1
ovulation
1
3
5
7
9
10
Day of oestrous cycle

Figure 2:3 Mare 3

Behavioural, cervical, ovarian and plasma progesterone changes in Mare 3 during one inter-oestrous period.

This mare demonstrated a period of basal progesterone 8-10 days before ovulation, 5 days prior to overt oestrus. Progesterone concentrations increased in the plasma 7 days before ovulation but had declined to baseline again 4 days before ovulation, the first day of standing oestrus. Ovulation in this mare occurred 24 hours prior to standing oestrus. A degree of cervical tone was still present during the first 3 days of standing oestrus. However the cervix became flaccid and fully dilated during the last three days of standing oestrus. No painful response was elicited at any stage in this mare.

Figure 2:3

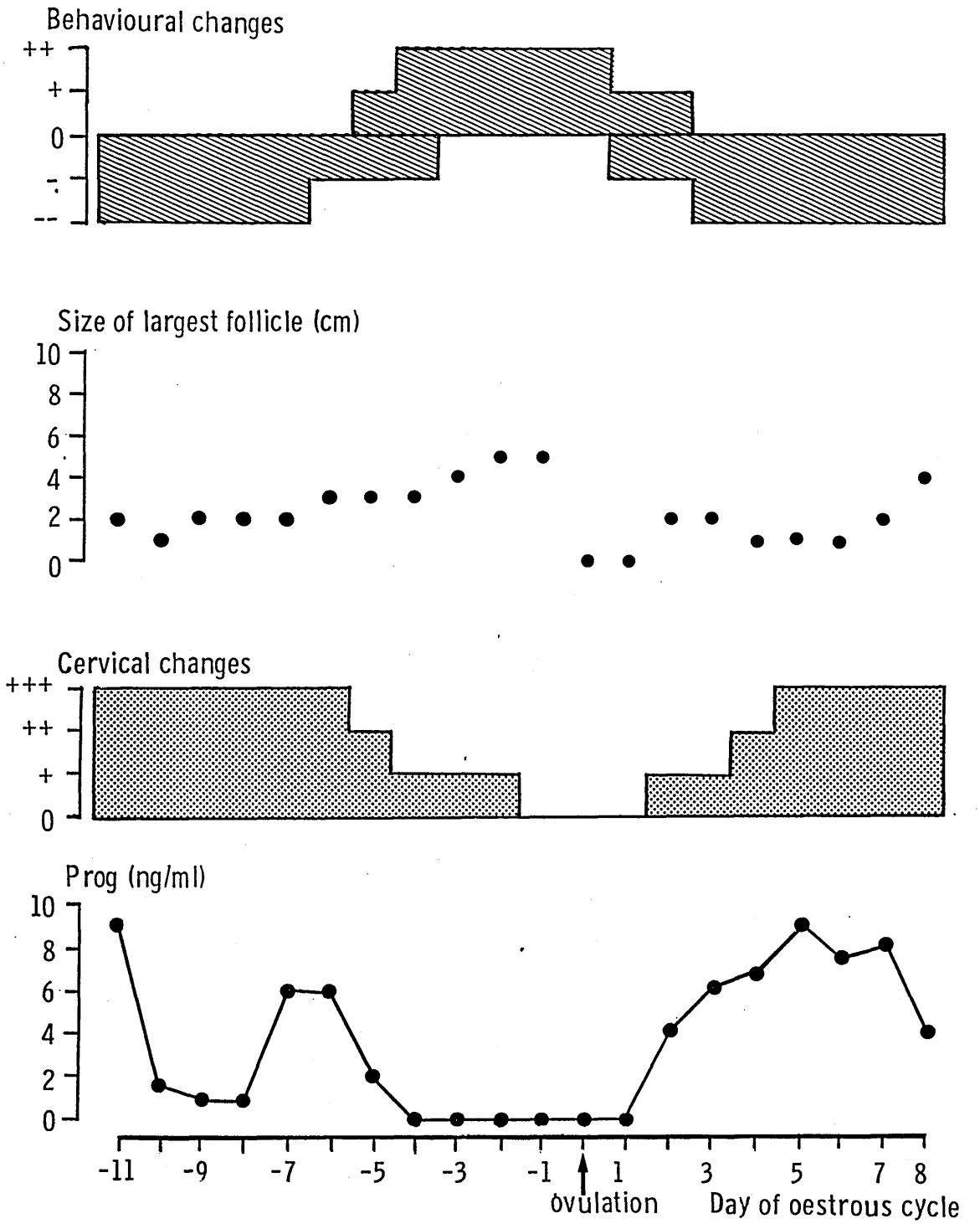
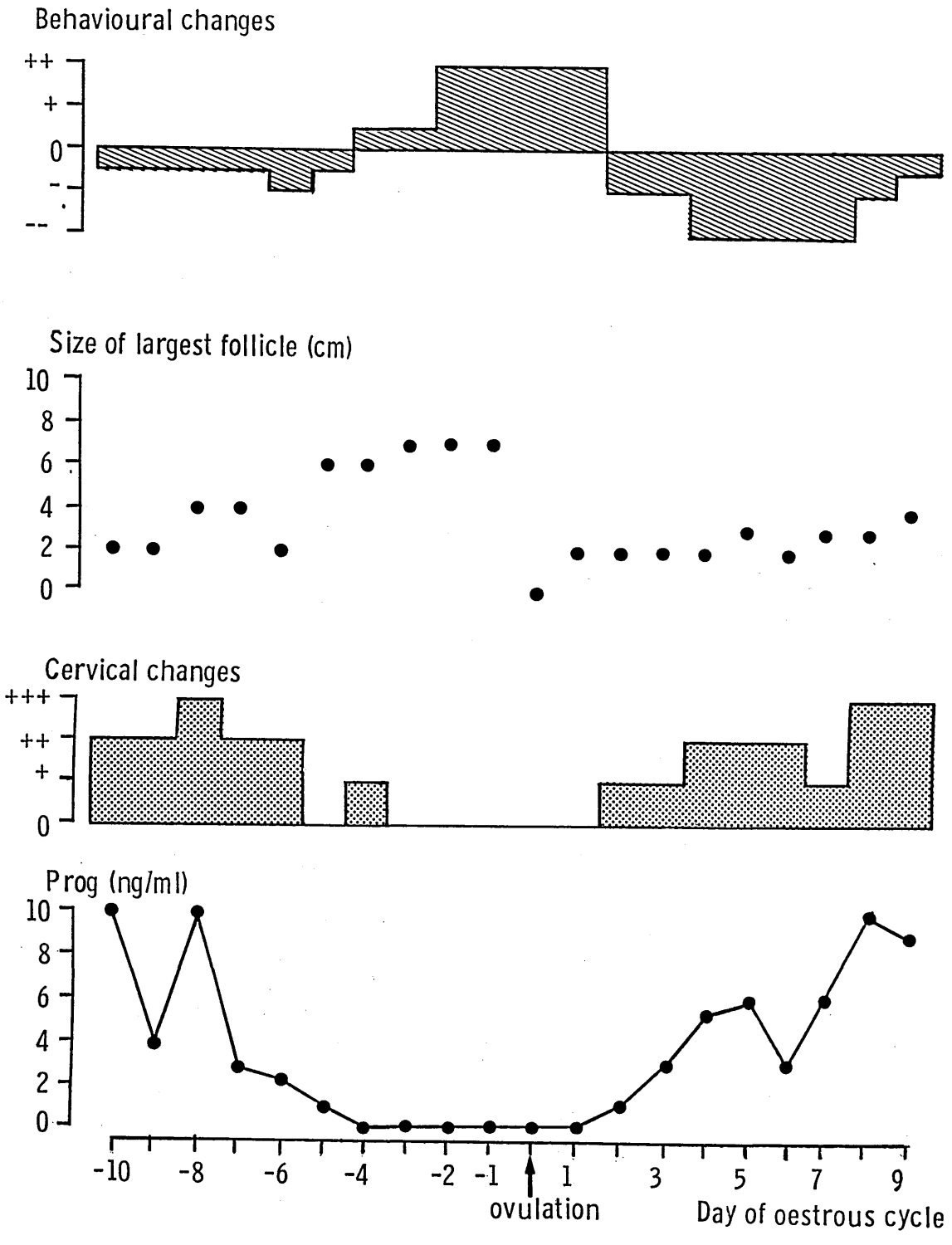

Behavioural changes
++
+
0
-
--
Size of largest follicle (cm)
10
8
6
4
2
0
Cervical changes
+++
++
+
0
Prog (ng/ml)
10
8
6
4
2
0
-11
-9
-7
-5
-3
-1
1
3
5
7
8
ovulation
Day of oestrous cycle

Figure 2:4 Mare 4

Behavioural, cervical, ovarian and plasma progesterone changes in Mare 4 during one inter-oestrous period.

This mare exhibited weak positive behavioural signs during the first two days of standing oestrus before full expression of oestrus. Ovulation in this mare occurred two days prior to the end of standing oestrus. This mare had a relatively relaxed cervix throughout the period of study and the borders were difficult to define, although variations were observed. Maximum dilation was recorded during the period of standing oestrus. No painful response was elicited on palpation of the ovary at any stage in this mare.

Figure 2:4


Behavioural changes
++
+
0
-
--
Size of largest follicle (cm)
10
8
6
4
2
0
Cervical changes
+++
++
+
0
Prog (ng/ml)
10
8
6
4
2
0
-10
-8
-6
-4
-2
-1
1
3
5
7
9
ovulation
Day of oestrous cycle

2.4 DISCUSSION

The four mares in this study exhibited negative behaviour during dioestrus and positive behaviour during oestrus, although very different patterns of expression were observed. Three mares in this group appeared to demonstrate a gradual transition from dioestrus to standing oestrus. In these mares the decrease in expression of negative behaviour appeared to be associated with a drop in progesterone concentrations. Although this would suggest a positive correlation between progesterone concentration and the intensity of expression, there is no firm evidence to date that this is the case. Munro *et al* (1979) found that variations in plasma progesterone concentrations were not correlated to the intensity of negative behavioural expression, although an inverse relationship between plasma progesterone concentrations and positive behaviour was recorded by these workers.

Progesterone concentrations declined rapidly at luteolysis in all four mares and are therefore one of the most accurate indicators that luteolysis has taken place. Mare 3 however demonstrated a period of basal progesterone 5 days prior to standing oestrus. As this is not physiologically likely to happen, it was assumed that either blood samples had been muddled or the progesterone assay had recorded wrong values. Stabenfeldt *et al* (1981) demonstrated that PGF-2α release precedes progesterone decline by 3-5 hours, therefore if a decline in progesterone concentrations is the only criterion used to determine luteolysis it will be too late to observe the luteolytic period. Also, as can be seen from Mare 4, marked fluctuations in plasma progesterone concentrations occurred prior to luteolysis therefore it would be difficult to predict the luteolytic-associated drop in progesterone concentrations by monitoring daily blood samples. It is also time consuming and expensive to assay samples on a daily basis to determine fluctuations in the progesterone concentrations, and as a result progesterone analysis tends to be retrospective.

During the preliminary clinical investigations one of the main areas of the reproductive tract examined *per rectum* was the cervix. Although previous studies of this aspect of the reproductive tract have been undertaken, these were performed on a purely clinical basis with no reference to associated progesterone concentrations. In this study clinical findings were related to plasma progesterone concentrations to determine if any correlation existed. Progesterone is the dominant hormone of dioestrus and is thought to be responsible for maintaining the cervix in a closed

state. As progesterone concentrations decline, during luteolysis, it was hypothesized that the cervix of the mare should gradually relax and become less obvious on rectal palpation. However the results from this study demonstrated that no correlation existed between the plasma progesterone and the cervical fluctuations. All four mares demonstrated a closed cervix during mid-dioestrus, although variations in degree were recorded. In particular Mares 2 and 3 displayed a gradual relaxation of the cervix as progesterone concentrations declined during luteolysis, suggesting a positive correlation between progesterone concentrations and the cervical state. Mare 1 however had a palpable cervix throughout the standing oestrous period and demonstrated marked dilation of the cervix at Day 5 post-ovulation, despite progesterone concentrations of 7ng/ml. Finally Mare 4 demonstrated a relatively relaxed cervix throughout the period of study. Fluctuations in degree were observed in this mare but these did not appear to be related to progesterone concentrations. The reasons for the fluctuations in cervical size and consistency in the mares are not apparent from this study. However factors other than progesterone may be involved in minor changes in the cervix, for example oestrogen, relaxin or possibly prostaglandins, such as PGE-2. None of these other hormones were examined during this study therefore it is not possible to determine if changes in these hormones could be responsible for the alterations in the cervical states in these mares. The results of both the behavioural and cervical studies undertaken in these investigations indicated that neither was useful in identifying when luteolysis was taking place. The most accurate means of identifying luteolysis in the studies described in this thesis was by determining when ovulation took place and presuming that luteolysis would occur around 14 days later.

It is relatively easy to palpate follicles developing on the surface of the ovaries. Any changes can be readily monitored and with experience it is possible to determine when ovulation of a follicle takes place. Once experience has been gained this is one of the most reliable guides to events in the oestrous cycle of the mare. Ovulation can be pin-pointed and luteolysis can then be estimated by counting forward 14 days. The projected date will only be an estimate of the time of luteolysis as Day 14 is only an average. This is demonstrated by the fact that two of the mares in this study exhibited a drop in progesterone at Day 14 post-ovulation (Mares 1 and 4) while the other two mares demonstrated a drop in progesterone on Day 15 post-ovulation (Mares 2 and 3). All the mares ovulated during the period of

study and their follicles attained a significant size prior to ovulation. Both Mares 1 and 2 demonstrated a painful response in the ovary on the day of and the day after ovulation. This response is thought to be due to palpation of a newly formed corpus haemorragicum in the ovary. Although all mares do not exhibit this reaction it is a useful additional parameter for determining the time of ovulation when present.

It is obvious from the above studies that luteolysis is not overtly expressed and, if each of the parameters discussed are taken in isolation, it is very difficult to assess accurately this stage of the cycle. However if all the parameters are assessed together a more accurate picture is obtained. Throughout the period of these studies, teasing of the mare was continued as a means of identifying when the mare came into standing oestrus. The mares were examined *per rectum* three times a week until behavioural oestrus, when rectal palpation was increased to daily examination, to try to assess the day of ovulation. Note was also made of any painful reactions elicited. The studies of cervical changes were discontinued as no useful information was available from the results. The onset of luteolysis was then estimated by counting forward from the day of ovulation to 14 days. Estimations of progesterone concentrations in daily blood samples continued to be carried out as they formed an essential part of the further investigations. These investigations were carried out in all the mares involved in the study.

3 CHAPTER THREE

PLASMA PROSTAGLANDIN F-2α METABOLITE AND PROGESTERONE CONCENTRATIONS IN CYCLING MARES AND MARES IN PROLONGED DIOESTRUS.

3.1 INTRODUCTION

The aims of this chapter were firstly to establish an adequate method of measuring 15 keto-13,14 dihydroprostaglandin F-2α (PGFM) and prostaglandin F-2α (PGF-2α) concentrations in the circulating plasma of the mares involved in this study. Secondly to determine the pattern of PGFM in the plasma of normal cycling mares during the period of luteolysis. Thirdly to determine the PGFM patterns in mares in prolonged dioestrus and to compare these patterns to the normal cycling mares. Fourthly to manipulate the mares in prolonged dioestrus back into standing oestrus by performing uterine biopsies. Finally to determine the effects of exogenous PGF-2α on the circulating plasma PGFM, progesterone and PGF-2α concentrations in normal cycling mares.

The radio-immunassay (RIA) method used for PGFM estimation in this study was that of Dobson (1983) with modifications by Escreet (1986). However several problems were encountered involving: the sensitivity of the assay; the percentage binding of the radio-active label and the repeatability of the standard curve. A reproducible RIA for identifying changes in the PGFM pattern in the plasma was defined. The modifications made to the original assay and the comparative results of the different assay methods are described and discussed. A second RIA to determine PGF-2α concentrations in the plasma and in culture medium from tissue samples was established and is also described and discussed.

3.2 MATERIALS AND METHODS

3.2.1 Mares

13 mares were used for the study. All the mares were bled during the normal breeding season. 11 of the mares were cycling normally prior to the study, although 1 mare failed to return to standing oestrus at the expected time and entered a phase of prolonged dioestrus. 2 of the mares were in a state of prolonged dioestrus prior to the start of the blood sampling regime. Details of the mares are given in Table 3:1.

Table 3:1

MARE	BREED	AGE (years)	PREVIOUS FOALS
1	NEW FOREST	20	several
2	EXMOOR	12	2
3	TB	12	3
4	WELSH	10	unknown
5	TB	10	2
6	TB	12	3
7	TB	15	several
8	DARTMOOR	14	2
9	ARAB X	14	several
10	ARAB X	14	several
11	NEW FOREST	10	2
12	WELSH	9	unknown
13	TB	17	several

3.2.2 Blood sampling regimes

The mares were divided into three groups:

1) Normal mares (8)
2) Mares in prolonged dioestrus (3)
3) Mares injected with 'Lutalyse' (2)

The details of each group are outlined below:-

Group 1: Normal cycling mares.

8 mares cycled normally throughout the sampling period and were subdivided into 3 small groups.

Group 1a

During October 1985 two mares (Mares 1 and 2) were bled twice daily from standing oestrus until Day 13 post ovulation when sampling was increased to four times daily, for a period of 4 days in Mare 1 and until standing oestrus in Mare 2.

Blood samples were collected from these two mares by venipuncture from the jugular vein using a 19G, 11/2" needle (Terumo) and heparinised Monovets (Sarstedt). Immediately after collection the samples were centrifuged (20 minutes, 4°*C*, 1200g), decanted into labelled plastic bottles and stored at -20°*C* until assayed. Mare 5 was also bled by this method.

Group 1b

During early September 1986 two mares (Mares 3 and 4) had blood samples taken every hour at what was calculated to be Day 13 post ovulation. Blood sampling started at 09.00 hours on the day designated Day 13 post ovulation. Due to the cannulae becoming blocked blood sampling was stopped after 28 hours in Mare 3 and 32 hours in Mare 4.

Group 1c

During the month of May 1987 3 mares (Mares 6, 7 and 8) were bled every hour for 57 hours. Mare 5 was bled every 3 hours for 57 hours. Blood sampling started at 09.00 hours on the day designated as Day 13 post ovulation.

Blood samples were collected from the jugular vein using a 19G (Terumo) indwelling cannula and heparinised monovets in the mares in Groups 1b and 1c, except Mare 5. The cannulae were stitched into place and patency was maintained by heparinised saline washes. Immediately after collection the samples were centrifuged (20 mins, 4°*C*, 1200G), decanted into labelled plastic bottles and stored at -20°*C* until assayed.

Group 2 : Mares in prolonged dioestrus

There were three mares in this group. Two of the mares were diagnosed as being in a state of prolonged dioestrus prior to the start of blood sampling. The third mare was cycling normally at the start of the investigations but failed to return to standing oestrus at the expected time. This mare remained in prolonged dioestrus for a further 3 months.

The bleeding regimes varied in each of these mares.

Mare 9: This mare was blood sampled twice daily, increasing to 4 times daily for a period of 4 days. Blood sampling started at Day 45 post-ovulation and occurred during October 1985. Day 45 was selected randomly.

Mare 10: this mare was blood sampled hourly for 28 hours starting at Day 13 post-ovulation during September 1986.

Mare 11: This mare was blood sampled hourly for 57 hours, starting at Day 55 post-ovulation during May 1987. Day 55 was selected as this would correspond approximately to Day 13 post-ovulation of the third oestrous cycle if a 21 day oestrous cycle is assumed.

Mares 9 and 11 returned to standing oestrus after an uterine biopsy was taken.

Group 3: Mares injected with 'Lutalyse'

Two mares (Mares 12 and 13) received a 1ml intramuscular injection of prostaglandin analog, 'Lutalyse' (5mg/ml) (Upjohn, Ltd). Indwelling cannulae were sutured into place as described above and blood samples were taken every 10 minutes for 30 minutes prior to the injection, then every 5 minutes for 45 minutes immediately after the injection. This was decreased to every 15 minutes for a further hour. A further 2 samples were taken at 4 and 5 hours post injection.

3.2.3 Uterine biopsy technique.

The mare was restrained in stocks. The perineum was washed with warm water and hibitane. The biopsy instrument was lubricated with Lubrel and inserted through the vulvar lips. The cervix was located and the instrument was passed gently into the uterus. The position of the instrument was identified digitally *per rectum.* The cutting edge of the biopsy instrument was placed against

the roof of the uterus and against the hand in the rectum. A small piece of endometrium was removed, placed into Bouin's fixative and stained by H and E.

Blood samples were collected prior to and following biopsy: Mare 9 had blood samples taken 4 times daily until she returned to standing oestrus. The samples were collected by venipuncture from the jugular vein. Mare 11 had an indwelling cannula sutured into place as described above and blood samples were taken every 15 minutes for one hour prior to biopsy. Sampling was increased to every 10 minutes for a period of 3 hours after the biopsy had been taken. Samples were taken every hour for 5 hours after this.

3.2.4 Teasing

as for Chapter 2.

3.2.5 Palpation of the genital tract *per rectum*

as for Chapter 2.

3.2.6 Plasma progesterone estimations

Plasma progesterone concentrations were estimated as for Chapter 2.

3.2.7 Estimation of 15 keto 13,14 dihydro prostaglandin F-2α (PGFM)

The method outlined below was that of Dobson (1983) with modifications by Escreet (1986)

Materials and Methods

1) Assay buffers

0.05M Tris-HCL (pH 7.0) was made up by mixing: 250ml 0.2M Tris (hydroxymethyl) methylamine (BDH Chemicals, Poole, Dorset.) (24.23g Tris/litre H_2O) and 450ml 0.1M Hydrochloric acid (4.364ml conc. HCL/500ml H_2O) and 0.1g sodium merthiolate. Water was added to make the volume up to 1 litre. Tris or HCL was added as necessary to adjust the pH to 7.0.

0.05M Tris-HCL-gel (pH 7.0) was made up by adding: 1g of gelatin to 1 litre 0.05M Tris-HCL (pH 7.0). The gelatin was dissolved by adding 1g of gelatin to a small quantity of Tris HCL and heating until dissolved. This solution was then added to the remainder of the Tris HCL and pH adjusted to 7.0 if necessary. The buffer solutions were stored at 4°C.

2) Radioactive Label.

15-keto 13, 14 dihydro [5,6,8,9,11,12,14,(n)-^{3}H] Prostaglandin F-2α (Amersham International plc, White lion road Amersham.) Specific activity 80 Ci/mmol.

An intermediate solution of radioactive label was prepared by dissolving 25uCi of freeze dried label in 20ml ethanol. This was stored at -20°*C* in a glass stoppered flask. A working solution was prepared by drying down 300ul at 50°*C* under a stream of air, adding 7.5ml Tris HCL gel buffer and vortex mixing to provide 4000c.p.m. in 100ul. This working solution was prepared fresh for each assay.

3) Antiserum.

Prostaglandin antiserum was a gift from Dr M.D. Mitchell (Univ. Texas S.W. Medical School, Dallas, Texas).

1mg freeze-dried antiserum was reconstituted with 5ml Tris-HCL-gel buffer to provide an intermediate dilution of 1:400. This was divided into 400ul aliquots and stored at -20°*C*. A working solution at a dilution of 1:8000 was prepared fresh for each assay by diluting one 400ul aliquot of intermediate solution with 7.6ml Tris-HCL-gel buffer.

The concentration of the working solution had previously been calculated as that which bound approximately 40% of the ^{3}H-PGFM. (Escreet, 1986)

4) PGFM-free plasma (Blank plasma).

Blank plasma was prepared by obtaining plasma from a gelding which was assumed to contain negligible quantities of PGFM (Barnes *et al,* 1978). This plasma was heated in a water bath at 45°*C* for 30 minutes, then stored at -20°*C*.

5) Standards.

A stock solution (1ug/ml) of PGFM in ethanol was prepared and stored at -20°*C*. 50ul of this solution was dried down at 50°*C* in a stream of air, then dissolved in 10ml of blank plasma. Serial dilutions with blank plasma gave standards of 2.0, 3.9, 7.8, 15.6, 31.2, 62.5, 125.0 and 250.0pg/100ul. The standard solutions were stored at -20°*C* and made up as necessary.

6) Controls

Three control plasma samples of high (500pg/ml), medium (250 pg/ml) and low (125 pg/ml) values were produced by adding blank plasma to known quantities of dried down stock PGFM standard solution. The control samples were stored at -20°*C*.

7) Extraction

The PGFM assay was carried out on unextracted plasma. However the samples were heated in a water bath at 45°*C* for 30 minutes to denature the protein, prior to incorporation into the assay.

8) Activated charcoal suspension

0.4g methanol-washed activated charcoal (BDH Chemicals, Poole, Dorset) was mixed with 80ml Tris-HCL buffer for at least one hour at 4°*C* prior to use. The suspension was made up fresh each week and was stored at 4°*C*.

9) Scintillation Fluid.

Ecosinct Fluid (National Diagnostics, Unit 3, Chamberlain Rd., Aylesbury, Buckinghamshire.)

10) Scintillator Vials.

(Canberra Packard, Brook House, Pangbourne, Berkshire.)

11) Assay tubes.

Soda glass 100mm x 16mm

Assay Protocol

The following tubes were set up in duplicate; Total counts (TC): 100ul Tris-HCL-gel buffer, Non-specific binding (NSB): 100ul Blank plasma, Total binding (Bo): 100ul Blank plasma. Standards: 100ul appropriate standard. Controls: High 100ul high control, Medium 50ul high control + 50ul blank plasma, Low 25ul high control + 75ul blank plasma. Unknowns: 100ul unknown sample. To all the tubes (except TC and NSB), 100ul of working antiserum solution was added. To the TC and NSB tubes 100ul Tris-HCL-gel buffer was

added. All the tubes then received 100ul radioactive label, were vortex mixed and incubated at 4°C for between 2 and 24 hours. After incubation 500ul charcoal suspension was added to all the tubes except the TC tubes which received 500ul Tris-HCL gel buffer. The charcoal suspension was added to separate the antibody-bound PGFM from the free PGFM. All the tubes were vortex mixed, incubated at 4°C for 15 minutes, then centrifuged at 1200g;4-8°C for 15 minutes. The supernatant, containing the antibody-bound fraction of the PGFM was then decanted into the scintillation vials and to each vial was added 2ml of scintillant. After thorough mixing the vials were left to equilibrate for at least 10 minutes. Counting was performed using a Tri-Carb 300C Liquid Scintillation System (Canberra Packard, Brook House, Pangbourne, Berks.). Results were determined by computer using a program based on that of Rodbard and Lewald (1970).

Assay Validation

1. Specificity

Cross reactions, calculated by Dr. Mitchell were as follows:

PGFM 100%

15-keto-F-2α 8%

13,14 dihydro F-2α 0.7%

PGF-1α; PGE-2α; PGE-1 0.5% (each)

2. Repeatability of Standard Curve

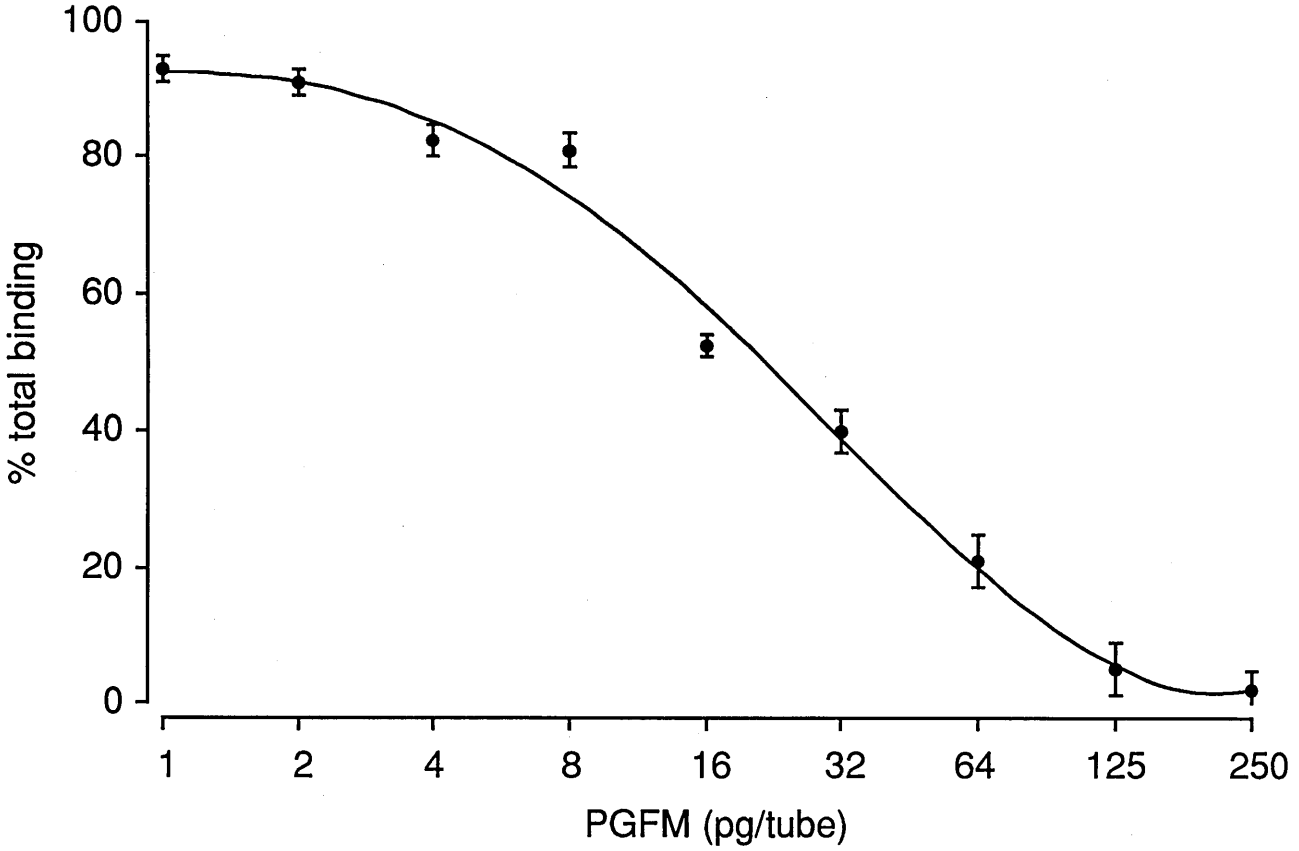
A composite standard curve (Figure 3:1) from 9 assays indicates that, using this method, the standard curve was not highly reproducible. The standard deviations are too great for each of the values of standard samples.

3. Precision

The intra-assay coefficient of variation (CV) was calculated using the equation:-

$$CV = SD \times \frac{100}{x}$$

Figure 3.1
PGFM standard curve in plasma 1st assay method



Where x = the mean concentration of randomly selected duplicate pairs of samples and the standard deviation (SD) between the duplicate pairs from 9 assays was calculated using the equation:

$$SD = \sqrt{\frac{\Sigma d^2}{2n-1}}$$

d = difference between 2 values of each duplicate pair n = the number of duplicate pairs. The intra-assay CV was 24% (n=45) for values < 50pg/ml. The inter-assay coefficient was calculated using the equation:

$$CV = SD \times \frac{100}{x}$$

where x = the mean concentration of randomly selected duplicate pairs of samples. SD = standard deviation. The inter-assay CV was 37% for a mean value of 412pg/ml (3 assays). 34% for mean values of 200pg/ml (9 assays) and 21% for mean values of 120pg/ml (9 assays).

4. Sensitivity

The limit of sensitivity of the assay, defined as the value at twice the standard deviation of blank values, was 6.0pg/tube, equivalent to 60pg/ml at a sample volume of 100ul.

5. Accuracy and Parallelism

Accuracy and Parallelism were not calculated for this assay as it was not used for the bulk of the work in this chapter.

Authors modifications to the assay described by Escreet

Materials and Methods

1) Assay buffers

The assay buffers used in the modified assay were the same as those in the first method except phosphate buffered saline with 1% added bovine serum albumin (PBS) was used. Phosphate buffered saline Mix: 4.0g NaCl, 0.1M Na $H_2 PO_4$, 0.1M $Na_2 H PO_4$, 0.5g Thiomersal and make up to 5 litres with distilled water. Adjust the pH to 7.4.

2) Radio-active label

The intermediate stock solution of radio-active label was made up as in the first method. However the working solution was prepared by drying down 600ul of intermediate stock solution at 50°*C* under a stream of air and adding 7.5ml Tris-HCL-gel buffer to provide 8000c.p.m. In 100ul. This volume of working solution was prepared fresh for each assay.

3) Antiserum

Prostaglandin antiserum was obtained from Steranti Research Ltd, London Rd, St. Albans, Herts. 1mg freeze-dried antiserum was reconstituted in 1ml Tris-HCL buffer to provide a stock solution at a dilution of 1:100. This was stored at -20°*C*. A working solution was prepared fresh for each assay by adding 10ml of Tris-HCL buffer to 100ul antiserum stock solution to give a working concentration of 1:10,000. The concentration of the working solution was determined by incubating various dilutions of antiserum with a fixed mass of radio-active labelled PGFM. The dilution of the working solution was that which bound approximately 50% ^{3}H-PGFM (Figure 3:2).

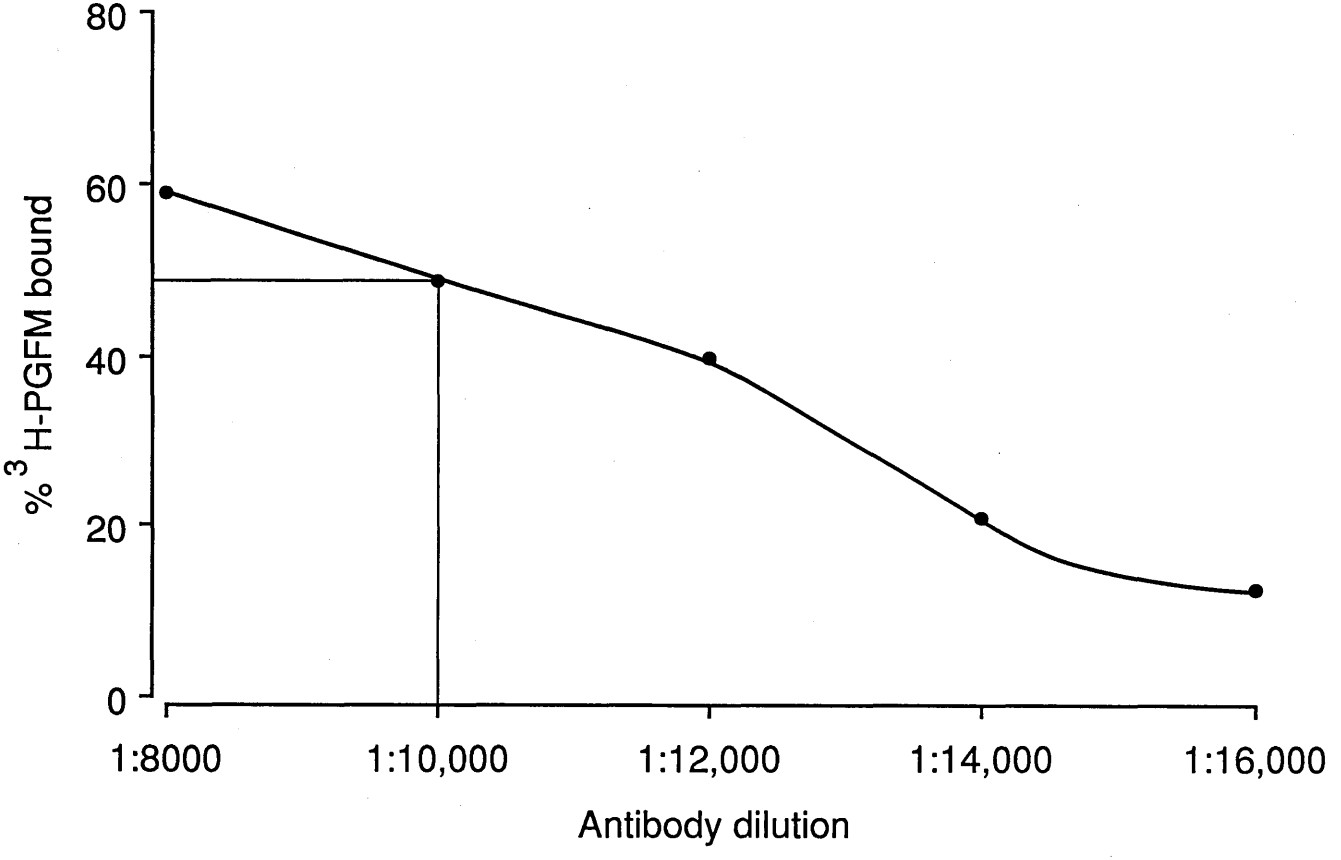
4) PGFM-free plasma (Blank plasma)

Blank plasma was prepared by obtaining plasma from a gelding which was assumed to have negligible quantities of PGFM. However to remove all PGF-2α and any non-specific binding which may lead to interference, the plasma was stripped. The plasma was mixed with 10g unwashed charcoal overnight at 4°*C*. The suspension was spun at 1200g for 1 hour and passed through a series of millipore filters, 7, 5, 2 and 0.45 um, to remove any remaining charcoal.

5) Standards

The stock solution of standards was as for the first method. 50ul of this solution was dried down at 50°*C* in a stream of air, then dissolved in 10ml PBS buffer. Serial dilutions with PBS buffer gave standards of 2.0, 3.9, 7.8, 15.6, 31.2, 62.5, 125.0 and 250.0pg/100ul. The standard solutions were stored at -20°*C* and made up as necessary.

Figure 3.2
PGFM antibody dilution curve (Steranti antiserum)



6) Controls

Three controls of high (500pg/ml), medium (250pg/ml) and low values (125pg/ml) were produced by adding PBS buffer to known quantities of dried-down stock PGFM standard solution. The controls were stored at -20°*C*.

7) Extraction

Extraction was carried out by adding 2ml diethyl ether to the unknown plasma samples. The samples and controls were vortex mixed for 10 minutes and spun down at 1200g for 15 minutes. The pellet was frozen by immersing the tube into methanol containing cardice and the supernatant was decanted into fresh assay tubes. The supernatant was dried down in a stream of air at 50°*C* and the tubes included in the assay procedure.

Percentage extraction was calculated by setting up 2 assay tubes with 20ul working radio-active label added to 100ul PBS buffer. This was vortexed for 10 seconds and incubated at room temperature for 30 minutes. Ether extraction, as described above was carried out on the tubes. No radio-active label or antibody was added and the tubes did not undergo the separation procedure. 1.1ml tris-HCL-gel buffer was added to the assay tubes.

Assay Protocol

The following tubes were set up in duplicate; Total counts (TC): 100ul PBS buffer, Non-specific binding (NSB): 100ul PBS buffer, Total binding (Bo): 100ul PBS buffer, Percentage Extraction (EXT): as above. Standards: 100ul appropriate standard

Controls: High 100ul high control, Medium 100ul medium control and Low 100ul low control. Unknowns: 100ul unknown sample. The plasma of all the unknown samples was extracted as described above. The assay procedure was then the same as that of the first method except the incubation time of the samples, antiserum and radio-active label was increased from 2-24 hours to 30-48 hours.

Assay Validation

1. Specificity

Cross reactions, calculated by Steranti Ltd., were as follows:

PGFM 100%

15-keto-F-2α 4.2%

PGF-2α; PGF-1α; PGE-2; PGEM; PGE-1

PGA-1; PGA-2; PGB-2; PGD-2; 15-ketoE-2 0.01% (each)

2. Repeatability of Standard Curve

A composite standard curve (Figure 3:3) from 15 assays indicates that the curve was highly reproducible using the modified assay technique.

3. Precision

The intra-assay coefficient of variation calculated as described previously was calculated to be 4% (n=76) for values <150pg/ml.
The inter-assay coefficient of variation calculated as described previously was calculated to be 16% for a mean value of 542pg/ml (15 assays). 18% for mean values of 200pg/ml (15 assays) and 14% for mean values of 112pg/ml (15 assays).

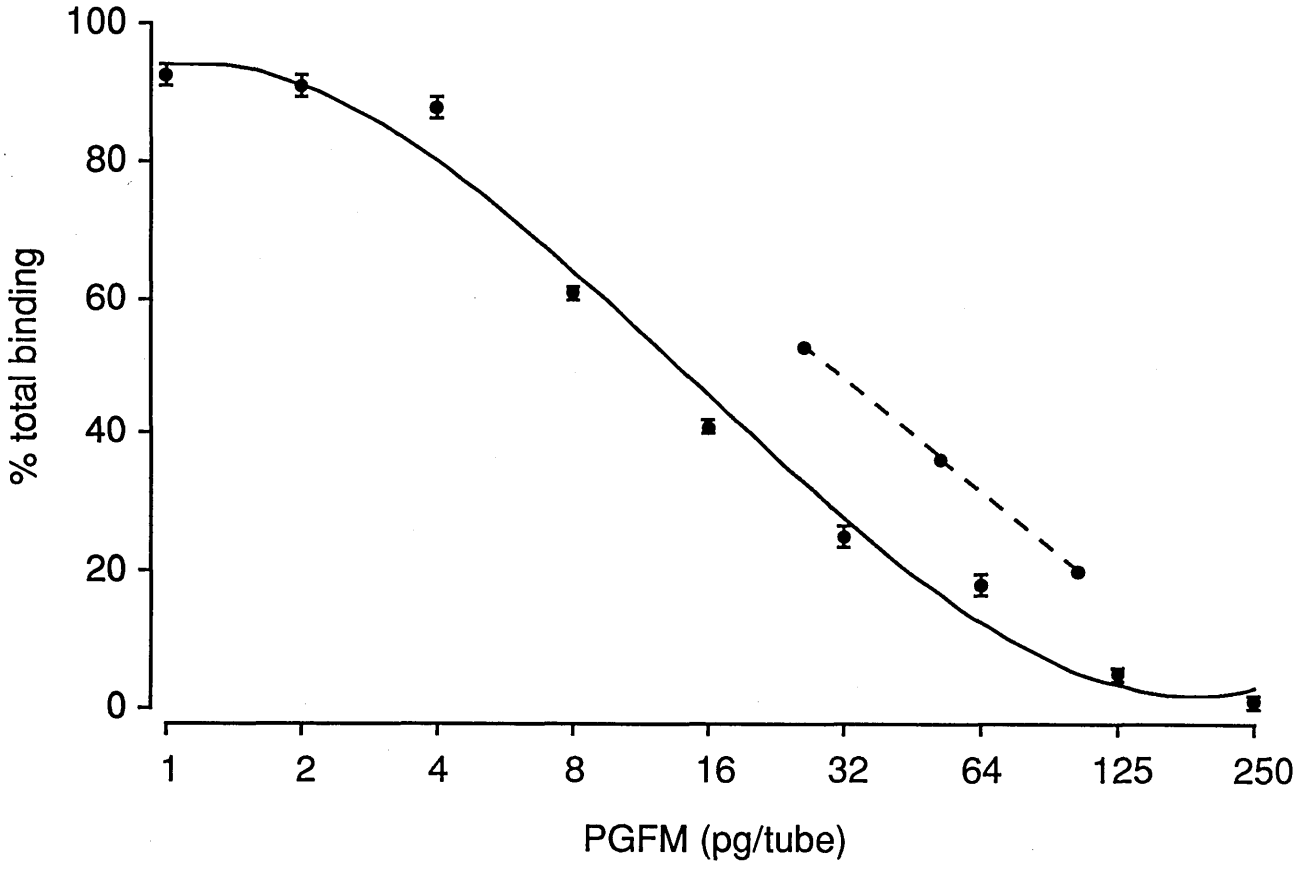
4. Sensitivity

The limit of sensitivity of the assay defined as the value at twice the standard deviation of blank values was 1.5pg/tube, equivalent to 15pg/ml at a sample volume of 100ul.

5. Accuracy

For an assessment of accuracy, known amounts of PGFM (between 2-250pg/ml) were added to plasma samples and included in several assays. After adjustment for PGFM concentration pool samples, the mean recovery figure +/- SEM was 113% +/- 8%.

Figure 3.3
PGFM standard curve in PBS buffer and parallelism curve



6. Parallelism

The standard curve was made up in PBS buffer and a curve of samples, measured at different dilutions appeared to be parallel (Figure 3:3).

7. Percentage Extraction

The percentage extraction of the samples was calculated to be 95 +/- 6%.

3.2.8 Prostaglandin F-2α (PGF-2α) estimations

A liquid phase radio-immunassay was established for the determination of PGF-2α in the supernatant of incubated tissue samples and in the circulating plasma from mares.

1) Assay buffers

i) 0.05M Tris-HCL (pH 7.4) prepared as described for PGFM assay

ii) Tris-HCL-gel buffer (pH 7.4) prepared as described for PGFM assay. The buffer solutions were made up as required and stored at -20°*C*.

2) Radioactive label (tracer).

[5,6,8,9,11,12,14,15,(n-^{3}H) Prostaglandin F-2α (Amersham International plc, Amersham, England. Specific activity 160-180 Ci/mmol.

An intermediate solution was prepared by mixing 25uCi of freeze-dried label with 25ml ethanol. This stock solution was stored at -20°*C*. A working solution was prepared fresh for each assay by drying down 500ul under a stream of air at 50°*C*. 5ml of Tris-HCL-gel buffer was added to provide 9000cpm in 50ul.

3) Antiserum.

Prostaglandin F-2α antiserum was donated by Dr. N.L. Poyser, Pharmacology Dept., Edinburgh University, Edinburgh, Scotland.

Aliquots of antiserum, at a dilution of 1:120 were stored at -20°C. A working solution was prepared fresh for each assay by adding 100ul of stock solution to 10ml Tris-HCL-gel buffer to give a final working solution of 1:12,000. An antibody dilution curve is demonstrated in Figure 3:4

4) Standards

1mg prostaglandin F-2 α (Sigma, Poole) was reconstituted with 1ml Tris-HCL-gel buffer and stored at -20°C. 41.5ml of Tris-HCL-gel buffer was added to 0.1ml of this solution to make up a stock solution which was stored at -20°C. A starting concentration of 2400pg/0.1ml was prepared by adding 100ul of stock to 10ml Tris-HCL-gel buffer. Serial dilutions with Tris-HCL-gel gave the following standards:

S6 2400pg/0.1ml Starting conc.
S5 800pg/0.1ml 1:3 dilution
S4 267pg/0.1ml 1:9 "
S3 89pg/0.1ml 1:27 "
S2 29.6/0.1 ml 1:81 "
S1 10pg/ml0.1ml 1:240 "
Standards were stored at -20°C.

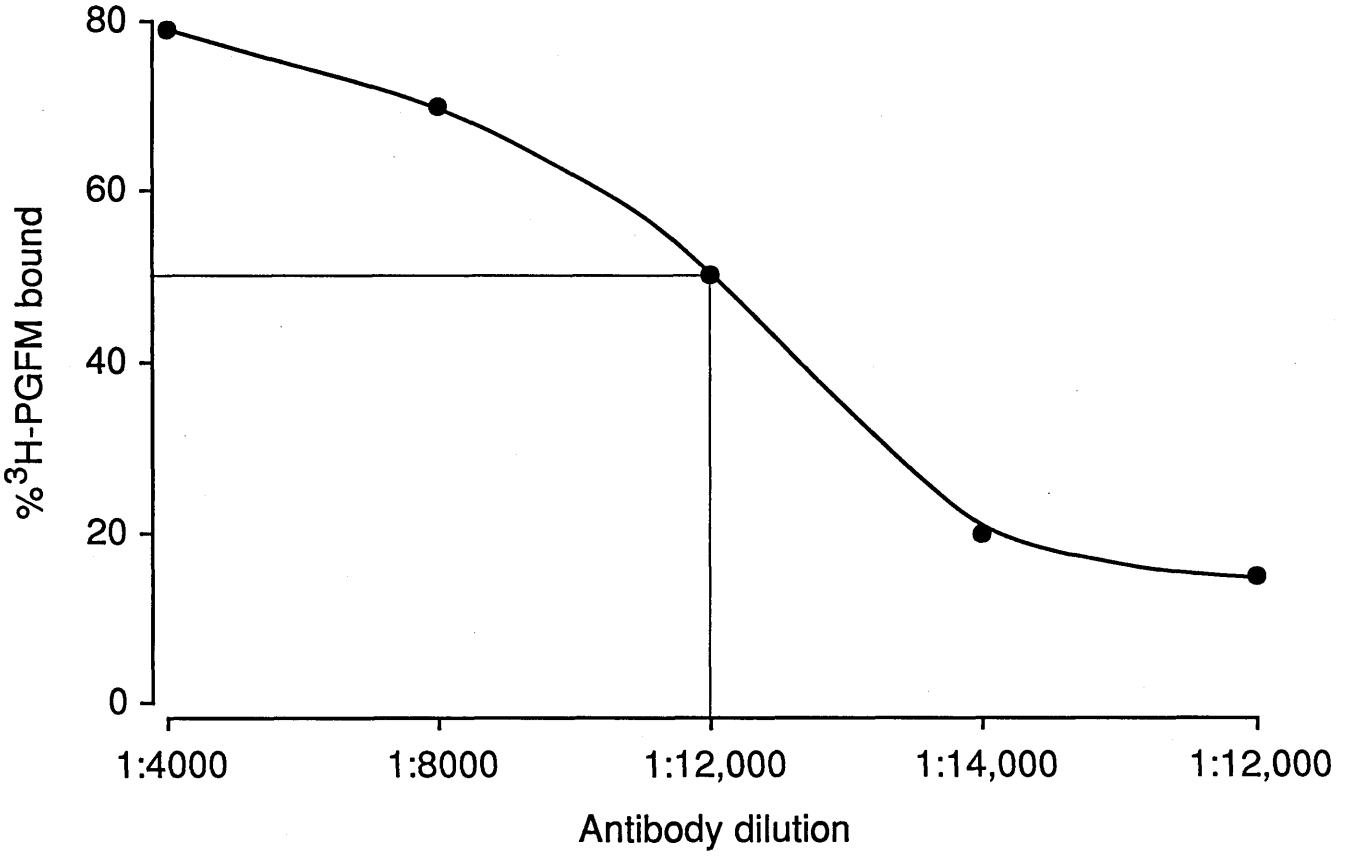
5) Controls

Three control samples of high (800pg/ml), medium (400pg/ml) and low (200pg/ml) were prepared by adding known quantities of PGF-2α stock to DMEM culture medium.

6) Extraction

No extraction was carried out on the samples. The assay was carried out directly on the tissue culture medium and the plasma samples. However the tissue culture samples were exposed to three cycles of freezing and thawing by placing the sample tubes in liquid nitrogen until frozen followed by immersion in warm water until thawed. This disrupted the cell membrane releasing any PGF-2α contained within the cells. The cell debris was removed by gentle spinning at 500g for 2 minutes.

Figure 3.4
PGF-2α antibody dilution curve



7) Separation

Separation of the antibody bound PGF-2α from the free PGF-2α was carried out using donkey anti-rabbit IgG precipitating serum and normal rabbit serum (SAPU, Carluke, Scotland). A working solution was prepared fresh for each assay by adding 0.2ml (1:20 dilution) anti-rabbit serum an 50ul (1:200) normal rabbit serum, to 5ml Tris-HCL-gel buffer.

8) Scintillation Fluid

As for PGFM assay

9) Scintillation Vials

As for PGFM assay

10) Assay tubes

Plastic LP3 tubes (Luckhams Ltd, Victoria Gdns, Burgess Hill, West Sussex).

Assay Protocol

The following tubes were set up in duplicate: Total counts (TC): 0.6ml Tris-HCL-gel buffer. Non-specific binding (NSB): 0.6ml Tris-HCL-gel buffer. Total binding (Bo): 0.6ml Tris-HCL-gel buffer. Standards: 0.5ml Tris-HCL-gel buffer. Controls: High 0.5ml Tris-HCL-gel buffer, Medium 0.5ml Tris-HCL-gel buffer and Low 0.5ml Tris-HCL-gel buffer. Unknowns: 0.4ml Tris-HCL-gel buffer. To each of the appropriate tubes was added: 100ul of each PGF-2α standard; the appropriate volume of sample and control to bring the total volume up to 0.6ml. To all the tubes 50ul of working tracer was added. All the tubes (except TC and NSB) received 50ul of working anti-serum and were mixed thoroughly on a vortex mixer. All the tubes were incubated at room temperature for a minimum of 60 minutes. After incubation 100ul of the working solution of second antibody was added to each of the tubes, except the TC tubes, to separate the antibody-bound PGF-2α from the free PGF-2α and the tubes were mixed thoroughly using a vortex mixer. All the tubes were then covered and incubated for 18-20 hours at 2-8°C. After this second incubation the tubes were centri-

fuged at 1200g for 30 minutes at 2-8°C. The supernatant of each tube, except TC, was decanted and the tubes allowed to drain onto absorbent paper, to remove any residual supernatant, leaving a precipitate containing the antibody-bound fraction. 1.0ml of 0.1M NaOH was added to all the tubes including the TC tubes and the tubes were vortex mixed to dissolve the precipitate. The solution was then decanted into scintillation vials and 10-11mls scintillation fluid was added to each vial. The vials were then counted in a Tri-Carb 300C Liquid Scintillation System. Results were determined using the programme used for the PGFM assay.

Assay Validation

1. Specificity

Cross-reactions determined by Dr. Poyser were as follows:

PGF-2α	100%
PGF-1α	100%
PGE-2	1.21%
PGE-1	0.52%
PGA-2	< 0.056%
PGB-2	0.0016%
PGFM	0.56%
PGF-2	0.198%
PGD-2	17.1%

2. Repeatability of Standard Curve

A composite standard curve (Figure 3:5) from 20 assays indicates that the curve was highly reproducible.

3. Precision

The intra-assay coefficient of variation (CV) and the inter-assay CV were calculated as for the PGFM assay. The intra-assay CV for this assay was calculated at 10% (n = 75). The inter-assay CV was calculated at 16% for a standard mean value of 852pg/ml (n = 12), 15% for a standard mean value of 378pg/ml (n = 12) and 13% for a standard mean value of 218pg/ml (n = 12).

4.Sensitivity

The limit of sensitivity of the assay defined as the value at twice the standard deviation of blank values, was 8.0pg/tube, equivalent to 40pg/ml at a sample volume of 200ul.

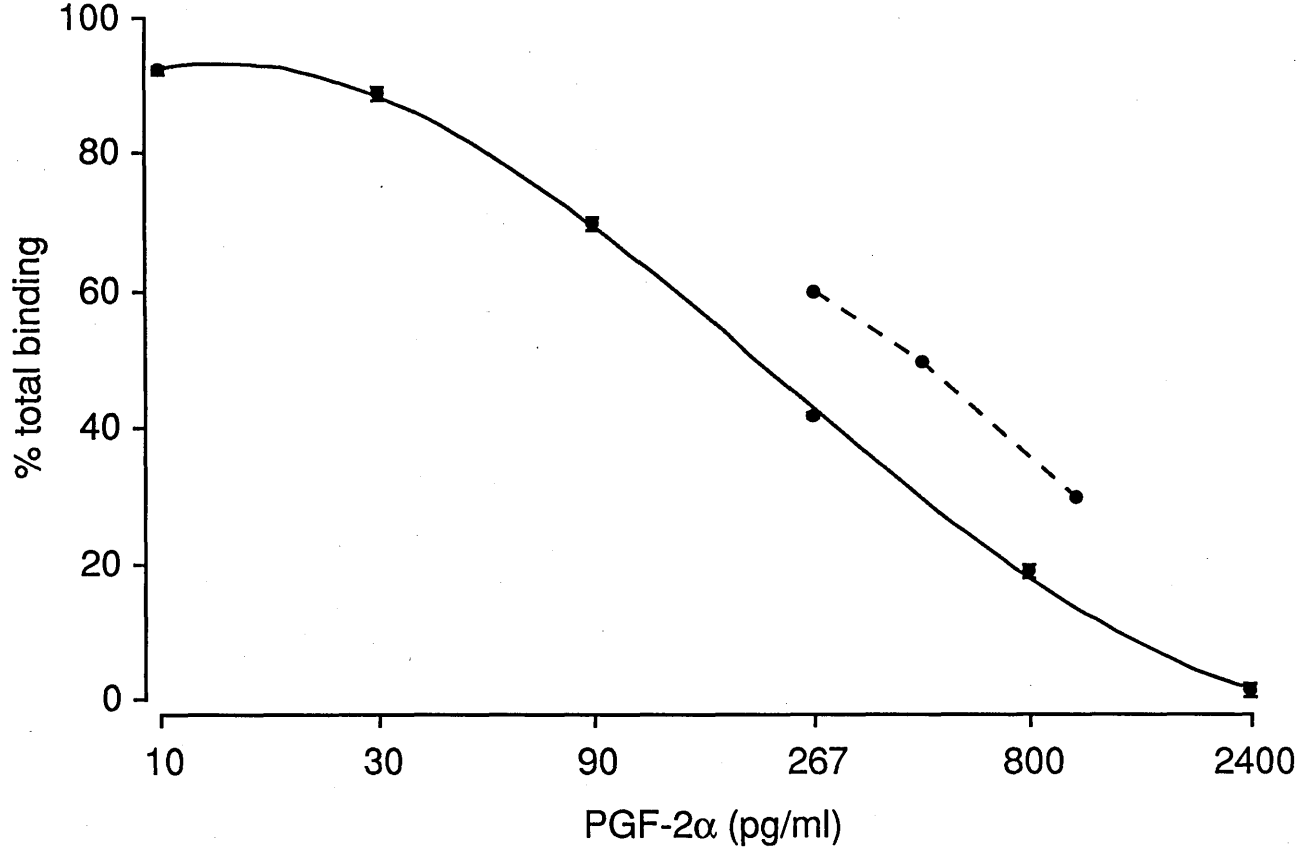
5. Accuracy

For an assessment of accuracy, known amounts of PGF-2α (between 10-240pg/ml) were added to samples and included in several assays. After adjustments for PGF-2α concentrations in pool samples the mean recovery figure +/- SEM was 105% +/- 10%.

6. Parallelism

The standard curve was made up in Tris-HCL-buffer and a curve of samples, measured at different dilutions appeared to be parallel (Fig 3:5)

Figure 3.5
PGF-2α standard curve + parallelism curve



3.3 RESULTS

The results are presented in two sections. The first deals with the results of the experiments to validate the assay developed to measure PGFM. The second relates to the measurement of PGFM in the three groups of mares defined in the materials and methods.

3.3.1 Assay to determine PGFM

Table 3:2

Comparisons of PGFM assay method 1 and PGFM assay method 2 after modifications were incorporated. The percentage binding in assay method 1 was very low. Control values did not determine the correct concentration of PGFM. The unknown sample concentration varied markedly between the 2 assay methods.

Table 3:2

	PGFM ASSAY METHOD 1	PGFM ASSAY METHOD 2
TOTAL COUNTS (cpm)	3781	7320
PERCENTAGE BINDING	28%	48%
CONTROL SAMPLE VALUES		
HIGH	338	450
MEDIUM	876	225
LOW	0	112
UNKNOWN SAMPLE VALUE (cpm)		
1	6	64
2	5	44
3	3	72
4	0	0
5	0	32
6	41	280
7	4	30
8	0	18
9	0	0
10	25	10

Table 3:3

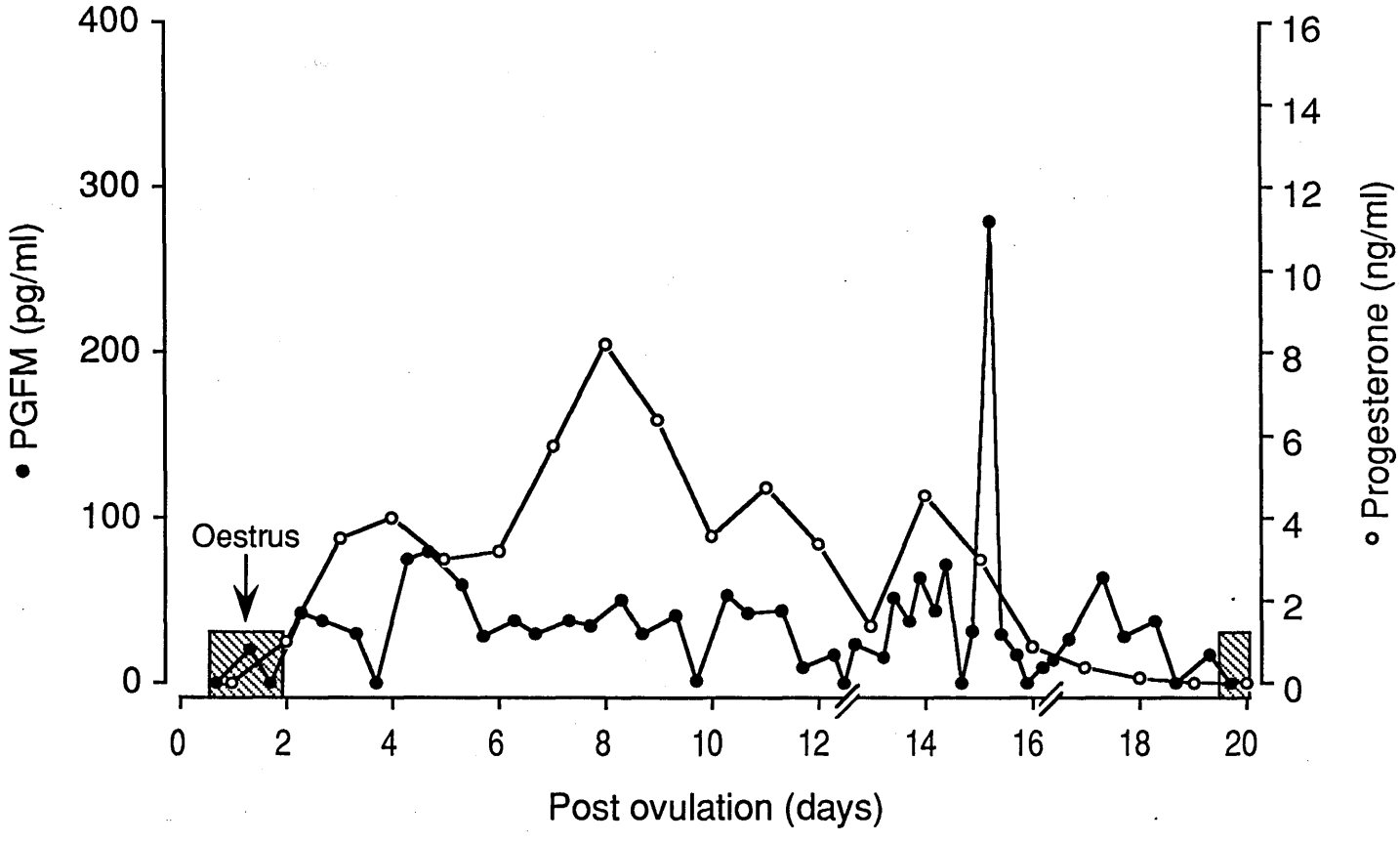
Effect of varying the incubation time on the percentage binding. Serial assays were set up and incubated for varying times. The percentage binding of each was calculated. The optimum binding of 50% was obtained after 46 hours incubation.

TIME (hours)	PERCENTAGE BOUND
24	38%
28	39%
30	46%
36	48%
46	50%
47	51%
48	53%
50	48%
55	46%

3.3.2 Group 1 Mares (Normal)

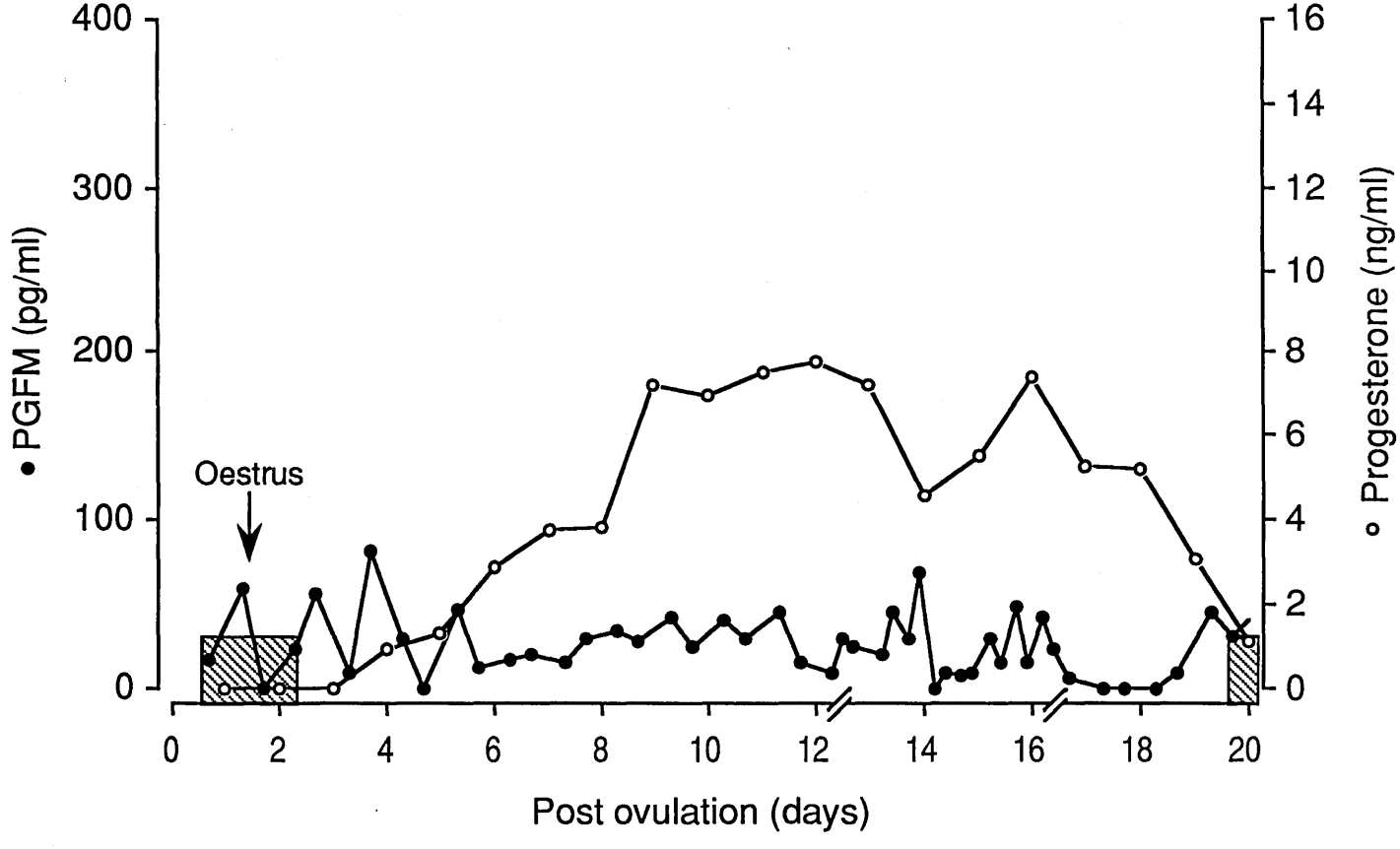
Mare 1 The circulating plasma PGFM and progesterone concentrations during one oestrous cycle of Mare 1 are shown in **Figure 3:6.** As can be seen PGFM concentrations fluctuated throughout the sampling period. One significant pulse was recorded on Day 15. This pulse occurred after plasma progesterone had begun to decline, prior to the onset of standing oestrus.

Figure 3.6
Circulating plasma PGFM and progesterone concentrations in daily blood samples taken from mare 1 during one inter-oestrous period



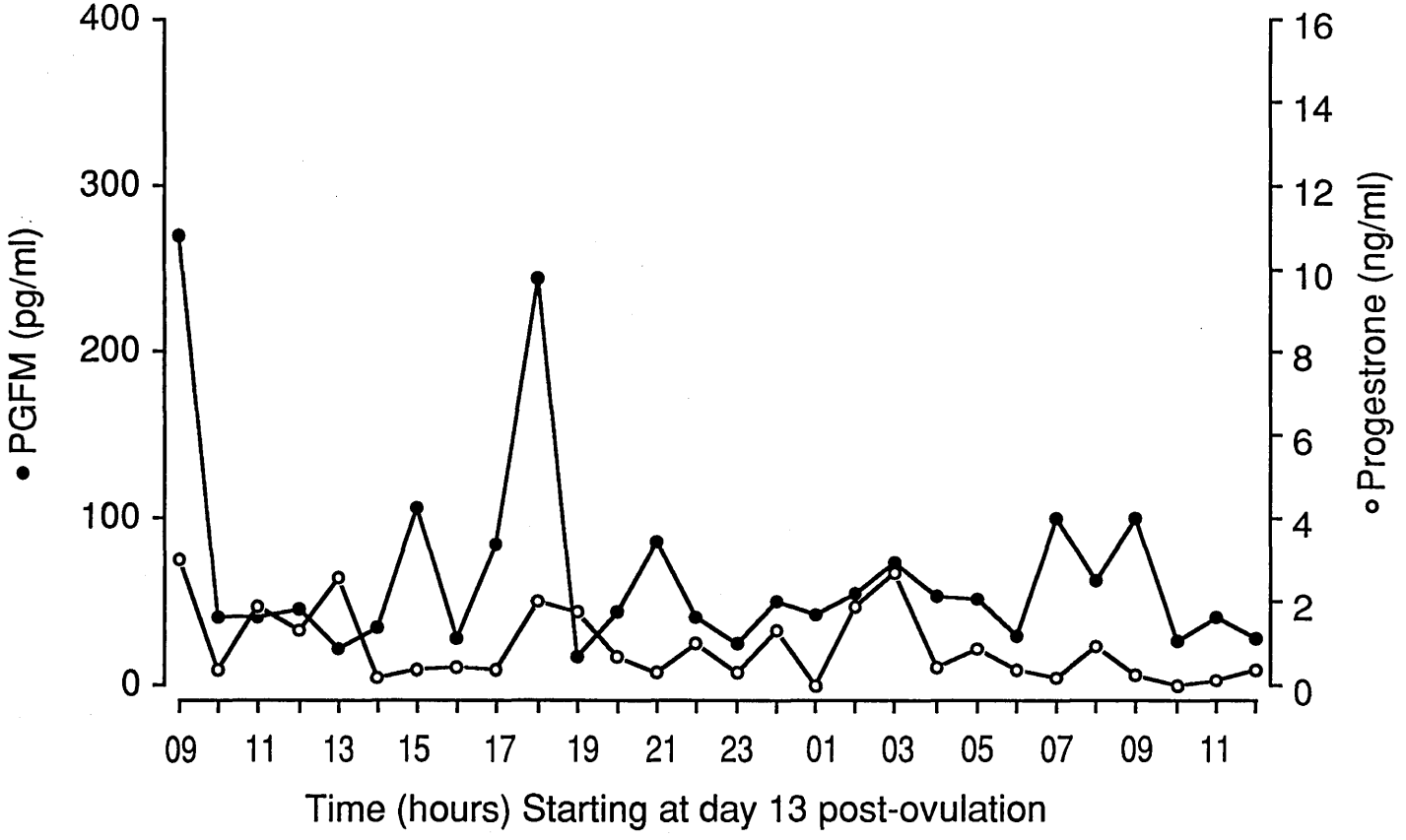
Mare 2 Plasma PGFM concentrations did not rise above 98pg/ml throughout the sampling period, although progesterone declined to baseline prior to the expression of standing oestrus. **[Figure 3:7]**

Figure 3.7
Circulating plasma PGFM and progesterone concentrations in daily blood samples taken from mare 2 during one inter-oestrous period



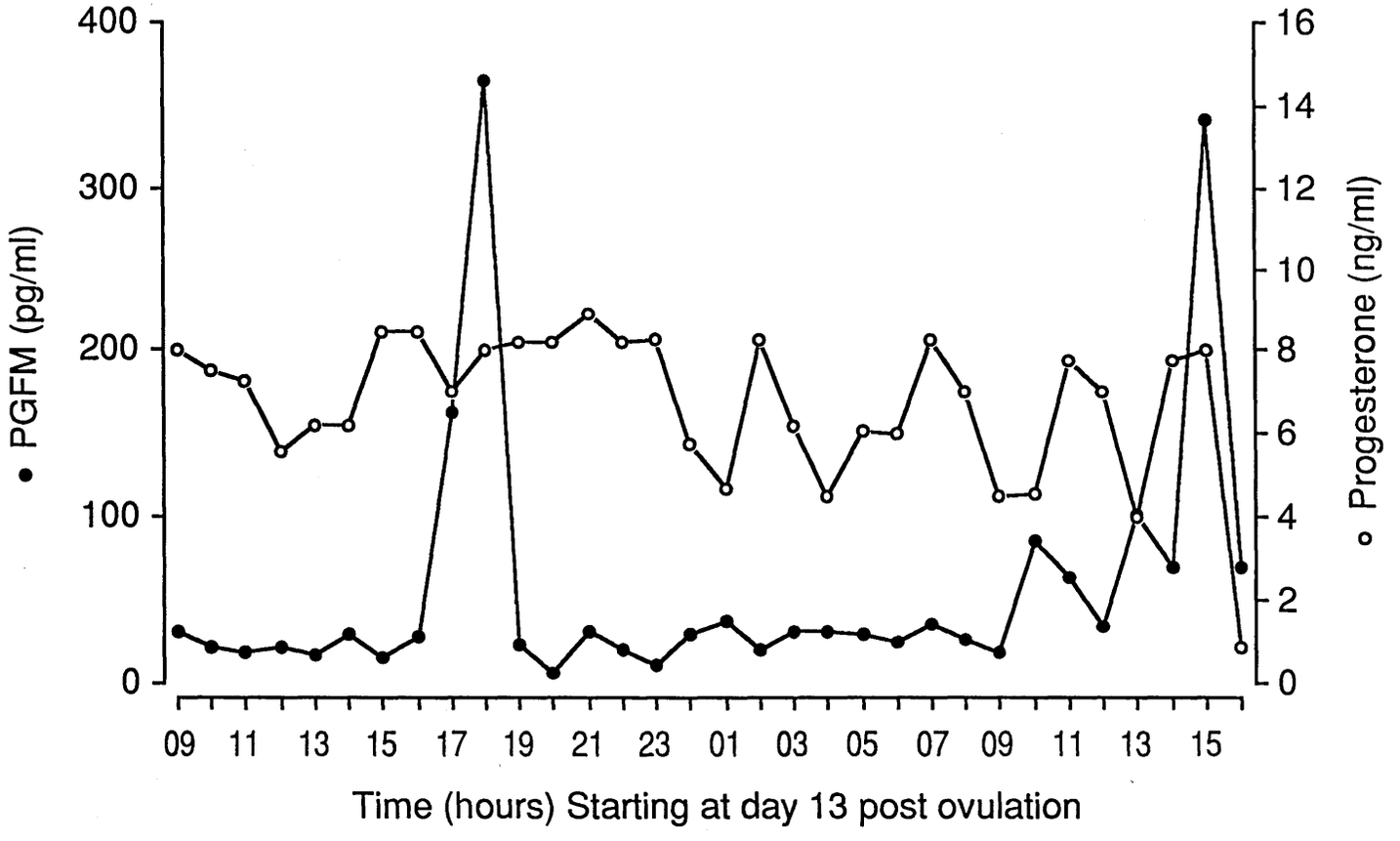
Mare 3 There was a pulsatile pattern of PGFM release with 4 pulses of PGFM observed within the first 14 hours of the sampling period (2 pulses of 250pg/ml and 2 of 100pg/ml). Plasma progesterone had already reached baseline prior to the initiation of blood sampling. The mare returned to standing oestrus 4 days after the end of the sampling period. **[Figure 3:8]**

Figure 3.8
Circulating plasma PGFM and progesterone concentrations in hourly blood samples from mare 3 at the expected time of luteolysis



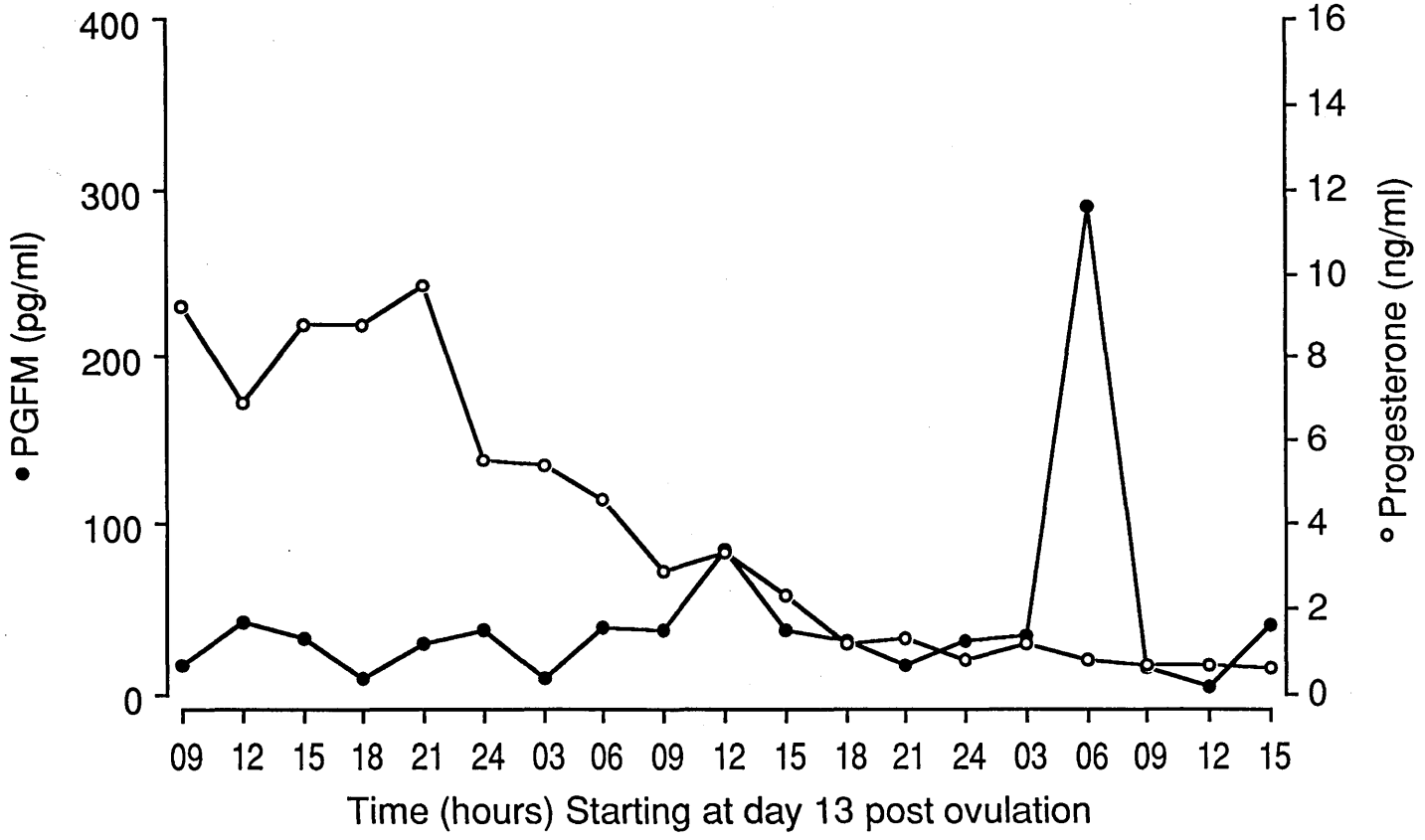
Mare 4 Two pulses of PGFM were recorded, 21 hours apart. In this mare progesterone remained high until after the second large pulse of PGFM when progesterone dropped abruptly to baseline. The mare returned to standing oestrus 6 days after the end of the sampling period. **[Figure 3:9]**

Figure 3.9
Circulating plasma PGFM and progesterone concentrations in hourly blood samples taken from mare 4 at the expected time of luteolysis



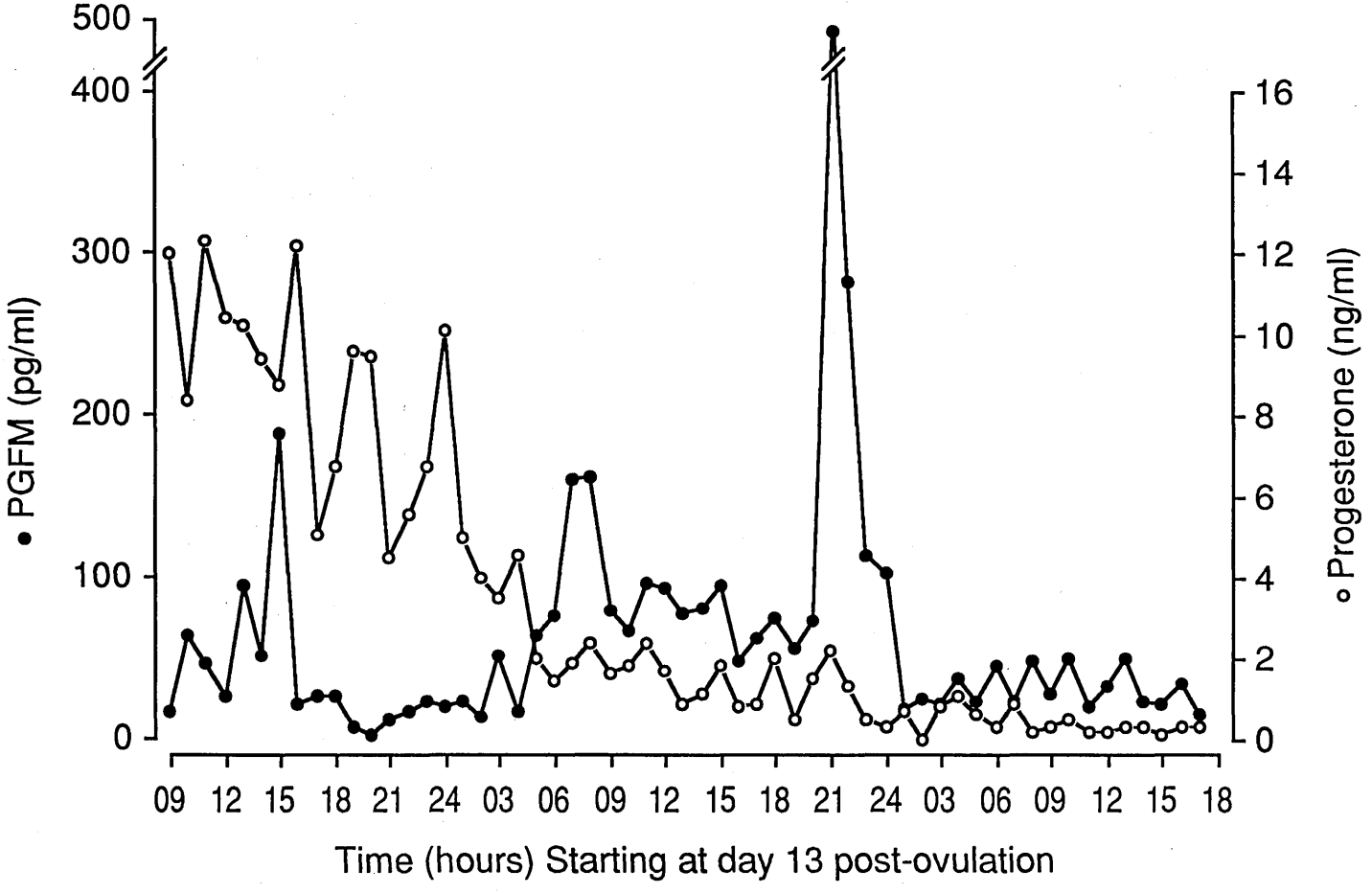
Mare 5 This mare was blood sampled once every 3 hours for 57 hours starting at Day 13 post-ovulation. Plasma progesterone concentrations declined from dioestrous concentrations to baseline during the sampling period. Only one PGFM pulse of real significance was recorded in this mare. This was observed in the last 24 hours of sampling and occurred after progesterone had declined. No pulses of PGFM were recorded prior to, or during, the drop in progesterone concentrations. This mare returned to standing oestrus 4 days after the end of the sampling period. **[Figure 3:10]**

Figure 3.10
Circulating plasma PGFM and progesterone concentrations in 3 hourly blood samples taken from mare 5 over a three day period at the expected time of luteolysis



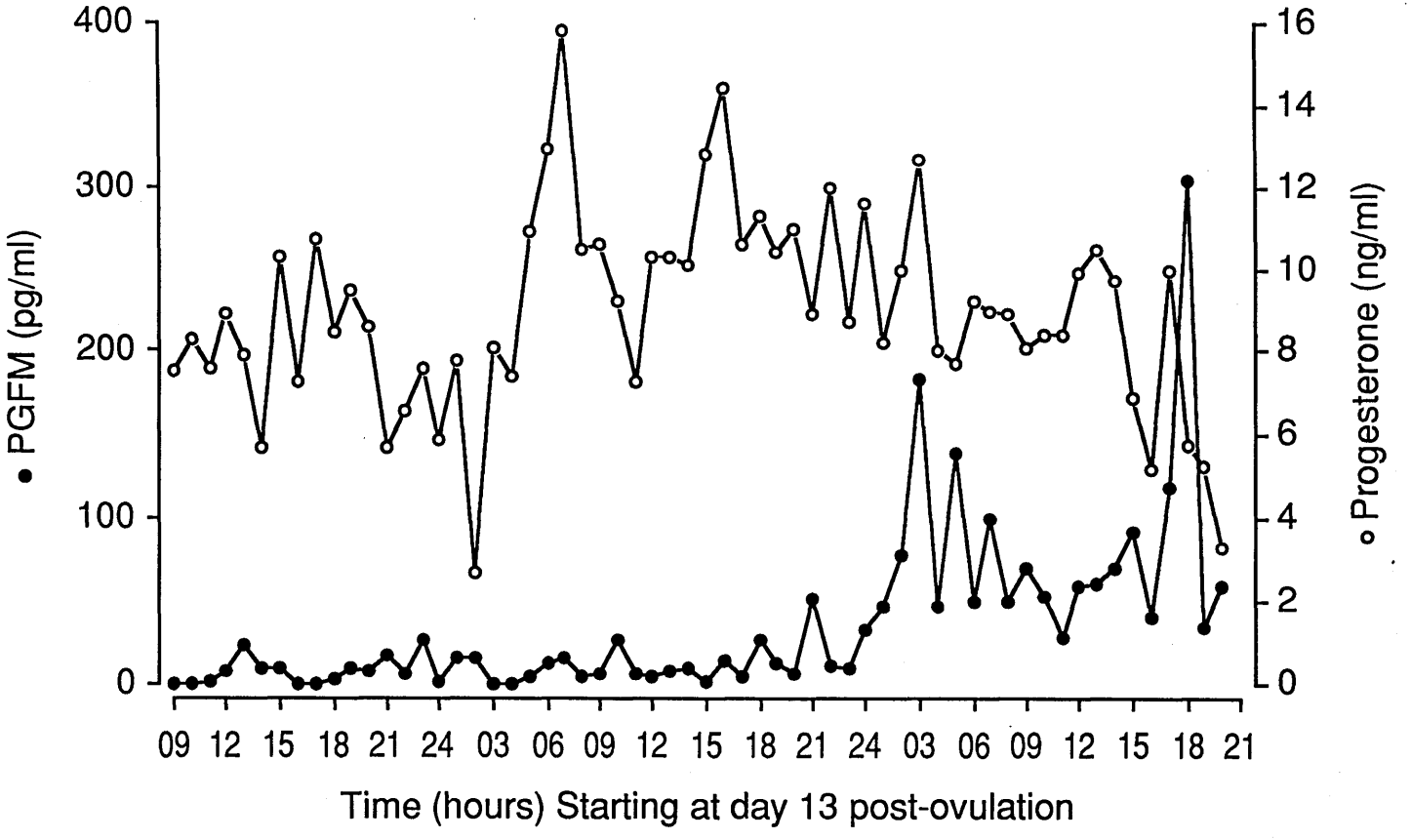
Mare 6 Plasma PGFM demonstrated three major pulses, 17 and 15 hours apart with several smaller pulses of rapid frequency. **[Figure 3:11]** The final pulse of PGFM was the largest pulse recorded. Progesterone concentrations dropped markedly 12 hours after the first pulse of PGFM and continued to drop to baseline during the rapid pulsatile PGFM release. The mare returned to standing oestrus 4 days after the end of the sampling period.

Figure 3.11
Circulating plasma PGFM and progesterone concentrations in hourly blood samples taken from mare 6 over a three day period at the expected time of luteolysis



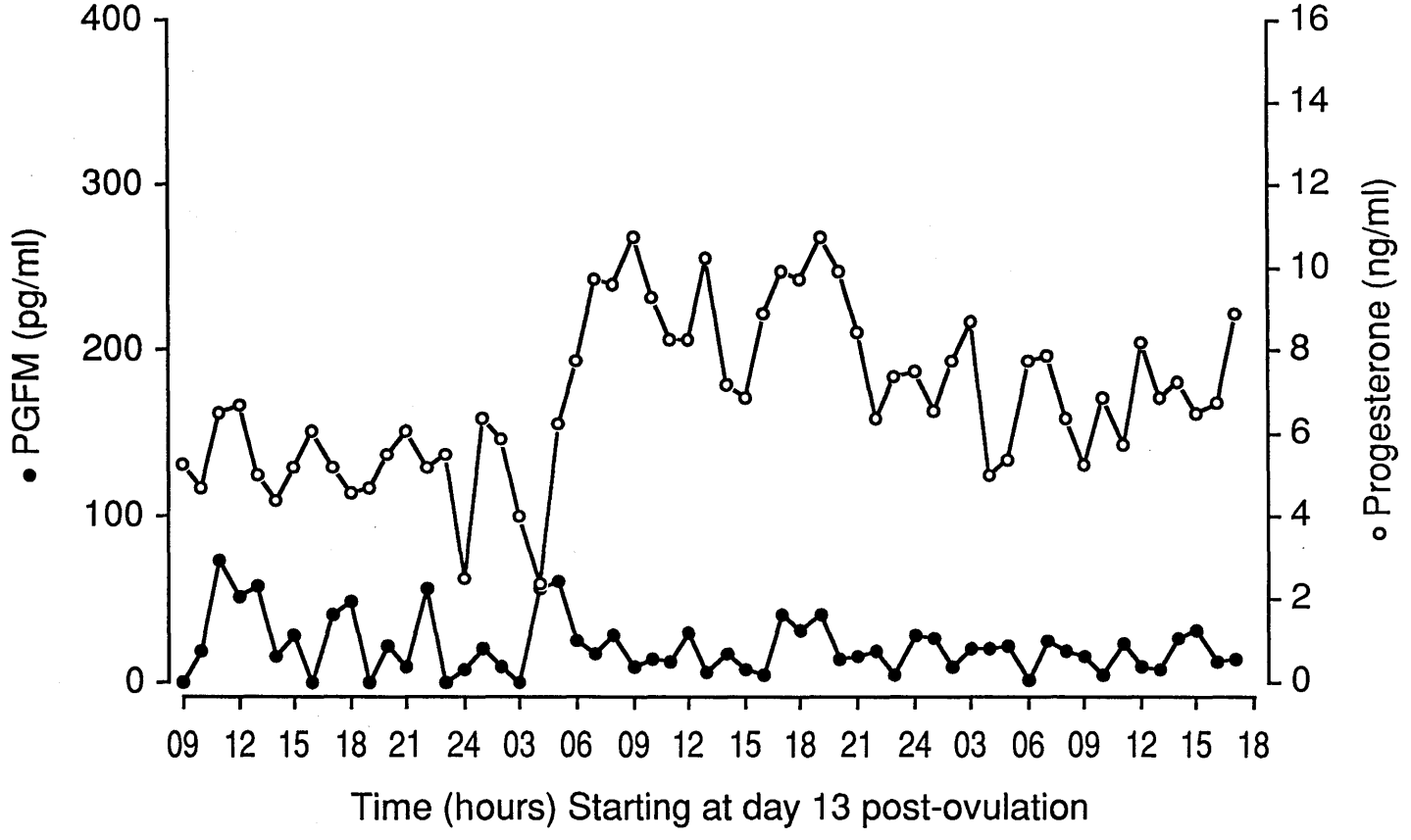
Mare 7 One large pulse of PGFM (183 pg/ml) was followed by a series of smaller but rapid pulses. **[Figure 3:12]** During this pulsatile release a decline in progesterone was observed. Progesterone appeared to recover after this but dropped rapidly to baseline as further pulses of PGFM occurred. The mare returned to standing oestrus 4 days after the end of the sampling period.

Figure 3.12
Circulating plasma PGFM and progesterone concentrations in hourly blood samples taken from mare 7 over a three day period at the expected time of luteolysis



Mare 8 Pulses of PGFM were not observed to increase above 70 pg/ml in this mare. Progesterone remained high throughout the sampling period. **[Figure 3:13]** This mare returned to standing oestrus 6 days after the end of the sampling period.

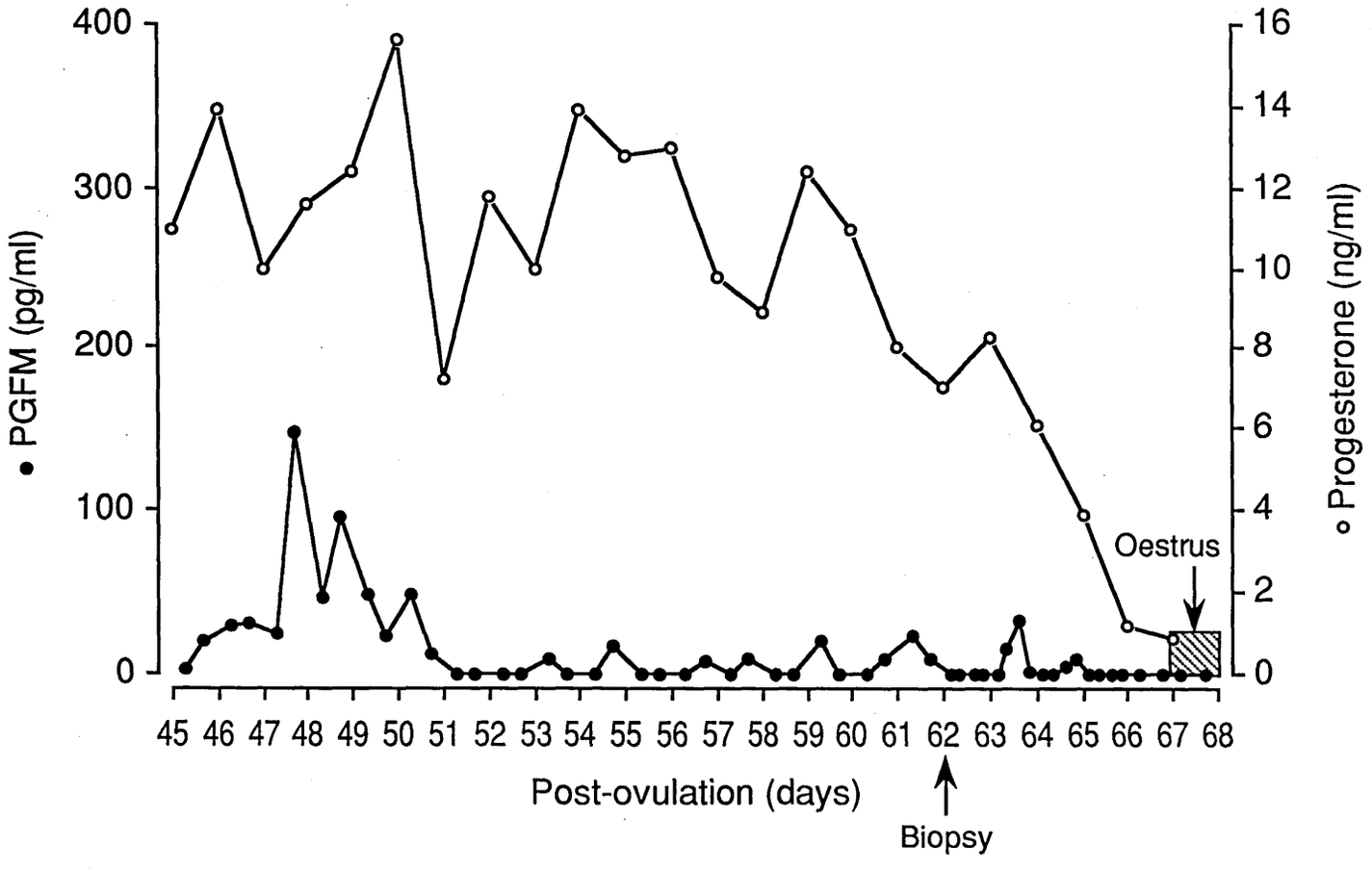
Figure 3.13
Circulating plasma PGFM and progesterone concentrations in hourly blood samples taken from mare 8 over a three day period at the expected time of luteolysis



3.3.3 Group 2 Mares (Prolonged dioestrus)

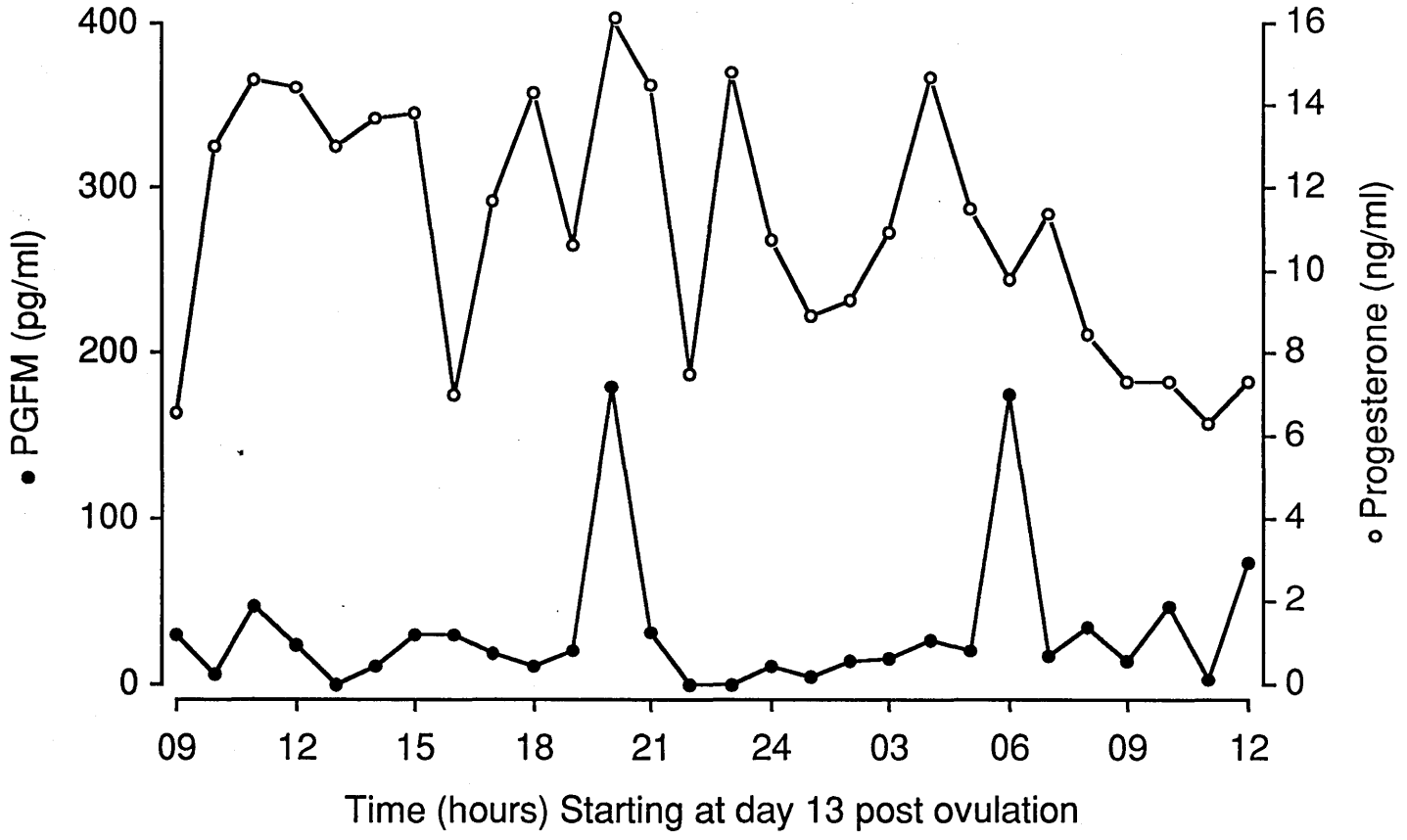
Mare 9 Two pulses of PGFM were observed during the sampling period, starting at Day 45 post-ovulation in a mare in prolonged dioestrus. **[Figure 3:14]** The pulses did not exceed 150 pg/ml and were not associated with a drop in progesterone concentrations. On Day 62 post-ovulation a uterine biopsy was taken and the mare returned to standing oestrus 5 days after the biopsy had been taken. No significant pulses of PGFM were detected in the plasma after the biopsy.

Figure 3.14
Circulating plasma PGFM and progesterone concentrations in daily blood samples from mare 9 in prolonged dioestrus



Mare 10 Two pulses of PGFM 8 hours apart were recorded during the sampling period, in a mare which had been cycling normally prior to the start of the study. **[Figure 3:15]** Neither of the pulses reached a magnitude greater than 180pg/ml. The second pulse appeared to be associated with a slight decrease in progesterone concentrations which did not reach baseline. This mare remained in a state of prolonged dioestrus for a period of 3 months before returning spontaneously to standing oestrus.

Figure 3.15
Circulating plasma PGFM and progesterone concentrations in hourly blood samples taken from mare 10 at the expected time of luteolysis



Mare 11 The circulating plasma progesterone and PGFM during a 57 hour sampling period is shown in **Figure 3:16**. Mare 11 had not demonstrated standing oestrus for 55 days prior to the start of the bleeding programme. PGFM was not observed to increase markedly in this mare throughout the sampling period. A series of very small but rapid pulses were detected during the final 24 hours of sampling. The largest pulse was 90 pg/ml and this appeared to persist for over one hour. During this period of increased PGFM release, progesterone appeared to be slightly depressed but did not reach baseline and the mare did not return to standing oestrus until after a uterine biopsy was taken. Timed blood samples were taken before and after the biopsy. Results are demonstrated in **Figure 3:17**. No significant pulses of PGFM were recorded during the sampling period, the largest pulse recorded was 93pg/ml. Progesterone concentrations did not drop during the sampling period but the mare returned to standing oestrus 4 days after the biopsy was taken.

Figure 3.16
Circulating plasma PGFM and progesterone concentrations in hourly blood samples taken from mare 11 in prolonged dioestrus over a three day period

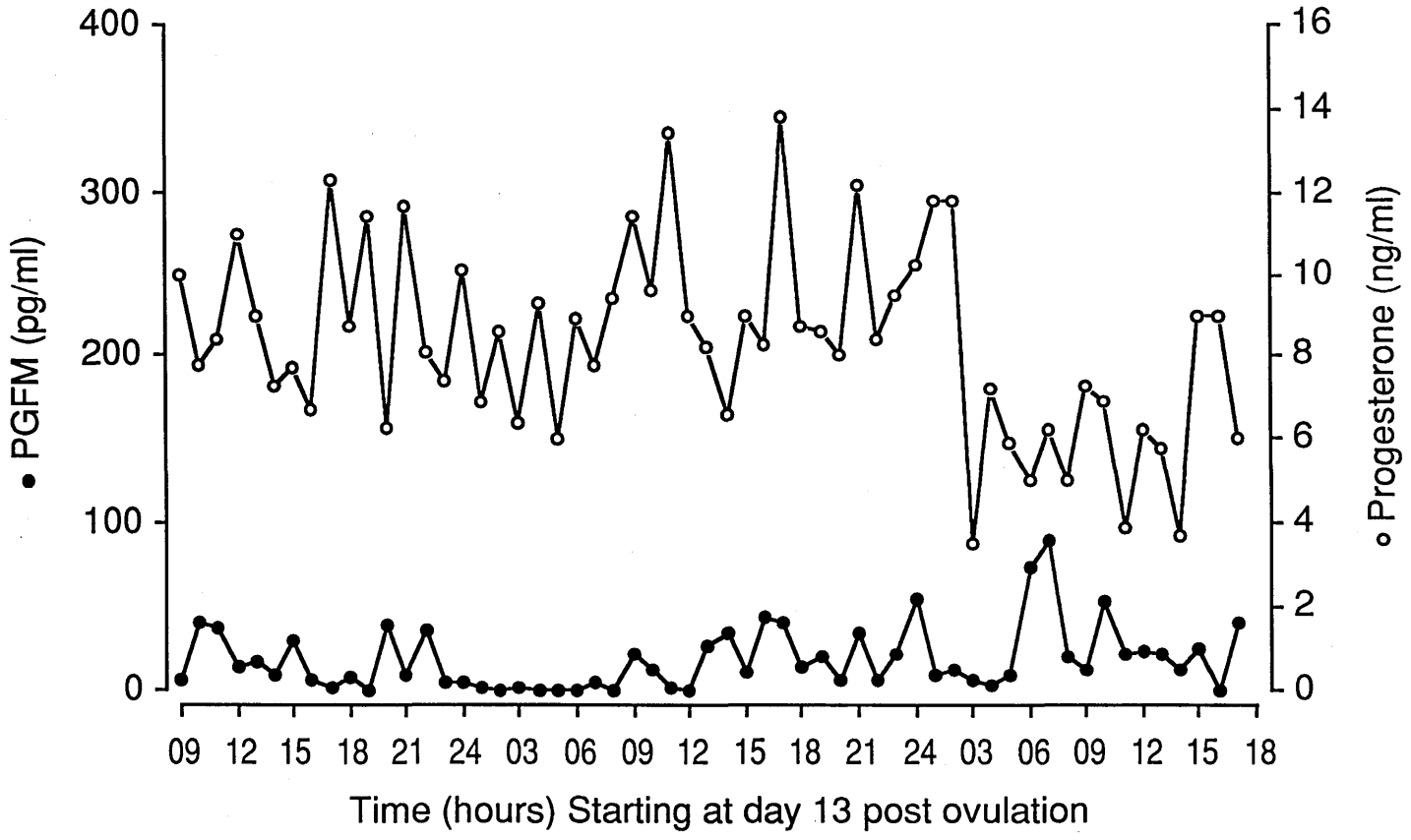
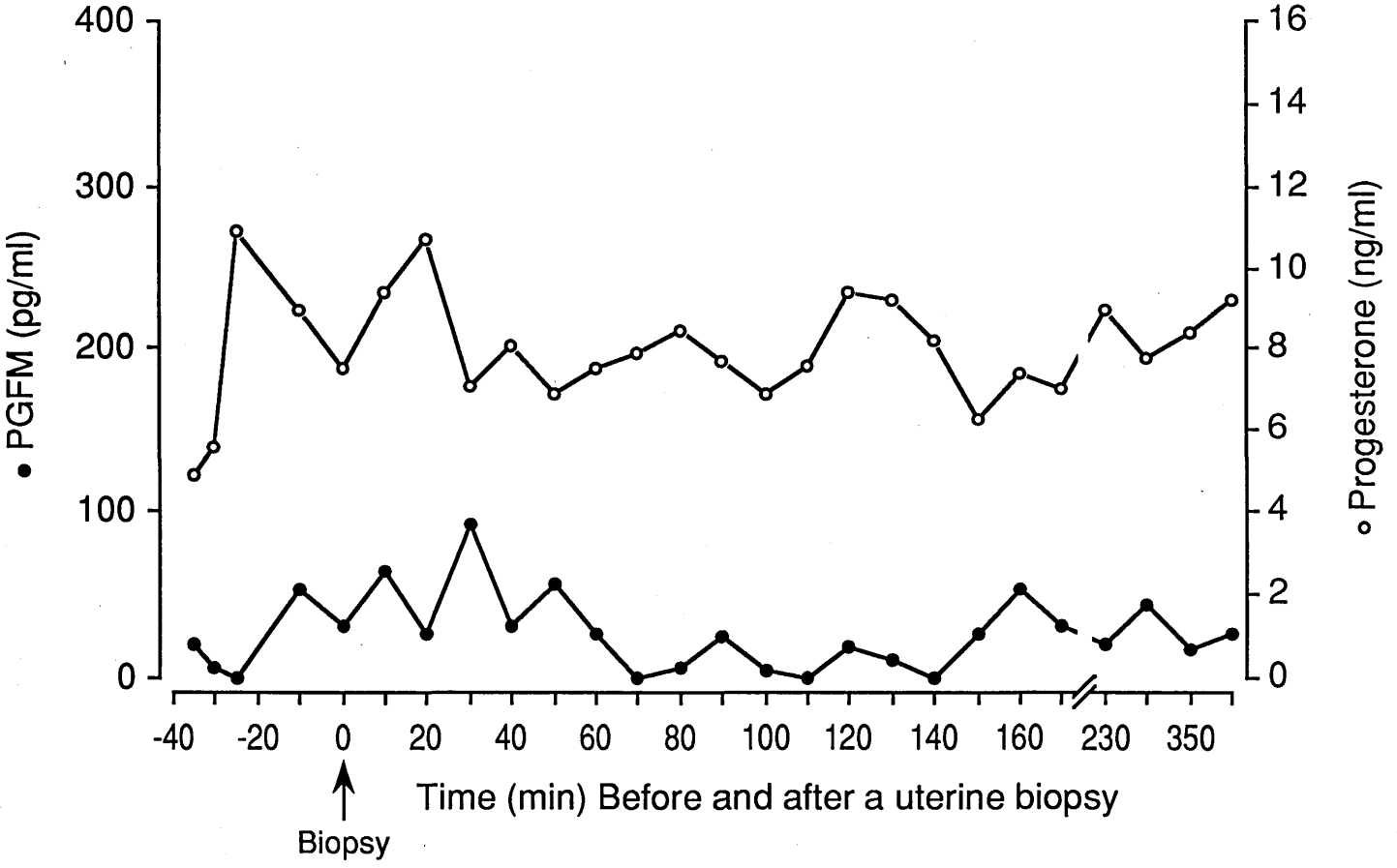


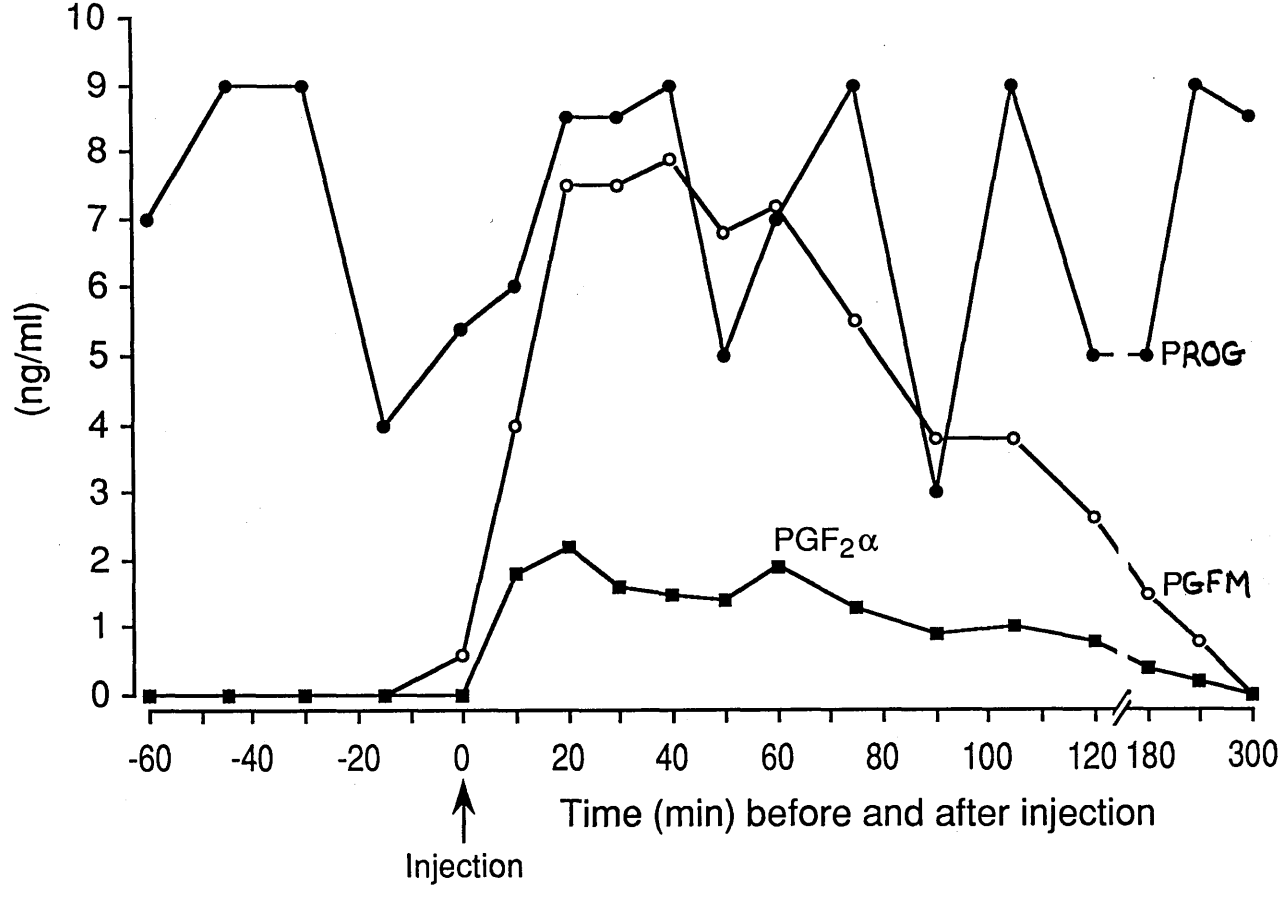
Figure 3.17
PGFM and progesterone concentration in timed plasma samples taken before and after a uterine biopsy from mare 11



3.3.4 Group 3 Mares (Injected with 'Lutalyse')

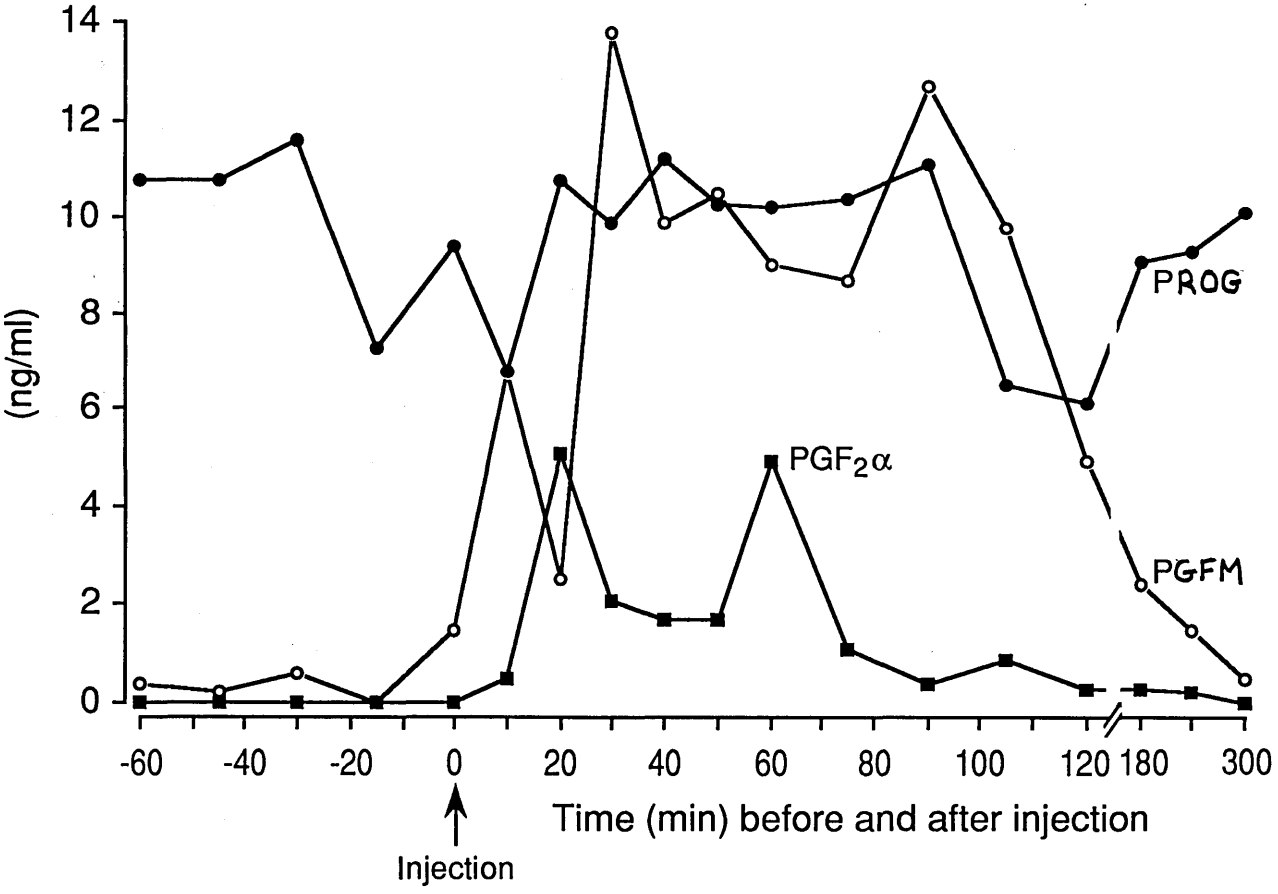
Mare 12 Raised plasma PGFM levels were observed in the plasma 10 minutes after the injection of 'Lutalyse' in Mare 12 **[Figure 3:18]**. The compound demonstrated a triphasic pattern in the plasma, with the three peaks all occurring within one and a half hours of the injection. Plasma PGF-2α concentrations mimicked the pattern of PGFM. An increase in PGF-2α was first observed 10 minutes after the injection, the same time as the PGFM increased. The magnitude of the PGF-2α peaks was not as great as those of the PGFM pulses and progesterone did not decrease during the sampling period. Progesterone concentrations remained high throughout the sampling period, although the mare returned to standing oestrus 3 days after the injection.

Figure 3.18
Plasma PGFM, $PGF_2\alpha$ and progesterone concentrations in timed blood samples from mare 13 before and after an injection of 'lutalyse'



Mare 13 PGFM increased in the plasma 10 minutes after the injection of 'Lutalyse' and rapidly reached a peak of 8 ng/ml at 30 minutes after the injection **[Figure 3:19]**. PGF-2α concentrations increased in the plasma to reach a gradual peak of 1 ng/ml 35 minutes after the injection. PGF-2α was still elevated in this mare up to 2 hours after the injection. Progesterone did not decline during the sampling period. Progesterone concentrations remained high throughout the sampling period although this mare returned to standing oestrus 3 days later.

Figure 3.19
Plasma PGFM, $PGF_2\alpha$ and progesterone concentrations in timed blood samples from mare 12 before and after an injection of 'lutalyse'



3.4 DISCUSSION

The major findings in this chapter were that adequate methods for the estimations of PGFM and PGF-2α in the circulating plasma of mares had been established. These methods were then used to detect a pulsatile pattern of PGFM in the plasma of eight normal cycling mares (Group 1) at Day 14 post-ovulation. This pulsatile pattern appeared to be associated with a drop in progesterone concentrations and the return to standing oestrus. There were three mares that remained in prolonged dioestrus (Group 2). Two of this group returned to standing oestrus after an uterine biopsy was taken. In two normal cycling mares given exogenous prostaglandin, raised PGF-2α concentrations in the plasma were detected despite the very short half-life of this compound.

The results in Table 3:2 indicate clearly that the first PGFM assay method was not measuring accurately the PGFM concentrations present in the plasma and control samples. In an attempt to try to solve these problems and allow a relatively accurate assessment of PGFM quantities in the plasma various changes to the assay were made.

Initially new radioactive label and antiserum were purchased in an attempt to increase the percentage binding. Although this only resulted in a slight improvement it was evident that the label, even when stored at -20°*C*, had to be purchased fresh every two months, if a drop in the percentage binding of the assay was to be avoided.

Some workers have increased the sensitivity of assays by decreasing the amount of radioactive label added to the samples. Alterations were made to the amount of label in this assay but optimum binding was found using 8000cpm. This was a slight increase in the concentration of working solution of label used in the original assay. In a further attempt to increase the percentage binding prior incubation of the samples with antiserum alone was investigated. The periods of incubation varied between one hour and overnight. In addition incubation was carried out at either 4°*C* or room temperature . No appreciable difference in percentage binding was found using any of these methods. This may be due to the fact that PGF-2α is a relatively small compound and pre-incubation with antiserum does not always increase the binding percentage when small compounds are involved. Therefore no prior incubation with antiserum was used.

After carrying out several assays using the first assay method an unexplained discrepancy was found between the cpm in the unknown samples, standards and total binding (Bo) samples. The unknown samples and the standards were reasonably high but the total binding (Bo) values were inexplicably low. Although this discrepancy is not fully understood it was thought that the unknown samples and standard samples may be reaching equilibrium before the total binding samples and so a greater incubation time was incorporated into the assay to allow the total binding values to reach equilibrium. The incubation time with both antiserum and radioactive label added to the sample was increased from 2-24 hours to 30-48 hours. Serial assays were set up and incubated for varying times. The results (Table 3:3) demonstrated that the optimum incubation period was 46 hours when 50% of the radioactive label was bound. However when compared with 48% bound after only 30 hours it was felt that the 16 hours delay required to gain an improvement of 2% binding was not sufficient to warrant the longer period of incubation. Thus in subsequent assays an incubation period of 30 hours was adopted.

According to McLaren *et al* (1985) a more reproducible standard curve could be obtained by making up the standard curve in phosphate buffered saline with added bovine serum albumen (PBS). The bovine serum albumen is required in the buffer as the protein will stop the PGFM from adhering to assay tubes, pipette tips etc. The total binding (Bo), non-specific binding (NSB) and control samples were also made up in this buffer. The results (Figure 3:4) demonstrate that the standard curve in PBS was comparable to that in stripped plasma and so PBS buffer was used in the assay instead of blank plasma.

An attempt was also made to improve the separation procedure using a double antibody method. This however was unsuccessful and the separation was performed using the charcoal method outlined above.

All the changes adopted greatly improved the efficiency and the sensitivity of the assay. However problems did still arise occasionally and the percentage binding would drop for no apparent reason. This would then correct itself despite all the ingredients being the same. No explicable reason can be given for these fluctuations, although other workers have experienced the same problems with prostaglandin assays (McLaren Per Comm)

In retrospect it now seems that it may have been possible to further increase the sensitivity and reproducibility of the assay in a number of ways:

1) Increasing the sample volume from 100ul to 200ul may have helped to resolve variability.
2) Increasing the volume of scintillation fluid; the 2mls used in the assay may have been insufficient for the sample volume used.
3) Maintaining the samples at 4°C throughout the separation procedure: vortex mixing of the samples was carried out at room temperature and a variable degree of warming may have occurred at this stage.

These additional modifications may have increased the sensitivity even further and resulted in a better inter-assay coefficient. However as only the trend of the PGFM pattern in the plasma and not the actual values were being monitored in this study the results demonstrated the expected trend in the mares, despite the discrepancies in the assay.

The PGF-2α assay raised few additional problems to those encountered in the PGFM assay. However when the ecosinct fluid was added to the samples frequently not all the sample would go into solution leaving white deposits which interfered with the counting of the samples. Therefore 10mls of scintillation fluidwere added to the sample, shaken and checked to determine if all the sample had dispersed. If not, a further 1ml was added. This volume proved to be adequate.

In the two mares in Group 1a (Mares 1 and 2) which had blood samples taken every 6 hours at what was calculated to be Day 13 post-ovulation, no clear pattern of PGFM was detected. Mare 1 (Fig 3:6) did demonstrate an increase in plasma PGFM concentrations at Day 15 post-ovulation. However progesterone concentrations had already begun to decline to baseline prior to the apparent PGFM release, suggesting that luteolysis had begun before the pulses of PGFM were observed in the blood samples. In Mare 2 (Fig 3:7) no pulses of PGFM were observed despite progesterone declining to baseline. However in this mare the circulating plasma progesterone concentrations suggest that the day of ovulation was miscalculated. Ovulation appeared to occur on Day 3. This mare often appeared indifferent to the stallion both during dioestrus and during standing oestrus, making it difficult to determine if the mare was in standing oestrus and so it is possible that the day of ovulation was miscalculated and inadequate blood samples were taken around the time of lysis.

The results from these two mares did demonstrate that the PGFM assay identified PGFM pulses in the plasma. The results also clearly demonstrated the difficulties involved in pin-pointing the day of luteolysis. The lack of information from these two mares was thought to be due to inadequate frequency of blood sampling, which led to false information about PGF-2α release in the mare during luteolysis. These findings are in agreement with Kindahl *et al* (1981) who suggested that blood samples had to be taken at least every hour before the true pattern of PGFM in the plasma could be observed. Although Neely *et al* (1979a) had suggested that 6 hourly blood sampling was adequate to determine the pattern of PGFM release during luteolysis, the results from these two mares suggest that a longer blood sampling period and more frequent blood samples were required. Stabenfeldt *et al* (1981) stated that luteolysis occurred over a 38 hour period. Therefore in Group 1b two normal mares (Mares 3 and 4) were blood sampled hourly during the expected time of luteolysis. Initially it was hoped to take blood samples over a 38 hour period but both mares lost the function of the cannula and blood sampling had to be stopped before the 38 hour period was complete.

Finally in four normal mares (Mares 5, 6, 7 and 8) in Group 1c the blood sampling period was increased to 57 hours to ensure that the period of luteolysis was fully covered. Mare 5 (Fig 3:10) was bled only every 3 hours, to determine if a longer period was adequate to demonstrate the PGFM pattern. Although pulses of PGFM were demonstrated in this mare the samples did not appear to give an accurate picture of the PGFM pattern in the plasma. This result confirmed the previous hypothesis that animals need to be bled at least every hour over the period of luteolysis before the pattern of PGFM in the plasma is revealed.

The results from the mares that were bled hourly suggest a pattern of increased PGFM release around Day 14-15 post-ovulation. There were several rapid pulses of PGFM released over approximately a 38 hour period. PGFM release appeared to be initiated in Mares 4, 6 and 7 by one large pulse followed by several pulses of smaller magnitude but increased frequency. In Mares 3 and 6 and possibly 7 this series of pulses appeared to be followed or terminated by the largest pulse of PGFM, often several times greater than the other pulses. In the mares which demonstrated a release pattern of PGFM, progesterone began to decline to baseline during the period of PGFM release, except Mare 3 which had baseline progesterone at the start of the blood sampling period. Luteolysis must have already begun prior to

Day 13 in this mare. Local conditions of the uterus have been associated with premature PGF-2α release. Animals with sub-acute endometritis can have shortened interoestrous periods. This effect is thought to be mediated by PGF-2α, released from the inflamed endometrium (Stabenfeldt *et al*, 1976). However Mare 3 demonstrated no signs of a uterine infection therefore it seems likely that the day of ovulation was again miscalculated in this mare and she was not at Day 13 as expected when blood sampling started. This was also thought to be the case in Mare 8 which demonstrated no drop in progesterone during the blood sampling period. These two mares, along with Mare 2 demonstrate the considerable problems associated with trying to pin-point accurately the time of luteolysis in the mare.

In Mares 4, 6 and 7 the first large pulse of PGFM preceded the drop in plasma progesterone concentrations by several hours. In Mare 4 the progesterone drop appeared to be associated with the second pulse of PGFM. This latter pattern agrees with Neely *et al* (1979a) who reported that the first pulse of PGFM initiated luteolysis but preceded the drop in progesterone by 3-5 hours. Only after progesterone concentrations had declined significantly was the largest pulse of PGFM recorded. Poyser (1981) suggested a negative feedback effect of progesterone on PGF-2α release and only when progesterone had reached a threshold level could PGF-2α be released in a large pulse. This may be a protection mechanism to protect the CL from slight releases of PGF-2α leading to premature onset of luteolysis. These results agree with those of other workers who demonstrated a complex pulsatile pattern of PGFM at the expected time of luteolysis in the mare and the other domestic species (Neely *et al*, 1979; McCracken *et al*, 1981). These workers suggested that PGF-2α release at this time was involved in the initiation and completion of luteolysis and that the pulsatile pattern of release was vital to the outcome of luteolysis. However 3 of the mares in Group 1c examined did not conform to this pattern of PGFM release. At the start of the investigation one of the mares (Mare 10) was cycling normally whilst the other two (Mares 9 and 11) were already in prolonged dioestrus.

Spontaneous persistence of the CL, or prolonged dioestrus is relatively common in the mare and is one of the major causes of infertility in the breeding mare (Stabenfeldt *et al*, 1974). Stabenfeldt *et al*, (1981) quoted the incidence at about 20% although Ginther (1979) reported an incidence of 33% in Thoroughbred mares. This is in direct contrast to the other species, particularly the ruminant in which the

incidence is as low as 2% (Bulman and Lamming, 1977). In the mare the CL can persist in the ovary for periods varying between 30-90 days. However the cause of prolonged dioestrus is not yet fully understood. Extensive damage to the uterine endometrium, where the endometrium is totally destroyed, can impair the synthesis and release of PGF-2α and the mare enters a period of prolonged dioestrus (Hughes *et al*, 1979). However pyometra due to β haemolytic streptococcus will lead to shortened interoestrus periods due to the irritant effect on the uterine endometrium.

A recognized approach to mares in prolonged dioestrus is to perform a uterine biopsy which results in the mare returning to standing oestrus within 2-5 days. It was decided to adopt this approach to the two mares already in prolonged dioestrus (Mares 9 and 11) and this had the desired effect in that both mares had returned to standing oestrus within 5 days after the biopsy. The mechanism responsible for returning mares to standing oestrus after a uterine biopsy is not fully understood but is thought to involve the release of PGF-2α from the traumatized uterus, leading to lysis of the CL and the return to standing oestrus. However although both these mares were in prolonged dioestrus raised PGFM concentrations were demonstrated in the plasma prior to the biopsy being taken. Mare 9 (Fig 3:14) demonstrated two pulses of PGFM during the blood sampling period. Both pulses were of considerable magnitude but neither led to a decrease in the circulating plasma progesterone concentrations. This mare had not been observed in standing oestrus for a period of 45 days prior to the onset of blood sampling. In Mare 11 (Fig 3:16) the pulses of PGFM recorded were not significant, only reaching a magnitude of 98pg/ml.

In the remaining mare in prolonged dioestrus (Mare 10), in which no uterine biopsy was taken, two pulses of PGFM were identified some 8 hours apart at the expected time of lysis. However neither pulse reached a magnitude greater than 180pg/ml although the second pulse did appear to cause a slight drop in progesterone concentrations. This mare, although apparently cycling normally at the start of the study, did not return to standing oestrus for a further 3 months. The mare was examined regularly throughout this period by teasing with a stallion and by palpation *per rectum*. Blood samples were also taken at irregular intervals and plasma progesterone concentrations remained above 1ng/ml so the mare had not entered anoestrus. In this mare either the uterus was capable of some but not enough prostaglandin production to bring about luteolysis or it was not released in the correct pattern. The other possibility is that a vital secondary source of prostaglandin was not

stimulated during this time in this mare. By three months it is probable that the CL came to the end of its effective lifespan and the mare returned to standing oestrus.

In none of these mares in prolonged dioestrus were the pulses of PGFM associated with a significant decline in progesterone. These findings are contrary to the findings of Stabenfeldt *et al* (1981) who demonstrated no PGFM concentrations in the plasma of mares in prolonged dioestrus unless associated with dioestrous ovulations. Stabenfeldt *et al* (1981) hypothesized that this condition represented a biochemical cellular defect, rendering the endometrial cells temporarily incapable of synthesising PGF-2α. Stull and Evans (1986) were also unable to demonstrate release of PGF-2α from endometrial tissue cultured *in vitro* from mares in prolonged dioestrus. The mares described in this thesis were examined *per rectum* on a daily basis and no apparent ovulations took place, therefore the PGFM patterns exhibited did not appear to be related to dioestrous ovulations as suggested by Stabenfeldt. However in the mares in prolonged dioestrus the overall magnitude of the pulses appeared to be reduced, markedly so in Mare 11. In the light of the rapid metabolism of PGF-2α and the anatomy of the uterine and ovarian vasculature in the mare it would seem likely that to maintain concentrations of unmetabolized PGF-2α at the ovary adequate to ensure that luteolysis proceeds as normal the concentration of PGF-2α synthesized and released from the uterus must be critical. If these concentrations decline below a certain level then the concentrations at the CL will be adversely affected and luteolysis will not proceed. However in both the mares biopsies in this study no pulses of PGFM were recorded in the plasma during the sampling period despite the mares returning to standing oestrus within 5 days of the biopsy. In Mare 9, which had blood samples taken only 4 times a day this could have been due to infrequent blood sampling. In Mare 11, which had increased frequency of blood sampling, the release of PGF-2α may have occurred after the blood sampling ceased and so the pulses were not picked up.

McCracken *et al,* (1981) demonstrated that it was not the overall amount of prostaglandin released that was essential for luteolysis to take place but the frequency of release that was critical. This was confirmed by the findings of Zarco *et al* (1984) who demonstrated that if the frequency was disrupted and the interval between the pulses extended in the ewe, the CL was able to recover from each attack of PGF-2α, allowing progesterone synthesis to be maintained. It would seem that the CL attempts to maintain production of progesterone and it is only if it is

continually bombarded with pulses of PGF-2α at a regular rate that the CL succumbs and luteolysis takes place. Therefore in the mares in prolonged dioestrus reported here the pattern of PGFM concentrations appeared to be very different from normal mares and this may have affected the outcome of luteolysis. However it is not possible to draw any firm conclusions from the small sample of mares in this study.

In Mares 9 and 11 the uterus appeared normal on histological examination, suggesting that no abnormality was present in the uterus preventing the synthesis and release of PGF-2α. Mares in prolonged dioestrus usually respond to exogenous prostaglandins by returning to standing oestrus 2-5 days after the injection (Allen and Cooper, 1975: Allen, 1981). The reasons why these mares will respond to exogenous prostaglandins and not to endogenous PGF-2α are not clear. To try to clarify this point, two mares in Group 3, Mares 12 and 13, were injected with therapeutic doses of an analog of PGF-2α, 'Lutalyse'. Both mares demonstrated baseline PGFM prior to the injection but in each case PGFM rose dramatically 10 minutes after the injection and by 20 minutes the parent compound appeared in the plasma and rose to high levels. Due to the very short half-life of the parent compound it is not usually evident in the plasma and PGFM is measured as a marker for PGF-2α release (Kindahl *et al*, 1981). However the uptake of PGF-2α by the lungs is an active and saturable process, such that, if the system is overloaded with an excessive amount of PGF-2α, the compound cannot be metabolized and so the concentrations in the plasma will build up (Poyser, 1981). Therefore it would seem that, in these two mares, the injection of 'Lutalyse' allowed the release of such a massive dose of prostaglandin into the plasma that the metabolic pathways were overloaded and unmetabolized prostaglandin was able to build up in the plasma to detectable concentrations. In both mares PGFM reached greater values than the normal physiological values observed in normal cycling mares at luteolysis. It would seem likely therefore that this injection produced such high concentrations of prostaglandin in the plasma that the CL was unable to protect itself and lysis occurred. Progesterone did not drop to baseline during the sampling period. This finding is consistent with the normally cycling mares which demonstrated a lag period between PGFM release and the drop in progesterone concentrations. Progesterone must have dropped to baseline after the end of the sampling period as the mares returned to standing oestrus.

Although plasma PGFM concentrations give an indication of PGF-2α concentrations they give no indication of the source of the PGF-2α. It is possible that in normal mares more than one source is stimulated to release PGF-2α during luteolysis. In mares in prolonged dioestrus it may be that although the uterus is able to synthesize and release PGF-2α the secondary sources of PGF-2α are not stimulated. Evidence has been growing from studies in the human (Shutt *et al*, 1976) and cattle (Shemesh and Hansell, 1975) that sources of PGF-2α other than the uterus may play a role in lysis of the CL in cycling mammals. PGF-2α has been demonstrated in increasing amounts in the ovarian tissue of these species as luteolysis approaches. A self-destruct mechanism may be involved in lysis of the CL.

The results from this study demonstrated the problems encountered if blood samples are taken too infrequently and the difficulties involved in pin-pointing accurately the day of luteolysis in the mare which has such a variable oestrous cycle. However a pulsatile pattern of PGFM was observed in the plasma of cycling mares around the expected time of luteolysis. The pattern of PGFM associated with lysis in the mares in this study was of a large initial pulse of PGFM. This was followed by small frequent pulses leading to a drop in progesterone to baseline. Once progesterone had dropped significantly the largest pulse of PGFM was observed. This appeared to terminate the release of PGFM. The mare returned to standing oestrus in 4-5 days after the end of PGFM release.

Some mares however do not return to standing oestrus but remain in a state of prolonged dioestrus. In this study these mares were found to exhibit a pulsatile pattern of PGFM in the circulating plasma but due to some fault in the mechanism lysis of the CL did not take place. The pattern of release did not correspond to that observed in the normal cycling mares and the magnitude of the pulses of PGFM in the plasma appeared to be reduced. These findings raised the possibility of a secondary source of PGF-2α being involved in luteolysis in the mare. It was decided therefore that this possibility of a secondary source of prostaglandin in the mare should be investigated.

4 CHAPTER FOUR
PROSTAGLANDIN F-2α
PRODUCTION BY ENDOMETRIAL AND LUTEAL CELLS CULTURED *in vitro*

4.1 INTRODUCTION

In several species including the pig (Patek and Watson, 1976), the rhesus monkey (Balmaceda *et al*, 1979) and the human (Shutt *et al*, 1976) it has been shown that the CL is capable of PGF-2α synthesis. During luteolysis in these species an increased production of PGF-2α by the luteal cells has been demonstrated. If the CL of the mare had similar capabilities this would abolish the need for the systemic transfer of prostaglandin from the uterus to the ovary, preventing metabolism of prostaglandin before it reached the ovary.

The aims of this chapter were firstly to investigate if the CL of the mare was capable of PGF-2α production and to determine if production was increased around the time of luteolysis. Secondly how this luteal PGF-2α production compared to that from the uterine endometrium of the same mare. Inhibition of production, to determine if PGF-2α concentrations were due to *de novo* synthesis, was attempted by the addition of aspirin. Finally stimulation of PGF-2α production by both luteal and endometrial tissues was attempted by adding oxytocin to the incubation medium.

4.2 MATERIALS AND METHODS

4.2.1 Source of luteal and endometrial tissue

Tissue was obtained from mares at the time of slaughter. In most cases luteal and endometrial tissue was removed from the same mare. Corpora lutea were carefully excised from the ovary, avoiding the surrounding ovarian tissue. Slices of approximately 2g each were removed. Endometrial tissue slices of about 4g each were removed, avoiding the underlying myometrial layers.

4.2.2 Preservation of tissue

Both endometrial and luteal tissue slices were placed into cryo-tubes (Nunc, Life Technologies Inc., Unit 4, Cawley Mill Trading Estate, Longbridge Way, Uxbridge), labelled and snap frozen in liquid nitrogen, using the method of King and Evans (1987). The time lapse between slaughter of the mare and snap freezing of the tissue was never more than 10 minutes.

4.2.3 Fresh and Frozen tissue samples

A very limited supply of fresh tissue became available from a local source. Sections of endometrial and luteal tissues were removed from 2 mares and stored in liquid nitrogen as described. Further endometrial and luteal sections were removed and stored on ice. On return to the laboratory these chilled sections were processed immediately. The frozen sections were processed at a later date and the results of the two were compared.

4.2.4 Determination of the stage of the oestrous cycle

a) Plasma progesterone analysis

Blood samples were taken immediately prior to slaughter and assayed for progesterone concentrations as described in Chapter 2. Blood samples were not available from the mares which had explant samples removed and so no progesterone estimations were possible.

b) Macroscopic examination of CL

Macroscopic examination of the CL was undertaken immediately after slaughter and the CL grouped into 4 different stages of the oestrous cycle:

STAGE	COLOUR	CONSISTENCY	STATE
early luteal	Very dark red	Liquid /sticky	Active
mid luteal	Brown /red	Fleshy	Active
late luteal	Brown /yellow	Firm /fibrous	Active /Inactive
follicular	Yellow /orange	Greasy /friable	Inactive

c) Microscopic examination

Small sections of luteal tissue, about 1cm, were removed from each CL. These sections were fixed immediately in Bouins Fixative solution. Paraffin sections of tissue samples were prepared and stained with Haematoxylin-eosin (H and E) on return to the laboratory.
Sections were examined with a light microscope and microscopic changes in appearance of the growing, secreting and degenerating CL noted. The features examined were:

1) Appearance and amount of luteal tissue.
2) Appearance of luteal cells
   a) size of cells
   b) size of nucleus
   c) density of staining
   d) state of cytoplasm

leading to determination if luteal cells were active, inactive or degenerating

3) Vascularity of structure

4) Presence and amount of connective tissue

5) Presence and amount of inflammatory cells, particularly eosinophils and macrophages.

The tissue was then categorised into the following groups:

a) Early luteal phase

b) Mid luteal phase

c) Late luteal phase

d) Follicular phase

4.2.5 Tissue culture methods

Two tissue culture methods were used. The medium used throughout was Dulbeccos Modified Eagles medium with 4500mg/L glucose and without pyruvate (DMEM; Gibco Ltd, Paisley).

a) Explant tissue culture method

Endometrial or luteal tissue was removed from the liquid nitrogen and thawed in water at 37°*C* tissue was then blotted dry and weighed. 10ml DMEM at room temperature was added to every one gram of tissue and placed in a glass stoppered flask. The medium and tissue were incubated at 37°*C* for up to 120 minutes and gassed for 10-20 seconds every hour with a mixture of 95% air and 5% CO^2. Aliquots of 300ul were removed at 0, 30, 60 and 120 minutes, decanted into labelled plastic bottles and stored at -20°*C* until assayed for PGF-2α concentration.

b) Single cell suspension method

Endometrial or luteal tissue was removed from liquid nitrogen and thawed in water at 37°*C* as before. The tissue was washed three times in ice-cold DMEM, blotted dry and weighed. Using sharp scissors the tissue was minced in ice-cold DMEM. The minced tissue was added to a fresh volume of medium in a glass flask (10ml of DMEM was added per 1g of tissue). Dispase II (Boehringer Mannheim) was added to the medium at a concentration of 2.0U/ml and the solution was incubated at 37°*C* in a shaking incubator for 120 minutes. The flask contents were then removed, centrifuged (5 minutes, 500g) and the super-

natant passed through a single layer of cheese-cloth to remove any residual debris. The cheese-cloth was rinsed through with fresh DMEM to remove any single cells attached to the cloth. The supernatant was centrifuged again (10 minutes, 1200g) and the pellet was washed with fresh DMEM. This step was repeated three times. Two millilitres of fresh medium was added to the final washed pellet. Cell numbers were determined by Coulter counter. Duplicate aliquots of 1 x10^3cells/ml were set up as follows:

a) controls: nothing added to the medium

b) oxytocin added to the medium at a final concentration of either 0.1iu/ml or 1.0iu/ml. Oxytocin was added to determine if PGF-2α production from the tissues was stimulated by the addition of oxytocin and if the effects were dose dependent.

c) aspirin added at a final concentration of 1ug/ml. Aspirin was added to inhibit PGF-2α production and so determine if PGF-2α production was due to *de novo* synthesis.

The aliquots were incubated at 37°*C* in a shaking incubator and gassed with 5% CO_2:95% air for 10-20 seconds every hour. 500 ul of the cell suspension were removed at 0, 30, 60 and 120 minutes, decanted into labelled plastic bottles and stored at -20°*C* until assayed for PGF-2α concentration.

4.2.6 Prostaglandin F-2α (PGF-2α) estimation

PGF-2α estimation was carried out as described in Chapter 3

4.2.7 Statistical analysis

Statistical analysis was carried out using the One Way Analysis of Variance test. However, as the variability in each of the samples in the different groups was not equal, analysis could not be carried out in all cases.

4.2.8 Tissue progesterone estimation

Estimation of progesterone concentrations was carried out on the supernatant from luteal cells at different stages of the cycle. This assay was not carried out in every case due to the lack of sample volume. The assay was carried out as in Chapter 2. DMEM culture medium was assayed for progesterone concentrations and found to have zero value. As it was the trend of the results from similar samples obtained from this assay which were being considered, no

corrections were made for the samples being in medium and not plasma. Also the baseline was considered to be the same as that for samples in plasma.

4.2.9 Identification of viable cells

A sample of cell suspension was mixed with an equal volume of 0.4% Trypan Blue in 0.9% saline. Those cells which excluded dye when observed under bright field microscopy were considered viable. However this factor was not taken into account in the calculations of PGF-2α production by the tissues.

4.3 RESULTS

Table 4:1

Table 4:1 demonstrates the comparison between the production of PGF-2α tissues which were either fresh or frozen prior to culture. In both cases examined the frozen tissue results were slightly depressed compared to the fresh results.

TABLE 4:1

Comparison of PGF-2α production (pg/10^3 cells) from fresh and frozen sections of luteal and endometrial tissue.

MARE IDENTITY	STAGE OF OESTROUS CYCLE	TYPE OF TISSUE	STATE OF TISSUE	PLASMA PROG (ng/ml)	INCUBATION TIME (mins) PGF-2α PRODUCTION (pg/10^3 cells)			
					0	**30**	**60**	**120**
1	EARLY	ENDOMET	FRESH	2.5	75	200	238	250
1	EARLY	ENDOMET	FROZEN	2.5	75	75	175	212
2	MID	LUTEAL	FRESH	10.7	62	119	222	193
2	MID	LUTEAL	FROZEN	10.7	50	75	169	126

Table 4:2

From the results of PGF-2α production by explants of luteal tissue taken from various mares at different stages of the oestrous cycle it would appear that there was a marked increase in PGF-2α production by the explants from the late luteal phase tissues compared to the early/mid luteal phase.

TABLE 4:2

PGF-2α production (ng/g tissue) by luteal tissue samples taken from mares at different stages of the oestrous cycle.

STAGE OF OESTROUS CYCLE	NO OF MARES SAMPLED	INCUBATION TIME (mins) PGF-2α (ng/g tissue)			
		0	**30**	**60**	**120**
EARLY	4	125 ± 216	565 ± 619	896 ± 768	1314 ± 1253
MID	3	478 ± 607	614 ± 797	877 ± 1083	1025 + 1142
LATE	3	2328 ± 2192	4504 ± 596	10319 ± 3319	16424 ± 8429
FOLLICULAR	1	6350	465	308	542

± SEM

Table 4:3

It is evident from the results of PGF-2α production by luteal cells in single cell suspension from the different stages of the oestrous cycle that there was a marked increase in PGF-2α production during the late luteal phase at all incubation times. This increase was found to be statistically significant ($P < 0.01$)

TABLE 4:3

PGF-2α production (pg/10^3 cells) by luteal cells in single cell suspension from mares at different stages of the oestrous cycle

STAGE OF OESTROUS CYCLE	NO. OF MARES SAMPLED	PLASMA PROG (ng/ml)	INCUBATION TIME (mins) PGF-2α (pg/10^3 cells)			
			0	30	60	120
EARLY	9	3.34 ± 1.9	56 ± 70	100 ± 93	176 ± 125	189 ± 126
MID	11	11.25 ± 3.9	90 ± 111	147 ± 160	248 ± 181	294 ± 131
LATE	6	16.7 ± 5.3	329 ± 303	504 ± 231	686 ± 106	1239 ± 454
FOLLICULAR	6	0.4 ± 0.17	56 ± 70	56 ± 59	114 ± 139	216 ± 112

± SEM

Table 4:4

Table 4:4 demonstrates progesterone production by luteal cells from different stages of the oestrous cycle. Most of the cell suspensions released concentrations of progesterone above baseline during early, mid and late luteal phases. Concentrations dropped to baseline in all the samples during the follicular phase of the cycle.

TABLE 4:4

Production of progesterone (ng/10^3 cells) by luteal cells in single cell suspension from mares at different stages of the oestrous cycle.

STAGE OF OESTROUS CYCLE	NO. OF MARES SAMPLED	PLASMA PROG (ng/ml)	INCUBATION TIME (mins) PROGESTERONE (ng/10^3 cells)			
			0	30	60	120
EARLY	4	3.0 ± 1.5	2.48 ± 1.5	2.82 ± 1.6	3.23 ± 2.0	2.43 ± 2
MID	4	9.27 ± 4.1	7.65 ± 5.1	8.45 ± 5.0	9.8 ± 4.5	10.64 ± 5.2
LATE	3	15.3 ± 4.7	6.64 ± 2.5	7.16 ± 2.6	7.55 ± 2.2	9.17 ± 3.2
FOLLICULAR	5	0.4 ± 0.17	0.152 ± 0.2	0.2 ± 0.2	0.24 ± 0.15	0.32 ± 0.2

± SEM

Table 4:5

Table 4:5 demonstrates the effects of aspirin on PGF-2α production by luteal cells in a single cell suspension at different stages of the oestrous cycle.

In each of the samples aspirin appeared to lead to an inhibition of PGF-2α production by the luteal cells. This effect was most marked after 60 minutes incubation.

TABLE 4:5

Effects of aspirin (1ug/ml) on PGF-2α production (pg/10^3 cells) by luteal cells in single cell suspension from mares at different stages of the oestrous cycle.

STAGE OF OESTROUS CYCLE	NO. OF MARES SAMPLED	INCUBATION TIME (Mins)	CONTROL	ASPIRIN (1ug/ml)
			PGF-2α (pg/10^3 cells)	
EARLY	3	**0**	5 ± 10	0 ± 4
		30	13 ± 9	14 ± 16
		60	176 ± 15	90 ± 9
		120	196 ± 163	77 ± 25
MID	4	**0**	5 ± 7	4 ± 8
		30	60 ± 34	40 ± 17
		60	605 ± 715	92 ± 62
		120	175 ± 180	66 ± 70
LATE	3	**0**	5 ± 9	3 ± 1
		30	29 ± 17	20 ± 7
		60	667 ± 234	478 ± 64
		120	918 ± 205	551 ± 60
FOLLICULAR	2	**0**	25 ± 17	7 ± 9
		30	226 ± 206	153 ± 55
		60	903 ± 1110	92 ± 90
		120	326 ± 117	162 ± 64

± SEM

Table 4:6

During the early luteal phase explants of endometrial tissue released relatively high concentrations of PGF-2α. Production apparently dropped off towards the mid luteal phase. A marked increase in PGF-2α production was then observed during the late luteal phase. This increase was greater than the production during the early luteal phase.

TABLE 4:6

PGF-2α production (ng/g tissue) by endometrial explant tissue samples from mares at different stages of the oestrous cycle

STAGE OF OESTROUS CYCLE	NO. OF MARES SAMPLED	INCUBATION TIME (mins) PGF-2α (ng/g endometrial tissue)			
		0	**30**	**60**	**120**
EARLY	4	2513 ± 2534	5127 ± 5030	5262 ± 5633	7819 ± 5347
MID	3	272 ± 285	1040 ± 1248	1398 ± 1432	1564 ± 1633
LATE	3	1129 ± 691	5946 ± 4346	10697 ± 4417	17266 ± 6511
FOLLICULAR	1	500	1600	1600	2500

± SEM

Table 4:7

Table 4:7 demonstrates PGF-2α production by endometrial cells in single cell suspension. The cells were taken from different areas of the uterus and at different stages of the oestrous cycle. Minor fluctuations in production between mares and between the different areas of the uterus occurred but these were not thought to be significant.

TABLE 4:7

PGF-2α production (pg/10^3 cells) by endometrial cells from different areas of the uterus from mares at different stages of the oestrous cycle

MARE IDENTITY	STAGE OF OESTROUS CYCLE	INCUB. (mins)	LEFT UTERINE HORN TIP	LEFT UTERINE HORN	RIGHT UTERINE HORN TIP	RIGHT UTERINE HORN	UTERINE BODY
3	EARLY	**0**	2710	2830	3325	2684	2494
		30	6946	7104	8142	3800	4843
		60	7195	11608	9789	6596	5892
		120	9192	4652	13868	25366	13357
4	EARLY	**0**	6660	988	3004	1528	2576
		30	13512	2295	8987	2290	5330
		60	10800	3891	18855	1956	7224
		120	14992	8481	14759	2186	18686
5	MID	**0**	0	0	NM	0	0
		30	1430	90	NM	0	0
		60	3780	4118	NM	78	1727
		120	5195	7609	NM	6518	4793
6	MID	**0**	1575	2377	1731	2598	NM
		30	3094	8431	3089	2772	NM
		60	3556	4723	4140	3951	NM
		120	3196	6154	9894	4755	NM
7	MID	**0**	1247	2246	NM	NM	NM
		30	2363	3368	NM	NM	NM
		60	4331	4370	NM	NM	NM
		120	6569	5131	NM	NM	NM

NM = No measurement taken

Table 4:8

During the late luteal phase there was a marked increase in PGF-2α production by the endometrial cells in single cell suspension. The concentrations released were significantly greater than those released during the early and mid luteal phases ($P < 0.001$)

TABLE 4:8

PGF-2α production (pg/10^3 cells) by endometrial cells in single cell suspension from mares at different stages of the oestrous cycle

STAGE OF OESTROUS CYCLE	NO. OF MARES SAMPLED	PLASMA PROG.(ng/ml)	INCUBATION TIME (mins) PGF-2α (pg/10^3 cells)			
			0	**30**	**60**	**120**
EARLY	8	3.01 ± 1.8	42 ± 60	98 ± 169	175 ± 212	301 ± 212
MID	8	13 ± 6.1	185 ± 143	217 ± 164	263 ± 152	339 ± 233
LATE	6	16.7 ± 5.3	437 ± 519	612 ± 387	1110 ± 556	2312 ± 1341
FOLLICULAR	5	0.51 ± 0.21	69 ± 124	400 ± 706	181 ± 285	278 ± 408

± SEM

Table 4:9

Table 4:9 demonstrates the effects of aspirin on PGF-2α production by endometrial cells in single cell suspension at 6 different stages of the oestrous cycle.

Aspirin appeared to lead to an inhibition of PGF-2α production from the endometrial cells. This effect was most marked after 60 minutes incubation.

TABLE 4:9

Effects of aspirin (1ug/ml) on PGF-2α production (pg/10^3 cells) by endometrial cells in single cell suspension from mares at different stages of the oestrous cycle.

STAGE OF OESTROUS CYCLE	NO. OF MARES SAMPLED	INCUBATION TIME (mins)	CONTROL	ASPIRIN (1ug/ml)
			PGF-2α (pg/10^3 cells)	
EARLY	3	0	0 ± 0	0 ± 0
		30	20 ± 25	0 ± 9
		60	21 ± 20	12 ± 10
		120	128 ± 114	60 ± 35
MID	3	0	13 ± 16	0 ± 2
		30	12 ± 11	7 ± 9
		60	93 ± 71	57 ± 11
		120	230 ± 204	180 ± 105
LATE	3	0	23 ± 27	14 ± 13
		30	313 ± 117	270 ± 91
		60	410 ± 205	260 ± 117
		120	978 ± 771	540 ± 404
FOLLICULAR	2	0	16 ± 12	30 ± 13
		30	90 ± 70	59 ± 27
		60	107 ± 112	70 ± 64
		120	130 ± 104	85 ± 80

± SEM

Table 4:10

Table 4:10 compares the production of PGF-2α released from the luteal tissue explants and the endometrial tissue explants.

There appears to be little variation in production between the two tissue explant culture except during the early luteal phase when the endometrial production of PGF-2α is significantly raised compared to the luteal prostaglandin production at each incubation time.

TABLE 4:10

Comparison of PGF-2α production (ng/g tissue) by explant tissue samples from luteal and endometrial tissues

STAGE OF OESTROUS CYCLE	NO. OF MARES SAMPLED	INCUBATION TIME (mins)	LUTEAL TISSUE	ENDOMETRIAL TISSUE
			PGF-2α(ng/g tissue)	
EARLY	4	0	125 ± 216	2513 ± 2534
		30	564 ± 619	5138 ± 5030
		60	896 ± 768	5262 ± 3633
		120	1314 ± 1253	7819 ± 5347
MID	3	0	478 ± 607	272 ± 285
		30	614 ± 797	1040 ± 1248
		60	877 ± 1083	1398 ± 1432
		120	1025 ± 1142	1564 ± 1638
LATE	2	0	2328 ± 2172	1129 ± 691
		30	4505 ± 596	5946 ± 4356
		60	10319 ± 3319	10697 ± 4417
		120	16424 ± 8429	17266 ± 6511

± SEM

Table 4:11

Table 4:11 compares PGF-2α production from single cell suspension cultures of luteal and endometrial tissues.

PGF-2α production by the two tissues appeared to be roughly comparable. It does appear as if endometrial production was slightly greater than the luteal production especially after 60 minutes incubation during the late luteal phase.

TABLE 4:11

Comparison of PGF-2α production (pg/10^3 cells) by single cell suspension cultures of luteal and endometrial tissues at different stages of the oestrous cycle.

STAGE OF OESTROUS CYCLE	NO. OF MARES SAMPLED		INCUBATION TIME (mins)	LUTEAL	ENDOMETRIAL
	LUTEAL	ENDOMET		PGF-2α CONCENTRATIONS (pg/10^3 cells)	
EARLY	9	8	**0**	56 ± 70	42 ± 60
			30	100 ± 93	98 ± 169
			60	176 ± 125	175 ± 212
			120	189 ± 125	301 ± 212
MID	11	8	**0**	90 ± 11	185 ± 413
			30	147 ± 160	217 ± 164
			60	248 ± 181	263 ± 152
			120	294 ± 131	339 ± 233
LATE	6	6	**0**	329 ± 303	437 ± 519
			30	524 ± 231	612 ± 386
			60	686 ± 160	1100 ± 556
			120	1239 ± 454	2312 ± 1341
FOLLICULAR	6	5	**0**	56 ± 70	69 ± 124
			30	54 ± 59	400 ± 706
			60	114 ± 139	181 ± 285
			120	216 ± 112	278 ± 408

± SEM

Table 4:12

In Table 4:12, during the early and late luteal phase, oxytocin appeared to exert a slight inhibitory effect on PGF-2α production by luteal tissue explant samples. During the early luteal phase at 30 minutes incubation, a marked increase in PGF-2α production was observed. This immediately dropped to baseline. During the mid luteal phase oxytocin appeared to exert a stimulatory effect on PGF-2α production.

TABLE 4:12

Effects of oxytocin (1iu/ml) on PGF-2α production (ng/g tissue) by luteal tissue explants removed from mares at different stages of the oestrous cycle.

STAGE OF OESTROUS CYCLE	NO. OF MARES SAMPLED	INCUBATION TIME (mins)	CONTROL	OXYTOCIN (1iu/ml)
			PGF-2α (ng/g tissue)	
EARLY	4	**0**	125 ± 216	10 ± 7
		30	564 ± 619	4987 ± 6997
		60	896 ± 768	134 ± 77
		120	1314 ± 1253	309 ± 268
MID	3	**0**	478 ± 607	690 ± 690
		30	614 ± 797	1122 ± 1118
		60	877 ± 1087	1376 ± 1334
		120	1025 ± 1142	1406 ± 1188
LATE	2	**0**	2328 ± 2172	349 ± 117
		30	4504 ± 596	1623 ± 1157
		60	10319 ± 3319	3675 ± 2965
		120	16425 ± 8429	10098 ± 6510

± SEM

Table 4:13

From this table it would appear that oxytocin did not have any effect on PGF-2α production by the luteal cells in single cell suspension at any stage of the incubation period.

TABLE 4:13

Effects of oxytocin at 2 dose rates on PGF-2α production (pg/10^3 cells) by luteal cells in single cell suspension from mares at different stages of the oestrous cycle.

STAGE OF OESTROUS CYCLE	NO. OF MARES SAMPLED	INCUBATION TIME (mins)	CONTROL	OXYTOCIN (0.1iu/ml)	OXYTOCIN (1.0iu/ml)
				PGF-2α(pg/10^3 cells)	
EARLY	7	**0**	56 ± 70	108 ± 120	64 ± 95
		30	100 ± 93	84 ± 83	80 ± 83
		60	176 ± 125	122 ± 119	118 ± 123
		120	189 ± 126	29 ± 205	180 ± 177
MID	11	**0**	90 ± 111	45 ± 34	39 ± 35
		30	147 ± 160	88 ± 72	79 ± 56
		60	248 ± 181	137 ± 60	152 ± 61
		120	294 ± 131	277 ± 120	245 ± 102
LATE	6	**0**	329 ± 303	203 ± 169	135 ± 220
		30	324 ± 231	470 ± 390	609 ± 457
		60	686 ± 106	779 ± 290	651 ± 286
		120	1239 ± 454	1164 ± 373	1189 ± 622
FOLLICULAR	6	**0**	56 ± 70	30 ± 30	32 ± 45
		30	54 ± 59	118 ± 119	393 ± 303
		60	114 ± 139	210 ± 142	344 ± 218
		120	216 ± 112	453 ± 398	1098 ± 1329

± SEM

Table 4:14

During the early and mid luteal phases oxytocin appeared to exert a slight stimulatory effect on PGF-2α production by the endometrial tissue explant samples. However an inhibitory effect was observed during the late luteal phase.

TABLE 4:14

Effects of oxytocin (1iu/ml) on PGF-2α production (ng/g tissue) by endometrial tissue explants removed from mares at different stages of the oestrous cycle.

STAGE OF OESTROUS CYCLE	NO. OF MARES SAMPLED	INCUB. TIME (mins)	CONTROL	OXYTOCIN (1iu/ml)
			PGF-2α (ng/g tissue)	
EARLY	4	**0**	2513 ± 2534	2922 ± 2549
		30	5127 ± 5030	6274 ± 4783
		60	5262 ± 3633	6989 ± 4892
		120	7819 ± 5347	10708 ± 7741
MID	3	**0**	272 ± 285	0 ± 0
		30	1040 ± 1248	1584 ± 1498
		60	1398 ± 1432	3056 ± 2930
		120	1564 ± 1633	4303 ± 4033
LATE	2	**0**	1129 ± 691	1251 ± 902
		30	5946 ± 4356	2792 ± 1170
		60	10697 ± 4417	5828 ± 2152
		120	17266 ± 6511	12482 ± 2384

± SEM

Table 4:15

From Table 4:15 it would appear that oxytocin had no marked effect on PGF-2α production by the endometrial cells in single cell suspension at any stage of the oestrous cycle.

TABLE 4:15

Effects of oxytocin at 2 dose rates on PGF-2α production (pg/10^3 cells) by endometrial cells in single cell suspension cultures from mares at different stages of the oestrous cycle.

STAGE OF OESTROUS CYCLE	NO. OF MARES SAMPLED	INCUBATION TIME (mins)	CONTROL	OXYTOCIN (O.1iu/ml)	OXYTOCIN (1iu/ml)
				PGF-2α(pg/10^3 cells)	
EARLY	8	**0**	42 ± 60	21 ± 29	32 ± 40
		30	98 ± 169	69 ± 58	95 ± 110
		60	175 ± 212	100 ± 91	147 ± 120
		120	301 ± 212	258 ± 180	365 ± 242
MID	8	**0**	185 ± 143	138 ± 188	52 ± 47
		30	217 ± 164	229 ± 172	204 ± 188
		60	263 ± 152	268 ± 150	323 ± 230
		120	339 ± 233	434 ± 221	498 ± 292
LATE	6	**0**	437 ± 519	664 ± 390	574 ± 532
		30	612 ± 386	992 ± 334	1412 ± 765
		60	1110 ± 556	1223 ± 157	1561 ± 567
		120	2312 ± 1341	1784 ± 402	2000 ± 388
FOLLICULAR	5	**0**	69 ± 124	2 ± 3	26 ± 35
		30	400 ± 706	210 ± 317	254 ± 202
		60	181 ± 285	216 ± 189	247 ± 164
		120	278 ± 408	705 ± 1078	298 ± 285
	± SEM				

Table 4:16

The concentrations of PGF-2α released from the tissues demonstrated in Table 4:16 do not demonstrate a gradual increase in concentrations. No apparent pattern to the release can be determined in these samples.

Table 4:16

Effects of freezing/storage on PGF-2α production by endometrial and luteal tissues.

MARE IDENTITY	STAGE OF OESTROUS CYCLE	TISSUE ORIGIN	INCUBATION TIME (mins) PGF2α production (pg/10^3 cells)			
			0	30	60	90
11	EARLY	ENDOMET.	161	11	91	112
12	MID	ENDOMET.	648	36	230	177
9	LATE	LUTEAL	214	102	109	24

Plate 4:1

Gross examination Early luteal phase

At this stage the CL consisted primarily of a large blood clot filling almost the whole of the ovarian substance (arrow). The structure was pliable when pressure was applied and on sectioning appeared either dark red/brown in colour and fluid-filled or dark red with a thick, sticky consistency.

Plates 4:2 and 4:3

Histological examination Early luteal phase

Histological examination demonstrated a lacey and insubstantial appearance to the luteal tissue which was found around the outer edges of the structure at this stage. The predominant cell type was a large, pale vacuolated cell (arrow). The central cavity full of debris and blood clot was very evident at low power and the tissue appeared highly vascular. At high power several large pale luteal cells can be seen with numerous blood cells throughout the section.

Plate 4:1

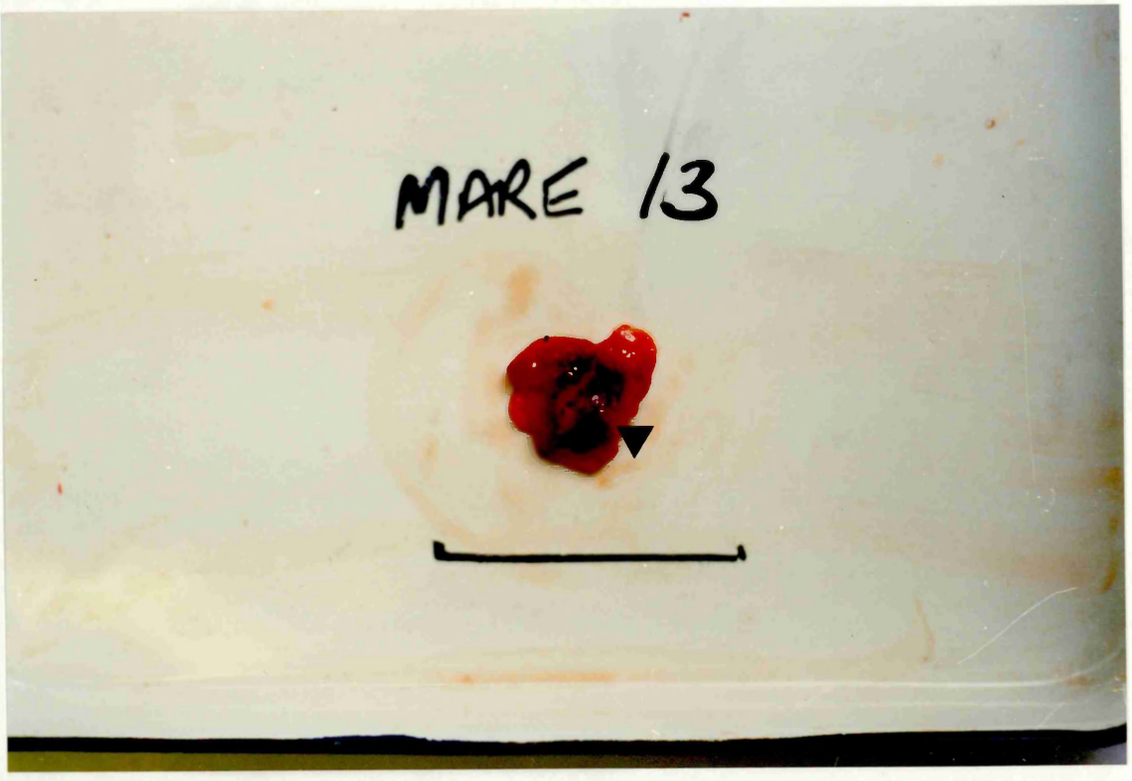


Plate 4:2 (Magnification X4)

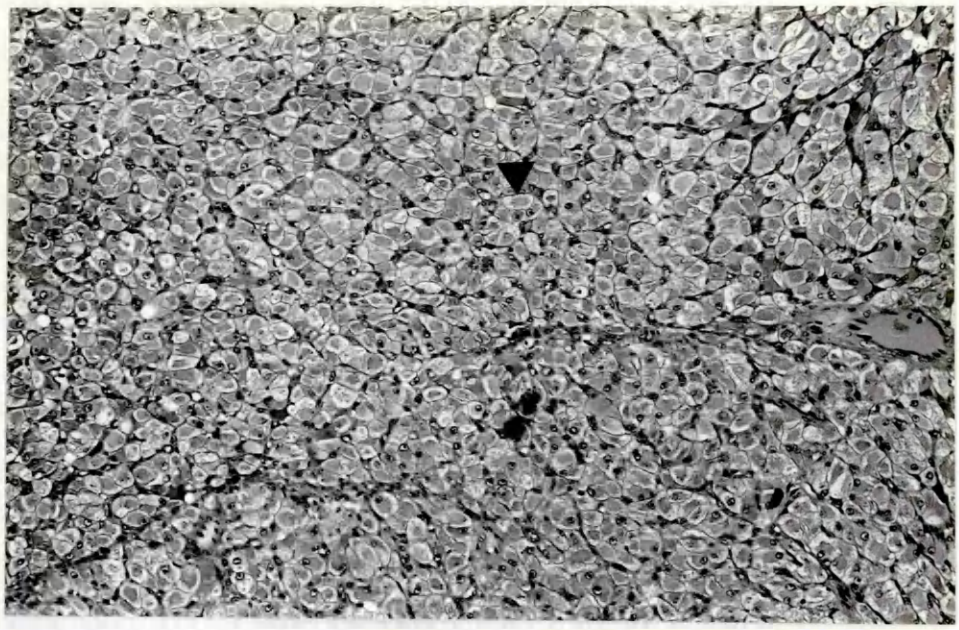


Plate 4:3 (Magnification X650)

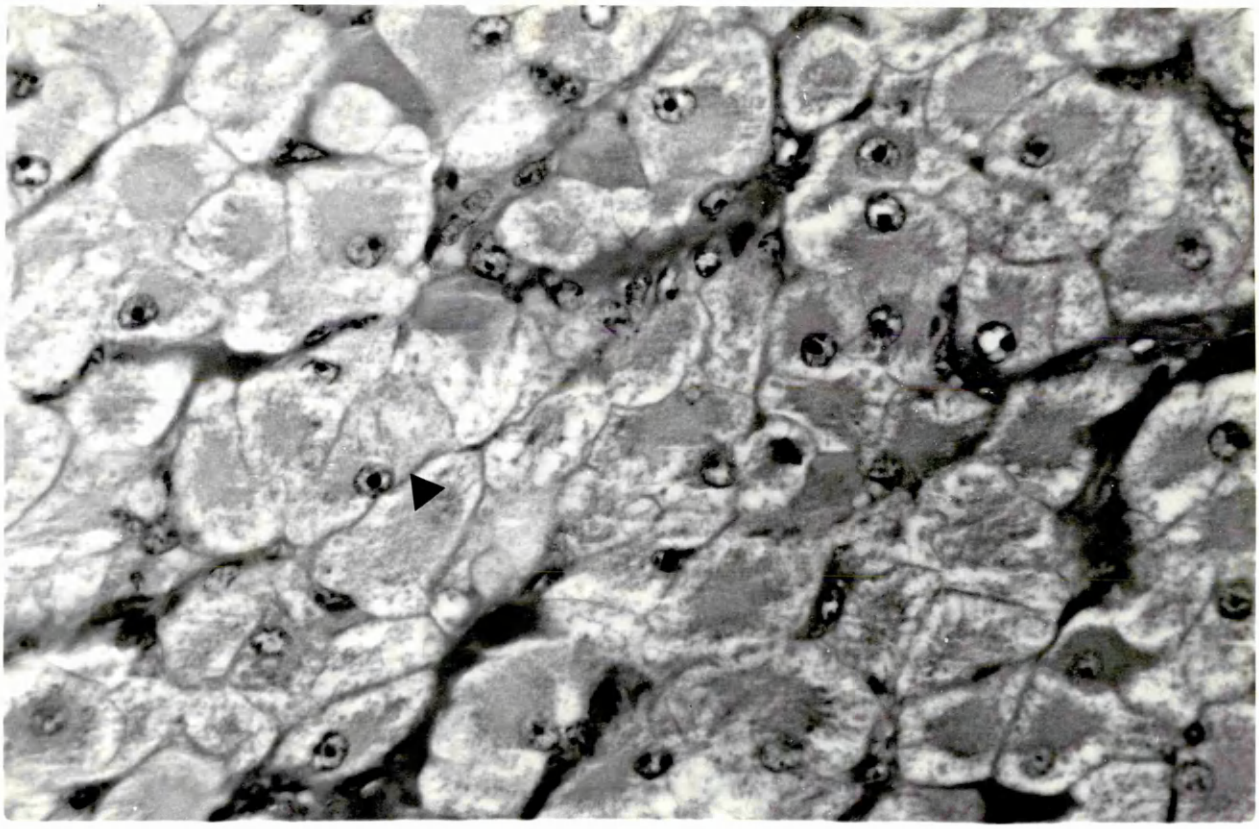


Plate 4:4

Gross examination Mid luteal phase

By the mid luteal phase of the oestrous cycle the CL appeared smaller and more compact than the early luteal phase due to a reduction in the central blood clot and an increase in the organised luteal tissue. On section the luteal tissue appeared red/brown in colour with a firm, fleshy consistency (arrow).

Plates 4:5 and 4:6

Histological examination Mid luteal phase

There appeared to be an increase in the total amount of luteal tissue present at this stage due to both hypertrophy and hyperplasia of the luteal cells and a reduction in the central cavity. Fine strands of connective tissue were evident giving the CL a lobullated appearance. The structure appeared very vascular. The luteal cells were primarily the large, pale vacuolated cells but small, dark cells with eosinophillic cytoplasms were evident along the edges of the connective tissue strands and along the edges of the CL. The small dark cells are readily seen at higher power (arrow).

Plate 4:4

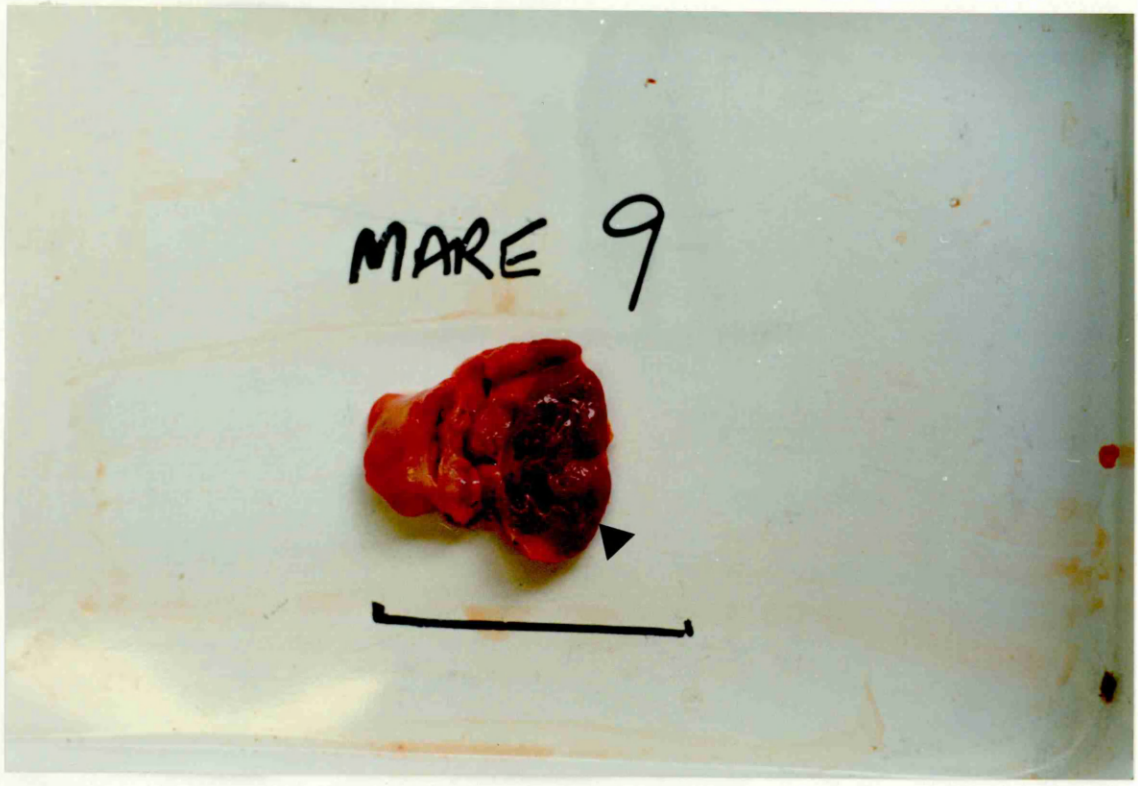


Plate 4:5 (Magnification X4)

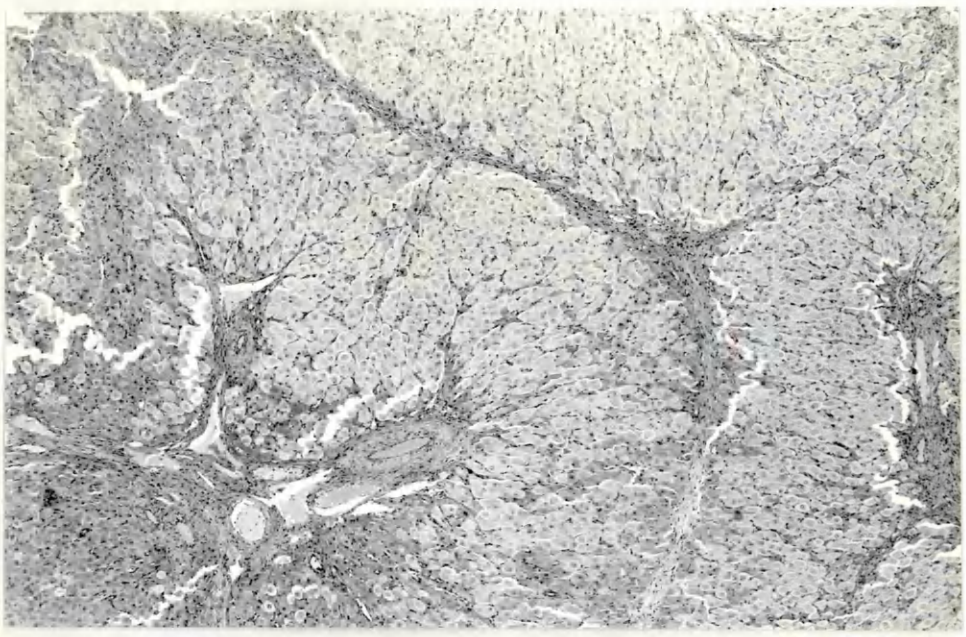


Plate 4:6 (Magnification X650)

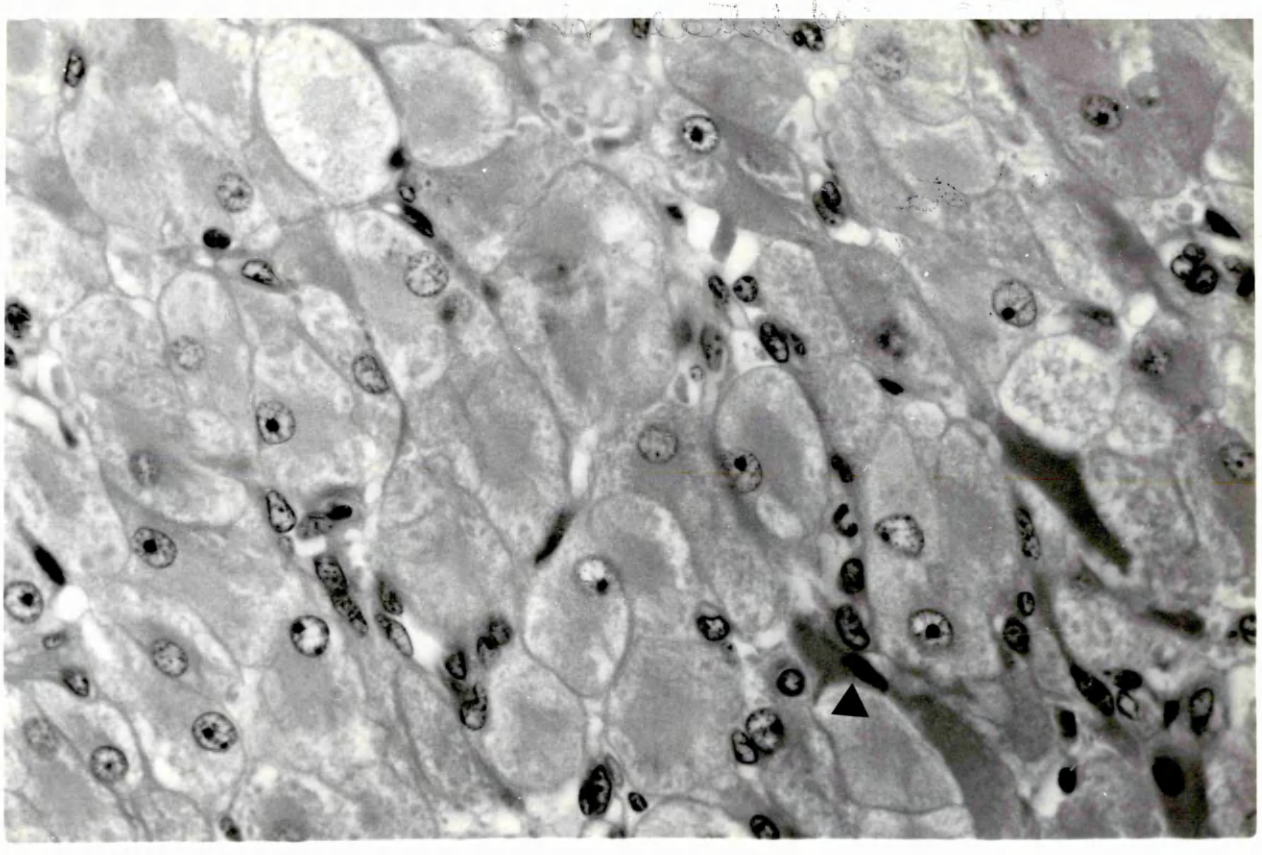


Plate 4:7

Gross examination Late luteal phase

By the late luteal phase of the oestrous cycle the CL appeared as a firm red/brown structure embedded in the substance of the ovary (arrow), often with an obvious brown tract leading from the CL through the pale ovarian stroma to the ovulatory fossa.

Plates 4:8 and 4:9

Histological examination Late luteal phase

Histologically there was an increase in the connective tissue component of the CL at this stage producing obvious lobullation. On low power the structure had a striated appearance as the cells appeared to radiate towards the centre of the gland. Small dark cells were evident throughout the luteal substance (arrow) although the majority of the cells appeared to be the large pale cells. There appeared to be a marked increase in the intracellular spaces leading to a less compact appearance.

Plate 4:7

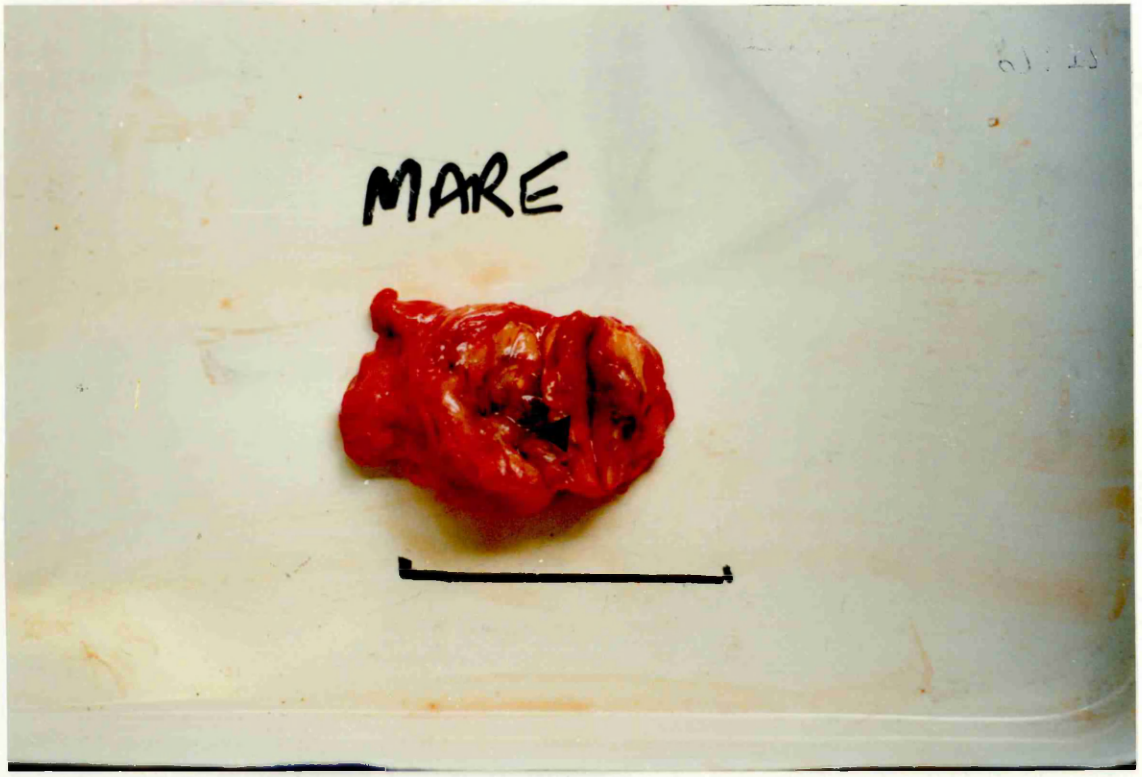


Plate 4:8 (Magnification X4)

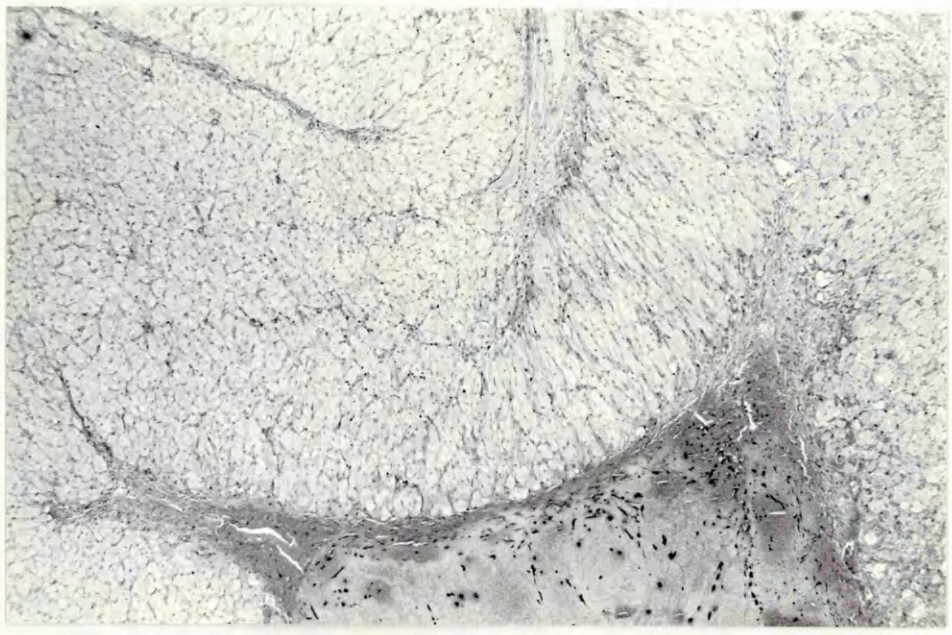


Plate 4:9 (Magnification X650)

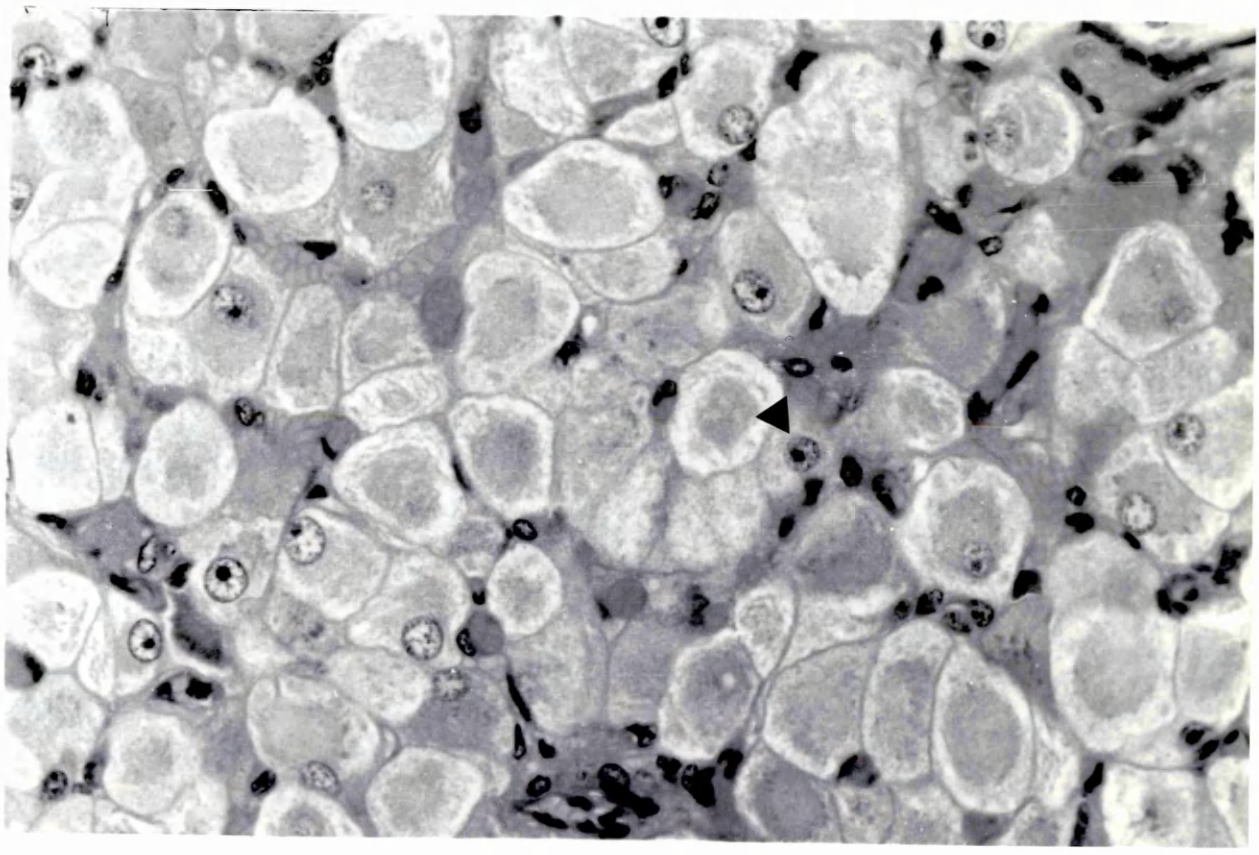


Plate 4:10

Gross examination Follicular phase

By the follicular phase of the oestrous cycle the regressing CL appeared small and yellow/orange in colour with a greasy, fat consistency (arrow).

Plates 4:11 and 4:12

Histological examination Follicular phase

The luteal tissue appeared less dense during this stage due to an increase in the intracellular spaces. All the luteal cells appeared small and dark on low power with marked evidence of degeneration of the cells. The cells appeared to be full of an amorphous substance, thought to be cholesterol stored in the cells for involvement in the synthesis of progesterone. At higher power the cytoplasm of the cells appeared shrunken and the nuclei pyknotic (arrow). The overall picture was of a dying, degenerating structure.

Plate 4:10

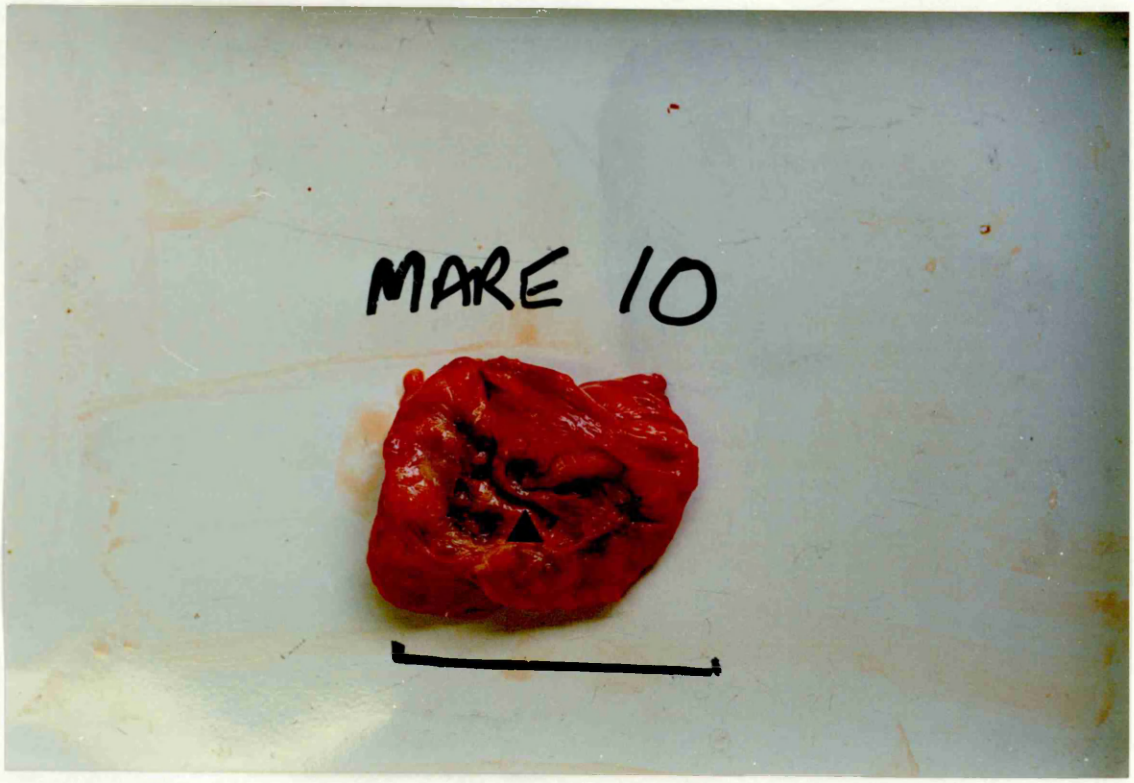


Plate 4:11 (Magnification X4)

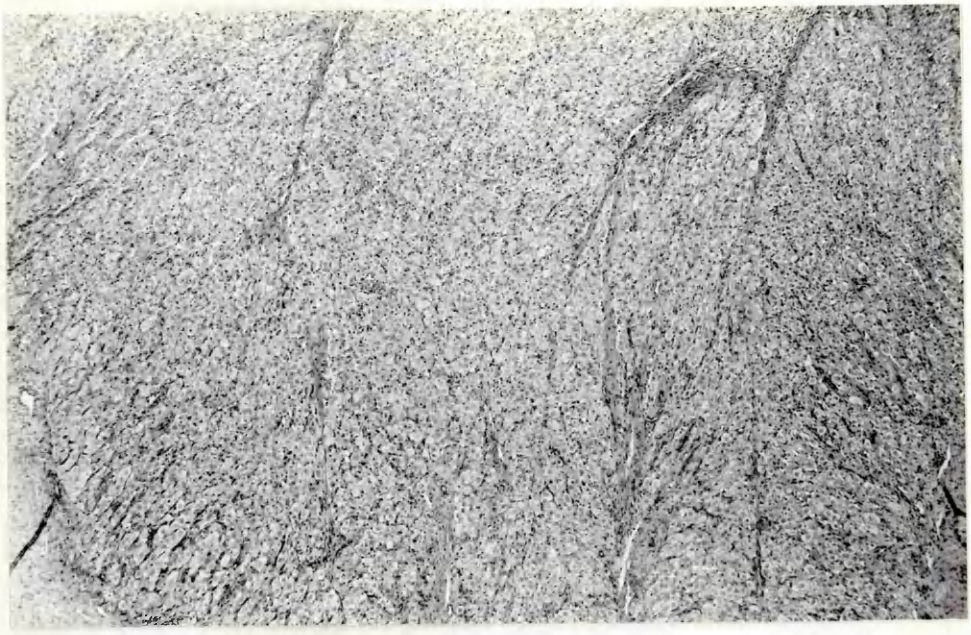
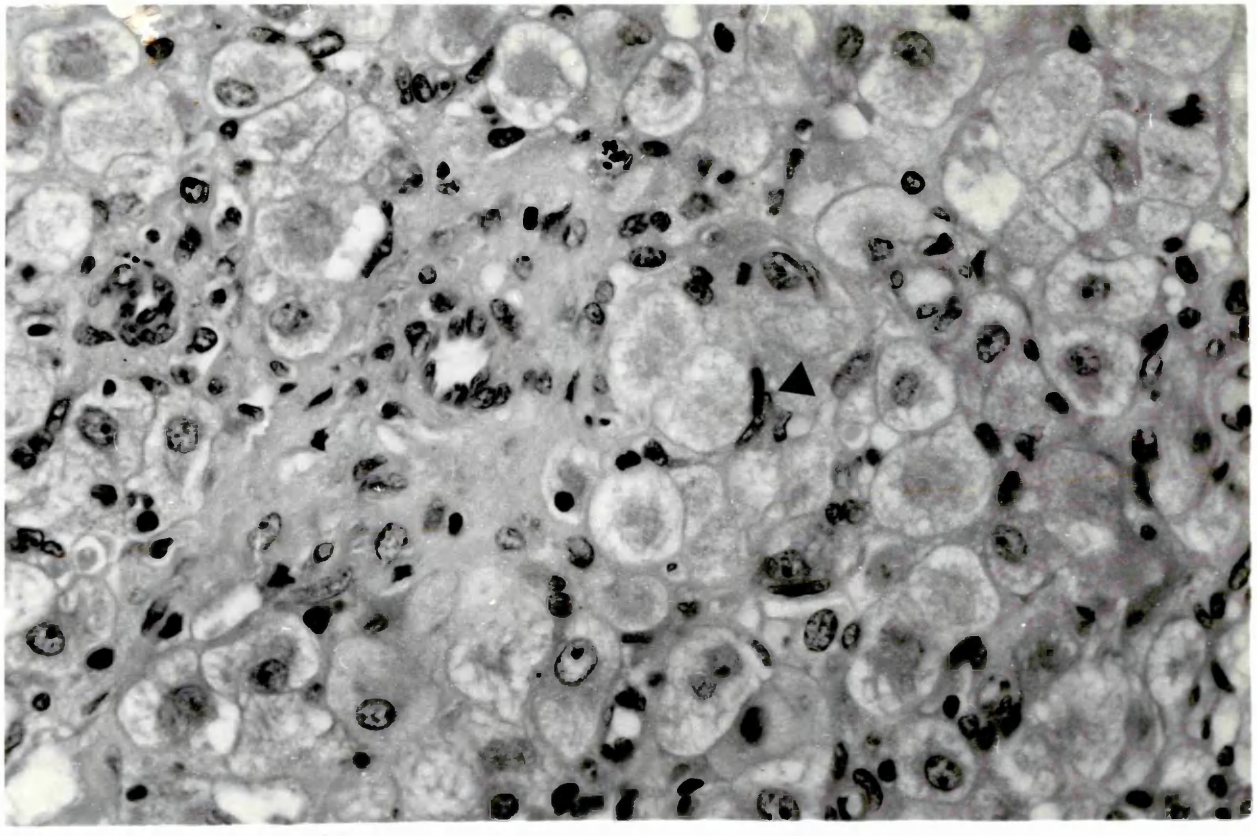


Plate 4:12 (Magnification X650)



4.4 DISCUSSION

To determine if a secondary source of PGF-2α was present in the mare, samples of endometrial and luteal tissues were obtained from mares immediately after slaughter. To allow transportation of samples from the slaughterhouse to the laboratory, a distance of over 200 miles, preservation of the tissue was required. The tissue was therefore placed in cryotubes and snap frozen in liquid nitrogen. Vernon *et al* (1981) had reported satisfactory results from freezing uterine tissue for receptor binding studies and King and Evans (1987) carried out snap freezing of endometrial and luteal tissue in liquid nitrogen prior to culturing for assessment of PGF-2α production. The results of the comparison of PGF-2α production by fresh and frozen tissue from a local source indicated that PGF-2α concentrations released from fresh and frozen tissue were comparable (Table 4:1). However it was found that some of the samples gave odd results or the tissue released no PGF-2α concentrations at all during the incubation period (Table 4:16). This suggested that the tissue was damaged either by the freezing process or by storing it in liquid nitrogen. As tissue was taken from more than one site of the uterus or CL, results were obtained from each mare.

The addition of a cryoprotectant to the tissues before freezing, such as dimethyl sulfoxide, might have increased preservation of the tissue. A more gradual freezing process, taking the temperature of the tissue down to -70°C prior to immersion in liquid nitrogen, may have minimised tissue damage due to the formation of ice crystals inside the cells. Also the addition of a solution of indomethacin in medium to the samples would have inhibited any release of prostaglandin from tissues damaged by freezing. However these procedures were not adopted in the studies reported.

As no reproductive history of the mares was available, an accurate method for determining the stage of the oestrous cycle had to be devised. Van Nierkerk (1973) categorised the CL of the mare by gross and histological changes of the CL at different stages of the oestrous cycle. Obviously the ideal method would have been to assess the stage of the oestrous cycle in the live mare, cull the mare and take various samples at the appropriate stage.

However in these investigations assessment of the stage of the oestrous cycle was determined by:

a) Macroscopic examination of the reproductive tract immediately after slaughter

b) Examination of histological sections from CL.

Initial gross examination allowed segregation into active and non-active CL. The newly ovulated CL appeared sticky and full of brown, viscous fluid and took up a large part of the ovarian substance. The ovary therefore appeared large and very soft. This was considered to be the Corpus haemorragicum. With increasing organisation the CL appeared red/brown in colour, with a fleshy/firm consistency. It appeared more compact and smaller but still occupied a large part of the ovary. As the CL aged it appeared increasingly firm and brown. The non-active CL tended to appear yellow/orange and greasy in consistency. The non-active CL tended to be smaller in size than the active CL.

Further segregation was carried out by examination of histological sections. This allowed segregation into the following categories:

a) Early luteal phase active
b) Mid luteal phase active
c) Late luteal phase active
d) Follicular phase non-active

During the early luteal stage ovulation had occurred relatively recently and luteal tissue was beginning to develop around the outer edge of the structure, therefore on histological section there appeared a large central area full of blood, debris and cells with lacey, insubstantial tissue around the outer edge. At this stage of the oestrous cycle the principal luteal cell type was a large, pale-staining cell with a vacuolated cytoplasm. The tissue was very vascular at this stage and there was little connective tissue apparent. By the mid luteal stage the luteal tissue had increased leading to a reduction in the central cavity. The luteal cells had become more organised with connective tissue strands penetrating the substance of the CL, leading to lobullation of the structure. Small dark cells had begun to appear along the connective tissue tracts. At this stage the tissue was still very vascular.

By the late luteal phase the CL appeared very organised with marked lobullation of tissue. The cells grew in tracts, leading to a striated appearance in the CL. Cell type was primarily large and pale but there was an increase in the numbers of small dark cells present. Evidence of degeneration of the luteal cells appeared at this stage with an increase in inflammatory cells, primarily macrophages and eosinophils.

By the follicular stage of the oestrous cycle luteolysis had taken place and the CL appeared as a dead and dying structure. Degenerating cells appeared full of fat and there was evidence of phagocytosis by increased numbers of inflammatory cells. Connective tissue was abundant at this stage.

The large pale-staining cells are thought to be the active progesterone producers. The vacuolated appearance was due to progesterone, stored in the cytoplasm, being washed out during the staining process (Van Nierkerk, 1973). They are abundant during the early and mid luteal phases when progesterone secretion by the cells is maximal. As the CL ages there is an increase in the numbers of small dark cells. Van Nierkerk thought that these cells represented a resting stage of the progesterone secretor cells and he reported that by Day 12 post-ovulation the major cell type of the CL was the small dark cell. In this study the numbers of these cells appeared to increase as the CL aged but by the late stage the large, pale-staining cells still appeared to be in the majority, although no actual count of cells was taken. Van Nierkerk suggested that by Day 14 post-ovulation the small dark cells did not revert to the pale-staining cells but degenerated. Evidence of this was not found in this study as it appeared that prior to degeneration the luteal cells became filled with fat, due to a build-up of cholesterol in the cytoplasm of the cells not synthesising progesterone (Challis *et al*, 1976). This build-up of fat led to the greasy, yellow consistency in the older, non-active structure. It is possible therefore that the small dark cells are physiologically distinct cells from the large pale cells. This would agree with the findings in the CL of the ewe, that the two cell types are physiologically distinct (O'Shea, 1987). Phagocytosis by the inflammatory cells followed by degeneration of the structure was then observed.

These studies allowed ageing of the various tissues obtained at slaughter and gave an indication of the stage of the oestrous cycle, prior to the assessment of PGF-2α production by the tissues. However this method of ageing was not ideal and some overlap of the stages obviously occurred, leading to inaccuracies in the results obtained. However under the conditions available, this method was considered to be satisfactory.

The initial attempts to assess PGF-2α production by the CL and endometrial tissues of the mares, where explants of tissue were incubated *in vitro* and assayed for PGF-2α production gave inconclusive results (Tables 4:2 and 4:6). However the results demonstrated that both the luteal and the endometrial tissue survived the freezing process and were capable of PGF-2α production at all stages of the oestrous cycle. During the early luteal stage endometrial tissue PGF-2α production was relatively high but a marked drop in production was observed during the mid luteal stage. Poyser (1981) suggested that progesterone inhibits PGF-2α production. The increased release of progesterone during the mid luteal phase, compared to the early luteal phase, may be the reason for the apparent decrease in PGF-2α production during the mid luteal phase. No progesterone results were available from the explant samples, however the effect was not demonstrated in the luteal tissue samples therefore it seems unlikely that the high concentrations of PGF-2α in the early luteal phase compared to the mid luteal phase was due to this effect. Red blood cells release significant amounts of PGF-2α and it maybe that the endometrial tissue had a high percentage of these cells present. However early luteal phase luteal tissue should have a high preponderance of red blood cells due to the corpus haemorragicum. The other possibility is that the endometrium still remains capable of a higher production of PGF-2α during the early luteal phase after the previous luteolysis. The other possibilty is that PGF-2α was released in response to endometrial tissue damage caused by the freezing and storing of the tissue.

During the late luteal phase there was a marked increase in PGF-2α from CL and endometrial tissue after 60 minutes incubation even compared to the early luteal phase. This tissue was taken from mares thought to be at the expected time of luteolysis and would suggest that an increased production of PGF-2α from the endometrium occurred in the mares during luteolysis.

In an attempt to determine if PGF-2α production by the endometrial and luteal explants was due to *de novo* synthesis from the tissue, indomethacin was added to the tissue samples. Indomethacin inhibits PGF-2α synthesis by inhibiting the enzyme cyclo-oxygenase, a vital enzyme in the synthetic pathway of PGF-2α (Vane, 1971). As prostaglandin is not stored in cells but synthesised and released as required, inhibition of the synthetic pathway will give an indication of whether or not PGF-2α is being actively synthesised by the cells (Smith and Lands, 1971). A concentration of 1ug indomethacin/ml and 10ug indomethacin/ml was added to the incubates. However the indomethacin did not go into solution but produced a precipitate. When samples with indomethacin were assayed for PGF-2α the concentration of PGF-2α was found to be greatly increased compared to the control samples. When the indomethacin was pelleted by centrifugation the PGF-2α concentrations declined but not to below control sample concentrations. An attempt was then made to dissolve the indomethacin in a small volume of absolute alcohol before adding to the culture. However this again apparently interfered with the assay.

Three standard curves were set up with the addition of:

1) indomethacin
2) indomethacin dissolved in absolute alcohol
3) absolute alcohol

to each of the standards.

In each case a straight line was produced.

Indomethacin is an acknowledged prostaglandin inhibitor and is the drug of choice in most *in vitro* experiments and so should not be capable of stimulating PGF-2α production from uterine and luteal tissues. The reason for the apparent increase in PGF-2α production was thought to be due to a physical interference of the indomethacin and alcohol with the Scintillation counter giving unrealistically high counts. The results from the standard curves with additional indomethacin and alcohol confirms this hypothesis. However the reasons why indomethacin was interfering in the assay remain unclear. Two different batches of indomethacin were tried and each gave the same results therefore the effect did not appear to be due to contamination of a batch. No reports of this problem could be found in the literature. In an attempt to overcome this problem, soluble aspirin was added to the incubates instead of indomethacin.

Several different concentrations of aspirin were tried but eventually a concentration of 1ug/ml was found to optimally inhibit PGF-2α production without causing any interference in the assay. Soluble aspirin was therefore used instead of indomethacin in this study.

To try to determine if the effects observed in the explant samples were real or not a single cell suspension culture was established. Initially the study set out to investigate if any difference between PGF-2α production from different areas of the uterus was apparent. Sections of endometrial tissue were removed from both the uterine horn tips, the horns themselves and the uterine body (Table 4:7). There appeared to be a marked increase in PGF-2α production from the right horn of Mare C at 120 minutes incubation but this was thought to be probably due to an artefactual effect. As the tissue culture method and the PGF-2α assay were not accurate enough to allow quantitative comparisons between the results obtained and, as no obvious differences between the different areas of the uterus were apparent, this part of the study was stopped and only endometrial sections from the body of the uterus were taken.

In both endometrial and luteal single cell suspensions (Tables 4:3 and 4:8) PGF-2α was released from the cells at all stages of the oestrous cycle with a gradual increase in PGF-2α production during the incubation period. A slight increase in PGF-2α production was observed between the early and mid luteal stages in both luteal and endometrial tissues but this increase was not statistically significant. In both the luteal and endometrial tissue samples the standard error was high. This effect was thought to be caused by the marked variations in the actual PGF-2α values, probably caused by variations in the exact stage of the oestrous cycle between the mares and also a natural variation in PGF-2α production between the relatively small numbers of mares examined.

During the early and mid luteal phases progesterone concentrations increased in the plasma as the CL became fully functional and luteal progesterone production increased during this period as expected. It appeared therefore that when progesterone production was maximal, PGF-2α production by both the luteal and endometrial cells was minimal. These results contrast with the explant results. However as the numbers of samples examined was increased in the single cell suspension and as this method was more sophisticated, it was

assumed that these results gave a more accurate picture of PGF-2α release from the luteal and endometrial tissues.

During the late luteal stage endometrial and luteal PGF-2α demonstrated a marked increase in production compared to the early and mid luteal stages. In both the endometrial and luteal samples the increase observed was statistically significant ($P > 0.01$ luteal, $P > 0.001$ endometrial). In each case aspirin led to inhibition of PGF-2α production, suggesting that the PGF-2α released was due to *de novo* synthesis by the tissues.

During the late luteal phase the standard error was within acceptable limits in both the luteal and endometrial samples, suggesting that the actual values of PGF-2α released by the cells of individual mares were more consistent.

During the late stage of the oestrous cycle lysis of the CL is thought to be taking place (Stabenfeldt *et al*, 1981). Neely *et al* (1979a) suggested that PGF-2α is released from the uterus during luteolysis, leading to raised PGFM concentrations in the circulating plasma and lysis of the luteal tissue. This study agrees with these findings. PGF-2α production by the endometrial tissue increased during the late luteal phase around the expected time of luteolysis and it would seem likely that this PGF-2α was involved in lysis of luteal tissue. However in this study PGF-2α production from luteal tissue was also demonstrated. By comparison of the results of PGF-2α production of the two different tissues (Table 4:10) it appeared that synthesis and release from the endometrial and luteal tissue was almost equivalent at the relevant stages of the oestrous cycle at all incubation times. Although there appeared to be a slight increase in endometrial production compared to luteal production after 60 minutes incubation at the late luteal phase, in the endometrial samples the standard error was greater, suggesting that PGF-2α production by the tissue from the different mares was not so consistent as that recorded in the luteal samples.

These results agree with the findings in other domestic species ie the rat (Olofssun *et al*, 1988), pig (Guthrie, Roxroad and Bolt, 1978), cow (Shemesh and Hansell, 1975) and rabbit (Daniels *et al*, 1988) that the CL is capable of synthesising and releasing PGF-2α at the expected time of luteolysis. Although the timing of the exact stage of the oestrous cycle was not accurate in this study, progesterone production by the luteal cells and plasma progesterone were still high. Therefore PGF-2α production by the luteal cells occurred prior to the

fall in progesterone concentrations associated with luteolysis. It would seem likely that this PGF-2α becomes actively involved in lysis. Workers suggested that PGF-2α released by the CL of the other domestic species was involved in lysis of luteal tissue and the return to standing oestrus. The pig is of particular interest as it resembles the mare in that no counter-current exchange mechanism exists (Harrison, 1979). To increase the concentration of unmetabolised PGF-2α at the CL it was hypothesized that not only PGF-2α released from the endometrium but PGF-2α released from the CL was also involved in luteolysis in this specis (Patek and Watson, 1976). In marsupials hysterectomy has no effect on luteolysis. The interoestrous periods are not disrupted. It would appear in these species that intraluteal prostaglandins are the sole source of luteolytic prostaglandins (Gemmell, 1984).

Ginther and First (1971) demonstrated that, in the mare, hysterectomy leads to prolonged inter-oestrous periods and irregular cycles. However although the uterus is vital to the regular cycle of the mare a more complicated interaction of events may occur, involving integration of uterine and luteal PGF-2α to ensure lysis proceeds to completion. Therefore PGF-2α production by the uterus maybe the trigger required to stimulate PGF-2α production from the luteal cells. This would solve the apparent anomaly of PGF-2α, a locally acting compound, travelling from the uterus to the ovary, via the systemic circulation, to bring about luteolysis. Luteal PGF-2α, released at the site of action, would not be subjected to metabolism by the lungs and so a high concentration of unmetabolised PGF-2α would be available at the CL to bring about luteolysis and the return to standing oestrus.

The histological investigations into the cells of the CL of the mare carried out in this study indicate that two distinct cells types may be present at different stages of the oestrous cycle.

During the mid luteal phase of the cycle small dark cells were evident along the edge and around the periphery of the CL. During the late luteal phase the numbers of dark cells present in the CL appeared to increase, although the actual percentage increase was not quantified. By relating the histological findings of the two distinct cell types to the PGF-2α production results from the luteal tissue at the different stages of the oestrous cycle, tends to suggest that PGF-2α

production from the luteal cells increased as the numbers of dark cells present in the luteal tissue increased.

In the ewe, Silvia *et al* (1984) reported that the two types of luteal cells were physiologically distinct. In the mare it is possible that the two cell types are also physiologically distinct and that the large luteal cells are concerned with progesterone production, while the small dark cells are involved with the synthesis and release of PGF-2α. This could explain the relatively low PGF-2α production during the early and mid luteal phases when the majority of the luteal cells are the large, pale progesterone secretors. As the CL ages and the number of dark cells present in the luteal tissue increases so the capacity of the CL for PGF-2α production markedly increases. Therefore, around the time of luteolysis, adequate numbers of dark cells are present, synthesising high concentrations of PGF-2α. However without an accurate method of ageing the luteal tissue and a method for identifying the different cell types and their relative percentages, no firm conclusions regarding the role of the different luteal cell types in the mare can be drawn from the findings in this study.

In an attempt to determine if oxytocin is involved in stimulating PGF-2α production from the luteal and endometrial tissues of the mare, exogenous oxytocin was added to the luteal and endometrial tissue incubates in this study. Oxytoxin has been reported to play an important role in the control of PGF-2α synthesis and release from the endometrium during luteolysis in several of the domestic species (Wathes, 1984), including the mare (Tetzke *et al*, 1987). Tan *et al* (1982) also demonstrated that oxytocin exerted a luteolytic effect on the CL of the human. King and Evans (1987) demonstrated an increased production of PGF-2α from endometrial cells removed from the uteri of mares and cultured *in vitro*, in response to oxytocin added to the incubation medium. These workers concluded that oxytocin played some role in luteolysis in the mare, being involved in the stimulation of PGF-2α production from the endometrium. In this study the effects of oxytocin on PGF-2α production by the luteal and endometrial tissues did not give consistent results (Tables 4:12-15). In the luteal tissue explants an inhibitory effect was observed during the early luteal phase, although at 30 minutes incubation a massive increase in PGF-2α production by the tissue was observed. This was caused by the very high result from one sample and if this sample was removed no increase at this stage was observed therefore this effect was

likely to be an artefact. During the mid luteal phase oxytocin did not have any effect on PGF-2α production at all. By the late luteal phase however oxytocin appeared to exert an inhibitory effect on PGF-2α production by the luteal and endometrial tissues. In the single cell suspension cultures oxytocin exerted no effect on PGF-2α production at any incubation time or at any dose rate, in either the luteal or endometrial cells.

Due to the contradictory results of the single cell suspensions and the tissue explants the results from the explant samples were placed in doubt. The explant tissue culture was a cruder method with smaller numbers of samples and only one dose rate of oxytocin used, therefore it was assumed that these results were not a true reflection of the effect of oxytocin on PGF-2α production by the luteal or endometrial tissues. The results from this study therefore suggest that exogenous oxytocin added to the tissue incubates does not exert a stimulatory or inhibitory effect on PGF-2α synthesis and release by either the luteal or the endometrial cells. This would suggest therefore that oxytocin is not involved in the events of luteolysis in the mare. These findings agree with those of Neely *et al* (1979b) and Arthur and Allen (1972) who were unable to induce precocious oestrus in mares in dioestrus with injections of exogenous oxytocin. In addition Flint and Sheldrick (Per Comm) were unable to demonstrate significant concentrations of oxytocin in the plasma of several mares throughout the oestrous cycle. These workers concluded that oxytocin was not involved in luteolysis in the mare.

It is now evident that there are several flaws in the single cell suspension method used in this study. The problem of preservation of the tissue has already been discussed. The concentration of cells in each aliquot was too low. The concentration should probably have been about 1 x 10^5cells/ml instead of 1 x 10^3cells/ml. This increased concentration may have lead to more consistent results and lowered the standard errors. Although the viability of the cells was checked by the addition of Trypan blue to a sample of the cells, this percentage was not taken into account. It was felt that as all the cells had been treated in the same manner and as the percentage was approximately the same in each case (about 60% viability. This is low compared to other workers who got > 75% viability), a correction for this percentage did not have to be taken into account. However it is now considered that a correction for viability should have been

included as, due to the freezing of the cells, a decreased and variable viability was likely to have occurred, as Table 4:16 demonstrates.

However despite these problems, the studies undertaken in this thesis do tend to suggest that in the mare the pattern of prostaglandin release from the luteal tissue at the different stages of the oestrous cycle mimics the pattern of prostaglandin release from the endometrium. Although a comparison of the actual values is difficult, considering the nature of the assay and tissue culture method, it does seem that the concentrations of PGF-2α released by the CL, compared to those released by the uterine endometrium, are equivalent at all incubation times, during all the stages of the oestrous cycle. This would suggest that the CL is an important source of PGF-2α during luteolysis. PGF-2α released by the uterine endometrium must first pass through the lungs, where extensive metabolism is thought to take place, before reaching the ovary. PGF-2α released from the CL is not subjected to this metabolism and so a high concentration of unmetabolised PGF-2α at the CL would be obtained by the addition of luteal prostaglandin to the unmetabolised PGF-2α reaching the ovary from the uterus. This would allow lysis of the CL despite extensive prostaglandin metabolism in the lungs. The control mechanisms for luteal prostaglandin release are unknown, even in the species where luteal prostaglandins are known to be involved. From this study it would appear that oxytocin is not involved in stimulating PGF-2α release from the endometrium or CL in the mare, as in other species such as the ewe. It was concluded therefore that oxytocin was not involved in the events of luteolysis in the mare.

The results from this study therefore suggest that the uterus of the mare is not the only source of PGF-2α involved in luteolysis in the mare, but a more complicated interaction of events is involved with input from both the ovary and the uterus bringing about the ultimate demise of the CL and the return to standing oestrus.

5 CHAPTER FIVE
GENERAL DISCUSSION

The work presented in this thesis represents the results of an investigation into the mechanisms involved in luteolysis in the non-pregnant, cycling mare and some of the effects of prolonged dioestrus in the mare.

Ovulation had to be accurately recorded to determine the approximate time of luteolysis. Initial investigations therefore involved monitoring four mares throughout one oestrous cycle by palpation *per rectum* to allow assessment of the state of the reproductive tract, in particular the ovary and the cervix. A suggestion that the cervix may relax during luteolysis was not confirmed by closer study, therefore changes in the cervix could not be used as an aid to predicting the time of luteolysis. Plasma progesterone estimations were carried out to allow correlation of the hormonal changes with changes in the reproductive tract. This allowed expertise to be established in the estimation of the time of ovulation. Luteolysis was assumed to occur fourteen days after ovulation.

During luteolysis the compound prostaglandin F-2 alpha (PGF-2α) is released from the uterus and is thought to bring about lysis of the corpus luteum (CL) and the return to standing oestrus (Neely *et al*, 1979a). To try to determine the patterns of PGF-2α release two mares were blood sampled throughout one oestrous cycle, initially twice daily, but increasing to four times daily as luteolysis approached. PGF-2α has a short half-life (Piper *et al*, 1970) therefore the metabolite 13,14 dihydro 15 keto prostaglandin F-2 alpha (PGFM), which has a longer half-life, can be assayed as a relatively accurate marker of PGF-2α release (Kindahl, 1981). As PGFM is a small compound, variations in the radio-immunassay results frequently appeared and several problems had to be resolved, but eventually a relatively consistent PGFM assay was established. However examination of the two mares gave little information regarding PGFM patterns in the plasma as the blood sampling had not been carried out frequently enough. It was found that 3-6 hourly sampling was not adequate to establish an accurate pattern of PGFM release. Hourly blood sampling was therefore performed for a 38 hour period at the expected time of luteolysis and pulses of PGFM were recorded in the plasma of two mares. However as the full luteolytic period was not covered using this method the duration of the blood sampling period

was increased to 57 hours. A pattern of PGFM in the plasma did then became apparent from the additional information that was available. A single large pulse of PGFM occurring prior to the drop in progesterone, appeared to initiate PGF-2α release at around Day 14 post-ovulation. There then followed several smaller pulses of increased frequency and a drop in progesterone to baseline. In most of the mares examined the largest pulse of PGFM recorded was not observed until progesterone concentrations had reached baseline, possibly due to a negative feedback of progesterone on prostaglandin release. This pulsatile release of PGF-2α appeared to be important to the outcome of luteolysis. Thorburn *et al* (1973) demonstrated that a series of surges of PGF-2α was required to bring about lysis of the CL in the ewe. Although the majority of the mares examined demonstrated a drop in progesterone concentrations and a return to standing oestrus three of the mares examined did not return to standing oestrus. They remained in a state of prolonged dioestrus with raised progesterone concentrations, despite two of the mares exhibiting raised PGFM levels. However the pulses of PGFM released appeared to be of a reduced magnitude compared to the normal cycling mares. Although PGFM is an accurate marker for PGF-2α it gives no indication as to the source of the PGF-2α. It was therefore considered that there may be a secondary source of PGF-2α other than the uterus. It was hypothesized that this secondary source was not stimulated to release PGF-2α in mares in prolonged dioestrus and so prostaglandin was unable to reach adequate concentrations at the CL during luteolysis, thereby preventing lysis from taking place. The ovary was considered the most likely secondary source of PGF-2α.

To determine if a secondary source of PGF-2α was present in the ovary of the mare, sections of luteal tissue and endometrial tissue were removed from freshly slaughtered mares and frozen in liquid nitrogen. The endometrial tissue was used for comparison purposes. The gross anatomy and histology of the ovary and, in particular the CL, had to be examined to estimate the approximate stage of the oestrous cycle, as the reproductive history of the mares was not available. The tissue was then grouped into four types:

1) Early luteal phase
2) Mid luteal phase
3) Late luteal phase
4) Follicular phase

A method was established for *in vitro* culture of the tissue from the mares and a radio-immunassay for PGF-2α estimations in the tissue culture supernatant was also set up. The differences in production of PGF-2α by the various tissues during the four stages of the oestrous cycle outlined above were compared. It appeared that PGF-2α was released by the luteal cells of the mare at all stages of the oestrous cycle, but that this production was markedly increased during the late luteal phase of the cycle, around the expected time of luteolysis. This luteal PGF α production compared closely to the PGF-2α production from the endometrial tissues. Aspirin added to the culture medium inhibited PGF-2α production indicating that the concentrations in the supernatant were due to *de novo* synthesis. It was hypothesized that this release of PGF-2α from the CL must be closely involved in luteolysis, as the tissue and plasma progesterone concentrations were increased during this time, suggesting that the CL was active and luteolysis was not yet complete. This luteal PGF-2α production was thought to raise concentrations of unmetabolised PGF-2α reaching the CL from the uterus, bringing about lysis of luteal tissue and the return to standing oestrus. Due to inaccuracies in ageing of the tissue it was not possible to determine which of the two sources of PGF-2α was released first. Ginther and First (1971) demonstrated the importance of the uterus in the cyclicity of the mare therefore it is possible that the uterus is the trigger for luteolysis to begin but the CL is the final effector in luteolysis.

To try to determine if oxytocin was involved in the stimulation of PGF-2α production from either the CL or the endometrium during luteolysis, exogenous oxytocin was added to the tissue culture medium and the effects observed. Oxytocin did not appear to lead to an increased PGF-2α production at any stage of the oestrous cycle from either the luteal or the endometrial tissue and it was concluded that oxytocin was not involved in luteolysis in the mare.

During the histological investigations it appeared that there were two distinct luteal cell types, large and small, present in the CL. It was hypothesized that these small cells were involved in the synthesis of PGF-2α during luteolysis as their numbers appeared to increase with the ageing of the CL, reaching maximum at expected time of luteolysis. The large luteal cells are thought to act as progesterone producers.

Investigations from this study revealed that luteolysis in the mare was more complicated than first anticipated. It appeared to involve the integration of uterine and luteal events leading to the release of adequate concentrations of PGF-2α in a crucial pattern of pulses at a crucial stage in the oestrous cycle, in order to bring about luteolysis and the return to standing oestrus. Any breakdown in communication between the two areas may prevent the mare from returning to standing oestrus and entering a state of prolonged dioestrus.

During the course of this study several areas were raised that require further investigation. To further clarify if the CL is an important source of PGF-2α it would be possible to determine if the enzymes for PGF-2α synthesis were present in the cells of the CL and if there was any difference between the two luteal cell types in this respect. By using monoclonal antibodies the heterogenicity and interdependence of the two luteal cells could be determined and the possibility of the small dark cells being responsible for PGF-2α production further investigated.

In addition, cannulation of the ovarian artery and vein of the live mare would allow assessment of the concentration of unmetabolized PGF-2α entering and leaving the ovary and these results, when compared to the jugular PGFM concentrations, may give some indication of the source of unmetabolised prostaglandin reaching the CL. One important area not yet investigated in the mare is the percentage of PGF-2α metabolised on one passage through the lungs. Davis *et al* (1980) reported differing rates of metabolism in different species. These workers reported a slower rate of metabolism in the sow than in the cow or sheep. This is interesting from the fact that the sow, like the mare, does not have a counter-current exchange mechanism bypassing the systemic circulation. Heep, Fleet and Hamon (1985) reported the transfer of PGF-2α from uterus to ovary in the ewe via the lymph channels thus bypassing the systemic circulation. Alawachi, Bland and Poyser (1981) reported that transfer of PGF-2α could also take place via the oviducal vein, suggesting that the counter-current mechanism is not the only pathway of exchange. Therefore the importance of these two routes to the transfer of PGF-2α from uterus to ovary in the mare must be calculated before the importance of a secondary source of PGF-2α can be fully elucidated.

It would also be interesting to determine the source of PGFM in the plasma of mares in prolonged dioestrus, as it may be that PGF-2α is released from the endometrium of these mares but not from the CL, causing a decreased concentration of unmetabolised PGF-2α at the CL, thus preventing lysis from taking place. To determine the source of the PGF-2α released in these mares, samples of endometrial and luteal tissue could be removed and cultured *in vitro* to determine production rates from both tissues and to compare to PGF-2α production from endometrial and luteal tissue from normal cycling mares. It would also be worthwhile to calculate the relative percentages of light and dark cells present in the CL of these mares and compare this to the relative percentages in normal cycling mares.

It thus appears that many aspects of luteolysis in the mare remain unclear and further intensive study is required to fully elucidate these points. An increased knowledge of the events of luteolysis and why they frequently breakdown in the mare would allow more control over the oestrous cycle of the mare thus increasing her productivity.

6
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