MATERNAL FACTORS AFFECTING EARLY PREGNANCY IN SHEEP

CHERYL J. ASHWORTH, B.Sc.

Thesis presented for the degree of

Doctor of Philosophy

University of Edinburgh

1985

TO MUM AND DAD.

DECLARATION

I declare that this thesis is my own composition, and has not been accepted in any previous application for a degree. The work it describes and the ideas it contains are those of the author, unless otherwise stated. All help given by other people has been acknowledged.

C.J.ASHWORTH

ACKNOWLEDGEMENTS

I am most grateful to Dr I. Wilmut (Animal Breeding Research Organisation (A.B.R.O.)) for supervising the work described in this thesis, and for the encouragement and willing help he offered throughout the duration of this study. Experimental animals and facilities were provided by A.B.R.O., and made available by kind permission of Dr R.B. Land. Sincere thanks also to Dr R.H.F. Hunter (Edinburgh University) for his continued interest during the project, and especially for reading this dissertation.

It is a pleasure to thank every-one at Dryden laboratory, especially Mr A. MacGregor and his staff for care of the experimental animals, Miss M.R. Thomson for expert assistance with surgical procedures, Mr J.C. Manson for anaesthesia and for drawing several figures presented in this thesis and Mrs B. MacKenzie for her thorough cleaning of laboratory glassware. The advice and encouragement given by Dr R. Webb and Mr G. Baxter during the establishment of the progesterone radio-immunoassay was much appreciated.

I am indebted to Mr D.I. Sales and Miss A.J. Springbett for their help and advice regarding experimental design and statistical analysis of data. Thanks to all the computer staff at A.B.R.O. and the Poultry Research Centre (PRC). I would also like to thank the A.B.R.O. librarians for their help in tracing sources of reference material.

Permission to use the continuous flow analyser was kindly granted by the Hill Farming Research Organisation. I wish to thank Mr E. Skedd and Miss P. Moberly for their assistance with these analyses.

Gifts of epostane (WIN 32,729) from Mrs H. Loizou (Sterling Winthrop Research and Development, Guildford, Surrey) of anti progesterone serum from Dr J.E.T. Corrie (MRC Immunoassay team, Edinburgh) and of iodonated progesterone tracer from Mr I. Swanston (MRC Centre for Reproductive Biology, Edinburgh) were much appreciated.

Finally, thanks to many friends and colleagues in Edinburgh; especially Dr. A.W. Stott, for all his help.

The work described in this thesis was supported by an AFRC postgraduate studentship.

CONTENTS

		PAGE
ABSTRACT		
CHAPTER 1	Introduction	
	(i) The establishment of pregnancy	1
	(ii) Hormonal requirements during early pregnancy	10
	(iii) Blastocyst endometrial interactions	14
	(iv) Embryonic mortality	21
	(v) Implications	22
CHAPTER 2	Validation of a radioimmunoassay to measure ovine plasma progesterone.	23
CHAPTER 3	Experiment 1: An investigation of the relationship between progesterone secretion and embryonic survival in Damline ewes.	33
CHAPTER 4	Experiment 2: The effect of epostane (WIN 32,729) on progesterone secretion and embryonic survival during early pregnancy.	52
CHAPTER 5	Experiment 3: Protein patterns in uterine secretions collected from cyclic Welsh Mountain ewes.	65
CHAPTER 6	Experiment 4: An investigation of the maternal and embryonic contribution to uterine fluids in Welsh Mountain ewes.	78
CHAPTER 7	Final Discussion	97
BIBLIOGRAPHY		101

ABSTRACT

Embryonic mortality imposes a severe limitation to reproductive efficiency in the domestic species. In sheep, 20% to 30% of conceptions are not represented by lambs at birth. Attempts to improve embryonic survival by alterations to nutritional regimes and by control of other environmental factors have achieved only limited success. It has been suggested that some embryos may die because of an inappropriate uterine environment.

The experiments described in this thesis investigated physiological mechanisms occurring during early pregnancy in the ewe. Knowledge of such factors may elucidate some causes of embryonic wastage and highlight potential means of improving embryonic survival. This thesis examined two aspects of maternal function during early pregnancy; luteal function, and the relationship between the developing embryo and the uterus.

An association between the progesterone profile after mating and embryonic survival was observed. Pregnancies during which all embryos survived had higher progesterone concentrations from the day after mating, relative to pregnancies associated with embryonic mortality. This experiment also revealed complex inter-relationships between several factors known to affect embryonic loss. Reduced fertility late in the breeding season could be explained entirely by differences in progesterone concentration.

The progesterone profile was highly variable both between ewes, and within ewes during successive pregnancies. The repeatability estimates for pre-luteal and luteal-phase progesterone concentrations suggested that the response to selection for a particular progesterone profile would be low.

Luteal-phase progesterone secretion was pharmacologically

suppressed in order to mimic the changes in progesterone concentration which may occur following environmental stress. Treatments which lowered progesterone levels also reduced embryonic survival.

The composition of uterine fluids was investigated in cyclic and pregnant ewes, and in ovariectomised ewes receiving exogenous steroids. Concentrations of several enzymes varied throughout the oestrous cycle. These changes were considered to reflect ovarian steroid concentrations. Differences in the levels of uterine proteins were observed between pregnant and non-pregnant ewes, and between the gravid and non-gravid horn of unilaterally pregnant ewes. Variations in the schedule of steroid administration to ovariectomised ewes affected the relative concentrations of several uterine components. The potential mechanisms by which such differences may arise were discussed.

The experiments described in this thesis have revealed associations between maternal factors and embryonic survival. Potential means of improving embryonic survival arising from these observations were described.

CHAPTER 1

INTRODUCTION

In this introduction the overall sequence of events in early pregnancy is considered briefly, before the hormonal requirements for the establishment of pregnancy are reviewed. There is then a consideration of the complex inter-relationships between the uterus, its secretions and the embryo during early pregnancy. Studies of these events also reveal the causes of some pregnancy failures. In a final section the known causes of embryonic loss are described.

The establishment of pregnancy

Ovine pregnancy is the product of a complex series of events, including folliculogenesis, ovulation, fertilisation, embryo transport and development, the maternal recognition of pregnancy and attachment of the embryo. Sheep are seasonally breeding animals. The transition from anoestrus to oestrus is stimulated by shortening periods of daylight, which alter the sensitivity of the hypothalamic pituitary axis to ovarian steroid feedback (Legan, Karsch and Foster, 1977).

Folliculogenesis

The ovary of the adult ewe contains between 12,000 and 86,000 small follicles, and between 100 and 400 larger developing follicles (Cahill, 1981). The development of primordial follicles through the pre-antral and antral phase, until they either undergo follicular maturation culminating in ovulation, or succumb to atresia takes approximately 6 months (Cahill and Mauléon, 1980). The stimulus for

a follicle to enter the growth phase is unknown, although the observation (Dufour, Cahill and Mauléon, 1979) that the number of non-atretic pre-antral and antral follicles is reduced following hypophysectomy suggests that gonadotrophins have some influence. The rate of follicular growth is independant of the stage of the oestrous cycle (Turnbull, Braden and Mattner, 1977). These authors estimated that approximately 3 follicles per day enter the antral phase, although the majority will undergo atretic degeneration.

Whether or not a follicle degenerates depends on several factors including the amount of gonadotrophin secreted by the pituitary, the sensitivity of the follicle to gonadotrophins and the androgen to oestrogen ratio in the follicular microenvironment (Moor, Hay, Cran and Dott, 1978). The physiological role for this perpetual growth and atresia of follicles throughout the oestrous cycle is unclear, although the steroidogenic activity of follicles destined to degenerate may be essential for the maturation of the few surviving follicles.

Hormone changes around the time of ovulation

Reduced progesterone concentration following the demise of the corpus luteum permits a rise in the basal secretion of luteinising hormone (LH) (Baird and Scaramuzzi, 1976) as the inhibitory effect of luteal phase progesterone on LH is removed (Karsch, Legan, Ryan and Foster, 1980). Follicle stimulating hormone (FSH) concentrations do not rise during the follicular phase, presumably due to increased oestradiol secretion (Baird and McNeilly, 1981) and follicular inhibin (Webb and Gauld, 1985). Accompanied by the increase in basal concentrations of LH is an increase in LH pulse frequency (Baird 1978a). By 24 hours after the decrease in

progesterone concentration the pulse frequency is approximately one per 75 minutes. LH induces the rise in intracellular steroid biosynthesis necessary for the complete maturation of the Graafian follicle by activation of the adenyl cyclase system. The cyclic AMP produced is primarily used in accelerating the conversion of cholesterol to pregnenolone (Marsh, 1976). Each pulse of LH stimulates an increased secretion of oestradiol from the largest non-atretic antral follicle(s) within 10 minutes (Baird, Swanston and McNeilly, 1981). Although LH pulse frequency increases, pulse amplitude decreases. The progressive increase in the amplitude of oestrogen secretion is due mainly to an increase in the number of LH receptors on the surface of granulosa cells (England, Webb and Dahmer, 1981). Consequently, intra-follicular and peripheral oestrogen concentration increases dramatically.

Although LH pulses also stimulate the rise in intra-follicular androgen concentration, during the 36 hours preceeding the pre-ovulatory surge of LH an increasing proportion of androgen produced from the pre-ovulatory follicle is converted to oestrogen by aromatase in the granulosa layer. This results in a progressive rise in the oestrogen to androgen ratio within the pre-ovulatory follicle (Baird and McNeilly, 1981). The pre-ovulatory rise in oestrogen concentration serves several functions including stimulation of oestrous behaviour and suppression of FSH secretion. Baird and McNeilly (1981) suggested that the decline in FSH prior to ovulation hastens atresia in antral follicles which will not ovulate, thereby providing an explanation for the wave of atresia observed at ovulation (Turnbull, Braden and Mattner, 1977). Any follicles destined to ovulate are probably protected from the deleterious effect of declining levels of FSH by their high

intrafollicular concentrations of FSH and oestradiol (McNatty, Gibb, Dobson, Thurley and Findlay, 1981). The major function of the pre-ovulatory rise in oestrogen levels is to stimulate a surge of LH and FSH approximately 60 hours following luteal regression (Goding, Buckmaster, Cerini, Cerini, Chamley, Cumming, Fell, Findlay and Jonas, 1973). A positive feedbach loop is therefore established whereby the increased plasma oestrogen levels increase both the sensitivity of the anterior pituitary to LH-RH and the secretion of LH-RH from the hypothalamus. In response to the pre-ovulatory gonadotrophin surge the Graafian follicles undergo a series of structural and functional changes (Hay and Moor, 1975) which culminate in ovulation about 24 hours later (Cumming, Brown, Blockey, Winfield, Baxter and Goding, 1971).

Follicular oestrogen and androgen secretion decreases during the pre-ovulatory surge, and remains low after ovulation (Baird, Swanston and McNeilly, 1981; Webb, England and Fitzpatrick, 1981; Webb and England, 1982). Paradoxically, it is the high LH concentration prevalent during the pre-ovulatory gonadotrophin surge that inhibits the production of these steroids (Moor, 1974). This reduction in steroidogenisis is thought to be due to a decrease in aromatase activity associated with a fall in the concentration of LH receptors (Webb and England, 1982). However, progesterone secretion may increase during this period, as suggested by the high follicular fluid (England, Dahmer and Webb, 1981; McNatty et al., 1981; Webb and Gauld, 1984) and ovarian vein (Wheeler, Baird, Land and Scaramuzzi, 1975) concentrations. This difference between the changes in progesterone and oestradiol concentration may reflect the activation of different enzyme systems following stimulation of the LH receptor. This would lead to differential steroid synthesis.

The period immediately prior to ovulation, therefore, is characterised by a gradual change in the pre-ovulatory follicle from predominantly oestrogen secretion by the thecal layer to progesterone secretion by the granulosa cells. Concentrations of LH and prolactin also decline following the pre-ovulatory gonadotrophin surge, but during this period there is a second surge of FSH (Baird and McNeilly, 1981). The exact cause and function of this surge is not known, although it may be due to a reduction in the negative feedback effect of oestrogen.

Ovulation and Fertilisation

The mechanisms involved in the release of the oocyte from the Graafian follicle include increased vascularisation of the pre-ovulatory follicle and the release of collagenase and other proteolytic enzymes (Espey, 1980). These enzymes cause a thinning of the connective tissue of the follicle wall. Essential to ovulation is the binding of LH to the peripheral layers of the granulosa cells, and to theca interna cells which have been previously primed with FSH and oestradiol (Lindner, Amsterdam, Salomon, Tsafriri, Nimrod, Lamprecht, Zor and Koch, 1977). LH stimulates the morphological changes in granulosa cells known as luteinisation. This involves an increase in cytoplasm and development of smooth endoplasmic recticulum and lipid inclusions.

The LH surge also stimulates the resumption of meiosis by the oocyte in the preovulatory follicle. Oocyte maturation can be considered as a series of interactions between the follicle cell and the oocyte (Crosby, Osbourn and Moor, 1981) with the oocyte remaining dependent on the follicle until almost the time of follicle rupture (Thibault, 1977). Gonadotrophic stimulation of

oocyte maturation is mediated by intra-follicular proteins, DNA (Osborn and Moor, 1983) and steroids; primarily oestradiol (Moor, 1978).

Fertilisation usually occurs in the ampullary-isthmic junction. Sperm transport is enhanced by the concentration of ovarian steroids present during the peri-ovulatory period (Hunter, Barwise and King, 1982). The likelihood of fertilisation of the secondary oocyte occurring before ageing is therefore enhanced.

Embryo transport and development

Circulating oestrogen levels after mating may affect the rate of transport of embryos along the oviduct (Moore, Miller and Trappl, 1983). The passage of embryos into the uterus normally occurs on the fourth day following oestrus (Holst and Braden, 1972). Embryo transfer studies (Moore and Shelton, 1964; Rowson and Moor, 1966) established that embryonic development proceeds normally only when the uterine environment is adequately prepared to receive a blastocyst from a donor ewe in oestrus at the same time as the recipient. Rowson and Moor, (1966) estimated the tolerance to asynchrony between donor and recipient to be no greater than $\pm$ 2 days if a pregnancy was to be established.

During the free living phase of embryonic development, conceptuses migrate between the uterine horns. This is important for embryonic growth, ensuring efficient utilisation of intra-uterine space. In super-ovulated ewes, there is a tendency to balance the number of foetuses between the uterine horns, despite the random distribution of corpora lutea. (Rhind, Robinson, Fraser and McHattie, 1980). The studies of Sittman (1972) predicted that intra-uterine migration would not contribute to embryonic loss, but

rather would tend to minimise loss due to crowding. There were no observed differences in embryonic survival between unilateral and bilateral twin ovulators.

The survival and development of embryos during the pre-attachment phase is dependent on the uterine environment. This is influenced by progesterone released during the luteal phase before oestrus, by oestrogen secreted immediately before and during the oestrus at which mating occurs, and by progesterone secretion during pregnancy (Miller and Moore, 1976; Miller, Moore, Murphy and Stone, 1977).

Luteal function

Progesterone secretion from the corpus luteum is stimulated by LH (Short, 1964; Hansel, Concannon and Lukaszawska, 1973) and prolactin (Denamur, Martinet and Short, 1974), which together constitute the luteotrophic complex. The mechanism by which LH exerts this effect is well documented. LH binds to its plasma membrane receptor and activates adenylate cyclase activity (Marsh, 1970). The cyclic AMP formed leads to the activation of protein kinases (Menon, 1973), one of which is involved in the cleavage of the side chain of cholesterol leading to formation of pregnenolone (Caron, Goldstein, Savard and Marsh, 1975). The conversion of pregnenolone to progesterone occurs in the microsomal fraction of luteal cells, and is catalysed by 3β-hydroxysteroid dehydrogenase (Caffrey, Nett and Niswender, 1979). Progesterone is then sequestered into protein-containing secretory granules (Gemmell, Stacy and Thorburn, 1974; Gemmell and Stacy, 1977; Sawyer, Abel, McClellan, Schmitz and Niswender, 1979) and released into the vasculature by calcium dependant active transport (Higuchi, Kaneko,

Abel and Niswender, 1976). In contrast, prolactin has no affect on in-vitro synthesis of progesterone in corpora lutea (Kaltenbach, Cook, Niswender and Nalbandov, 1967). It has been suggested (Martal, 1981) that prolactin is necessary for the storage of cholesterol by the luteal cell.

Progesterone concentrations during the luteal phase and early pregnancy are highly variable, with considerable daily fluctuations (McNatty, Revfeim and Young, 1973; Quirke, Hanrahan and Gosling, 1979). The reasons for these within-ewe, within-day variations are not known. Progesterone concentrations were normal until day 9 in ewes hypophesectomised on days 2 to 5 (Denamur, Martinet and Short, 1966), suggesting that circulating gonadotrophin concentrations are adequate to maintain luteal function. There is no evidence of an association between LH pulse frequency and the pattern of progesterone release from the corpus luteum during the luteal phase (Baird, Swanston and Scaramuzzi, 1976). This may be due to the two cell types (large and small) which have been identified in ovine corpora lutea (Fitz, Mayan, Sawyer and Niswender, 1982). It has been suggested (Hixon, Pijanowski, Weston, Skanks and Wagner, 1983) that while basal concentrations of progesterone are secreted by small luteal cells in response to LH, progesterone pulses may originate from large luteal cells. As large luteal cells have more prostaglandin-$F_{2\alpha}$ (PG-$F_{2\alpha}$) and prostaglandin-E_2 (PG-E_2) receptors, (Fitz, et al., 1982), a prostaglandin may be the oscillator controlling progesterone pulses.

As the ovine corpus luteum develops both the number of receptors for LH and the proportion of total receptors occupied by LH, are highly correlated with the peripheral progesterone concentration (Diekman, O'Callaghan, Nett and Niswender, 1978). LH

receptor populations are under inhibitory control from circulating LH concentrations. Experimental elevation of plasma LH concentrations (Niswender, Suter and Sawyer, 1981) was followed by decreased numbers (down regulation) of luteal receptors for LH. Both the basal levels and pulse frequency of plasma LH concentrations are regulated by the negative feedback effects of ovarian progesterone (Karsch, et al., 1980).

Maternal recognition of pregnancy

Essential to the establishment and maintenance of pregnancy is the recognition of a conceptus by the maternal endocrine system. In the absence of a normal embryo, endometrial PG-$F_{2\alpha}$ secretion induces luteal regression (Goding, 1974). The conceptus must overcome the effects of uterine PG-$F_{2\alpha}$ secretion, in order to ensure the maintenance of luteal function. As judged from transplantation studies, an embryo must be in the uterus by day 12 to prevent luteolysis (Moor and Rowson, 1966a), and intra-uterine infusions of embryonic extracts extend luteal lifespan in previously cyclic animals (Moor and Rowson, 1966b). This mechanism ensures that the lifespan of the corpus luteum is extended to at least day 50, by which time the placenta can usually synthesise sufficient progesterone to maintain pregnancy (Amoroso and Perry, 1977).

The experiments described in this thesis were performed to investigate two of the components important in the establishment of pregnancy, namely the effect of luteal function on embryonic development, and the relationship between the developing conceptus and the uterine environment.

Hormonal requirements during early pregnancy

The qualitative and quantitative changes in steroid hormone levels necessary for the establishment of pregnancy have been determined following embryo transfer to ovariectomised ewes (Miller and Moore, 1976). The maximum number of pregnancies is obtained in ewes receiving a particular pattern of ovarian steroid replacement therapy. This consists of a schedule of oestradiol and progesterone injections designed to simulate ovarian secretion during the luteal phase of the cycle before mating, oestradiol around the time of oestrus, and progesterone during early pregnancy. In order to mimic the pattern of progesterone secretion during the oestrous cycle, it is necessary to administer increasing doses of progesterone between days 0 and 8 (Miller and Moore, 1976). More recently it has been shown that pregnancy can be maintained by a simpler schedule of progesterone in ovariectomised ewes. This consists of a low dose between oestrus and day 4, followed by a higher dose typical of luteal phase concentrations from day 4 or 5 (Wilmut, Sales and Ashworth, 1985a).

Omission of either progesterone before oestrus or oestrogen around the time of oestrus reduces the proportion of ovariectomised ewes with normal embryos (Miller and Moore, 1976; Miller *et al.*, 1977a). The mechanism by which progesterone administered during the previous luteal phase affects reproductive performance is not known. However, oestradiol administered around oestrus increases the progesterone receptor concentration in the uterus during the first week of pregnancy (Murphy, Stone, Miller and Moore, 1977).

After mating, progesterone is the major ovarian steroid affecting embryonic survival. In ewes ovariectomised on day 3 after mating, pregnancy can be maintained by the administration of

progesterone alone (Cumming, Baxter and Lawson, 1974). Administration of oestradiol after mating was without effect on survival of embryos transferred to the uterus of intact ewes on day 4, although protein synthesis and mean cell content of RNA were increased (Miller et al., 1977a).

Experiments in which differing amounts of progesterone have been administered to ewes after mating have revealed that both the plasma concentration of progesterone and the time when it increases to a level typical of the luteal phase, are critical for embryonic survival (Wilmut, Sales and Ashworth, 1985a). The proportion of pregnant ewes following luteal phase injections of 5mg progesterone was 69%, whereas survival was 90% after injection of 25mg of progesterone (Parr, Cumming and Clarke, 1982). These observations are supported by experiments in which silastic implants expected to generate circulating progesterone concentrations of either 0.5ng/ml or 2.0ng/ml were administered to ovariectomised ewes from day 4 or 5 before embryos were transferred on day 6. The number of pregnancies was significantly greater in ewes receiving the higher dose of progesterone (69%) than in those receiving 0.5ng/ml (20%) (Wilmut, Sales and Ashworth, 1985a).

Progesterone administration affects both uterine function and embryonic development. Treatment of mated ewes with 10, 20 or 40mg of progesterone from day 3 resulted in a dose-related increase in the rate of embryonic cell division during the second week of pregnancy (Wintenberger-Torrès, 1967). In 1976 Miller and Moore showed that the timing of changes in uterine function could be modified by altering the time of progesterone administration. Two groups of long term ovariectomised ewes received a sequence of steroid injections designed to simulate the pattern of ovarian

secretion in intact animals. In one group of ewes (group 1) the sequence of injections was administered 1.5 days earlier than in group 2. Ewes in both groups received a day 4 embryo on days 2.5, 4 or 5.5 after oestrus. Maximal embryonic survival was observed following synchronous transfer of a day 4 embryo in group 2. However, in ewes in which the injection schedule had been advanced by 1.5 days, maximal survival occurred when the day 4 embryos were transferred to day 2.5 recipients. The timing of embryo transfer had to be advanced by exactly the same interval as the injection schedule.

Table 1.1

Proportion of ewes with normal embryos at slaughter

Days after oestrus	Group 1	Group 2
2.5	81.2%	35.3%
4.0	41.1%	70.6%
5.5	11.8%	29.4%

Although the uterine requirement for progesterone is well documented, Miller et al., (1977a) failed to detect any metabolic activity in the endometrium which is controlled specifically by progesterone. However, ovariectomised ewes receiving progesterone in the absence of any previous oestradiol treatment exhibited increased numbers of oestradiol receptors, a higher RNA:DNA ratio and a greater rate of protein synthesis (Stone, Murphy and Miller, 1978). Luteal phase progesterone concentrations induce the synthesis of several endometrial proteins including succinate

dehydrogenase and alkaline phosphatase (Murdoch and White, 1968). The latter enzyme is thought to serve a histotrophic role during blastocyst development (Murdoch, 1971).

The absence of progesterone between days 0 and 4 of pregnancy reduces the number of pregnant ewes (Wilmut, Sales and Ashworth, 1985a). Plasma progesterone concentrations at this stage of pregnancy are considered to affect oviductal transport (Winteberger-Torrès, 1961) and may negate an embryotoxic uterine factor present around the time of oestrus. One candidate for such an embryotoxic effect is oestradiol (Miller, Wild and Stone, 1979). The administration of luteal phase concentrations of progesterone immediately after oestrus does not jeopardise embryonic survival providing that embryos are transferred at an appropriate age. Transfer of day 10 embryos to such ewes on day 6 resulted in a normal proportion of pregnancies (Lawson and Cahill, 1983). This suggests that, at oestrus, the uterus is able to respond to concentrations of progesterone typical of those observed during the luteal phase (Wilmut, Sales and Ashworth, 1985a).

Several experiments have indicated that environmental stress may affect luteal phase plasma progesterone concentrations. Ewes fed twice maintenance rations exhibited depressed plasma progesterone concentrations, whereas undernutrition is associated with increased progesterone concentration (Williams and Cumming, 1982). Similarly, plasma progesterone levels are higher mid-season than at the begining of the breeding season (Rhind, Chesworth and Robinson, 1978).

These observations show that pregnancy depends upon a sequence of hormonal changes begining before oestrus. The patterns of secretion of these hormones are variable. In particular, changes in

environmental factors such as season and nutrition alter the plasma progesterone profile. Such changes may be associated with embryonic loss. These conclusions prompt several questions which have been considered in this thesis. How variable is the progesterone profile in typical ewes under standard conditions of husbandry? Is there sufficient variation in progesterone levels to jeopardise embryonic survival? Do differences in progesterone concentration account for any of the influence of the environment on embryonic survival?

Blastocyst endometrial interactions

It is necessary for the conceptus and the uterus to communicate with each other during early pregnancy to support development of the embryo, and to ensure intra-uterine migration, prevention of luteolysis, attachment and placentation. Prior to blastulation, embryonic development proceeds normally in a range of environments, including the rabbit oviduct (Lawson, Adams and Rowson, 1972) and in the oviducts of ewes irrespective of either the time of the breeding season or the stage of the oestrous cycle (Willadsen, 1982). However, ovine embryos fail to develop beyond the blastocyst stage when they are confined to the oviductal environment (Wintenberger-Torrès, 1956). This suggests that some aspect of the uterine environment is essential for further embryonic development.

The contribution of the blastocyst

Secretions of the early embryo are considered to fulfill two major roles; inhibition of luteolysis and prevention of immunological rejection of the conceptus. Several embryonic products have been proposed as antiluteolytic or luteotrophic agents. In humans (Catt, Dufau and Vaitukatis, 1975) and non-human

primates (Hearn, 1976) the corpus luteum is maintained by chorionic gonadatrophin released from the developing blastocyst. There is some evidence of 'hCG like' activity in ovine blastocysts (Wintenberger-Torrès, 1978), and the luteotrophin ovine chorionic somatomammotrophin (oCS) has been detected in the embryonic cotyledons from days 16-17 (Martal and Djiane, 1977). However, these proteins do not provide adequate luteotrophic support during the preimplantation period. Administration of oCS to cyclic ewes does not alter the time of luteolysis (Martal, Lacroix, Loudes, Saunier and Wintenberger-Torrès, 1979). These authors demonstrated that intra-uterine infusion of day 14-16 trophoblastic homogenates extended the lifespan of the corpus luteum by 30 to 60 days. Homogenates of day 21-23 embryos and their membranes did not inhibit luteolysis. It was concluded that day 14-16 conceptuses released a proteinaceous antiluteolysin, which was termed trophoblastin. Proteins from day 14-15 embryos have little affinity for LH or prolactin receptors, and do not stimulate progesterone or cAMP synthesis in dispersed luteal cells (Ellinwood, Nett and Niswender, 1979a). These authors speculated that the embryo does not exert a direct effect on the corpus luteum, but that such effects are mediated via the uterus. Trophoblastin was isolated by Masters, Roberts, Lewis, Thatcher, Bazer and Godkin (1982) and Godkin, Bazer, Moffat, Sessions and Roberts (1982). This protein is taken up by endometrial epithelial cells in vitro (Godkin et al., 1982) supporting the notion that it exerts its luteotrophic effects via the uterus.

It has been proposed that in sheep (Baird, Abel, Brown, Kelly and Wilmut, 1981; Findlay, 1981) trophoblastin may modify endometrial prostaglandin (PG) synthesis to increase the relative

abundance of PG-E_2 over PG-$F_{2\alpha}$, perhaps by increasing the activity of 9-keto reductase, converting PG-$F_{2\alpha}$ to PG-E_2 (Watson, Shepherd and Dodson, 1979). There are conflicting reports regarding the pattern of PG-$F_{2\alpha}$ release in cycling and pregnant ewes. Some authors have observed that the rise in PG-$F_{2\alpha}$ concentrations between days 14 and 17 in cyclic ewes, fails to occur during pregnancy (McCracken, Schramm and Okuliez, 1984). Conversely, reports indicating that uterine vein PG-$F_{2\alpha}$ concentrations are similar in pregnant and cycling ewes between days 14 and 17 (Silvia, Ottobre and Inskeep, 1981) suggest that the embryo has a luteoprotective, rather than an antiluteolytic role. This hypothesis is supported by the findings that the dose of exogenous PG-$F_{2\alpha}$ required to induce luteolysis is considerably higher in pregnant than non-pregnant ewes after day 13, and is proportional to the number of embryos present (Silvia, Fitz, Mayan and Niswender, 1984). The discrepency in PG-$F_{2\alpha}$ concentrations between pregnant and cycling ewes may arise because PG-$F_{2\alpha}$ is secreted in a pulsatile manner (Thorburn, Cox, Currie, Restall and Schneider, 1973) and is rapidly cleared by the lungs (Davis, Fleet, Harrison and Maule Walker, 1980). Additionally, the increased endometrial blood flow during pregnancy (Greiss and Anderson, 1970) may have a diluting effect on PG-$F_{2\alpha}$ concentrations. The capacity of the endometrium to produce PG-$F_{2\alpha}$ <u>in vitro</u> does not appear to be affected by pregnancy (Findlay, Ackland, Burton, Davis, Maule Walker, Walters and Heap, 1981). This may be explained by the re-direction of prostaglandins by the endometrium, as proposed by Bazer and Thatcher (1977). Further support for this hypothesis is provided by the experiments of Ellinwood, Nett and Niswender (1979b) who reported increased concentrations of PG-$F_{2\alpha}$ and PG-E_2 in uterine flushings of pregnant

ewes on days 13-17. However, such measurements in the uterus would be difficult, as stimulation of smooth muscle induces the release of prostaglandins.

Day 12-15 ovine embryos synthesise PG-$F_{2\alpha}$ and PG-E_2 *in vitro* (Marcus, 1981; Hyland, Manns and Humphrey, 1982). PG-E is a direct antagonist of PG-$F_{2\alpha}$ (Henderson, Scaramuzzi and Baird, 1977) and has been shown to stimulate progesterone secretion by ovine luteal cells *in vitro* (Silvia *et al.*, 1984).

In pigs, preimplantation trophectoderm tissue produces oestrone and oestradiol-17β *in vitro* (Heap, Perry, Gadsby and Burton, 1975). Aromatase activity is detectable around the time of maternal recognition of pregnancy (Flint, Burton, Gadsby, Saunders and Heap, 1979) which begins around day 12. Oestrogens released by the blastocyst are considered to have an antiluteolytic role. In pigs, this effect is thought to be achieved by a re-direction of PG-$F_{2\alpha}$ secretion causing an accumulation in the uterine lumen rather than release into the uterine vein (Bazer and Thatcher, 1977). Attempts to locate aromatase activity in ovine embryos were unsuccessful (Gadsby, Heap and Burton, 1980). This may have been due to the meagre amounts of tissue availiable.

The antiluteolytic embryonic products discussed so far exert a local influence, as during unilateral pregnancy, the sterile horn remains strongly luteolytic (Moor, 1968).

Blastocyst products are also thought to play a role in the immunological adjustments occurring during pregnancy to prevent maternal rejection of the embryo. Masters *et al.*, (1982) observed that a very large glycoprotein (m.w. >660,000) was released from developing pig, cow and sheep blastocysts. These authors suggested that *in vivo* glycoprotein may be deposited on the surface of the

trophoblast. Such a glycoprotein surface coat could act as a barrier against the immune response of the maternal organism (Guillomot, Fléchon and Wintenberger-Torrès, 1982).

The contribution from the uterus.

The secretions of the endometrium nurture development of the ovine embryo. The critical factors have not been defined, and are difficult to study. Observations have been made by direct studies of proteins recovered from the lumen, during culture of the endometrium or embryos and by embryo transfer.

It has been reported that the rate of total protein synthesis in both caruncular and inter-caruncular tissue is higher in pregnant than in non-pregnant ewes, on day 15 (Findlay et al., 1981), although this observation was not repeated in a subsequent experiment (Findlay, Clarke, Swaney, Colvin and Doughton, 1982). Electrophoretic studies of uterine flushings collected on day 15 of pregnancy revealed 10 uterine specific bands, of which 5 were characteristic of pregnant animals (Roberts, Symonds and Parker, 1976). The functions of some uterine specific proteins occurring in other species, including blastokinin in rabbits (Daniel, 1972) and porcine uteroferrin (Murray, Bazer, Wallace and Warnick, 1972) have been described. Laster, Maurer and Chenault, (1978) reported that uterine proteins from day 10 cow flushings were superior to bovine serum albumin (BSA) in promoting the expansion and survival of cultured day 12 bovine embryos. This evidence suggests that uterine specific proteins may play a role in the development of the conceptus.

Pregnancy associated proteins may originate directly from the blastocyst, or be due to the effect of ovarian steroids on the

endometrium (Finn and Porter, 1975) or the blastocyst may exert a local influence on endometrial protein synthesis. In pigs, blastocyst oestrogens stimulate the secretion of a group of proteins including an iron transport protein, uteroferrin (Bazer and Roberts, 1983). The synthesis of this protein in the endometrium is controlled by progesterone (Knight, Bazer and Wallace, 1973). The increase in protein synthesis during pregnancy is thought to be due to increased metabolic activity in the endometrium, reflected by caruncular hyperaemia on day 16 (Boshier, 1970). There is an increase in blood flow to the gravid horn, particularly to its endometrium, begining on day 12-13 (Greiss and Anderson, 1970). This may result from a local influence of the blastocyst on the uterine vascular bed. The binding of catecholoestrogens to alpha adrenergic receptors on the smooth muscle cells of the uterine arterioles leads to vasodilation and increased uterine blood flow (Ford, 1982).

An important function of proteins released by the endometrium during pregnancy is in the immunological recognition of the conceptus allograft. Although immunoprotective glycoproteins are released by both the endometrium and trophoblast (Guillomot, Flechon and Wintenberger-Torrès, 1982) there is not a complete immunological barrier between the maternal and embryonic systems (Beer and Billingham, 1979). The maternal system must respond to a variety of conceptus antigens and histocompatability antigens in order to achieve immunological tolerance. An inability to respond to such antigens may be a cause of embryonic loss. Ovine pregnancy associated antigen (oPAA) was detected in 94% of uterine flushings from day 14-19 pregnant ewes (Staples, Lawson and Findlay, 1978). In 1981 Sergerson observed immunosuppressive factors consisting of

one or more endometrial proteins. Concentrations of these factors were highest on day 14 of pregnancy, leading to the suggestion of an association between these factors and oPAA. Immunosuppressive activity was greater in pregnancy than at a comparable period during the oestrous cycle. The conceptus may enhance immunosuppressive activity in ovine uterine flushings (Sergerson, 1981) or, differences in circulating concentrations of plasma hormones during pregnancy may modify immunosuppressive activity.

Progesterone and/or progesterone metabolites may serve an immunoprotective role to ensure conceptus survival. Concentrations of progesterone in utero-ovarian lymph in ducts ipsilateral to the ovary bearing a corpus luteum were 10 to 1000 fold higher than in jugular vein plasma between days 15 and 45 of gestation (Staples, Fleet and Heap, 1982). It was noted that cell numbers in such lymph were low (200 leucocytes/ml) and that they were mainly of lymphocytes (94%). These cells would be exposed to concentrations of progesterone which are sufficiently high to suppress mitogen induced transformation of lymphocytes in vitro. It was suggested that such an effect could occur in vivo.

Analysis of uterine secretions may be used to study several aspects of early pregnancy. In ovariectomised animals, a comparison of different steroid regimes may reveal the endocrine control of uterine secretions. A contrast of fluids from non-pregnant and pregnant animals will highlight the uterine changes associated either with the presence of a blastocyst or the prolonged luteal phase. If serial samples can be collected from the same animals, it may be possible to relate the sequence of uterine changes to steroid profiles in individual ewes.

Embryonic Mortality

Despite the numerous regulatory mechanisms involved in the establishment of pregnancy, embryonic mortality causes a considerable reduction in reproductive efficiency. It is generally agreed that in mated animals 10% to 40% of ovulations are not represented by lambs at birth (Edey, 1969). In sheep, the vast majority of prenantal loss occurs during the first three weeks of pregnancy (Quinlivan, Martin, Taylor and Cairney, 1966). The possible causes of embryonic mortality have been comprehensively described, by Edey (1969), and more recently by Wilmut, Sales and Ashworth (1985b). For the purposes of this review, these factors are summarised in Table 1.2. In 1961, Hanly indicated that even when all these factors were accounted for, it was still impossible to eliminate embryonic loss. The remaining basal embryonic mortality must be due either to some innate failure of the developing embryo, or of the utero-tubal system to support that development.

It has been proposed that considerable embryonic mortality may be due to genetic abnormalities either inherited or arising during gametogenesis (Bishop, 1964). He suggested that such embryonic loss was a desirable means of eliminating unsuitable genotypes from the population. In sheep, chromosomal abnormalities have only been observed in 6% of embryos collected on day 2 or 3 post coitum (Long and Williams, 1980). In mice selected for reproductive performance, the genetic determinants of embryonic survival were defined by crossing lines and by embryo transfer (Bradford, 1979). The maternal genotype accounted for considerabely more of the genetic variation in prenatal mortality than the embryonic genotype. These observations suggest that maternal factors are critical in

TABLE 1.2

ENVIRONMENTAL AND PHYSIOLOGICAL FACTORS KNOWN TO DECREASE EMBRYONIC SURVIVAL IN SHEEP

	Source
1. Nutrition	
Decreasing plane, pre-mating	Gunn, Doney and Russel (1969 & 1972)
Increasing plane, pre-mating	Gunn, Doney and Russel (1969 & 1972)
Decreasing plane, post-mating	Edey (1966 & 1970) Cumming et al. (1975) Hamra and Bryant (1982)
Increasing plane, post-mating	Cumming et al. (1975) Brien, Cumming and Baxter (1977)
2. Increased number of ovulations	Edey (1966) Cumming et al. (1975) MacKenzie and Edey (1975)
3. Early season mating	Hulet et al. (1956) Oldham, Knight and Lindsay (1976)
4. Late season mating	Ashworth, Sales and Wilmut (1984)
5. Cold stress	Griffiths, Gunn and Doney (1970) Doney, Smith and Gunn (1976) MacKenzie, Thwaites and Edey (1976)
6. Heat stress	Dutt, Ellington and Carlton (1959) Dutt (1963 & 1964) Alliston, Elgi and Ulberg (1961)
7. Handling stress	Kilgour and De Langen (1970)
8. Young ewes/Ewe lambs	Quirke and Hanrahan (1977) Blockey, Parr and Restall (1975) Restall et al. (1976a & 1976b)
9. Genetic abnormalities	Bishop (1964) Long and Williams (1980)
10. Lactating ewes	Cognie and Pelletier (1976)

determining the success of a pregnancy.

In sheep, embryonic mortality occurs sufficiently early in pregnancy to allow at least one more mating before the rams are removed from the flock. Embryonic mortality does not therefore usually cause a dramatic reduction in the lambing percentage. The effects of embryonic mortality in naturally mated ewes tend to be insidious. Embryonic loss is associated with repeat matings at unpredictable times, and a consequent spread in lambing dates. Additionally, the number of multiple births is reduced, and some ewes remain barren.

In embryo transfer regimes techniques to increase ovulation rate and to facilitate embryo transfer and recovery are well developed. Therefore, embryonic mortality increasingly becomes a major limiting factor to reproductive efficiency following embryo transfer in the domestic species. In these situations, embryonic loss also represents a significant economic loss. The majority of the investment in terms of synchronising heats in both donor and recipient ewes, surgical facilities and the time of experienced personnel has already been made.

Implications

Knowledge of the mechanisms involved in the establishment of pregnancy and in the loss of embryos could lead to developments to reduce embryonic mortality. This might be achieved by selection for improved survival or by pharmacological intervention. As embryonic loss occurs in many species including humans, an understanding acquired in sheep may have application to other species.

CHAPTER 2

VALIDATION OF A RADIOIMMUNOASSAY TO MEASURE OVINE PLASMA PROGESTERONE.

Introduction

Variations in maternal plasma progesterone concentrations may affect embryonic survival. In order to test this, it was necessary to use an assay system that could accurately detect small differences between low progesterone levels. At the onset of this work there was no assay of such sensitivity available in the laboratory. This chapter describes the validation and routine procedure of a suitable progesterone radioimmunoassay (RIA).

A high proportion of circulating progesterone is bound to plasma proteins, especially albumin and cortisol binding globulin (CBG) (Dunn, Nisula and Rodbard, 1981). A direct method to displace progesterone from these binding proteins has recently been described (Ratcliffe, Corrie, Dalziel and Macpherson, 1982). Such assays have used either steroids with high affinity for CBG such as Danazol (McGinley and Casey, 1979) and cortisol (Haynes, Corcoran, Eastman and Doy, 1980), or 8-anilo-1-naphthalenesulphonic acid, (ANS) (Ratcliffe et al., 1982). An assay to measure ovine plasma progesterone using ANS in a citric acid buffer system was initially developed. However, it was not possible to achieve satisfactory precision at the low progesterone concentrations found in ovine plasma during the follicular phase of the oestrous cycle. Therefore, an assay system involving solvent extraction of progesterone from plasma was established. This assay was based on the method of Corrie, Ratcliffe and Macpherson, (1981) which uses a

radioiodonated progesterone 11α-glucuronide-tyramine conjugate with antiserum raised against progesterone 11α-hemisuccinate-bovine serum albumin. Such combinations of tracer and antiserum are described as heterologous bridge assays. The immunogen and tracer possess different protein bridges, but they are attached to the same position (in this case the 11α position) on the steroid (Fig. 2.1). Corrie et al. (1981a) observed that such systems produce specific and sensitive standard curves to a range of antisera.

Assay Materials.

Glassware: All glassware used for the progesterone RIA was soaked in Haemo-sol regular (A & J Beveridge, Edinburgh) for at least 2 hours, and thoroughly washed. It was then rinsed in several changes of double distilled deionised water, and baked at 200°C for 2 hours. Extraction of plasma progesterone was performed in 20mm x 150mm glass culture tubes (Corning Ltd., Stone, Staffordshire). 125mm x 15mm and 12mm x 75mm glass test tubes (Glass Wholesale Supplies, London) were used respectively for reconstitution and assay of plasma progesterone.

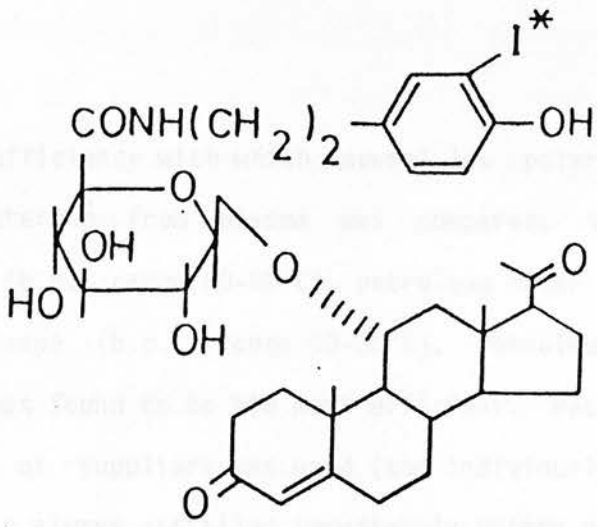
Buffers: A stock 0.5M Phosphate Buffer was prepared by dissolving 716g di-sodium hydrogen orthophosphate in 4 litres double distilled deionised water. Seventy eight grammes of sodium dihydrogen orthophosphate were dissolved in 1 litre double distilled deionised water. Approximately 640ml of this solution were added to the di-sodium hydrogen orthophosphate solution to adjust the pH to 7.5. The buffer was then stored at room temperature.

A 0.05M Assay Buffer (PBS-Gel) was prepared by diluting 200ml stock phosphate buffer in 1.8 litres double distilled deionised

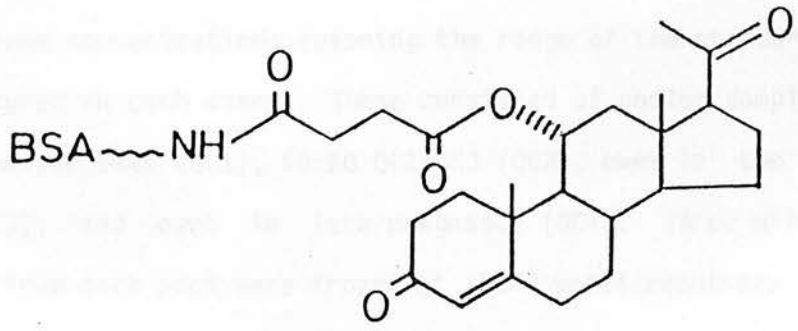
Fig. 2.1

Structure of the iodinated progesterone tracer and the progesterone anti-serum conjugate used in the present assay system.

FIG. 2.1. STRUCTURE OF THE IODINATED PROGESTERONE TRACER AND THE PROGESTERONE ANTI-SERUM CONJUGATE (FROM CORRIE, RATCLIFFE+ MacPHERSON 1981a)



RADIOIODINATED PROGESTERONE 11∝ GLUCURONIDE - TYRAMINE CONJUGATE



PROGESTERONE ANTI-SERA RAISED AGAINST 11∝ HEMISUCCINATE BOVINE SERUM ALBUMIN IMMUNOGENS

water. Eighteen grammes of NaCl and 200mg of Thiomersal were dissolved in 0.05M phosphate buffer. Two grammes of gelatin (swine skin, Sigma Ltd., Poole, Dorset.) were dissolved in 400ml phosphate buffered saline (PBS) at 50°C before adding the remainder of the PBS. The buffer was mixed for at least 2 hours, filtered and stored at room temperature between assays. A new batch was made up each week.

Solvents: The efficiency with which several low polarity solvents extracted progesterone from plasma was compared. These included petroleum ether (b.p. range 60-80°C), petroleum ether (b.p. range 40-60°C) and hexane (b.p. range 60-80°C). Petroleum ether (b.p. range 60-80°C) was found to be the most efficient. Petroleum ether from a variety of suppliers was used (see individual experimental chapters) and was always distilled immediately before use.

Progesterone standard solution: Progesterone (Sigma Ltd., Poole, Dorset) was dissolved in 'Aristar' ethanol (BDH Ltd., Poole, Dorset) to give a concentration of 59ng/ml. This was further diluted to 2ng/ml with PBS-Gel. Seven millilitre aliquots of this solution were frozen at -50°C until use.

Quality Control Plasma: Four quality control plasma samples having progesterone concentrations spanning the range of the standard curve were measured in each assay. These consisted of pooled samples from ovariectomised ewes (QC1), 50:50 QC1:QC3 (QC2), ewes in the luteal phase (QC3), and ewes in late pregnancy (QC4). Three millilitre aliquots from each pool were frozen at -50°C until required.

Progesterone antiserum: Several progesterone antisera, raised either in sheep or rabbits were compared. Of these, a rabbit antiserum to progesterone 11α-hemisuccinate-bovine serum albumin gave the highest binding. This antiserum was provided by Dr. J.E.T. Corrie, (MRC Immunoassay Team, Edinburgh.) and was raised in New Zealand White rabbits as described by Dighe and Hunter (1974). The antiserum was titred and found to have a suitable working dilution of 1:8000 in PBS-Gel (Fig. 2.2). One hundred microlitre aliquots of neat antiserum were stored at -50°C until required, at which time they were diluted with 800ml PBS-Gel to give a dilution of 1:8000.

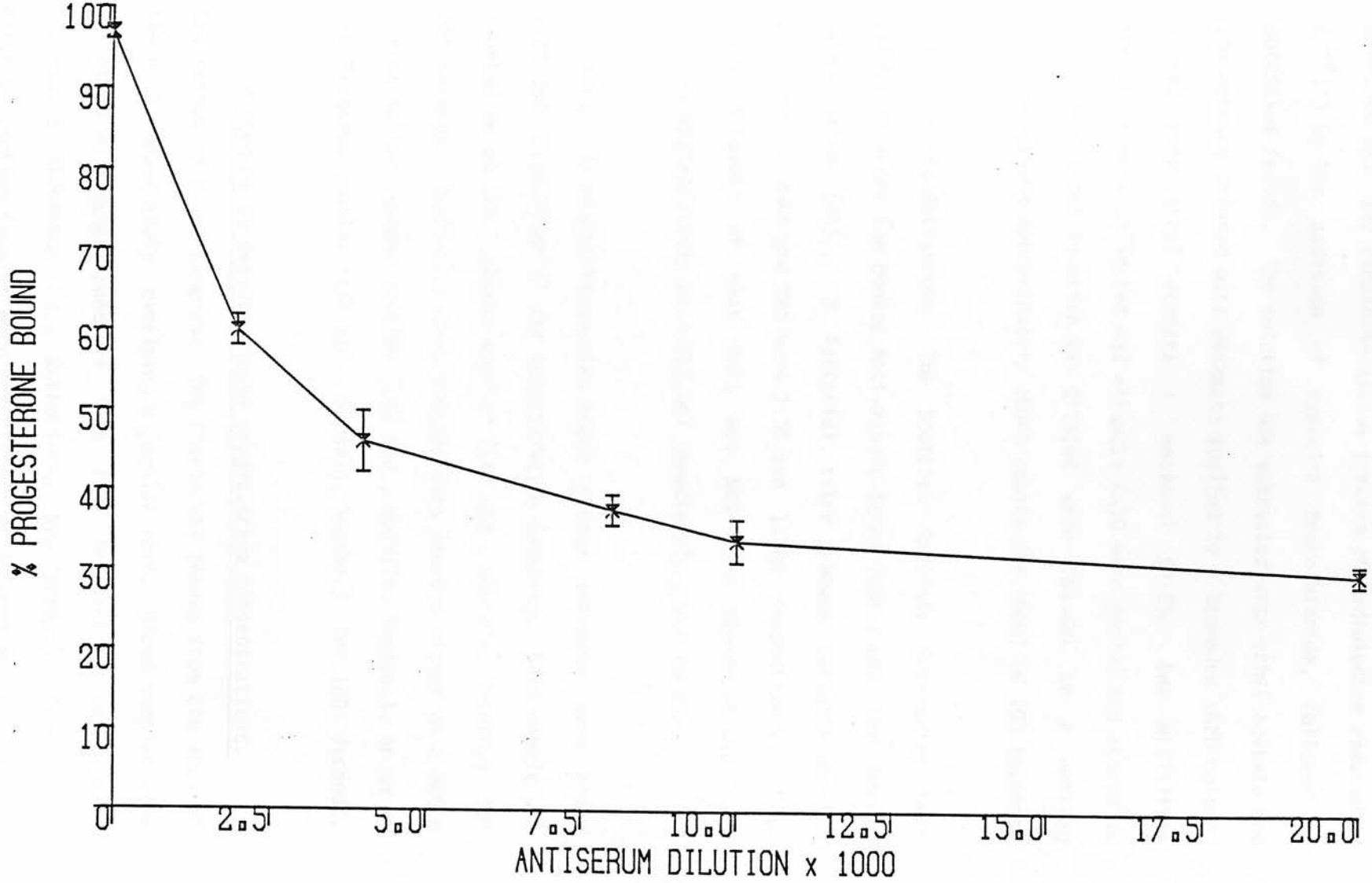
Radiochemicals: 1,2,6,7-[^{3}H]-progesterone (85 Ci/mmol) was supplied by the Radiochemical Centre, Amersham. This was purified by passing it through an LH20 Sephadex column eluted with ethyl alcohol : methanol (3:2). Five hundred microlitre aliquots were collected in glass test-tubes. Ten microlitres of each aliquot were mixed with 3ml 'Fiso Fluor 1' (Fisons Ltd., Loughborough, Leics.), and counted for 60secs on an LKB Rackbeta counter. Aliquots with the highest counts were pooled, dried down over nitrogen and reconstituted in 5ml 'Aristar' ethanol. This stock solution was stored at 4°C. An appropriate dilution with PBS-Gel was prepared prior to use, to deliver approximately 500 counts per 20ul in 300 seconds.

Radioiodonation of the progesterone 11α-glucuronide-tyramine conjugate was performed as described by Corrie, Hunter and Macpherson, (1981b). This method uses chloramine-T to oxidise the radioiodide and yield an active form of iodine which substitutes into the phenolic groups of tyramine. The reaction was then

Fig. 2.2

Titre of progesterone antiserum (31/8). The X-axis shows the proportion of assay buffer (PBS-Gel) to one unit of antisera. Each point represents the mean ± s.e.m. of 4 determinations.

FIG 2.2: TITRE OF PROGESTERONE ANTISERUM (31/8)



quenched and any remaining active species of radioiodine reduced to [$^{125}I^-$] by the addition of cysteine hydrochloride, followed by potassium iodide. The solution was extracted with ethyl acetate and the extract diluted with methanol applied to a Sephadex LH20 column, eluted with ethyl acetate : methanol (3:2). One millilitre fractions were collected and aliquots 8-10 were pooled and stored at 4°C. This stock solution was diluted with PBS-Gel to a working dilution giving approximately 15000 counts per 100ul in 100 seconds.

Precipitating Antiserum: The Scottish Antibody Production Unit (SAPU) provided the Donkey Anti-Rabbit Serum (DARS) and the Normal Rabbit Serum (NRS). A factorial titre showed suitable working dilutions of DARS and NRS were 1:35 and 1:300 respectively, (Fig. 2.3). Aliquots of neat DARS and NRS were stored at -50°C and diluted appropriately with PBS-Gel immediately prior to use.

Counters: To assess recoveries 500ul plasma extracts were mixed with 3ml 'Fiso-Fluor 1' for scintillation counting. Each sample was counted on an LKB Rackbeta counter (LKB Ltd., Uppsala, Sweden.) for 300 seconds. Duplicate assay samples were counted either on a 80000 series Wallac gamma counter (LKB Ltd., Uppsala, Sweden.), or on an LKB Riagamma counter (LKB Ltd., Uppsala, Sweden.) for 100 seconds.

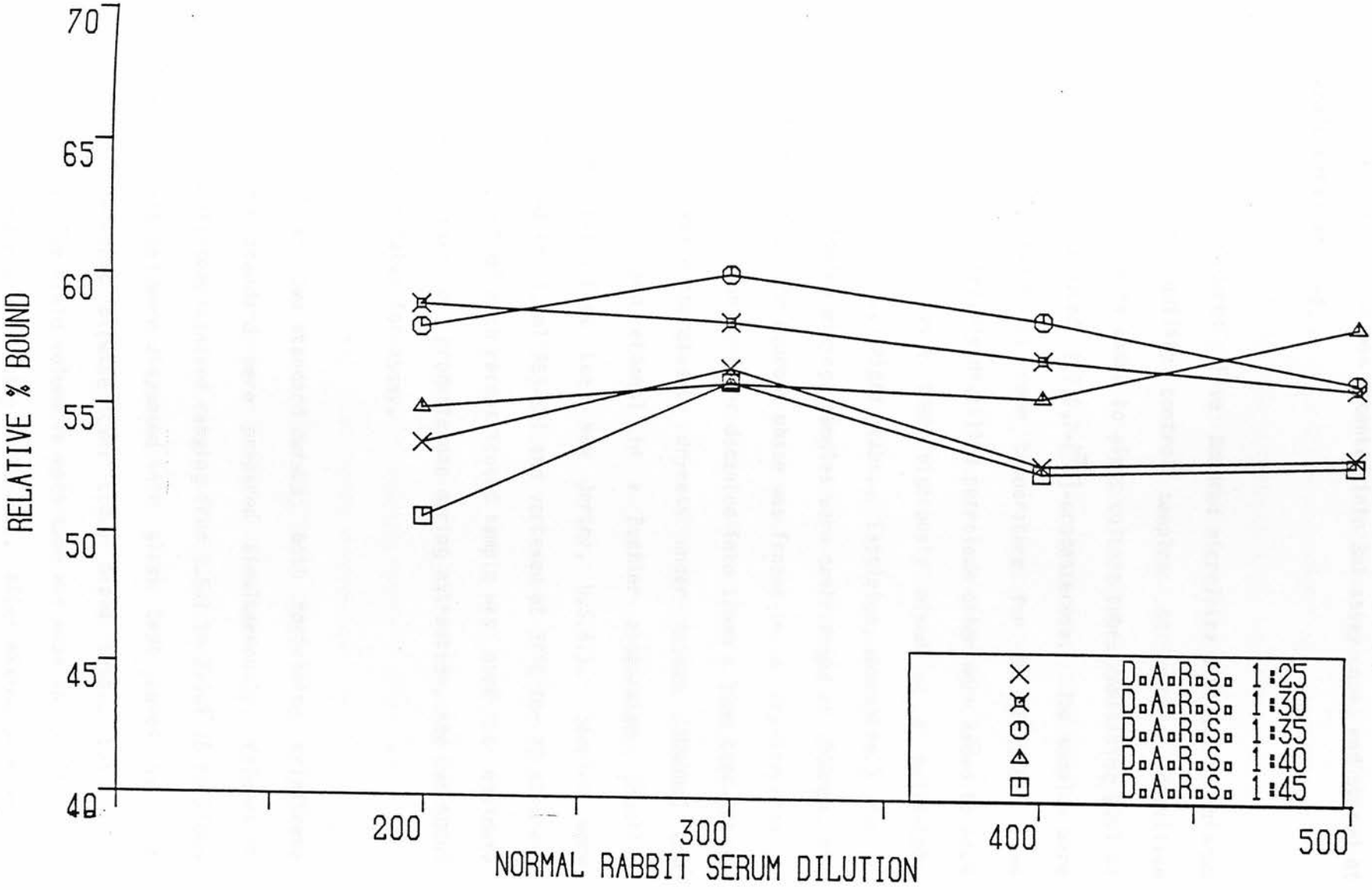
Procedure to measure plasma progesterone concentrations.

Collection of blood samples: The fleece was shaved from the area of the neck immediately overlying a jugular vein. Blood samples were collected by jugular puncture into 10ml heparinised vacutainers (Beckton & Dickenson Co., Rutherford, New Jersey.). Immediately after collection samples were centrifuged at 3000rpm and 4°C for 20

Fig. 2.3

Titre of donkey anti-rabbit serum and normal rabbit serum. The X-axis shows the proportion of assay buffer (PBS-Gel) to one unit of normal rabbit serum. Each point shows the mean of 3 determinations.

FIG 2.3: TITRE OF DONKEY ANTI-RABBIT SERUM AND NORMAL RABBIT SERUM



minutes. The plasma was decanted into 3ml assay cups, and stored at -20°C until assayed.

Extraction Procedure: Five hundred microlitre aliquots of unknown plasma samples, quality control samples or double distilled deionised water were added to glass culture tubes containing 20ul of appropriately diluted 1,2,6,7-[^{3}H]-progesterone. The samples were mixed and incubated at room temperature for 30 minutes. Five millilitres of freshly distilled petroleum ether were added to each tube. The samples were then vigorously mixed on a multi-tube vortexer (SMI, Ltd., Alpha Labs., Eastleigh, Hampshire.) for 15 minutes. Following mixing, samples were centrifuged at 1000rpm, 4°C for 10 minutes. The aqueous phase was frozen in a dry-ice/ethanol bath and the organic layer decanted into 125mm x 15mm test-tubes. The extract was evaporated to dryness under vacuum (20mmHg) and using dry-ice and ethanol in a Buchler evaporater (Buchler Instruments Inc., Fort Lee, New Jersey, U.S.A.). Samples were reconstituted in 1.8ml PBS-Gel and vortexed at 37°C for 20 minutes. A 500ul aliquot of each reconstituted sample was used to estimate procedural losses of progesterone during extraction, and two 500ul aliquots were taken for assay.

Assay Procedure: Two standard curves, both containing triplicate sets of each standard were prepared simultaneously. Volumes of 2ng/ml progesterone standard ranging from 2.5ul to 375ul (5 to 750pg progesterone/tube) were dispensed into glass test tubes using a direct displacement pipette (SMI Ltd., Alpha Labs., Eastleigh, Hampshire.). The fluid volume in each tube was made up to 500ul by adding an appropriate quantity of PBS-Gel. After mixing on a vortex

mixer the tubes were incubated at room temperature for 30 minutes. A complete standard curve and set of duplicate quality control samples were placed at the begining and end of each assay to allow an assessment of within-assay drift.

Duplicate 500ul aliquots of the reconstituted unknown and quality control plasma extracts in PBS-Gel were dispensed into assay tubes. Two hundred microlitres of a 1:8000 dilution of progesterone antiserum (31/8) was added to all tubes containing standards and plasma extracts. Three tubes received 0.2ml PBS-Gel in place of antibody to estimate the extent of any non-specific binding to the tracer. One hundred microlitres of [^{125}I]-progesterone in PBS-Gel, containing approximately 15000 counts per 100 seconds, was added to all tubes. Three tubes received progesterone tracer only to give an estimate of the total counts added. The assay was vortexed on a multi-vortex shaker (Denley V100 Multivortexer, Denley Instruments Ltd., Billinghurst, Sussex.), and incubated at room temperature for 2 hours.

Separation of antibody bound and unbound progesterone was achieved by adding 0.1ml of a 1:300 dilution of NRS in PBS-Gel, followed by 0.1ml of a 1:35 dilution of DARS in PBS-Gel to each tube. After mixing on a multi-vortex shaker, the tubes were incubated overnight at 4°C. Following incubation 1ml of PBS-Gel (4°C) was added to all tubes except total count tubes, which were then centrifuged at 3000rpm and 4°C for 40 minutes. The supernatant was decanted by inversion and any excess fluid aspirated. Each tube was then counted for 100 seconds on one of the LKB gamma counters.

Gamma counts from the 80000 series Wallac gamma counter or the LKB Riagamma counter were captured on punched paper tape or a Sirrius micro computer (Computer Services (Scotland) Ltd.)

respectively, and transferred to a 'main frame' computer (Prime Computer Inc.,Framingham Ma., Type 400). These data were processed through a radioimmunoassay data processing program package (Wilson, Maxwell and Sales, 1979) which calculates a calibration curve for each assay using the log-logit transformation and iterative weighted least squares regression analysis (Rodbard and Lewald, 1970). A calibration curve was calculated for each set of standards, and for the overall standard curve. Multiple standard curves within an assay were compared to monitor within assay variation. Potency estimates and confidence limits were calculated for each sample. Progesterone concentrations generated by this package were corrected for individual procedural losses during extraction. Unknown plasma samples were re-assayed in a subsequent assay if: (1) the potency estimate was below 20% or above 80% binding on the standard curve, (2) there was a significant difference in potency estimates between duplicate pairs, or (3) there were less than two estimates for each sample.

Assay Reliability Criteria.

The minimum detectable dose was defined as the smallest dose for which the response was significantly different from the zero standard value. The sensitivity was calculated individually for each assay, as was the reproduceability of the standard curve, extraction efficiencies, water blanks, and inter- and intra-assay coefficients of variation. Mean values for these criterea were calculated individually for each experiment, and are discussed in the relevant chapters.

An important aspect of the validation of the assay method involved the reduction of assay blank levels to insignificant values

(that is, less than the minimum detectable dose). During the initial validation, both charcoal treated plasma and double distilled deionised water samples were used to estimate assay blanks. Both samples gave estimates which were less than the minimum detectable dose for the assay. Double distilled deionised water was therefore used routinely to provide an estimate of assay blank. This consistently produced blank values which were lower than the minimum detectable dose. This is preferable to the practice of subtracting blank values from estimates of unknown samples for several reasons. Firstly, blanks are likely to vary between samples, secondly they cannot be estimated acurately for each sample and thirdly, subtraction of blank values assumes that the blank produces radiommunoassay curves that are parallel to the standard curve (Painter and Niswender, 1979).

Comparison of linear regression lines revealed no significant differences between standard curves following incubation for 2 or 4 hours (Fig. 2.4).

The specificity of this antiserum has been described by Corrie, Ratcliffe and Macpherson (1982; Table 2.1). Further validation was performed by preparing standard curves with progesterone and several other steroids. The cross-reactions of these steroids at 50% displacement of tracer were calculated by the method of Abraham (1969). All steroids tested showed negligable cross-reactivities of <2% except 4-pregnen-11α-ol-3,20-dione (11α-hydroxyprogesterone), 5α-pregnane-3,20-dione and 4-pregnen-17α-ol-3,20-dione (17α-hydroxyprogesterone) (Table 2.1). It must be accepted that this antiserum could not discriminate between 11α-hydroxyprogesterone and progesterone.

Volumes of 0.1, 0.25, 0.5, 0.75 and 1ml of QC2, QC3 and QC4

Fig. 2.4

Effect of varying incubation time of first antibody. Each point represents the mean ± s.e.m. of 3 determinations.

FIG 2.4: EFFECT OF VARYING INCUBATION TIME OF FIRST ANTIBODY

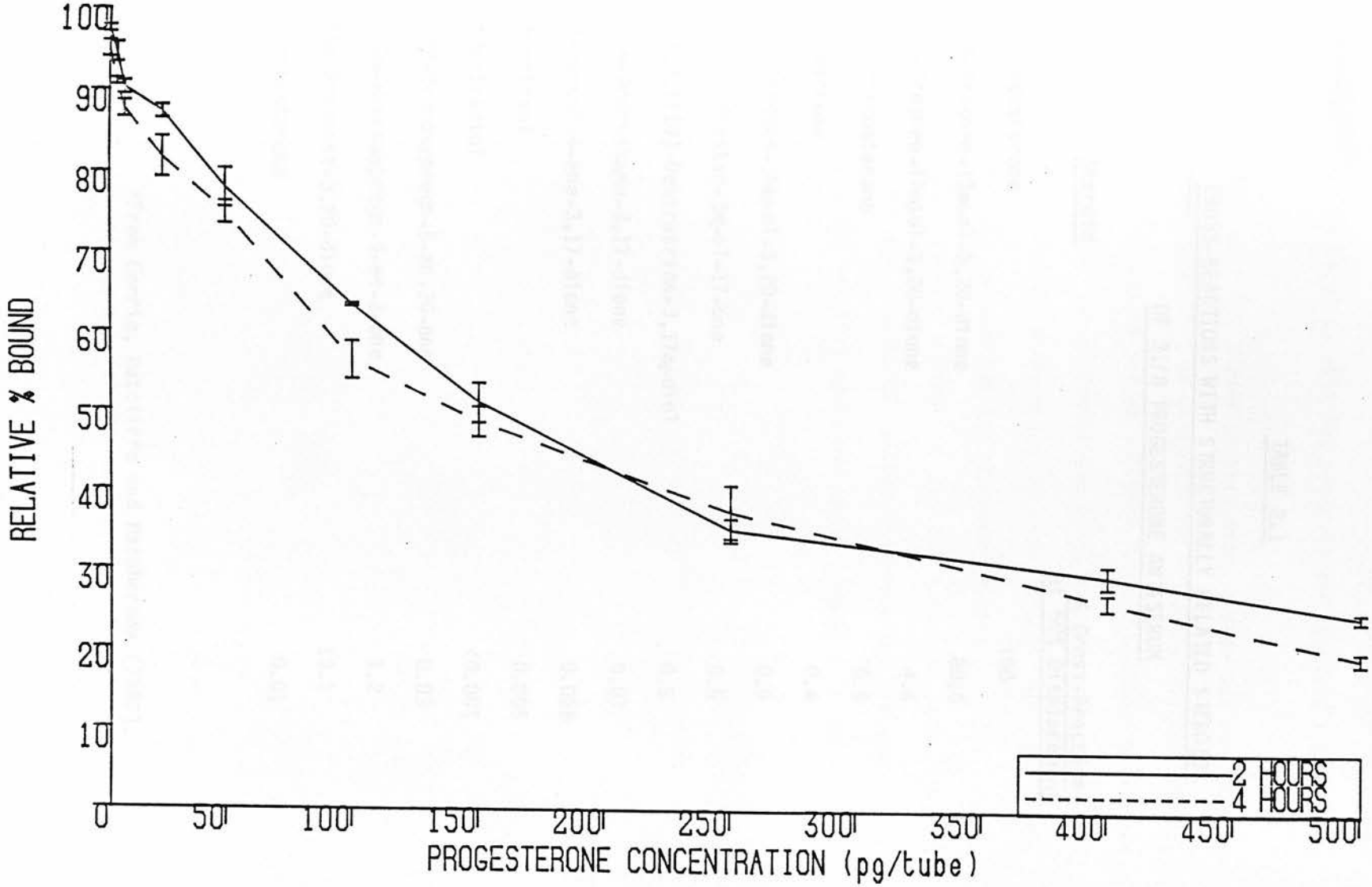


TABLE 2.1

CROSS-REACTIONS WITH STRUCTURALLY RELATED STEROIDS

OF 31/8 PROGESTERONE ANTISERUM

Steroid	% Cross-Reaction at 50% Displacement
Progesterone	100
4-Pregnen-11α-ol-3,20-dione	80.6
4-Pregnen-17α-ol-3,20-dione	4.6
Corticosterone	0.6
Oestrone	0.4
4-Pregnen-16α-ol-3,20-dione	0.9
5α-Androstan-3α-ol-17-one	0.6
1,3,5(10)-Oestratrien-3,17α-diol	0.5
5α-Androstane-3,17-dione	0.07
*Androst-4-ene-3,17-dione	0.025
*Cortisol	0.005
*Oestradiol	<0.001
*3β-Hydroxypregn-5-en-20-one	0.03
*20α-Hydroxypregn-4-en-3-one	1.2
*5α-Pregnane-3,20-dione	13.1
*Testosterone	0.01

*From Corrie, Ratcliffe and Macpherson (1982).

plasma samples were assayed, and found by comparison of linear regression to be parallel with the progesterone standard curve (Fig. 2.5).

The effect of ovine plasma on this assay system was assessed by measuring known quantities of progesterone in assay buffer alone, and in 0.5ml aliquots of plasma obtained from an ovariectomised ewe (Fig. 2.6). Comparison of regression lines by analysis of variance showed no significant differences in the slopes or the 50% displacement point between the two standard curves.

This assay system proved to be both robust and highly repeatable over the range of venous progesterone concentrations present during the oestrous cycle and early pregnancy in sheep.

Fig. 2.5

Parallelism check of standard curve (each point represents the mean of 3 determinations) and quality control plasma (each point represents the mean of 4 determinations).

FIG 2,5 : PARALLELISM CHECK OF STANDARD CURVE AND QUALITY CONTROL PLASMA

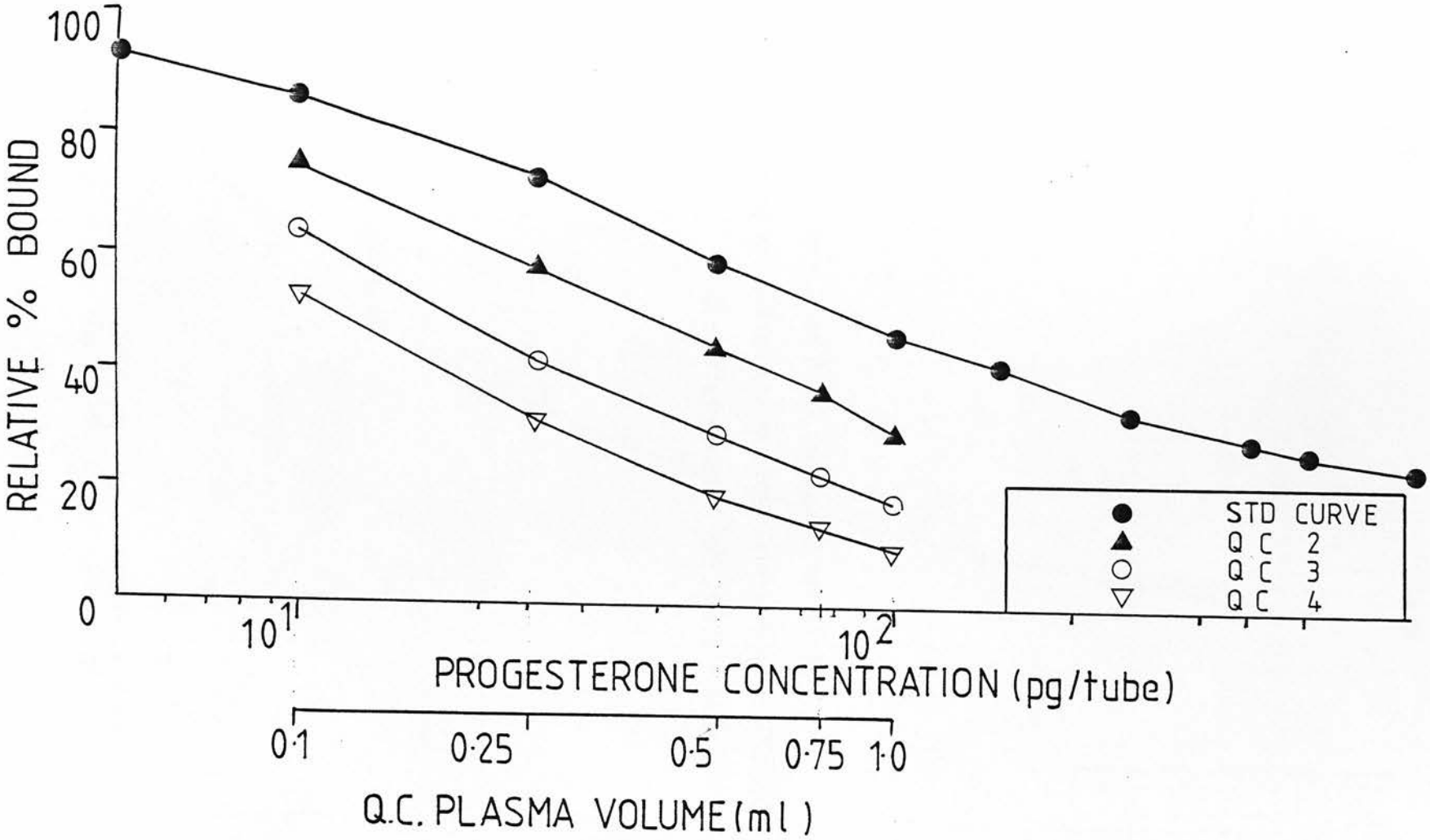
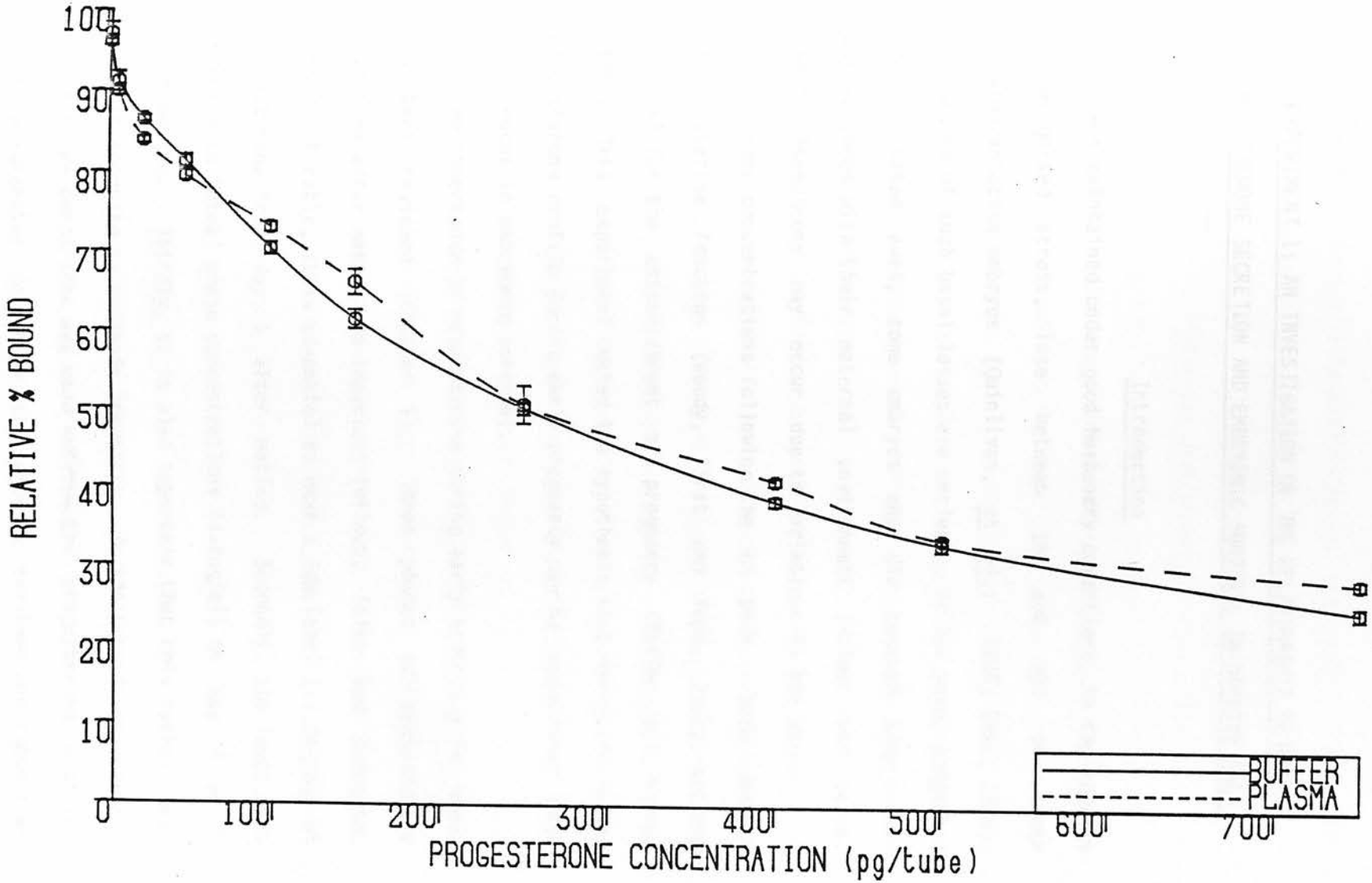


Fig. 2.6

Effect of ovariectomised ewe plasma (each point represents the mean ± s.e.m. of 4 determinations) on standard curve (each point represents the mean ± s.e.m. of 3 determinations).

FIG 2.6: EFFECT OF OVARIECTOMISED EWE PLASMA ON STANDARD CURVE



CHAPTER 3

EXPERIMENT 1: AN INVESTIGATION OF THE RELATIONSHIP BETWEEN PROGESTERONE SECRETION AND EMBRYONIC SURVIVAL IN DAMLINE EWES.

Introduction

Ewes maintained under good husbandry conditions, in the absence of recognised stress, lose between 15 and 30% of their pre-implantation embryos (Quinlivan, et al.; 1966, Edey, 1979). The causes of such basal losses are unclear. It has been suggested that in mated ewes, some embryos may die because they become asynchronous with their maternal environment (Wilmut and Sales, 1981). Asynchrony may occur due to variations in the sequence of progesterone concentrations following the LH peak. Such changes affect uterine function (Woody, First and Pope, 1967), and are critical for the establishment of pregnancy (Miller and Moore, 1976). This experiment tested the hypothesis that variation in the progesterone profile during early pregnancy may be associated with differences in embryonic survival.

The importance of progesterone during early pregnancy in sheep has been reviewed (Chapter 1). Three phases of progesterone secretion after mating are important (Wilmut, Sales and Ashworth, 1985a). Firstly, it is essential to have a low level (<1.0ng/ml) of progesterone from day 1 after mating. Secondly, the level must increase to luteal phase concentrations (4-6ng/ml) on day 4 or 5 after mating. Thirdly, it is also important that this luteal phase level is adequate to maintain pregnancy. In the present experiment, a within-ewe comparison was made between the progesterone profiles during pregnancies in which all embryos survived and those that

suffered loss.

A brief account of this experiment has been published elsewhere (Ashworth, Sales and Wilmut, 1984).

Materials and Methods

Experimental Animals

Thirty ABRO Damline ewes were used in this experiment. The Damline ewe was produced by crossing of Finnish Landrace (47%), East Friesland (24%), Border Leicester (17%) and Dorset Horn (12%) breeds. Selection was carried out within the flock for 8 week litter weight of lambs weaned (Smith, King, Nicholson, Wolf and Bampton, 1979). The ewes for the present experiment were culled from the selection flock, but were all of proven fertility.

Ewes were housed indoors during periods of blood collection, and for 24 hours before and after surgery. Whilst indoors, they were fed on hay supplemented with a proprietary complete diet (Ruminant A, Seafield Mill). Paddock feeding consisted of ad libitum hay and turnips. Oestrous cycles were synchronised by two 2ml intra-muscular injections of 100ug of cloprostenol, a prostaglandin analogue (Estrumate, I.C.I. plc, Macclesfield, Cheshire) given 9 days apart. Oestrous behaviour was confirmed by twice daily heat detection using vasectomised rams. At the second oestrus following synchronisation, the ewes were hand mated by at least two rams from a group of seven.

Collection of blood samples.

Blood samples were collected by jugular puncture, as described in the previous chapter. Samples were obtained at 7am, 2pm, and 9pm for 7 days around the time of mating, and subsequently twice daily,

at 8am and 4pm until day 16. A vasectomised ram was kept with the flock, to mark any ewes returning to heat.

Surgical procedures.

The numbers and distribution of corpora lutea and foetuses were recorded during a mid-ventral laparotomy. Surgery was performed within 2 days of an observed return to oestrus, or 30 days after mating in the absence of oestrus.

Ewes were fasted for 24 hours prior to surgery. Anaesthesia was induced by a single intra-venous injection of 8-20ml 10% w:v sodium thiopentone (Intraval, May and Baker Ltd., Dagenham.) An endotracheal tube was inserted and anaesthesia maintained by a mixture of oxygen, nitrous oxide and halothane (Fluothane, ICI plc, Macclesfield, Cheshire) in a semi-closed system. Ewes were placed in a recumbent position and the ventral abdominal area shaved and disinfected. Under aseptic conditions, an incision was made parallel with, and 1cm lateral to, the mid-line. Fascia and peritoneum were incised at the mid-line, and the reproductive tract exteriorised. Ovulation sites were recorded, and foetal numbers determined by gentle uterine palpation. To minimise adhesions, 10ml of sterile, isotonic saline were introduced into the pelvic cavity.

Following surgery, 100ug of cloprostenol were administered to pregnant ewes to induce abortion.

Experimental Design

The regime described above was repeated on the same animal population on four separate occasions during the 1982-1983 breeding season. Blood samples were collected from only those ewes which showed oestrous behaviour within a pre-defined 5 day interval.

Observations were recorded during September, November, January and March. In this way, ewes were allowed at least one cycle free from experimentation between successive sampling occasions.

Only 10 ewes showed oestrus within the prescribed 5 day period during November. It was presumed that the products of conception had not been removed, and that embryonic remains were preventing a return to regular cyclicity. The interval between matings was therefore increased. Only those ewes which had not been mated in November were synchronised for January mating. Consequently, the maximum number of sampling occasions per ewe was 3. After observations in November or January, a second injection of 100ug cloprostenol was given 8 days after surgery to promote the return to normal cycles.

One ewe did not show oestrus within the pre-defined 5 day interval during any of the 4 sampling occasions. A second ewe died during the experimental period, and was only represented on one sampling occasion. Blood samples were collected on 3 occasions from 16 ewes, and on 2 occasions from the remaining 12 ewes.

This design permitted several pregnancies to be monitored in the same ewes during one breeding season. Both the repeatability and the relation to embryonic survival of various aspects of the progesterone profile were determined.

Radioimmunoassay of plasma samples

Only the following plasma samples were assayed.

All samples from:

(1)Ewes which experienced embryonic mortality at any occasion during the breeding season.

(2)Ewes which were bled on three occasions.

Plasma samples representing 51 cycles from a total of 18 animals were assayed.

LH concentrations were measured in a series of 8 consecutive plasma samples per cycle. The third sample was closest to the time of first mating. All samples were measured in the same assay, using a double antibody precipitation method. The assay protocol was as first described by Martensz, Baird, Scaramuzzi and Van Look, (1976) and incorporated the modifications described by Webb, Baxter, Preece, Land and Springbett (1985). The minimum detectable dose was 0.443ng LH/ml. The intra-assay coefficient of variation was 4.9%.

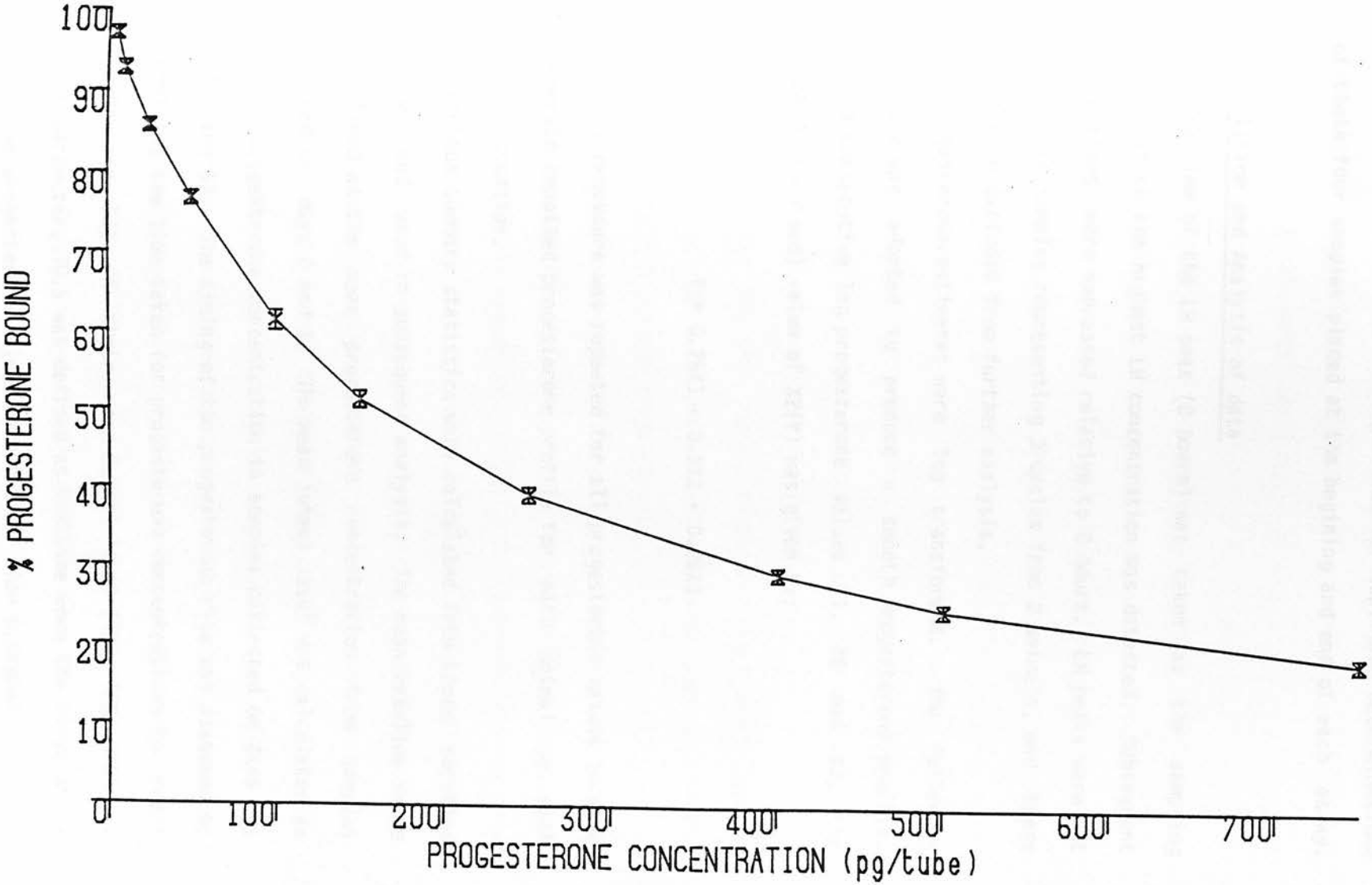
Progesterone assays were performed as described in Chapter 2. All samples collected from a ewe during one sampling occasion were measured in the same assay. No assay contained samples from the same animal acquired during different sampling occasions. A total of 19 assays, including 2 containing repeat samples were performed. Plasma samples were randomised within assays.

Progesterone was extracted from plasma using freshly distilled petroleum ether (Fisons Ltd., Loughborough, Leics.). The mean extraction efficiency was 76.8 $\pm$ 1.96% (s.e.m.). The mean minimum detectable dose was 16.302 $\pm$ 1.61pg/tube (s.e.m.) (150pg/ml). Water blanks were less than the minimum detectable dose in all assays. Within each assay, the overall standard curve was used to estimate progesterone concentrations. These curves were highly reproduceable between assays (Fig. 3.1), with no significant differences in slope (mean slope = -0.939 $\pm$ 0.196 (s.e.m.)). The inter-assay coefficients of variation using four standard quality control samples with mean progesterone concentrations of 0.19$\pm$0.08ng/ml, 1.56$\pm$0.27ng/ml, 2.54$\pm$0.42ng/ml and 6.84$\pm$0.96ng/ml were 44.9%, 14.1%, 16.7% and 17.3% respectively. The intra-assay

Fig. 3.1

Composite standard curve from progesterone assays performed in experiment 1. Each point represents the mean of 114 determinations $\pm$ 1 s.e.m.

FIG 3.1: COMPOSITE STANDARD CURVE FROM PROGESTERONE ASSAYS IN EXPERIMENT 1



coefficient of variation averaged 6.3% for duplicate determinations of these four samples placed at the begining and end of each assay.

Assimilation and Analysis of data

The time of the LH peak (0 hours) was taken as the sampling time at which the highest LH concentration was detected. Subsequent sample times were expressed relative to 0 hours. LH peaks were not detected in samples representing 3 cycles from 2 animals, and these cycles were excluded from further analysis.

Progesterone estimates were log transformed. The following procedure was adopted to produce a smooth progesterone profile. Given 3 consecutive log progesterone values X1, X2 and X3, the weighted (smoothed) value of X2(Y) was given by:

$$Y = 0.25X1 + 0.5X2 + 0.25X3$$

This procedure was repeated for all progesterone values to give a separate smoothed progesterone profile for each animal on each sampling occasion.

Various summary statistics were calculated from these smoothed profiles, and used in subsequent analysis. The mean baseline value was defined as the mean progesterone concentration from samples collected on days 0 and 1. The mean luteal level was calculated as the mean progesterone concentration in samples collected on days 9, 10, 11 and 12. The timing of the progesterone rise was assessed by determining the time taken for progesterone concentrations to reach 25%, 50% and 75% of their mean luteal level (T25, T50, T75). A further parameter, T0.5 was defined as the time when the first of 2 consecutive progesterone values were greater than 0.5ng/ml.

The progesterone variables were analysed using least squares and analysis of variance. The main (fixed) effects and dependent variables used in the model were:

Main (fixed) effects	Dependent varaibles
Individual ewe	Mean baseline level
Ovulation numbers	Mean luteal level
Season	T25
Assay	T50
	T75
	T0.5

The repeatabilities of the mean baseline and mean luteal progesterone values were estimated; these are a measure of the within-ewe variation between pregnancies.

The embryonic survival data from all ewes in the experiment were analysed using a general linear model with a logit link function (Nelder and Wedderburn, 1972). The contribution of the main effects listed above to the proportion of ovulations surviving was determined. By sequentially removing and replacing each variable to the statistical model, the relative contribution of each to the likelihood of an individual embryo surviving could be determined. The effects of ovulation number and of the stage of the breeding season were determined after allowing for the substantial variation between ewes in embryonic survival. Further analysis was done on the subset of the survival data for which progesterone information was available, adding the progesterone profile traits to the model.

Results

Numerous environmental and physiological factors affect embryonic survival. This experiment revealed complex inter-relationships between these factors (Fig. 3.2).

Between ewes, there was considerable variability in both circulating progesterone concentrations, and in the amounts associated with survival of all embryos (Fig. 3.3). Significant differences between animals in the proportion of embryos surviving were observed ($p<0.01$). There was also significant variation in progesterone concentrations between the same ewes during different pregnancies. The repeatabilities of the mean baseline and mean luteal progesterone values were 15 $\pm$ 20% (s.e.m.) and 17 $\pm$ 20% (s.e.m.) respectively.

In the within-animal analysis, periods in which partial or total embryonic loss occurred were associated with lower baseline progesterone levels ($p<0.05$; Fig. 3.4), and a tendency towards lower luteal phase levels ($p<0.10$). These significant differences in baseline progesterone concentration were observed despite the high coefficient of variation observed amongst the quality control samples at this part of the standard curve. There were no significant differences in the four timing components between ewes experiencing embryonic loss, and those in which all embryos survived.

Means derived from log transformed progesterone values are presented as both geometric means; to highlight significant differences, and as arithmetic means; for comparison with published values (Tables 3.1 and 3.2). The only significant effects were an increase in luteal phase progesterone concentrations with more

Fig. 3.2

Diagramatic representation of the relationship between several factors influencing embryonic loss. The dotted arrow indicates that not all of the effect of ovulation rate on embryonic survival was mediated by differences in progesterone concentration. In contrast, the effect of season on embryonic survival could be explained entirely by differences in progesterone concentration (shown by a solid arrow).

FIG 3.2:
INTER RELATIONSHIPS BETWEEN FACTORS INFLUENCING EMBRYO LOSS

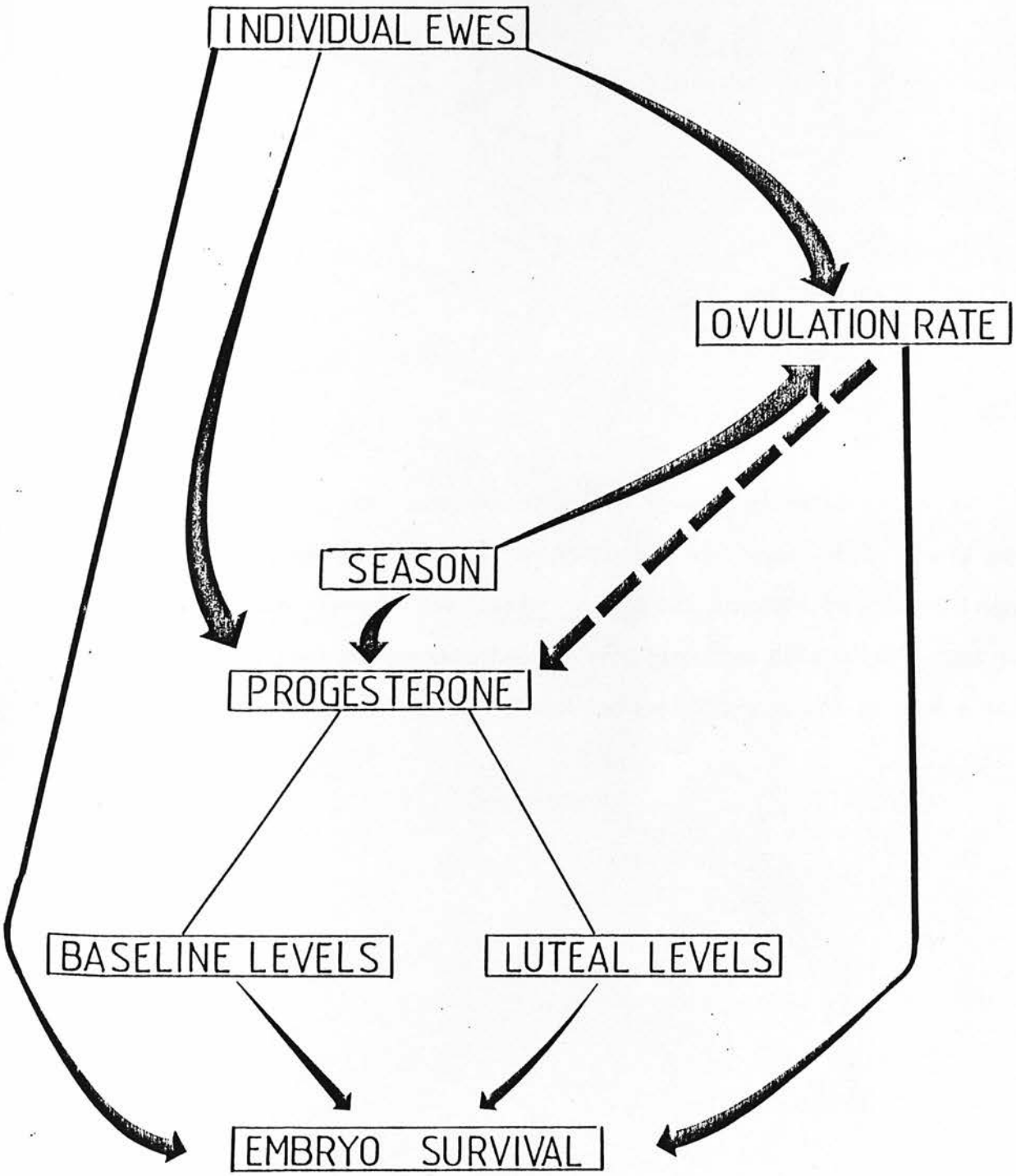


Fig. 3.3

All ewes mated in January 1983 were pregnant 30 days later. The black lines show the progesterone profiles from ewes having equal numbers of foetuses and corpora lutea. Progesterone secretion in ewes experiencing embryonic loss is indicated by red lines. Both these ewes had three ovulations and two foetuses.

FIG 3.3:
PROGESTERONE LEVELS FOLLOWING JANUARY MATING

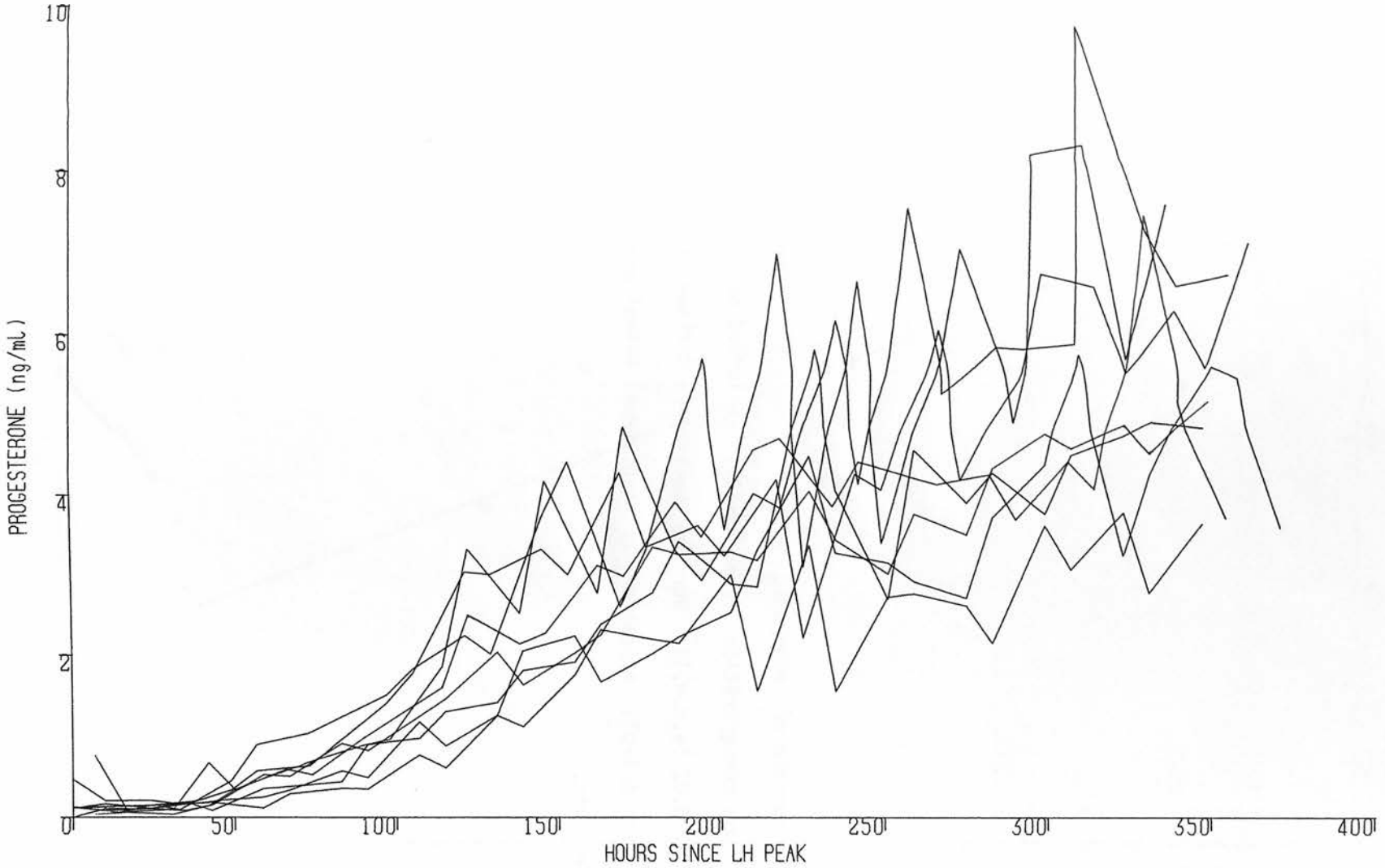


Fig. 3.4

Relationship of embryonic survival to the mean plasma progesterone concentration from samples collected on days 0 and 1 ($r=0.076 \pm 0.02$ (s.e.m.)). Working logits were derived from logit values ($\log (p/1-p)$) adjusted for individual animal and ovulation number effects.

FIG 3.4: OBSERVED EMBRYO LOSS:
ANIMAL & OVULATION RATE EFFECTS vs LOG BASELINE PROGESTERONE

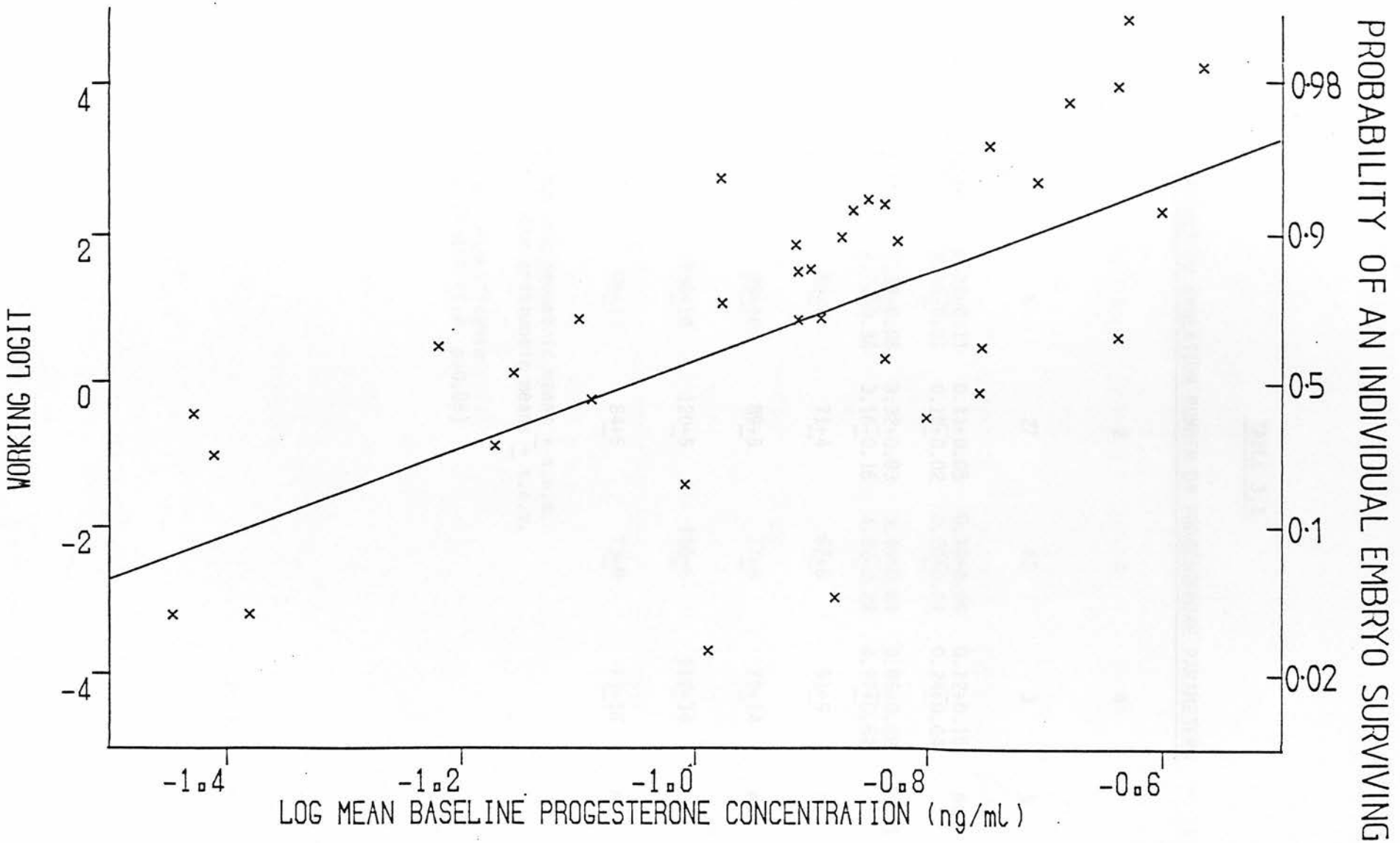


TABLE 3.1

EFFECT OF OVULATION NUMBER ON PROGESTERONE PARAMETERS

No. of ovulations	1	2	3	4	
No. of observations	6	27	11	3	s
Baseline level (ng/ml)*	0.12±0.11 0.15±0.01	0.11±0.05 0.18±0.02	0.10±0.09 0.20±0.04	0.12±0.15 0.24±0.04	ns
Luteal level (ng/ml*)	2.09±0.06 2.74±0.36	3.32±0.03 3.16±0.16	3.49±0.04 4.02±0.23	3.85±0.08 4.27±0.52	p<0.01
T25(hours)	65±7	71±4	62±6	51±9	ns
T50(hours)	83±10	89±5	77±8	72±14	ns
T75(hours)	130±10	120±5	110±8	112±14	ns
T0.5(hours)	96±12	84±6	73±9	73±16	ns

* Upper values are geometric means ± s.e.m.
  Lower values are arithmetic means ± s.e.m.

s = Statistical significance
ns = Not significant (i.e. p>0.05)

TABLE 3.2

EFFECT OF THE STAGE OF THE BREEDING SEASON ON PROGESTERONE PARAMETERS

	September	November	January	March	
No. of observations	16	7	8	16	s
Baseline level (ng/ml*)	0.15±0.07 0.21±0.03	0.14±0.08 0.18±0.02	0.12±0.10 0.20±0.03	0.07±0.06 0.15±0.02	p<0.01
Luteal level (ng/ml*)	2.69±0.04 3.07±0.23	3.61±0.04 3.98±0.19	3.47±0.05 4.01±0.34	2.77±0.03 2.97±0.21	ns
T25(hours)	65±4	66±6	59±6	61±4	ns
T50(hours)	87±7	81±8	78±9	76±6	ns
T75(hours)	125±7	110±8	124±9	112±6	ns
T0.5(hours)	87±8	69±9	81±11	84±7	ns

* Upper values are geometric means ± s.e.m.
  Lower values are arithmetic means ± s.e.m.

s = Statistical significance
ns = Not significant (i.e. p>0.05)

ovulations ($p<0.01$; Table 3.1) and lower baseline progesterone levels following March mating ($p<0.01$; Table 3.2).

The overall results on embryonic survival are summarised with respect to the number of ovulations in Table 3.3; the differences are significant ($p<0.01$) and may be summarised as a linear decline in survival with increasing numbers of corpora lutea (Fig. 3.5). The effect of the stage of the breeding season was also significant ($p<0.05$) with lower embryonic survival in March (Table 3.4). When progesterone was included in the statistical model, this effect of season was no longer significant, suggesting that seasonal differences in embryonic survival may be mediated by changes in plasma progesterone concentration. The effect of the number of corpora lutea on embryonic survival however, was only partially attributable to differences in progesterone concentration.

The above analyses have shown significant variation in embryonic survival and progesterone between ewes, and an association of these within animals. In the between-animal analysis of embryonic survival, there were again indications that ovulation numbers and baseline progesterone levels were important, but these were not significant. There was a tendency for lower baseline progesterone values and greater numbers of corpora lutea to be associated with an increased likelihood of embryonic survival. In each case, therefore, the effects were in the opposite direction to those in the within-animal analysis.

Discussion

The experimental procedure has allowed the study of the first 30 days of up to 3 pregnancies in a single breeding season. In view of the considerable variation between ewes in embryonic survival

TABLE 3.3

EFFECT OF OVULATION NUMBER ON EMBRYONIC SURVIVAL AND PREGNANCY

		Number of Ovulations					
		1	2	3	4	5	s
Foetal Number	0	3	5	0	1	1	–
	1	8	7	0	0	0	
	2		26	10	2	0	
	3			8	0	1	
	4				0	0	
	5					0	
No. of observations		11	38	18	3	2	
%Ewes pregnant on day 30		73	87	100	67	50	
%Ovulations surviving to day 30		73	79	81	33	30	$p<0.01$

s = Statistical significance
ns = Not significant (i.e. $p>0.05$)

Fig. 3.5

Relationship of embryonic survival to the number of corpora lutea ($r = -0.26 \pm 0.05$ (s.e.m.)). Working logits were derived from logit values ($\log (p/1-p)$) adjusted for individual animal and baseline progesterone effects.

FIG 3.5: OBSERVED EMBRYO LOSS:
ANIMAL & PROGESTERONE EFFECTS vs OVULATION RATE

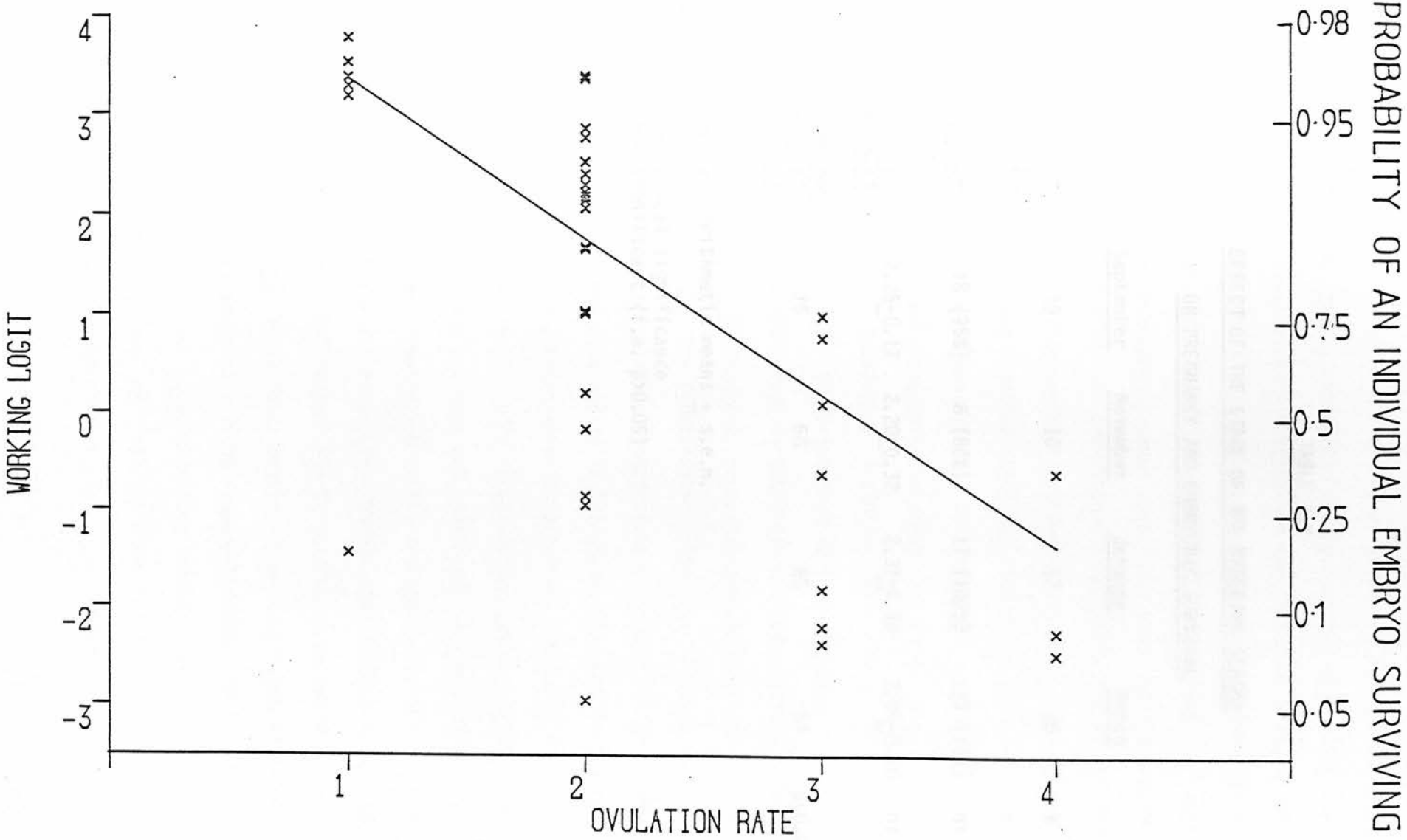


TABLE 3.4

EFFECT OF THE STAGE OF THE BREEDING SEASON ON PREGNANCY AND EMBRYONIC SURVIVAL

	September	November	January	March	
No. of observations	19	10	17	26	s
No. pregnant on day 30	18 (95%)	8 (80%)	17 (100%)	19 (73%)	ns
Mean no. of ovulations*	2.25±0.17	2.20±0.32	2.37±0.18	2.05±0.18	ns
% Ovulations represented by a foetus	75	68	85	51	$p<0.05$

* Values are arithmetic means ± s.e.m.

s = Statistical significance
ns = Not significant (i.e. $p>0.05$)

(Hanrahan, 1982) this represents a major increase in sensitivity compared with the typical one pregnancy per season, and allows the study of within animal effects free of the confounding effects of years, age and parity. This approach has revealed an association between differences in plasma progesterone concentration and embryonic survival in naturally mated ewes. The experiment has also provided new information on the pattern of progesterone secretion and variation in fertility during the breeding season.

This experiment has provided the first direct evidence of an association between lower progesterone concentration around the time of mating and reduced embryonic survival in mated ewes under standard conditions of husbandry. A similar association has also been observed in cattle; the proportion of pregnancies was greater in cows exhibiting higher progesterone concentrations on days 1 to 3 (Lee and Ax, 1984). During the periovulatory period the ovary secretes <0.05ug/min of progesterone (Baird, 1978a). The contribution to the circulating progesterone concentration from the adrenal cortex during this period is not known, although during mid pregnancy the autotransplanted adrenal secretes around 0.03ug/min (Harrison and Heap, 1978). The preovulatory gonadotrophin surge inhibits intrafollicular androgen and oestrogen production (Baird and McNeilly, 1981). However, progesterone secretion in ovulatory follicles may increase at this time (Webb and Gauld, 1984) as indicated by higher follicular fluid (England, Dahmer and Webb, 1981; McNatty et al. 1981) and ovarian venous plasma (Wheeler, Baird, Land and Scaramuzzi, 1975) concentrations. This suggests that some luteinisation of the granulosa cells occurs prior to ovulation, although there is apparently no morphological evidence for this (Bjersing, Hay, Kann, Moor, Naftolin, Scaramuzzi, Short and

Younglai, 1972).

Although the differences in progesterone during the luteal phase were not significantly associated with embryonic survival, it is likely that progesterone concentration at this time is important. Embryo transfer to ovariectomised ewes receiving progesterone has demonstrated the influence of variation in progesterone concentration (Chapter 1). Similarly, in dairy cattle injection of progesterone during the luteal phase has sometimes led to an increase in embryonic survival (reviewed by Sreenan and Diskin, 1983). Differences in luteal phase progesterone levels between pregnant and non-pregnant ewes have been observed prior to luteolysis. In 1981 Brien, Cumming, Clarke and Cocks demonstrated higher levels of progesterone in pregnant ewes on day 12. At this stage differences in progesterone concentration may arise from the embryo directly stimulating progesterone secretion from the corpus luteum (Godkin, Cote and Duby, 1978). It has been suggested that such a mechanism accounts for differences in luteal phase progesterone between pregnant and non-pregnant cows (Hansel, 1981).

The mechanisms behind the association between progesterone profile and embryonic survival require further study. The observed differences between pregnancies in which embryos were lost and those in which all embryos survived could reflect several effects including: a) inadequate uterine function in the absence of progesterone, b) a lack of synchrony between the uterine environment and the embryo, c) differences in the metabolism of progesterone, d) a luteotrophic effect from the embryo, e) inadequate follicular development, or f) that progesterone is a good indicator of a ewe's general fitness and ability to carry a pregnancy.

Embryo transfer experiments involving ovariectomised ewes have

suggested that progesterone concentrations between days 1 and 4 influence the proportion of embryos surviving (Wilmut et al. 1985a). Embryonic survival was 19% (3 out of 16) following transfer to ewes receiving no exogenous progesterone before luteal phase levels of plasma progesterone were generated from day 4 or 5 onwards. Survival was 56% (9 out of 16) in recipients fitted with silastic implants releasing low levels of progesterone between days 1 and 4 before luteal phase levels were generated. As the embryos were transferred to the uterus on Day 6, it seems probable that baseline progesterone exerts its effects on embryonic survival via the uterus. Inadequate progesterone concentrations after mating may induce a uterine environment which is unfavourable for embryonic development, or they may affect the timing of changes in uterine function, and promote asynchrony between the embryo and the uterus.

The work on asynchrony that partly prompted this experiment (Wilmut and Sales, 1981), had predicted that the timing of the switch from baseline levels to luteal levels of progesterone would influence survival. There was no evidence of such an association in the present study. This may indicate that, in the present experiment, there was insufficient variation in the timing of the progesterone rise to affect embryonic survival. The lack of association between timing components and embryonic survival could also reflect the statistical difficulty in establishing the existence of an intermediate optimum. Differences in progesterone concentration around the time of mating may alter the uterine environment or the timing of changes in the environment. In 1983, Lawson and Cahill demonstrated that injections of 25mg of progesterone early in the oestrous cycle advanced uterine function. Day 10 embryos transferred to such ewes on day 6 were able to

implant normally. Embryos in ewes with low progesterone concentrations around the time of heat, may be at an inappropriate stage of development relative to the uterus. Such asynchrony is unlikely to affect embryonic development during the first week of pregnancy (Wilmut and Sales, 1981). Subsequent development would be impaired if the stage of embryonic development was advanced relative to the uterine biochemistry.

No significant differences in the production rate (PR) or metabolic clearance rate (MCRb) from the blood of progesterone between cyclic and pregnant ewes have been observed prior to placentation (Bedford, Harrison and Heap, 1972). In one ewe PR and MCRb were compared on day 7 of the oestrous cycle and of pregnancy. PR was higher during pregnancy (7.08ug/min) than the oestrous cycle (3.67ug/min). During pregnancy, an increased proportiion of MCRb is attributable to uterine uptake (Bedford, Harrison and Heap, 1974).

There are no reports of the embryo affecting maternal function during the first week of ovine pregnancy. The earliest effects are considered to occur on day 9, when embryos exert a local influence on endometrial oestrogen levels (Findlay et al., 1982). As differences in progesterone concentration were observed as early as days 0 and 1 in this experiment, it seems unlikely that this is caused by the embryo. The possibility of the embryo signalling its presence to its mother shortly after fertilisation has been suggested by the isolation of an early pregnancy factor (EPF) with immunosuppressive properties in maternal blood (Morton, Nancarrow, Scaramuzzi, Evison and Clunie, 1979). The authors suggest that a protein 'zygotonin' may be released from the fertilised egg to stimulate the production of maternal EPF. However, this protein has not been isolated from the conceptus. EPF is apparently not

essential for the maintenance of pregnancy (Nancarrow, Wallace and Grewal, 1981).

It is also possible that these results reflect events in follicular maturation. Corpora lutea which secrete low levels of progesterone may be formed from inadequate follicles, which also failed to support normal oocyte development. In these circumstances the embryo may not have the potential for normal development. Although such anomalies have not been reported in cyclic ewes, inadequate corpora lutea are formed during anoestrus following LH-RH treatment to induce ovulation (Haresign and Lamming, 1978; McNeilly, Hunter, Land and Fraser, 1981). This suggestion could be tested by determining whether exogenous progesterone increased embryonic survival.

It has been suggested (Wilmut, Sales and Ashworth, 1985b) that some embryonic mortality has an evolutionary advantage, as it may be associated with an animals ability to live and reproduce in a wide variety of environments. Several environmental factors such as nutrition (Williams and Cumming, 1982) and the stage of the breeding season (Rhind, Chesworth and Robinson, 1978) affect both progesterone concentration and embryonic survival. The poor repeatability of progesterone level observed in the present study suggest that variation in progesterone secretion is primarily of environmental rather than genetic origin. In this way, progesterone concentration may be a reflection of both external conditions and of an animals own body condition and may influence the number of embryos carried. The levels of embryonic survival observed in field situations may reflect the best compromise between the ability to breed in a range of environments and the need for the majority of eggs to develop to term.

This experiment did not suggest that excessive secretion of progesterone may be detrimental to embryonic survival. Elevated progesterone levels from days 7 to 16 after mating were associated with increased embryonic loss in ewes fed sub-maintenance rations (Williams and Cumming, 1982). This may provide evidence of an intermediate optimum level of progesterone, or it may reflect a direct effect of under-nutrition on embryonic development.

The observed effects of ovulation rate on luteal phase progesterone concentrations are in agreement with those of Quirke, Hanrahan and Gosling, (1979). It has been suggested (Plotka, Erb and Harrington, 1970) that the progesterone content of ovine luteal tissue is related to the weight rather than the number of corpora lutea. It appears paradoxical that whilst a relatively higher progesterone profile is associated with embryonic survival, an increase in the number of ovulations, and coincident increase in luteal phase progesterone concentrations, is associated with embryonic loss. Since the marginal increase in progesterone concentration decreases as the number of ovulations increases, the progesterone concentration per embryo is reduced. If each embryo has a specific requirement either for progesterone, or more likely, for a progesterone induced component of uterine fluids, an increased number of corpora lutea may prove detrimental. Two experiments performed in pigs provide some support for this idea. Knight, Bazer, Wallace and Wilcox (1974) reported a dose response relationship between the dose of progesterone administered and the quantity of uterine protein recovered from both ovariectomised and intact gilts. In an experiment in which the numbers of porcine embryos and corpora lutea were either increased using PMSG, or decreased by hemi-ovariectomy embryonic survival was significantly

lower in the treatment group with the lowest plasma progesterone concentration : embryo ratio (Webel, Reimers and Dzuik, 1975). This may explain why partial embryonic loss occurred more frequently than total loss in the present experiment.

The effect of ovulation rate on embryonic survival could only be partially explained by differences in progesterone concentration. Ovulation extends over a longer period of time in multiple ovulators (Whyman, Johnson, Knight and Moore, 1979). In twin ovulating ewes, the mean interval between ovulations was 1.2 $\pm$ 0.6 hours, although in about 5% of ewes the interval exceeded 7 hours (D.I.Sales, personal communication). This spread in the timing of ovulation will increase the range of developmental ages between embryos within a female. The chance of asynchrony between a ewe and one of her embryos will therefore be greater. The likelihood of an embryo being at an appropriate stage to establish a successful pregnancy will also be increased. In this way, a greater number of ovulations leads to an increased proportion of pregnancies, but decreases in individual embryonic survival.

The present observations provide new information on seasonal effects on fertility. Optimal embryonic survival is achieved during the middle of the breeding season. Depressed fertility is evident both early in the season (Hulet, Voitlander, Pope and Casida, 1956) and following spring mating (Oldham, Knight and Lindsay, 1976). It has been suggested that decreased conception late in the breeding season is due to the increasing proportion of infertile ewes within the non-pregnant group. The results from this experiment do not support such a view, as the same population of ewes was used throughout the experimental period. Seasonal differences in embryonic survival could be explained entirely by variations in the

pattern of progesterone secretion. The lower progesterone levels following March mating are in agreement with those of Rhind, Robinson, Fraser and Phillippo (1977). In 1978 Rhind, Chesworth and Robinson reported prolactin levels which were five times greater in March than December. Elevated prolactin levels may reduce progesterone concentrations. In women, high prolactin levels suppressed progesterone production from granulosa cells cultured in vitro (McNatty, Sawers and McNeilly, 1974).

Alternatively, in the present experiment, the lower progesterone concentrations towards the end of the breeding season may reflect lower adrenal progesterone secretion as the ewes became accustomed to the experimental procedures required for the collection of blood samples. It would be necessary to monitor plasma cortisol levels around the time of mating throughout the experiment to determine if the ewes were less stressed as the experiment proceeded. Both handling stress (Kilgour and De Langen, 1970) and post-mating injection of ACTH (Doney, Smith and Gunn, 1976) reduce the number of pregnant ewes. It would therefore be expected that as ewes perceived less stress as the experimental procedures were repeated, embryonic survival would increase. However, in the present study, embryonic survival was lowest following the final mating. The stress of surgery would not have affected embryonic survival, as laparotomy was performed either 30 days after mating, or after the ewe had returned to oestrus.

Seasonal changes affect both male and female reproductive performance, and the possibility that the seasonal affect on embryonic survival is a result of changes in male function cannot be ignored. In 1981, Colas observed lower sperm motility and a higher percentage of nigrosin-eosin stained spermatozoa in ejaculates

collected from Ile-de-France rams in Spring than those collected in Autumn.

Seasonal differences in reproductive performance have been attributed to changes in responsiveness to the negative (Karsch, Goodman and Legan, 1980) and positive (Land, Wheeler and Carr, 1976) feedback action of oestradiol. Legan, Karsch and Foster (1977) suggested that an increased response to oestradiol negative feedback terminates ovarian cyclicity by inhibiting an increase in tonic LH secretion. Seasonal differences in the timing of the preovulatory oestradiol-17B rise, and the LH peak led Wheeler, Baird, Land and Scaramuzzi (1977) to propose a decreased availability of maturing follicles in the late breeding season (February) compared to the mid season (December). All these seasonal effects are associated with a reduction in the number of ovulations. This was not observed in the current study. The reduction in circulating progesterone levels observed in March may therefore be a consequence of decreases in cell number, weight, or secretory capacity of the corpora lutea.

There is much between-ewe variation in both embryonic survival and progesterone concentrations. Genetic variation in embryonic survival may be a trait of either the ewe, the embryo, or both. Embryo transfer experiments suggest that the genotype of the dam is important in determining embryonic survival. There were no differences in survival between embryos transferred from high and low prolificacy breeds (Bradford, 1972). The repeatability of variation in embryonic survival is low (8.0% $\pm$ 9.8% (s.e.m.)) (Hanrahan, 1982). As any trait is less heritable than it is repeatable, embryonic survival per se is not a good selection criterion. It was hoped that a physiological trait associated with embryonic survival might prove a suitable criterion. In the present

experiment, repeatabilities of components of the progesterone profile were also low. This may reflect the range of environmental and physiological factors which affect progesterone concentrations.

This experiment has shown that the progesterone profile during pregnancies in which all embryos survive is relatively higher from days 0 to 1 than the profile during pregnancies associated with embryonic loss. Although the present observations do not suggest a novel method of selection for embryonic survival, they do create new opportunities for pharmacological intervention.

EXPERIMENT 2: THE EFFECT OF EPOSTANE (WIN 32,729) ON PROGESTERONE SECRETION AND EMBRYONIC SURVIVAL DURING EARLY PREGNANCY

Introduction

The experiment described in Chapter 3 showed that circulating progesterone concentration differs between pregnancies in which all embryos survive, and those which suffer loss. Although several physiological and environmental factors contribute to embryonic loss (Edey, 1969) the mechanism by which these factors exert this effect is not known. It has been suggested that such factors may increase embryonic loss by altering the progesterone profile, and hence disrupting the relationship between the uterus and the embryo (Wilmut, Sales and Ashworth, 1985b). This review indicated that many factors which cause embryonic loss also alter progesterone concentrations. In the present experiment, luteal phase progesterone concentrations were suppressed in a controlled manner and any effect on embryonic survival determined.

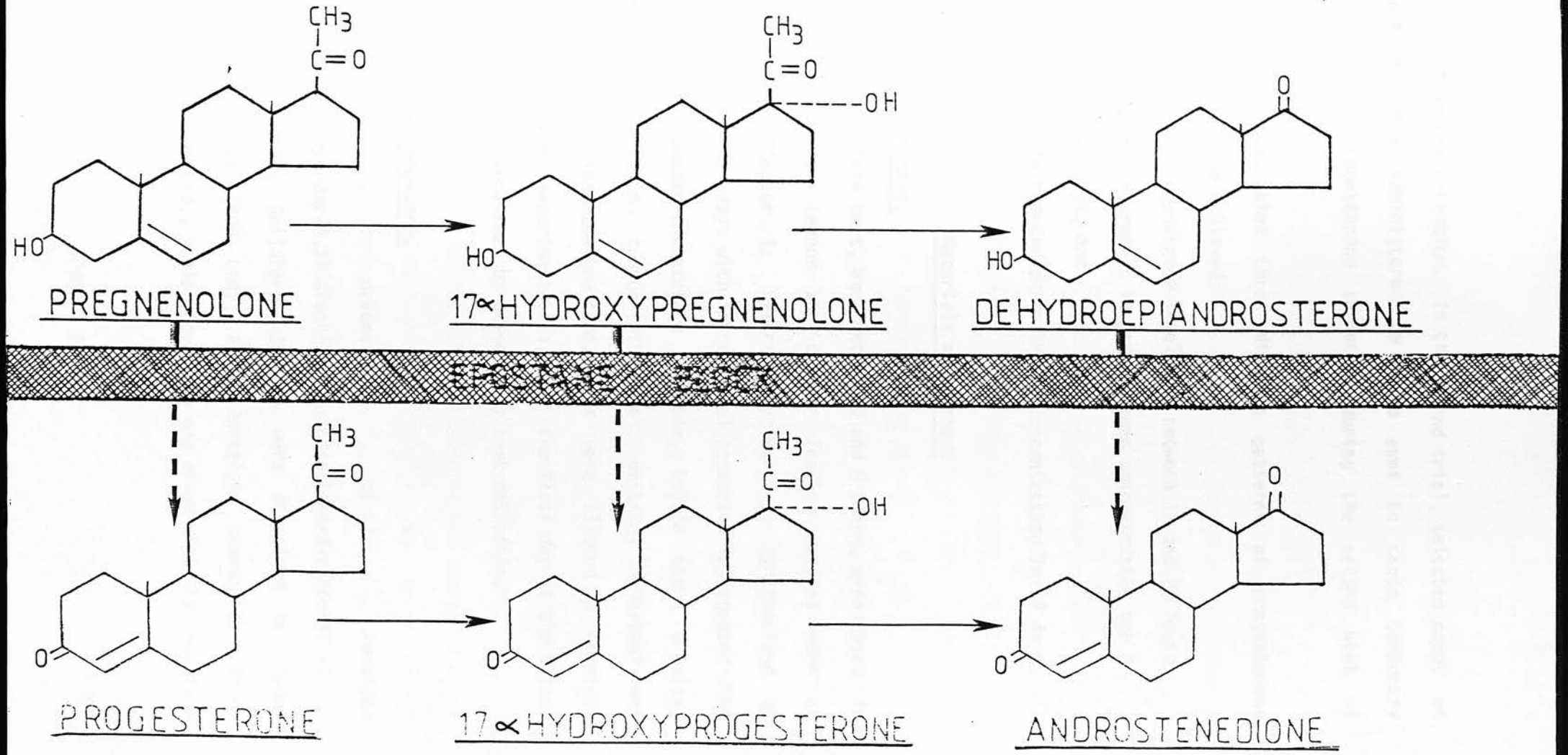
Such decreases in steroid concentration were induced by injections of epostane, a competitive inhibitor of 3β-hydroxysteroid dehydrogenase (3β-HSD) and thus of progesterone secretion (Fig. 4.1). Previous administrations of epostane to sheep have been during late pregnancy. Single 100mg intra-venous injections decreased progesterone levels within an hour after treatment and induced parturition approximately 30 hours later (Ledger, Anderson and Turnbull, 1982).

A dose response trial (Trial 1) was performed to determine the doses of epostane required to cause particular decreases in

Fig. 4.1

Diagram showing the location of the epostane block (shown by the shaded area) in ovarian steroid biosynthesis. Epostane inhibits the oxidation of 3β-hydroxy-Δ^5-steroids to Δ^4-3-oxosteroids by inhibiting 3β-hydroxysteroid dehydrogenase activity.

Fig 4:1 DIAGRAM SHOWING THE LOCATION OF THE EPOSTANE BLOCK IN OVARIAN STEROID BIOSYNTHESIS



progesterone concentration. In the second trial, selected doses of epostane were administered to mated ewes to cause temporary decreases in progesterone secretion during the second week of pregnancy.

It was hoped that three distinct patterns of progesterone secretion would be achieved:

(1) suppressed progesterone levels for between 12 and 24 hours,

(2) intermittent decreases in progesterone concentration for 3 consecutive days, and

(3) continuously reduced progesterone concentration for 3 days.

Materials and Methods

Experimental Animals.

Welsh Mountain ewes, aged between 2 and 9 years, were used in this experiment. Indoor and outdoor feeding regimes were as described in Chapter 3. Oestrous cycles were synchronised by treatment for 12 days with intravaginal pessaries impregnated with 60mg medroxyprogesterone acetate (Veramix, Upjohn Ltd., Crawley, Sussex.). Following sponge withdrawal, oestrous behaviour was monitored using vasectomised rams. Ewes were allowed a complete cycle free from experimentation, and the first day of the second oestrus (day 0) confirmed by twice daily heat detection.

Preparation of epostane.

Five grammes of epostane (4,5-Epoxy-17-hydroxy-4,17-dimethyl-3-oxandrostane-2-carbonitryl, Sterling Winthrop, Guildford, Surrey) were dissolved in 200ml 'Aristar' ethanol (BDH Ltd., Poole Dorset.) overnight. 200ml arachis oil (BDH Ltd., Poole, Dorset.) was mixed with the epostane

solution prior to injection. A fresh solution of epostane was made up each evening before an injection.

Determination of Plasma Progesterone Concentrations.

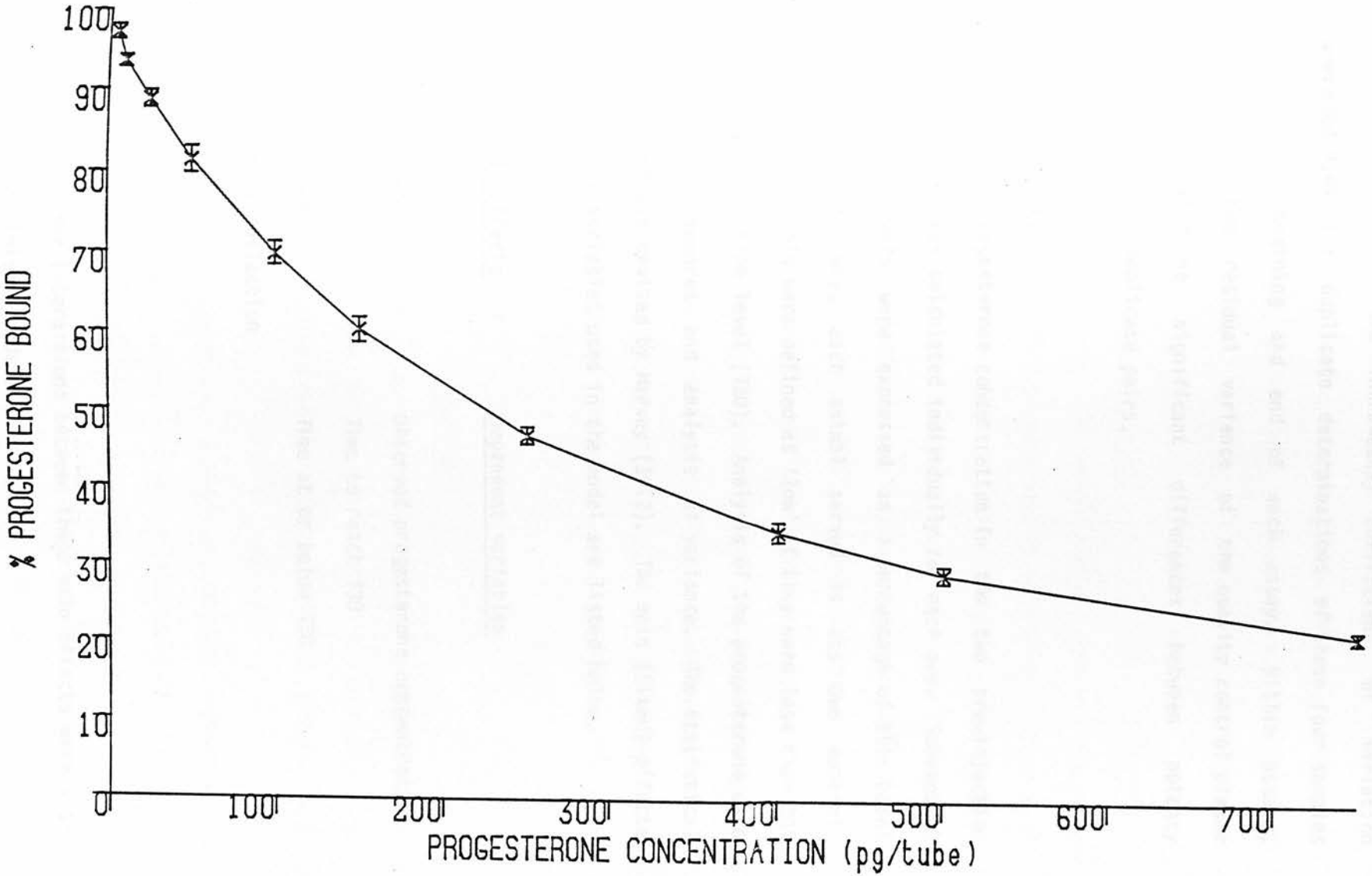
Both the collection and radioimmunoassay of plasma samples for progesterone estimation were performed as described in Chapter 2. Negligable (<0.001%) cross-reaction was observed between epostane and progesterone antisera (31/8) at 50% displacement of tracer. To ensure that within-animal effects were not confounded with between assay differences, plasma samples from two animals in each treatment group were measured in each assay. Eleven assays, including one containing repeat samples, were performed. Within assays, the samples were randomised to compensate for any assay drift.

No significant differences between progesterone assays performed in trials 1 and 2 were apparent. The assay reliability criteria from both sets of assays were therefore pooled. Progesterone was extracted from plasma using freshly distilled petroleum ether (BDH Ltd., Poole, Dorset.). The mean extraction efficiency was 67.0 $\pm$ 1.70%(s.e.m.). The mean minimum detectable dose was 26.73 $\pm$ 3.21pg/tube (s.e.m.) (287pg/ml). Water blanks were less than the minimum detectable dose in all assays. Within each assay, both standard curves were parallel. The overall standard curve was used to estimate progesterone concentrations. Between assays, the overall standard curves were highly reproduceable, (Fig. 4.2), with no significant differences in slope (mean slope= -1.073 $\pm$ 0.023 (s.e.m.)). The inter-assay coefficients of variation using four standard quality samples with mean progesterone concentrations of 0.21 $\pm$ 0.09ng/ml, 2.18 $\pm$ 0.27ng/ml, 3.32 $\pm$ 0.28ng/ml and 9.21 $\pm$ 0.97ng/ml were 46.7%, 12.6%, 8.3% and

Fig. 4.2

Composite standard curve from progesterone assays performed in experiment 2. Each point represents the mean of 66 determinations ± 1 s.e.m.

FIG 4.2: COMPOSITE STANDARD CURVE FROM PROGESTERONE ASSAYS IN EXPERIMENT 2



10.5% respectively. The intra-assay coefficient of variation averaged 9.4% for duplicate determinations of these four samples placed at the begining and end of each assay. Within assays, analysis of the residual variance of the quality control plasma samples revealed no significant differences between potency estimates within duplicate pairs.

Analysis of Data.

The mean progesterone concentration in the two pre-injection plasma samples was calculated individually for each ewe. Subsequent progesterone levels were expressed as a percentage of this basal value. In this way, each animal served as its own control. Progesterone levels were defined as 'low' if they were less than 30% of the pre-injection level (T30). Analysis of the progesterone data was by least squares and analysis of variance. The statistical package used was devised by Harvey (1972). The main (fixed) effects and dependent variables used in the model are listed below.

Main (fixed) effects	Dependent variables
Individual ewe	Observed progesterone concentration
Age of ewe	Time to reach T30
Treatment group	Time at or below T30
Time of sample collection	
Assay	

All possible 2-way interactions between these main effects were also included in the initial model.

The effect of epostane on embryonic survival was determined using the Chi-squared test. The percentage embryonic survival in the control ewe groups was defined as the 'expected' embryonic survival.

Trial 1:

Of 50 ewes synchronised, 30 which showed oestrus behaviour within a 36 hour time interval were used in this trial. These ewes were randomly assigned to 5 treatment groups. Subcutaneous injections of epostane were administered to 4 of the 5 groups of ewes at 8am on day 9. Ewes received 0.8,4,8 or 20ml of the 12.5g/l epostane solution to deliver 10, 50, 100 or 250mg epostane respectively. The remaining 6 ewes served as controls, receiving an injection of 20ml 50:50 ethanol:arachis oil on day 9. Jugular venous samples were collected one hour before and immediately before injection, and then at 1, 1.5, 2.5, 4, 6 and 12 hours after injection, thereafter, every 12 hours until 48 hours following injection.

Results

Injections of varying doses of epostane significantly lowered progesterone concentrations in all four groups of ewes. Increasing doses of inhibitor affected both the magnitude and the duration of this response.

There were no significant differences between progesterone concentrations in the five treatment groups prior to injection (time -1 and 0 hours), or at 36 and 48 hours after treatment. In the control ewes there was no significant difference between progesterone levels in samples collected at different times relative

to the control injection. Progesterone profiles in ewes receiving epostane were therefore compared to the overall control ewe profile.

There was a significant ($p<0.001$) effect of epostane on progesterone concentrations in the 4 treatment groups. Significant ($p<0.001$) differences in progesterone concentrations were observed between treatment groups receiving different doses of epostane (Table 4.1).

In addition to the differences in the extent of progesterone reduction, the time over which the level was depressed also varied with treatment. This interaction was significant ($p<0.001$; Fig. 4.3). Control ewes had significantly higher ($p<0.05$) progesterone levels than those receiving 250mg epostane from 1 to 24 hours after injection, and than ewes given 100mg epostane from 1.5 to 24 hours following injection. Progesterone levels in ewes receiving 50mg epostane were significantly lower than control values at 2.5, 4, 6, 8 and 12 hours. A 10mg dose of epostane only significantly reduced progesterone concentrations 4 hours after administration.

Injections of 1x250mg, 3x50mg and 3x250mg of epostane respectively were therefore selected to elicit the three responses required for trial 2.

Trial 2:

Oestrous cycles of 80 Welsh Mountain ewes were synchronised. Of these, 52 which showed a 2nd oestrus within a 36 hour time interval of each other were mated and randomly assigned to four treatment groups.

Ewes received subcutaneous injections of an appropriate volume of a 12.5g/l epostane solution. The injection and bleeding regime for each treatment group is shown in Table 4.2. A blood sample was

TABLE 4.1

EFFECT OF VARYING DOSES OF EPOSTANE

ON PROGESTERONE CONCENTRATION ($p<0.001$).

Dose of epostane (mg)	Least-squares mean progesterone concentration (ng/ml) ± s.e.m.
0 (controls)	6.20 ± 0.20
10	4.68 ± 0.18
50	3.43 ± 0.21
100	3.03 ± 0.19
250	2.83 ± 0.19

Fig. 4.3

Effect of varying doses of epostane on plasma progesterone concentrations. Epostane injections were administered at 8am on day 9 (day 0 = day of oestrus). Each point represents the mean of 6 determinations.

FIG 4.3: EFFECT OF VARYING DOSES OF EPOSTANE ON PLASMA PROGESTERONE CONCENTRATION

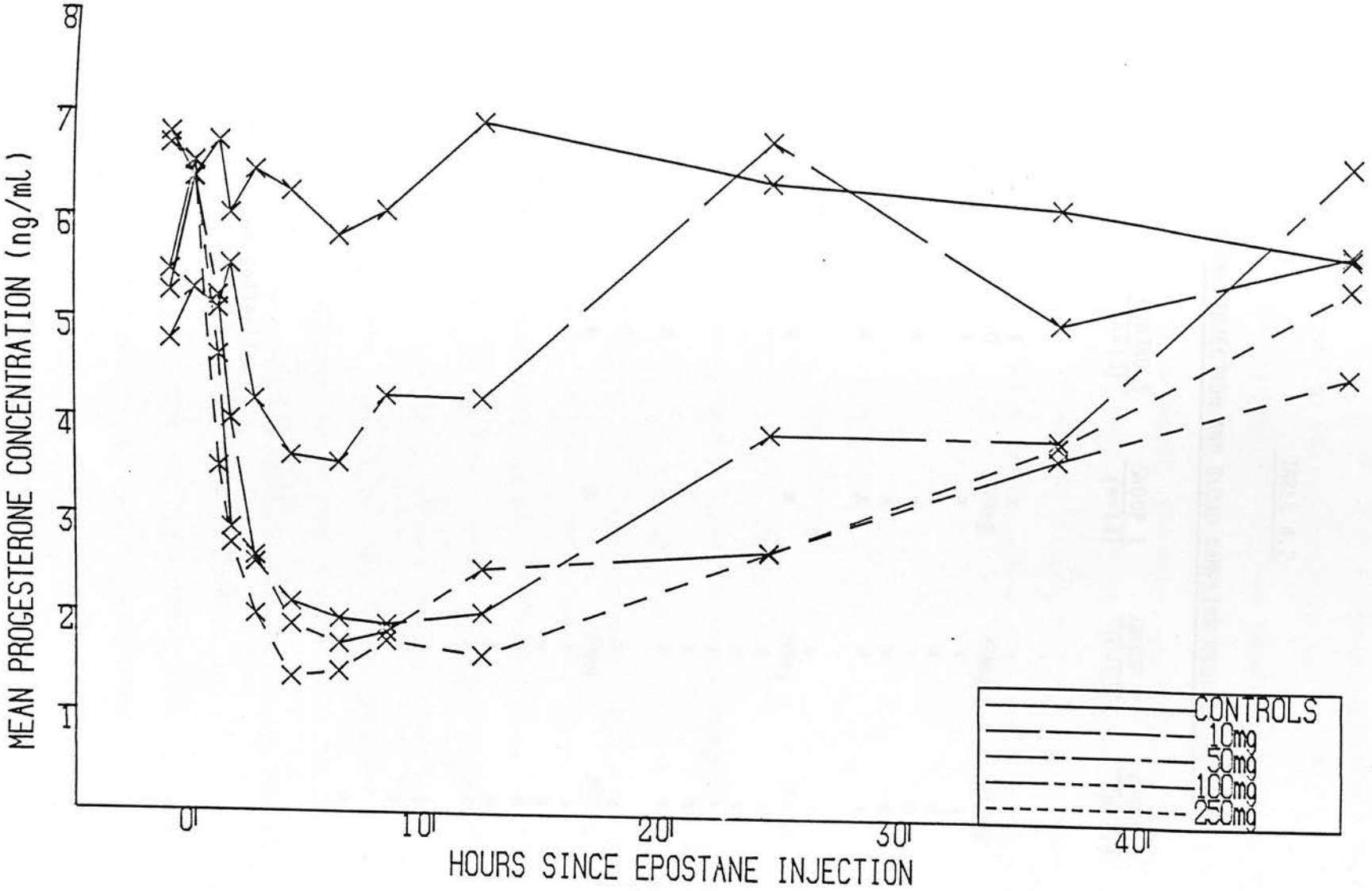


TABLE 4.2

EPOSTANE INJECTION AND BLOOD SAMPLING REGIME: TRIAL 2

	Time	CONTROLS (n=13)	GROUP 1 (n=13)	GROUP 2 (n=13)	GROUP 3 (n=11)
Day 9	7.00	x	x	x	x
	8.00	0	250mg	50mg	250mg
	10.00	x	x	x	x
	12.00		x	x	x
	14.00	x	x	x	x
	16.00		x	x	x
	20.00	x	x	x	x
Day 10	7.00			x	x
	8.00	x	x	50mg	250mg
	10.00			x	x
	12.00			x	x
	14.00			x	x
	16.00			x	x
	20.00	x	x	x	x
Day 11	7.00			x	x
	8.00	x	x	50mg	250mg
	10.00			x	x
	12.00			x	x
	14.00			x	x
	16.00			x	x
	20.00			x	x
Day 12	8.00			x	x
	20.00			x	x
Day 13	8.00			x	x

x = Time of blood collection

also collected immediately prior to each epostane injection. The control ewes received 20ml of the injection vehicle.

A vasectomised ram was kept with the ewes throughout the trial, in order to mark any return heats. A mid-ventral laparotomy was performed on all ewes which had not returned to oestrus by day 30 to determine corpora lutea and foetal numbers. The number of ovulations in ewes which returned to oestrus was not determined. The anaesthesia and surgical procedures were as described in Chapter 3.

Results.

Injections of epostane reduced both embryonic survival and progesterone concentrations. The treatments had different effects on the duration of low progesterone levels, and on the proportion of pregnant ewes.

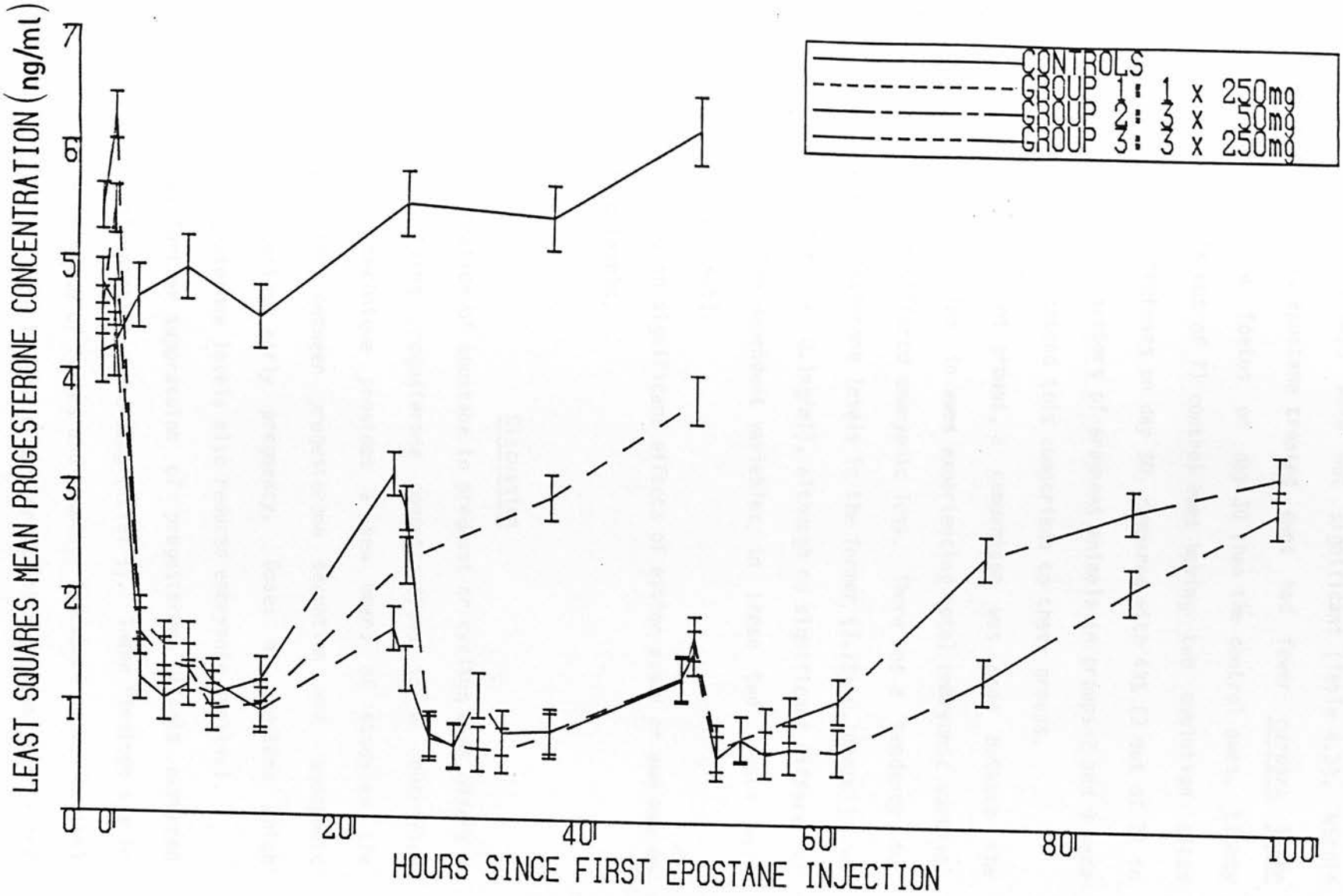
There was a significant ($p<0.001$) effect of epostane on progesterone concentration in all three treatment groups (Fig. 4.4). In the control ewes there were no significant differences in the progesterone level at different times relative to the sham injection. Individual progesterone profiles did vary within group 1 ($p<0.01$), and groups 2 and 3 ($p<0.001$). Progesterone concentrations were at or below the T30 level for between 12 and 24 hours in group 1, periods of approximately 18 hours during 3 consecutive days in group 2, and for three consecutive days in group 3.

The proportion of pregnancies was significantly ($p<0.05$) lower in ewes experiencing continuously low levels of progesterone for three days (group 3) than in either the controls, or ewes in which progesterone concentrations were suppressed for less than 24 hours (group 1). Differences in the proportion of pregnancies between

Fig. 4.4

Effect of epostane on progesterone concentrations during pregnancy. The first epostane injection was administered at 8am on day 9 (day 0 = day of mating). Control and group 1 ewes received an injection at 0 hours, and those in groups 2 and 3 at 0, 24 and 48 hours.

FIG 4.4: EFFECT OF EPOSTANE ON PROGESTERONE CONCENTRATIONS DURING PREGNANCY



other treatment groups were not significant (Table 4.3). Within pregnant animals, epostane treated ewes had fewer corpora lutea represented by a foetus on day 30 than the control ewes. Eighty five percent (6 out of 7) control ewes having two ovulation sites also had two foetuses on day 30, compared with 43% (3 out of 7) in group 1. The low numbers of pregnant animals in groups 2 and 3 made it unrealistic to extend this comparison to these groups.

Within treatment groups, a comparison was made between the progesterone profiles in ewes experiencing total embryonic survival with those that suffered embryonic loss. There was a tendency for higher mean progesterone levels in the former (1.78± 0.18ng/ml) than the latter (1.61 ± 0.1ng/ml), although no significant differences between any of the dependent variables in these two groups were observed (Fig. 4.5).

There were no significant effects of either assay or ewe age on progesterone levels.

Discussion

Administration of epostane to pregnant or cycling ewes offers a method of modifying progesterone concentrations in a controlled manner. This technique provides a new means of studying the inter-relationship between progesterone secretion and embryonic development during early pregnancy. Doses of epostane which depressed progesterone levels also reduced embryonic survival.

Minimal further suppression of progesterone levels occurred with doses of epostane above 50mg (trial 1). These findings are in agreement with those of Peters and Lamming, (personal communication) in which progesterone levels in Hereford x Friesian cows stabilised at 5-6ng/ml following injections of 4x250 and 4x125mg of epostane.

TABLE 4.3

EFFECT OF EPOSTANE ON EMBRYONIC SURVIVAL

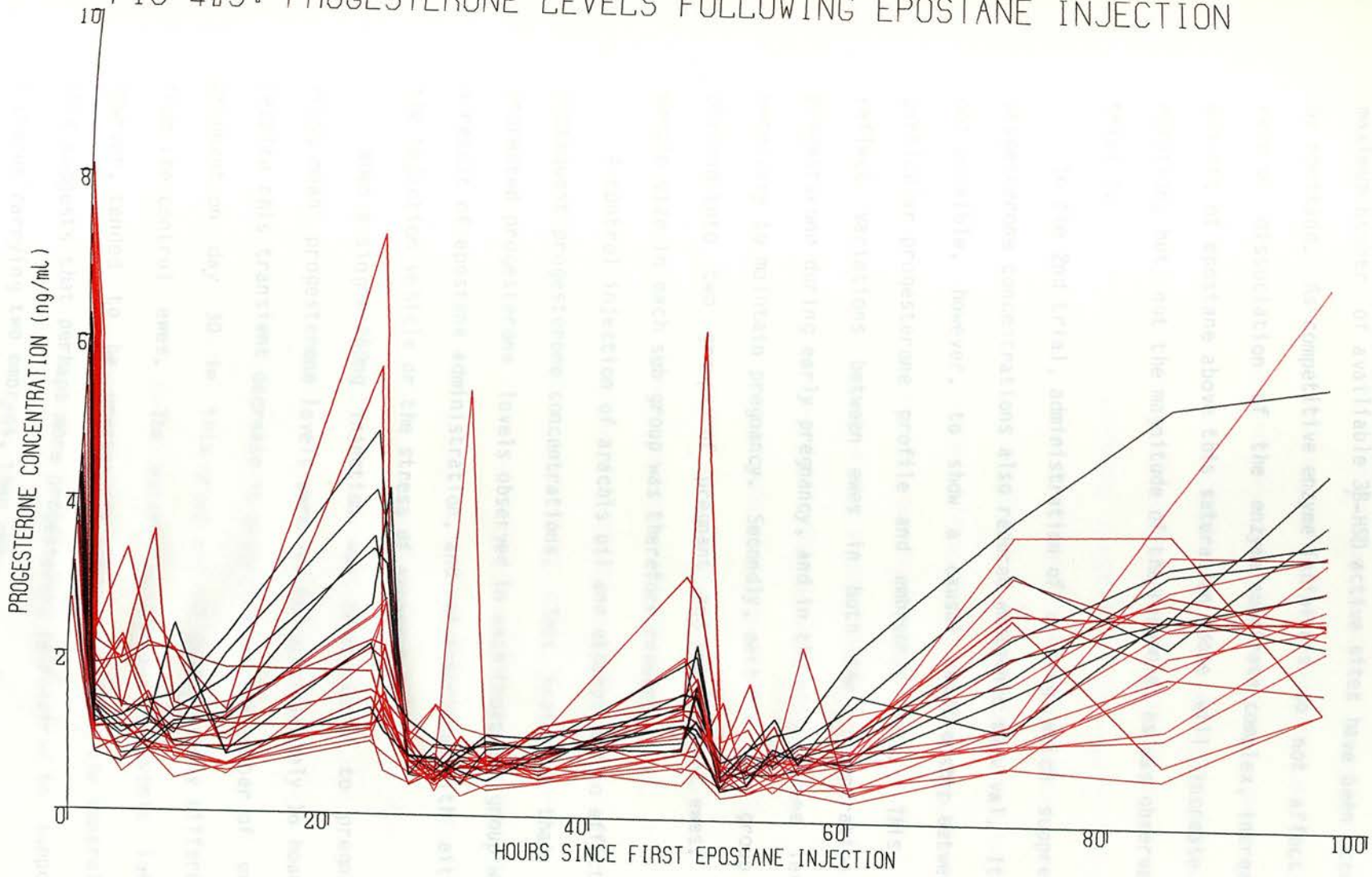
	Controls	Group 1	Group 2	Group 3	Statistical significance
All ewes					
No. of ewes	13	13	13	11	
No. pregnant on Day 30	11	12	7	2	
% Pregnant	84.6	92.3	53.8	18.2	$p<0.05$
Ewes pregnant on Day 30					
No. of *corpora lutea*	18	19	10	3	
No. of foetuses	17	15	9	2	
%Embryonic loss	5.5	21.0	10.0	33.3	ns

ns = Not significant (i.e. $p>0.05$)

Fig. 4.5

Individual progesterone profiles from ewes in treatment groups 2 and 3. Injections of 50mg or 250mg of epostane were administered at 0, 24 and 48 hours. The black lines show progesterone patterns in ewes experiencing total embryonic survival. Red lines represent progesterone concentrations in ewes which suffered either total or partial embryonic loss.

FIG 4.5: PROGESTERONE LEVELS FOLLOWING EPOSTANE INJECTION



It would appear that at the substrate concentration present, the maximum number of availiable 3β-HSD active sites have been occupied by epostane. As competitive enzyme inhibitors do not affect the rate of dissociation of the enzyme substrate complex, increasing amounts of epostane above this saturation dose will increase the duration, but not the magnitude of the response, as was observed in trial 1.

In the 2nd trial, administration of a drug which suppressed progesterone concentrations also reduced embryonic survival. It was not possible, however, to show a causal relationship between a particular progesterone profile and embryonic loss. This may reflect variations between ewes in both the concentration of progesterone during early pregnancy, and in the progesterone levels necessary to maintain pregnancy. Secondly, each treatment group was divided into two groups of pregnant and non-pregnant ewes. The sample size in each sub group was therefore reduced.

A control injection of arachis oil and ethanol had no effect on subsequent progesterone concentrations. This indicates that the depressed progesterone levels observed in each treatment group were a result of epostane administration, and not associated with either the injection vehicle or the stress of experimentation.

When a single 250mg injection was administered to pregnant ewes, mean progesterone levels were low for approximately 16 hours. Despite this transient decrease in progesterone, the number of ewes pregnant on day 30 in this group was not significantly different from the control ewes. The extent of partial embryonic loss, however, tended to be greater in group 1 ewes than the controls. This suggests that perhaps more progesterone is required to support a uterus carrying two embryos, than one.

Ovarian production of 17α-hydroxyprogesterone and androstenedione is also inhibited by epostane. These steroids may be implicated in the observed effect of epostane on embryonic survival. Exogenous progesterone implants reversed the interceptive effect of epostane in rats (data on file at Sterling Winthrop Research and Development). This provides evidence for a causal relationship between suppressed progesterone and embryonic loss in rats. A similar association has recently been demonstrated in sheep (C.J.Ashworth, I.Wilmut, R.Webb and A.J.Springbett, unpublished data). The doses of epostane administered to ewes in group 3 in the present experiment (250mg on days 9,10 and 11) were given to ewes which had a progesterone releasing silastic implant inserted on Day 8. Embryonic survival was 72% in untreated ewes, 78% in ewes given progesterone and epostane and 0 in ewes given epostane alone. Not only does this experiment suggest causality between depressed progesterone concentration and lower embryonic survival, it also indicates that, at the doses given, epostane is not embryotoxic.

The effect of epostane administration on adrenal biosynthesis in sheep is not known. Epostane is a more potent inhibitor of ovarian and placental biosynthesis than of adrenal synthesis in women (Spuy, Jones, Wright, Piura, Paintin, James and Jacobs, 1983) and non-human primates (Creange, Anzalone, Potts and Schane, 1981). However, in rats epostane appears to be an equipotent adrenal and ovarian/placental inhibitor (Creange et al., 1981). The reason for this species difference is not known. In higher mammals, the differences in adrenal and ovarian potencies may reflect multiple enzyme systems which respond differently to the same agent (Gibb and Hagerman, 1976). Azastene, an alternative inhibitor of the 3B-HSD system, is also equipotent in rats (Schane and Creange, 1980) but

was ineffective in suppressing the stimulatory action of ACTH on ovine adrenal biosynthesis (Singh-Asa, Jenkin and Thorburn, 1982). It would seem likely that epostane is a less potent inhibitor of adrenal than ovarian/placental biosynthesis in sheep. It would be necessary to measure cortisol and dehydroepiandrosterone concentrations following epostane administration in order to confirm this.

Several environmental factors which contribute towards embryonic loss also affect progesterone concentrations. There is a seasonal effect on both progesterone concentrations (Rhind, Chesworth and Robinson, 1978; Quirke, Hanrahan and Gosling, 1979) and embryonic survival (Cumming, Blockey, Winfield, Parr and Williams, 1975). In the preceeding chapter it was shown that the lower proportion of embryos surviving late in the breeding season could be explained entirely by differences in the progesterone profile. The likelihood of an individual embryo surviving within a ewe is inversely related to the number of corpora lutea (Cumming et al., 1975). The marginal increase in progesterone concentration decreases with increasing ovulations (Quirke, Hanrahan and Gosling, 1979). Therefore, the concentration of progesterone per embryo is reduced. While such effects have not previously been shown, this observation suggests that each embryo may have a specific progesterone requirement. This may explain the results observed in group 1, where most ewes retained one of their two embryos following a temporary decrease in progesterone levels. The effects of varying nutritional regimes on early pregnancy provide evidence for an intermediate optimum level of luteal phase progesterone. Both under- and over-fed ewes have depressed embryonic survival (Edey, 1970). Ewes fed on lupin supplements at mating had lower

progesterone levels than controls (Brien et al., 1981). A 25% ration, however, induced higher progesterone levels (Williams and Cumming, 1982).

Many environmental stresses, including heat stress (Alliston, Elgi and Ulberg, 1961; Dutt, 1964), cold stress (MacKenzie, Thwaites and Edey, 1975), and sheep handling procedures (Kilgour and De Langen, 1970) reduce embryonic survival. Such effects are thought to be due to increased adrenal activity (Howarth and Hawk, 1968) and can be mimicked by injections of ACTH (Doney, Smith and Gunn, 1976). A study performed by Rhind, Doney, Gunn and Leslie (1984) did not detect any effect of environmental stress on progesterone concentrations. However, as plasma progesterone levels were only measured daily, diurnal fluctuations would be missed. In the present experiment, ewes which experienced repeated daily variations in progesterone levels (group 2) did exhibit reduced embryonic survival.

The results observed in group 3 were as expected. Continuously low progesterone for four consecutive days dramatically reduced embryonic survival. In mice, postcoital injections of anti-progesterone monoclonal antibody had a similar effect on pregnancy (Wang, Rider, Heap and Feinstein, 1984). Suppression of progesterone was associated with the lack of an implantation response, and arrested embryonic development.

The present experiment highlights the importance of an appropriate concentration of progesterone to ensure the maintenance of pregnancy. Although ewes appeared tolerant to a brief reduction in progesterone concentrations, repeated decreases were associated with embryonic mortality. There are several possible explanations for the observed reductions in plasma progesterone concentrations

and embryonic survival. The lower progesterone concentrations generated during the luteal phase may trigger the prostaglandin mediated luteolytic response resulting in abortion. It was not possible to monitor cycle lengths following epostane injection during trial 1. However, luteal phase administration of epostane to cattle (Peters and Lamming, personal communication) or of trilostane (an alternative inhibitor of the 3β-HSD system) to ewes on days 8 to 11 (Jenkin, Gemmell and Thorburn, 1984) did not affect cycle length. In the present experiment plasma progesterone concentrations declined rapidly following epostane injections on the morning of day 9. The uterus, therefore, would not have had the 7-10 day period of progesterone priming necessary before it can synthesise adequate quantities of PG-$F_{2\alpha}$ to induce luteolysis (Baird, 1978b). Alternatively, changes in progesterone concentrations affect uterine function, and could disrupt the relationship between the embryo and the uterus and thereby increase the likelihood of embryonic loss.

CHAPTER 5

EXPERIMENT 3: PROTEIN PATTERNS IN UTERINE SECRETIONS COLLECTED FROM CYCLIC WELSH MOUNTAIN EWES

Introduction

Uterine secretions provide the immediate environment for the developing conceptus. From the second week of pregnancy, the blastocyst becomes increasingly dependent on substrates secreted by the endometrium (Heap, Flint and Gadsby, 1979). Embryo transfer studies have shown the need for a strict synchrony between an embryo and its mother (Rowson and Moor, 1966). When ovariectomised ewes are used as recipients, embryonic survival is optimal if they receive a precise pattern of progesterone injections (Miller and Moore, 1976). These observations suggest that uterine fluid has a constantly changing composition, and that such changes are induced primarily by differences in the concentration of circulating progesterone. Variation in both the timing and extent of changes in uterine secretions will induce a range of uterine environments, only some which are favourable for embryonic development.

It was hoped to establish a technique whereby successive uterine secretions could be collected from the same ewe. This would enable the pattern of uterine changes within a ewe to be defined, and the extent of between-ewe variations in such profiles to be determined. It would also be possible to supply hormones to ovariectomised ewes, and assess the effects on uterine secretions.

The secretion of uterine proteins is regulated by ovarian steroids. However, the specific steroid requirements and mode of secretion of these proteins vary. Circulating progesterone levels

affect uterine concentrations of ovine alkaline phosphatase (Murdoch and White, 1967) and β.glucuronidase (Murdoch and O'Shea, 1977). In pigs, progesterone induces uterine leucine amino-peptidase (Roberts, Bazer, Baldwin and Pollard, 1976). Beier (1974) showed that rabbit uterine albumin : total protein ratio is dependent upon the dominant ovarian steroid during the oestrous cycle. Although the precise functions of these particular proteins within the ovine uterus are not known, a knowledge of temporal changes in their uterine concentrations may provide a marker of the physiological or functional stage of the uterine environment. The total protein in the uterus, together with the four uterine proteins described above were measured in the present experiment.

Uterine secretions were initially collected from in-dwelling uterine cannulae, as described by Lane and Cook (personal communication). Poly vinyl cannulae were placed at each end of one uterine horn, and secured by ligatures. This technique enables successive samples to be collected throughout the oestrous cycle, in the same animal.

The presence of such cannulae was associated with disturbances to cycle length. Of 14 ewes fitted with a cannula; one did not exhibit oestrus throughout the four week experimental period, 6 had cycle lengths greater than 30 days, and 5 had cycles lasting 6 days or less. The remaining two ewes had 10 and 12 day oestrous cycles. It seems likely that the cannulae were preventing normal luteal function, as occurs following insertion of an intra-uterine device (IUD) (Hawk, 1968). This effect is thought to be mediated by activation of the uterine luteolytic mechanism, as levels of PG-$F_{2\alpha}$ were elevated in IUD bearing ewes (Spilman and Duby, 1972; Wilson, Cenedella, Butcher and Inskeep, 1972).

In view of this, it was concluded that the uterine environment in these animals would be atypical. A surgical procedure to collect uterine secretions was therefore developed.

Materials and Methods

Experimental Animals.

Oestrous cycles of thirty six Welsh Mountain ewes were synchronised by two 2ml intra-muscular injections containing 100ug of cloprostenol given 9 days apart. The occurrence of the second oestrus following synchronisation was monitored twice daily by vasectomised rams. Ewes showing a second oestrus within a pre-defined 10 day interval of each other were randomly assigned to six treatment groups.

Design of experiment

Uterine flushings were collected on days 0,3,6,9,12,15 of the cycle, and at the subsequent return to oestrus. Flushings from both uterine horns were collected on two consecutive occasions for each ewe. Uterine secretions were collected at each point designated by an X on Table 5.1.

Table 5.1: Regime used to collect uterine secretions.

		Day of cycle						
		0	3	6	9	12	15	Return
Group (n)	A(4)	X	X					
	B(6)		X	X				
	C(5)			X	X			
	D(6)				X	X		
	E(6)					X	X	
	F(5)						X	X

This design permits successive sampling of uterine secretions from each ewe, whilst minimising the surgical trauma to that animal. Secondly, it was possible to determine if there is a 'carry-over' effect from one operation to the next, and hence if successive sampling is possible.

Collection of uterine flushings

Uterine secretions were collected during a mid-ventral laparotomy performed under general anaesthesia. Anaesthetic and surgical procedures were performed as described in Chapter 3. Fluid collection was facilitated by means of a 2.1mm external diameter intra-venous catheter (Portex Ltd., Hythe, Kent) trimmed to a length of approximately 10cm. Catheters were gas sterilised using ampoules containing ethylene oxide ('Anprolene', H.W. Anderson Products, Oyster Bay, New York) released in a closed system. A small stab wound was made in the tip of each uterine horn, through which the catheter was introduced into the uterus. The lumen was occluded at the base of the appropriate horn (to prevent irrigation of the opposite horn) whilst sterile 0.33M NaCl was rinsed through the uterine lumen. Care was taken to avoid distending the uterine lumen. Consequently, differing volumes of saline (between 6-10ml) were introduced.

The flushings were withdrawn into sterile test-tubes, and centrifuged at 4°C, 3000rpm for 30 minutes. An aliquot of the supernatant was frozen to await albumin and protein analyses. The remainder was used for enzyme determinations, which were performed on the day of collection.

Assay procedures.

The concentrations of each of the five uterine proteins investigated were determined using colormetric analyses. Quality control samples for the three enzymes measured consisted of known quantities of enzyme diluted with appropriate volumes of isotonic saline.

β.Glucuronidase activity was measured by colormetric determination of the amount of phenolphthalein liberated following incubation of the uterine flushing with phenolpthalein glucuronic acid at 56°C for one hour (Fishman, Kato, Anstiss and Green 1967). The intra- and inter-assay co-efficients of variation were 1.4% and 6.0% respectively.

The activity of leucine amino-peptidase in the flushings was determined by the method of Nagal, Willig and Schmidt (1964). Leucine amino-peptidase catalyses the dissociation of L-leucine-p-nitranilide into leucine and p-nitraniline. The amount of p-nitraniline liberated in a given time is proportional to the leucine amino-peptidase concentration, and can be measured at 405nm. Variation within- and between-assays was 16.4% and 23.5% respectively.

Alkaline phosphatase levels were measured by the method of Price and Woodman (1971), on a continuous flow analyser. The sample was incubated with buffer substrate at 37°C for approximately 9 minutes. The liberated phenol was then dialysed into bicarbonate and determined colormetrically at 505nm. Intra- and inter-assay co-efficients of variation were 2.1% and 4.0% respectively.

All albumin and total protein determinations were performed in the same assay on a continuous flow analyser. Protein levels were measured by the biuret method (Failing, Buckley and Zak, 1960). The

albumin assay was based on the method of Doumas, Watson and Biggs (1971), which was modified for use on an continuous flow analyser by Spencer and Price (1977). Assay variation was assessed using Welcomtrol Normal Quality Control Sera (Wellcome Reagents Ltd., London) diluted 1:100 with isotonic saline. Within-assay co-efficients of variation were 4.0% and 1.7% for albumin and total protein respectively.

The specific activity of each enzyme was expressed as a proportion of the total protein recovered for each flush sample.

Statistical Procedures

Enzyme values were log transformed. A within-animal comparison between the first and second uterine fluid collection was performed. Between animals, the effects of day of cycle, number of ovulations, proximity to an ovary bearing a *corpus luteum*, side of uterus and individual animals on the concentration of uterine proteins were determined using general linear models (Nelder and Wedderburn, 1972).

Results

The five uterine components investigated were present in uterine rinsings at varying concentrations throughout the oestrous cycle. However, a substantial proportion (43%) of the leucine amino-peptidase determinations were below the minimum detectable dose (0.54mU/ml) for the assay.

Within-animals, there were significant ($p<0.05$) differences in 4 of the proteins measured between samples collected during the first and second flushes (Figs. 5.1, 5.3, 5.4 and 5.5). Levels of β.glucuronidase were not significantly different between the first

Fig. 5.1

Total protein levels in uterine flushings collected throughout the oestrous cycle. Each point represents the mean concentration ± 1 s.e.m. The number of observations each day is shown on table 5.1.

FIG 5.1: TOTAL PROTEIN LEVELS DURING THE OESTROUS CYCLE

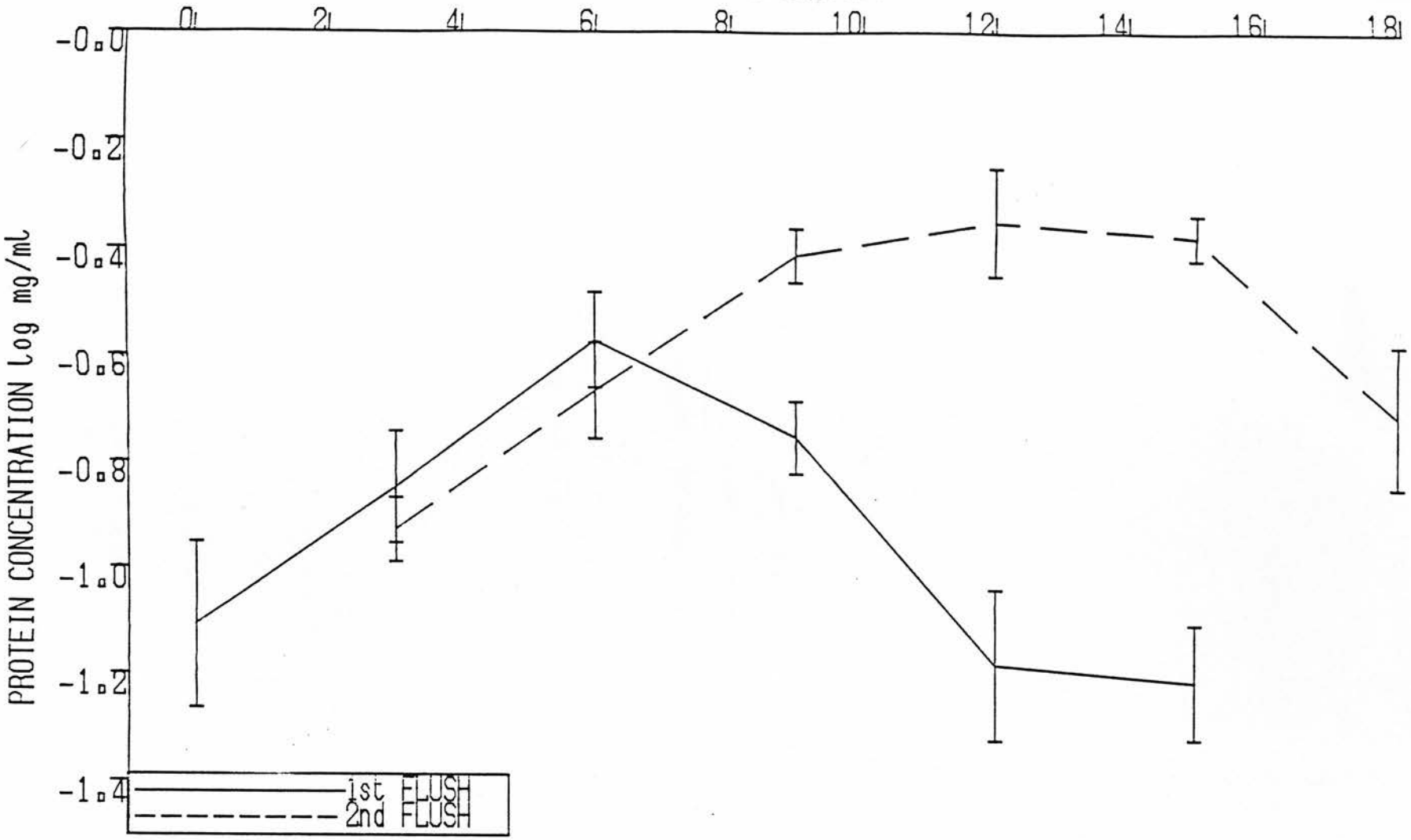


Fig. 5.2

β.Glucuronidase levels in uterine flushings collected throughout the oestrous cycle. Each point represents the mean concentration ± 1 s.e.m. The number of observations each day is shown on table 5.1.

FIG 5.2: β.GLUCURONIDASE LEVELS DURING THE OESTROUS CYCLE

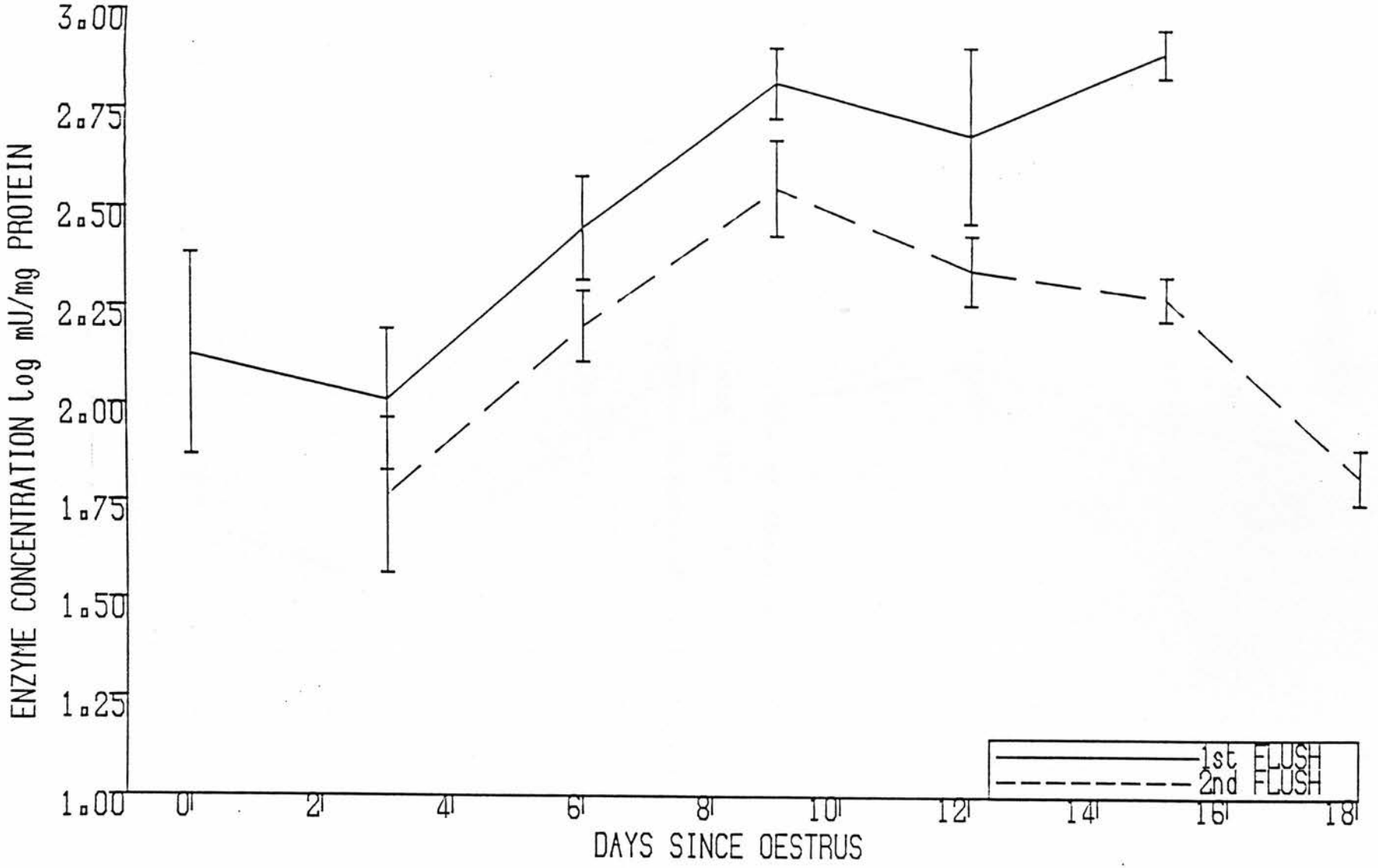


Fig. 5.3

Alkaline phosphatase levels in uterine flushings collected throughout the oestrous cycle. Each point represents the mean concentration $\pm$ 1 s.e.m. The number of observations each day is shown on table 5.1.

FIG 5.3: ALKALINE PHOSPHATASE LEVELS DURING THE OESTROUS CYCLE

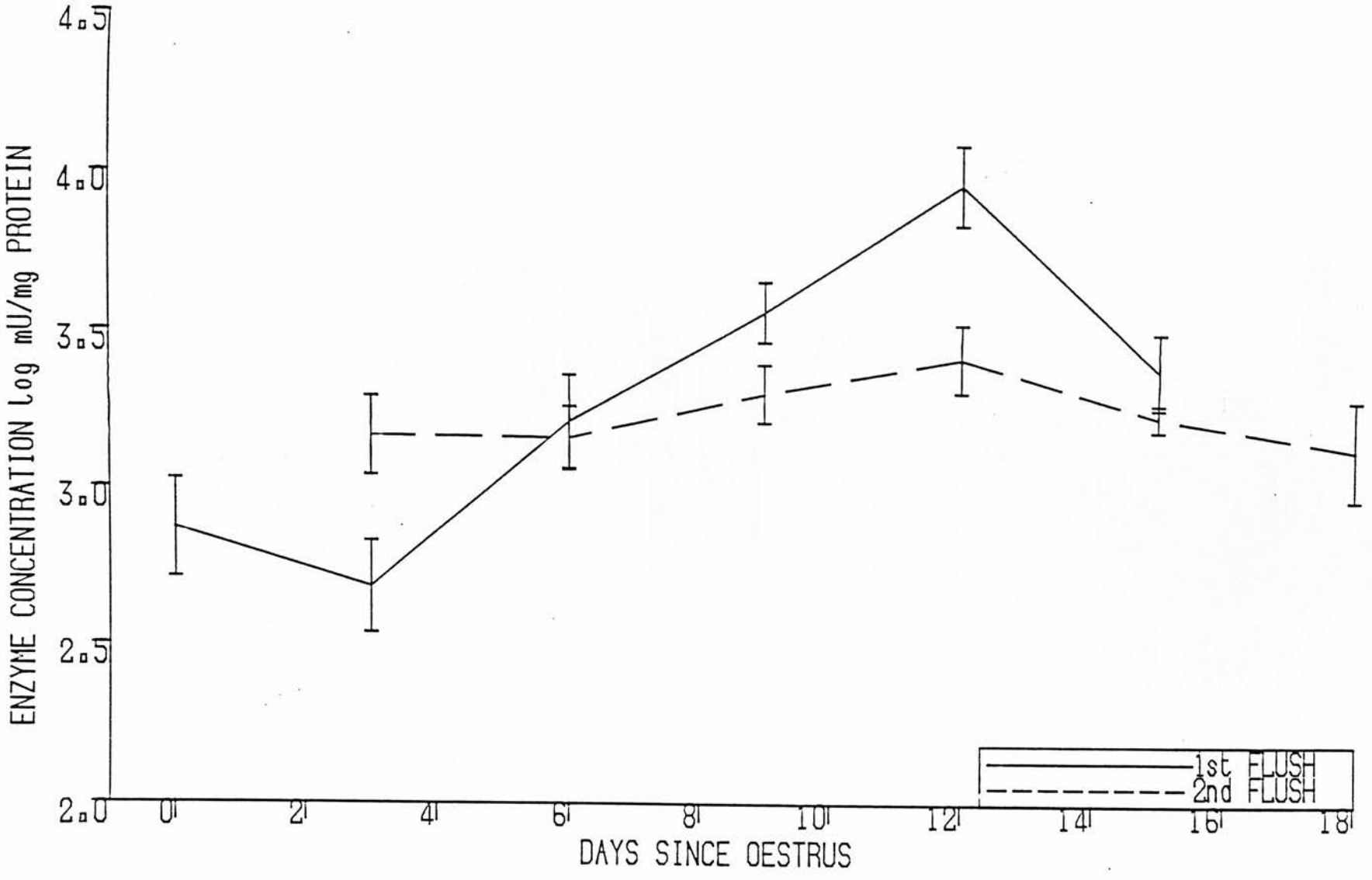


Fig. 5.4

Albumin : total protein ratio in uterine flushings collected throughout the oestrous cycle. Each point represents the mean concentration $\pm$ 1 s.e.m. The number of observations each day is shown on table 5.1.

FIG 5.4: ALBUMIN : TOTAL PROTEIN RATIO
DURING THE OESTROUS CYCLE

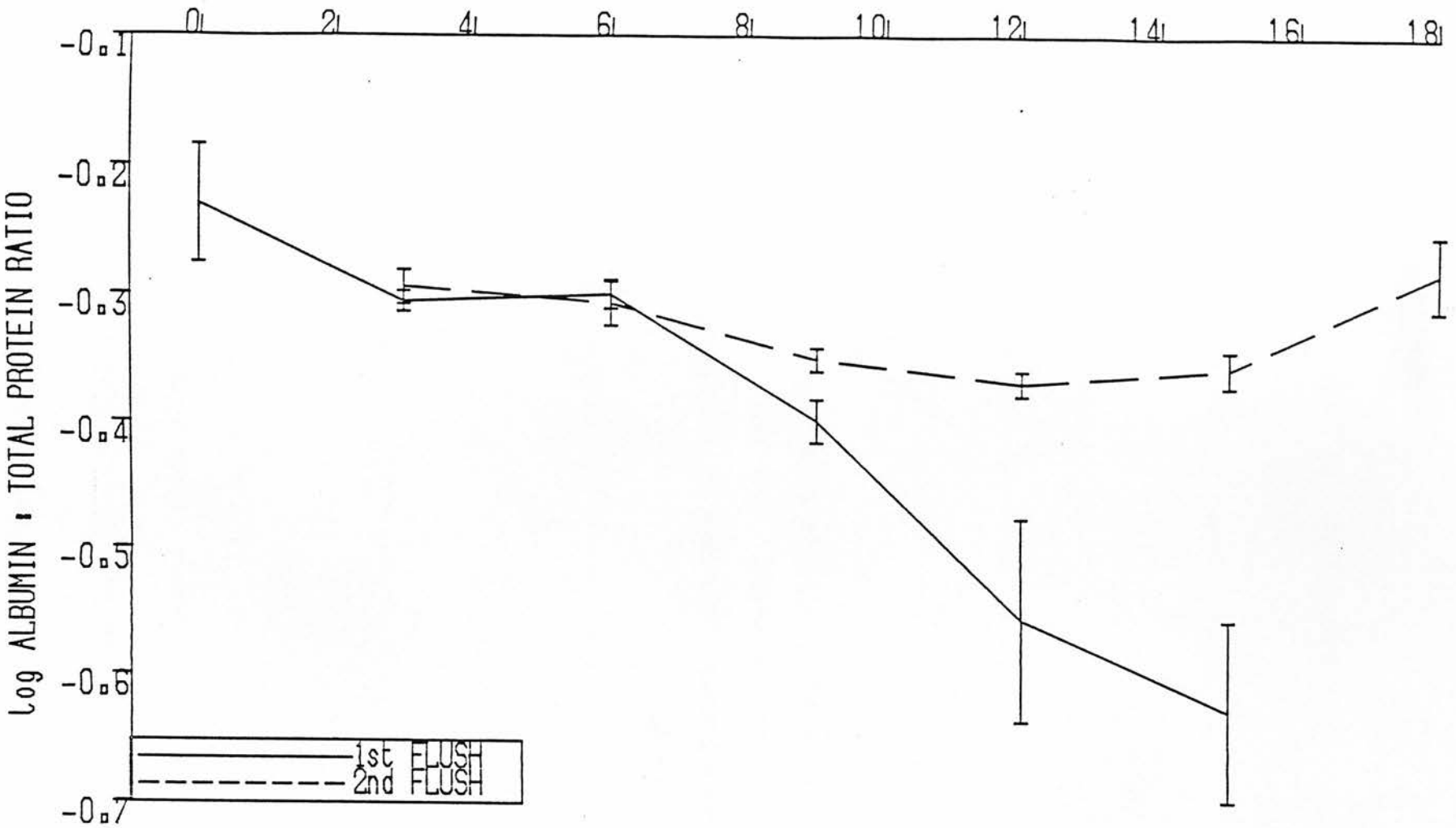
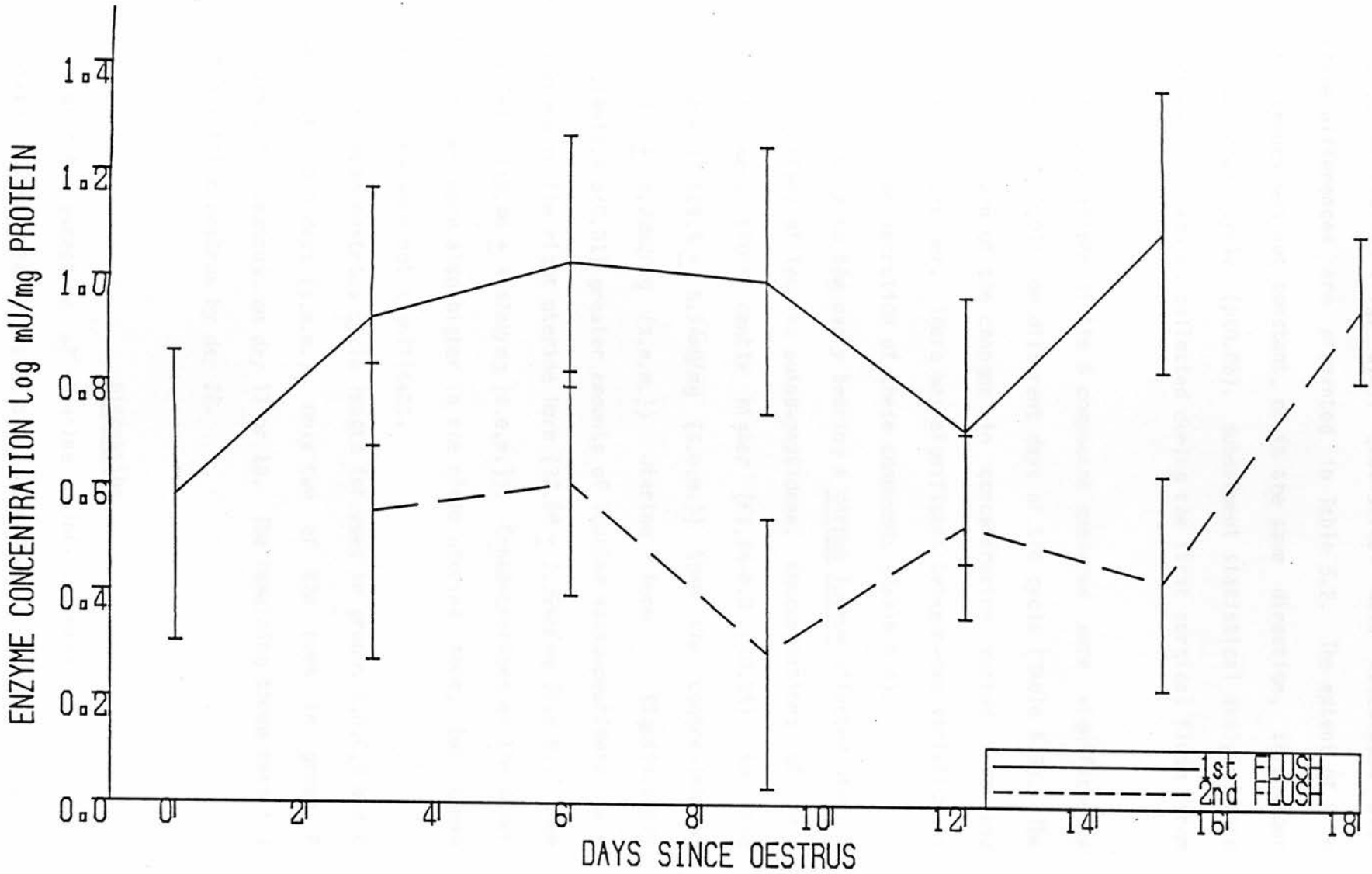


Fig. 5.5

Leucine amino-peptidase levels in uterine flushings collected throughout the oestrous cycle. Each point represents the mean concentration $\pm$ 1 s.e.m. The number of observations each day is shown on table 5.1.

FIG 5.5: LEUCINE AMINO-PEPTIDASE LEVELS DURING THE OESTROUS CYCLE



and second flushes (Fig. 5.2). Statistical data associated with these differences are presented in Table 5.2. The extent of this discrepency was not constant, or in the same direction, throughout the oestrous cycle ($p<0.05$). Subsequent statistical analyses were performed on samples collected during the first surgical flush from each ewe.

Concentrations of the 5 compounds measured were significantly different ($p<0.01$) on different days of the cycle (Table 5.3). The precise pattern of the changes in concentration varied from one protein to another. There was significant between-ewe variation in the pattern of secretion of these compounds (Table 5.4).

Proximity to the ovary bearing a corpus luteum affected uterine concentrations of leucine amino-peptidase. Concentrations of this enzyme were significantly higher ($F_{1,74}=6.8$ $p<0.05$) in the ipsilateral (29.5 $\pm$ 5.74mU/mg (s.e.m.)) than the contra-lateral (22.08 $\pm$ 5.22mU/mg (s.e.m.)) uterine horn. Significantly ($F_{6,74}=15.9$ $p<0.01$) greater amounts of leucine amino-peptidase were observed in the right uterine horn (32.68 $\pm$ 7.32mU/mg (s.e.m.)) than the left (19.46 $\pm$ 4.47mU/mg (s.e.m.)). Concentrations of the other two enzymes were also higher in the right uterine horn, but these differences were not significant.

The mean oestrous cycle length for ewes in groups A,B,C,D and E was 17.6 $\pm$ 0.3 days (s.e.m.). Only two of the ewes in group F returned to oestrus on day 17 or 18. The remaining three ewes had not exhibited oestrus by day 22.

Discussion

With the exception of leucine amino-peptidase, the uterine proteins investigated in this study were present in measurable, but

TABLE 5.2

EFFECT OF A SURGICAL FLUSH ON SUBSEQUENT FLUSHES

Analysis of deviance table

	DF	Mean Deviance	F-Value	Statistical Significance
β.Glucuronidase				
Sample x Day	4	2.7		
Anim/Day+Sample+Sample.Day	26	43.6	1.677	ns
Leucine amino-peptidase				
Sample x Day	4	2.1		
Anim/Day+Sample+Sample.Day	26	16.5	3.315	$p<0.05$
Alkaline Phosphatase				
Sample x Day	4	5.4		
Anim/Day+Sample+Sample.Day	26	39.2	3.597	$p<0.05$
Albumin				
Sample x Day	4	1.4		
Anim/Day+Sample+Sample.Day	26	7.9	4.531	$p<0.05$
Total Protein				
Sample x Day	4	5.7		
Anim/Day+Sample+Sample.Day	26	24.0	6.135	$p<0.05$

DF = Degrees of freedom
ns = Not significant (i.e. $p>0.05$)

TABLE 5.3

EFFECT OF DAY OF CYCLE ON PROTEIN LEVELS

Analysis of deviance table

	DF	Mean Deviance	F-Value	Statistical Significance
β.Glucuronidase				
Animal	6	20.8		
Animal+Day	38	79.8	9.92	p<0.001
Leucine amino-peptidase				
Animal	6	22.9		
Animal+Day	38	204.2	4.26	p<0.01
Alkaline Phosphatase				
Animal	6	20.9		
Animal+Day	38	63.4	12.6	p<0.001
Albumin				
Animal	6	2.6		
Animal+Day	38	7.5	13.4	p<0.001
Total Protein				
Animal	6	22.7		
Animal+Day	38	51.3	16.8	p<0.001

DF = Degrees of freedom

TABLE 5.4

BETWEEN ANIMAL DIFFERENCES IN PROTEIN LEVELS

Analysis of deviance table

	DF	Mean Deviance	F-Value	Statistical Significance
β.Glucuronidase				
Sample	1	16.7		
Animal/Day.Sample	30	46.3	10.82	p<0.01
Leucine amino-peptidase				
Sample	1	22.0		
Animal/Day.Sample	30	88.3	7.47	p<0.05
Alkaline Phosphatase				
Sample	1	4.6		
Animal/Day.Sample	30	60.88	2.27	ns
Albumin				
Sample	1	1.2		
Animal/Day.Sample	30	3.4	11.1	p<0.01
Total Protein				
Sample	1	13.0		
Animal/Day.Sample	30	46.7	8.4	p<0.01

DF = Degrees of freedom
ns = Not significant (i.e. p>0.05)

variable quantities throughout the oestrous cycle. Uterine flushings give a good recovery of uterine luminal fluid contents (Heap, 1962). The chronological changes in protein concentrations observed in this experiment therefore reflect the immediate environment which would confront a developing embryo during the first two weeks of pregnancy. Variations in these compounds may jeopardise embryonic development.

Significant between-ewe variations exist in both substrate concentration, and in the timing of changes in these concentrations in uterine secretions. Uterine secretions are the product of several maternal factors including ovarian steroids (Finn and Porter, 1975; Yochim, 1975). Variations in concentrations of uterine proteins may reflect differences in circulating steroid concentrations, cytoplasmic receptor populations, gene transcription of particular proteins and the secretory capacity of the endometrium. An investigation of the relationship between these uterine proteins and the embryo could determine their usefulness as an index of the functional stage of early pregnancy.

The reason for the discrepency in uterine protein concentrations collected during the first and second surgical flush is not known. Such differences may arise from a distension of the uterine lumen during the flushing procedure, from surgical intervention or as a result of anaesthesia. In 1953 Moore and Nalbandov suggested that experimental distension of the uterine lumen by means of plastic beads induced neurally mediated changes in reproductive function resulting in shortened cycle lengths. The effect of such uterine distension on oestrous cycle length depended on the stage of the cycle at which it was applied. Insertion of plastic beads on day 3 reduced cycle length, whereas ewes receiving

beads on day 8 exhibited a delayed return to oestrus (Nalbandov, Moore and Norton, 1955; Inskeep, Oloufa, Howland, Pope and Casida, 1962). The results from the present experiment follow a different pattern, as normal cycle lengths were observed in all ewes except those flushed very late in the cycle (group F). Recent data obtained from mice suggest that uterine flushing may split the uterine epithelium and rupture the underlying stroma and blood vessels (Martin, 1984). It has been suggested that contaminants appear in the uterine rinsings due to leaching of plasma and interstitial fluid into the uterine lumen from damaged epithelial tissue (Milligan and Martin, 1984). Such a phenomenon may explain a carry-over effect between successive uterine secretions collected from a species such as the mouse. The close apposition of the opposing epithelial walls of the mouse uterus (Martin and Finn, 1970) necessitates forceful flushing procedures generating relatively high hydrostatic pressures (Milligan and Martin, 1984). In this experiment care was taken to ensure that no more saline was introduced into the uterus as soon as resistance was felt.

Surgical handling of the reproductive tract may have altered endometrial protein synthesis or secretory capacity. Such changes may have contributed to the observed carry-over effect. Anaesthesia affects pituitary production of gonadotrophins (Clarke and Doughton, 1983). It is difficult to see how such a mechanism would affect progesterone induced factors in the uterine environment during the earlier part of the cycle as luteal function progressed normally in hypophysectomised ewes from days 2 to 9 (Denamur, Martinet and Short, 1966). Such anaesthesia induced suppression of pituitatry gonadotrophins may explain the delayed return to oestrus observed in group F. However, ewes in group E also received anaesthetic on day

15, but had typical cycle lengths ranging from 17 to 21 days. Although the cause of such a carry-over effect cannot be satisfactorily explained, results from serial collection of uterine secretions should be interpreted with caution. Several conclusions can be drawn from the protein concentrations in the first uterine flush collected from each ewe.

Uterine concentration of total protein is maximal during the luteal phase of the oestrous cycle in sheep (Murdoch and White, 1967; Eyestone and French, 1981), pigs (Murray et al., 1972) and mares (Zavy, Sharp, Bazer, Fazeleabas, Sessions and Roberts, 1982). The results from the present experiment support these findings, but not those of Iritani, Gomes and Vandermark, (1969) who did not observe any cyclic changes in sheep uterine protein concentrations. Protein secretion appears to be under the control of circulating progesterone levels. Ovariectomised ewes receiving daily injections of progesterone experience increased endometrial protein synthesis (Brinsfield and Hawk, 1974). In pigs, the peak of protein secretion occurs whilst the uterus is under progestational control (Roberts et al. 1976). It is generally agreed that in sheep the rate of synthesis is greater in inter-caruncular, rather than caruncular endometrium (Findlay et al., 1982; Miller and Moore, 1983). Rates of protein synthesis in inter-caruncular endometrium are highest at or shortly after oestrus (Miller, Murphy and Stone, 1977) at which time the rate of synthesis is about 45 times greater than the rate of inter-caruncular protein secretion (Miller, Tassel and Stone, 1983). Both uterine progesterone receptors and RNA:DNA ratios are highest around the time of oestrus (Miller et al., 1977b), and are considered to be stimulated by oestradiol (Miller, et al., 1977a). These observations suggest that endometrial synthesis is maximal at

oestrus, but that the secretory capacity is stimulated by progesterone concentrations.

The cyclic changes in uterine alkaline phosphatase concentration in this experiment were similar to those obtained from serial slaughter experiments by Murdoch and White (1967). The pattern of secretion of alkaline phosphatase from both the endometrium and the uterine caruncles follows the growth and regression of the corpora lutea (Murdoch and O'Shea, 1978). In 1968 Murdoch and White showed that this enzyme was under progestational control as alkaline phosphatase levels increased dramatically in uterine tissue obtained from ovariectomised ewes given progesterone. Support for these observations can be obtained from an experiment in which ovariectomised ewes received silastic implants to generate luteal phase progesterone concentrations (C.J.Ashworth, I.Wilmut and D.I.Sales, unpublished observations). Implants were inserted on either day 0 or day 4. The rise in uterine fluid alkaline phosphatase concentration occurred earlier in the former group than the latter. Distinct species differences exist in the mechanism of stimulation of this enzyme. Oestrogen appears to stimulate uterine alkaline phosphatase in women (Gautray, Condere, Colomb, Sibut and Maurel, 1969) and sows (Goode, Warnick and Wallace, 1965). The function of ovine endometrial alkaline phosphatase is not known. Murdoch (1971) showed that such alkaline phosphatase was non-specific in activity, and concluded that it may have a histotrophic role during early pregnancy in the ewe. Later in ovine pregnancy, alkaline phosphatase is involved in the transfer of material across the placenta (Borghese, 1957).

β.Glucuronidase levels in this experiment were similar to those found in uterine extracts from ewes slaughtered at differing stages

of the oestrous cycle (Murdoch and O'Shea, 1978). Ovariectomy significantly reduced β.glucuronidase actvity in caruncular and inter-caruncular homogenates (Findlay et al., 1981). Such results suggest that this enzyme may also be under progestational control. However, these authors observed that the presence of a silastic implant containing 100mg of oestradiol in ovariectomised ewes from day 0 stimulated elevated levels of β.glucuronidase in day 15 caruncular, inter-caruncular and myometrial tissues. Groups of ovariectomised ewes given progesterone implants from day 3 to day 15 either alone, or in conjunction with oestradiol releasing implants exhibited higher β.glucuronidase levels than ovariectomised animals, but lower levels than the group receiving oestradiol alone. Endometrial β.glucuronidase production is clearly not totally controlled by progesterone.

In contrast to the other uterine proteins investigated, the albumin : total protein ratio declined throughout the oestrous cycle. These data are in agreement with those of Beier (1974) in which ovariectomised rabbits had higher albumin : protein levels when under a progesterone dominated regime, compared to an oestrogenic regime.

The results from the leucine amino-peptidase assays should be interpreted with caution, because of the many near zero values. The present results suggest that the activity of this enzyme was greatest in the mid-luteal phase of the cycle. This is in agreement with the observations from uterine secretions obtained from cycling mares (Zavy, Bazer, Sharp and Wilcox, 1979). Beier (1974) indicated that leucine amino-peptidase in rabbit uterine flushings is modulated by maternal progesterone levels. In 1976 Roberts et al. demonstrated that leucine amino-peptidase levels in non pregnant

gilts were controlled by progesterone. The role of this enzyme in the non-pregnant uterus is not known. In pregnant gilts leucine amino-peptidase accumulates in the allantoic and amniotic fluid, reaching peak concentrations at day 80 (Roberts et al., 1976).

The specific functions of the individual uterine proteins investigated are unclear. The primary function of uterine secretions is to provide nutritive support for the conceptus (Bazer, Roberts and Sharp, 1978). Such proteins also serve as enzymes, carrier molecules for steroid hormones (Beato and Baier, 1975), and are involved in the maternal recognition of pregnancy (Bazer and Thatcher 1977).

Neither of the techniques employed in this study were found to be suitable for the collection of successive uterine fluid samples from the same ewe. Despite this, interesting observations have been made by collecting single samples from each ewe. This procedure has been used to study uterine changes during early pregnancy.

CHAPTER 6

EXPERIMENT 4: AN INVESTIGATION OF THE MATERNAL AND EMBRYONIC CONTRIBUTION TO UTERINE FLUIDS IN WELSH MOUNTAIN EWES.

Introduction

Species in which implantation occurs at a relatively late stage of gestation, rely on the fluids of the uterine lumen for embryonic nutrition. In this way, these fluids can be considered as a complex culture medium (Bazer and Roberts, 1983). The total protein concentration in ovine uterine fluids increases between days 13 and 17 of pregnancy (Ellinwood, Nett and Niswender, 1979a), and is higher on day 15 than at the comparable stage of the oestrous cycle (Findlay et al., 1981). Electrophoretic studies on uterine flushings collected on day 15 of pregnancy revealed 10 uterine specific bands, of which 5 were characteristic of pregnant animals (Roberts, Symonds and Parker, 1976).

The proteins present during pregnancy may originate from the blastocyst or the endometrium. The synthesis and release of protein may be stimulated by ovarian steroids or by a local or systemic action of the blastocyst on endometrial protein synthesis. The relative contribution of these three systems to the overall protein milieu was investigated.

The present experiments were carried out to determine the effect of both pregnancy and of variation in steroid levels on uterine secretions. In order to define the effects of pregnancy, fluids were recovered from pregnant and non-pregnant ewes. The latter ewes were ovariectomised animals receiving steroid hormones capable of maintaining pregnancy (Miller and Moore, 1976; Chapter

1). In the pregnant ewes, the embryo was confined to one uterine horn by a ligature so that fluid could be collected from both a gravid and a non-gravid horn. In this way it was possible to differentiate between local and systemic effects of the embryo. Observations were made at 3 intervals after mating; before the maternal recognition of pregnancy, after luteolysis would occur in cyclic animals, and at the time of implantation.

Whilst it is well known that ovarian steroids affect embryonic survival (Miller and Moore, 1976) and endometrial protein synthesis (Finn and Porter, 1975; Yochim, 1975), the role of each of the steroids in the sequence described in Chapter 1 is not known. The timing of changes in steroid concentrations affect uterine receptor concentrations (Stone et al., 1978). The effect of such changes in the receptor population on the composition of the uterine environment is also unknown. The effect of omitting a particular steroid treatment on subsequent uterine concentrations of enzymes known to vary throughout the oestrous cycle was determined on day 12 after oestrus.

Uterine leucine amino-peptidase concentrations were not determined in this experiment, due to the unacceptable high minimum detectable dose of the assay system. The four other uterine proteins measured in experiment 3 were monitored during the present experiment. In the light of the 'carry-over' effect between successive flushes performed on the same animal (see Chapter 5) only one surgical uterine flush was performed on each ewe.

Materials and Methods

Experimental Animals

One hundred Welsh Mountain ewes were used in this experiment.

Thirty ewes (group 1) were naturally mated. A further 60 ewes (groups 2 to 5) were ovariectomised and received exogenous steroid implants. Ewes in group 6 received no treatment after ovariectomy.

Mated ewes: Oestrous cycles were synchronised by treatment for 12 days with intravaginal pessaries impregnated with 60mg medroxyprogesterone acetate (Veramix, Upjohn Ltd., Crawley, Sussex). These ewes were naturally mated at the second oestrus following synchronisation. The number and distribution of ovulations was determined during a mid-ventral laparotomy performed on the second or third day after mating. Unilateral pregnancy was achieved by ligating one oviduct in ewes having a corpus luteum on both ovaries. A ligature was also secured at the base of each uterine horn to prevent migration of the embryo and its associated membranes. Anaesthesia, and procedures to exteriorise the genital tract were performed as described in Chapter 3.

Ovariectomised ewes: Ovariectomy was performed at least 6 weeks prior to collection of uterine fluids. Seventy ewes were randomly assigned to 5 treatment groups. Group 2 (30 ewes) received a sequence of exogenous steroids to mimic the pattern of ovarian secretion occurring during the oestrous cycle and early pregnancy. The remaining forty ewes received incomplete steroid regimes. The steroid treatments administered to ewes in groups 2 to 6 are shown in Table 6.1.

All ewes, apart from those in group 6, also received one 25ug injection of oestradiol, followed by a progestagen releasing pessary for 14 days prior to oestrus. The peak of oestradiol secretion occurring at oestrus in intact animals, was mimicked by three

TABLE 6.1

SCHEDULE OF STEROID TREATMENTS ADMINISTERED TO OVARIECTOMISED EWES

	Treatment group				
	2	3	4	5	6
Number of ewes	30	10	10	10	10
Oestradiol *	+	+	+	-	-
Tube progesterone implant *	+	+	-	+	-
Sachet progesterone implant *	+	-	+	+	-

* See text for concentration and duration of steroid treatments.

injections of increasing doses of oestradiol (3.5ug, 7.0ug, 14.0ug) administered at 0900, 1400 and 1700 hours respectively, on the day following sponge withdrawal. The first day of oestrous behaviour was detected by vasectomised rams, and defined as day 0. Plasma progesterone concentrations typical of those observed during early pregnancy in intact ewes were generated by subcutaneous silastic implants. Early pre-luteal phase concentrations were achieved using implants prepared using a modification of the technique described by Dziuk and Cook (1965). One end of a 4.5cm length of silastic medical grade tubing (0.132 inches i.d. x 0.183 inches o.d., Dow Corning Corp., Medical Products, Midland, Michigan, U.S.A.) was sealed with a 0.5cm plug of silastic medical grade elastomer (Dow Corning Corp., Michigan, U.S.A.). Progesterone (Sigma Ltd., Poole, Dorset) was packed into the tube, and the open end of the tube sealed as before. Luteal-phase progesterone levels were generated from 'sachet' implants prepared as described by Karsch, Legan, Hauger and Foster, (1977). Briefly, 80cm x 50cm packets of silastic sheeting (Dow Corning Corp., Michigan, U.S.A.) were prepared using silastic medical grade adhesive (Dow Corning Corp., Michigan, U.S.A.). It is important that these implants remain flat in situ, to ensure the appropriate surface area for diffusion. To this end, a plastic 'X' shape was placed in the packet. The packet was filled with approximately 5g of progesterone and the opening sealed. A 5cm tab of silastic tubing was attached to one end of the sachet to facilitate implant removal.

Two tube implants were inserted on day 1, and removed on day 4 (groups 2 and 5) or day 12 (group 3). A blood sample was collected at the time of tube implant removal, to measure progesterone concentrations. Ewes in groups 2, 4 and 5 received two sachet

implants which remained in position until immediately after the last blood sample was collected (Table 6.2). Both types of implants were gas sterilised using ampoules containing ethylene oxide ('Anprolene', H.W. Anderson Products, Oyster Bay, New York) in a closed system, soaked in alcohol for 15 minutes and then immersed in double distilled deionised water immediately before insertion. Implants were inserted and removed under general anaesthesia, induced by a single intra-venous injection of 8-20ml 10% w:v sodium thiopentone (Intraval, May and Baker Ltd., Dagenham.) and maintained by a mixture of halothane (Fluothane, I.C.I., plc, Macclesfield, Cheshire), nitrous oxide and oxygen in a closed system. The appropriate area of skin was shaved and disinfected. Tube implants were placed at the top of the fore-limb, and sachet implants in the ventral abdomen. An incision was made in the subcutaneous fascia, and a suitably sized pocket prepared. Following implant insertion, the wound was closed using Michel wound clips (The Holborn Surgical Instrument Co., London).

The skin immediately overlying the implants was disinfected prior to their removal. The original incision was re-opened to remove both tube implants, and sachet implants which had been in position for less than two weeks. Sachet implants in situ for a longer time were removed by cutting into the silastic tab at the top of the sachet. The wound was left open for a few minutes to allow drainage, excess fascia was removed, and the wound closed using Michel clips.

Collection of uterine secretions.

The schedule for the collection of uterine fluids is shown in Table 6.2. Secretions were collected from both uterine horns as

TABLE 6.2

REGIME USED TO COLLECT BLOOD SAMPLES AND UTERINE FLUIDS

		TREATMENT GROUP									
		1a	1b	1c	2a	2b	2c	3	4	5	6
Number of ewes		10	10	10	10	10	10	10	10	10	10
Days since oestrus	12	BF	B	B	BF	B	B	BF	BF	BF	BF
	20	B	BF	B	B	BF	B				
	30	B	B	BF	B	B	BF				

Blood samples and uterine fluids were collected at times marked by B and F, respectively

described in Chapter 5, with the exception that larger volumes (up to 20ml) of isotonic saline were introduced into the uterine lumen of pregnant ewes. This corresponds to the increased uterine size during pregnancy. Pregnancy diagnosis of mated ewes was performed during surgery.

Determination of uterine protein concentrations.

Enzyme and albumin assays were performed as described in Chapter 5. The biuret method was not sufficiently sensitive to detect protein concentrations in some of the samples collected during this experiment. This is thought to be due to the increased volume of saline used to rinse gravid uteri. Total protein determinations were performed according to the method described by Lowry, Rosebrough and Randal (1955). Quality control samples were as used in the biuret assay system. Both assays were comparable over the range of protein concentrations detected in experiment 3 (between method co-efficient of variation = 6.91%). The inter- and intra-assay co-efficients of variation for each of the four assays are shown in Table 6.3.

Table 6.3: Protein assay reliability criteria

	Co-efficients of variation (%)	
	Intra-assay	Inter-assay
Total Protein	3.2	7.2
Albumin	1.3	21.5
β.Glucuronidase	3.2	13.0
Alkaline Phosphatase	2.3	6.0

Determination of Plasma Progesterone Concentrations.

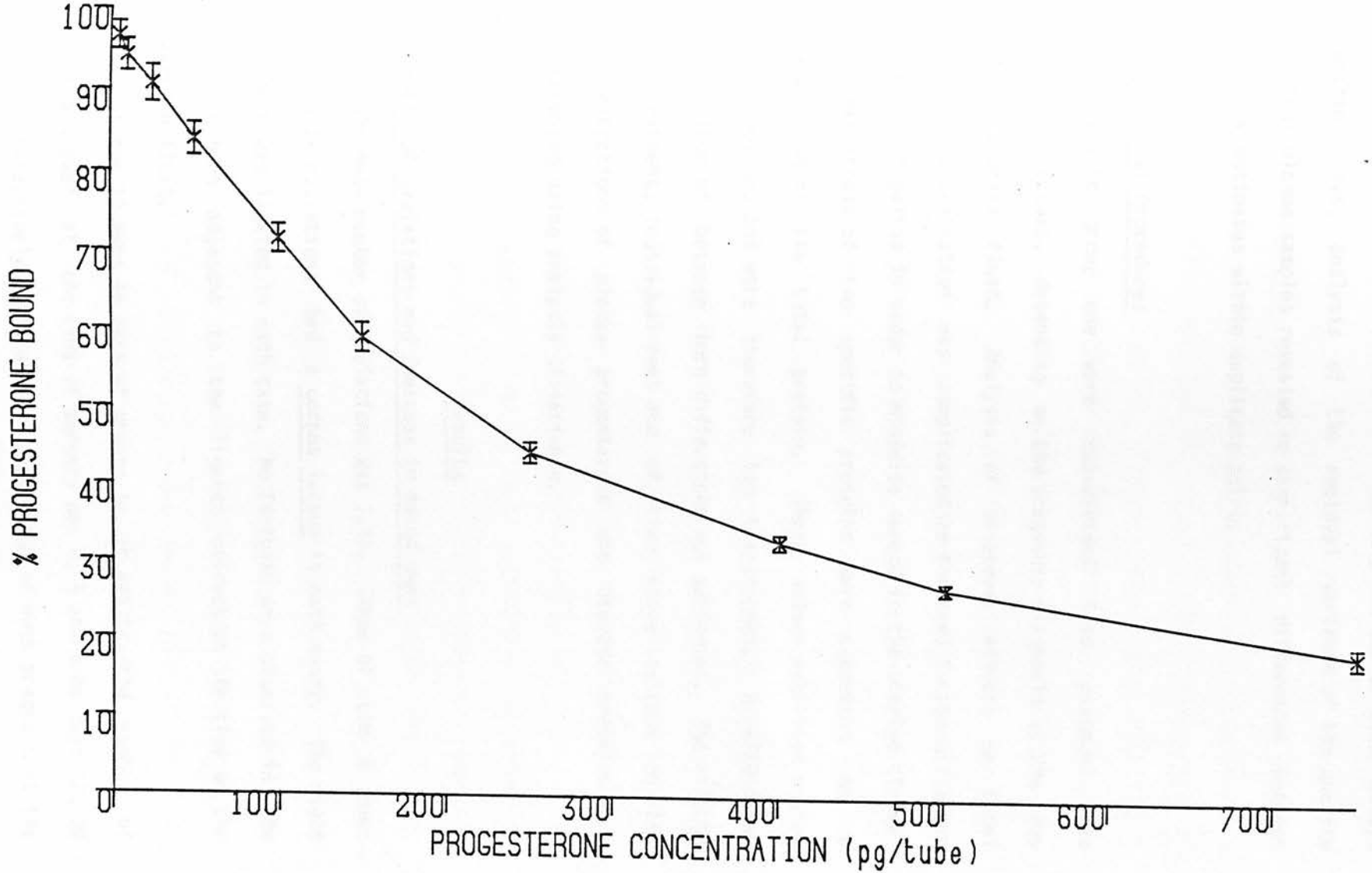
The schedule for the collection of blood samples is shown in Table 6.2. Both the collection and radioimmunoassay of plasma samples for progesterone estimation were performed as described in Chapter 2. As so few samples were collected for each ewe, it was impossible to make realistic within-animal comparisons. Therefore, plasma samples were organised to provide reliable estimates of within-day differences between animals, and between treatment groups. All samples collected at the time of small implant removal were assayed with all the day 12 samples. Samples collected on days 20 and 30 were also measured in the same assay. Three assays, including one containing repeat samples were performed. Within assays, the samples were randomised to compensate for any assay drift.

Progesterone was extracted from plasma using freshly distilled petroleum ether (Koch-Light Laboratories Ltd., Colnbrook, Bucks.). The mean extraction efficiency was 63.2 $\pm$ 4.88%(s.e.m.). The mean minimum detectable dose was 27.73 $\pm$ 8.19pg/tube (s.e.m.) (316pg/ml). Water blanks were less than the minimum detectable dose in all three assays. Within each assay, both standard curves were parallel. The overall standard curve was used to estimate progesterone concentrations. Between assays, the overall standard curves were highly reproduceable, (Fig. 6.1), with no significant differences in slope (mean slope= -1.149 $\pm$ 0.0275 (s.e.m.)). The inter-assay coefficients of variation using four standard quality control samples with mean progesterone concentrations of 0.18 $\pm$ 0.06ng/ml, 2.33 $\pm$ 0.08ng/ml, 3.19$\pm$ 0.21ng/ml and 9.42 $\pm$ 0.83ng/ml were 23.0%, 5.8%, 11.5% and 15.3% respectively. The intra-assay coefficient of variation averaged 8.7% for duplicate determinations

Fig. 6.1

Composite standard curve from progesterone assays performed in experiment 4. Each point represents the mean of 18 determinations ± 1 s.e.m.

FIG 6.1: COMPOSITE STANDARD CURVE FROM PROGESTERONE ASSAYS IN EXPERIMENT 4



of these four samples placed at the begining and end of each assay. Within assays, analysis of the residual variance of the quality control plasma samples revealed no significant differences between potency estimates within duplicate pairs.

Statistical Procedures

Ewes in group one were sub-divided into pregnant and non-prenant ewes, depending on the pregnancy diagnosis at the time of the uterine flush. Analysis of treatment effects on total protein concentration was complicated by the need to use different volumes of saline in order to minimise damage to the uterine tissue. Concentrations of the specific proteins were expressed as a proportion of the total protein. These values exhibited a skew distribution, and were therefore log transformed. A within-ewe comparison of between horn differences was performed. The effects of treatment, individual ewes and of time since oestrus on the concentrations of plasma progesterone and uterine proteins were determined using analysis of variance.

Results

Numbers of ovulations and foetuses in mated ewes

The mean number of ovulations was 1.16. Three of the 6 ewes having 2 ovulations had a corpus luteum in each ovary. The right oviduct was ligated in each case. No foetuses were observed in the uterine horn adjacent to the ligated oviduct at the time of the uterine flush.

Of the 10 ewes in each of groups 1a, 1b and 1c, the number of pregnant ewes at the time of surgery was 8, 5 and 4 on days 12, 20 and 30 respectively. The overall percentage of ewes pregnant at the

time of surgery was therefore 57%.

Progesterone concentrations

Differences between ewes in progesterone concentrations approached significance ($p<0.1$). Within ewes, differences in progesterone concentrations on different days were significant ($p<0.01$). Progesterone levels were significantly affected by the combination of both treatment and days since oestrus ($p<0.01$; Fig. 6.2). There were no significant differences between progesterone concentrations in implant ewes (group 2) and in pregnant ewes on any of the three sampling occasions. Progesterone levels were significantly lower on day 20 in non-pregnant ewes ($p<0.01$) but not on days 12 or 30.

The mean plasma progesterone concentration at the time of tube implant removal on day 4 was 0.93 $\pm$ 0.048ng/ml (s.e.m.). Concentrations tended to be lower in ewes which had received oestradiol around the time of oestrus (0.88 $\pm$ 0.05ng/ml (s.e.m.) vs 1.05 $\pm$ 0.12ng/ml (s.e.m.)), but this difference was not significant. Progesterone concentrations on day 12 in each of the ovariectomised ewe treatment groups are shown in Fig. 6.3. Progesterone level was lower in the ewes that received no treatment after ovariectomy (group 6) than in groups 2 ($p<0.01$), 3 ($p<0.05$), 4 ($p<0.01$) and 5 ($p<0.01$). An intermediate concentration was generated by the tube implants (group 3), and a significantly higher level by the sachet implant ($p<0.05$). The absence of oestradiol around the time of oestrus, or of the tube progesterone implant, did not result in significantly different progesterone concentrations on day 12.

Fig. 6.2

Plasma progesterone concentrations in mated ewes (group 1) and in ewes receiving the full sequence of steroid supplementation designed to simulate hormone changes occurring in intact ewes during early pregnancy (group 2). Each point represents the mean $\pm$ 1 s.e.m.

FIG 6.2: PLASMA PROGESTERONE CONCENTRATIONS IN EWES IN GROUPS ONE AND TWO

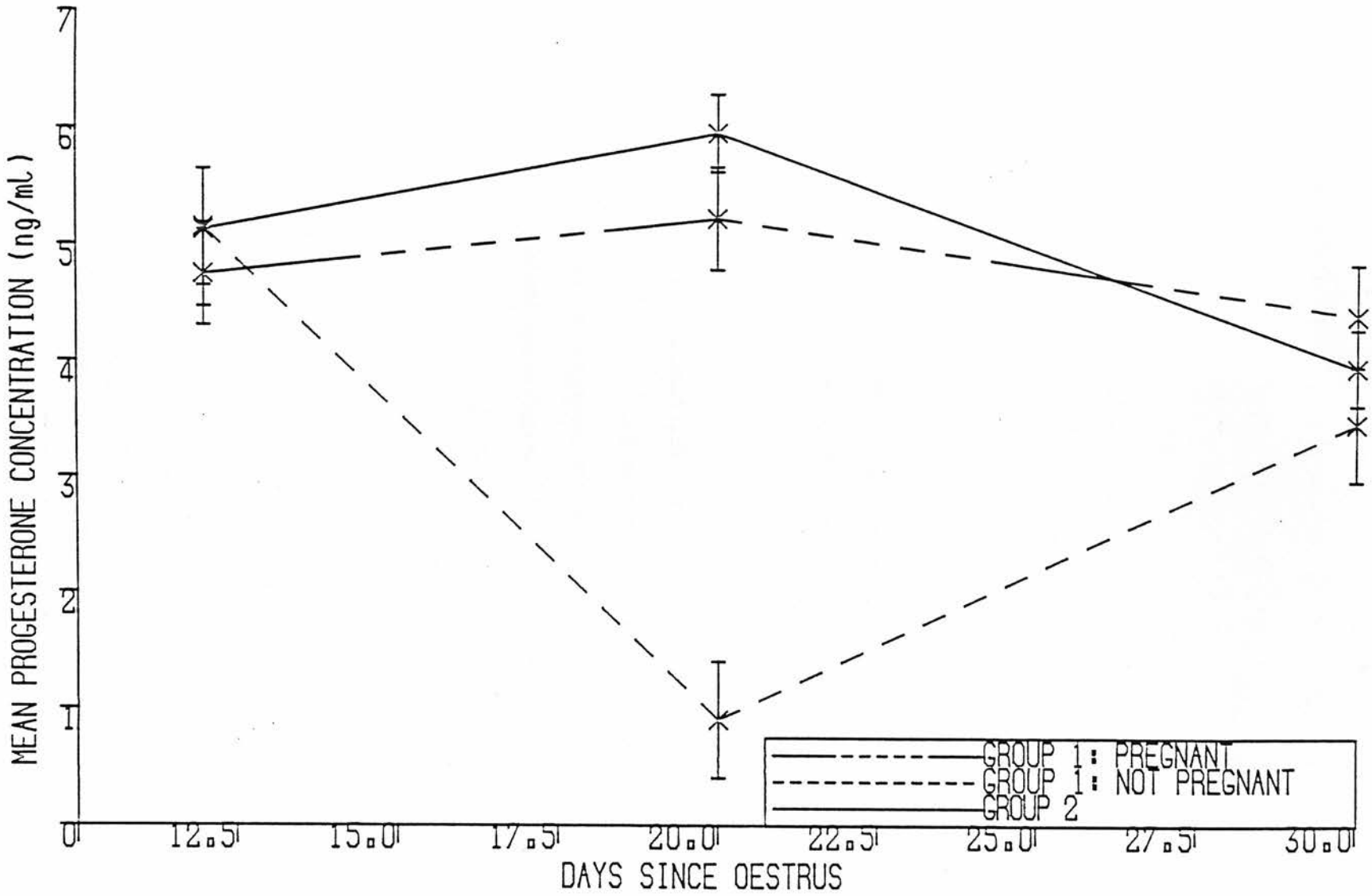


Fig. 6.3

Plasma progesterone concentrations on day 12 in ovariectomised ewes. Each bar represents the mean of 10 determinations ± 1 s.e.m.

Treatment groups:

2: Full hormone regime (oestradiol, tube progesterone implant and sachet progesterone implant).

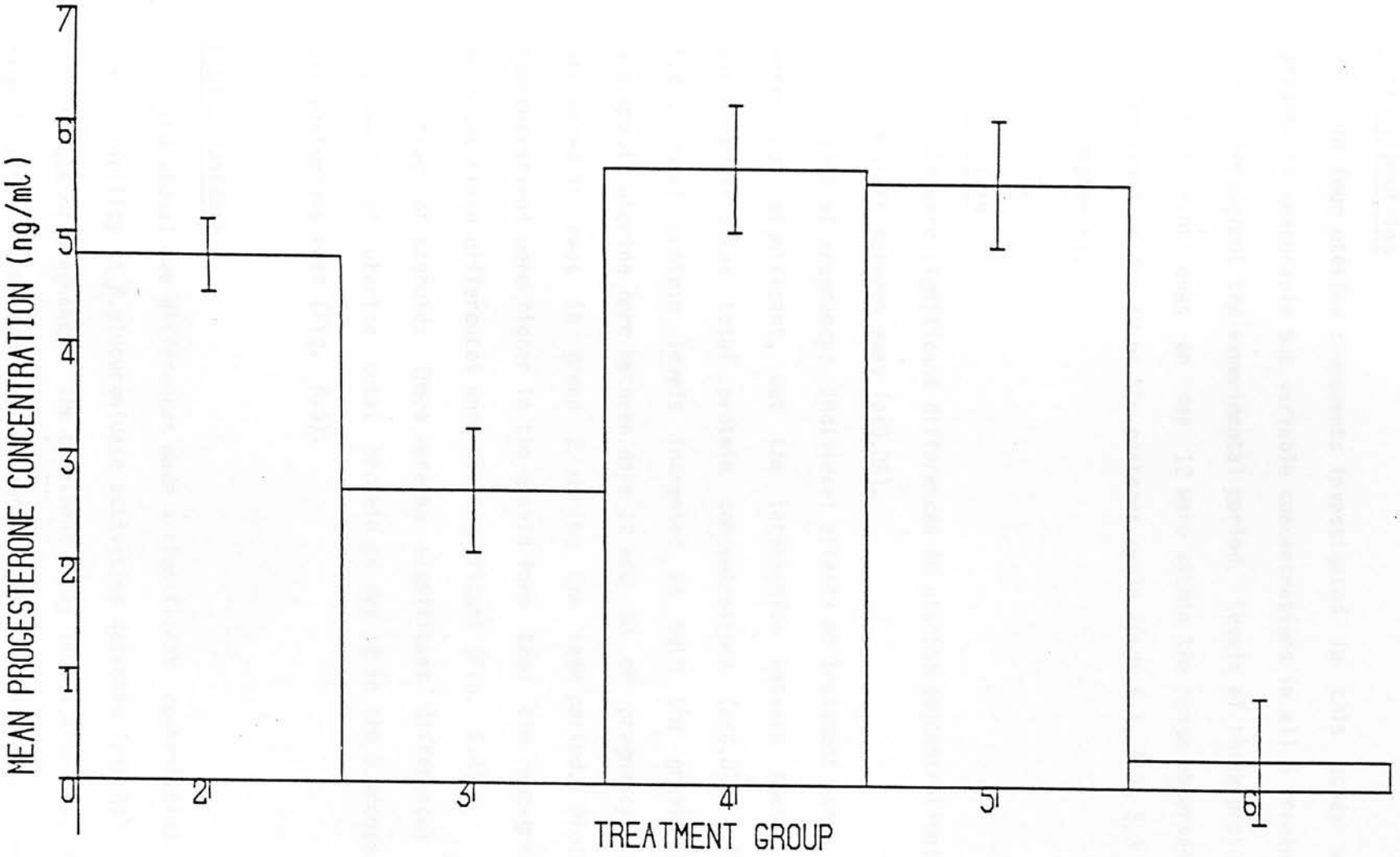
3: As group 2 but without sachet progesterone implant.

4: As group 2 but without tube progesterone implant.

5: As group 2 but without oestradiol.

6: No exogenous steroids.

FIG. 6.3: PLASMA PROGESTERONE CONCENTRATIONS ON DAY 12 IN OVARIECTOMISED EWES



Uterine proteins

The four uterine components investigated in this study were present in measurable but variable concentrations in all 6 treatment groups throughout the experimental period. Levels of these proteins in non-pregnant ewes on day 12 were within the range observed in unmated ewes on day 12 of the oestrous cycle (Figs 5.1, 5.2, 5.3 and 5.4; Chapter 5).

Total Protein

There were significant differences in uterine concentrations of total protein between ewes ($p<0.05$).

Effect of pregnancy: Individual effects of treatment and day were not significant, but the interaction between these two variables affected total protein concentrations ($p<0.01$; Fig. 6.4). Total protein levels increased in both the gravid and non-gravid uterine horn between days 12 and 30 of pregnancy, but decreased in ewes in group 2 during the same period. Protein concentrations were higher in the gravid horn than the non-gravid horn but these differences were not significant (Fig. 6.4).

Effect of steroid: There were no significant differences in the levels of uterine total protein on day 12 in the 5 groups of ovariectomised ewes (Fig. 6.5).

β.Glucuronidase

Individual ewe differences made a significant contribution to the variability in β.glucuronidase activities detected ($p<0.01$).

Effect of pregnancy: The treatment x day interaction was not significant. Consequently, treatment means across days were calculated. There was a significant effect of treatment on

Fig. 6.4

Uterine total protein concentrations in mated ewes (group 1) and in ewes receiving the full sequence of steroid supplementation designed to simulate hormone changes occurring in intact ewes during early pregnancy (group 2). Each point represents the mean $\pm$ 1 s.e.m.

FIG 6.4: UTERINE TOTAL PROTEIN CONCENTRATIONS IN EWES IN GROUPS ONE AND TWO

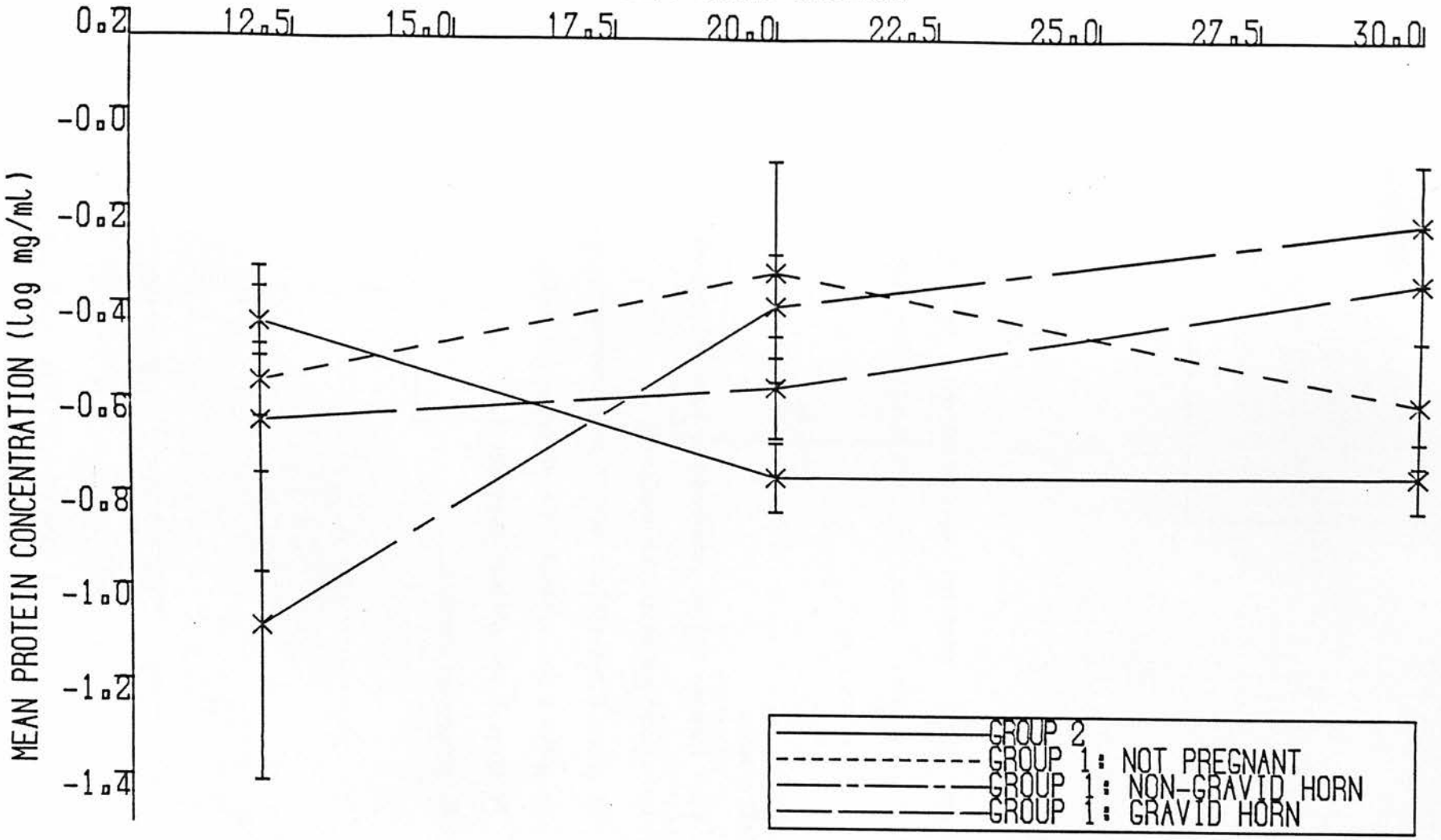


Fig. 6.5

Uterine total protein concentrations on day 12 in ovariectomised ewes. Each bar represents the mean of 10 determinations ± 1 s.e.m.

Treatment groups:

2: Full hormone regime (oestradiol, tube progesterone implant and sachet progesterone implant).

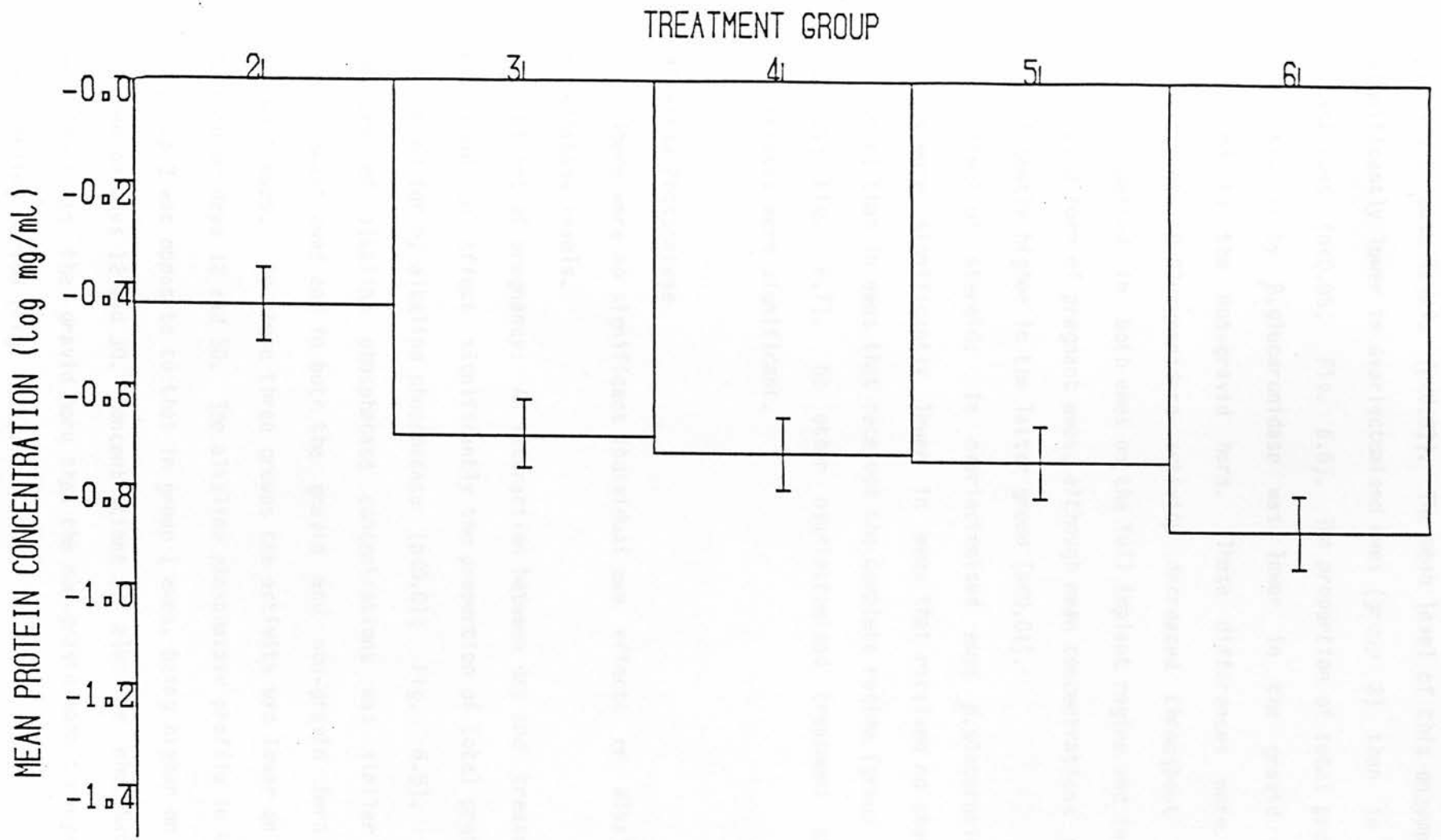
3: As group 2 but without sachet progesterone implant.

4: As group 2 but without tube progesterone implant.

5: As group 2 but without oestradiol.

6: No exogenous steroids.

FIG. 6.5: UTERINE TOTAL PROTEIN CONCENTRATIONS ON DAY 12 IN OVARIECTOMISED EWES



β.glucuronidase levels ($p<0.01$). The mean level of this enzyme was significantly lower in ovariectomised ewes (group 2) than in the intact ewes ($p<0.05$; Fig. 6.6). The proportion of total protein accounted for by β.glucuronidase was lower in the gravid horn compared to the non-gravid horn. These differences were not significant. β.Glucuronidase activity decreased throughout the sampling period in both ewes on the full implant regime and in the non-gravid horn of pregnant ewes, although mean concentrations were significantly higher in the latter group ($p<0.01$).

Effect of steroid: In ovariectomised ewes β.glucuronidase levels were significantly lower in ewes that received no steroid (group 6) than in ewes that received the complete regime (group 2), ($p<0.05$; Fig. 6.7). No other ovariectomised treatment group differences were significant.

Alkaline Phosphatase

There were no significant individual ewe effects on alkaline phosphatase levels.

Effect of pregnancy: An interaction between day and treatment was found to affect significantly the proportion of total protein accounted for by alkaline phosphatase ($p<0.01$; Fig. 6.8). The pattern of alkaline phosphatase concentrations was similar in non-pregnant ewes and in both the gravid and non-gravid horn of pregnant ewes. In these three groups the activity was lower on day 20 than on days 12 and 30. The alkaline phosphatase profile in ewes in group 2 was opposite to that in group 1 ewes, being higher on day 20 than on days 12 and 30. Concentrations of alkaline phosphatase were lower in the gravid horn than the non-gravid horn throughout the sampling period (Fig. 6.8).

Fig. 6.6

Uterine β-glucuronidase concentrations in mated ewes (group 1) and in ewes receiving the full sequence of steroid supplementation designed to simulate hormone changes occurring in intact ewes during early pregnancy (group 2). Each point represents the mean ± 1 s.e.m.

FIG 6.6: UTERINE β.GLUCURONIDASE CONCENTRATIONS IN EWES IN GROUPS ONE AND TWO

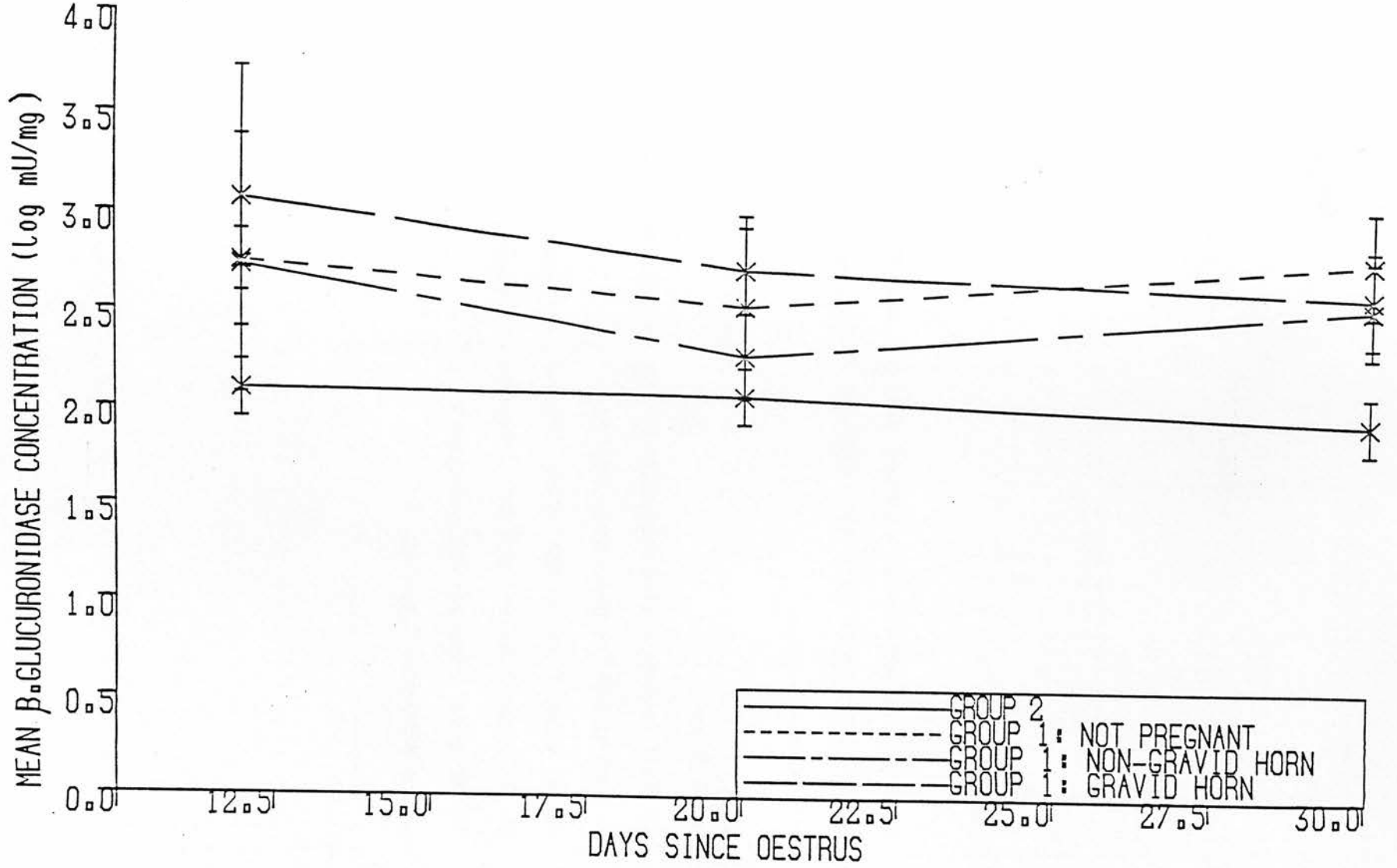


Fig. 6.7

Uterine β.glucuronidase concentrations on day 12 in ovariectomised ewes. Each bar represents the mean of 10 determinations ± 1 s.e.m.

Treatment groups:

2: Full hormone regime (oestradiol, tube progesterone implant and sachet progesterone implant).

3: As group 2 but without sachet progesterone implant.

4: As group 2 but without tube progesterone implant.

5: As group 2 but without oestradiol.

6: No exogenous steroids.

FIG. 6.7: UTERINE β.GLUCURONIDASE CONCENTRATIONS ON DAY 12 IN OVARIECTOMISED EWES

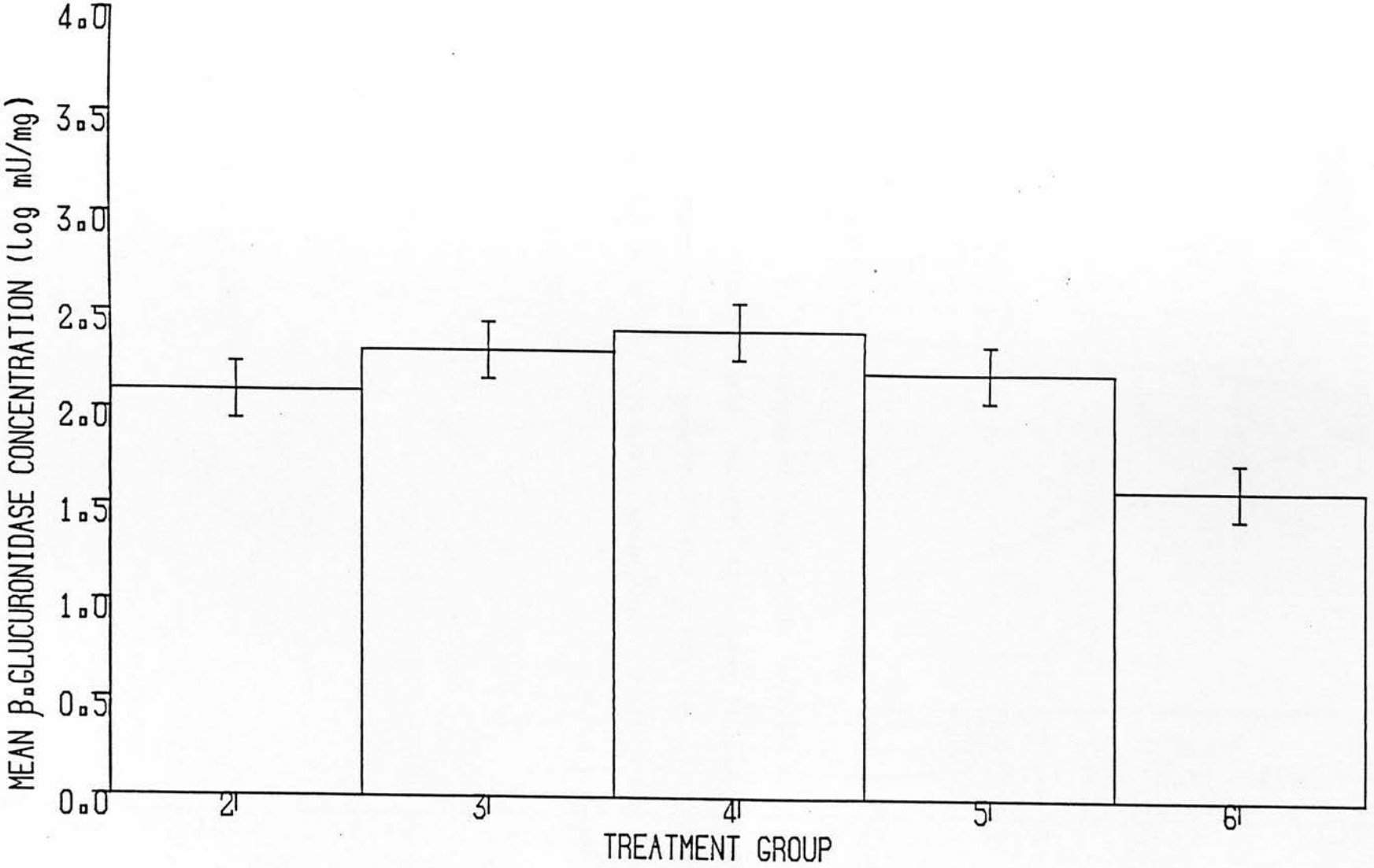
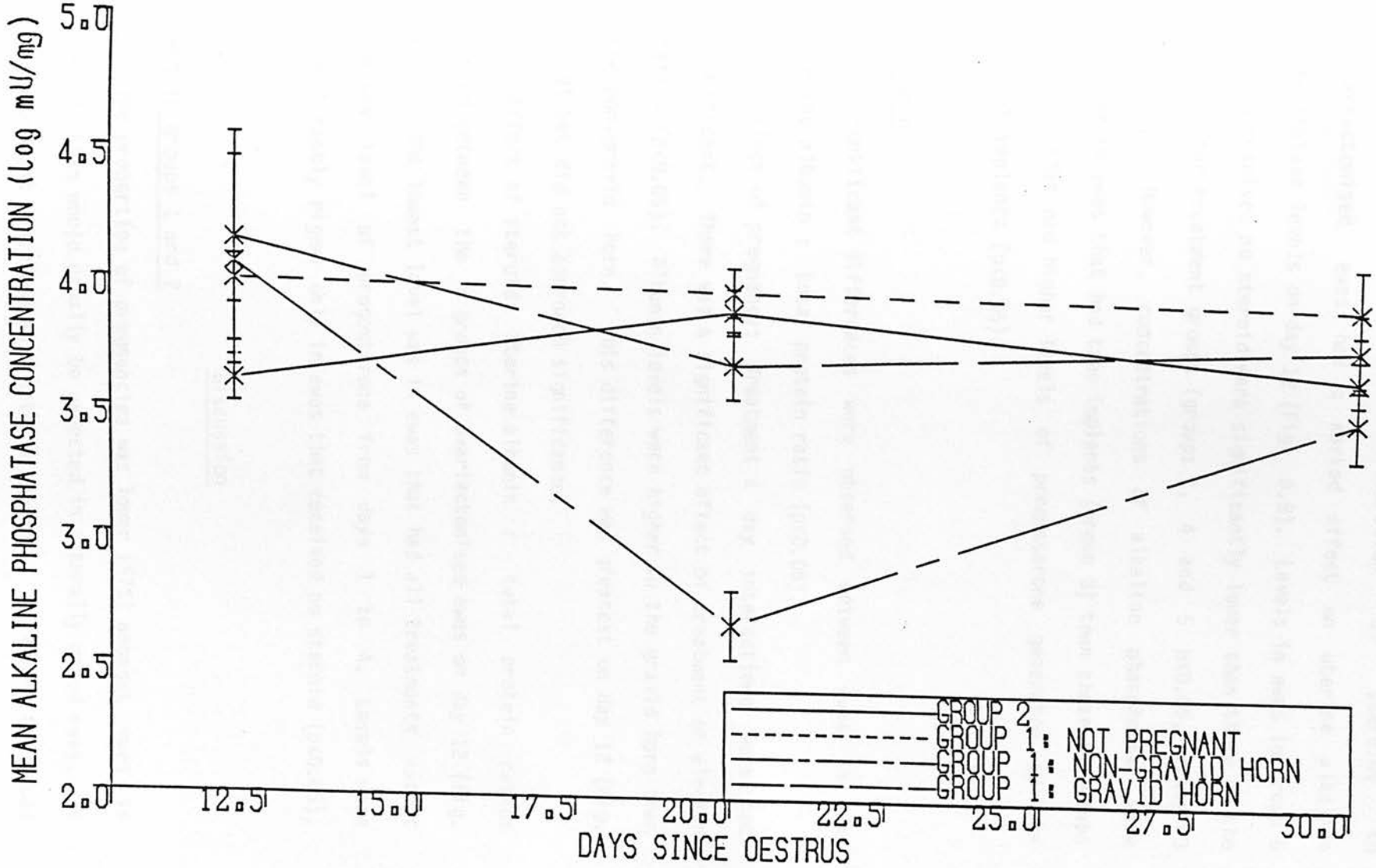


Fig. 6.8

Uterine alkaline phosphatase concentrations in mated ewes (group 1) and in ewes receiving the full sequence of steroid supplementation designed to simulate hormone changes occurring in intact ewes during early pregnancy (group 2). Each point represents the mean $\pm$ 1 s.e.m.

FIG 6.8: UTERINE ALKALINE PHOSPHATASE CONCENTRATIONS IN EWES IN GROUPS ONE AND TWO



Effect of steroid: Administration of steroids to ovariectomised ewes had a marked effect on uterine alkaline phosphatase levels on day 12 (Fig. 6.9). Levels in ewes in group 6 which received no steroid were significantly lower than those in the other four treatment groups (groups 2, 4 and 5 $p<0.05$, group 3 $p<0.01$). However, concentrations of alkaline phosphatase were higher in ewes that had tube implants (group 3) than those in groups 5 and 2 that had higher levels of progesterone generated by the sachet implants ($p<0.05$).

Albumin

Significant differences were observed between ewes in the uterine albumin : total protein ratio ($p<0.05$).

Effect of pregnancy: Treatment x day interactions were not significant. There was a significant effect of treatment on albumin ratios ($p<0.05$). Albumin levels were higher in the gravid horn than the non-gravid horn. This difference was greatest on day 12 (Fig. 6.10) but did not approach significance.

Effect of steroid: Uterine albumin : total protein ratios varied between the 5 groups of ovariectomised ewes on day 12 (Fig. 6.11). The lowest level was in ewes that had all treatments except the low level of progesterone from days 1 to 4. Levels were significantly higher only in ewes that received no steroid ($p<0.05$).

Discussion

Ewes in groups 1 and 2

The proportion of pregnancies was lower (57%) amongst ewes in group 1 than would usually be expected in naturally mated ewes. In the experiment described in Chapter 4 embryonic survival in mated

Fig. 6.9

Uterine alkaline phosphatase concentrations on day 12 in ovariectomised ewes. Each bar represents the mean of 10 determinations ± 1 s.e.m.

Treatment groups:

2: Full hormone regime (oestradiol, tube progesterone implant and sachet progesterone implant).

3: As group 2 but without sachet progesterone implant.

4: As group 2 but without tube progesterone implant.

5: As group 2 but without oestradiol.

6: No exogenous steroids.

FIG. 6.9: UTERINE ALKALINE PHOSPHATASE CONCENTRATIONS ON DAY 12 IN OVARIECTOMISED EWES

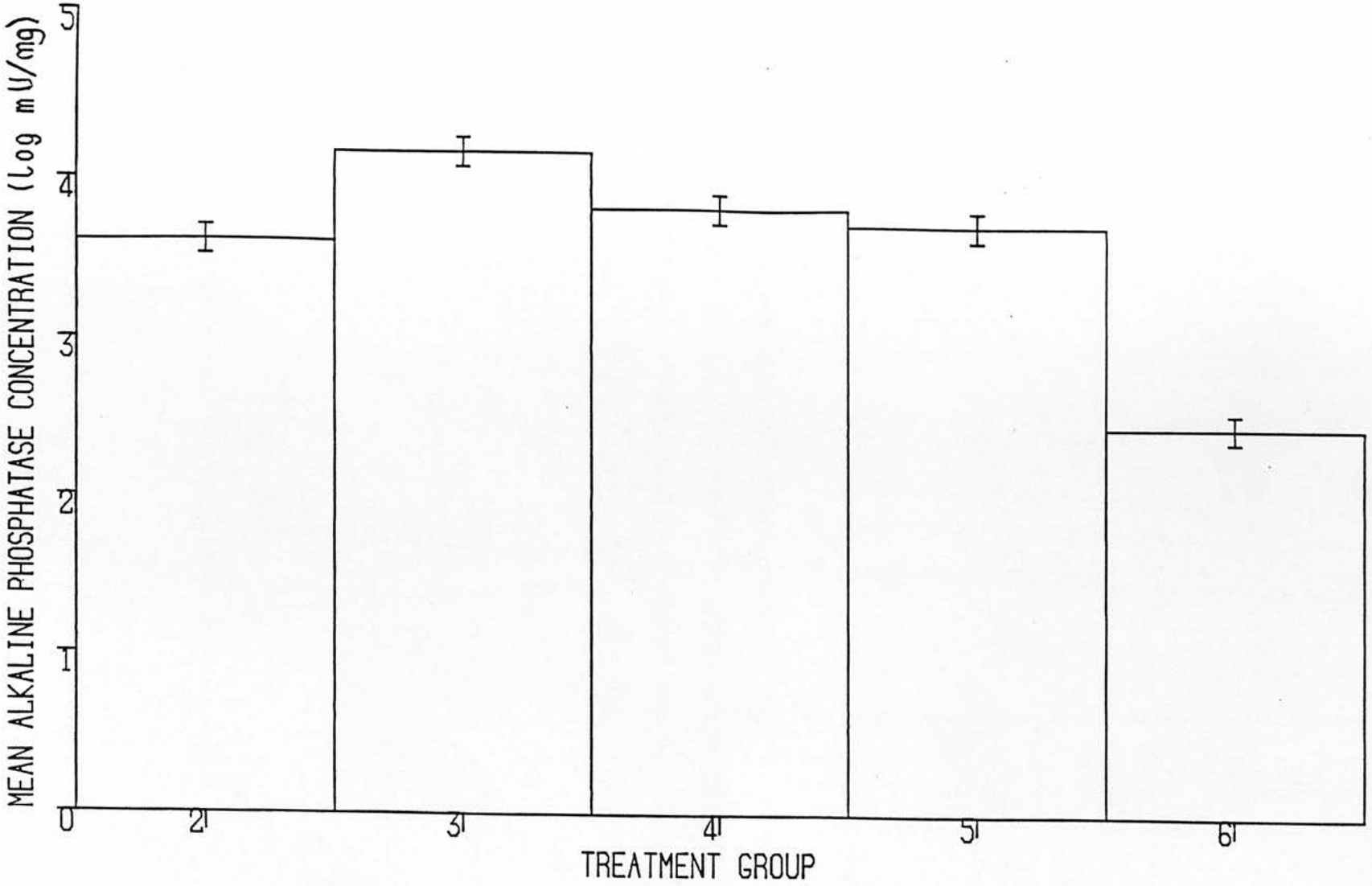


Fig. 6.10

Uterine albumin : total protein ratio in mated ewes (group 1) and in ewes receiving the full sequence of steroid supplementation designed to simulate hormone changes occurring in intact ewes during early pregnancy (group 2). Each point represents the mean $\pm$ 1 s.e.m.

FIG 6.10: UTERINE ALBUMIN : TOTAL PROTEIN RATIO IN EWES IN GROUPS ONE AND TWO

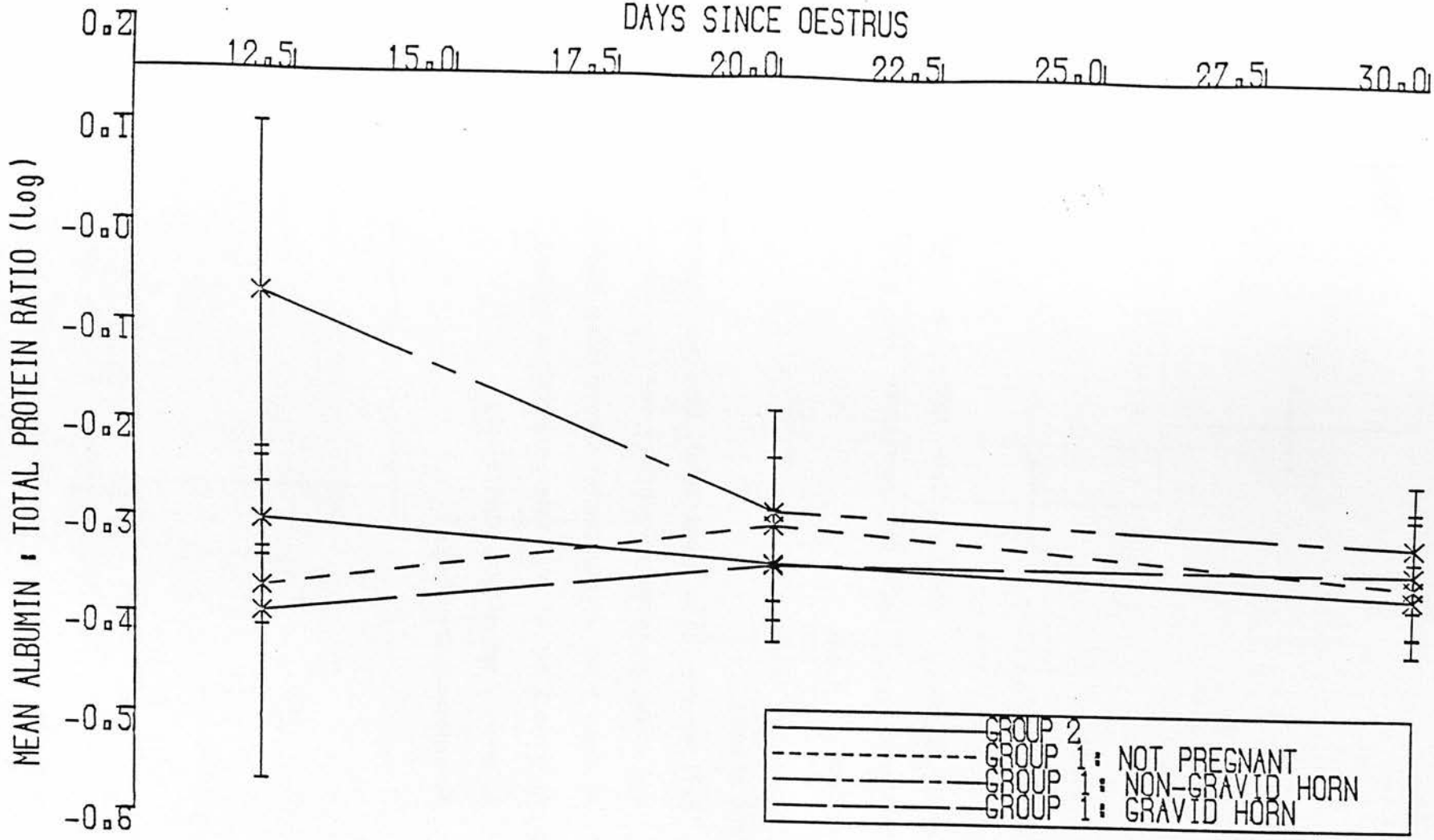


Fig. 6.11

Uterine albumin : total protein ratio on day 12 in ovariectomised ewes. Each bar represents the mean of 10 determinations ± 1 s.e.m.

Treatment groups:

2: Full hormone regime (oestradiol, tube progesterone implant and sachet progesterone implant).

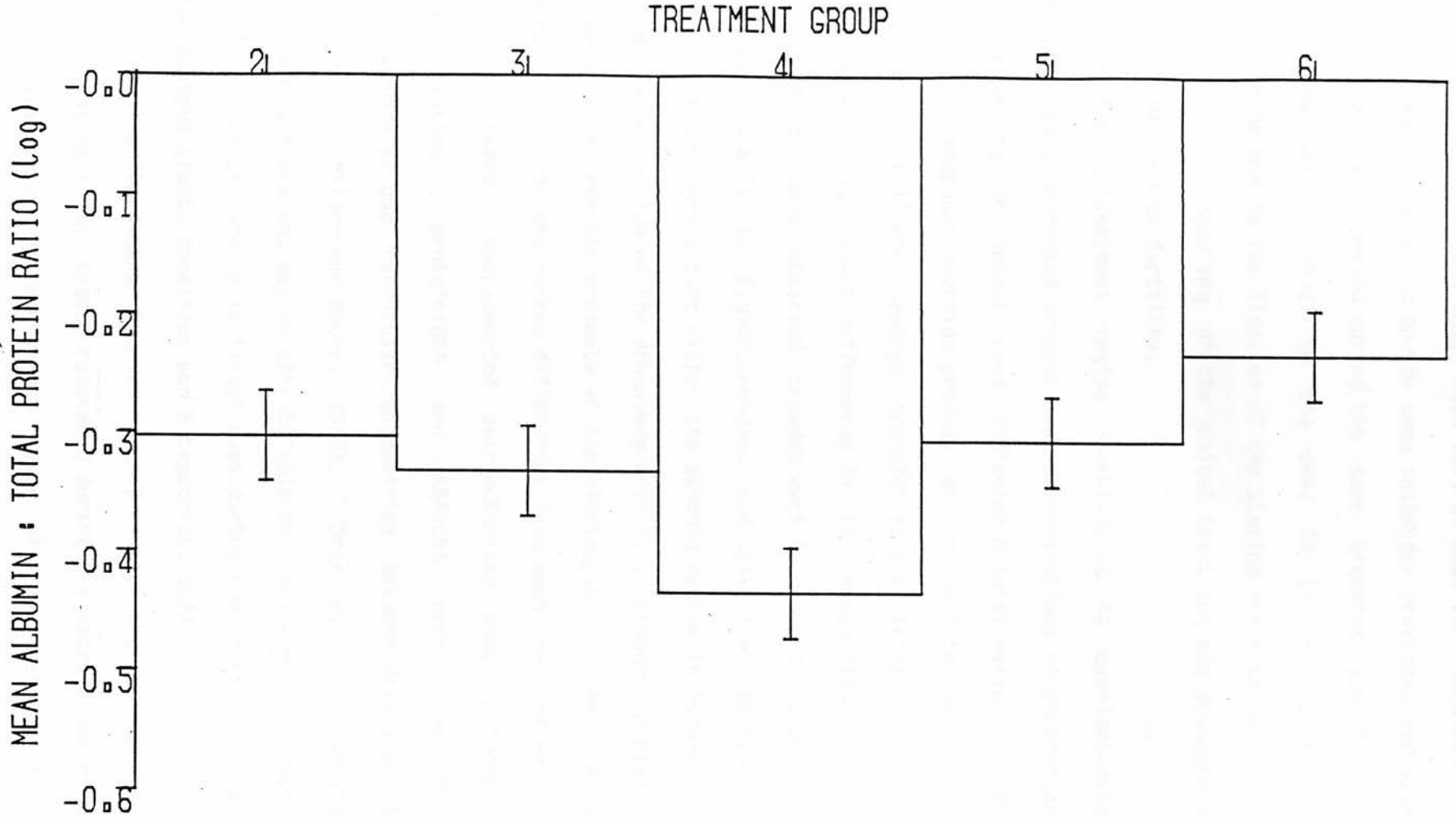
3: As group 2 but without sachet progesterone implant.

4: As group 2 but without tube progesterone implant.

5: As group 2 but without oestradiol.

6: No exogenous steroids.

FIG. 6.11: UTERINE ALBUMIN : TOTAL PROTEIN RATIOS
ON DAY 12 IN OVARIECTOMISED EWES



Welsh Mountain ewes was 85%. Both sets of ewes were obtained from the same source, subjected to the same husbandry practices and mated within a three week period during the same breeding season. The reduced embryonic survival in the ewes in the present study is thought to be due to the ligation of the uterine horns on day 2 or 3. Anaesthesia, handling of the genital tract and the presence of ligatures may reduce fertility.

The steroid treatment regime administered to ovariectomised ewes in group 2 generated progesterone concentrations which mimicked those occurring in intact ewes following natural mating. Such a sequence of exogenous steroids provide an appropriate environment for pregnancy following embryo transfer to the uterus (Miller and Moore, 1976). Significant differences in the composition of the uterine fluids were observed between ewes in group 2 and pregnant ewes, particularly in β.glucuronidase and alkaline phosphatase levels. This suggests that either the steroid regime is incomplete, or that a proportion of the pregnancy associated changes in uterine proteins arise from the presence of the blastocyst in the uterine lumen. Consistent and marked differences have been reported between entire and steroid supplemented ovariectomised ewes in terms of protein synthesis, protein:DNA and RNA:DNA ratios in both inter-caruncular and caruncular endometrium between days 4 and 10 after oestrus (Miller and Moore, 1983). These authors suggested that such differences may be attributable to continuing oestrogenic stimulation of the uterus in intact ewes during the first 10 days after oestrus (Pant, Hopkinson and Fitzpatrick, 1977).

Several authors have observed significantly higher rates of protein synthesis in tissue recovered during pregnancy compared to tissue recovered at an equivalent stage of the oestrous cycle

(Findlay et al., 1981; Sergerson, 1981; Miller and Moore, 1983). The results from the present study on day 12 do not support these findings. There are several possible explanations for such a discrepency. Firstly, the rate of protein synthesis is higher in the inter-caruncular than the caruncular endometrium (Findlay et al., 1982; Miller and Moore, 1983). The technique employed to collect uterine fluids may have rinsed the caruncular endometrium more thoroughly than the inter-caruncular spaces, and therefore failed to reveal the increased synthesis from the inter-caruncular endometrium. Secondly, the volume of the uterine lumen is affected by pregnancy status and ovarian steroids. To ensure that the uterine lumen was effectively rinsed, but that uterine distension was minimised, differing volumes of saline were used to rinse the uterus. As a result, comparisons between treatment groups should be interpreted with caution. The two studies described above investigated protein synthesis in endometrial explants of constant weight. Changes in the relative proportion of the other proteins investigated were not a result of differences in the overall total protein concentration.

The observed increase in total protein concentrations during pregnancy supports the data of Ellinwood, Nett and Niswender, (1979a) and those of Miller, Tassel and Stone, (1983). A part of this increase is attributable to pregnancy associated proteins in the uterine lumen during the pre-implantation period (Roberts, Parker and Symonds, 1976; Staples, Lawson and Findlay, 1978). However, such pregnancy specific proteins account for a very small proportion of the total uterine protein.

The observation that total protein levels are higher in the gravid uterine horn than the non-gravid horn on days 20 and 30

suggests that the conceptus may trigger the release of additional endometrial proteins, or secrete a significant amount of protein into the lumen. In culture, ovine blastocysts release at least two proteins between day 12 and 20 (Masters et al., 1982). One of these is a low molecular weight protein thought to have antiluteolytic properties, and the other molecule is a large glycoprotein. It has been suggested that the glycoprotein may be released in an insoluble form in vivo, and so would not contribute to the uterine luminal fluids.

The presence of an embryo may affect uterine function by day 20 by several mechanisms, including an effect on uterine blood flow, or on protein synthesis per cell by the local action of oestrogen. There is an increase in blood flow to the gravid horn, particularly the endometrium, begining on day 12-13 of pregnancy (Greiss and Anderson, 1970). This may result from a local influence of the blastocyst on the uterine vascular bed. The binding of catecholoestrogens to alpha adregenergic receptors on the smooth muscle cells of the uterine arterioles leads to vasodilation and increased uterine blood flow (Ford, 1982). The increase in the protein concentration in the gravid horn occurred after the reported increase in blood flow to the endometrium. This would seem a likely mechanism whereby the blastocyst exerts a local influence on endometrial protein synthesis. Although oestradiol can mimic the effects of pregnancy on endometrial protein synthesis (Miller, Wild and Stone, 1979; Findlay et al., 1981), and uterine blood flow (Huckabee, Crenshaw, Curet, Mann and Barron, 1970) there is no conclusive evidence that this steroid is involved. Neither the trophoblast (Gadsby, Heap and Burton, 1980) nor the endometrium (Findlay et al., 1981) have the capacity to produce oestrogens

before implantation.

In 1977 Murdoch and O'Shea reported two peaks of β.glucuronidasse activity occurring on days 8 and 30 in pregnant endometrial homogenates. These authors suggested that the second peak was due to the release of acid hydrolases within epithelial cells, and was associated with implantation. The lower β.glucuronidase concentrations in the gravid horn observed during the present experiment would not support such a function. Findlay et al., (1981) showed that β.glucuronidase levels were higher in caruncular tissue than inter-caruncular tissue on day 15 in both pregnant and non-pregnant ewes. These authors did not observe significant differences between activity in pregnant and non-pregnant ewes on day 15. These findings are supported by the observations in the present experiment, in which there was no significant effect of pregnancy status on β.glucuronidase levels on days 12, 20 and 30. Ovariectomised ewes receiving silastic implants containing 100mg oestradiol from day 0 to 15 exhibited higher β.glucuronidase concentrations on day 15 (Findlay et al., 1981). This may explain the significantly lower pattern of β.glucuronidase concentrations in ovariectomised ewes receiving only progesterone supplementation compared to the intact ewes. Levels of β.glucuronidase tended to be lower in the gravid horn than the non-gravid horn throughout the experimental period. This may be a reflection of the decrease in nuclear, cystolic and total oestrogen receptors in the caruncular endometrium of the gravid horn during unilateral pregnancy (Findlay et al., 1982).

The only apparent differences in alkaline phosphatase concentrations were between ovariectomised ewes receiving the full implant regime, and intact ewes. This suggests that in mated ewes

alkaline phosphatase concentrations are not affected by progesterone levels alone. As the changes in alkaline phosphatase observed in intact ewes were neither consistent nor significant, it is difficult to envisage which other factors may be important.

Ovariectomised ewes

With the exception of the albumin : total protein ratio, levels of uterine proteins were lower in ovariectomised ewes receiving no exogenous steroids. This supports the belief that ovarian steroids stimulate endometrial proteins (Finn and Porter, 1975; Yochim, 1975).

Total protein levels were lower, but not significantly so, on day 12 in ewes that had not received oestradiol around the time of oestrus (group 5), compared to ewes on the full implant regime (group 2). This is in agreement with the findings of Miller et al., (1977b). These authors observed that omission of oestradiol around the time of oestrus caused significant decreases in uterine weight, DNA:RNA ratios, oestrogen and progesterone receptor concentrations, the rate of protein synthesis and total protein concentration on day 4. Embryos transferred to such ewes ceased to develop within 1-2 days. Oestradiol present during oestrus is thought to regulate the concentration of cystolic progesterone receptors (Murphy, Stone, Miller and Moore, 1979) and may modify embryonic development by affecting endometrial sensitivity to progesterone. Such an effect can be seen in the present study. Progesterone concentrations on day 12 were similar in groups 2 and 5, however, uterine levels of alkaline phosphatase, an enzyme known to be controlled by progesterone (Murdoch and White, 1967 and 1968), were significantly lower in ewes that did not receive oestradiol (group 5) than ewes

that received the complete regime (group 2) on day 12.

The function of the progesterone concentrations generated by tube implants in this experiment is not known. Embryonic survival in recipient ewes receiving the steroid regime given to ewes in group 2 and in group 4 was 19% and 56% respectively (Wilmut, Sales and Ashworth, 1985a). It has been suggested that progesterone immediately after oestrus may inhibit an embryotoxic effect in the uterine lumen during early pregnancy. One candidate for such a toxin is oestradiol. Progesterone is known to reduce the cytosol concentrations of oestrogen receptors in the uterus (Miller, Wild and Stone, 1979). The observation that albumin : total protein ratios were lowest in ewes in group 4 supports this idea, as such ratios are reduced in oestrogen dominated regimes (Beier, 1974). Similarly, β.glucuronidase concentrations were higher (although not significantly so) in this group of ewes. Uterine secretion of this enzyme may require oestradiol (Findlay et al., 1981).

In the light of the numerous reports showing that steroid hormones affect both uterine receptor concentrations and specific components of the luminal fluids, it was surprising that so few significant differences between treatment groups were observed in the present study. The considerable between ewe variation in each group may have masked some between treatment group effects. The studies of Miller et al., (1977b) suggest that most of the uterine effects following omission of steroid treatments occur within 3-4 days of oestrus, and that by day 10 these effects are less significant. The observation that uterine protein concentrations between groups 2, 4 and 5 on day 12 were not significantly different supports this finding. The reason for such changes in uterine sensitivity or function is not known. In order to gain more

information on the effects of the steroid treatments administered in this trial, it would be worth-while to investigate specific uterine proteins soon after oestrus, and to determine if the steroid receptor populations differed between groups.

The present observations show that the uterine environment is highly variable, even between ewes on a constant steroid regime. Such variation may be a cause of embryonic loss if the uterine environment is inappropriate for a developing embryo.

CHAPTER 7

FINAL DISCUSSION

The experiments described in this thesis have provided new understanding of the maternal factors which influence early pregnancy, and have highlighted opportunities for the treatment of some forms of infertility. These studies have revealed variation in both progesterone concentration and in uterine function during the first weeks of pregnancy. Such variability could induce a range of uterine environments, only some of which are appropriate for development of the embryo (Wilmut and Sales, 1981). Although such variation may be responsible for embryonic loss, it also provides flexibility with which individual ewes can respond to their environment.

The pattern of progesterone secretion during early pregnancy may be sufficiently variable to jeopardise embryonic survival (Chapter 3). Two possible means of improving embryonic survival arise from this observation. It may prove beneficial to supplement progesterone after mating in order to improve the likelihood of embryonic survival. Supplementary progesterone administered during the second and third weeks after mating increased the proportion of pregnant beef (Sreenan and Diskin, 1983) and dairy (Shemesh, Ayalon, Lavi, Mileguir, Shore and Toby, 1983) heifers. In view of the evidence that embryonic survival is reduced in animals with low progesterone levels during the first few days of pregnancy (Chapter 3; Lee and Ax, 1984) it may be appropriate to provide supplementary progesterone throughout the first two weeks of pregnancy. Providing that the dose of progesterone administered immediately after oestrus

is not so great as to advance uterine function (Lawson and Cahill, 1983), this may prove an effective means of increasing fertility. A second approach may involve genetic selection for a progesterone related trait associated with embryonic survival. Although the heritability of the pattern of progesterone secretion is likely to be poor (Chapter 3), it may be worth-while to select for a progesterone related trait, such as uterine responsiveness to progesterone. The importance of other steroid hormones during early pregnancy is not so clearly defined. It is important to determine any effects these hormones and their inter-relationship with progesterone may have on embryonic survival.

These experiments did not determine whether an association between a particular uterine milieu and embryonic survival exists. It is possible that specific uterine factors are required at particular stages of embryonic development in order for pregnancy to continue. In vitro culture of ovine blastocysts of known developmental age in a variety fluids may provide greater understanding of substrate requirements.

In addition to the contribution to embryonic mortality caused by variation in maternal hormones and uterine secretions, variation in the stage of embryonic development may affect the success of a pregnancy. Differences in the developmental age of an embryo may arise due to variation in the timing of ovulation (Whyman et al., 1979), in the timing of sperm penetration, and the subsequent cleavage rate. In mice, a gene 'Ped' (preimplantation embryonic development) influences the time of the first cleavage and the subsequent rate of cell division (Goldbard, Varbanac and Warner, 1982). Strains of mice possessing the 'fast' allele of this gene exhibit a more rapid rate of cleavage (Warner, Gollnick and Golbard,

1984). An experiment has recently been performed in pigs to determine the effect of variation in the stage of embryonic development on pregnancy (Wilmut, Sales and Ashworth, 1985a). Embryos were recovered from naturally mated sows on day 6 after mating, and classified into groups according to their stage of development. These groups of blastocysts were then returned to ligated regions of the original uterus. Embryonic survival was assessed following slaughter on days 13 to 15. Embryos which were relatively more advanced on day 6 were more likely to survive. Embryonic survival may be improved if the spread of ovulation time was reduced so that less variation in egg stage may arise. Unfortunately the likelihood of fertilisation would also be reduced if oestrous behavior was modified.

The hypothesis that some embryonic mortality may arise due to an asynchrony between the ewe and her embryo (Wilmut and Sales, 1981) suggests that embryonic loss need not be due to an inadequacy in either maternal or embryonic function, but due to an incompatability in the timing of changes occurring in the two systems.

It has been observed that natural selection may favour an intermediate level of embryonic survival (Land, Gauld, Lee and Webb, 1982). Several explanations for this phenomenon have been proposed. Embryonic mortality may be a method of eliminating unfit genotypes from the population (Bishop, 1964). It also provides a means of prolonging the interval between successive births, to ensure that the survival and development of the older offspring is not jeopardised (Short, 1978). More recently Wilmut, Sales and Ashworth (1985b) suggested that embryonic mortality may be a consequence of an animal having retained the ability to live and reproduce in a

variety of environments. The levels of embryonic survival observed in the field situation may reflect the best compromise between the ability to breed in a range of environments and the need for a great majority of eggs to develop to term. For example, it is potentially beneficial for an animal to respond to nutritional changes by having more ovulations, although the response may alter the progesterone profile.

This theory would suggest that embryonic survival could only be improved by introducing more uniformity in either the physiological mechanisms involved in early pregnancy, or in the environment. Animals selected to breed very efficiently in one environment might be less suited to surviving in alternative environments. It is therefore questionable as to whether such a long term artificial intervention would be beneficial.

BIBLIOGRAPHY

Abraham, G. E. (1969) Preliminary communication: Solid phase radioimmunoassay of Estradiol-17β. J. Clin. Endocrinol. Metab. 29: 866-870.

Alliston, C.W., Elgi, G.E., Ulberg, L.C. (1961) Loss of potential young in the ewe due to high ambient temperatures. J.Appl.Physiol. 16: 253-256.

Amoroso, E.C., Perry, J.S. (1977) Ovarian activity during gestation. In 'The Ovary' 2nd edition, vol. II, Physiology, pp 315-398. Ed. S. Zuckerman and B.J. Weir, Academic Press, New York.

Ashworth, C.J., Sales, D.I., Wilmut, I. (1984) Patterns of progesterone secretion and embryonic survival during repeated pregnancies in Damline ewes. Proc. 10th Int. Congr. Anim. Reprod. and A.I., Illinois 2: 74.

Baird, D.T. (1978a) Pulsatile secretion of LH and ovarian oestradiol during the follicular phase in the sheep. Biol. Reprod. 18: 359-364.

Baird, D.T. (1978b) Local utero-ovarian relationships. In 'Control of Ovulation', pp 217-233. Eds D.B.Crighton, G.R.Foxcroft, N.B.Haynes and G.E.Lamming. Butterworths, London.

Baird, D.T., Abel, M.H., Brown, A., Kelly, R.W., Wilmut, I. (1981) Synthesis and release of prostaglandins by the uterus during the oestrous cycle and early pregnancy. In 'Uterus et Fecondite' pp 69-82. Colloque de la Société Nationale pour l'étude de la Sterilite et de la Fecondite, Paris.

Baird, D.T., McNeilly, A.S. (1981) Gonadotrophic control of follicular development and function during the oestrous cycle of the ewe. J.Reprod.Fert. Suppl. 30: 119-133.

Baird, D.T., Scaramuzzi, R.J. (1976) Changes in the secretion of ovarian steroids and pituitary luteinising hormone in the peri-ovulatory period in the ewe: the effect of progesterone. J.Endocr. 70: 237-245.

Baird, D.T., Swanston, I.A., McNeilly, A.S. (1981) Relationship between LH, FSH and prolactin concentration and the secretion of androgens and estrogens by the preovulatory follicle in the ewe. Biol.Reprod. 24: 1013-1025.

Baird, D.T., Swanston, I., Scaramuzzi, R.J. (1976) Pulsatile release of LH and secretion of ovarian steroids in the sheep during the luteal phase of the estrous cycle. Endocrinology 98: 1490-1496.

Bazer, F.W., Roberts, R.M. (1983) Biochemical aspects of conceptus-endometrial interactions. J.Exp.Zool. 228: 373-383.

Bazer, F.W., Roberts, R.M., Sharp, D.C. (1978) Collection and analysis of female genital tract secretions. In 'Methods in Mammalian Reproduction' pp 503-538 Ed. J.C. Daniels. Academic Press, New York.

Bazer, F.W., Thatcher, W.W. (1977) Theory of maternal recognition of pregnancy in swine based on estrogen controlled endocrine versus exocrine secretion of prostaglandin-$F_{2\alpha}$ by the uterine endometrium. Prostaglandins 14: 379-401.

Beato, M., Baier, R. (1975) Binding of progesterone to proteins of the uterine luminal fluid. Identification of uteroglobin as the binding protein. Biochim. Biophys. Acta. 392: 346-356.

Bedford, C.A., Harrison, F.A., Heap, R.B. (1972) The metabolic clearance rate and production rate of progesterone and the conversion of progesterone to 20α-Hydroxypregn-4-en-3-one in the sheep. J.Endocr. 55: 105-108.

Bedford, C.A., Harrison, F.A., Heap, R.B. (1974) Splanchnic, uterine, ovarian and adrenal uptake of progesterone and 20α-Dihydroprogesterone in the pregnant and non-pregnant sheep. J.Endocr. 62: 277-290.

Beer, A.E., Billingham, R.E. (1979) Maternal immunological recognition mechanisms during pregnancy. In 'Maternal Recognition of Pregnancy'. Ciba Fdn. Colloq. No. 64 Ed. J. Whelan. Elsevier Excerpta Medica - North Holland, Amsterdam.

Beier, H.M. (1974) Oviductal and uterine fluids. J.Reprod.Fert. 37: 221-237.

Bishop, M. (1964) Paternal contribution to embryonic death. J.Reprod.Fert. 7: 383-369.

Bjersing, L., Hay, M.F., Kann, G., Moor, R.M., Naftolin, F., Scaramuzzi, R.J., Short, R.V., Younglai, E.V. (1972) Changes in gonadotrophins, ovarian steroids and follicular morphology in sheep at oestrus. J.Endocr. 52: 465-479.

Blockey, M.A.deB., Parr, R.A., Restall, B.J., (1975) Wastage of ova in young Merino ewes. Aust.Vet.J. 51: 298-302.

Borghese, E. (1957) Recent histochemical results of studies on embryos of some birds and mammals. Int.Rev.Cytol 6: 289-337.

Boshier, D.P. (1970) The pontamine blue reaction in pregnant sheep uteri. J.Reprod.Fert. 19: 51-61.

Bradford, G.E. (1972) Genetic control of litter size in sheep. J.Reprod.Fert., Suppl. 15: 23-41.

Bradford, G.E. (1979) Genetic variation in prenatal survival and litter size. J.Anim.Sci. 49: Suppl. II 66-74.

Brien, F.D., Cumming, I.A., Baxter, R.W. (1977) Effect of feeding a lupin grain supplement on reproductive performance of maiden and mature ewes. J.Agric.Sci.Camb. 89: 437-443.

Brien, F.D., Cumming, I.A., Clarke, I.J., Cocks, C.S. (1981) Role of plasma progesterone concentrations in early pregnancy of the ewe. Aust. J. Exp. Agric. Anim. Husb. 21: 562-565.

Brinsfield, T.H., Hawk, H.W. (1974) Ultrastructure of sheep endometrial cells after ovariectomy and hormone treatment. Biol. Reprod. 10: 98-102.

Caffrey, J.L., Nett, T.M., Abel, J.H., Niswender, G.D. (1979) Activity of 3β-Hydroxy-Δ^5-steroid dehydrogenase/Δ^5-Δ^4-isomerase in the ovine corpus luteum. Biol.Reprod. 20: 279-287.

Cahill, L.P. (1981) Folliculogenisis in the sheep as influenced by breed, season and oestrous cycle. J.Reprod.Fert. Suppl. 30: 135-142.

Cahill, L.P., Mauléon, P. (1980) Influences of season, cycle and breed on follicular growth rates in sheep. J.Reprod.Fert. 58: 321-328.

Caron, M.G., Goldstein, S., Savard, K., Marsh, J.M. (1975) Protein kinase stimulation of a reconstituted cholesterol side chain cleavage enzyme system in the bovine corpus luteum. J.biol.Chem. 250: 5137-5143.

Catt, K.J., Dufau, M.L., Vaitukatis, J.L. (1975) Appearance of hCG in pregnancy plasma following the initation of implantation by the blastocyst. J.Clin.Endocr.Metab. 40: 537-540.

Clarke, I.J., Doughton, B.W. (1983) Effects of various anaesthetics on resting plasma concentrations of luteinising hormone, follicle stimulating hormone and prolactin in ovariectomised ewes. J.Endocr. 98: 78-89.

Cognie, Y., Pellitier, J. (1976) Pre-ovulatory LH release and ovulation in dry and in lactating ewes after progestagen and PMSG treatment during seasonal anoestrum. Ann.Biol.anim.Bioch.Biophy. 16: 529-536.

Colas, G. (1981) Variations saisonnieres de la Qualite du sperme chez le belier Ile-de-France. II. Fecondance: relation avec les criteres qualitatifs observes in vitro. Reprod.Nutr.Develop. 21: 399-407.

Corrie, J.E.T., Ratcliffe, W.A., Macpherson, J.S. (1981a) Generally applicable ^{125}iodine-based radioimmunoassays for plasma progesterone. Steroids 38: 709-717.

Corrie, J.E.T., Hunter, W.M., Macpherson J.S. (1981b) A strategy for radioimmunoassay of plasma progesterone with the use of a homologous site ^{125}I-labelled radioligand. Clin. Chem. 27: 594-599.

Corrie, J.E.T., Ratcliffe, W.A., Macpherson, J.S. (1982) The provision of ^{125}I-labelled tracers for radioimmunoassay of haptens: A general approach? J. Immunol. Meths. 51: 159-166.

Creange, J.E., Anzalone, A.J., Potts, G.O., Schane, H.P. (1981) WIN 32,729, A new, potent interceptive agent in rats and rhesus monkeys. Contraception 24: 289-299.

Crosby, I.M., Osborn, J.C., Moor, R.M. (1981) Follicle cell regulation of protein synthesis and developmental competence in sheep oocytes. J.Reprod.Fert. 62: 575-582.

Cumming, I.A., Baxter, R., Lawson, R.A.S. (1974) Steroid hormone requirements for the maintenance of early pregnancy in sheep: A study using ovariectomised adrenalectomised ewes. J.Reprod.Fert. 40: 443-446.

Cumming, I.A., Blockey, M.A.deB., Winfield, C.G., Parr, R.A., Williams, A.H. (1975) A study of relationships of breed, time of mating, level of nutrition, live weight, body condition, and face cover to embryo survival in ewes. J. Agric. Sci. Camb. 84: 559-565.

Cumming, I.A., Brown, J.M., Blockey, M.A. de B., Winfield, C.G., Baxter, R., Goding, J.R. (1971) Constancy of interval between luteinising hormone release and ovulation in the ewe. J.Reprod.Fert. 24: 134-135.

Daniel, J.C. (1972) Local production of protein during implantation in the rabbit. J.Reprod.Fert. 31: 303-306.

Davis, A.J., Fleet, I.R., Harrison, F.A., Maule Walker, F.M. (1980) Pulmonary metabolism of prostaglandin $F_{2\alpha}$ in the concious non-pregnant ewe and sow. J.Physiol.London 301: 86P.

Denamur, R., Martinet, J., Short, R.V. (1966) Secretion de la progesterone par les corps jaunes de le brebis apres hypophysectomie section de la tige pituitaire et hysterectomie. Acta Endrocrinologica 52: 72-90.

Denamur, R., Martinet, J., Short, R.V. (1973) Pituitary control of the ovine corpus luteum. J.Reprod.Fert. 32: 207-220.

Diekman, M.A., O'Callaghan, P.L., Nett, T.M., Niswender, G.D. (1978) Validation of methods for quantification of luteal receptors for LH throughout the estrous cycle and early pregnancy. Biol.Reprod. 19: 999-1009.

Dighe, K.K., Hunter, W.M. (1974) A solid-phase radioimmunoassay for plasma progesterone. Biochem. J. 143: 219-231.

Doney, J.M., Smith, W.F., Gunn, R.G. (1976) Effects of post mating environmental stress or administration of ACTH on early embryo loss in sheep. J. Agric. Sci. Camb. 87: 133-136.

Doumas, B.T., Watson, W.A., Biggs, H.G. (1971) Albumin standards and the measurement of serum albumin with bromocresol green. Clin.Chim.Acta 31: 87-96.

Dufour, J., Cahill, L.P., Mauléon, P. (1979) Short- and long-term effects of hypophesectomy and uni-lateral ovariectomy on ovarian follicular populations in sheep. J.Reprod.Fert. 57: 301-309.

Dunn, J.F., Nisula, B.C., Rodbard, D. (1981) Transport of Steroid Hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. J. Clin. Endocrinol.Metab. 53: 58-68.

Dutt, R.H. (1963) Critical period for early embryo mortality in ewes exposed to high ambient temperature. J.Anim.Sci. 22: 713-719.

Dutt, R.H. (1964) Detrimental effects of high ambient temperature on fertility and early embryo survival in sheep. Int. J. Biometeor. 8: 47-56.

Dutt, R.H., Ellington, E.F., Carlton, W.W. (1959) Fertilisation rate and early embryo survival in sheared and unsheared ewes following exposure to elevated air temperature. J.Anim.Sci. 18: 1308-1317.

Dziuk, P.J., Cook, B. (1966) Passage of steroids through silicone rubber. Endocrinology 78: 208-211.

Edey, T.N. (1966) Nutritional stress and preimplantation embryonic mortality in Merino sheep. J.Anim.Sci.Camb. 67: 287-302.

Edey, T.N. (1969) Prenatal mortality in sheep: A review. Anim. Breed. Abstr. 37: 173-190

Edey, T.N. (1970) Nutritional stress and pre-implantation embryonic mortality in Merino Sheep 1967. J.Agric.Sci. Cambridge. 74: 193-198.

Edey, T.N. (1979) Embryo Mortality. In 'Sheep Breeding', pp 315-325. Eds Tomes, Robertson, Lightfoot and Haresign, Butterworths, London.

Ellinwood, W.E., Nett, T.M., Niswender, G.D. (1979a) Maintenance of the corpus luteum of early pregnancy in the ewe. I. Luteotrophic properties of embryo homogenates. Biol.Reprod. 21: 281-288.

Ellinwood, W.E., Nett, T.M., Niswender, G.D. (1979b) Maintenance of the corpus luteum of early pregnancy in the ewe. II. Prostaglandin secretion by the endometrium in vitro and in vivo. Biol.Reprod. 21 845-856.

England, B.G., Dahmer, M.K., Webb, R. (1981) Relationships between follicular size and antral fluid steroid concentrations at three stages of the estrous cycle in the ewe. Biol.Reprod. 24: 1068-1075.

England, B.G., Webb, R., Dahmer, M.K. (1981) Follicular steroidogenisis and gonadotrophin binding to ovine follicles during the estrous cycle. Endocrinology 109: 881-887.

Espey, L.L. (1980) Ovulation as an inflammatory response - a hypothesis. Biol.Reprod. 22: 73-106.

Eyestone, W.H., French, L.R. (1981) Uterine specific antigens in the ewe. Theriogenology 16: 359-371.

Failing, J.F., Buckley, M.T., Zak, B. (1960) Automatic determination of serum proteins. Am.J.Clin.Path. 33: 83-88.

Ford, S.P. (1982) Control of uterine and ovarian blood flow throughout pregnancy in ewes, sows and cows. J.Anim.Sci. 55 (Suppl. II) 32-42.

Findlay, J.K. (1981) Blastocyst-endometrial interactions in early pregnancy in the sheep. J.Reprod.Fert. Suppl. 30: 171-182.

Findlay, J.K., Ackland, N., Burton, R.D., Davis, A.J., Maule Walker, F.W., Walters, D.E., Heap, R.B. (1981) Protein, prostaglandin and steroid synthesis in caruncular and intercaruncular endometrium of sheep before implantation. J.Reprod.Fert. 62: 361-377.

Findlay, J.K., Clarke, I.J., Swaney, J., Colvin, N., Doughton, B. (1982) Oestrogen receptors and protein synthesis in caruncular and intercaruncular endometrium of sheep before implantation. J.Reprod.Fert. 64: 329-339.

Finn, C.A., Porter, D.G. (1975) Handbooks of Reproductive Biology. Vol. 1: 'The Uterus' Elek Science, London.

Fishman, W.H., Kato, K., Anstiss, C.L., Green, S. (1967) Human serum β.glucuronidase, its measurement and some of its properties. Clin.Chim.Acta. 15: 435-447.

Fitz, T.A., Mayan, M.H., Sawyer, H.R., Niswender, G.D. (1982) Characterisation of two steroidogenic cell types in the ovine corpus luteum. Biol. Reprod. 27: 703-711.

Flint, A.P.F., Burton, R.D., Gadsby, J.E., Saunders, P.T.K., Heap, R.B. (1979) Blastocyst oestrogen synthesis and the maternal recognition of pregnancy. In 'Maternal Recognition of Pregnancy'. Ciba Fdn. Colloq. No. 64. Ed. J. Whelan. Elsevier Excerpta Medica - North Holland, Amsterdam.

Gadsby, J.E., Heap, R.B., Burton, R.D. (1980) Oestrogen production by blastocyst and early embryonic tissue of various species. J.Reprod.Fert. 60: 409-417.

Gautray, J.P., Condere, P., Colomb, M.C., Sibut, H., Maurel, C. (1969) Biochemical determination of alkaline phosphatase activity in the human endometrium during the menstrual cycle. Am.J.Obstet.Gynecol. 104: 818-822.

Gemmell, R.T., Stacy, B.D. (1977) Effects of colchicine on the ovine corpus luteum: role of microtubules in the secretion of progesterone. J.Reprod.Fert. 49: 115-117.

Gemmell, R.T., Stacy, B.D., Thorburn, G.D. (1974) Ultrastructural study of secretory granules in the corpus luteum of the sheep during the oestrous cycle. Biol.Reprod. 11: 447-462.

Gibb, W., Hagerman, D.D. (1976) The specificity of the 3β-hydroxysteroid dehydrogenase activity of bovine ovaries towards dihydroepiandrosterone and pregnenolone: evidence for multiple enzymes. Steroids 28: 31-41.

Goding, J.R. (1974) The demonstration that PG-$F_{2\alpha}$ is the uterine luteolysin in the ewe. J.Reprod.Fert. 38: 261-271.

Goding, J.R., Buckmaster, J.M., Cerini, J.C., Cerini, M.E.D., Chamley, W.A., Cumming I.A., Fell, L.R., Findlay, J.K., Jonas, H. (1973) Gonadotrophins in the ovine oestrous cycle. J.Reprod.Fert. Suppl. 18: 31-37.

Godkin, J.D., Bazer, F.W., Moffat, J., Sessions, F., Roberts, R.M. (1982) Purification and properties of a major, low molecular weight protein released by the trophoblast of sheep blastocysts. J.Reprod.Fert. 65: 141-150.

Godkin, J.D., Cote, C., Duby, R.T. (1978) Embryonic stimulation of ovine and bovine corpora lutea. J.Reprod.Fert. 54: 375-378.

Goldbard, S.B., Verbanac, K.M., Warner, C.M. (1982) Role of the H-2 complex in pre-implantation mouse embryo development. Biol.Reprod. 26: 591-596.

Goode, L., Warnick, A.C., Wallace, H.D. (1965) Alkaline and acid phosphatase activity in the endometrium and ovary of swine. J.Anim.Sci. 24: 955-958.

Greiss, F.C.Jr, Anderson, S.G. (1970) Uterine blood flow during early ovine pregnancy. Am.J.Obstet.Gynec. 106: 30-38.

Griffiths, J.G., Gunn, R.G., Doney, J.M. (1970) Fertility in Scottish Blackface ewes as influenced by climatic stress. J.Agric.Sci.Camb. 75: 485-488.

Guillomot, M., Flechon, J. -E., Wintenberger-Torrès, S. (1982) Cytochemical studies of uterine and trophoblastic surface coats during blastocyst attachment in the ewe. J.Reprod.Fert. 65: 1-8.

Gunn, R.G., Doney, J.M., Russel, A.J.F. (1969) Fertility in Scottish Blackface ewes as influenced by nutrition and body condition at mating. J.Agric.Sci.Camb. 73: 289-294.

Gunn, R.G., Doney, J.M., Russel, A.J.F. (1972) Embryo mortality in Scottish Blackface ewes as influenced by body condition at mating and by post mating nutrition. J.Agric.Sci.Camb. 79: 19-25.

Hamra, A.M., Bryant, M.J. (1982) The effects of level of feeding during rearing and early pregnancy upon reproduction in young female sheep. Anim.Prod. 34: 41-48.

Hanly, S. (1961) Prenatal mortality in farm animals. J.Reprod.Fert. 2: 182-194.

Hanrahan, J.P. (1982) Selection for increased ovulation rate, litter size and embryo survival. Proc. 2nd World Congress on Genetics Applied to Livestock Production V 294-309.

Hansel, W. (1981) Plasma hormone concentrations associated with early embryo mortality in heifers. J.Reprod.Fert. Suppl. 30: 231-239.

Hansel, W., Concannon, P.W., Lukaszewska, J.H. (1973) Corpora lutea of the large domestic animal. Biol.Reprod. 8: 222-245.

Haresign, W., Lamming, G.E. (1978) Comparison of LH release and luteal function in cyclic and LH-RH treated anoestrus ewes pretreated with PMSG or oestrogen. J.Reprod.Fert. 52: 349-353.

Harrison, F.A., Heap, R.B. (1978) Progesterone secretion during pregnancy in sheep with an autotransplanted adrenal and an autotransplanted ovary. J.Reprod.Fert. 54: 153-157.

Harvey, W.R. (1972) Least squares and maximium likelihood general purpose program. Ohio State University. (Mimeograph).

Hawk, H.W. (1968) Effect of intra-uterine devices on corpus luteum function. J.Anim.Sci. 27: 119-123.

Hay, M.F., Moor, R.M. (1975) Functional and structural relationships in the Graafian follicle population of the sheep ovary. J.Reprod.Fert. 45: 267-275.

Haynes, S.P., Corcoran, J.M., Eastman, C.J., Doy, F.A.(1980) Radioimmunoassay of progesterone in unextracted serum. Clin. Chem. 24: 1607-1609.

Heap, R.B. (1962) Some chemical constituents of uterine washings: a method for analysis with results from various species. J.Endocr. 24: 367-378.

Heap, R.B., Flint, A.P.F., Gadsby, J.E., Rice, C. (1979) Hormones, the early embryo and the uterine environment. J.Reprod.Fert. 55: 267-275.

Heap, R.B., Perry, J.S., Gadsby, J.E., Burton,R.D. (1975) Endocrine activities of the blastocyst and early embryonic tissue in the pig. Biochem.Soc.Trans. 3: 1183-1188.

Hearn, J.P. (1976) Immunization against pregnancy. Proc.R.Soc.Lond. 195: 149-160.

Henderson, K.M., Scaramuzzi, R.J., Baird, D.T. (1977) Simultaneous infusion of PG-E_2 antagonises the luteolytic action of prostaglandin $F_{2\alpha}$ *in vivo*. J.Endocr. 72: 379-383.

Higuchi, T., Kaneko, A., Abel, J.H., Niswender, G.D. (1976) Relationship between membrane potential and progesterone release in ovine *corpora lutea*. Endocrinology 99: 1023-1032.

Hixon, J.E., Pijanowski, G.J., Weston, P.G., Shanks, R.D., Wagner, W.C. (1983) Evidence for an oscillator other than luteinising hormone controlling the secretion of progesterone in cattle. Biol.Reprod. 29: 1155-1162.

Holst, J.P., Braden, A.W.H. (1972) Ovum transport in the ewe. Aust.J.Biol.Sci. 25: 167-173.

Howarth, B. Jr., Hawk, H.W. (1968) Effect of hydrocortisone on embryo survival in sheep. J. Anim. Sci. 27: 117-121.

Huckabee, W.E., Crenshaw, C., Curet, L.B., Mann,L., Baron, D.L. (1970) The effect of exogenous oestrogen on the blood flow and oxygen consumption of the uterus of the non-pregnant ewe. Q.J.Exp.Physiol. 55: 16-24.

Hulet, C.V., Voitlander, H.P.(Jr), Pope, A.L., Casida, L.E. (1956) The nature of early season infertility in sheep. J.Anim.Sci. 15: 607-616.

Hunter, R.H.F., Barwise, L., King, R. (1982) Sperm transport, storage and release in the sheep oviduct in relation to time of ovulation. Br.vet.J. 138: 225-232.

Hyland, J.H., Manns, J.E., Humphrey, W.D. (1982) Prostaglandin production by ovine embryos and endometrium *in vitro*. J.Reprod.Fert. 65: 299-304.

Inskeep, E.K., Oloufa, M.M., Howland, B.E., Pope, A.L., Casida, L.E. (1962) Effect of experimental uterine distension on estrual cycle length in ewes. J.Anim.Sci. 21: 331-332.

Iritani, A., Gomes, W.R., Vandermark, N.L. (1969) Secretion rates and chemical composition of oviduct and uterine fluids in ewes. Biol. Reprod. 1: 72-76.

Jenkin, G., Gemmell, R.T., Thorburn, G.D. (1984) Induction of transient functional luteolysis in cyclic sheep by a 3β-hydroxysteroid dehydrogenase inhibitor. J.Endocr. 100: 61-66.

Kaltenbach, C.C., Cook, B., Niswender, G.D., Nalbandov, A.V. (1967) Effect of pituitary hormones on progesterone synthesis by ovine luteal cells *in-vitro*. Endocrinology 81: 1407-1409.

Karsch, F.J., Goodman, R.L., Legan, S.J. (1980) Feedback basis of seasonal breeding: test of a hypothesis. J.Reprod.Fert. 58: 521-535.

Karsch, F.J., Legan, S.J., Hauger, R.L., Foster, D.L. (1977) Negative feedback action of progesterone on tonic luteinising hormone secretion in the ewe: Dependence on the ovaries. Endocrinology 101: 800-806.

Karsch, F.J., Legan, S.L., Ryan, K.D., Foster, D.L. (1980) Importance of estradiol and progesterone in regulating LH secretion and estrous behaviour during the sheep estrous cycle. Biol.Reprod. 23: 404-413.

Kilgour, R., De Langen, H. (1970) Stress in sheep resulting from management practices. Proceedings of the New Zealand society of Animal Production 30: 65-76.

Knight, J.W., Bazer, F.W., Wallace, H.D. (1973) Hormonal regulation of porcine uterine protein secretions. J.Anim.Sci. 36: 546-553.

Knight, J.W., Bazer, F.W., Wallace, H.D., Wilcox, C.J. (1974) Dose-response relationships between exogenous progesterone and estradiol and porcine uterine protein secretions. J.Anim.Sci. 39: 747-751.

Land, R.B., Gauld, I.K., Lee, G.J., Webb, R. (1982) Further possibilities for manipulating the reproductive process. In 'Future developments in the genetic improvement of animals' pp 59-82, Ed J.S.F.Barker, K.Hammond and A.E.McClintock, Academic Press, Australia.

Land, R.B., Wheeler, A.G., Carr, W.R. (1976) Seasonal variation in the oestrogen induced LH discharge of ovariectomised Finnish Landrace and Scottish Blackface ewes. Annls Biol.anim.Biochim.Biophys. 16: 521-528.

Laster, D.B., Maurer, R.R., Chenault, J.R. (1978) The effect of uterine flushings on in vitro growth of bovine embryos. J.Anim.Sci. 46 (Suppl. I) 395 (Abstract).

Lawson, R.A.S., Adams, C.E., Rowson, L.E.A. (1972) The development of sheep eggs in the rabbit oviduct and their viability after re-transfer to ewes. J.Reprod.Fert. 29: 105-116.

Lawson, R.A.S., Cahill, L.P. (1983) Modification of the embryo maternal relationship in ewes by progesterone treatment early in the oestrous cycle. J.Reprod.Fert. 67: 473-475.

Ledger, W.L., Anderson, A.B.M., Turnbull, A.C. (1982) The success of labour induced by progesterone withdrawal in pregnant sheep. VI International Congress on Hormonal Steroids. (Abstract).

Lee, C.N., Ax, R.L. (1984) Milk progesterone of dairy cows injected with gonadotrophin releasing hormone at the first post partum breeding. Proc. 10th Int. Cong. Anim. Reprod. and A.I., Illinois 2: 401.

Legan, S.J., Karsch, F.J., Foster, D.L. (1977) The endocrine control of seasonal reproductive function in the ewe: A marked change to the negative feedback action of estradiol on luteinising hormone secretion. Endocrinology 101: 818-824.

Lindner, H.R., Amsterdam, A., Salomon, Y., Tsafriri, A., Nimrod, S.A., Lamprecht, S.A., Zor, U., Koch, Y. (1977) Intraovarian factors in ovulation: determinants of follicular response to gonadotrophins. J.Reprod.Fert. 51: 215-235.

Long, S.E., Williams, C.V. (1980) Frequency of chromosomal abnormalities in early embryos of domestic sheep (Ovis Aries). J.Reprod.Fert. 58: 197-201.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randal, R.J. (1951) Protein measurement with the protein phenol reagent. J.Biol.Chem. 193: 265-275.

MacKenzie, A.J., Edey, T.N. (1975) Effects of premating undernutrition on oestrus, ovulation and prenatal mortality in Merino ewes. J.Agric.Sci.Camb. 84: 119-124.

MacKenzie, A.J., Thwaites, C.J., Edey, T.N. (1975) Oestrous, ovarian and adrenal response of the ewe to fasting and cold stress. Aust. J. Agric Res. 26: 545-551.

Marcus, G.J. (1981) Prostaglandin formation by the sheep embryo and endometrium as an indication of maternal recognition of pregnancy. Biol.Reprod. 25: 56-64.

Marsh, J.M. (1970) The stimulatory effect of luteinising hormone on adenyl cyclase in the bovine corpus luteum. J.biol.Chem. 245: 1596-1603.

Marsh, J.M. (1976) The role of cyclic AMP in gonadal steroidogenisis. Biol.Reprod. 14: 30-53.

Martal, J. (1981) Luteal function during early sheep pregnancy. J.Reprod.Fert. Suppl. 30: 201-210.

Martal, J., Djiane, J. (1977) The production of chorionic somatomammotrophin in sheep. J.Reprod.Fert. 49: 285-289.

Martal, J., Lacroix, M.C., Loudes, C., Saunier, M., Wintenberger-Torrès, S. (1979) Trophoblastin, an antiluteolytic protein present in early pregnancy in sheep. J.Reprod.Fert. 56: 63-73.

Martensz, N.D., Baird, D.T., Scaramuzzi, R.J., Van Look, P.F.A. (1976) Androstenedione and the control of luteinising hormone in the ewe during anoestrus. J.Endocr. 69: 227-237.

Martin, L. (1984) On the source of uterine 'luminal fluid' proteins in the mouse. J.Reprod.Fert. 71: 81-87.

Masters, R.A., Roberts, R.M., Lewis, G.S., Thatcher, W.W., Bazer, F.W., Godkin, J.D. (1982) High molecular weight glycoproteins released by expanding pre-attachment sheep, pig and cow blastocysts in culture. J.Reprod.Fert. 66: 571-583.

Martin, L., Finn, C.A. (1970) The effects of an intra-uterine device on uterine cell division and epithelial morphology during early pregnancy in the mouse. J.Endocr. 48: 347-354.

McCracken, J.A., Schramm, W., Okuliecz, W.C. (1984) Hormone receptor control of pulsatile secretion of PG-$F_{2\alpha}$ from the ovine uterus during luteolysis and its abrogation in early pregnancy. Anim.Reprod.Sci. 7: 31-55.

McGinley, R., Casey, J.H. (1979) Analysis of progesterone in unextracted serum: a method using danazol (17α-pregn-4-en-20-yno(2,3-d)isoxazol-17-ol) a blocker of steroid binding to proteins. Steroids 23: 127-138.

McNatty, K.P., Gibb, M., Dobson, C., Thurley, D.C., Findlay, J.K. (1981) Changes in the concentration of gonadotrophic and steroidal hormones in the antral fluid of ovarian follicles throughout the oestrous cycle of the sheep. Aust.J.Biol.Sci. 34: 67-80.

McNatty, K.P., Revfeim, K.J.A., Young, A. (1973) Peripheral plasma progesterone concentrations in sheep during the oestrous cycle. J.Endocr. 58: 219-225.

McNatty, K.P., Sawers R.S., McNeilly, A.S. (1974) A possible role for prolactin in the control of steroid secretion by the human Graafian follicle. Nature. Lond. 250: 653-655.

McNeilly, A.S., Hunter, M., Land, R.B., Fraser, H.M. (1981) Inadequate corpus luteum function after the induction of ovulation in anoestrus ewes by LH-RH or an LH-RH agonist. J.Reprod.Fert. 63: 137-144.

Menon, K.M.J. (1973) Purification and properties of a protein kinase from bovine corpus luteum that is stimulated by cyclic AMP and luteinising hormone. J.biol.Chem. 248: 494-501.

Miller, B.G., Moore, N.W. (1976) Effects of progesterone and oestradiol on RNA and protein metabolism in the genital tract and on survival of embryos in the ovariectomised ewe. Aust.J.Biol.Sci. 29: 565-573.

Miller, B.G., Moore, N.W. (1983) Endometrial protein secretion during early pregnancy in entire and ovariectomised ewes. J.Reprod.Fert. 68: 137-144.

Miller, B.G., Moore, N.W., Murphy, L., Stone, G.M. (1977a) Early pregnancy in the ewe: effects of oestradiol and progesterone on uterine metabolism and embryo survival. Aust.J.Biol.Sci. 30: 279-288.

Miller, B.G., Murphy, L., Stone, G.M. (1977b) Hormone receptor levels and hormone RNA and protein metabolism in the genital tract during the oestrous cycle of the ewe. J.Endocr. 73: 91-98.

Miller, B.G., Tassel, R., Stone, G.M. (1983) Growth of the endometrium and cotyledons during early pregnancy in the ewe: rates of protein secretion and synthesis and nuclear and cytosol steroid hormone receptor levels. J.Endocr. 96: 137-146.

Miller, B.G., Wild, J., Stone, G.M. (1979) Effects of progesterone on the oestrogen-stimulated uterus: a comparative study of the mouse, guinea pig, rabbit and sheep. Aust.J.Biol.Sci. 32: 549-560.

Milligan, S.R., Martin, L. (1984) The resistance of the mouse uterine lumen to flushing and possible contamination of samples by plasma and interstitial fluid. J.Reprod.Fert. 71: 81-87.

Moor, R.M. (1968) Effect of an embryo on corpus luteum function. J.Anim.Sci. Suppl. 1: 97-118.

Moor, R.M. (1974) Ovarian follicle of the sheep: inhibition of oestrogen secretion by luteinising hormone. J.Endocr. 61: 455-463.

Moor, R.M. (1978) Role of steroids in the maturation of ovine oocytes. Ann.Biol.anim.Bioch.Biophys. 18: 477-482.

Moor, R.M., Hay, M.F., Dott, H.M., Cran, D.G. (1978) Macroscopic identification and steroidogenic function of atretic follicles in sheep. J.Endocr. 77: 309-318.

Moor, R.M., Rowson, L.E.A. (1966a) The effect of removal of embryos on corpus luteum function. J.Endocr. 34: 497-502.

Moor, R.M., Rowson, L.E.A. (1966b) Local maintenance of the corpus luteum in sheep with embryos transferred to isolated portions of the uterus. J.Reprod.Fert. 12: 539-550.

Moore, N.W., Miller, B.G., Trappl, M.N. (1983) Transport and development of embryos transferred to the oviducts and uteri of entire and ovariectomised ewes. J.Reprod.Fert. 68: 129-135.

Moore, W.W., Nalbandov, A.V. (1953) Neurogenic effects of uterine distention on the estrous cycle of the ewe. Endocrinology 53: 1-11.

Moore, N.W., Shelton, J.N. (1964) Egg transfer in sheep. Effect of degree of synchronisation between donor and recipient, age of egg and size of transferred eggs. J.Reprod.Fert. 7: 145-152.

Morton, H., Nancarrow, C.D., Scaramuzzi, R.J., Evison, B.M., Clunie, G.J.A. (1979) Detection of early pregnancy by the rosette inhibition test. J.Reprod.Fert. 56: 75-80.

Murdoch, B.E., O'Shea, T. (1977) β.Glucuronidase in the reproductive tract of the ewe during early pregnancy. Theriogenology. 8: 140.

Murdoch, B.E., O'Shea, T. (1978) Activity of enzymes in the mucosal tissues and rinsings of the reproductive tract of the naturally cyclic ewe. Aust.J.Biol.Sci. 31: 345-354.

Murdoch, R.N. (1971) Some biochemical and kinetic properties of sheep uterine endometrial alkaline phosphatase. Aust.J.Biol.Sci. 24: 331-334.

Murdoch, R.N., White, I.G. (1967) Activity of enzymes in the endometrium, caruncles, and uterine rinsings of progesterone treated and naturally cycling ewes. Aust.J.Biol.Sci. 21: 123-131.

Murdoch, R.N., White, I.G. (1968) The effect of oestradiol and progesterone on the activity of enzymes in the endometrium and caruncles of the ovariectomised ewe. J.Endocr. 42: 187-192.

Murphy, L., Stone, G.M., Miller, B.G., Moore, N.W. (1977) Oestradiol and progesterone: soluble receptor levels and metabolism in the uterus of the ovariectomised ewe. Aust.J.Biol.Sci. 30: 225-228.

Murray, F.A., Bazer, F.W., Wallace, H.D., Warnick, A.C. (1972) Quantitative and qualitative variation in the secretion of protein by the porcine uterus during the estrous cycle. Biol.Reprod. 7: 314-320.

Nagel, W., Willig, F., Schmidt, F.H. (1964) Über die Aminosäurearylamidase-(sog.Leucinaminopeptidase-)Aktivität im menschlichen Serum. Klin. Wschr. 42: 447-449.

Nalbandov, A.V., Moore, W.W., Norton, H.W. (1955) Further studies on the neurogenic control of the estrous cycle by uterine distention. Endocrinology 56: 223-231.

Nancarrow, C.D., Wallace, A.L.C., Grewal, A.S. (1981) The early pregnancy factor in sheep and cattle. J.Reprod.Fert. Suppl. 30: 191-199.

Nelder, J.A., Wedderburn, R.W.N. (1972) Generalised linear models. J.R.Statist. Soc.A.135: 370-384.

Niswender, G.D., Suter, D.E., Sawyer, H.R. (1981) Factors regulating receptors for LH on ovine luteal cells. J.Reprod.Fert. Suppl. 30: 183-189.

Oldham, C.M., Knight, T.W., Lindsay, D.R. (1976) An explanation for the reduced fertility in Merino ewes at the first oestrus of the breeding season. Proc.Aust.Soc.Anim.Prod. 11: 129-132.

Osborn, J.C., Moor, R.M. (1983) Time-dependent effects of α-amanitin on nuclear maturation and protein synthesis in mammalian oocytes. J.Embryol.exp.Morph. 73: 317-338.

Painter, K., Niswender, G.D. (1979) Radioiodinated steroid hormones - general principles. In 'Methods of hormone radioimmunoassay', 2nd Ed. Eds B.Jaffe and H.R. Beham, Academic Press.

Pant, H.C., Hopkinson, C.R.N., Fitzpatrick, R.J. (1977) Concentration of oestradiol, progesterone, luteinizing hormone and follicle stimulating hormone in the jugular venous plasma of ewes during the oestrous cycle. J.Endocr. 73: 247-255.

Parr, R.A., Cumming, I.A., Clarke, I.J. (1982) Effects of maternal nutrition and plasma progesterone concentrations on survival and growth of the sheep embryo in early gestation. J.agric.Sci.Camb. 98: 39-46.

Plotka, E.D., Erb, R.E., Harrington, R.B. (1970) Female sex steroid relationships during the oestrous cycle of the ewe. J.Anim.Sci. 30: 412-419.

Price, C.P., Woodman, D.D. (1971) A comparison of two colorimetric procedures for the assay of 5'-nucleotidase. Clin.Chim.Acta 33: 260-263.

Quinlivan, T.D., Martin, C.A., Taylor, W.B., Cairney, I.M. (1966) Estimates of pre- and perinatal mortality in the New Zealand Romney Marsh ewe. 1.Pre- and perinatal mortality in those ewes that conceived to one service. J.Reprod.Fert. 11: 379-390.

Quirke, J.F., Hanrahan, J.P. (1977) Comparison of the survival in the uteri of adult ewes of cleaved ova from adult ewes and ewe lambs. J.Reprod.Fert. 51: 487-489.

Quirke, J.F., Hanrahan, J.P., Gosling, J.F. (1979) Plasma progesterone levels throughout the oestrous cycle and release of LH at oestrus in sheep with different ovulation rates. J. Reprod. Fert. 55: 37-44.

Ratcliffe, W.A., Corrie, J.E.T., Dalziel, A.H., Macpherson, J.S. (1982) A comparison of direct assays for serum progesterone using a ^{125}I- radioligand with assays involving serum extraction. Clin. Chem. 28: 1314-1318.

Restall, B.J., Brown, G.H., Blochey, M.A.deB., Cahill, L., Kearins, R. (1976a) Assessment of reproductive wastage in sheep. 1. Fertilisation failure and early embryonic survival. Aust.J.Exp.Agric.Anim.Husb. 16: 329-335.

Restall, B.J., Wilkins, J., Kilgour, R., Tyrell, R.N., Hearnshaw, H. (1976b) Assessment of reproductive wastage in sheep. 3. An investigation of a commercial sheep flock. Aust.J.Exp.Agric.Anim.Husb. 16: 344-352.

Rhind, S.M., Chesworth, J.M., Robinson, J.J. (1978) A seasonal difference in ovine peripheral plasma prolactin and progesterone concentrations in early pregnancy and in the relationship between the two hormones. J. Reprod. Fert. 52: 79-81.

Rhind, S.M., Doney, J.M., Gunn, R.G., Leslie, I.D. (1984) Effects of body condition and environmental stress on ovulation rate, embryo survival, and associated plasma follicle stimulating hormone, luteinizing hormone, prolactin and progesterone profiles in Scottish Blackface ewes. Anim. Prod. 38: 201-209.

Rhind, S.M., Robinson, J.J., Fraser, C., McHattie, I. (1980) Ovulation and embryo survival rates and plasma progesterone concentrations of prolific ewes treated with PMSG. J.Reprod.Fert. 58: 139-144.

Rhind, S.M., Robinson, J.J., Fraser, C., Phillippo, M. (1977) Effects of season and lactation on ovulation rate, plasma progesterone concentrations in early pregnancy and lamb production in Finnish Landrace x Dorset Horn ewes. Anim.Prod. 24: 128 (Abstract).

Roberts, M.R., Bazer, F.W., Baldwin, N., Pollard, W.E. (1976) Progesterone induction of lysozyme and peptidase activities in the porcine uterus. Archs.Biochem.Biophys. 177: 499-507.

Roberts, G.P., Parker, J.M., Symonds, H.W. (1976) Macromolecular components of genital tract fluids from the sheep. J.Reprod.Fert. 48: 99-107.

Rodbard, D., Lewald, J.E. (1970) Computer analysis of radio-ligand assay and radio-immunoassay data. Acta Endocr. 64 Suppl. 147: 79-103.

Rowson, L.E.A., Moor, R.M. (1966) Embryo transfer in sheep: the significance of synchronising oestrus in donor and recipient animal. J.Reprod.Fert. 11: 207-212.

Sawyer, H.R., Abel, J.H., McClellan, M.C., Schmitz, M., Niswender, G.D. (1979) Secretory granules and progesterone secretion by ovine corpora lutea in-vitro. Endocrinology 104: 476-486.

Schane, H.P., Creange, J.E. (1980) An overview of luteal phase contraception. In 'Research frontiers in fertility regulation' Ed G.L. Zatuchi, M.L. Labbok and J.J. Sciarra, pp 330-334. Harper and Row, Hagerstown.

Sergerson, E.C. Jr. (1981) Immunosuppressive effect of ovine uterine secretory protein upon lymphocytes in vitro. Biol.Reprod. 25: 77-84.

Shemesh, M., Ayalon, N., Lavi, S., Mileguir, F., Shore, L.S., Toby, D. (1983) A new approach to the use of progesterone levels for pregnancy determination. Br.vet.J. 139: 41-48.

Short, R.V. (1964) Ovarian steroid synthesis and secretion in-vivo. Recent Prog.Horm.Res. 20: 303-339.

Short, R.V. (1978) When a conception fails to become a pregnancy. In 'Maternal Recognition of Pregnancy'. Ciba Fdn. Colloq. No. 64 Ed. J.Whelan. Elsevier Excerpta Medica - North Holland, Amsterdam.

Silvia, W.J., Fitz, T.A., Mayan, M.H., Niswender, G.D. (1984) Cellular and molecular mechanisms involved in luteolysis and maternal recognition of pregnancy in the ewe. Anim.Reprod.Sci. 7: 57-74.

Silvia, W.J., Ottobre, J.S., Inskeep, E.D. (1981) Concentrations of prostaglandins E_2, $F_{2\alpha}$ and 6-keto-prostaglandin — $F1\alpha$ in the utero-ovarian venous plasma of early pregnant and cycling ewes. Biol.Reprod. 24: 99A.

Singh-Asa, P., Jenkin, G., Thorburn, G.D. (1982) Effects of hydroxysteroid dehydrogenase inhibitors on in vitro and in vivo steroidogenesis in the ovine adrenal gland. J.Endocr. 92: 205-212.

Sittman, K. (1972) Intrauterine migration and mortality of sheep embryos. Can.J.Anim.Sci. 52: 195-196.

Smith, C., King, J.W.B., Nicholson, D., Wolf, B.T., Bampton, P.R. (1979) Performance of crossbred sheep from a synthetic dam line. Anim.Prod. 29: 1-9.

Spencer, K., Price, C.P. (1977) Influence of reagent quality and reaction conditions on the determination of serum albumin by the Bromcresol green dye binding method. Ann.clin.Biochem. 14: 105-115.

Spilman, C.H., Duby, R.T. (1972) Prostaglandin mediated luteolytic effect of an intra-uterine device in sheep. Prostaglandins 2: 159-168.

Spuy, Z.M. van der, Jones, D.L., Wright, C.S.W., Piura, B., Paintin, D.B., James, V.H.T., Jacobs, H.S. (1983) Inhibition of 3-beta-hydroxysteroid dehydrogenase activity in first trimester human pregnancy with Trilostane and WIN 32,729. Clin.Endocr. 19: 521-532.

Sreenan, J.M., Diskin, M.G. (1983) Early embryonic mortality in the cow: its relationship with progesterone concentration. Vet.Rec. 112: 517-521.

Staples, L.D., Fleet, I.R., Heap, R.B. (1982) Anatomy of the utero-ovarian lymphatic network and the composition of afferent lymph in relation to the establishment of pregnancy in the sheep and goat. J.Reprod.Fert. 64: 409-420.

Staples, L.D., Lawson, R.A.S., Findlay, J.K. (1978) The occurrence of an antigen associated with pregnancy in the ewe. Biol.Reprod. 19: 1076-1082.

Stone, G.M., Murphy, L., Miller, B.G. (1978) Hormone receptor levels and metabolic activity in the uterus of the ewe: regulation by oestradiol and progesterone. Aust.J.Biol.Sci. 31: 395-403.

Thibault, C. (1977) Are follicular maturation and oocyte maturation independent processes? J.Reprod.Fert. 51: 1-15.

Thorburn, G.D., Cox, R.I., Currie, W.B., Restall, B.J., Schneider, W. (1973) Prostaglandin $F_{2\alpha}$ and progesterone concentrations in the utero-ovarian venous plasma of the ewe during the oestrous cycle and early pregnancy. J.Reprod.Fert. Suppl. 18: 151-158.

Turnbull, K.E., Braden, A.W.H., Mattner, P.E. (1977) The pattern of follicular growth and atresia in the ovine ovary. Aust.J.Biol.Sci. 30: 229-241.

Wang, M.-Y., Rider, V., Heap, R.B., Feinstein, A. (1984) Action of anti-progesterone monoclonal antibody in blocking pregnancy after postcoital administration in mice. J. Endocr. 101: 95-100.

Warner, C.M., Gollnick, S.O., Goldbard, S.B. (1984) Genetic control of early embryonic development by gene(s) in the MHC (H-2 complex). Proc. 10th Int. Cong. Anim. Reprod. and A.I., Illinois 4: XIII 10-15.

Watson, J., Shepherd, T.S., Dodson, K.S. (1979) Prostaglandin E-2-9-ketoreductase in ovarian tissues. J.Reprod.Fert. 57: 489-496.

Webb, R., Baxter, G., Preece, R.D., Land, R.B., Springbett, A.J. (1985) Control of gonadotrophin release in Scottish Blackface and Finnish Landrace ewes during seasonal anoestrus. (In press).

Webb, R., England, B.G. (1982) Relationship between LH receptor concentrations in thecal and granulosa cells and in-vivo and in-vitro steroid secretion by ovine follicles during the preovulatory period. J.Reprod.Fert. 66: 169-180.

Webb, R., England, B.G., Fitzpatrick, K.E. (1981) Control of the preovulatory gonadotrophin surge in the ewe. Endocrinology 110: 873-881.

Webb, R., Gauld, I.K. (1984) Final maturation of the preovulatory follicle in the ewe. In 'Colloque de la société Français pour l'étude de la fertilité'.

Webb, R., Gauld, I.K. (1985) Folliculogenisis in sheep: control of ovulation rate. In 'The genetics of reproduction in sheep', pp 261-274, Ed. R.B.Land and D.Robinson, Butterworths, London.

Webel, S.K., Reimers, T.J., Dzuik, P.J. (1975) The lack of relationship between plasma progesterone levels and number of embryos and their survival in the pig. Biol.Reprod. 13: 177-186.

Wheeler, A.G., Baird, D.T., Land, R.B., Scaramuzzi, R.J. (1975) Increased secretion of progesterone from the ovary of the ewe during the preovulatory period. J.Reprod.Fert. 45: 519-522.

Wheeler, A.G., Baird, D.T., Land, R.B., Scaramuzzi, R.J. (1977) Seasonal variation in oestrus, the secretion of oestrogen and progesterone and LH levels before ovulation in the ewe. J.Reprod.Fert. 51: 427-432.

Whyman, D., Johnson, D.L., Knight, T.W., Moore, R.W. (1979) Intervals between multiple ovulations in PMSG-treated and untreated ewes and the relationship between ovulation and oestrus. J.Reprod.Fert. 55: 481-488.

Willadsen, S.M. (1982) Micromanipulation of embryos of the large domestic species. In 'Mammalian Egg Transfer', Ed. C.E. Adams, pp185-210. CRC Press Inc., Boca Raton, Florida.

Williams, A.H., Cumming, I.A. (1982) Inverse relationship between concentration of progesterone and nutrition in ewes. J. Agric. Sci. Camb. 98: 517-522.

Wilmut, I., Sales, D.I. (1981) Effect of an asynchronous environment on embryonic development in sheep. J.Reprod.Fert. 61: 179-184.

Wilmut, I., Sales, D.I., Ashworth, C.J. (1985a) The influence of variation in embryo stage and maternal hormone profiles on embryo survival in farm animals. Theriogenology 23: 107-119.

Wilmut, I., Sales, D.I., Ashworth, C.J. (1985b) Physiological criteria for embryo mortality- is asynchrony between embryo and ewe a significant factor? 'The genetics of reproduction in sheep' pp 275-289, Ed. R.B.Land and D.Robinson, Butterworths, London.

Wilson, R.J., Cenedella, R.L., Butcher, R.L., Inskeep, E.K. (1972) Levels of prostaglandins in the uterine endometrium during the ovine oestrous cycle. J.Anim.Sci. 34: 93-99.

Wilson, D.T., Maxwell, D.A.G., Sales, D.I. (1979) Radioimmunoassay Data processing program package. (Mimeograph), Department of Statistics and Computing, AFRC Animal Breeding Research Organisation, Edinburgh.

Wintenberger-Torrès, S. (1956) Les rapports entre l'oeuf en segmentation et la tractus maternal chez les brebis. Proc. 3rd Int. Cong. Anim. Reprod. and A.I., Cambridge 1: 62-90.

Wintenberger-Torrès, S. (1961) Mouvements des trompes et progression des oeufs chez la brebis. Annls.Biol.anim.Biochim.Biophys. 1: 121-133.

Wintenberger-Torrès, S. (1967) Étude expérimentale de la migration tubaire des oeufs et de leur segmentation chez la brebis. These de Doctorat D'Etat es Sciences Naturelles, Paris.

Wintenberger-Torrès, S. (1978) Role actif de l'embryon avant l'implantation. In 'Implantation de l'oeuf' pp 181-192. Eds. F. du Mesnil du Buisson, A. Psychoyos and K. Thomas. Masson, Paris.

Woody, C.D., First, N.L., Pope, A.L. (1967) Effect of exogenous progesterone on estrous cycle length. J.Anim.Sci. 26: 139-140.

Yochim, J.M., (1975) Development of a progestational uterus: Metabolic effects. Biol.Reprod. 12: 106-133.

Zavy, M.T., Bazer, F.W., Sharp, D.C., Wilcox, C.J. (1979) Uterine luminal fluids in the cycling mare. Biol.Reprod. 20: 689-698.

Zavy, M.T., Sharp, D.C., Bazer, F.W., Fazeleabas, A., Sessions, F., Roberts, R.M. (1982) Identification of stage-specific and hormonally induced polypeptides in the uterine protein secretions of the mare during the oestrous cycle and early pregnancy. J.Reprod.Fert. 64: 199-207.