The Role of Follicular Fluid Proteins in the Control of Gonadotrophin Secretion and Follicular Development in the Heifer

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Thesis submitted to the University of Edinburgh
for the degree of Doctor of Philosophy

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April 1991

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Abstract

Previous studies in this and other laboratories have, to date, failed to result in the development of a commercially useful technique for the reliable induction of twinning in cattle. The use of methods known to be efficacious in sheep have not resulted in repeatable effects in the cow. It would appear that the cow differs from the sheep in some important aspect of the control of the reproductive processes, particularly those governing follicular growth and dominance. The aim of these studies was to clarify the effects of follicular fluid proteins on follicular growth and gonadotrophin secretion.

A previous study in this laboratory (Price, 1987) demonstrated that LH and FSH concentrations were grossly elevated following immunisation against a porcine follicular fluid preparation. We sought to confirm this observation and extend our understanding of the underlying processes involved. Heifers immunised against an ovine follicular fluid preparation displayed abnormally elevated peripheral FSH and LH concentrations. However, no difference in the response of heifers to an exogenous bolus of GnRH during the luteal phase of the oestrous cycle was observed. Analysis of the patterns of gonadotrophin secretion during the luteal and follicular phases of the cycle suggested that the immunised animals were unresponsive to endogenous oestradiol concentrations. Following ovariectomy, differences between immunised and control heifers were abolished, but the differences between treatment groups were restored following the insertion of a s.c. oestradiol implant, confirming our hypothesis. It would appear that the normal functioning of the oestradiol-mediated negative feedback control of gonadotrophins is dependent on the action of a follicular fluid protein.

Having demonstrated the existence of such a follicular protein, it was of interest to examine the effects of the direct administration of follicular fluid proteins on gonadotrophin secretion and ovulation rate. Treatment

failed to reduce peripheral FSH concentrations, although a significant dose-dependent hypersecretion of FSH was observed following the cessation of treatment. In addition, treatment with bovine or ovine follicular fluid proteins led to a significant increase in LH pulse amplitude. These effects on gonadotrophin secretion again occurred in the absence of any change in oestradiol concentrations, suggesting that the effects of treatment were most probably mediated via direct pituitary or hypothalamic effects.

The treatment of heifers with steroid-free bovine follicular fluid resulted in a marked suppression of follicular development. Since the administration of follicular fluid proteins had previously failed to suppress peripheral FSH concentrations, and given that other authors had previously failed to suppress peripheral FSH concentrations even when using relatively large doses of follicular fluid (Johnson & Smith, 1985), it seemed unlikely that the observed effects of follicular fluid were mediated via reduced FSH concentrations. A further study confirmed that the follicular fluid-induced suppression of follicular development was not associated with any such alteration in FSH secretion, nor was it dependent on the inhibin-content of the follicular fluid. Incidentally, it was also observed that the hypersecretion of FSH following the cessation of treatment was not dependent on the inhibin content of the follicular fluid.

Collectively, we have demonstrated that follicular fluid proteins are important components in the control of the reproductive system of the cow. Such proteins are involved in the oestradiol-mediated negative feedback regulation of FSH and LH, the determination of LH pulse amplitude and the regulation of follicular growth. This latter role may represent the mechanism by which follicular dominance is effected and may present a useful target for future research attempting to develop techniques for the induction of twinning in cattle.

Declaration

I hereby declare that this thesis has been composed by myself, and has not been submitted for any other degree, in Edinburgh or elsewhere. The work presented herein is my own, and all work of other authors is duly acknowledged. I also acknowledge all assistance given to me during the planning, execution and presentation of this thesis.

Andrew Stephen Law

Publications arising from the Thesis

a) Oral communications

1) **Law, A.S.**, Price, C.A., Morris, B.A. & Webb, R. (1987) Effects of passive immunisation against testosterone following active immunisation against 'inhibin' preparations from two species in the cyclic heifer. *Proceedings of the Society for the Study of Fertility Winter Meeting (Abstract).*

2) **Law, A.S.**, Logue, D.N., O'Shea, T. & Webb, R. (1990). Evidence for a novel factor in steroid-free bovine follicular fluid (bFF) which acts to directly suppress follicular development. *J. Reprod. Fert. Abstract Series* **5**, Abstract No. 7.

3) Turzillo, A.M., **Law, A.S.**, McNeilly, J.R. & Webb, R. (1991) Pituitary concetrations of LH and mRNA for LH subunits in heifers immunized against partially purified follicular fluid. *Proceedings of the Society for the Study of Reproduction Summer Meeting.*

b) Refereed poster presentations

1) **Law, A.S.** & Webb, R. (1990) Partially purified inhibin fails to suppress peripheral FSH concentrations but still induces a 'rebound' in intact heifers. *J. Reprod. Fert. Suppl.* **43**, Abstract No. 38.

c) Magazine articles

1) **Law, A.S.** & Webb, R. (1989). Aiming towards physiological control of twinning. *Scottish Farmer* **96**, Livestock Supplement (30/9/89), pp. 3-4.

d) Refereed publications

1) **Law, A.S.**, O'Shea, T. & Webb, R. (1991) Evidence for a direct action of bovine follicular fluid in suppressing follicular development and delaying estrus in heifers. *Endocrinology* (submitted)

2) **Law, A.S.**, Price, C.A., Turzillo, A.M., McNeilly, J.R. & Webb, R. (1991). The effect of immunisation against a protein fraction of ovine or porcine follicular fluid on peripheral gonadotrophin concentrations and pituitary gonadotrophin and mRNA content in the cyclic heifer. (in preparation).

3) **Law, A.S.** & Webb, R. (1991) The effect of partially purified protein fractions of follicular fluid on gonadotrophin secretion in the intact, cyclic heifer. (in preparation).

Acknowledgements

Firstly, I wish to thank my supervisors, Dr. S.G. Hillier (Dept. of Obstetrics and Gynaecology) and Dr. R. Webb (Institute of Animal Physiology and Genetics Research; IAPGR) for their continued support throughout the considerable duration of this project. I am also indebted to Dr. R. Webb for the arrangement of the funds to support the studentship and to the Agricultural and Food Research Council for allowing me to conduct this work using their facilities.

Secondly, I wish to thank all those who were of assistance during the experimental work itself. Much of the animal work would have been impossible without the extraordinary organisational abilities of Mrs. M. Ritchie and the expert assistance of Mr. S.B. Wilson, Mr. W.A. Ritchie, Mr. D. McGavin and Miss. M. Thompson. Thanks must also go to the farm staff, initially under the command of Mr. A.D. McGregor, and latterly Mr. H. Bowran. The assistance of Dr. P. Dixon (Royal [Dick] School of Veterinary Studies) with the ovariectomies and the advice and training in the mysteries of sub-lumbar laparoscopy received from Dr. C.A. Price is also gratefully acknowledged. Thanks also to Dr. D.N. Logue (Scottish Agricultural College) and especially Mr. J. Gong (IAPGR) for their invaluable help in carrying out the ultrasound examinations.

I would also like to thank all those who were of assistance in the laboratory, including Ms. C. Anderson, Miss A. Clarkson, Miss S. Rusbridge and Miss C. Warren and especially Messrs. G. Baxter and D. McBride for their help with assays, electrophoresis, chromatography, cell culture and, indeed, all aspects of laboratory work. Special thanks must go to Mrs. B. McKenzie for her unsurpassed devotion to the task of cleaning the glassware.

I am indebted to Dr. D.J. Bolt (United States Department of Agriculture) for the generous provision of antisera, standard preparations and purified hormones for iodination and to the staff of the Medical Research Council's Reproductive Biology Unit , for the numerous gifts of radiolabelled progesterone and for performing some of the inhibin bioassay work. Thanks also to Drs. T. O'Shea (University of New England) and K. Miyamoto (Gunma University School of Medicine, Japan) for the gift of the 256H monoclonal antibody against bovine inhibin and to Dr. Goddard (IAPGR) for the monoclonal antibody raised against chicken growth hormone. The immunisation studies were also greatly facilitated by the gift of Non-Ulcerative Freund's Adjuvant received from Mr. B.A. Morris (University of Surrey).

I am grateful to Mr. D. Waddington and Ms. A.J. Springbett for statistical advice, to all those in the library, including Mr. M. McKeen, Mrs. R. McDonald and Mrs. L. Rhodes who continued to be of assistance far beyond the normal call of duty and to Messrs. N. Russell, E. Armstrong and R. Field for photographic assistance. In addition, several people provided useful criticisms of draft manuscripts. I thank Drs. R. Webb and J. McNeilly (IAPGR) in particular for this service.

I am deeply indebted to Apple Computer, Inc., Microsoft (Word, Excel), Zaristow Software (AssayZap, Munro), Silicon Beach Software (Superpaint), Aldus (Freehand) and Abelbeck Software (Kaleidagraph) without whom the final stages of this thesis would have been much more traumatic.

Life outside of work has also played its part throughout the duration of this project. I thank all the friends that I have accumulated over the years for their help when beer money was tight, particularly those at the Penicuik Rugby and Cricket Clubs.

Finally I would like to thank my family, especially my parents, without whose continued encouragement and emotional and financial support, none of this would have been possible. Special thanks must go to Miss D.B. Reeves for being there when things seemed to be going wrong and for understanding my not being there when things were going well.

There is insufficient space available here to fully express my gratitude to all those who have helped me over the past four years. I apologise if I have omitted any individual name; it was by no means intentional. I thank you all.

Dedication

This thesis is dedicated to the memory of

Cecil Billings

(1907-1988)

whose hard work, honesty,
pride in himself and his family,
and love for us all
will forever be an inspiration.

Introduction

The study of the reproductive endocrinology of the domestic species has led to the development of commercially viable techniques for the improvement of fecundity in sheep. However, similar techniques for the improvement of the number of calves born per dam have not been developed. Recent political decisions have made the current economic climate conducive to the development of these methods in cattle.

Unfortunately, the direct transfer of the current sheep technology has met with limited success. It would appear that our understanding of the processes involved in the control of reproductive function in cattle, and possibly sheep also, is incomplete. This series of studies was therefore performed in an attempt to further examine the role of the protein components of follicular fluid in the control of reproduction in the heifer.

CHAPTER 1

Literature Review:

The Reproductive Physiology of the Cow

1.1. General

The cow is a polyoestrous, principally monotocous, species. Puberty is reached between 9-14 months of age, depending on breed and plane of nutrition (Wiltbank, Gregory, Swiger, Ingalls, Rothlisberger & Koch, 1966; Wiltbank, Kassons & Ingalls, 1969; Short & Bellows, 1971; Laster, Glimp & Gregory, 1972), although there are recorded instances of individual animals reaching sexual maturity as early as 5 months of age (Craig, 1930). Following the attainment of sexual maturity, regular cyclic oestrous activity continues until slaughter, except during pregnancy and the immediate post-partum period. Despite reports of seasonal variation in the length of time from calving to fertile service (Hammond, 1927), the length of the oestrous cycle (Hammond, 1927) and conception rate (Mercier & Salisbury, 1947) and photoperiod-dependent changes in the endocrine response of ovariectomised heifers to exogenous oestradiol (Hansen, Kamwanja & Hauser, 1982), the domestic cow is generally accepted to be an aseasonal breeder (but see Tucker, 1982, for a review of seasonality in cattle), in contrast to certain breeds of wild cattle (Zuckerman, 1953) and related domestic species such as the sheep (Marshall, 1937).

The reproductive system of the cow, like the sheep, is fully developed at birth and large antral follicles may be found in the ovaries of many prepubertal animals (Hammond, 1927; Casida, Chapman & Rupel, 1935; Perry & Rowlands, 1962; Desjardins & Hafs, 1969). Similar prepubertal follicular development has been reported in many other species e.g. mouse (Brambell, 1927), rat (Lane, 1935) and giraffe (Kellas, van Lennep & Amoroso, 1958). However, these developing follicles fail to reach preovulatory size before puberty, and no luteal tissue is found in the ovaries of immature animals. The later stages of follicular development and ovulation are only possible following puberty.

1.2. The Oestrous Cycle

1.2.1. Temporal Characteristics

The oestrous cycle of the cow is characterised by short periods of intense proceptive sexual behaviour or oestrus, separated by much longer periods of quiescence. There has been much debate as to the precise length of the cycle. Hammond (1927) reported a mean cycle length of around 19 days for both cows and heifers, but commented on the large variation both within and between animals which was also influenced by season. Analysis of a large series of data by Asdell, de Alba and Roberts (1949) revealed an average cycle length of 20.23 days for heifers and 21.28 days for cows, although there was no consistent relationship between age *per se* and cycle length. Approximately 85% of all cycles in this analysis were between 18 and 24 days in length; an observed cycle length in this range may therefore be considered to be "normal".

Likewise, there is a large amount of variation in the duration of the period of oestrus. Hammond (1927) observed a mean duration of 16.43 hours with a range between 6 and 30 hours. Other workers' reports vary within this general range. There is a suggestion that the duration of

oestrus may be shorter in heifers than in cows (Hammond, 1927; Trimberger, 1948) and correlated with the length of the previous cycle (Hammond, 1927).

The onset of oestrus is marked by an increase in restlessness in many animals. This is followed by a period of proceptive behaviour when the cow will lick, butt and mount other cows and the bull, and will stand when mounted by either. There is also a distinctive arching of the back and straining during this period, particularly after a mounting. A flow of mucus from the vulva is apparent throughout oestrus; initially it is clear and fluid, but becomes thicker and yellowy-white as oestrus progresses. However, the intensity of all the symptoms of oestrus vary markedly from individual to individual (Hammond, 1927). Frequently blood is observed in the vulval discharge some 1-5 days after oestrus (Trimberger, 1941; Weber, Morgan & McNutt, 1948). The origin and significance of this bleeding is uncertain, although it does not appear either to be related to, or to influence fertility.

The timing of ovulation in relation to oestrus has also been examined. Most workers agree that ovulation occurs somewhere between 24-48 hours after the onset (Zupp, 1926; Hammond, 1927; Hansel & Trimberger, 1952; Quirk, Hickey & Fortune, 1986), or 7-16 hours after the end of oestrus (Nalbandov & Casida, 1942; Hammond, 1946; Trimberger, 1948). There is evidence that ovulation may be hastened by the act of copulation (Marion, Smith, Wiley & Barrett, 1950a,b) although the cow is more normally considered to be a spontaneous ovulator. More ovulations occur on the right-hand ovary than the left (Reece & Turner, 1938; Rajakoski, 1960), although the physiological significance of this observation is unclear.

1.2.2. Nomenclature

The absence of any obvious external signs of oestrous cyclicity other than the overt signs of oestrus itself in large animals such as the cow and the sheep, coupled with the length of the oestrous cycle in these species, make identification of the stage of the cycle using the classical terminology (pro-oestrus, oestrus, met-oestrus & di-oestrus) difficult. It is therefore more convenient to consider the cycle as consisting of two distinct phases, the follicular and the luteal, based on the dominant structure present on the ovaries during each stage, and to consider the day of oestrus (or occasionally the day of ovulation) as the day of transition from one phase to the other (Day 0). Unless otherwise stated, this nomenclature will be used throughout the remainder of this thesis.

1.2.3. Ovarian Dynamics

1.2.3.1. Characteristics of follicular development

In mammals, with only a few exceptions, the number of primordial follicles present in the ovaries of an individual is fixed at birth or in the immediate post-natal period depending on the species concerned. The cow is born with approximately 150,000 primordial follicles, but this figure is reduced by natural wastage to approximately 3,000 by 15-20 years of age (Erickson, 1966). A large majority of the follicles remaining in the aged animals are abnormal (Erickson, 1966). However, a detailed discussion of the origin of primordial follicles and the factors influencing their development is beyond the scope of this review. The reader is referred to the general review by Peters (1978) and that of Rajakoski (1960) for a review of the early literature regarding this subject.

Following the initiation of ovarian function (which occurs during foetal development in the cow; see above), the primordial follicles start to develop either at random, or in response to an unknown trigger. This

early stage of follicular development is characterised by the enlargement of the oocyte, proliferation of the granulosa cells and the development of a thecal cell layer (Peters, 1979). The rate at which the non-growing follicles are stimulated to initiate their development appears to be partly dependent on the size of the pool of primordial follicles (Krohn, 1967; Krarup, Pederson & Faber, 1969), although other factors, including factors present in follicular fluid (Peters, Byskov & Faber, 1973) may act to reduce the numbers commencing their development. Once the development of an individual follicle is begun, it is continuous and sequential until the follicle either ovulates or becomes atretic (Peters & Levy, 1966; Peters, 1969). The majority of developing follicles are destined to become atretic (Brand & de Jong, 1973).

Although the processes of primordial follicle formation are dependent on gonadotrophic support (see Peters, 1979), the initial further development of these follicles is less so, since pre-antral follicular development is largely unaffected up to 10 weeks after hypophysectomy (Dufour, Cahill & Mauléon, 1979). Indeed, antral follicles up to 2 mm in diameter were observed in hypophysectomised animals in this and other studies (Driancourt, Fry, Clarke & Cahill, 1979; McNatty, Heath, Hudson & Clarke, 1990). Similar follicular development is observed in sheep following blockade of gonadotrophin secretion by means of chronic Gonadotrophin-Releasing Hormone (GnRH) agonist administration (McNeilly & Fraser, 1987; Picton, Tsonis & McNeilly, 1990) or immunisation against GnRH (McNeilly, Jonassen & Fraser, 1986). Further development of multiple follicles to normal preovulatory size may be stimulated in these animals by infusion of Follicle-Stimulating Hormone (FSH) alone (Picton, Tsonis & McNeilly, 1990), although immuno–neutralisation of basal Luteinising Hormone (LH) secretion during FSH infusion blocks this development (McNeilly, Picton, Campbell & Baird,

1991). Paradoxically, the restoration of LH pulses during the period of FSH infusion also reduces the number of developing large follicles (McNeilly *et al.*, 1991). Following the cessation of FSH infusion, or hypophysectomy, all large antral follicles regress rapidly (Dufour, Cahill & Mauléon, 1979; Driancourt *et al.*, 1979; Picton, Tsonis & McNeilly, 1990). Thus the latter, preovulatory, stages of follicular development are under acute gonadotrophic support and control with both basal LH and FSH required for full follicular development and LH pulses seeming to act either directly or indirectly to regulate the numbers of follicles developing.

Much of the literature regarding the developmental changes in follicular function has been derived from studies of rats or mice. Consequently, much of the data in the remainder of this section will be concerned with these species. Initially the granulosa cell layer of the follicle contains only receptors for FSH (Eshkol & Lunenfeld, 1972; Richards, 1980). Similarly, the theca cells contain only LH receptors, a situation that persists throughout the development of the follicle (Richards & Kersey, 1979; Richards, 1980). Stimulation of the granulosa cells by FSH induces cellular proliferation (Eshkol & Lunenfeld, 1972; Richards, 1980), the development of aromatase enzyme activity (Dorrington, Moon & Armstrong, 1975; Moon, Dorrington & Armstrong, 1975; Armstrong & Papkoff, 1976; Erickson & Hsueh, 1978a; Dorrington & Armstrong, 1979) and the formation of functional LH receptors in the granulosa cells (Zeleznik, Midgley & Reichert, 1974; Channing, 1975; Richards, Ireland, Rao, Bernath, Midgley & Reichert, 1976; Erickson, Wang, Casper, Mattson & Hofeditz, 1982; Adashi, Resnick, Svoboda & Van Wyk, 1985a; Amsterdam, May & Schomberg, 1988). FSH is also capable of stimulating the further production of FSH receptors on granulosa cells (Richards *et al.*, 1976; Ireland & Richards, 1978a). There is evidence to suggest that differentiation of the granulosa layer gives rise to two

subpopulations of cells (Erickson, Hofeditz, Unger, Allen & Dulbecco, 1985; Kasson, Meidan, Davoren & Hsueh, 1985), but there is, as yet, little evidence of their differing function. Stimulation of the differentiated granulosa cells by LH enhances the FSH induction of LH receptors (Ireland & Richards, 1978b) and stimulates the already developed aromatase activity and hence increases oestrogen production (Wang, Hsueh & Erickson, 1981). However, the granulosa cells lack the 17α-hydroxylase and 17-20 desmolase enzymes necessary for the production of androgens, the precursors of the oestrogens (Bjersing & Carstensen, 1967; see below). The substrate for this oestrogen production is therefore provided by the theca cells which produce androgens in response to LH stimulation (Erickson & Ryan, 1976; Tsang, Moon, Simpson & Armstrong, 1979; Tsang, Armstrong & Whitfield, 1980; Makris & Ryan, 1980; Bogovich & Richards, 1982; Erickson, Magoffin, Dyer & Hofeditz, 1985). In most species, the theca cells are unable to aromatise the androgens thus produced (Leung & Armstrong, 1980). Therefore both theca and granulosa cells, in the presence of both LH and FSH, are required for the full expression of follicular aromatase activity as originally demonstrated by Falck (1959).

The oestrogens and androgens resulting from this ordered follicular development also interact with the gonadotrophins in the functioning and differentiation of the follicular cells. Oestradiol synergises with FSH in the formation of FSH and LH receptors (Richards & Midgley, 1976; Richards *et al.*, 1976; Ireland & Richards, 1978a; Richards, 1979; Farookhi & Desjardins, 1986), the induction of aromatase activity (Zhuang, Adashi & Hsueh, 1982; Daniel & Armstrong, 1983) and apparently acts independently to stimulate granulosa cell proliferation (Goldenberg, Vaitukatis & Ross, 1972). Oestradiol also reduces the thecal cell production of androgens in response to LH (Leung, Goff, Kennedy & Armstrong, 1978;

Leung & Armstrong, 1979; Magoffin & Erickson, 1981,1982), an action which may at first sight appear to be detrimental to follicular function. However, one of the early symptoms of atresia in antral follicles is an increase in the androgen : oestrogen ratio (Ireland & Roche, 1982a; Ireland & Roche, 1983a,b) and injections of exogenous androgen induce atresia (Payne & Runser, 1958; Maraček, Tokoš & Halagan, 1977). If this increase in androgen : oestrogen ratio is indeed a cause of, rather than a symptom of atresia then an oestrogen-induced reduction in androgen production may represent an intra-follicular protection mechanism against such an atretic process.

It has been estimated that the time taken for a follicle to develop from a primordial follicle into a large dominant follicle is approximately 6 months in the sheep (Cahill & Mauléon, 1980). The resulting dominant follicle, having undergone the functional changes described briefly above, is characterised by its high oestradiol production and large number of granulosa LH receptors (sheep: England, Webb & Dahmer, 1981; Webb & England, 1982a,b; cattle: Webb & Bellows, 1980; Staigmiller, England, Webb, Short & Bellows, 1982). However, if the development of the follicle is not coincident with the onset of luteal regression then it will become atretic and regress itself (Ireland & Roche, 1987; see section 1.2.3.2 below). If however, it is destined to be the ovulatory follicle, then luteolysis will allow the increased LH secretion to drive the final stages of maturation and ovulation (see below). Increased LH pulsatile secretion stimulates the further production of androgen by the theca and its conversion to oestradiol by the granulosa and the further development of LH receptors until the triggering of the LH surge and ovulation. The processes of ovulation and corpus luteum formation which have been reviewed elsewhere (Rajakoski, 1960) are beyond the scope of this review and will not be considered here.

The multifarious processes and hormonal interactions involved in the development of dominant ovulatory follicles are far too complex for this necessarily brief review to do them justice. The reader is referred to one of the many excellent volumes available on this subject for further information (e.g. Midgley & Sadler, 1979; Franchimont & Channing, 1981; Rolland, van Hall, Hillier, McNatty & Schoemaker, 1982; Greenwald & Terranova, 1983; Roche & O'Callaghan, 1987)

1.2.3.2. Patterns of follicular development

Early observations of the patterns of follicular growth and development in cattle were based on the histological examination of the ovaries from a relatively small number of cattle slaughtered on a known day of the oestrous cycle. They demonstrated that the dominant follicle present on a pair of ovaries was larger on the days immediately before oestrus than at any other stage of the cycle and it was concluded that follicles developed over one or two cycles, culminating in ovulation (Hammond, 1927). Later studies, involving greater numbers of animals, also demonstrated that the dominant follicle was largest at this time, but that there was also another peak in follicular diameter occurring at approximately mid-cycle (Rajakoski, 1960). This was interpreted as implying that there were two waves of follicular development, the first wave commencing at the day of oestrus and resulting in a large dominant follicle by Day 13 which became atretic and regressed, and a second wave that developed from Day 13 and which resulted in the ovulation of the largest dominant follicle at the subsequent oestrus. Following this study, several other studies using similar techniques were reported, some of which supported the theory of follicular waves (Hackett & Hafs, 1969; Swanson, Hafs & Morrow, 1972; Ireland, Coulson & Murphree, 1979), and others which were unable to demonstrate any such pattern and concluded that follicular development was continuous and independent of the stage

of the oestrous cycle (Choudary, Gier & Marion, 1968; Donaldson & Hansel, 1968; Lobel & Levy, 1968; Marion & Gier, 1971). However, these studies relied entirely on the statistical interpretation of single time-point data obtained from a large number of animals, and were unable to follow the growth and fate of single follicles throughout the oestrous cycle within an individual animal. The first studies to address this question in cattle and sheep relied on the marking of the two largest follicles with carbon or India ink at laparotomy on known days of the oestrous cycle. They demonstrated that the ovulatory follicle only becomes the largest follicle within 2-3 days of oestrus, confirming the dynamic nature of ovarian turnover (Smeaton & Robertson, 1971; Dufour, Whitmore, Ginther & Casida, 1972; Matton, Adelakoun, Couture & Dufour, 1981). In addition, Matton *et al.* (1981) observed that nearly 90% of the second largest follicles marked on days 3, 8 or 13 of the oestrous cycle failed to become the largest follicle over the next five days, and the majority regressed over this time period, concurrent with the largest follicle, as would be characteristic of distinct waves of follicular growth. Smeaton & Robertson (1971) also suggested that there were three distinct waves of follicular growth throughout the oestrous cycle of the sheep.

Although these methods allowed the study of the fate of individual follicles over a fixed time period, they were still restricted in their ability to follow shorter term, daily, within animal changes in follicular growth. More recent studies employing real-time B-mode ultrasound techniques have permitted the repeated examination of ovaries necessary for such detailed analysis, without the associated trauma of surgical procedures. Initial reports were unable to follow the development of individual follicles throughout the cycle, but confirmed the previous histological data in that there appeared to be two major phases of growth of large follicles (Pierson & Ginther, 1984, 1987a,b,c) and that the ovulatory

follicle was selected around 3 days prior to ovulation (Pierson & Ginther, 1984, 1988; Quirk, Hickey & Fortune, 1986). Other investigations used video-recordings to facilitate the repeated identification of follicles by their position in relation to other follicles and observed that there were 3 waves of follicular development in the majority of bovine oestrous cycles (Sirois & Fortune, 1988; Savio, Keenan, Boland & Roche, 1988; Savio, Boland & Roche, 1990). Extension of the length of the cycle by the administration of exogenous progestagen increased the number of waves of follicular development within the cycle (Fortune, Sirois, Turzillo & Lavoir, 1991). Given the regularity of the waves, and the presence of waves of follicular development throughout pregnancy as well as through the oestrous cycle (Pierson & Ginther, 1986, 1987d), it seems likely that the number of waves per cycle is merely determined by the length of the cycle, rather than being an intrinsic characteristic of the cycle.

Each wave of follicular development is characterised by the simultaneous emergence from the pool of small follicles of a number (5-7) of larger, growing follicles > 5 mm in diameter. One of this group rapidly emerges as the "dominant" follicle and continues to develop, whilst the others become atretic and regress. The dominant follicle normally reaches a maximum size of 10-15 mm in diameter and remains the dominant follicle for a few days, then it too becomes atretic and regresses, to be replaced within five days by a further dominant follicle grown from the next wave of follicles. If luteal regression occurs during the growth phase or early period of dominance, then the dominant follicle, free from the restrictive hormonal milieu imposed by the corpus luteum upon the hypothalamus/pituitary gland, will continue to develop to preovulatory size (up to 20 mm) and will eventually trigger the hormonal cascade leading to ovulation.

1.2.4. The Hormones of the Oestrous Cycle

1.2.4.1. Gonadotrophins

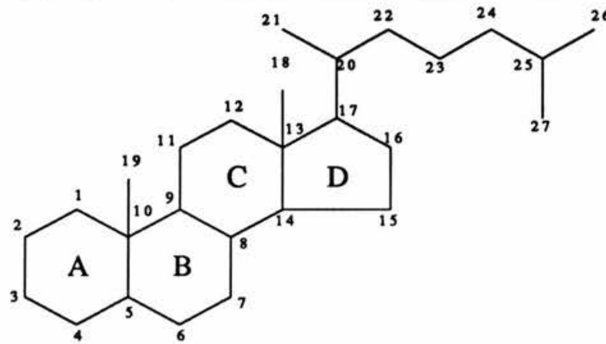
The gonadotrophins, FSH and LH, are produced and secreted by the anterior pituitary gland. They are two-subunit glycoproteins with approximate molecular weights of 30kDa. The α subunit is common to both (along with Thyroid Stimulating Hormone [TSH] and human Chorionic Gonadotrophin [hCG]), whilst the β subunit confers hormonal specificity (Liao & Pierce, 1970; Pierce, 1971; Pierce, Liao, Carlsen & Reimo, 1971). The release of both LH and FSH is stimulated by GnRH, a decapeptide hormone secreted by the hypothalamus, although the cyclic patterns of peripheral LH and FSH concentrations suggest that the overall control of the secretion of each hormone differs (see sections 1.2.4.3 & 1.2.4.4). The biochemistry of the gonadotrophins and the role of GnRH have been extensively reviewed elsewhere (e.g. Pierce & Parsons, 1981; Ryan, Keutmann, Charlesworth, McCormick, Milius, Calvo & Vutyavanich, 1987; Everett, 1988) and will not be considered further in this thesis.

1.2.4.2. Steroids

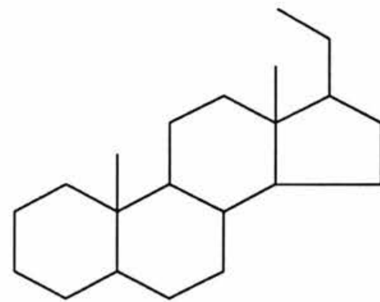
The steroids are a ubiquitous class of hormones, with their role in mammals varying from involvement in stress responses to the stimulation of secondary sexual characteristics and the coordination of the events leading to conception and parturition. All steroid hormones share a common core structure namely the perhydrocyclopentano-phenanthrene nucleus (or "steroid nucleus") which consists of three cyclohexane rings (A, B and C) and a cyclopentane ring (D) shown in Figure 1.1. Biological activity and specificity is conferred by the attachment of various groupings at specific sites on the steroid nucleus.

The ovarian sex steroids are all derived from the parent steroid compound, cholesterol, and may be classified by biological activity or

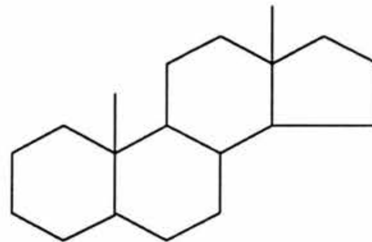
Figure 1.1 Structure of the parent steroid (cholestane) and the three major categories of ovarian steroids, namely the progestins, androgens and oestrogens.



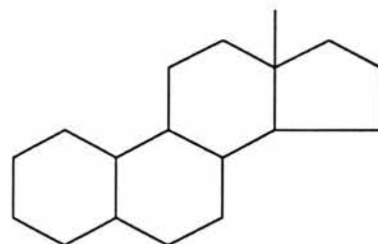
Cholestane (C_{27})



Pregnane (C_{21})



Androstane (C_{19})



Oestrane (C_{18})

basic structure into one of three groups, namely the progestins (C_{21}), androgens (C_{19}) and oestrogens (C_{18}). The basic structure of the parent compounds of each of these groups is shown in Figure 1.1. The major progestin, androgens and oestrogen produced by the bovine ovary are progesterone, androstenedione and testosterone, and oestradiol-17β respectively (Short, 1962).

The consensus pathway of sex steroid biosynthesis has been elucidated by careful consideration of a large amount of analytical and enzymatic data. Briefly, it involves the removal of the side chain of cholesterol to yield a C_{21} progestin, pregnenolone, by the action of a P_{450} cytochrome-linked enzyme complex. This is then either converted to 17α-hydroxy-pregnenolone and then to a C_{19} steroid, dehydroepiandrosterone, by a 17α-hydroxylase/$C_{17,20}$-lyase enzyme complex through the so called "Δ^5 pathway", or to progesterone (C_{21}) by a 3β-hydroxysteroid de-hydrogenase/$\Delta^{5,4}$-isomerase enzyme and hence via 17α-hydroxy-progesterone to androstenedione (C_{19}) via the "Δ^4 pathway". Dehydroepi-androsterone resulting from the Δ^5 pathway conversion may subsequently be converted to androstenedione by the action of the 3β-hydroxysteroid dehydrogenase/$\Delta^{5,4}$-isomerase enzyme complex. Either androstenedione or testosterone, a product of the action of 17β-hydroxysteroid dehydrogenase on androstenedione, may then be converted to oestrone or oestradiol-17β respectively by the action of an aromatase enzyme complex.

The reader is referred to one of the the many excellent reviews available (e.g. Gore-Langton & Armstrong, 1988) for a more detailed consideration of steroidogenesis in the ovary.

1.2.4.3. Patterns of hormonal concentrations throughout the oestrous cycle

Early studies of LH secretion during the oestrous cycle utilised relatively insensitive bioassays (e.g. Ovarian Ascorbic Acid Depletion assay; Parlow, 1961), and were largely restricted to the measurement of pituitary gland content. These demonstrated that pituitary LH content changed little throughout the cycle, with the exception of a short period around the time of oestrus when concentrations fell sharply (Rakha & Robertson, 1965; Desjardins & Hafs, 1968; Hackett & Hafs, 1969). This was interpreted as being indicative of a period of release of LH into the general circulation. Analysis of daily blood samples either by bioassay of acetone-concentrated samples (Anderson & McShan, 1966; Karg, Aust & Böhm, 1967) or by radioimmunoassay of untreated samples (Schams & Karg, 1969; Hansel & Snook, 1970; Snook, Saatman & Hansel, 1971) confirmed that peripheral concentrations of LH were low throughout the oestrous cycle and that a major increase was observed around oestrus. Throughout the luteal phase, other small peaks in peripheral concentrations (Day 8-9, Day 16-19: Schams & Karg, 1969; Day 14-17: Snook, Saatman & Hansel, 1971) or reductions in pituitary content (Day 10-13: Desjardins & Hafs, 1968; Day 11-18: Hackett & Hafs, 1969) were also observed, but these could not be related to any physiological changes in ovarian function. However, the observation that LH concentrations in ovariectomised Rhesus monkeys showed short-term fluctuations suggestive of a pulsatile mode of release (Dierschke, Battacharya, Atkinson & Knobil, 1970) and subsequent similar observations in rats (Gay & Sheth, 1972), sheep (Butler, Malven, Willett & Bolt, 1972) and cows (Beck & Convey, 1976; Griffin & Randel, 1978; Forrest, Fleeger, Long, Sorenson & Harms, 1980) led to much more detailed study of the control of

LH secretion. The frequency and amplitude of LH pulses in the peripheral circulation is considered in more detail below.

Similarly, early observations of the patterns of secretion of FSH were restricted to inferences derived from studies of pituitary gland content, which showed a marked decrease between Day 18 and Day 0, concomitant with, but commencing earlier than, the reduction in pituitary LH (Rakha & Robertson, 1965; Hackett & Hafs, 1969). Further reductions in pituitary FSH content were also observed at around days 4-6 of the cycle (Desjardins & Hafs, 1968; Hackett & Hafs, 1969). However, these studies were flawed by their assumption of a constant rate of gonadotrophin synthesis, and subsequent radioimmunoassay of daily blood samples revealed that the preovulatory release of FSH did occur at the same time as the LH peak (Akbar, Reichert, Dunn, Kaltenbach & Niswender, 1974; Schams & Schallenberger, 1976; Schams, Schallenberger, Hoffmann & Karg, 1977) and not before. Unfortunately, due to problems encountered in developing a sufficiently specific and sensitive radioimmunoassay for bovine FSH, many of the reports detailing cyclic changes in FSH secretion are conflicting. Some authors report that FSH concentrations rise over the last four days preceding oestrus (Schallenberger, Schöndorfer & Walters, 1985), whilst others report a decrease (Peters, 1985). Other reports still demonstrate a significant reduction in a proportion of animals, but some animals showed no change, and in others, FSH concentrations actually rose (Schallenberger, Schams, Bullerman & Walters, 1984). There does appear to be a secondary rise of FSH, independent of LH, some 24 hours after the preovulatory surge (Dobson, 1978; Walters & Schallenberger, 1984), but the pattern of FSH concentrations throughout the remainder of the oestrous cycle is ill-defined. Some workers have demonstrated significant peaks of FSH at various other times throughout the cycle (Schams *et al.*, 1977). There are

reports of pulsatile FSH release (Ireland & Roche, 1982b; Walters, Schams & Schallenberger, 1984; Schallenberger *et al.*, 1984; Walters & Schallenberger, 1984; Schallenberger, Schöndorfer & Walters, 1985), but other workers using an RIA based on a different antiserum failed to observe such a phenomenon, possibly because of the use of a more stringent definition of a pulse (Peters, 1985).

Much data exists concerning the concentrations of progesterone in relation to day of oestrous cycle. Initial analysis by ultra-violet spectrophotometry or gas-liquid-chromatography was cumbersome and required large sample volumes. However, the development of competitive protein-binding assays (Murphy, 1969), in addition to the subsequent development of suitable RIA methods, allowed the peripheral levels of progesterone to be determined. Essentially, progesterone concentrations are low throughout the period of oestrus, but rise from around Day 3 to reach a peak at about Day 10-14, thereafter remaining elevated until luteolysis at around Day 18. Following the onset of luteal regression, progesterone concentrations fall precipitously to values below the limits of detection of most assay methods and remain so until Day 3 of the subsequent oestrous cycle (Donaldson, Bassett & Thorburn, 1970; Henricks, Dickey & Niswender, 1970; Henricks, Dickey & Hill, 1971; Shemesh, Lindner & Ayalon, 1971; Snook, Saatman & Hansel, 1971; Robertson, 1972; Glencross, Munro, Senior & Pope, 1973; Smith, Fairclough, Payne & Peterson, 1975). Pulses of progesterone may be detected in jugular venous plasma during the mid-luteal phase of the oestrous cycle, but are generally ill-defined. Analysis of serum samples obtained from either the ovarian vein or caudal vena cava during the luteal phase of the oestrous cycle reveal pulses of progesterone release from the ovary bearing the corpus luteum (Walters, Schams & Schallenberger, 1984).

Descriptions of peripheral oestradiol concentrations in the cow, like those for FSH, have also been limited by the lack of sufficiently sensitive assays, although this has been due to the exceedingly low peripheral concentrations prevalent for much of the oestrous cycle. Oestradiol concentrations in jugular plasma begin to rise following the onset of luteolysis and reach a peak at about the time of oestrus (Shemesh, Ayalon & Lindner, 1972; Hansel & Echternkamp, 1972; Dobson & Dean, 1974; Walters & Schallenberger, 1984). Following this, oestradiol concentrations fall rapidly, but show further smaller peaks at various times throughout the luteal phase of the oestrous cycle; particularly prominent is a peak of oestradiol secretion around days 4-6 (Dobson & Dean, 1974). Pulses of oestradiol are clearly detectable in vena cava, but not jugular venous blood (Walters, Schams & Schallenberger, 1984; Schallenberger *et al.*, 1984; Walters & Schallenberger, 1984).

Reports of androstenedione and testosterone plasma concentrations throughout the oestrous cycle are fewer in number. Kanchev & Dobson (1976) reported that androstenedione concentrations fluctuated throughout the oestrous cycle, with no discernable, physiologically significant, pattern. Wise, Caton, Thatcher, Rami Lehrer & Fields (1982) similarly failed to demonstrate any significant day to day changes in peripheral or ovarian vein concentrations of androstenedione or dehydroepiandrosterone, although testosterone secretion was significantly elevated during the late luteal phase. Kanchev & Dobson (1976) also observed an increase in plasma testosterone concentrations at about Day 13-16 of a 21 day oestrous cycle. Similar increases in testosterone concentrations have been reported to occur prior to oestrus (Kanchev, Dobson, Ward & Fitzpatrick, 1976). Some authors have suggested that the patterns of testosterone concentrations are similar to

those observed for oestradiol (Shemesh & Hansel, 1974; Kanchev & Dobson, 1976).

The patterns of release of the gonadotrophins vary markedly with the stage of the oestrous cycle in the cow (Rahe, Owens, Fleeger, Newton & Harms, 1980). During the mid-luteal phase, pulses of LH are infrequent, occurring approximately every 3-4 hours, and of relatively high amplitude, each pulse being between 0.7 and 7.0 ng/ml, depending on the assay used. Mean LH concentrations during this period are relatively low, averaging around 0.5-2.0 ng/ml (Rahe *et al.*, 1980; Walters, Schams & Schallenberger, 1984; Price, Morris & Webb, 1987). Data concerning the FSH concentrations during this phase of the oestrous cycle are highly variable, but most authors report that they are similarly relatively low (50 ng/ml: Walters, Schams & Schallenberger, 1984) with evidence of some fluctuations between days. Some authors report a pulsatile mode of FSH release with pulses occurring at a rate of approximately 1 pulse every 2 hours, somewhat more frequently than those of LH, and with an amplitude of around 17 ng/ml (Walters, Schams & Schallenberger, 1984). During this period, oestradiol concentrations are low in jugular venous plasma, averaging approximately 4 pg/ml, but pulses of approximately 6 pg/ml are detectable in vena cava blood, at a similar rate to those of LH observed in jugular plasma (Walters, Schams & Schallenberger, 1984). Progesterone concentrations, meanwhile, are elevated, averaging 4.0 ng/ml in jugular plasma, and 12.3 ng/ml in vena cava plasma, with pulses of secretion observed at a rate of approximately 1 pulse every 2 hours, similar to those of FSH (Walters, Schams & Schallenberger, 1984). Following the onset of the regression of the corpus luteum, progesterone concentrations fall rapidly, reaching 1.0 ng/ml within 24-36 hours (Schallenberger *et al.*, 1984). During this period, the frequency of LH pulses increases to approximately one pulse every 40-60 minutes, whilst

the amplitude of each pulse reportedly decreases to between 0.3 and 1.8 ng/ml (Rahe *et al.*, 1980). However, other workers report that LH pulse amplitude actually increases during luteal regression (Schallenberger *et al.*, 1984; Schallenberger, Schöndorfer & Walters, 1985), although pulse amplitude around the time of the LH surge was reduced (Walters & Schallenberger, 1984). In some studies no differences in LH pulse amplitude were observed between luteal and follicular phases (Peters, 1985). FSH concentrations reportedly change little during this period, although some changes in pulse amplitude were observed in certain individual animals, and there was a tendency for basal FSH concentrations to decrease slightly (Butler, Katz, Arriola, Milvae & Foote, 1983; Schallenberger *et al.*, 1984; Peters, 1985; Parfet, Smith, Cook, Skyer, Youngquist & Garverick, 1989). Ireland & Roche (1982a) reported an increase in FSH concentrations over this period. Regardless of the exact nature of the changes in gonadotrophin secretion during luteolysis and the follicular phase, most authors are in agreement that the removal of progesterone from the peripheral circulation allows the release of the pituitary-ovarian system and the development of the large follicle destined to ovulate. It is therefore not surprising to find that the changes in oestradiol secretion observed during the early stages of luteal regression reportedly mirror those of LH, with an increase in pulse frequency to approximately 1 pulse per hour and a significant rise in overall mean oestradiol concentrations, although pulse amplitude remains unchanged (Schallenberger *et al.*, 1984). However, these authors reported that patterns of oestradiol secretion during the later stages of luteal regression varied markedly between animals, with one animal continuing to display a pulsatile release, whilst this was abolished in two other animals. Most of the oestradiol secreted by the bovine ovary is produced by the single largest follicle (Staigmiller *et al.*, 1982) and the

disappearance of the ovarian oestradiol response to LH pulses presumably reflects a loss of responsiveness of this follicle to gonadotrophins, possibly through a reduction in the number of LH receptors (Ireland & Roche, 1982a) which may further reflect the age and health of this follicle. Scaramuzzi, Turnbull & Nancarrow (1980) and Sirois & Fortune (1988) have previously concluded that the length of time from luteal regression to oestrus and ovulation is dependent on the stage of development of the largest dominant follicle at the time of luteolysis.

As luteal regression continues, progesterone concentrations fall below 0.5 ng/ml and mean oestradiol concentrations continue to rise to above 12 pg/ml in the jugular vein (Chenault, Thatcher, Kalra, Abrams & Wilcox, 1975; Walters & Schallenberger, 1984). This rise in oestradiol concentrations is due mainly to an increase in the amplitude of oestradiol pulses, rather than any change in frequency (Walters & Schallenberger, 1984) and is presumably mediated through the increase in LH receptors in the largest dominant follicle observed during this period (Staigmiller *et al.*, 1982; see above). The amplitude and frequency of LH and FSH pulsatile release remain similar to those observed during the early stages of luteal regression until approximately 60 hours after the onset of regression when a sudden increase in LH release occurs. Jugular LH concentrations reach a peak of between 7 and 50 ng/ml and remain elevated for approximately 6 hours (Snook, Saatman & Hansel, 1971; Walters & Schallenberger, 1984). Pulses of LH are observed throughout this LH surge at a rate of approximately 2 pulses per hour and pulse amplitude is greater at this time than at any other stage of the oestrous cycle (Walters & Schallenberger, 1984). A coincident surge release of FSH is also observed at this time with peak values reaching 120 ng/ml, although no significant increase in FSH pulse amplitude has been demonstrated (Walters & Schallenberger, 1984). Some workers report a

small rise in ovarian progesterone secretion at the same time as the gonadotrophin surge (Walters & Schallenberger, 1984) and similar observations have been made in sheep (Wheeler, Baird, Land & Scaramuzzi, 1975), although the significance of this finding is unclear. Following the gonadotrophin surge, pulsatile LH release is abolished for a period of around 12 hours, although FSH release continues with approximately the same frequency as before the surge. After this period, low-amplitude LH pulses are once again discernable at a rate of around 1 pulse per hour (Walters & Schallenberger, 1984). A secondary rise in FSH secretion alone is observed commencing between 4 and 12 hours after the end of the preovulatory LH/FSH surge, principally due to an increase in pulse amplitude, and reaching a peak of around 80-90 ng/ml within 24 hours (Dobson, 1978; Walters & Schallenberger, 1984). By four days after the gonadotrophin surge, the rates of LH and FSH pulsatile release are reduced to around 1 pulse every 90 minutes (Walters, Schams & Schallenberger, 1984). The amplitude of each LH or FSH pulse, and the mean concentrations of both hormones are similar during this early luteal phase to those observed during the mid-luteal phase (Walters, Schams & Schallenberger, 1984; see above).

1.2.4.4. Interactions between gonadal and pituitary hormones

The interactions between the hypothalamus/pituitary gland and the ovaries/testes have long been postulated. Hypophysectomy results in a failure of the development of the ovaries in rats, but this may be reversed by the daily administration of pituitary tissue (Smith, 1926a). Furthermore, administration of pituitary extracts to intact rats results in precocious puberty and stimulates follicular development (Smith, 1926b). Similarly administration of pituitary tissue to cattle results in superovulation (Casida, Meyer, McShan & Wisnicky, 1943). The gonad-stimulating activity has been separated into two fractions, namely a

follicle-stimulating fraction (Thylakentrin, Prolan A, Folliculin, FSH) and a luteinising fraction (Interstitial Cell-Stimulating Hormone [ICSH], Metakentrin, Prolan B, LH) (Fevold, Hisaw & Leonard, 1931; Greep, van Dyke & Chow, 1942). Conversely, surgical castration leads to characteristic histological changes in the pituitary gland, which may be reversed, at least in part, by the administration of testicular extracts (McCullagh, 1932; Mottram & Cramer, 1923). Over the last twenty years, study of the temporal changes in hormone concentrations throughout the oestrous cycle, together with the hormonal consequences of surgical castration and pituitary extract administration in various species have allowed a much greater understanding of the feedback mechanisms, both positive and negative, which act to regulate the endocrine reproductive system.

The majority of pulses of LH are followed between 5 and 30 minutes later by pulses of oestradiol in the ovarian vein in the sheep and the cow (Baird, Swanston & Scaramuzzi, 1976; Scaramuzzi & Baird, 1977; Baird & McNeilly, 1981; Walters, Schams & Schallenberger, 1984; Schallenberger *et al.*, 1984; Walters & Schallenberger, 1984). Similarly the administration of a bolus of exogenous LH results in a release of oestradiol, confirming this action of LH on the ovary (McCracken, Uno, Goding, Ichikawa & Baird, 1969; Baird & McNeilly, 1981; Campbell, McNeilly & Baird, 1989). Exogenous LH also results in increased progesterone production by luteal tissue both *in vivo* and *in vitro* (Armstrong & Black, 1966; Ursely & Leymarie, 1979; Litch & Condon, 1988), although Baird, Swanston & Scaramuzzi (1976) failed to demonstrate a correlation between fluctuations in progesterone concentrations and LH pulses in the sheep. There is evidence that FSH, not LH, may be more important in stimulating luteal progesterone production *in vivo* in the cow (Schallenberger, Rampp & Walters, 1983; Walters, Schams & Schallenberger, 1984) as these workers observed pulses of FSH, separate from LH pulses, which were

associated with progesterone release. In addition, specific receptors for FSH have been reported to be present in the bovine corpus luteum (Manns & Niswender, 1983) and in low levels in the human corpus luteum (Bramley, Stirling, Swanston, Menzies, McNeilly & Baird, 1987), although no such receptors could be found in porcine corpora lutea (Ziecik, Esbenshade & Britt, 1988). A later study in cattle also failed to demonstrate any correlation between FSH binding and physiological function (Manns, Niswender & Braden, 1984). Furthermore, other workers have failed to demonstrate specific FSH pulses in the cow (Peters, 1985), and in the study of Walters, Schams & Schallenberger (1984), the "separate" FSH pulses were associated with low-amplitude pulses of LH which may have confounded the interpretation of the results. Foster, Lamming & Peters (1980) concluded that any association between FSH release and progesterone production in the cow was due to the coincidental release of FSH with LH. More recent work using GnRH-agonist treated sheep indicates that pulsatile progesterone release from the corpus luteum may be totally independent of pulsatile gonadotrophin secretion (McNeilly & Fraser, 1987). Clearly the role of FSH in the functioning of the bovine corpus luteum merits further investigation. FSH is also instrumental in the induction of aromatase activity in the developing follicle since the infusion of FSH increases oestradiol concentrations in sheep (Hudson, McNatty, Ball, Gibb, Heath, Lun, Kieboom & Henderson, 1985; see above).

Measurement of gonadotrophin concentrations reveals that the changes observed in the pituitary following castration are also reflected by increases in peripheral concentrations of LH and FSH (Hobson & Hansel, 1972; Convey, Beck, Neitzel, Bostwick & Hafs, 1977). This rise in gonadotrophins is initially mediated through an increase in both pulse frequency and amplitude, and then later by a further rise in pulse amplitude despite a gradual decrease in pulse frequency (Schallenberger

& Peterson, 1982; Martin, Scaramuzzi & Henstridge, 1983). The role of the ovarian steroids in this regulation of gonadotrophin secretion has been extensively investigated.

Injection of oestradiol alone fails to suppress peripheral LH concentrations in ovariectomised cattle (Hobson & Hansel, 1972). In fact administration of exogenous oestradiol rapidly induces a surge-like release of LH and FSH, similar to that observed prior to ovulation, in cows (Hobson & Hansel, 1972; Short, Howland, Randel, Christensen & Bellows, 1973; Short, Randel, Staigmiller & Bellows, 1979; Schoenemann, Humphrey, Crowder, Nett & Reeves, 1985), prepubertal heifers (Swanson & McCarthy, 1978; Staigmiller, Short & Bellows, 1979), cyclic heifers (Peters, 1984), ovariectomised heifers (Butler *et al.*, 1983), and sheep (Goding, Catt, Brown, Kaltenbach, Cumming & Mole, 1969), although LH concentrations are initially suppressed (Schoenemann *et al.*, 1985). This surge is mediated initially through an increase in pituitary sensitivity to GnRH and subsequently by an action at the level of the hypothalamus to increase GnRH secretion (Drouin, Lagacé & Labrie, 1976; Beck & Convey, 1977; Kesner, Convey & Anderson, 1981; Schoenemann *et al.*, 1985). Oestradiol has also been reported to increase the pituitary content of both LH and FSH (Schoenemann *et al.*, 1985) and the secretion of FSH from cultured rat pituitaries (Farnworth, Robertson, de Kretser, Findlay & Burger, 1989), to increase the mean concentrations of LH (Critser, Miller, Gunsett & Ginther, 1983) and to increase the amplitude of LH pulses in ovariectomised cattle (Kinder, Garcia-Winder, Imakawa, Day, Zalesky, D'Occhio, Kittok & Schanbacher, 1983; Day, Imakawa, Pennel, Zalesky, Clutter, Kittok & Kinder, 1986; Imakawa, Day, Zalesky, Garcia-Winder, Kittok & Kinder, 1986; Stumpf, Day, Wolfe, Wolfe, Clutter, Kittok & Kinder, 1988). This increase in LH pulse amplitude has been reportedly associated

with a concomitant decrease in pulse frequency (Kinder *et al.*, 1983; Imakawa *et al.*, 1986)

Conversely, oestradiol reportedly increases LH pulse frequency (Karsch, Foster, Bittman & Goodman, 1983) and is capable of reducing overall mean FSH and LH concentrations in sheep (Rawlings, Jeffcoate & Rieger, 1984), although the concentrations of oestradiol attained during this latter study (11-98 pg/ml) were high. Other workers have also reported that oestradiol, at more physiological levels, is capable of suppressing basal LH and FSH concentrations in sheep (Goodman, Legan, Ryan, Foster & Karsch, 1980; Clarke, Funder & Findlay, 1982; Martin, Scaramuzzi & Henstridge, 1983) and cattle (Butler *et al.*, 1983; Price & Webb, 1988). Oestradiol has also been reported to decrease LH pulse amplitude (cattle: Price & Webb, 1988; sheep: Goodman & Karsch, 1980; Karsch *et al.*, 1983) through a decrease in sensitivity to GnRH (Goodman & Karsch, 1980) and to decrease the secretion of FSH by ovine pituitary cells in culture (Miller, Knight, Grimek & Gorski, 1977; Tsonis, McNeilly & Baird, 1986). Since rising concentrations of oestradiol are associated with an increase in LH pulse frequency and a decrease in pulse amplitude (see above) during the early follicular phase of the bovine oestrous cycle, these latter effects of oestradiol on gonadotrophin secretion would appear to be the more physiologically significant observations of oestradiol negative feedback.

Early observations of the role of progesterone in the control of the oestrous cycle in cattle demonstrated that progesterone was capable of blocking or delaying the external signs of oestrus (Christian & Casida, 1948), suggesting that progesterone was antagonistic to the effects of oestrogens. Support for this hypothesis came with the observation that progesterone was capable of blocking the oestradiol-induced gonadotrophin surge in cattle (Hobson & Hansel, 1972; Barnes, Kazmer,

Bierley, Richardson & Dickey, 1980; Roche & Ireland, 1981a; Kesner, Padmanabhan & Convey, 1982; Schoenemann *et al.*, 1985) and sheep (Scaramuzzi, Tillson, Thorneycroft & Caldwell, 1971). However, some authors failed to block the LH surge with exogenous progesterone, although the presence of high endogenous concentrations of progesterone were effective in this respect (Hausler & Malven, 1976; Short *et al.*, 1979). Further investigation of this phenomenon reveals that progesterone is unable to prevent the oestradiol-induced increase in GnRH receptor numbers in cattle (Schoenemann *et al.*, 1985) and sheep (Moss, Crowder & Nett, 1981), although it is capable of inhibiting the increase in pituitary cell responsiveness to GnRH (Hsueh, Erickson & Yen, 1979; Padmanabhan & Convey, 1981) and prevents the increase in pituitary gonadotrophin concentration following oestradiol treatment (Schoenemann *et al.*, 1985). No effect of progesterone alone could be demonstrated in these studies. Accordingly there are a number of reports detailing a failure of progesterone to influence either LH or FSH secretion in ovariectomised animals (sheep: Goding, Blockey, Brown, Catt & Cumming, 1970; Scaramuzzi *et al.*, 1971; Rawlings, Jeffcoate & Rieger, 1984; cattle: Barnes *et al.*, 1980; Schoenemann *et al.*, 1985; rats: Welschen, Dullaart & de Jong, 1978). However, other studies report that progesterone reduces tonic LH secretion (sheep: Karsch, Legan, Hauger & Foster, 1977; Hauger, Karsch & Foster, 1977; Karsch, Legan, Ryan & Foster, 1978; cattle: Price & Webb, 1988) and LH pulse frequency (sheep: Goodman & Karsch, 1980; cattle: Ireland & Roche, 1982b; Imakawa *et al.*, 1986; Price & Webb, 1988) together with mean FSH concentrations in cattle (Price & Webb, 1988). Ireland and Roche (1982b) observed that removal of progesterone from the peripheral circulation by PRID removal or luteolysis, resulted in an increase in the amplitude of both LH and FSH pulses together with an increase in the frequency of LH pulses in cattle, further suggesting a

role for progesterone in the negative feedback control of these hormones. Similar, careful study of the temporal relationships between the steroids and the gonadotrophins throughout the oestrous cycle of the sheep had previously led Hauger, Karsch & Foster (1977) to propose that progesterone was the major "controller" of the oestrous cycle. Indeed, progesterone treatment has been developed as a commercial method of oestrous cycle control and synchronisation in cattle (Roche, 1978; Roche & Ireland, 1981b). However, in spite of the weight of evidence suggesting that progesterone is a negative feedback agent in the control of gonadotrophin secretion, there are reports in the literature that record an increase in FSH and LH secretion following progesterone treatment of cultured rat pituitaries (Lagacé, Massicotte & Labrie, 1980; Farnworth *et al.*, 1989). This may reflect a species difference, as the oestrous cycle of this species differs greatly from those of the large domestic animals, not least in the significant rise in progesterone concentrations observed immediately prior to the gonadotrophin surge; progesterone stimulation of gonadotrophin production may therefore be of importance in the preovulatory surge in this species.

The scarcity of data concerning the peripheral concentrations of androgens in the cyclic cow is reflected in the equal lack of data concerning the role of the androgens in the feedback control of gonadotrophin secretion. The majority of the available reports suggest that androgens play little role in negative feedback with exogenous androgens reportedly unable to affect any parameter of LH or FSH secretion (ewe: Martin, Scaramuzzi & Henstridge, 1983; steer: McCarthy & Swanson, 1976; D'Occhio, Kinder & Schanbacher, 1982; cow: Butler *et al.*, 1983), although Kennedy and Rawlings (1984) reported that administration of androgens led to a reduction in mean plasma FSH concentrations in steers and testosterone reportedly reduces the

sensitivity of cultured pituitary cells to GnRH (Drouin, Lagacé & Labrie, 1976). Other authors have qualified this observation in that the administration of testosterone to cows reduces the magnitude of the LH response, but not the FSH response, to exogenous GnRH, although no effect of treatment on basal gonadotrophin concentrations could be demonstrated (Thompson, Voelkel, Reville-Moroz, Godke & Derrick, 1984). However, in spite of the apparent lack of effect of exogenously administered androgens, immunisation against androgens patently *does* alter gonadotrophin secretory patterns. Immunisation against androstenedione leads to an increase in LH pulse frequency in sheep (Martensz & Scaramuzzi, 1979). Immunisation of heifers against testosterone resulted in significantly reduced mean LH and FSH concentrations in one animal that remained cyclic, but an increase in gonadotrophin concentrations in five other animals that became anoestrous as a result of treatment (Price, Morris & Webb, 1987). Despite these observations, it is difficult to draw meaningful conclusions from such immunisation studies without further information on the specificity of the antibodies raised since any observed effects may be mediated through the immuno-neutralisation of steroid moieties other than those of the initial androgen immunogen, or alternatively may act through the removal of androgen as a substrate for oestrogen production and hence oestrogen negative feedback.

In summary, both progesterone and oestradiol are capable of inducing profound changes in gonadotrophin secretion in the cyclic cow through a variety of pituitary and hypothalamic influences. The role of androgens in this respect is less clear, but is likely to be negligible at best.

The effects of progesterone and oestradiol are also known to be synergistic. In isolation, progesterone and oestradiol are unable to account for the cyclic changes in LH secretion in the sheep, but when

combined in physiological concentrations, they are sufficient to maintain normal levels of LH in ovariectomised animals (Goodman *et al.*, 1980; Karsch *et al.*, 1980; Martin, Scaramuzzi & Henstridge, 1983; Rawlings, Jeffcoate & Rieger, 1984). Similar observations have been reported in monkeys (Karsch, Weick, Hotchkiss, Dierschke & Knobil, 1973) and cattle (Beck, Smith, Seguin & Convey, 1976; Price & Webb, 1988). However, although Price & Webb (1988) have reported that oestradiol and progesterone in physiological concentrations are also capable of maintaining normal levels of FSH in short-term ovariectomised heifers, other authors have reported that steroid treatment of ovariectomised cattle (Roche & Ireland, 1981b), sheep (Goodman, Pickover & Karsch, 1981) and rats (Campbell & Schwartz, 1977) fails to suppress FSH concentrations to those levels observed in intact controls, suggesting that a further, non-steroidal component of negative feedback may exist. Indeed, there clearly exist several other components in the system controlling gonadotrophin secretion. One such component is evident during the immediate post-partum period in cattle, when the suckling stimulus is capable of inhibiting the oestradiol-induced gonadotrophin surge (Radford, Nancarrow & Mattner, 1978; Smith, Payne, Tervit, McGowan, Fairclough, Kilgour & Goold, 1981). Ovariectomy at this time also results in a slower increase in gonadotrophins than observed in non-suckling animals (Schallenberger & Peterson, 1982). There also appears to be a further ovarian component involved in that LH pulse amplitude reportedly increases with time after ovariectomy in sheep, regardless of the presence or absence of steroids (Martin, Scaramuzzi & Henstridge, 1983). Indeed, several authors have reported that steroid negative-feedback is completely abolished in long-term ovariectomised animals (Goding *et al.*, 1970; Scaramuzzi *et al.*, 1971). Ovarian factors have also been reported to be involved in the regulation of pulse amplitude in rats

(Nagesh Babu, Bona-Gallo & Gallo 1986), and in protecting the hypothalamic "pulse-generator" from the negative feedback effects of oestradiol, in the Rhesus monkey (Kesner, Wilson, Kaufman, Hotchkiss, Chen, Yamamoto, Pardo & Knobil, 1987). This factor may also be involved in the attainment of puberty, since this reportedly corresponds to a loss of sensitivity of the hypothalamus/pituitary gland complex to the negative feedback effects of oestradiol in the heifer (Day, Imakawa, Garcia-Winder, Zalesky, Schanbacher, Kittok & Kinder, 1984). In addition, there is evidence to suggest that the absence of the ovaries leads to an increase in the half-life of both LH (Montgomery, Crosbie, Martin & Pelletier, 1984) and FSH (Fry, Cahill, Cummins, Bindon, Piper & Clarke, 1987) in sheep.

The richest source of ovarian follicular products is follicular antral fluid. The major constituents of follicular fluid that have been identified and characterised to some degree will be considered in detail in the next section of this review.

1.3. The nature of follicular fluid

Follicular fluid is a complex mixture of proteins and steroids, the composition of which varies with, and reflects, the health of the individual follicle (Short, 1962; Andersen, Krøll, Byskov & Faber, 1976; see Edwards, 1974 for review of the early literature). Its proximity to the oocyte provides a rich microenvironment essential for full development and maturation, and many follicular products have been implicated in the overall regulation of ovarian follicular function and reproduction. This section of the literature review will consider the nature and physiological significance of several of the proteinaceous follicular products isolated over the last few years. By far the most extensively studied of these proteins is inhibin.

1.3.1. Inhibin

Inhibin has been defined as "a glycoprotein hormone consisting of two dissimilar disulfide-linked subunits, which inhibits pituitary gonadotropin production and/or secretion, preferentially that of FSH" (Burger & Igarashi, 1988). This definition is the result of many years of purification, isolation and study of the active principles of gonadal fluids and extracts. The field of inhibin research has received much attention over recent years and several excellent reviews of the literature are available (de Jong, 1988; Ying, 1988, 1989; de Kretser & Robertson, 1989). The reader is referred to one of these texts for a more extensive review of this area.

1.3.1.1. The history of the inhibin concept

The concept of inhibin is not a new one. Indeed, the term "inhibin" was first coined nearly 60 years ago by McCullagh (1932) to describe an active, water-soluble extract of bulls' testes which caused the dissappearance of the characteristic pituitary "castration cells" when injected into previously castrated rats. Organic extracts of bulls' testes containing a factor which McCullagh termed "androtin" (testosterone) stimulated the development of the secondary sexual organs, but were unable to effect this pituitary reversal (McCullagh & Walsh, 1934). Mottram & Cramer (1923) had previously observed the development of such cells in the pituitary gland without concomitant degeneration of the accessory sex organs following exposure of rats to radiation. The principal site of testicular damage in these animals was the seminiferous tubules, suggesting that they were the site of production of inhibin. McCullagh and his coworkers continued to investigate this phenomenon and extended their observations by demonstrating that injections of "inhibin-containing" preparations interrupted regular oestrous

cyclicity in female rats (McCullagh & Schneider, 1940), although other groups were unable to confirm his findings (Nelson, 1934; Nelson & Gallagher, 1935; Vidgoff & Vehrs, 1940; Rubin, 1941). However, little further progress was possible due to the limited techniques then available to investigators. Following the development of methods for the assay of gonadotrophins, a good deal of evidence was accumulated, principally from the study of cases of reproductive dysfunction in men, but also including some data derived experimentally in rats, which supported the hypothesis that the testes produced a factor from the seminiferous epithelium which influenced pituitary gland activity, and suggested that the absence of this factor resulted in abnormally elevated urinary levels of FSH (see Baker, Bremner, Burger, de Kretser, Dulmanis, Eddie, Hudson, Keogh, Lee & Rennie, 1976, for review). The first direct evidence for an FSH-suppressing testicular factor was provided by Franchimont (1972) who demonstrated that extracts of human seminal plasma were capable of suppressing FSH concentrations when injected into rats. Subsequently several other workers reported the presence of FSH-suppressing activity in rete-testis fluid (Setchell & Sirinathsinghji, 1972; Setchell & Jacks, 1974; Baker *et al.*, 1976) and testicular extracts (Lee, Keogh, de Kretser & Hudson, 1974; Keogh, Lee, Rennie, Burger, Hudson & de Kretser, 1976) and inhibin activity was demonstrated in bull seminal plasma (Franchimont, Chari, Hagelstein & Duraiswami, 1975). At around this time, it was also reported that the menopausal transition in women was associated with an increase in FSH, but not LH concentrations, suggesting that an inhibin-like entity may be present in the female as well as the male (Sherman, 1976), and bovine follicular fluid was demonstrated to contain inhibin-like activity in high concentrations (de Jong & Sharpe, 1976). Inhibin activity has subsequently been demonstrated in the ovarian follicular fluid of all species studied thus far,

including rat (Fujii, Hoover & Channing, 1983), sheep (Scott, Quigg, Trounson, Tsonis & Findlay, 1980), pig (Marder, Channing & Schwartz, 1977; Schwartz & Channing, 1977), human (Chari, Hopkinson, Daume & Sturm, 1979), monkey (Channing, Tanabe, Hahn, Philips & Carraher, 1984), hamster (Chappel, 1979) and horse (Miller, Wesson & Ginther, 1979). Further investigation has also revealed that inhibin is identifiable in other tissues with several reports detailing activity in placental extracts (rabbit: Hochberg, Weiss & Richman, 1981; human: McLachlan, Healy, Robertson, Burger & de Kretser, 1986; Merchenthaler, Culler, Petrusz & Negro-Vilar, 1987; rat: Apte & Sheth, 1982). Some reports even suggest the presence of an inhibin-like substance in gastric juice (Sheth, Vaze & Sheth, 1982; Shanbhag, Sheth, Nanivadekar & Sheth, 1984), although the relevance of this observation is unclear.

In summary, it took over 50 years from the initial observation of non-steroidal gonadal control of pituitary function to demonstrate the endocrine mechanisms underlying this phenomenon. Having identified both the action of the inhibin molecule, and a potent source of raw material in follicular fluid, the stage was set for rapid progress in the further characterisation of inhibin and the elucidation of its physiological role in both male and female.

1.3.1.2. The isolation and purification of inhibin

1.3.1.2.1. Early methods for the detection of inhibin

Having identified the underlying endocrine action of inhibin, the model systems used in this identification were rapidly developed into methods for its detection. The absence of suitably advanced techniques for the assay of FSH in blood plasma dictated that early assay systems measured inhibin activity by indirect "double" bioassay, relying on the inhibin content of the test fluid to suppress endogenous FSH scretion in rats or mice, which could then be detected by the increase in ovarian or

uterine weight following hCG administration, the so-called "reversed Steelman-Pohley" bioassay (Setchell & Siranathsinghji, 1972). Simpler, more direct methods for the detection of inhibin were subsequently developed, based on the direct measurement of the suppression of FSH concentrations in various test animals including castrated rats and mice (de Jong & Sharpe, 1976; Davies, Main, Young & Setchell, 1976; Nandini, Lipner & Moudgal, 1976; Hopkinson, Fritz, Chari, Sturm & Hirschäuser, 1977; Setchell, Davies & Main, 1977; Davies, Main & Setchell, 1978), intact male rats (Setchell & Jacks, 1974; Franchimont *et al.*, 1975; Hopkinson *et al.*, 1977; de Jong, Welschen, Hermans, Smith & van der Molen, 1978), anaesthetised female rats (Schwartz & Channing, 1977), castrated sheep (Keogh *et al.*, 1976) and cryptorchid sheep (Blanc, Cahoreau, Courot, Dacheux, Hochereau-de-Reviers & Pisselet, 1978). Other workers utilised *in vitro* methods to achieve the same end, measuring instead the suppression of basal or GnRH-induced FSH release from rat pituitary halves (Davies *et al.*, 1976; Davies, Main & Setchell, 1978) or from rat pituitary cells in culture (Baker *et al.*, 1976; Steinberger & Steinberger, 1976; de Jong *et al.*, 1978; Franchimont, Demoulin, Verstraelen-Proyard, Hazee-Hagelstein, Walton & Waites, 1978). This latter technique was subsequently developed into a quantitative assay method for the determination of inhibin in relatively concentrated biological fluids (Eddie, Baker, Higginson, Hudson, Keogh, de Kretser & Burger, 1977; Scott, Burger & Quigg, 1980; Lee, Colvin, Quigg, Atley, McMaster, Leversha & Burger, 1987) as a more sensitive alternative to the hCG uterine weight bioassay proposed by other authors (Chari, Duraiswaimi & Franchimont, 1976; Ramasharma, Murthy & Moudgal, 1979; Murthy, Ramasharma & Moudgal, 1979). The use of cultured ovine pituitary cells further improved the sensitivity of the procedure to the point whereby inhibin bioactivity was detectable in serum samples from cyclic sheep (Tsonis, McNeilly &

Baird, 1986). The validity and specificity of most of these assay techniques has been discussed elsewere (Hudson, Baker, Eddie, Higginson, Burger, de Kretser, Dobos & Lee, 1979; de Jong, Welschen, Hermans, Smith & van der Molen, 1979). Together these assay methods provided the basic tools for the monitoring of the procedures for the isolation of inhibin.

1.3.1.2.2. The purification of inhibin

Prior to the observation that bovine follicular fluid was a potent source of FSH-suppressing activity, inhibin had been considered to be a testicular hormone. Therefore, it is not surprising that the initial reports claiming the partial isolation of inhibin utilised male testicular fluids as the source. However, there was much controversy as to the correct size of the "inhibin" molecule. Principally by the use of dialysis techniques to exclude either low or high molecular weight components, but also with some application of gel permeation studies, the molecular weight (MW) of the male inhibin was variously reported as being around 1400-1500 (Sheth, Joshi, Moodbidri & Rao, 1979), less than 5000 (Franchimont, Demoulin, Verstraelen-Proyard, Hazee-Hagelstein & Tunbridge, 1979), between 10,000 and 20,000 (Baker *et al.*, 1976), approximately 19,000 (Thakur, Vaze, Dattatreyamurty, Arbatti & Sheth, 1978; Vaze, Thakur & Sheth, 1979), between 20,000 and 90,000 (Davies *et al.*, 1976; Davies, Main & Setchell, 1978) or greater than 100,000 (Cahoreau, Blanc, Dacheux, Pisselet & Courot, 1979). One of these studies yielded the first claimed radioimmunoassay for inhibin utilising the purified preparation obtained from human seminal plasma as immunogen, tracer and standard (Vaze, Thakur & Sheth, 1979) and, although neither ovine testicular extracts nor bovine seminal plasma cross-reacted in this system, significant quantities of immuno-activity could be detected in monkey semen, bovine, ovine and porcine follicular fluids, and in rat serum. Continued study led to the isolation and sequencing of two forms of

inhibin derived from seminal plasma and designated as α-inhibin and β-inhibin (Ramasharma, Sairam, Seidah, Chrétien, Manjunath & Schiller, 1984; Yamashiro, Li, Ramasharma & Sairam, 1984; Seidah, Arbatti, Rochemont, Sheth & Chrétien, 1984; Li, Hammonds, Ramasharma & Chung, 1985) which were described as single chain polypeptides variously containing 31, 52, 92 or 94 amino acids. Unfortunately, these α and β peptide sequences were subsequently shown to be degradation products of a gel-forming protein in seminal fluid and a sperm-coating antigen respectively (Johansson, Sheth, Cederlund & Jörnvall, 1984; Lilja & Jeppsson, 1985; Gordon, Liu, Akiyama, Tsuda, Hara, Schmid & Ward, 1987) and to have little inhibin-like activity except under the specific conditions employed by their proponents during their purification process (very short-term incubation of pituitary cells). Interestingly, however, the administration of antibodies raised against these proteins was capable of eliciting a rise in plasma FSH concentrations in rats (Thakur *et al.*, 1978; Vaze, Thakur & Sheth, 1979). The physiological mechanisms through which this increase in FSH secretion is mediated have not been defined.

The isolation of inhibin from follicular fluid was first reported by Robertson, Foulds, Leversha, Morgan, Hearn, Burger, Wettenhall & de Kretser (1985) who observed that the molecule obtained had an apparent MW of approximately 58,000 and could be dissociated into two subunits of approximate MW 43,000 and 15,000. The same laboratory subsequently reported that the 43,000 MW subunit was further split under acid conditions to yield an inhibin molecule of MW 32,000, consisiting of two subunits of MW 20,000 and 15,000 (Robertson, de Vos, Foulds, McLachlan, Burger, Morgan, Hearn & de Kretser, 1986). These observations were confirmed by other groups working with bovine, porcine and ovine follicular fluid. (Miyamoto, Hasegawa, Fukada, Nomura,

Igarashi, Kangawa & Matsuo 1985; Ling, Ying, Ueno, Esch, Denoroy & Guillemin, 1985; Rivier, Spiess, McClintock, Vaughan & Vale, 1985; Fukada, Miyamoto, Hasegawa, Nomura, Igarashi, Kangawa & Matsuo 1986; Miyamoto, Hasegawa, Fukada & Igarashi, 1986; Leversha, Robertson, de Vos, Morgan, Hearn, Wettenhall, Findlay, Burger & de Kretser, 1987). Ling *et al.* (1985) also observed two forms of inhibin in porcine follicular fluid which differed in the structure of the smaller, rather than the larger, subunit. To clarify discussion, the two subunits have now been designated α and β for the larger and smaller subunit respectively, and the two isoforms of the β subunit have further been described as β_A and β_B subunits. The partial amino acid sequences obtained from these initial purification studies were subsequently used to construct DNA probes which were then employed to isolate the cDNA sequences for each of the three subunits (α, β_A and β_B) from a porcine ovarian DNA library (Mason, Hayflick, Ling, Esch, Ueno, Ying, Guillemin, Niall & Seeburg, 1985) and the full amino acid sequences for the relevant precursor proteins were determined. Similar studies were performed using a bovine cDNA library, although no β_B subunit sequence could be detected (Forage, Ring, Brown, McInerney, Cobon, Gregson, Robertson, Morgan, Hearn, Findlay, Wettenhall, Burger & de Kretser, 1986). The cDNA sequences resulting from these investigations were also used to identify the comparable sequences in human (Mason, Niall & Seeburg, 1986; Stewart, Millborrow, Ring, Crowther & Forage, 1986) and rat cDNA libraries (Esch, Shimasaki, Cooksey, Mercado, Mason, Ying, Ueno & Ling, 1987; Woodruff, Meunier, Jones, Hsueh & Mayo, 1987) and to determine the primary protein sequences of each subunit of inhibin. There is a high degree of homology, in terms of the location of cysteine residues, between the α and β subunits. This has been interpreted as suggesting that both subunits are derived from a single ancestral gene. In addition there is a good deal of

sequence homology between the two β subunits. Across species, there is also a high degree of conservation in terms of the sequence of the α subunit. The β_A subunit is reportedly identical in cattle, human, rat and pig inhibin, whilst the β_B subunit differs by only 3 amino acids in those species where it is present. The β subunit also shows a high degree of homology with transforming growth factor β (TGFβ) and other factors involved in growth and differentiation suggesting that inhibin (or its subunits or processed products thereof) may play a role in these processes.

1.3.1.3. The physiology of inhibin

1.3.1.3.1. Production and regulation

Inhibin is produced by the granulosa cells in the female as demonstrated by the presence of inhibin-like activity in the spent media from *in vitro* cultures of these cells from a variety of species (Erickson & Hsueh, 1978b; Henderson & Franchimont, 1981,1983; Hermans, van Leeuwen, Debets, Sander & de Jong, 1982; Croze & Franchimont, 1984a,b; Tsonis, Sharp & McNeilly, 1988; Luck, Rodgers & Findlay, 1989; Campbell, 1989). Further support for this site of production is provided by the immunohistochemical localisation of inhibin in the granulosa cells of bovine and porcine ovaries (Rokukawa, Inoue, Miyamoto, Kurosumi & Igarashi, 1986) and the demonstration of inhibin α subunit gene expression in rat granulosa cells (Davis, Dench, Nikolaidis, Clements, Forage, Krozowski & Burger, 1986). Ovariectomy of rats results in the disappearance of assayable inhibin activity in serum (Lee, McMaster, Quigg & Leversha, 1982; Robertson, Hayward, Irby, Jacobsen, Clarke, McLachlan & de Kretser, 1988), thus confirming the gonadal origin of inhibin. Alternatively, the administration of either pregnant mare serum gonadotrophin (PMSG) or FSH results in an increase in peripheral inhibin concentrations (Lee, McMaster, Quigg, Findlay & Leversha, 1981;

Lee *et al.*, 1982; Tsonis, McNeilly & Baird, 1986,1988; Robertson *et al.*, 1988; Carson & McMaster, 1988; Buckler, Healy & Burger, 1989; McNeilly, Swanston, Crow, Tsonis & Baird, 1989) and in the concentrations of inhibin activity in granulosa cell culture media (Bicsak, Tucker, Cappel, Vaughan, Rivier, Vale & Hsueh, 1986; Suzuki, Miyamoto, Hasegawa, Abe, Ui, Ibuki & Igarashi, 1987; Zhang, Carson, Herington, Lee & Burger, 1987a; Zhang, Lee, Carson & Burger, 1988a), apparently through a cAMP-mediated system (Bicsak *et al.*, 1986; Suzuki *et al.*, 1987), as would be expected if inhibin is indeed part of the hormonal complex involved in the negative feedback regulation of FSH. However, McNeilly & Baird (1989) were unable to show any relationship between LH, FSH or oestradiol with inhibin secretion into the ovarian vein of sheep, and other workers failed to demonstrate any FSH stimulation of bovine granulosa cell inhibin production in cells cultured in defined media (Henderson & Franchimont, 1981; Klein, Robertson, Shukovski, Findlay & de Kretser, 1990), although both LH and FSH stimulated inhibin secretion when serum was present (Henderson & Franchimont, 1981). These authors also reported that steroids were capable of modulating inhibin production, with androgens stimulating and progesterone inhibiting secretion (Henderson & Franchimont 1981,1983) and suggested that these actions may respectively be involved in the promotion of follicular atresia and the loss of inhibin-secreting activity following ovulation, since bovine luteal tissue does not secrete inhibin in culture (Henderson & Franchimont 1983). However, other authors have been unable to repeat this work, although they concur that inhibin production from spontaneously luteinised bovine granulosa cells is minimal (Luck, Rodgers & Findlay, 1989). More recently, human and rat luteal tissue has been demonstrated to express at least the α subunit of inhibin (Davis *et al.*, 1986; Davis, Krozowski, McLachlan & Burger, 1987) and immuno- and

bio-active inhibin has been isolated from these tissues (Davis *et al.*, 1987; Cuevas, Ying, Ling, Ueno, Esch & Guillemin, 1987; Merchenthaler *et al.*, 1987; Smith, Millar, McNeilly, Illingworth, Fraser & Baird, 1990). Human granulosa cells also produce inhibin following spontaneous luteinisation in culture (Tsonis, Hillier & Baird, 1987) and may be stimulated by LH and testosterone, but not FSH. In addition evidence was presented by this group for the secretion of inhibin by the sheep corpus luteum *in vivo* (Tsonis, Baird, Campbell, Leask & Scaramuzzi, 1988a). However, the ovine CL, like the bovine CL, does not contain inhibin in measurable amounts (A.S.McNeilly *cited by* Smith *et al.*, 1990), and no appreciable inhibin subunit gene expression could be detected in bovine or ovine corpora lutea (Rodgers, Stuchberry & Findlay, 1989) suggesting that the initial observations of Tsonis *et al.* (1988a) were erroneous and that species differences in luteal function may exist. Others have reported circumstantial evidence for LH-stimulated corpus luteum production of inhibin in the macaque, further strengthening the hypothesis that the primate corpus luteum is functionally different from that of the ruminant (Fraser, Robertson & de Kretser, 1989).

The cAMP mediated stimulation of inhibin secretion also suggests that LH may stimulate the production of inhibin from large follicles with functional LH receptors, although Campbell, McNeilly & Baird (1989) failed to demonstrate an increase in ovarian inhibin secretion following an exogenous LH bolus, albeit in anoestrous ewes. Carson & McMaster (1988) similarly observed no increase in inhibin concentrations following LH treatment of hypophysectomised rats. Zhang *et al.* (1988a) also reported that LH alone had no effect on granulosa cell inhibin production and was actually inhibitory to PMSG-stimulated inhibin secretion, whereas Bicsak *et al.* (1986) and (Suzuki *et al.*, 1987) have previously reported a stimulatory effect of LH treatment following FSH

priming. Lee (1983) observed that LH (hCG) treatment reduced inhibin concentrations in PMSG-primed rats, although the possibility remains that this may due to the luteinisation of follicles, rather than a direct suppression of granulosa cell activity.

In addition to the documented response of granulosa/lutein cells to the gonadotrophins and steroids, other factors reportedly are capable of influencing inhibin production. In particular insulin-like growth factor-1 (IGF1) stimulates the production of inhibin from granulosa cell cultures in defined media (Bicsak *et al.*, 1986; Zhang *et al.*, 1987a), as does insulin (Suzuki *et al.*, 1987; Campbell, 1989), whilst epidermal growth factor (EGF) suppresses inhibin production in a similar model (Bicsak *et al.*, 1986; Franchimont, Hazee-Hagelstein, Charlet-Renard & Jaspar, 1986; Suzuki *et al.*, 1987; Zhang, Herington, Carson, Findlay & Burger, 1987b). Alternatively, fibroblast growth factor (FGF) neither stimulates nor suppresses inhibin production (Bicsak *et al.*, 1986). The same authors also reported that GnRH inhibited and vasoactive intestinal polypeptide (VIP) stimulated granulosa cell inhibin secretion. In addition, there is evidence to suggest that inhibin production may be regulated by other members of the inhibin/TGFβ family of proteins since activin (a dimer of inhibin β subunits; see below) and TGFβ both stimulate granulosa cell inhibin production *in vitro* (Sugino, Nakamura, Hasegawa, Miyamoto, Abe, Igarashi, Eto, Shibai, & Titani, 1988a; Xiao, Findlay & Robertson, 1990; Zhang, Findlay, Carson, Herington & Burger, 1988b) whilst follicle-stimulating hormone-suppressing protein (FSP; see below) suppresses inhibin production (Xiao, Findlay & Robertson, 1990). The extent to which all these mechanisms operate *in vivo*, and their relative importance, remain to be determined.

1.3.1.3.2. The physiological significance of inhibin

The study of the physiological significance of inhibin has been hampered by a lack of sufficient quantities of purified material to allow infusion studies to be carried out. Many studies have therefore used follicular fluid, from a variety of species, as a crude source of "inhibin-like activity". Other inferences have been drawn from statistical analyses and correlations observed following the measurement of hormonal changes throughout the oestrous cycle. More recently, as more purified preparations of inhibin have become available and the full amino acid sequence of the subunits has been elucidated, studies employing immunological approaches have been performed. The reader is again referred to one of the many reviews listed above for fuller details of this rapidly expanding area of research.

The initial observations that led to the hypothesis of inhibin suggested that it was responsible for the suppression of pituitary function, a definition that was later narrowed to the specific suppression of pituitary FSH release (see above). This concept was initially confirmed in ruminants by the demonstration that steroid-free follicular fluid preparations were capable of suppressing peripheral FSH concentrations in castrate (Cummins, O'Shea, Bindon, Lee & Findlay, 1983; Findlay, Gill & Doughton, 1985; Clarke, Findlay, Cummins & Ewens, 1986; Martin, Taylor & McNeilly, 1987; Knight & Castillo, 1988) and intact sheep (Miller, Critser & Ginther, 1982; McNeilly, 1984,1985; Wallace & McNeilly, 1985; Martin, Wallace, Taylor, Fraser, Tsonis & McNeilly, 1986; Martin, Taylor & McNeilly, 1987; McLeod & McNeilly, 1987; Knight & Castillo, 1988; Wallace, Martin & McNeilly, 1988; Baird, Campbell & McNeilly, 1990). Replacement of steroids alone in ovariectomised ewes failed to suppress FSH concentrations to physiological levels, although LH concentrations were normal (Goodman, Pickover & Karsch, 1981). However, FSH

concentrations could be reduced to within the normal range by means of a combination of steroids and follicular fluid (inhibin) injections (Martin, Price, Thiery & Webb, 1988). The mode of action of follicular fluid treatment appears to involve a reduction in pituitary FSH production (Martin *et al.*, 1986) due to a decrease in FSH β subunit gene expression (Mercer, Clements, Funder & Clarke, 1987) at a locus distal to the GnRH receptor (Wang, Farnworth, Burger & Findlay, 1990), although there is evidence that part of the response may involve a reduction in the number of GnRH receptors on the pituitary (Martin *et al.*, 1986). This latter action may explain the reduction in LH concentrations observed following treatment of long-term ovariectomised ewes with high doses of bovine follicular fluid (Findlay, Gill & Doughton, 1985; Clarke *et al.*, 1986), and the reduction in GnRH-induced LH release in bFF treated rats (Franchimont, Verstraelen-Proyard, Hazee-Hagelstein, Renard, Demoulin, Bourguignon & Hustin, 1979).

The role of inhibin in cattle is less clear. Follicular fluid treatment of ovariectomised heifers reduces peripheral FSH concentrations (Ireland, Curato & Wilson, 1983; Kiracofe, Ramirez-Godinez, McGowan & Bolt 1983; Beard, Castillo, Glencross, McLeod & Knight, 1988; Beard, Savva, Glencross, McLeod & Knight, 1989) and prevents the transient rise in FSH concentrations following unilateral ovariectomy (Johnson, Smith & Elmore, 1985). Highly purified inhibin is also effective in reducing FSH concentrations in ovariectomised heifers (Beard *et al.*, 1988). This reduction in systemic FSH concentrations apparently results from a specific reduction in pituitary FSH-β subunit mRNA content, similar to that observed in sheep (Beard *et al.* 1989). However, although Quirk & Fortune (1986) reported that follicular fluid treatment of intact cattle suppressed peripheral FSH concentrations, their data are far from conclusive, and Johnson & Smith (1985), using a slightly different

experimental protocol, failed to suppress FSH concentrations in treated animals below control values. In addition, unlike the sheep, treatment of short-term ovariectomised heifers with implants which produce physiological concentrations of steroids in the peripheral circulation is sufficient to maintain both LH and FSH concentrations within the expected, normal range (Price & Webb, 1988), suggesting that the role of inhibin in the normal intact cyclic heifer may be, at best, slight.

Analysis of plasma FSH and inhibin concentrations in the peripheral serum of rats suggests that an inverse relationship exists between the two (Hamada, Watanabe, Kokuho, Taya, Sasamoto, Hasegawa, Miyamoto, Igarashi, 1989). However, studies in sheep have shown that inhibin concentrations are higher in Booroola ewes carrying a copy of the fecundity (F) gene than non-carriers of the gene (Tsonis, Baird, Campbell, Downing & Scaramuzzi, 1988b). Since the F gene-carriers have previously been shown by some groups to have elevated FSH concentrations (Bindon, 1984; McNatty, Hudson, Henderson, Gibb, Morrison, Ball & Smith, 1987; McNatty & Henderson, 1987), this concomitant increase in inhibin concentrations argues against the importance of inhibin in the negative feedback regulation of FSH in the sheep. The situation is further complicated by the fact that other authors have reported that highly prolific Booroola ewes have significantly *lower* ovarian inhibin content than their contemporaries (Cummins *et al.*, 1983).

Inhibin concentrations in follicular fluid increase with follicular diameter (Scott *et al.*, 1980; Tsonis, Quigg, Lee, Leversha, Trounson & Findlay, 1983), and there is a correlation between inhibin concentrations and indicators of follicular health (Scott *et al.*, 1980; Channing, Gagliano, Hoover, Tanabe, Batta, Sulewski & Lebech, 1981; Tsonis *et al.*, 1983; Henderson, Franchimont, Charlet-Renard & McNatty, 1984a). However,

there is a large variation between individual follicles, and this has been interpreted as reflecting differences in both granulosa cell number and activity, and the rate of exit of inhibin from the follicle (Tsonis *et al.*, 1983). Mann, McNeilly & Baird, (1989) have also reported that the majority of the inhibin secreted into the ovarian venous effluent is derived from the largest antral follicles. It might therefore be expected that inhibin secretion should reach a peak just prior to ovulation, when follicular diameter and health is at a maximum. However, studies of inhibin secretion throughout the oestrous cycle are inconclusive in this respect. Some workers concur with this hypothesis, and report a follicular phase increase in serum inhibin concentrations (Findlay, Clarke, Quigg, Katsahambas, Juhola & de Blasiis, 1988; Findlay, Quigg, Juhola, Katsahambas, Clarke, Doughton & Robertson, 1988; Findlay, Clarke & Robertson, 1990), whilst others report no change in inhibin secretion (McNeilly *et al.*, 1989; Mann, McNeilly & Baird, 1989), and others still report a decrease in inhibin over this period (Fujii, Hoover & Channing, 1983; Padmanabhan, Convey, Roche & Ireland, 1984; Hasegawa, Miyamoto, Iwamura & Igarashi, 1988; Tsonis *et al.*, 1988a; Tsonis, McNeilly & Baird, 1988). Analysis of the pattern of inhibin subunit gene expression in the rat ovary throughout the oestrous cycle suggests that there is a loss of both β_A and β_B gene expression early in pro-oestrus, which could account for any loss of inhibin activity observed during the follicular phase (Meunier, Cajander, Roberts, Rivier, Sawchenko, Hsueh & Vale, 1988). However expression of the α subunit gene increases throughout pro-oestrus (Meunier *et al.*, 1988), and since many of the immunoassays utilised in the study of inhibin secretory patterns cross react fully with the free α subunit molecule (see Robertson, 1990 for review), this may be expected to result in an increase in apparent inhibin activity during this period. Indeed, the presence in bovine follicular fluid of monomeric α

inhibin subunit has been demonstrated (Knight, Beard, Wrathall & Castillo, 1989; Robertson, Giacometti, Foulds, Lahnstein, Goss, Hearn & de Kretser, 1989; Sugino, Nakamura, Takio, Titani, Miyamoto, Hasegawa, Igarashi & Sugino, 1989), and granulosa cells reportedly secrete free α subunit into the culture media (Bicsak, Cajander, Vale & Hsueh, 1988). In addition, inhibin α subunit gene expression has been detected in the adrenal cortex and shown to be controlled by ACTH (Crawford, Hammond, Evans, Coghlan, Haralambidis, Hudson, Penschow, Richards & Tregear, 1987). These observations together with the differing bioactivity : immunoactivity ratio observed with the different molecular weight forms of inhibin known to exist (Grootenhuis, Steenbergern, Timmerman, Dorsman, Schaaper, Meloen & de Jong, 1989) necessitate the use of extreme caution when ascribing any physiological significance to data derived from such immunoassay techniques.

Attempts to elucidate the role of inhibin by means of immunisation have likewise proved inconclusive. Immunisation of prepubertal lambs against a partially purified inhibin-enriched bovine follicular fluid preparation (bovine ppff) results in an advancement of puberty, and a significant increase in subsequent ovulation rate (O'Shea, Al-Obaidi, Hillard, Bindon, Cummins & Findlay, 1984; Al-Obaidi, Bindon, Hillard & O'Shea, 1987). Immunisation of adult ewes and heifers against similar ppff preparations also results in significant increases in ovulation rate (sheep: O'Shea, Cummins, Bindon & Findlay, 1982; Henderson, Franchimont, Lecomte-Yerna, Hudson & Ball, 1984; Cummins, O'Shea, Al-Obaidi, Bindon & Findlay, 1986; cattle: Price, Morris, O'Shea & Webb, 1987). However, the expected increases in FSH are variable, with increases apparent in some animals, and not in others (Al-Obaidi, Bindon, Findlay, Hillard & O'Shea, 1987), although plasma from immunised ewes is capable of neutralising the FSH-suppressing activity of bovine follicular fluid in

ovariectomised animals (Al-Obaidi, Bindon, Hillard, O'Shea & Piper, 1986). Channing, Tanabe, Turner & Hodgen (1982) have previously observed the development of similar antibodies to inhibin following porcine follicular fluid administration to monkeys. Despite the ability of these antibodies to neutralise inhibin bioactivity in cell culture systems *in vitro*, and, therefore, presumably also in the peripheral circulation *in vivo*, no increase was observed in peripheral FSH concentrations in the immune animals, supporting the observations in immunised sheep and cattle. The use of more highly purified inhibin preparations (O'Shea, Bindon, Hillard, Piper, Findlay & Miyamoto, 1989; Hillard, Bindon, King, O'Shea, Andrews & Hinch, 1990) or recombinantly derived inhibin subunits or fragments thereof (Forage, Brown, Oliver, Atrache, Devine, Hudson, Goss, Bertram, Tolstoshev, Robertson, de Kretser, Doughton, Burger & Findlay, 1987; Anderson, Gilchrist, Hinch, Johnston, Munro & O'Shea, 1990a; O'Shea, Anderson, Bindon, Hillard & Sinosich, 1990; Wrathall, McLeod, Glencross, Beard & Knight, 1990) as antigens also results in an increase in ovulation rate, but the effects of treatment on FSH concentrations are again unclear with some workers reporting consistent increases in basal FSH concentrations during the breeding season (Findlay, Doughton, Robertson & Forage, 1989a; Wrathall *et al.*, 1990), and others observing either transient increases which were not related to subsequent ovulation rate (O'Shea *et al.*, 1989) or no changes at all (O'Shea *et al.*, 1990). Anderson, Bindon, Hillard, Munro & O'Shea (1990b) reported that immunisation of lambs against a fragment of porcine inhibin hastened the onset of puberty with no changes in gonadotrophin concentrations whilst immunisation against an immuno-affinity purified porcine inhibin preparation increased gonadotrophins with no effect on reproductive function. In contrast, administration of antisera raised against inhibin results in a definite increase in FSH concentrations in

female rats (Rivier, Rivier & Vale, 1986; Culler & Negro-Vilar, 1988,1989), suggesting that inhibin may play an important role in the regulation of FSH secretion in this species. Similar treatment of intact adult male rats failed to increase FSH concentrations (Culler & Negro-Vilar, 1988; Rivier, Cajander, Vaughan, Hsueh & Vale, 1988), although increases in FSH concentration were observed after antiserum administration to animals previously treated with ethane dimethane sulphonate to selectively destroy the Leydig cells (Culler & Negro-Vilar, 1990), which implies that the effect of inhibin negative feedback is "masked" in this sex by other, more potent Leydig cell-derived negative feedback agents. Interestingly, during this latter study, it was observed that immunisation against the inhibin preparation also resulted in elevated LH secretion, suggesting that inhibin is capable of suppressing LH secretion during the rat oestrous cycle. However, studies using ovine pituitary cells suggest the opposite; treatment of cultured cells with crude or highly purified inhibin resulted in an increased LH response to challenge with GnRH (Muttukrishna & Knight, 1990).

It is possible, however, that the role of inhibin may be a local ovarian control of follicular development, rather than as a component of a long-loop negative feedback mechanism, although the evidence to support this hypothesis is scarce. This area of the literature has recently been reviewed (Findlay, Xiao & Shukovski, 1990). Porcine inhibin reportedly suppresses the ability of FSH to induce oestrogen production by rat granulosa cells *in vitro* (Ying, Becker, Ling, Ueno & Guillemin, 1986), although other authors were unable to duplicate these findings using bovine inhibin preparations (Hutchison, Findlay, de Vos & Robertson, 1987; Sugino, *et al.*, 1988a). Possibly the observed suppression may have been due to a contamination of the porcine inhibin preparation with other components, as porcine follicular fluid has been shown to contain

several factors that inhibit the actions of FSH *in vitro* (see below). Roles for inhibin in potentiating the LH-induced androgen secretion by cultured rat thecal cells (Hsueh, Dahl, Vaughan, Tucker, Rivier, Bardin & Vale, 1987) and in inhibiting the maturation of rat oocytes (O, Robertson & de Kretser, 1989) have also been proposed, but these hypotheses require further investigation.

In conclusion, the rapid expansion in the field of inhibin research has demonstrated the existence of large between species, between sex, and, possibly, between breed differences in the functioning of the follicular-fluid protein-mediated negative feedback regulation of gonadotrophin secretion. Despite the volume of work performed in this area, the physiological significance of inhibin in the large domestic species remains uncertain.

1.3.2. Follistatin

During the purification of inhibin, several other proteins bearing either structural or functional similarity were isolated. One such protein with inhibin-like activity was isolated from porcine (Ueno, Ling, Ying, Esch, Shimasaki & Guillemin, 1987) and bovine (Robertson, Klein, de Vos, McLachlan, Wettenhall, Hearn, Burger & de Kretser, 1987) follicular fluid and was named FSH-suppressing protein (FSP) or follistatin. It is a monomeric protein secreted by granulosa cells in response to FSH, but not LH (Klein *et al.*, 1990), which exists in two or three molecular weight forms (porcine: 31kDa, 35kDa; bovine 31kDa, 35kDa, 39kDa) and which acts to suppress pituitary FSH secretion (Robertson *et al.*, 1987; Ueno *et al.*, 1987; Ying, Becker, Swanson, Tan, Ling, Esch, Ueno, Shimasaki & Guillemin, 1987; Wang, Farnworth, Robertson & Findlay, 1989a,b), although its specific activity is only between one-tenth and one-third that of inhibin (Ying *et al.*, 1987; Robertson, Farnworth, Clarke, Jacobsen, Cahir, Burger & de Kretser, 1990). A similar monomeric FSH-suppressing

protein of approximate MW 36kDa was also observed in ovine follicular fluid (Leversha *et al.*, 1987). The primary sequence of the porcine molecules was deduced from cDNA cloning and DNA sequencing using probes based on trypsin digests of the follistatin molecule (Esch, Shimasaki, Mercado, Cooksey, Ling, Ying, Ueno & Guillemin, 1987) and the same probes were subsequently used to identify the corresponding gene sequence in human (Shimasaki, Koga, Esch, Cooksey, Mercado, Koba, Ueno, Ying, Ling & Guillemin, 1988) and rat (Shimasaki, Koga, Buscaglia, Simmons, Bicsak & Ling, 1989) ovarian cDNA libraries. These studies revealed that the lower molecular weight form of the molecule was apparently identical to the higher molecular weight form but was truncated by 27 amino acid residues at the carboxyl-terminal end. The rat apparently only expresses the larger molecular weight form of the molecule. The deduced amino acid sequences were highly conserved between the species, with only 6 "conservative" substitutions distinguishing human from porcine follistatin. A degree of homology with EGF was also noted. This latter observation may be of some physiological significance as follistatin gene expression was also detected in the brain and kidney of rats, suggesting that it may also play a role in the differentiation and function of these tissues (Shimasaki *et al.*, 1989).

The effects of follistatin on pituitary cell FSH release *in vitro* are additive to those of inhibin (Ying, 1988; Robertson *et al.*, 1990) and may be neutralised by antisera specific to follistatin, but not by antisera raised against inhibin (Robertson *et al.*, 1990). Ying (1988, 1989) has suggested that the action of follistatin may be more at the level of FSH release, rather than synthesis, although the data to support this hypothesis was not elaborated. However, in the absence of data to demonstrate its presence in the systemic circulation, the role of follistatin in the regulation of FSH secretion and release must remain purely speculative.

It has been suggested that follistatin may play a local paracrine or autocrine role in follicular development. Follistatin reportedly stimulates FSH-induced progesterone production by rat granulosa cells, whilst suppressing aromatase activity and inhibin production (Xiao, Findlay & Robertson, 1990) and either stimulates (O, Robertson & de Kretser, 1989) or inhibits (Buscaglia, Fuller, Mazzola, Bacigalupi, Shimasaki, Ui, Simmons, Castillo, Schroeder & Ling, 1989) oocyte maturation *in vitro*. These observations clearly require further study.

1.3.3. Activin

Also during the purification of inhibin, two groups of workers isolated an inhibin-related protein from porcine follicular fluid which stimulated, rather than suppressed the release of FSH from pituitary cells *in vitro*. This was identified as a dimer of the β-inhibin subunits (Ling, Ying, Ueno, Shimasaki, Esch, Hotta & Guillemin, 1986a,b; Vale, Rivier, Vaughan, McClintock, Corrigan, Woo, Karr & Speiss, 1986). Due to its opposite action to inhibin, this protein was named activin, but studies of its role have been hampered by a lack of a suitable assay system. It is assumed that the production of activin is regulated by similar mechanisms to inhibin, although the differential control of inhibin subunit expression and the process of subunit-subunit association is poorly understood. The observation that activin is identical to an erythroid differentiation factor (EDF; Eto, Tsuji, Takezawa, Takano, Yokogawa & Shibai, 1987; Murata, Eto, Shibai, Sakai & Muramatsu, 1988) and highly homologous to a factor responsible for determining the developmental fate of *Xenopus* blastula cells (XTC-MIF; Smith, Price, Van Nimmen & Huylebroeck, 1990; van den Eijnden-Van Raaij, van Zoelent, van Nimmen, Koster, Snoek, Durston & Huylebroeck, 1990; Green & Smith, 1990), and the structural homology of the inhibin β-subunit to TGFβ (see above) suggest that activin may be a local modulator of cellular function

and differentiation. Indeed, recent evidence suggests that its mode of action in stimulating pituitary cell FSH secretion *in vitro* may be to stimulate the differentiation of stem cells present in the pituitary gland (Childs, Ellison, Foster & Ramaley, 1981; Naor, Childs, Leifer, Clayton, Amsterdam & Catt, 1982) into FSH secreting cells (Katayama, Shiota & Takahashi, 1990), although a previous study suggested that the increase in FSH secretion was due to an initial increase in release, followed by an increase in FSH production (Schwall, Nikolics, Szonyi, Gorman & Mason, 1988). However, there has been no report to date demonstrating the presence of activin in the peripheral circulation. Until ovarian activin secretion is conclusively shown, reports of the actions of ovarian activin on pituitary function must be treated with caution, particularly since activin is apparently unable to stimulate FSH release *in vivo* (Miyamoto, Hasegawa, Fukada & Igarashi, 1988)

Activin reportedly also influences ovarian cellular function, causing a dose-dependent increase in inhibin secretion from rat granulosa cells *in vitro* (Hutchinson *et al.*, 1987; Sugino *et al.*, 1988a; LaPolt, Soto, Su, Campen, Vaughan, Vale & Hsueh, 1989; Xiao, Findlay & Robertson, 1990). This appears to be a specific effect as receptors for EDF/activin have been demonstrated to be present on granulosa cells and to be produced in response to FSH (Sugino, Nakamura, Hasegawa, Miyamoto, Igarashi, Eto, Shibai & Titani, 1988b). This may represent a system for the amplification of FSH action on granulosa cells as activin has also been shown to induce FSH receptors in granulosa cells (Hasegawa, Miyamoto, Abe, Nakamura, Sugino, Eto, Shibai & Igarashi, 1988). Activin has previously been shown to synergise with FSH in the stimulation of progesterone production and aromatase activity in rat granulosa cells in culture (Xiao, Findlay & Robertson, 1990) and the induction of LH receptors (Sugino *et al.*, 1988a), although Hutchinson *et al.*, (1987) demonstrated a decrease in FSH-

stimulated progesterone production in the presence of activin. In addition, bovine granulosa cell progesterone production is reportedly reduced, along with that of oxytocin, by incubation with activin (Shukovski & Findlay, 1989) leading these authors to suggest that the role of activin may be to prevent the premature luteinisation of the granulosa cells of the developing follicle. This differing effect on progesterone secretion may therefore reflect the differing importance of progesterone in preovulatory endocrine events between the two species. The field of activin research seems certain to provide much information in the next few years.

1.3.4. Other Inhibin-related peptides

The production of the 32 kDa form of inhibin involves the post-translational proteolytic cleavage of the α subunit. This is a two stage process, initially resulting in the formation of two fragments of the subunit, one from the N-terminus end of the molecule (the α_N peptide) and the other (the pro-α_C peptide) which is further processed to give rise to the mature α_C peptide which, together with a β subunit, forms the inhibin molecule. The α_N, pro-α_C, and α_C peptides have all been isolated and purified from bovine follicular fluid (see section 1.3.1.2.2 above). However, although the α_C peptide has been investigated *de facto* during the examination of the role of inhibin, little study has been made of the α_N peptide. Immunisation of ewes against an α_N peptide sequence resulted in an increase in the number of corpora lutea per ewe, but a decrease in the number of lambs born, apparently due to a failure of the developing follicles to ovulate (Findlay, Tsonis, Doughton, Brown, Bertram, Braid, Hudson, Tierney, Goss & Forage, 1989b; Findlay, Tsonis, Doughton, Pearson, Borchers, Hungerford, Greenwood & Forage, 1989c). Anderson *et al.* (1990a) have similarly observed a reduction in fertilisation rate in ewes immunised against a bovine inhibin preparation. These observations may

imply a role for the α_N peptide in the process of ovulation as Findlay and his coworkers have hypothesised (Findlay *et al.*, 1989c; Findlay, Xiao & Shukovski, 1990), or alternatively may be the result of a premature luteinisation of follicles, suggesting that the α_N peptide may possibly be the luteinisation inhibitor described by Channing and coworkers (Ledwitz-Rigby, Stetson & Channing, 1973; Channing, 1979). As is the case for all the protein components of follicular fluid, much more research is required before definitive conclusions as to the role of the α_N peptide may be drawn.

1.3.5. Follicle-regulatory protein

The mechanisms by which the dominant ovarian follicle is initially selected and by which it continues to exert its dominance are still unclear. The ovaries are perfused with the same peripheral blood and hence all follicles are presumably exposed to similar concentrations of gonadotrophins. Either the dominant follicle reaches a stage in its development whereby it can continue to develop without the influence of gonadotrophins, or it must actively suppress the development of other follicles in the face of an adequate gonadotrophic drive. Early studies of the processes of folliculogenesis in the primate demonstrated that the injection of steroid-free porcine follicular fluid suppressed both FSH and follicular development, suggesting that even the dominant follicle requires FSH support (diZerega, Turner, Stouffer, Anderson, Channing & Hodgen, 1981). However, studies of the primate ovarian cycle revealed a clear commencement of follicular dominance that was not associated with a fall in FSH (diZerega, Marut, Turner & Hodgen, 1980). Furthermore, during studies of the effects of exogenous gonadotrophin administration (human menopausal gonadotrophin; hMG) on ovarian function, it was observed that the responsiveness of the follicular population was reduced both in the presence of a dominant follicle and after ablation of that

follicle (diZerega & Hodgen, 1980) when compared to the response prior to the selection of the dominant follicle. Injection of protein fractions of ovarian venous blood plasma collected from women at laparotomy into hypophysectomised rats also resulted in a reduction in the ovarian response to exogenous hMG, provided that the blood was taken from the vein draining the ovary containing the dominant follicle (diZerega, Goebelsmann & Nakamura, 1982). This led to speculation that a protein secreted by the dominant follicle may be responsible for the phenomenon of follicular dominance by reducing the ability of other follicles to develop further, despite the presence of sufficient gonadotrophic stimulation. The active factor was named follicle-regulatory protein (FRP). The postulated role of follicular proteins (especially FRP) in the control of folliculogenesis has been discussed elsewhere (diZerega, Campeau, Ujita, Kling, Marrs, Lobo & Nakamura, 1983a; diZerega, Tonetta & Westhof, 1987).

The substance responsible for this inhibition of FSH action has since been extensively studied and isolated from porcine follicular fluid (Holmberg, Campeau, Devereaux, Ono & diZerega, 1986a,b; Ono, Campeau, Holmberg, Nakamura, Ujita, Devereaux, Tonetta, DeVinna, Ugalde & diZerega, 1986). It is protein of MW 12-16,000kDa, secreted by human (diZerega, Marrs, Campeau & Kling, 1983b) and porcine (Tonetta, Yanagihara, DeVinna & diZerega, 1988) granulosa cells. FRP (or FRP-like) activity has also been detected in human (diZerega, Campeau, Nakamura, Ujita, Lobo & Marrs, 1983c; diZerega, Marrs, Roche, Campeau & Kling, 1983d), porcine (Kling, Roche, Campeau, Nishamura, Nakamura & diZerega, 1984) and bovine (Hillier, van Hall, van den Boogaard, de Zwart & Keyzer, 1982) follicular fluids and also in fluid from atretic but not viable equine follicles (Channing, Anderson, Hoover, Kolena, Osteen, Pomerantz & Tanabe, 1982). There is also evidence to suggest that the

human corpus luteum may produce FRP (Katt, Fujimori, Yanagihara, Campeau, Numazaki, Holst, Tonetta, Rodgers, Westhof, Mishell, Horenstein & diZerega, 1988; Fujimori, Rodgers, Nakamura, Katt, Yanagihara & diZerega, 1988).

The factors that control the secretion of FRP have not been fully elucidated. Porcine granulosa cells from small and medium follicles produce much more immunoactive FRP than do cells taken from large follicles. However, the production of FRP by granulosa cells from medium and large follicles increases with the length of time in culture (Tonetta *et al.*, 1988). This is in direct contrast to the observation that human granulosa cells secrete less FRP with time in culture (diZerega *et al.*, 1983b); possibly this may reflect the difference between ovulation rate control in polytocous and monotocous species. In this latter study the FRP activity in media was inversely correlated with the progesterone production of the cells. However, Katt *et al.* (1988) have demonstrated that FRP concentrations rise prior to the LH surge and then continue to be elevated until the late luteal period. This would tend to suggest a luteal source of production and hence a coproduction of progesterone and FRP and therefore support the observations in porcine granulosa cells (Tonetta *et al.*, 1988). Neither FSH nor steroids alone alter FRP secretion from porcine granulosa cells. However, in conjunction with steroids, FSH stimulates the production of FRP by granulosa cells from small follicles, but inhibits the production by cells from large follicles (Tonetta *et al.*, 1988).

Injection of FRP disrupts folliculogenesis and the ovarian cyclicity of menstruating monkeys (diZerega & Wilks, 1984) and suppresses follicular growth in cyclic guinea pigs (Fujimori, Nakamura, Tonetta & diZerega, 1987) *in vivo*. In addition, treatment of intact sheep with ovine follicular fluid (Cahill, Driancourt, Chamley & Findlay, 1985) or treatment of

hypophysectomised sheep with bovine follicular fluid (Cahill, 1984; Larson, Mallory, Lewis, Dailey & Inskeep, 1987) reduces the ability of PMSG to stimulate follicular development. Porcine follicular fluid also antagonises the effects of exogenous FSH on follicular recruitment in pigs (Guthrie, Bolt, Kiracofe & Miller, 1986). Studies *in vitro* have shown that FRP antagonises the actions of FSH on aromatase activity and progesterone secretion from granulosa cells (Battin & diZerega, 1985a,b; Schreiber & diZerega, 1986) and inhibits microsomal 3β-hydroxysteroid dehydrogenase activity in granulosa (Battin & diZerega, 1985a) and placental cells (Chicz, Nakamura, Goebelsmann, Campeau, Tonetta, Frederick & diZerega, 1985). In addition, it is reported that FRP prevents the FSH-induced development of LH/hCG receptors in granulosa cells (Montz, Ujita, Campeau & diZerega, 1984). However, this antagonism of FSH action does not involve an interference with FSH binding to its receptor (diZerega *et al.*, 1983b). In fact FSH has been shown to potentiate the FSH-antagonising effects of FRP in reducing aromatase activity and progesterone production (Westfhof, Westhof, Ahmad & diZerega, 1988). These authors also observed an increased incidence of the cellular signs of atresia in cells treated with FRP. They therefore suggested that FRP was responsible for the initiation of atresia. However, further studies are required to determine the exact nature and significance of the action of FRP.

1.3.6. Other inhibitors of FSH action

Although the FSH-antagonising action of FRP appears to be via a mechanism distal to the FSH receptor (see above), there are in addition several inhibitors of FSH binding to its receptor described in the follicular fluid of cattle (Darga & Reichert, 1978; Sluss, Fletcher & Reichert, 1983) and pigs (Sluss & Reichert, 1984a). This FSH-binding inhibitor (FSH-BI) activity may be split into three categories, namely a

high molecular weight fraction (> 8,000) which may be a secretory product of a Serratia bacteria contamination (Sluss & Reichert, 1984b), a very low molecular weight fraction (< 500) which is most likely to be due to non-specific ionic effects (Fletcher, Dias, Sanzo & Reichert, 1982; Sluss & Reichert, 1984a), and an intermediate molecular weight fraction, not attributable to either of the above artifactual explanations (Darga & Reichert, 1978; Fletcher *et al.*, 1982; Sluss & Reichert, 1984a). The significance of this FSH-BI activity has not been demonstrated. However, Sluss & Reichert (1984a) have shown that only around 15% of the total FSH-BI activity may be ascribed to the medium molecular weight range fraction. At least part of this activity has subsequently been ascribed to the action of a hydrophilic peptide composed of 10 or 11 amino acids (Reichert, Andersen, Branca, Fletcher & Sluss, 1984). An assay method for the detection of a low molecular weight FSH-BI from porcine and bovine follicular fluid was recently reported, and initial investigations suggest that, in the cow, the highest concentrations of inhibitor are found in small follicles during the luteal phase of the oestrous cycle (Sluss, Branca, Ford, Krishnan & Reichert, 1989). An earlier report had suggested that the inhibitor concentration was correlated with the state of atresia within individual follicles (Sluss, Fletcher & Reichert, 1983). However, the cellular origins, hormonal control and absolute identitity of these binding inhibitors have not yet been established, making speculation about their physiological role difficult.

To further complicate the issue, it has been reported that there exist both FSH agonist and antagonist activities within separate fractions of the FSH-BI activity (Sluss, Schneyer, Franke & Reichert, 1987). The agonist activity is not due to pituitary FSH, or a fragment thereof, but has been attributed to two protein products of approximate MW 58,000 and 45,000. Unlike pituitary FSH, these proteins do not dissociate into subunits, but

are recognised by antibodies raised against pituitary FSH (Schneyer, Reichert, Franke, Ryan & Sluss, 1988). These authors have suggested that the presence of such agonist and antagonist activities is indicative of a gonadal level of modulation of pituitary stimulation of reproductive function. Again, this theory requires much further testing.

1.3.7. Other follicular fluid factors

Porcine follicular fluid also contains specific gonadotrophin binding activity, which may be expected to influence the potency of the gonadotrophins in the follicular microenvironment. These binding factors have been tentatively identified as fragments of gonadotrophin receptor complexes resulting from proteolytic activity (Kolena & Šebőková, 1986; Kolena, Šebőková & Horkovics-Kováts, 1986; Yarney, Sairam, Bhargavi, Downey & Srikandakumar, 1990). In addition, many other factors are capable of influencing the responsiveness of follicular cells to the gonadotrophins (see Tonetta & diZerega, 1986,1990 for a review of paracrine control of follicular function). For example, insulin and IGF have been shown both to act alone and to synergise with FSH in promoting granulosa cell development and steroidogenesis (Adashi, Resnick, Svoboda & Van Wyk, 1984; Adashi *et al.*, 1985a,b; Adashi, Resnick, Brodie, Svoboda & Van Wyk, 1985c; Adashi, Resnick, D'Ercole, Svoboda & Van Wyk, 1985d; Lino, Baranao & Hammond, 1985; Dorrington, Bendell, Chuma, & Lobb, 1987; Adashi, Resnick, Hernandez, May, Knecht, Svoboda & Van Wyk, 1988; Amsterdam, May & Schomberg, 1988; Skinner & Osteen, 1988), and both insulin and IGF-1 receptors have been identified in porcine and human ovarian tissue (Baranao & Hammond, 1984; Poretsky, Grigorescu, Siebel, Moses & Flier, 1985). In addition, insulin infusion alters the follicular size distribution within the ovary and stimulates aromatase activity in pigs (Matamoros, Cox, Moore, 1990), resulting in an increase in ovulation rate in both pigs (Cox, Stuart, Althen, Bennett &

Miller, 1987) and sheep (Teleni, Rowe & Croker, 1984). In sheep, both increased dietary energy in the form of lupin grain supplementation and infusion of glucose lead to elevated plasma insulin concentrations and increased ovulation rate (Downing & Scaramuzzi, 1991). Conversely, human, ovine and porcine follicular fluid are known to contain insulin-like growth factor-binding proteins (Holly, Eden, Alaghband-Zadeh, Carter, Jemmott, Chard & Wass, 1989; Falconer, Bindon, Piper & Hillard, 1989; Shimasaki, Shimonaka, Ui, Inouye, Shibata & Ling, 1990) which have been demonstrated to be capable of inhibiting the action of FSH in rat granulosa cell cultures (Shimasaki *et al.*, 1990), presumably by reducing the availability of IGF. In addition, FSH has recently been demonstrated to reduce the secretion of IGF binding proteins by rat granulosa cells (Adashi, Resnick, Hernandez, Hurwitz & Rosenfeld, 1990). However, *in vivo* studies have shown that follicular development and oestradiol concentrations in follicular fluid are not correlated with IGF-1 concentrations in cattle (Spicer, Echternkamp, Canning & Hammond, 1988) and there is no difference in IGF-1 concentrations in follicular fluid between Booroola and control Merino ewes (Falconer *et al.*, 1989) Furthermore, the concentration of IGF-1 binding protein is significantly higher in the follicular fluid of highly prolific Booroola Merino ewes compared to controls (Falconer *et al.*, 1989). There are reports of binding proteins potentiating the effects of IGF-1 *in vitro* (Elgin, Busby & Clemmons, 1987); this may indicate a possible reason for the increased prolificacy of the Booroola. However, since the follicles of the Booroola ewe mature at a smaller size and produce less oestradiol than those follicles in control sheep (Baird, Ralph, Seamark, Amato & Bindon, 1982; Driancourt, Cahill & Bindon, 1985; McNatty, Henderson, Lun, Heath, Ball, Hudson, Fannin, Gibb, Kieboom & Smith, 1985; McNatty & Henderson, 1987), a reduced activity of intrafollicular IGF-1 in this breed is not

inconceivable. Recently, however, it has been reported that cattle selected for twins have a higher concentration of IGF-1 in both peripheral plasma and in the two largest follicles (Echternkamp, Spicer, Gregory, Canning & Hammond, 1990a).

In addition to the effects of IGF-1, other growth factors are known to influence follicular development. TGFβ is known to synergise with FSH in rat granulosa cells (Dodson & Schomberg, 1987), whilst the structurally related epidermal growth factor (EGF) reduces FSH-induced granulosa cell function and LH receptor induction (Hsueh, Welsh & Jones, 1981; Knecht & Catt, 1983; Dodson & Schomberg, 1987; Zhang *et al.*, 1987b). Infusion of EGF into sheep results in a reduction in ovarian oestradiol and inhibin secretion (Scaramuzzi, Murray, Campbell, Downing, Evans & Panaretto, 1988; Murray, Downing, Evans, Findlay & Scaramuzzi, 1989) and blocks oestrus, the LH surge and ovulation (Shaw, Jorgensen, Tweedale, Tennison & Waters, 1985; Radford, Avenell & Panaretto, 1987; Radford, Panaretto, Avenell & Turnbull, 1987). Recent studies have shown that transforming growth factor α (TGFα), which is highly homologous to EGF, also acts to reduce follicular oestradiol secretion and delays the preovulatory LH surge (Murray, Downing, Evans & Scaramuzzi, 1990).

There are many other factors which have been reported to be present in ovarian tissues and fluids, and/or to influence follicular development. These include GnRH-like proteins (Ying & Guillemin, 1981; Dekel, Sherizly, Phillips, Nimrod, Zilberstein & Naor, 1985; Aten, Ireland, Weems & Behrman, 1987; Ledwitz-Rigby, 1987), vasoactive intestinal polypeptide (VIP; Fredericks, Lundquist, Mathur, Ashton & Landgrebe, 1983; Kasson *et al.*, 1985; Törnell, Carlsson & Hillensjö, 1988; Schmidt, Jörgensen, Kannisto, Liedberg, Ottesen & Owman, 1990), thyroid hormone (Maruo, Hayashi, Matsuo, Yamamoto, Okada & Mochizuki, 1987), relaxin (Bagnell, Greenwood & Bryant-Greenwood, 1984), gonadotrophin-surge inhibiting factor

(Danforth, Sinosich, Anderson, Cheng, Bardin & Hodgen, 1987; Knight, Lacey, Peter & Whitehead, 1990; Fowler, Messinis & Templeton, 1990; see Whitehead, 1990 for review), a thecal cell-derived growth factor (Dorrington *et al.*, 1987; Bendell, Lobb, Chuma, Gysler & Dorrington, 1988; Lobb, Skinner & Dorrington, 1988; Tsonis, Sharp & McNeilly, 1988) and interleukins (Gottschall, Uehara, Hoffmann & Arimura, 1987; Khan, Schmidt, Hallin, Di Pauli, De Geyter & Nieschlag, 1988; Fukuoka, Yasuda, Taii, Takakura & Mori, 1989; Nakamura, Kato & Terranova, 1990). However, the evidence for the involvement of these factors is largely preliminary and limited.

In conclusion, follicular fluid is a complex fluid containing numerous factors which may modulate the responsiveness of the individual follicle to an apparently uniform external gonadotrophic stimulus. This review has not attempted to consider the intra- and inter-ovarian role of steroids, which may further alter the ability of follicles to respond to both FSH and LH. Clearly, the study of follicular function will necessitate many more years of painstaking research before any definite conclusions as to the overall control of follicular development, recruitment, selection and dominance may be reasonably drawn.

1.4. The control of ovulation rate and calving rate

1.4.1. The incidence of twinning in cattle

As mentioned briefly at the start of this review, the cow is a monotocous species. The low incidence of multiple births in the cow is due, in the main, to a relatively low incidence of multiple ovulations, compared with that observed in other ruminants, e.g. the sheep. The literature surrounding the phenomenon of twinning in cattle has been extensively reviewed elsewhere (Hendy & Bowman, 1970; Rutledge, 1975; Anderson, 1978; Morris, 1984,1990; Morris & Day, 1986).

Estimates of the frequency of twinning under normal production conditions vary widely with breed, location and study. Figures quoted by Rutledge (1975), summarising many other authors' findings, range from 0 to 11.3%. However, the more extreme values tend to occur in those studies of smaller herds, where familial influences and the small sample size may be expected to distort the results. When studies considering fewer than 500 births are excluded from the analysis, the overall mean incidence of twinning is 0.894%.

There is evidence that the occurence of multiple births is higher in the dairy breeds than the beef breeds, and more frequent in older cattle than younger (see reviews by Hendy & Bowman, 1970 and Rutledge, 1975). It has also been suggested that season may influence the twinning rate (Gregory, Echternkamp, Dickerson, Cundiff, Koch & Van Vleck, 1990). However, in contrast to the sheep, there are no breeds of cattle in which multiple birth is considered to be a normal occurrence.

1.4.2. The desirability of twinning in cattle

There has long been debate as to the desirability of multiple births in cattle and there are clearly documented disadvantages associated with twin calvings. Cattle giving birth to twins reportedly have a higher incidence of retained placentae than those carrying a single foetus (Turman, Laster, Renbarger & Stephens, 1971; Vincent & Mills, 1972; Hamori, 1975; Johnson, Turman & Stephens, 1975), in addition to increased calving difficulties due to malpresentation of the foetuses (Cady & Van Vleck, 1978; Reid, Wilton & Walton, 1986; Diskin, McEvoy, Hickey & Sreenan, 1987). Furthermore, the birthweight of twins is significantly lower than that of single calves and gestation length is shorter in dams carrying multiple foetuses (Vincent & Mills, 1972; Johnson, Turman & Stephens, 1975; Cady & Van Vleck, 1978; Diskin *et al.*, 1987; de Rose & Wilton, 1988). These factors all contribute to the increased rate of

neonatal mortality frequently associated with multiple calvings (Cady & Van Vleck, 1978) and have led several authors to suggest that twinning may be an undesireable trait in the commercial farm situation (Erb & Morrison, 1959; Hamori, 1975; Cady & Van Vleck, 1978). However, Diskin *et al.* (1987), using embryo transfer (see below) to repeatably obtain high twinning rates, have observed that the overall incidence of calving difficulties is not increased in cows bearing twins; cows bearing single calves tended to suffer from a higher incidence of calving problems due to oversize foetuses. In addition, although the size of individual calves is reduced with multiple births, the total weight of calf produced and weaned per cow is significantly increased (Vincent & Mills, 1972; Johnson, Turman & Stephens, 1975; Smith, Pollak & Anderson, 1982; Diskin *et al.*, 1987; de Rose & Wilton, 1988). Estimates of increased gross margins based on varying incidences of twinning suggest that the financial advantages far outweigh the disadvantages (Lamond, 1974; Dickerson, 1978; Diskin *et al.*, 1987), even after allowing for the costs of embryo transfer (see below). Furthermore, several authors have pointed out that the reports of increased calving difficulties and associated problems are frequently the result of the unplanned nature of twinning, and suggest that the development of new management and feeding practices for twin-bearing cattle may reduce the observed level of difficulties (Lamond, 1974; Morris, 1984). Indeed, feed supplementation during the last two months of pregnancy increases the length of gestation and also the birth weight of twin calves and reduces the incidence of retained placental membranes (Gordon, Williams & Edwards, 1962; Chupin, Huy, Azan, Mauléon & Ortavant, 1976), suggesting that this may be a successful approach.

One further complication associated with twinning is the occurence of freemartins in mixed sex twins (Erb & Morrison 1959; Ohno, 1969; Dunn,

McEntee, Hall, Johnson & Stone, 1979). This seriously reduces the usefulness of twinning in the dairy herd where heifer calves are required as herd replacements. It has been estimated that only 9% of heifer calves born co-twin to a bull are fertile (Gilmore, 1949; Erb & Morrison 1959); clearly the costs involved in rearing these animals to such an age at which their reproductive competence may be assessed are prohibitive, given such a low potential success rate. However, the development of techniques for the sexing of embryos (Anderson, 1987; Leonard, Kirszenbaum, Cotinot, Chesné, Heyman, Stinnakre, Bishop, Delouis, Vaiman & Fellous, 1987; Rall & Leibo, 1987; Bondioli, Ellis & Pryor, 1989; Xu, Picard, King & Goff, 1989; Kerr, Matthaei, Bradley & Reed, 1990) and semen (Johnson, Flook & Hawk, 1989; Kovacs & Foote, 1989; West, West & Aitken, 1989; see Amann, 1989 for review) may provide the answer to this problem.

1.4.3. The induction of twinning in cattle

The many studies detailed above clearly demonstrate that the cow is fully capable of carrying twin foetuses to term and successfully raising them to a suitable weaning age. The major limitation to the twinning rate in cattle is therefore not uterine in nature, rather it is likely to be the low incidence of double ovulations in the bovine population. Over the years, many investigators have attempted to overcome this problem using a variety of approaches.

1.4.3.1. Genetic selection

Potentially the simplest method for the reliable induction of a high incidence of twinning in a herd is to practice a selective breeding program. Morris, (1990) has recently reviewed the literature concerning the genetic improvement of litter size in domestic species. This approach has previously proved successful in sheep and has resulted in the

development of several breeds in which multiple ovulations are the norm (e.g. Booroola: Turner, 1978). Indeed, several experimental selection herds have been established in Australia (Bindon, Piper, Cheers, Curtis, Nethery & Holland, 1982), New Zealand (Morris, 1984), France (Frebling, Gillard & Menissier, 1982), Reading (Hendy & Bowman, 1970) and the United States (Mechling & Carter, 1964; Gregory *et al.*, 1990). However, Mechling & Carter (1964) reported that the reponse to selection was minimal, and the mean twinning rate of an unselected neighbouring herd was no different after some thirty years of selection; initial results from the later studies suggest they have enjoyed little more success. The principal cause of this lack of success is undoubtedly the low heritability of twinning which is generally held to be around 0.03-0.06 in cattle (Maijala & Syväjärvi, 1977; Morris & Day, 1986; Gregory *et al.*, 1990). It has recently been suggested that repeated measurements of ovulation rate in pubertal heifers may provide a more reliable criterion for selection in cattle as the heritability of this trait is reportedly much higher (0.34) than that for twinning rate (Echternkamp *et al.*, 1990b). The success or otherwise of this approach will be awaited with interest.

1.4.3.2. Gonadotrophin treatment

The use of pituitary extracts to induce multiple ovulations in cattle and sheep is long established (e.g. see Casida *et al.*, 1943). However, the response to a given dose of gonadotrophin is highly variable. The reasons for this variability have been reviewed elsewhere (cattle: Monniaux, Chupin & Saumande, 1983; Moor, Kruip & Green, 1984; sheep: Driancourt, 1987). Some authors suggest that season may influence the ovarian response to stimulation (Hasler, McCauley, Schermerhorn & Foote, 1983; Massey & Oden, 1984; Almeida, 1987; Gordon, Boland, McGovern & Lynn, 1987), although others have found no evidence to support this (Shea, Janzen & McDermand, 1984). In addition, any effect of season is most

likely not mediated through temperature effects since the superovulatory response of heat-stressed cattle is not different from that of cattle maintained under cool conditions (Page, Jordan & Johnson, 1989). Stage of the oestrous cycle is also reported to influence the ovulatory response to exogenous gonadotrophin (Sreenan & Gosling, 1977; Hasler *et al.*, 1983; Price & Webb, 1989) which Moor *et al.* (1984) have interpreted to be due to the operation of the normal hormonal mechanisms involved in follicular selection and dominance, although some authors have failed to observe any effect of stage of oestrous cycle in FSH/PMSG treated sheep (McMillan & Hall, 1990). Recent data tend to confirm the hypothesis of Moor *et al.* (1984) in that the superovulatory response is reduced in the presence of a dominant follicle in cattle (Grasso, Guilbault, Roy, Matton & Lussier, 1989; Guilbault, Lussier, Grasso & Roy, 1989). A further component of the variability of the response may lie in the composition of the gonadotrophin preparations utilised (Murphy, Mapletoft, Manns & Humphrey, 1984). The response to gonadotrophin treatment is reduced with increasing LH bioactivity (Chupin, Combarnous & Procureur, 1984; Donaldson & Ward, 1985,1987; Donaldson, Ward & Glenn, 1986; Beckers, 1987; Chupin, Cognié, Combarnous, Procureur & Saumande, 1987; Gonzalez, Lussier, Carruthers, Muphy & Mapletoft, 1990), an effect which apparently is quantitatively breed dependent (Chupin, Combarnous & Procureur, 1985). In addition, the dose of FSH is reported to be critical, with an optimum level of stimulation producing the maximum number of ovulations and doses below or above the optimum resulting in a reduced response (Donaldson, Ward & Glenn, 1986). Furthermore, the treatment regime also influences the superovulatory response and there are numerous reports in the literature detailing the effects of subtle changes in the superovulatory regime. However, these reports are almost invariably conflicting. For example, the effect of FSH priming at the start

of the oestrous cycle is variously reported to improve (Touati, van der Zwalmen, Ectors, Beckers & Ectors, 1989; Petr, Míka & Jílek, 1990), have no effect on (Rieger, Desaulnier & Goff, 1988), or reduce (Guilbault *et al.*, 1989; Totey, Singh & Talwar, 1989) the subsequent response to superovulatory treatments. Similarly, the use of anti-PMSG antibodies around the time of the preovulatory LH surge has been reported to increase the effectiveness of PMSG-based superovulatory treatments by some authors (Dieleman & Bevers, 1987; Dieleman, Bevers & Gielen, 1987; Kim, Rorie, Youngs, White & Godke, 1987; Saumande & Chupin, 1987; Wang, Wu, Xu, Hagele & Mapletoft, 1987), whilst others suggest that this treatment has no such effect (Alfuraiji, Broadbent, Hutchinson, Dolman & Atkinson, 1989; Callesen, Bak, Greve, Avery, Gotfredsen, Holm, Hyttel, Pedersen, Schmidt, Smith & Svanborg, 1989).

The long term effects of superovulation on the performance of dams has not been fully investigated. Those reports available suggest that gonadotrophin stimulation does not influence subsequent normal fertility or milk production (Bak, Greve & Schmidt, 1989; Bak, Greve, Schmidt & Liboriussen, 1989), although animals thus treated have a tendency to develop temporary cystic ovarian conditions (Bak, Greve & Schmidt, 1989) and the fertilisation rate may be reduced at later superovulations (Hasler *et al.*, 1983).

Unfortunately, to date, no consistent treatment has been developed to reliable induce the twin ovulations required for a practical on farm application. Until such a time as this problem is overcome, superovulation will simply remain a technique to improve the efficiency of embryo transfer and selection programmes.

1.4.4. Other endocrine manipulations

The knowledge that endogenous gonadotrophin concentrations are controlled by gonadal negative feedback (see previous sections) and that

exogenous gonadotrophins may be used to override the mechanisms normally controlling ovulation rate (see section above) have led many investigators to attempt to increase ovulation rate by judicious manipulation of negative feedback. These investigations may be grouped into two categories, namely those involving manipulation of steroid negative feedback and those which attempt to alter the production and actions of gonadal protein feedback agents.

1.4.4.1. Manipulation of steroid negative feedback

The modulation of the actions of endogenous steroids in sheep by immunological means is well documented (Scaramuzzi & Hoskinson, 1984; Scaramuzzi, Hoskinson, Radford, Hinks & Turnbull, 1984). Active immunisation of ewes against androstenedione increases gonadotrophin secretion during both anoestrus and the breeding season (Scaramuzzi & Martensz, 1975; Martensz, Baird, Scaramuzzi & Van Look, 1976) and results in increased ovulation and lambing rates (Scaramuzzi, Davidson & Van Look, 1977; Van Look, Clarke, Davidson & Scaramuzzi, 1978; Scaramuzzi, 1979; Croker, Cox, Johnson & Wilson, 1982; Scaramuzzi & Hoskinson, 1984; Philipon & Terqui, 1987; Cognie, 1988). Although, in the early studies, conception rate was reduced in immunised ewes (Van Look *et al.*, 1978; Scaramuzzi, Martin, Hoskinson, Downing, Gow, Turnbull & Hinks, 1982), the technique has subsequently been refined (see Scaramuzzi & Hoskinson, 1984) and is now marketed as a commercially patented technique for increasing the fecundity of sheep (Cox, Hoskinson, Scaramuzzi, Wilson & George, 1982). Active immunisation of ewes against other ovarian steroids e.g. testosterone (Scaramuzzi, 1979), oestradiol-17β or oestrone (Rawlings, Kennedy & Henricks, 1978; Scaramuzzi, Martensz & Van Look, 1980; Croker *et al.*, 1982; Scaramuzzi *et al.*, 1982; Smith, Cox, McGowan & Wilson, 1982) or progesterone (Hoskinson, Scaramuzzi, Downing, Hinks & Turnbull, 1982) also results in

an increase in ovulation rate in those ewes which ovulated, although a large proportion of treated animals failed to do so. Immunisation of ewes against progesterone also resulted in profound disturbances to the length of the oestrous cycle (Hoskinson *et al.*, 1982). However, this incidence of anovulation has been interpreted as being due to an excessive immune response and the use of a refined immunisation protocol is claimed to eliminate this problem (Scaramuzzi & Hoskinson, 1984). Indeed, Pathiraja (1982) has demonstrated that the ovulation rate in response to a given dose of steroid antisera is reduced above an optimum antibody titre. Controlled increases in ovulation and lambing rate may be induced in sheep following passive immunisation, using carefully optimised dose rates, against androstenedione, oestradiol, oestrone or testosterone or a mixture of a quarter dose of all four (Pathiraja, 1982; Land, Morris, Baxter, Fordyce & Forster, 1982; Pathiraja, Carr, Fordyce, Forster, Land & Morris, 1984; Webb, Land, Pathiraja & Morris, 1984). Active immunisation of ewes against a combination of androgens and oestrogens also reportedly increases lambing rate in a more controlled manner than immunisation against one or other steroid, suggesting that this may be a more effective overall strategy (Wilson, Cox, Wong & Paull, 1986).

Immunisation of cattle against steroids has been less successful. Active immunisation of cows against oestradiol-17β either results in blocked ovulation and disrupted oestrous cycles with a high incidence of ovarian cysts (Martin, Henricks, Hill & Rawlings, 1978) or has no effect on ovarian function (Wise & Schanbacher, 1983). Immunisation against oestrone likewise reportedly fails to alter ovulation rate (Sreenan, Diskin, Morris, Tait & Kilpatrick, 1983; Sreenan, Morris, Tait & Diskin, 1987). Immunisation against testosterone reportedly results in an enhanced ovulatory response to PMSG but a reduction in the number of ova subsequently recovered (Boland, Nancarrow, Hoskinson, Murray,

Scaramuzzi, Radford, Avenell & Bindon, 1985). Other workers have also reported an increase in ovulation rate following immunisation against testosterone (D'Occhio, Gifford, Cox, Weatherly & Setchell, 1986; Price, Morris & Webb, 1987), androstenedione (Wise & Schanbacher, 1983; Walton, 1985), or dehydroepiandrostenedione (Sreenan *et al.*, 1987), and an advancement of the onset of puberty in calves immunised against androstenedione (D'Occhio, Gifford, Hoskinson, Weatherley & Setchell, 1989). However, there is a general disagreement about the repeatability of an individual treatment regime (Sreenan, 1984; Sreenan *et al.*, 1987), and, in those studies which have demonstrated changes in ovulation rate, there is a high frequency of abnormal ovarian function and anovulation (Walton, 1985; Price, Morris & Webb, 1987; Sreenan *et al.*, 1987). The response of cattle to passive immunisation against a variety of ovarian steroids is equally variable, with an increased ovulation rate in some animals, and blocked ovulations in others (Sreenan, 1984; Webb *et al.*, 1984). It is certain that immunisation of cattle against steroids is not yet a practical technique for increasing ovulation rate.

An alternative approach to the disruption of steroid mediated negative feedback has also been investigated, namely the use of stereochemical blockers of steroid synthesis or action. For example, Land & Scaramuzzi, (1979) have reported an increase in the ovulation rate of sheep treated with the weak oestrogen, clomiphene citrate. Similar treatment of sheep with the 3β-hydroxysteroid dehydrogenase enzyme inhibitor, Epostane, also results in increased ovulation and lambing rates (Webb, 1987). Unfortunately, the responses obtained in both these studies were variable or associated with silent heats and blocked ovulations in some animals. Much further study is required before these may be considered as practical techniques for increasing ovulation rate. However, the results are consistent with previous observations of the effect of the

administration of steroids on ovarian function. Exogenous oestradiol reduces ovulation rate in sheep (Webb & Gauld, 1985; Adams & Atkinson, 1986) and cattle (Price & Webb, 1988) and suppresses follicular development in Rhesus monkeys (Zeleznik, 1981), although silastic implants containing oestrone or androstenedione were unable to alter ovulation rate in a separate sheep study (Scaramuzzi & Hoskinson, 1984). It has also been suggested that luteal progesterone may act locally to suppress follicular development in the primate (diZerega & Hodgen, 1982), although other authors have suggested that the presence of a corpus luteum may be conducive to ipsilateral follicular development in the cow (Matton *et al.*, 1981; Pierson & Ginther, 1987c). Alternatively, the results of steroid immunisation and steroid synthesis disruption on ovarian function are seemingly in conflict with the proposed role of FRP, a naturally occurring aromatase and 3β-HSD enzyme inhibitor (see section 1.3.5 above), in regulating follicular development.

Immunisation against the ovarian steroids reportedly increases the peripheral concentrations and secretion rate of ovarian steroids (Martensz & Scaramuzzi, 1979; Scaramuzzi, Martensz & Van Look, 1980; Scaramuzzi, Baird, Clarke, Martensz & Van Look, 1980). However, it is unclear if this increase in steroidogenesis is due to an increase in the steroidogenic capacity of individual follicles or if it is indicative of an increased number of steroidogenic follicles. Alternatively, it may merely reflect the presence of a quantity of antibody-bound steroid which is unavailable to the tissues, and therefore inactive. No conclusions as to the relevance of these increased steroid concentrations may usefully be drawn without further investigation.

Active or passive immunisation of ewes against oestradiol-17β leads to elevated plasma LH concentrations (sheep: Pant & Rawlings, 1973; Rawlings, Kennedy & Henricks, 1978; Martensz, Scaramuzzi & Van Look,

1979; Scaramuzzi, Martensz & Van Look, 1980; Pathiraja *et al.*, 1984; Webb *et al.*, 1984; cattle: Martin *et al.*, 1978) principally through an increase in LH pulse frequency (Martensz, Scaramuzzi & Van Look, 1979; Thomas, Martin & Pearce, 1982; Pathiraja *et al.*, 1984; Webb *et al.*, 1984). Similar effects on LH secretion are observed following immunisation against oestrone (Martensz, Scaramuzzi & Van Look, 1979; Scaramuzzi, Martensz & Van Look, 1980; Webb *et al.*, 1984; Pathiraja *et al.*, 1984), androstenedione (Martensz *et al.*, 1976; Martensz & Scaramuzzi, 1979; Scaramuzzi, 1979; Webb *et al.*, 1984; Pathiraja *et al.*, 1984) or testosterone (sheep: Martensz & Scaramuzzi, 1979; Martensz, Scaramuzzi & Van Look, 1979; Scaramuzzi, 1979; Webb *et al.*, 1984; Pathiraja *et al.*, 1984; cattle: Price, Morris & Webb, 1987), although some authors have failed to influence LH concentrations in cattle immunised against androstenedione (D'Occhio *et al.*, 1989). Immunisation against oestradiol or oestrone also results in elevated peripheral plasma concentrations of FSH in some animals (Pant & Rawlings, 1973; Martensz, Scaramuzzi & Van Look, 1979; Scaramuzzi, Martensz & Van Look, 1980; Webb *et al.*, 1984; Pathiraja *et al.*, 1984) which may be expected to stimulate follicular development. However, immunisation against androgens has been shown to either have no effect or to reduce FSH concentrations (sheep: Martensz & Scaramuzzi, 1979; Martensz, Scaramuzzi & Van Look, 1979; Scaramuzzi, 1979; Webb *et al.*, 1984; cattle: Price, Morris & Webb, 1987; D'Occhio *et al.*, 1989).

The observations of increased LH:FSH ratios in animals immunised against androgens are of particular interest given that LH is known to reduce the ovulatory response to FSH (see section 1.4.3.2 above) and that LH pulses antagonise FSH-induced follicular growth in ewes chronically treated with a potent GnRH agonist (Picton, Tsonis & McNeilly, 1990). Indeed treatment of sheep (Fitzgerald, Ruggles & Hansel, 1985), hamsters (Greenwald & Terranova, 1981) and guinea-pigs (Terranova & Greenwald,

1981) with antiserum to LH results in an increase in ovulation rate. It would therefore appear that the increases in ovulation rate observed following immunisation against ovarian steroids are not mediated through changes in gonadotrophins alone. Indeed, the observation that the superovulatory response to PMSG is enhanced by immunisation against testosterone (Boland *et al.*, 1985) or oestrone (Hoskinson, Hinks & Scaramuzzi, 1982), suggesting that ovarian sensitivity to gonadotrophins is increased, may be an indicator of the key action of successful steroid immunisation protocols.

1.4.4.1.1. Manipulation of protein negative feedback

The availability of inhibin preparations of increasing purity has, in recent years, allowed the manipulation of protein-mediated gonadotrophin negative feedback. The effect of immunisation against gonadal proteins (inhibin) on ovarian function and gonadotrophin secretion has been discussed in section 1.3.1.3.2 above.

An alternative approach to the modulation of the ovarian protein negative feedback is the use of follicular fluid to temporarily down-regulate the selective negative feedback control of FSH secretion. The direct administration of steroid-free follicular fluid to sheep results in a marked reduction in peripheral FSH concentrations with little or no effect on LH concentrations (see section 1.3.1.3.2 above). Following the cessation of follicular fluid treatment, FSH concentrations increase and subsequently "rebound" above the levels observed prior to treatment, before falling back to control values once more (Miller, Critser & Ginther, 1982; McNeilly, 1984,1985; Wallace & McNeilly, 1985; Henderson, Prisk, Hudson, Ball, McNatty, Lun, Heath, Kieboom & McDiarmid, 1986; McNeilly & Wallace, 1987; Wallace, Martin & McNeilly, 1988). This rebound in FSH concentrations is associated with a concomitant increase in ovulation rate (McNeilly, 1985; Wallace & McNeilly, 1985; McNeilly & Wallace, 1987;

Henderson *et al.*, 1986) leading McNeilly & Wallace (1987) to suggest that this may represent the basis for a practical application for increasing ovulation rate. However, to date, there are no reports of increased ovulation rate following such treatment of cattle. The nature of the rebound of FSH secretion is not fully understood.

1.4.4.2. Nutritional considerations

The role of nutrition in the control of reproductive function is one which has not been extensively studied by reproductive endocrinologists. However, the effects of insulin and the insulin-like growth factors on granulosa cell function (see section 1.3.7 above) suggest that energy balance may play a major role in determining the level of ovarian activity in mammals. Supplementation of the diet of sheep with lupin grain leads to an increase in ovulation rate (Knight, Oldham & Lindsay, 1975) which has been associated with changes in gonadotrophin secretion (Davis, Brien, Findlay & Cumming, 1981; Nottle, Seamark & Setchell, 1988), although Downing & Scaramuzzi, (1991) failed to observe any changes in either LH or FSH. In this study, increases in ovulation rate were associated with increases in plasma insulin concentrations and elevation of insulin concentrations by the infusion of glucose or branched chain amino acids similarly resulted in an increase in ovulation rate (Downing, Scaramuzzi & Joss, 1989; Downing & Scaramuzzi, 1991). The direct infusion of insulin to pigs likewise results in a stimulation of follicular development (Matamoros, Cox & Moore, 1990) and an enhanced ovulation rate (Cox *et al.*, 1987). One report detailing the effects of the use of bovine somatotrophin (BST) in dairy cattle to increase milk production has documented an increased incidence of twin calvings in treated animals (Butterwick, Rowlinson, Weekes, Parker & Armstrong, 1988), although others have failed to replicate these observations (Peel & Bauman, 1987; Phipps, Weller, Austin, Craven & Peel,

1988; Whitaker, Smith, Kelly & Hodgson-Jones, 1988) despite a significant increase in the number of antral follicles in the ovaries (Gong, Bramley & Webb, 1990). The infusion of recombinant BST also reportedly increases the superovulatory response of cattle (Herrier, Farries & Niemann, 1990; Rieger, Walton, Goodwin & Johnson, 1990). Administration of porcine growth hormone to gilts resulted in an increased ovulation rate (Kirkwood, Thacker, Gooneratne, Guedo & Laarveld, 1988), although this was associated with a high degree of anoestrus and other workers have reported reduced ovarian activity following similar treatment (Bryan, Hammond, Canning, Moudshein, Carbaugh, Clark & Hagen, 1989). It seems likely that nutritional factors play a modulatory role in the normal functioning of the reproductive system. However, the nature of the interactions have yet to be determined.

1.4.4.3. Embryo transfer

In the absence of a simple technique to reliably induce twin ovulations, the only proven method for inducing twinning in cattle is embryo transfer. The economics of this approach have been investigated both theoretically (Guerra-Martinez, Anderson & Dickerson, 1987) and in practice (Diskin *et al.*, 1987; Davis, Harvey, Bishop & Gearheart, 1989) and twinning by means of embryo transfer shown to be a viable economic proposition. The problems associated with the induction of twins have been discussed in section 1.4.2 above and will not be considered here. The reader is referred to one of the reviews of twinning in cattle listed above for a more detailed review of the literature concerning the use of embryo transfer to induce twins in cattle.

1.5. Overall conclusions

In conclusion, despite considerable research effort, no practical application has yet been developed to reliably induce the incidence of

twin ovulations and calvings in cattle. Attempts to transfer to cattle techniques which are effective in sheep have met with limited success. Yet the overall aim of such techniques, i.e. to produce more calves per cow calving, is an attractive financial proposition. It would appear that the endocrine mechanisms which control reproduction in cattle differ in a quantitative or qualitative manner from those which act in the sheep. Before a useful technique to control ovulation rate in cattle may be developed, these endocrine differences must be more fully delineated.

CHAPTER 2

Materials & Methods

2.1. Animals

All animals used in the experimental studies were Hereford x Friesan heifers, between 18 months and 3 years of age. Within an individual experiment, animals were age-matched contemporaries. Animals were purchased as calves and raised at the Institute's farms at Blythbank and Stanhope, prior to transport to the Dryden Farm where the intensive experimental work was performed. A "settling in" period of at least one month was allowed between transport and the commencement of any experiment to allow for acclimatisation and halter training. During the intensive phases of the experiments, animals were housed in a covered steading on straw bedding. They were fed a diet of hay and concentrates and water was available *ad libitum.*

2.2. Surgical procedures

2.2.1. Blood sampling

Infrequent blood samples were taken by jugular venepuncture. During periods of intensive "window" sampling, animals were fitted with stoppered indwelling jugular cannulae inserted as described below. The animals were restrained in a conventional crush, haltered and the area of the neck overlying the jugular vein clipped. The skin surrounding the site was thoroughly scrubbed with a Savlon solution (ICI Ltd.,

Macclesfield, Cheshire) and disinfected with Hibitane (ICI Ltd.) and the area anaesthetised with lignocaine (Lignovet: C-Vet Ltd., Bury St. Edmunds). A small incision was made through the skin, and the plastic cannula introduced to the jugular vein by means of a protruding central needle. The needle was withdrawn and the cannula stoppered and sutured in place. The animals showed no sign of distress either during or after the procedure, and, with regular flushing with heparinised saline, the cannulae remained patent for up to one week. Any bleeding following the removal of the cannulae was rapidly stopped by direct pressure. No cannula-related problems were encountered in any of the experimental series.

Infrequent blood samples were allowed to clot overnight at room temperature and serum was harvested by aspiration of the supernatant following centrifugation at 1,000 g for 30 minutes. Serial blood samples taken via the cannula were taken into heparinised tubes and centrifuged within 30 minutes of collection. Plasma was similarly obtained by aspiration of the supernatant. All serum/plasma samples were stored frozen at -20°C until required for hormone assay.

2.2.2. Immunisation

Animals were immunised and subsequently boosted by the s.c. injection of an emulsion containing 4 mg antigen in approximately 1.5 ml sterile saline plus 1.0 ml of a suspension of *Corneybacterium parvum* (Wellcome Biotechnology, Beckenham, Kent) emulsified in non-ulcerative Freund's adjuvant (Guildhay Antisera, University of Surrey, Guildford). At the time of the primary immunisation, but not at subsequent booster injections, treated animals were also injected s.c. into the brisket with 2.5 ml heat inactivated *Bordetella pertussis* (Wellcome Foundation Ltd., Dartford, Kent). The emulsions were injected at four sites, at the top of each leg. This protocol has previously been shown to be the

optimum for producing the maximum immune response (Price *et al.*, 1987). Some temporary discomfort was observed in a few animals, and some developed small sterile swellings at the site of injection, but these rapidly regressed.

2.2.3. Laparoscopy

Ovulation rate was measured by direct observation at laparoscopy, using a modification of the sub-lumbar method of Holland, Bindon, Piper, Thimonier, Cornish & Radford, (1981). To minimise problems due to rumen size during the procedure, animals were fasted from food for 48 hrs, initially in the straw yard, and, for the final 24 hrs, in a concrete floored starving pen. Water was also withheld during the latter 24 hr period.

Animals were led up a short ramp into a crush which was tilted forwards at an angle of approximately 10° to further minimise rumen problems and to present the uterus and ovaries to best effect for observation. All animals were sedated with Rompun (Intervet: Bayer UK Ltd., Bury St. Edmunds) and the right sub-lumbar fossa region clipped and scrubbed with Savlon solution and sterilised with Hibitane. Two incision sites, one approximately 10 cm ventral and dorsal to the other, were injected with 10 ml lignocaine (Lignovet: C-Vet Ltd., Bury St. Edmunds), introduced at varying depths to anaesthetise skin, muscle and peritoneum. Two short (1.5 cm) incisions were made, one at each site, and trocars and cannulae were inserted in a slightly ventro-caudal direction to avoid puncturing the rumen. The peritoneal cavity was inflated with medical grade nitrous oxide, and the endoscope and manipulator introduced through their respective cannulae. The ovaries were viewed directly and permanent records obtained by photography when required. Following examination, excess peritoneal gas was vented and the cannulae removed. A topical antibiotic was applied to the incision sites (Terramycin Spray: Pfizer Ltd., Sandwich, Kent) and each animal

received a single i.m. injection of long-acting penicillin (Duphapen: Duphar Veterinary Ltd., Southampton). No sutures were required to close the wound and animals recovered rapidly. Most could be observed feeding within 10 minutes of the end of the procedure; any delay in feeding was due to excessive sedation, rather than surgical trauma. No infections as a result of this procedure were recorded, and the operation could be repeated on the same animal over several consecutive oestrous cycles.

2.2.4. Ovariectomy

Ovariectomies were also performed under sedation in the inclined crush described above using the methods detailed by Price & Webb (1988). Anaesthesia was induced by para-vertebral blocking with lignocaine (Lignovet: C-Vet Ltd., Bury St. Edmunds) and a 20-30 cm incision made ventrally from the sub-lumbar fossa. The ovary was exposed and the blood vessels supplying it ligated. The ovary was then excised and the wound closed and sutured with degradeable sutures (Dexon: Davis & Geck, Gosport). The procedure was subsequently repeated on the other flank for the removal of the other ovary. Wounds were again treated with a topical antibiotic spray and animals received a single i.m. injection of long acting penicillin as described for the laparoscopy. Some animals suffered minor infections at the incision sites and were treated as required under veterinary supervision.

2.2.3. Implant insertion

Oestradiol implants were prepared and inserted s.c. in the axilla region under general anaesthesia as previously described by Price & Webb (1988). The size of implant used corresponded to the largest (4 x 6.5 cm) implant used by these authors.

2.3. Non-surgical procedures

2.3.1. Ultrasound examination

The ovaries of heifers were examined trans-rectally using a real-time B-mode ultrasound scanner and 7.5 MHz probe (Aloka Co. Ltd., Tokyo, Japan). The resulting images were recorded on videotape (Kodak plc.) for later analysis.

2.4. Radioimmuno-Assays

2.4.1. Buffers

Assay buffers were prepared weekly, or as required, using RIA grade chemicals obtained from SIGMA Chemical Company, Poole, Dorset. The water used in the buffers in the early experiments was double distilled and de-ionised; in later experiments, purification was by means of reverse osmosis using a Waters Milli-Q purification system (Millipore Corporation, Milford, MA).

Two assay buffers were routinely in use in the laboratory. Both were based on a 0.05 M phosphate buffer pH 7.5 which was prepared in 5 litre batches at 10 x working strength. The buffer used for gonadotrophin assays contained 1g bovine serum albumin (Fraction V) and 9g NaCl per litre. Bacterial contamination was prevented by the addition of 0.01% thimerosal. This buffer will be referred to as 0.1%-BSA buffer. The buffer utilised in steroid assays also contained 9g NaCl per litre, but the non-specific protein used in this buffer was swine skin gelatin (300 bloom). The gelatin was dissolved in 200 mls of buffer at 50°C prior to dilution to give a final concentration of 1g gelatin per litre of buffer. Following final dilution, this buffer was also filtered through filter paper (Whatman Qualitative No. 1; W. & R. Balston Ltd.) to remove suspended particles. Bacterial contamination was again prevented by the addition of 0.01% thimerosal. This buffer will be referred to as 0.1%-gelatin buffer.

2.4.2. Assay Calculations

All assays were analysed on a Macintosh computer using the AssayZap software (Zaristow Software, Haddington, East Lothian). The assay sensitivities, and within and between assay coefficients of variation are detailed in the relevant chapters.

2.4.3. Luteinising Hormone

Samples were assayed for LH using the radioimmunoassay described by Price, Morris & Webb, (1987). Briefly, the procedure was as follows. Standard samples (USDA-bLH-B5; biopotency 2.1 x NIH-LH-B9) ranging from 0.05 to 2.0 ng per tube and serum/plasma samples (100 or 200 µl), were diluted to 500 µl in 12 x 75 mm polyethylene assay tubes with 0.1%-BSA buffer. Specific antibody (R.B.Staigmiller, USDA, ARS, Montana Agric. Exp. Stn., Miles City, MT 59301) raised in rabbits and affinity purified on Sephadex 4B was added (200 µl per tube) at an initial dilution of 1:20,000 in 0.1%-BSA. The assay was then incubated at 4°C for 48 hrs prior to the addition of 12-15,000 cpm ^{125}I-labelled LH (USDA-bLH-I1; iodinated by the method of Greenwood, Hunter & Glover, 1963) in 100 µl and a further 48 hr incubation at 4°C. Antibody-bound and free LH were separated by the addition of 100 µl normal rabbit serum (NRS) at an initial dilution of 1:400 and 200 µl donkey anti-rabbit serum (DARS) at an initial dilution of 1:45 (both obtained from Scottish Antibody Production Unit, Carluke; SAPU) incorporating 10% 0.1 M EDTA, followed by an overnight incubation at 4°C, the addition of 0.8-1.0 ml 0.1%-BSA buffer pre-wash and centrifugation at 2,000 g for 30 minutes, again at 4°C. The unbound LH was poured off in the supernatant, the tubes allowed to drain, the rims aspirated, and the activity remaining in the pellet counted on a gamma counter.

2.4.4. Bovine Follicle-Stimulating Hormone

FSH was determined using a modification of the homologous double antibody radioimmunoassay system described by Bolt & Rollins (1983). The final assay procedure was as follows. Standards (USDA-bFSH-B1) and unknowns (250µl) were diluted to 500µl in 0.1%-BSA in 12 x 75 mm polystyrene tubes. First antibody (USDA-5-POOL; 200µl) at an initial dilution of 1:10,000 was added, the tubes were vortexed and incubated at 4°C in a draught-free cupboard for 48 hrs. Following this two day incubation, 100µl of highly purified bovine FSH (bFSH-BP3) iodinated by the lactoperoxidase method of Thorrel & Johansson, (1971) was added to all tubes at a rate of 12-15,000 counts per minute. The tubes were vortexed and incubated at 4°C in a draught-free cupboard for a further 48 hrs. NRS (100µl) and DARS (200µl) were then added to all tubes except totals at initial dilutions of 1:400 in 0.1%-BSA and 1:45 in 0.1%-BSA with 10% EDTA added respectively. The tubes were again vortexed and incubated at 4°C in a draught-free cupboard overnight. Following the addition of 1 ml 0.1%-BSA as a pre-wash, the tubes were centrifuged at 2,000 g for 30 minutes at 4°C, the supernatant decanted and the pellet subsequently counted in a gamma counter following thorough aspiration of the tube rims.

When used at an initial dilution of 1:10,000, the antibody bound 16-20% of radio-labelled tracer added. The standard curve was highly reproducible ranging from 0.5 to 32 ng/tube with an ED_{50} of approximately 10 ng/tube. Samples from a number of animals showed excellent parallelism with the standard curve when assayed over a wide range of dilutions and FSH added to plasma samples was recovered with an accuracy of 92.5% (± 2.3 sem). Cross reactivities of various bovine pituitary hormones calculated as the ratio of hormone required to suppress binding of radiolabelled FSH to 50% of maximum binding to the amount of FSH standard required to produce a similar effect were assessed

Figure 2.1 Specificity of the FSH antiserum. The antiserum was tested against bovine Growth Hormone, Prolactin and LH. Samples taken from heifers at various stages of the oestrous cycle and measured at several dilutions showed excellent parallelism to the standard preparation.

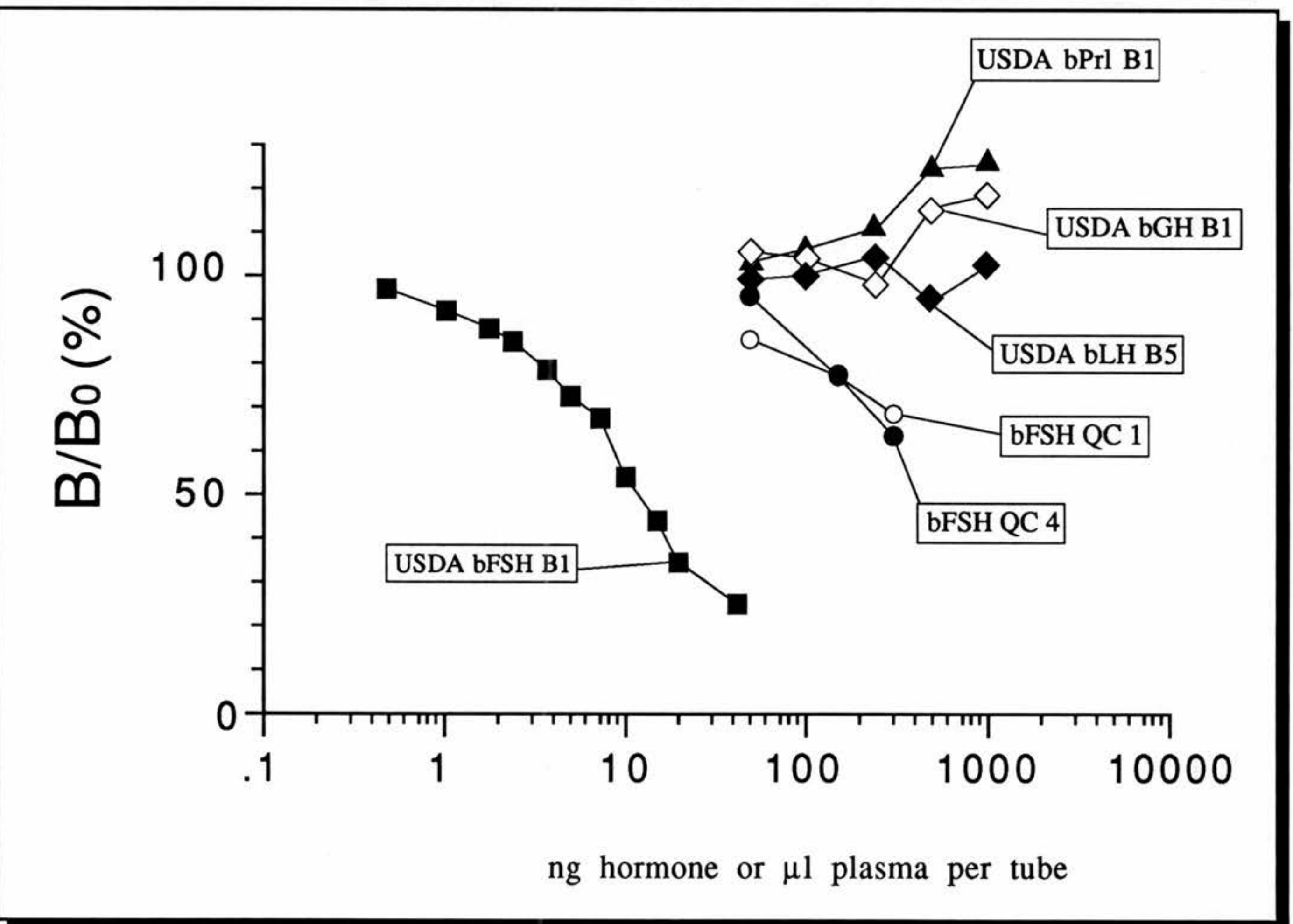
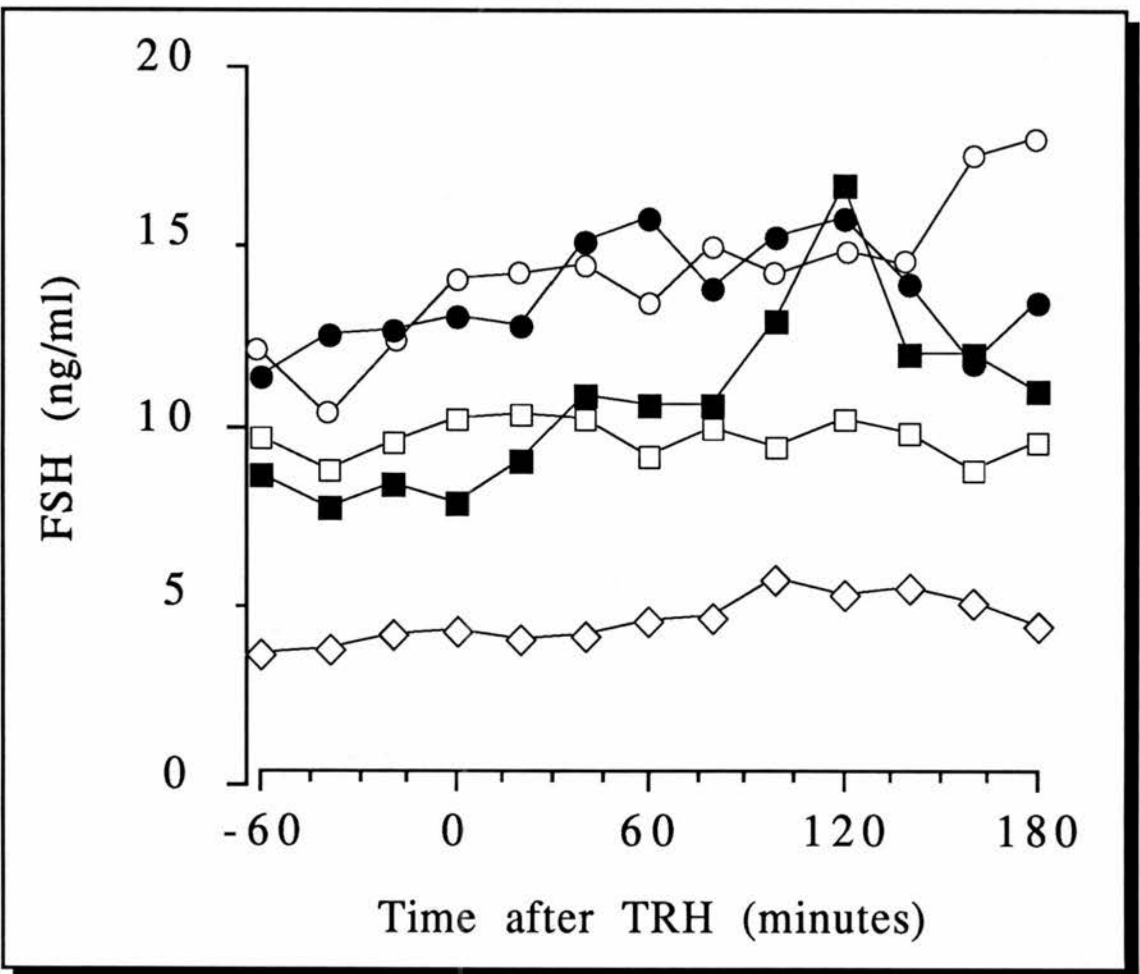


Figure 2.2 FSH concentrations in the sera of five ovariectomised oestradiol-implanted heifers following a bolus injection of TRH at time 0.



as follows :- USDA-bPRL-I-1, USDA-bGH-B-1, USDA-bLH-B1: < 0.25% (see Figure 2.1). An apparently high cross reactivity with a TSH preparation (NIADDK-bTSH-B11) appears to be due to contamination of this particular TSH preparation with FSH since no significant increase in immunoreactive FSH concentrations was observed in serial blood samples taken from cattle following a bolus TRH injection (Figure 2.2).

2.4.5. Ovine Follicle-Stimulating Hormone

Ovine FSH was measured directly in culture media using the radioimmunoassay reagents supplied by NIAMDD. This assay is routinely in use in this laboratory (McNeilly, Fordyce, Land, Martin, Springbett & Webb, 1988). Standards (NIAMDD-oFSH-RP1) covering the range 0.124 to 5 ng per tube and samples were diluted to 500 µl with 0.1%-BSA in plastic assay tubes. Specific antisera (R-anti-oFSH-I-1; 200 µl) at an initial dilution of 1:20,000 in 0.1%-BSA and approximately 12-15,000 cpm radiolabelled ovine FSH (NIAMMD-oFSH-I-1; iodinated by the method of Thorrel & Johansson, 1971) in 100 µl were added and the tubes vortexed and incubated overnight at 27°C. The antibody bound and free FSH were separated by the addition of 100 µl of NRS (SAPU) and 200 µl DARS (SAPU) at initial dilutions of 1:300 and 1:45 in 0.1%-BSA and 0.1%-BSA plus 10% 0.1 M EDTA respectively, followed by incubation overnight at 4°C, centrifugation, decanting and rim aspiration as described for the bovine assay. The activity of the pellet in each assay tube was counted on a gamma counter. The between and within assay coefficients of variation are detailed in the experimental chapters.

2.4.6. Inhibin

Inhibin was assayed using the radioimmunoassay described by McNeilly, Swanston, Crow, Tsonis & Baird, (1989) using reagents generously supplied by Dr A.S. McNeilly. Briefly, standards (3.9-1,000 pg

per tube) and samples were diluted to a volume of 300 µl with 0.1%-BSA. Specific first antibody (R150) raised in rabbits against a synthetic peptide corresponding to the 1-26 N-terminus of the α-chain of 32 kDa porcine inhibin (p1-26α inhibin) was added (100 µl per tube at an initial dilution of 1:15,000 in 0.1%-BSA) and the tubes were incubated for 24 hrs at 4°C. Tracer (^{125}I-p1-26α inhibin) was added at a rate of 100 µl per tube to give approximately 20,000 cpm and the tubes were again incubated at 4°C for a further 24 hrs. The antibody bound and free fractions were separated by the addition of 100 µl each of NRS (SAPU) and DARS (SAPU) at initial dilutions of 1:500 and 1:40 in 0.1%-BSA and 0.1%-BSA plus 10% 0.1 M EDTA respectively, followed by incubation overnight at 4°C, centrifugation, decanting and rim aspiration as described for the previous assays. The activity of the pellet in each assay tube was counted on a gamma counter.

2.4.7. Oestradiol

Oestradiol was assayed using the method of Webb, Baxter, McBride, Nordblom & Shaw, (1985) which is routinely in use in this laboratory. The procedure is as follows. Samples (2-3 mls) were dispensed into 16 x 125 mm screw-capped glass culture tubes, together with approximately 1,500 d.p.m. 2,4,6,7,16,17-^{3}H-oestradiol-17β (Amersham Int. plc) in 10 µl HPLC-grade absolute ethanol (FSA Laboratory Supplies, Loughborough) to allow estimation of recovery efficiency, 7 mls water and 400 µl sepharose-linked ovine antibody to oestradiol. The tubes were then capped and mixed end over end overnight at room temperature.

Chromatography columns (10 x 120 mm soda glass) fitted with porosity 1 sinter discs (Schott Glass, UK) were washed with 3 ml 90% methanol followed by 10 mls water. This cycle was repeated 3 times. The contents of the extraction tubes were then tipped directly on to the column, the tubes rinsed with 10 mls water and the washings subsequently added to the column. Each column was then washed with

three cycles of 7 mls water. Following these washing cycles, excess moisture retained on the column was removed with light suction and the antibody-bound extracted oestradiol eluted with 3 ml 90% methanol into 16 x 125 mm glass test tubes. The ethanol was evaporated under vacuum in a Buchler heated vortex evaporator, and the extracted steroid reconstituted into 1.8 mls 0.1%-gelatin.

The extraction efficiency for each sample was estimated by counting the ^{3}H activity remaining in 500 µl reconstituted oestradiol sample. Estimates of total activity added and the level of background activity were also obtained and the recovery efficiency calculated according to the following equation:-

$$\text{Recovery Efficiency} = \frac{(\text{sample counts - background}) * \left(\frac{\text{reconstituted volume}}{\text{counted volume}}\right)}{(\text{total counts - background})}$$

The remainder of the reconstituted oestradiol sample was taken for radioimmunoassay. Standards, in triplicate, ranging from 0.5 to 48 pg per tube and samples (usually 500 µl of the reconstitute per tube) were prepared in a total volume of 500 µl 0.1%-gelatin buffer in glass assay tubes (12 x 75 mm). Tracer (approx. 12-15,000 cpm in 100 µl; ^{125}I-17β-oestradiol-11α-tyrosinemethylester iodinated by the method of Hunter, Nars & Rutherford, 1975) and specific antisera (1:20,000 initial dilution in 200 µl buffer; R48; raised in rabbits against oestradiol-11β-succinyl-bovine serum albumin) were added and the tubes vortexed and incubated at room temperature for 3 hrs. The antibody bound and free oestradiol were separated by the addition of 100 µl each of NRS (SAPU) and DARS (SAPU) at initial dilutions of 1:400 and 1:40 in 0.1%-gelatin and 0.1%-gelatin plus 10% 0.1 M EDTA respectively, followed by incubation overnight at 4°C, centrifugation, decanting and rim aspiration as

described for the gonadotrophin assays. The activity remaining in each assay tube was again counted on a gamma counter.

Control samples measured in each assay included samples from cattle in known physiological states which covered a wide range of oestradiol concentrations. Water blanks were also included to estimate the amount of interference due to water and glassware contamination. The quantity of oestradiol added as recovery label was also assayed directly to allow for correction. Sample potencies were calculated in terms of pg oestradiol per assay tube and then corrected for assay blank and recovery efficiency according to the following equation:-

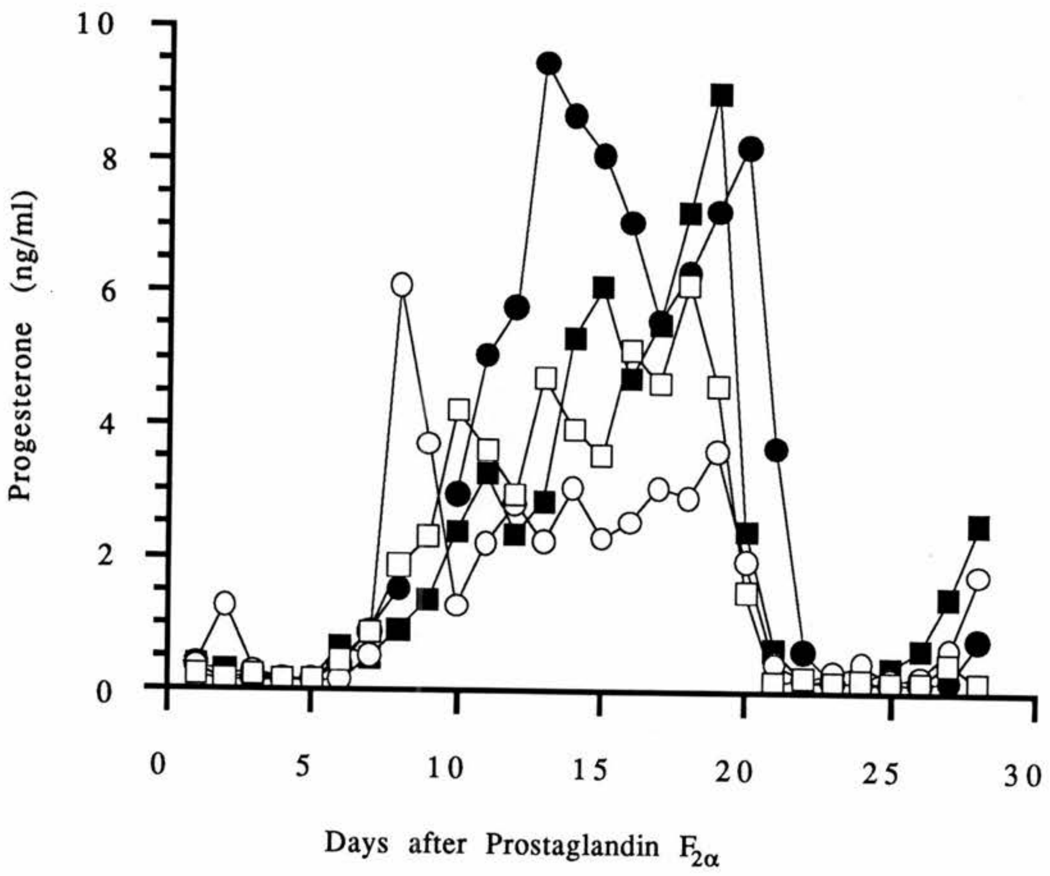
$$\text{Corrected Potency} = \frac{\left(\dfrac{(\text{pg/tube} - H_2O\ \text{blank}) * \left(\dfrac{\text{reconst. vol.}}{\text{assayed vol.}}\right)}{\text{recovery efficiency}} - \text{recovery label} \right)}{\text{volume extracted}}$$

The within and between assay coefficients of variation and the assay sensitivities for each experiment are detailed in the individual experimental chapters.

2.4.8. Progesterone

Progesterone was assayed by direct assay of non-extracted samples using 8-anilino-naphthalene-sulphonic acid (ANS) to prevent the interference of plasma steroid-binding proteins. The final assay procedure was as follows. Standards covering the range 7.8 to 1,000 pg per assay tube were diluted to 450 µl in 0.1%-gelatin buffer in glass assay tubes. Samples (50 µl) were diluted to 500 µl in 0.1%-gelatin and ovariectomised heifer serum (50 µl) was added to each standard tube. Progesterone tracer (^{125}I-progesterone-11α-glucuronide-tyramine iodinated by the method of Corrie, Ratcliffe & Macpherson, 1982 and generously supplied by the MRC Reproductive Biology Unit [RBU]

Figure 2.3 Progesterone concentrations in the sera of four heifers blood sampled daily for a period of twenty eight days following an i.m. injection of prostaglandin $F_{2\alpha}$



Edinburgh) was added to each tube at a rate of approximately 12-15,000 cpm per tube in a volume of 100 µl 0.1%-gelatin containing 1 mg ANS per ml immediately prior to the addition of 200 µl specific antisera (R31/8; raised in rabbits against progesterone-11α–hemisuccinate-bovine serum albumin and provided by Dr. Corrie of the RBU) at an initial dilution of 1:8,000 and incubation at room temperature for 3 hrs. The bound and free fractions were separated by the addition of 100 µl NRS (SAPU) and DARS (SAPU) at initial dilutions of 1:300 and 1:35 in 0.1%-gelatin and 0.1%-gelatin plus 10% 0.1 M EDTA respectively. Tubes were incubated overnight at 4°C, centrifuged, decanted, aspirated and counted as described previously.

When used at an initial dilution of 1:8,000, the antibody bound approximately 70% of added tracer. The cross-reactivities of other steroids in the assay were not different from those previously described for the same antisera used in an ether-extracted assay system (Corrie, Ratcliffe & Macpherson, 1982; Ashworth, 1984). To ensure that the assay was capable of measuring progesterone in the peripheral circulation of normal cattle, several heifers were blood sampled daily throughout a synchronised oestrous cycle and the samples assayed for progesterone. The results are shown in Figure 2.3. Progesterone added to ovariectomised heifer serum could be assayed quantitatively with a mean recovery of 102% (± 1.38 sem). No correction for recovery efficiency was required due to the direct nature of the assay.

2.5. Bioassays

2.5.1. Assay calculations

Potencies of samples measured by bioassay were calculated by comparison of parallel regression lines generated using the Genstat statistical package (Rothamstead Experimental Station). Samples were compared against either a pool of steroid-stripped bovine follicular fluid

or a steroid-free ovine rete testis fluid sample which has previously been standardised against an ovine testicular lymph preparation of arbitrary potency 1 u. activity per mg (Eddie, Baker, Higginson & Hudson, 1979).

2.5.2. Inhibin

Inhibin bioactivity was estimated by the ability of a sample to suppress basal FSH release from dispersed ovine pituitary cells in culture, using a modification of the procedure of Tsonis, McNeilly & Baird, (1986). All media used in the cell culture procedure were obtained from Northumbria Biologicals Ltd. The lamb serum and donor bovine serum were obtained from Flow Laboratories, Rickmansworth, Hertfordshire. The assay procedure was as follows.

Sheep heads were obtained from wethers at slaughter. The pituitaries were removed within 10 minutes of death and placed in sterile phosphate-buffered saline (PBS) containing 7.5 mmol glucose per litre for transportation to the laboratory. Each pituitary was subsequently dissected free from connective tissue and chopped into 3-4 mm blocks on a siliconised glass plate. The tissue was transferred to a 20 ml plastic Universal container and washed 6-8 times with PBS containing 7.5 mmol glucose per litre and 0.1% BSA. Following washing, the tissue was transferred into a spinner flask containing 10 ml 0.5% trypsin in PBS with 0.1% BSA and stirred gently for 30 minutes at 37°C in an atmosphere containing 2% CO_2. After the 30 minute incubation, the enzyme solution was removed and replaced with supplemented medium (Dulbecco's Modified Eagle's Medium [DMEM] supplemented with 20 mmol HEPES, 10% lamb serum, 2.5% donor calf serum, 2 mmol glutamine and 1 mmol $NaHCO_3$ per litre, 50 U penicillin per ml, 50 mg streptomycin per ml, 2.5 µg amphotericin per ml together with 10 ng RU486 per ml plus a highly specific oestradiol antisera at a dilution of 1:50,000 to prevent any possible steroid interference). The tissue was then stirred gently under

the same conditions for a further 30 minutes. The supplemented DMEM was then removed and replaced with 10 ml Ca^{2+} and Mg^{2+}-free PBS (PBS^-), supplemented with 2 mmol EDTA per litre and 0.1% BSA, and the tissue stirred for a further 10 minutes to encourage the dissociation of gap junctions. The supplemented PBS^- was subsequently removed, the tissue fragments transferred to a fresh Universal container and washed 3-4 times with unsupplemented PBS^- prior to dispersal in 3-5 ml PBS^- using a series of siliconised glass pipettes of graded diameter. The resultant dispersed cell suspension was separated from cell debris and other detritus by aspiration and the cells washed thoroughly by centrifugation and resuspension in fresh supplemented DMEM. After two cycles of centrifugation, the cells from each pituitary were pooled and the number of viable cells, as estimated by their ability to exclude Trypan blue dye, counted in a haemocytometer. Cells were seeded in 24 well plastic culture plates at a rate of 150,000 cells per well in 50 µl supplemented DMEM, following which a further 550 µl supplemented DMEM was added. Cells were allowed to attach to the plastic wells by incubation at 37°C in an atmosphere of 95% air, 2% CO_2 for 48 hours prior to the addition of test substances.

The media was removed and the cells washed with supplemented DMEM, following which test substances were added to each well. The reference preparation was a pool of charcoal-extracted bovine follicular fluid. All samples, including the reference preparation, were stripped of steroids by incubation with 1 mg/ml charcoal plus 0.1 mg/ml dextran overnight at 4°C and centrifugation, a process that removes approximately 99% of steroids (Wallace & McNeilly, 1985). This was followed by passage down a Sep-Pak C_{18} cartridge (Waters Division, Millipore Corp.) which removed a further 96.3% (± 0.008%) of the remaining steroids. All were subsequently sterilised by ultra-filtration

through a 0.22 micron filter (µStar: Costar Corporation, Cambridge, MA) and diluted as required in supplemented DMEM. The reference preparation was assayed in quadruplicate at each of five dilutions on at least two plates. Each sample was assayed in quadruplicate at three separate dilutions on at least two plates. All plates had at least four control wells (no test substance or reference preparation added) and those plates on which test samples were assayed also carried two separate dilutions of the reference preparation in quadruplicate. Following the addition of test samples, the cells were incubated for a further 48 hrs. The media was harvested and stored at -20°C until assayed for FSH content.

2.6. *Follicular fluid processing*

2.6.1. Collection of follicular fluid

Follicular fluid was collected by aspiration of all visible follicles of macroscopically normal appearance present on ovaries obtained from the local slaughterhouse. Ovaries were collected on ice and transported to the laboratory within four hours of death. No reproductive history of the donor animals was available. Immediately following collection, the follicular fluid was centrifuged at 1,000 g for twenty minutes to remove cellular debris and the supernatant stored at -20°C until required for further treatment.

Batches of follicular fluid sufficient for the completion of an individual experiment or series of experiments were subsequently thawed, pooled and stirred overnight at 4°C with 1 mg charcoal (Norit SX 1: Hopkin & Williams, Chadwell Heath, Essex) per ml plus 0.1 mg dextran (Dextran-T70: Pharmacia Fine Chemicals, Milton Keynes, Bucks.) per ml to remove steroids. The following day, the follicular fluid was centrifuged at 14,000 g for 1 hr to separate charcoal from fluid and the supernatant stored in aliquots as steroid-free follicular fluid. Similar

treatment has previously been demonstrated to remove 99% of steroids from follicular fluid (Wallace & McNeilly, 1985).

2.6.2. *Red Sepharose affinity chromatography*

Inhibin-enriched fractions of follicular fluid were obtained for the immunisation (Chapter 3) and direct administration studies (Chapters 4 & 5) by the Red Sepharose affinity chromatography method of Jansen, Steenbergen, de Jong & van der Molen, (1981) using the modified buffers described by Cummins *et al.*, (1986).

Typically, 50 mls steroid-free follicular fluid were applied to a column containing 240 mls Red Sepharose CL-4B (Pharmacia Ltd., Milton Keynes, Bucks.) which had previously been equilibrated with buffer A (0.35M-KCl, 1M Urea in 25 mM-Tris-HCl at pH 7.0). The non-bound proteins were washed through the column with buffer A. The inhibin-containing fraction of follicular fluid was eluted with buffer B (1.2M KCl, 1M Urea in 25 mM Tris-HCl at pH 7.0). The column was regenerated with 1M KCl.

The inhibin-enriched partially purified follicular fluid (ppff) fraction was then concentrated and dialysed against sterile physiological saline under pressure in a stirred ultrafiltration cell. The membrane used in the cell had a molecular weight cut-off point of approximately 5kDa (YM5; Amicon Ltd., Stonehouse, Cheshire). Concentrated column eluates were subsequently pooled and sterilised by exposure to 25kRads from a ^{137}Cs source and protein concentrations were determined by the method of Lowry, Rosebrough, Farr & Randall, (1951). The inhibin activity (Tsonis, McNeilly & Baird, 1986; see section 2.5.2 above) of a typical fraction of bovine follicular fluid prepared in the above manner was 1221 ± 3.4 U/mg protein.

2.6.3. Immunoaffinity chromatography

2.6.3.1. Preparation of immunoaffinity columns

The immunoaffinity gels were prepared by coupling the ammonium sulphate precipitated fraction of ascitic fluid to an activated agarose gel (Affigel 10; Bio-Rad Laboratories Ltd., Watford, Hertfordshire) in accordance with the manufacturers instructions. Briefly the protein fraction was exhaustively dialysed against the coupling buffer (0.1 M $NaHCO_3$ pH 8.5) and diluted to a concentration of approximately 15 mg protein/ml. An equal volume of gel was washed thoroughly with ice-cold coupling buffer and immediately mixed with the dialysed antibody solution in an ice bath. The reaction mixture was left to stand in the ice bath for 4 hrs, following which it was removed and kept at room temperature overnight with gentle agitation. The coupling buffer was removed over a sintered glass funnel and any remaining active ester binding sites were blocked with an excess of 1M Ethanolamine-HCl solution pH 8.0. The gel was subsequently washed thoroughly with coupling buffer prior to packing in a short chromatography column (C10/40: Pharmacia Ltd.).

2.6.3.2. Immunoaffinity processing of follicular fluids

Inhibin was removed from follicular fluid by affinity extraction using a monoclonal antibody to the 32K bovine inhibin (256H; Miyamoto *et al.*, 1986) coupled to an inert gel as described above. The extraction procedure was as follows. The column was equilibrated with a 5 times bed volume of 0.05 M phosphate buffer pH 7.5 containing 9g NaCl per litre (PBS). Typically 25 mls follicular fluid were loaded onto the column and washed through with PBS, with the protein containing fraction being retained as inhibin-free follicular fluid. The inhibin bound to the column was eluted with a 0.1 M Glycine-HCl solution pH 2.0 containing 0.01% PMSF and was

immediately neutralised with a one fifth volume of 2 M Tris-HCl pH 8.5. The column was immediately neutralised with a twice gel volume of 0.1 M Tris-HCl solution pH 9.0 and subsequently regenerated by washing with a three times gel volume of 6 M Guanidine-HCl followed by re-equilibration with 0.1 M Tris-HCl pH 9.0. The column was then stored at 4°C overnight. These latter regeneration steps are important in maintaining the efficacy of the column.

Control follicular fluid was processed through a similar immunoaffinity column to which a monoclonal antibody to chicken growth hormone (6F5; Goddard, Houston & Gray, 1987) had been coupled using the above procedure. This antibody has been extensively characterised and shown not to bind bovine growth hormone. Subsequent elution of this column failed to elicit a protein peak.

Follicular fluids were pooled and stored frozen at -20°C until required for injection.

2.7. Antibody analysis

The response of individual animals to immunisation procedures or the administration of follicular fluid proteins was monitored by double diffusion gel precipitation analysis as described by Ouchterlony & Nilsson (1978). Agar (1.5% solution w:v in 0.05 m PO_4 buffer containing 9 g NaCl per litre) was poured to just cover a glass slide (3" x 3"). After the gel had solidified, six wells (1.5 mm diameter) were punched in the agar, radial to a single similar central well, using a template and capillary tube linked to a vacuum pump. Approximately 20 μl of antigen were placed into the central well and serial dilutions of cattle serum were placed into each of the surrounding wells. The plates were then incubated overnight at room temperature in a humid environment. The following day, the plates were throughly washed and incubated overnight at room temperature in water. Lines of precipitation were subsequently visualised by staining

the gel with amido black (1% w:v solution in a 10:2:15 solution of distilled H_2O, acetic acid and methanol respectively) for two minutes followed by destaining for 24 hrs in a 5:5:1 solution of the same reagents. The antibody titre was defined as the highest dilution of antiserum required to produce a visible line of antibody:antigen precipitation.

2.8. *Electrophoresis*

The protein content of the fraction of follicular fluid retained on the anti-bovine inhibin immunoaffinity column was examined by gel electrophoresis. SDS-reduced samples were run on a pre-cast 10-15% poly-acrylamide gradient gel (PhastGel: Pharmacia Ltd.) using the PhastSystem (Pharmacia). The gel was developed by silver staining in the PhastSystem Development Unit prior to photography.

2.9. *Statistical analyses*

Data is presented as arithmetic means with standard errors unless stated otherwise. Comparisons of continuous data between treatment groups were made by Student's t-test or analysis of variance allowing for multiple measurements. Non-parametric data was compared by Mann-Whitney ranked test. Pulsatile LH secretion was analysed using a pulse detecting program (Munro: Zaristow Software) on a Macintosh. The algorithm chosen as best suited to the data was the regional coefficient of variation method of Veldhuis, Weiss, Mauras, Rogol, Evans & Johnson (1986). This method defines a pulse as a peak which exceeds the previous and following nadirs by more than a pre-defined multiple of the mean coefficient of variation of the samples immediately surrounding the peak. The majority of the statistical comparisons were performed using the Minitab statistical package (Pennsylvania State University, USA) either on a VAX mainframe or, latterly, on a Macintosh. The more complex analyses were performed on the VAX mainframe using the Genstat package (Rothamstead Experimental Station).

Chapter 3

Ovarian and endocrine effects of active immunization against porcine or ovine follicular fluid preparations in the cyclic heifer

3.1. Introduction

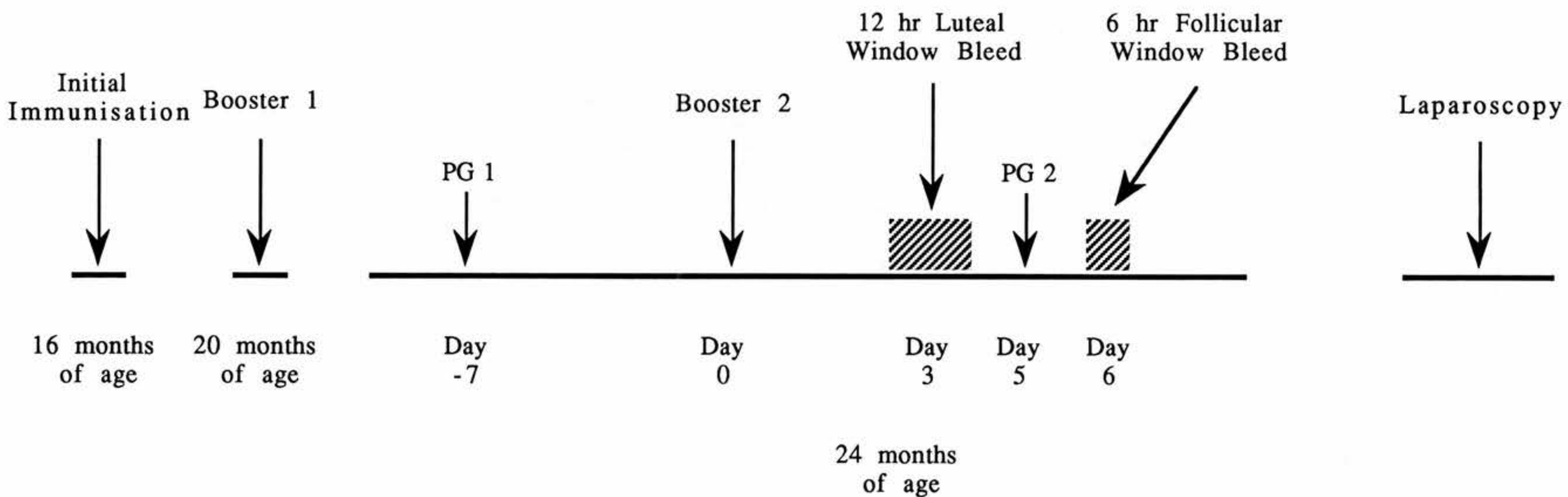
Previous studies attempting to increase the incidence of twin ovulations in cattle by immunisation against hormones involved in the ovarian negative feedback regulation of gonadotrophin secretion have met with limited success (see sections 1.3.1.3.2 and 1.4.4.1). In particular immunisation of heifers against follicular fluid proteins reportedly results either in an increase in ovulation rate with no consistent changes in gonadotrophin concentrations (Price *et al.*, 1987) or, conversely, in no change in ovulation rate despite marked disturbances of the normal pattern of gonadotrophin secretion, particularly that of LH (Price, 1987). The mechanism by which these latter effects were mediated is not clear. Therefore we undertook the following series of experiments in order to clarify the effects of such immunisation procedures on the components of LH secretion.

3.2. Materials and methods

3.2.1. Animals

A total of thirty two Hereford x Friesan heifers were used in the following studies under the conditions described in Chapter 2.

Figure 3.1 Experimental protocol



3.2.2. Preparation of antigens

The partially purified fractions of ovine and porcine follicular fluid were obtained by Red Sepharose affinity chromatography as described in Chapter 2.

3.2.3. Experiment 1

3.2.3.1. Experimental protocol

The experimental protocol is shown in Figure 3.1. Animals were assigned at random to one of two experimental groups at sixteen months of age. Animals in group 1 (n=14) were actively immunised against 4 mg of an inhibin-enriched ovine follicular fluid fraction as previously described (Price *et al.*, 1987), whilst those in group 2 (n=12) received adjuvant alone. One animal in group 2 was later determined to be a freemartin and was removed from the trial. The animals were subsequently boosted at twenty months of age and again at twenty-four months.

Seven days prior to the second booster injection, all animals received an i.m. injection of a synthetic analogue of Prostaglandin $F_{2}\alpha$ (Estrumate: ICI plc., Macclesfield) to enable synchronisation of oestrous activity. Five days after the second booster injection, all animals received a second injection of prostaglandin to induce luteolysis and were allowed to ovulate. Serial blood samples were taken every ten minutes for twelve hours during the luteal phase of the cycle, and again every ten minutes for 6 hours during the follicular phase. Ovulation rate was determined by sub-lumbar laparoscopy at a suitable time after oestrus.

3.2.3.2. Hormone Assays

3.2.3.2.1. LH Assay

Samples from this study were assayed for LH in five assays. The mean sensitivity of the assays was 0.05 ng/tube (0.5 ng/ml) and the within and

between assay coefficients of variation were 5.73% and 12.27% respectively.

3.2.3.2.2. FSH Assay

FSH was estimated in several samples from each phase of the oestrous cycle in a single assay. The sensitivity of the assay was 1.4 ng/tube (4.2 ng/ml) and the within assay coefficient of variation was 12.98%.

3.2.3.2.3 Oestradiol Assay

Oestradiol was measured in several samples taken during each phase of the oestrous cycle. The sensitivity of the assay was 1.2 pg/tube and the within assay coefficient of variation was 13.43%.

3.2.3.3 Estimation of gonadotrophin binding

The presence of gonadotrophin binding activity was measured in plasma samples from control and immunised animals. Plasma (200 μl) was incubated with assay buffer (500 μl) and ^{125}I-labelled bovine FSH (12,000 cpm in 100 μl) for 48 hrs at 4°C. Following this incubation period, antibody bound iodinated FSH was precipitated by the addition of 1 ml of a 20% solution of polyethylene glycol prior to vortexing and centrifugation at 2,000 g for 30 minutes at 4°C. The unbound FSH was decanted in the supernatant and the activity remaining in the pellet counted on a gamma counter. The binding capacity of the plasma was calculated as the percentage of the total activity added after correction for non-plasma associated binding.

3.2.4. Experiment 2

3.2.4.1. Experimental protocol

Seven animals previously immunised against an ovine ppff fraction and five control animals were ovariectomised. Four weeks later, all animals were fitted with s.c. silastic implants containing crystalline

oestradiol-17β (see Chapter 2). Blood samples were taken throughout the experiment to analyse the effect of immunisation on the steroid-mediated negative feedback regulation of gonadotrophin secretion.

3.2.4.2. Hormone Assays

3.2.4.2.1. LH Assay

All samples from this experiment were assayed at two dilutions in a single assay. The sensitivity of the assay was 0.05 ng/tube (0.25 ng/ml) and the within assay coefficient of variation was 9.33 %.

3.2.5. Experiment 3

3.2.5.1. Experimental protocol

Oestrous activity was synchronised in six animals previously immunised against a porcine ppff fraction and six controls by means of two i.m. injections of a prostaglandin $F_{2\alpha}$ analogue twelve days apart. On day 9 of the synchronised oestrous cycle all animals were fitted with indwelling jugular cannulae. The following day, blood samples were taken every fifteen minutes for two hours via the cannula. Immediately following this, all animals were challenged with a bolus i.v. injection of 25 μg GnRH (Gonadorelin: Ayerst Laboratories Ltd., Andover, Hants.) in 2 ml saline. Blood samples were then taken every ten minutes for one hour, every fifteen minutes for the next hour, every twenty minutes for the third hour and subsequently every thirty minutes for a further three hours for LH analysis. The next day, all animals were slaughtered and the pituitaries recovered and weighed. Fragments of individual pituitaries were subsequently homogenised in 0.05 M phosphate buffer and pituitary LH content estimated by radioimmunoassay.

3.2.5.2. Hormone Assay

3.2.5.2.1. LH Assay

All samples from this experiment were assayed in a single assay. The sensitivity of the assay was 0.056 ng/tube and the within assay coefficient of variation was 16.03 %.

3.3. Results

3.3.1. Experiment 1

3.3.1.1. Oestrus and ovulation rate

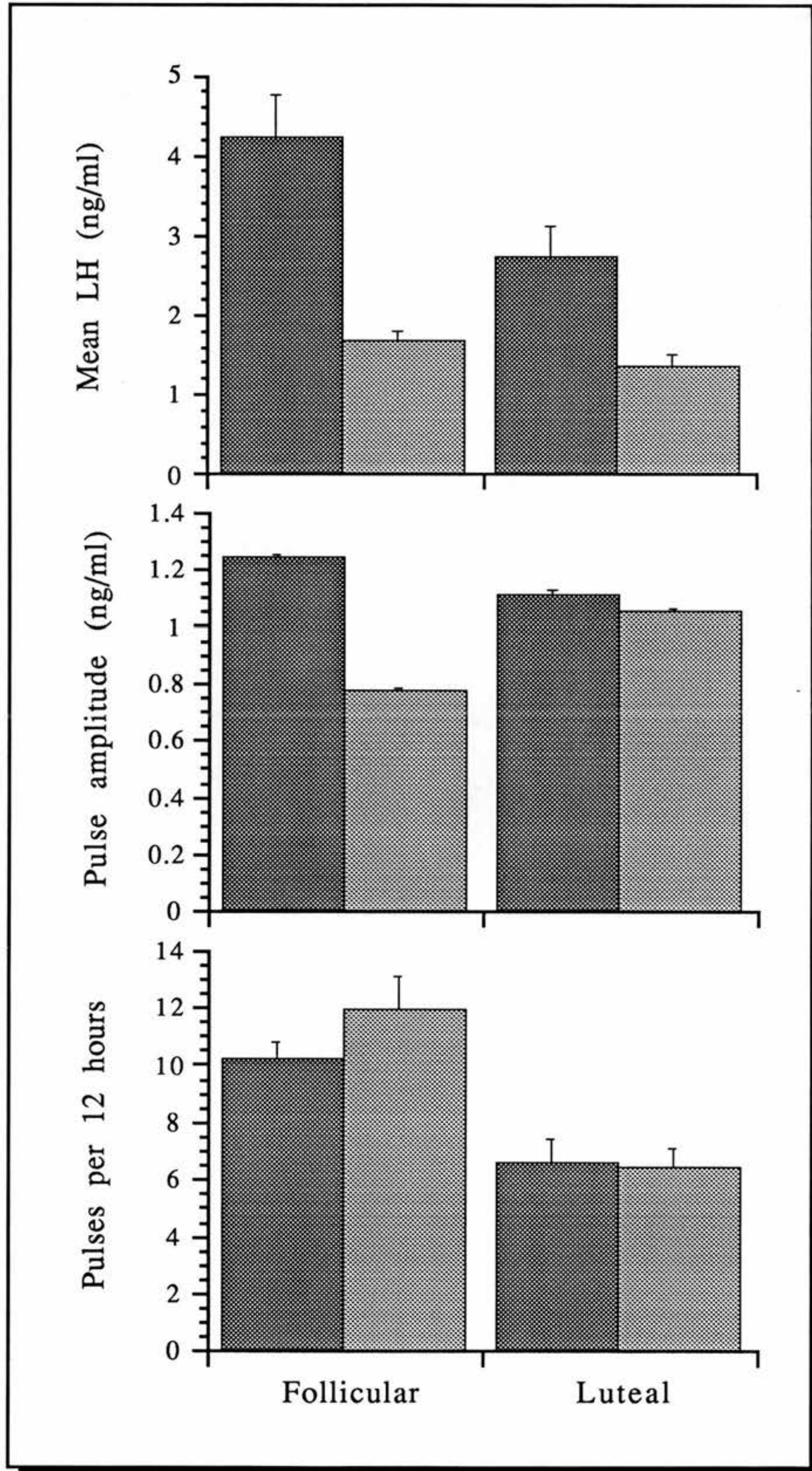
All animals continued to display oestrus regularly following immunisation. The length of the oestrous cycle was not altered by treatment. No increase in ovulation rate was observed in any animal.

3.3.1.2. LH concentrations

Mean LH concentrations in immunised and control animals during the follicular and luteal phases of the oestrous cycle are shown in Figure 3.2 (arithmetic means ± sem). The variability between animals and phases of the oestrous cycle were such that the data were transformed logarithmically for the purposes of statistical analysis. Mean LH concentrations were significantly elevated in immunised animals ($p < 0.001$) during both phases of the oestrous cycle. Follicular phase LH concentrations were also higher than luteal phase concentrations in both treatment groups ($p < 0.005$). Although the analysis of variance suggested that the differences in gonadotrophin concentrations between follicular and luteal phases were not significantly affected by treatment, this was confounded by large between animal variation which remained after transformation. A comparison of the absolute differences between follicular and luteal phase concentrations within individual animals demonstrated that the increase in LH concentrations during the follicular phase was significantly greater in the immunised animals ($P < 0.005$). The

Figure 3.2

Mean LH concentration, pulse amplitude and pulse frequency during the luteal and follicular phases of the oestrous cycle in control animals (▨) and animals immunised against an ovine follicular fluid preparation (▩)



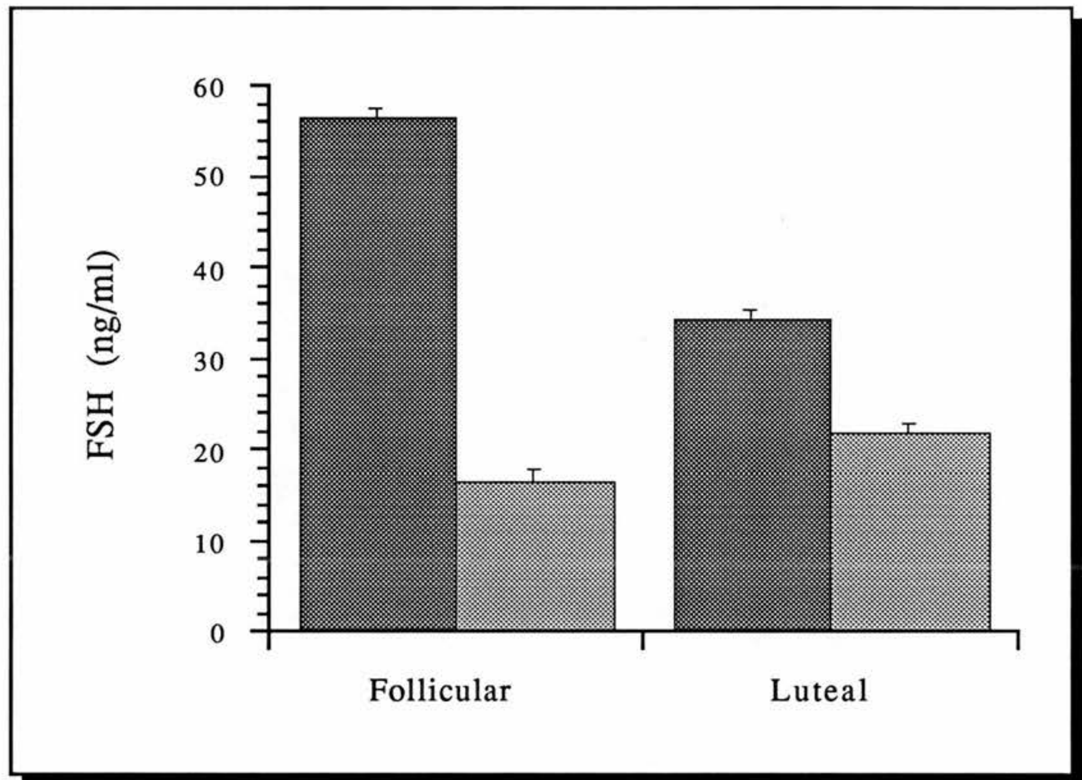
increase in LH concentrations in immunised animals appeared to be due to an increased basal secretion, rather than any change in pulsatile secretion as there was no difference between treatment groups ($p > 0.05$) in either pulse amplitude or frequency during the luteal phase of the oestrous cycle. Pulse amplitude was significantly greater in immunised than control heifers during the follicular phase ($p < 0.005$). However, there were no differences between groups in LH pulse frequency ($p > 0.05$) during this phase of the oestrous cycle.

Pulse frequency was significantly higher during the follicular phase than during the luteal phase in both immunised ($p < 0.005$) and control heifers ($p < 0.001$). However, although pulse amplitude was significantly reduced in control animals during the follicular phase when compared to the luteal phase ($p < 0.05$), there was no significant difference in pulse amplitude between phases in immunised animals ($p > 0.05$).

3.3.1.3. FSH concentrations

Mean FSH concentrations in immunised and control animals during the follicular and luteal phases of the oestrous cycle are shown in Figure 3.3 (arithmetic means ± sem). Again, the large variability between animals and phases of the oestrous cycle necessitated the logarithmic transformation of the data prior to statistical analysis. Mean FSH concentrations were significantly elevated in immunised animals ($p < 0.001$) during both phases of the oestrous cycle. However, the difference was most marked during the follicular phase. There was also a highly significant interaction between treatment and phase of the oestrous cycle ($p < 0.01$). This resulted in an altered pattern of FSH concentrations across the oestrous cycle in immunised animals; FSH levels were significantly elevated during the follicular phase when compared to the luteal phase in immunised animals ($p < 0.05$) whereas in control animals the reverse was the case (luteal > follicular; $p < 0.05$).

Figure 3.3 Mean FSH concentrations during follicular and luteal phases of the oestrous cycle in control animals (▩) and animals immunised against a protein fraction of ovine follicular fluid (▩).



3.3.1.4 Oestradiol concentrations

Mean oestradiol concentrations in control and immunised animals during the luteal and follicular phase of the oestrous cycle are shown in Table 3.1. There was no difference in oestradiol concentrations between treatment groups in either phase of the cycle ($p > 0.05$).

Table 3.1 The effect of immunisation against an ovine follicular fluid preparation on mean oestradiol concentrations.

	Oestradiol Concentrations (pg/ml)	
	Luteal	Follicular
Control	5.57 ± 0.411	8.95 ± 1.549
Immunised	5.53 ± 0.504	11.51 ± 2.237

3.3.1.5 Gonadotrophin binding

There was no significant difference in the gonadotrophin binding capacity of plasma from control or immunised animals (3.96 ± 0.40% vs 4.77 ± 0.64%, control vs. immunised; $p > 0.05$)

3.3.2. Experiment 2

3.3.2.1. LH concentrations

The LH data from this experiment are summarised in Table 3.2. Mean LH concentrations were significantly higher in immunised animals than in controls ($p < 0.005$) prior to treatment. Following ovariectomy, LH concentrations rose in all animals to levels significantly higher than those seen in intact animals ($p < 0.001$). However, the peak LH concentration following ovariectomy did not differ between treatment groups ($p > 0.05$). Mean serum LH concentrations 18 days after implant

insertion were significantly higher in immunised animals than controls ($p < 0.01$).

Table 3.2 The effect of ovariectomy and subsequent insertion of a silastic implant containing oestradiol on mean LH concentrations in control animals and animals previously immunised against a protein fraction of ovine follicular fluid. Within columns, differing superscripts indicate significant differences (a vs b; $p < 0.05$: x vs y; $p < 0.01$)

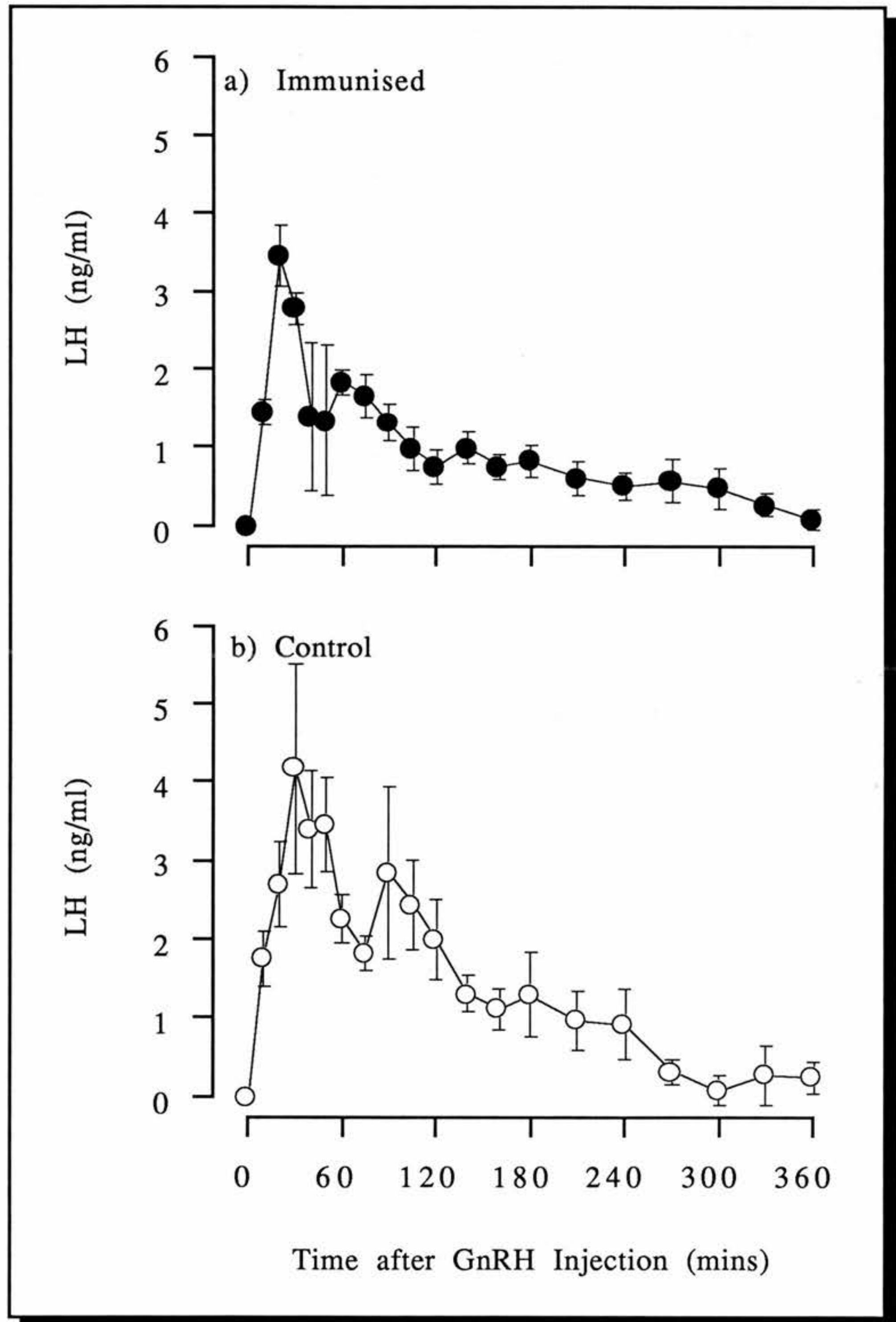
	Mean LH (ng/ml)		
	Before Ovariectomy	After Ovariectomy	After Implant Insertion
Control	0.47 ± 0.05^{a}	3.77 ± 0.92	0.68 ± 0.09^{x}
Immunised	0.96 ± 0.13^{b}	3.43 ± 0.46	1.50 ± 0.51^{y}

3.3.3. Experiment 3

3.3.3.1. Response to GnRH

Data for this study were transformed logarithmically prior to analysis to reduce between animal variability. Although the last booster immunisation injection was administered some four months prior to the execution of this study, basal LH concentrations remained significantly higher in immunised than control animals ($p < 0.05$). However, the response to a single injection of 25 µg synthetic GnRH did not differ between treatment groups ($p > 0.01$) neither in the amplitude of the resulting peak of release nor in the total area under the LH profile curve after correction for initial baseline. The mean LH profiles in response to GnRH injection are shown in Figure 3.4.

Figure 3.4 Mean response to a single injection of 25μg synthetic GnRH at time 0 in animals immunised against porcine follicular proteins (a) and control animals (b).



3.3.3.2. Pituitary LH content

A summary of the mean pituitary weights and LH contents are shown in Table 3.3. There was no significant difference in pituitary LH content whether expressed in terms of µg LH per mg wet weight or as total pituitary content. Pituitary weight did not differ between treatment groups

Table 3.3 Summary of mean pituitary weight and pituitary LH content in control animals and animals previously immunised against a protein fraction of ovine follicular fluid.

	Pituitary weight (g)	Pituitary LH content (µg LH/mg wet wt.)	Total LH content (mg)
Control	2.324 ± 0.134	22.83 ± 5.085	51.63 ± 13.01
Immunised	2.038 ± 0.122	21.98 ± 5.061	44.86 ± 10.81

3.4. Discussion

Immunisation of heifers against either a partially purified follicular fluid preparation of porcine or ovine origin resulted in marked increases in immunoactive gonadotrophin concentrations. Similar effects have previously been reported following immunisation against a porcine follicular fluid preparation (Price, 1987). Another earlier study in which heifers were immunised against an ovine preparation reported transient changes in gonadotrophin concentrations in some animals and increased ovulation rate in a proportion of heifers (Price *et al.*, 1987). No such increases in ovulation rate were observed in this study, in spite of the marked increases in FSH and LH. Furthermore, no changes in peripheral oestradiol levels were observed as a result of the elevated immunoassayable gonadotrophin concentrations.

The changes in gonadotrophin secretion were particularly marked during the follicular phase of the oestrous cycle. Indeed, neither LH pulse

amplitude nor frequency were altered in immunised animals during the luteal phase, the difference in mean LH concentrations between treatment groups apparently being due solely to an elevated basal secretion during this period. In addition, no significant difference in the LH response to a single i.v. injection of synthetic GnRH during the luteal phase of the oestrous cycle was observed between animals immunised against a porcine immunogen and controls.

Differences between treatment groups were abolished following ovariectomy, but were restored after the subsequent insertion of a s.c. oestradiol implant, suggesting that the observed differences were the result of a disturbance of oestradiol-mediated negative feedback. However, the differences in peripheral gonadotrophin concentrations were not reflected in changes in either pituitary size or gonadotrophin content in porcine immunised animals.

The initial intention of immunisation against follicular fluid preparations was to eliminate inhibin-mediated negative feedback regulation of FSH and hence increase ovulation rate (see sections 1.3.1.3.2 and 1.4.4 above). The increase in FSH concentrations in immunised animals is consistent with this aim. However, the observed changes in LH concentrations suggest that the effect of immunisation is not a direct inhibin-mediated phenomenon, given that exogenous steroid-free follicular fluid does not suppress LH concentrations in ovariectomised heifers or sheep, unless administered at high doses (cattle: Ireland, Curato & Wilson, 1983; Kiracofe *et al.* 1983; Beard *et al.*, 1988,1989,1990; sheep: Cummins *et al.*, 1983; Findlay, Gill & Doughton, 1985; Clarke *et al.*, 1986; Martin, Taylor & McNeilly, 1987; Knight & Castillo, 1988), and the definition of inhibin precludes any such major effects on LH secretion (Burger & Igarashi, 1988). The mechanism by which gonadotrophin concentrations are elevated by the above immunisation protocol

therefore seems likely to involve phenomena other than the neutralisation of direct effects of inhibin on the pituitary gland.

The possibility exists that the observed changes in gonadotrophin concentrations are the result of antibodies raised in response to components of the follicular fluid preparation (e.g. porcine or ovine gonadotrophins, FSH binding inhibitors [see section 1.3.6] or gonadotrophin receptor fragments [see section 1.3.7]) which may be interfering in the immunoassays employed in this laboratory and hence resulting in artificially elevated immunoactive LH or FSH levels. However, this seems unlikely for several reasons. Firstly, although the immunoactive gonadotrophin contents of the follicular fluid preparations used as antigens in this study were not measured, similar preparations utilised in previous experiments contained negligible LH/FSH activity (Price, 1987). Secondly, samples from immunised animals assayed at multiple dilutions did not depart significantly from parallelism with the standard curve and no significant increase in gonadotrophin binding could be detected in plasma from immunised animals. Thirdly, if such antibody interference were responsible for the elevated gonadotrophin concentrations then it would be expected that the differences in LH and FSH concentrations between immunised and control animals would remain relatively constant throughout the oestrous cycle. This was clearly not the case; LH pulse amplitude was significantly greater in immunised than in control animals during the follicular phase, the difference in mean LH concentrations between groups was considerably greater during the follicular phase and the changes in FSH secretion between luteal and follicular phase were divergent between groups. Therefore, it would seem that the changes in gonadotrophin secretion observed in immunised animals reflect a genuine disruption of some component of ovarian negative feedback. In

addition, the observed differences in mean LH concentrations were abolished following ovariectomy, further suggesting that immunisation had affected some aspect of the normal physiological negative feedback regulation of gonadotrophin secretion. Following the insertion of oestradiol-containing silastic capsules, the differences were restored. Therefore, the effect of immunisation most probably involves a reduction in the ability of the pituitary gland or hypothalamus to "read" the ovarian oestradiol negative feedback signal.

This hypothesis is entirely consistent with the changes in the patterns of gonadotrophin secretion observed in the luteal and follicular phases of the oestrous cycle in Experiment 1. Oestradiol reportedly reduces mean LH and FSH concentrations and LH pulse amplitude in both sheep (Goodman *et al.*, 1980; Goodman & Karsch, 1980; Clarke, Funder & Findlay, 1982; Karsch *et al.*, 1983; Martin, Scaramuzzi & Henstridge, 1983) and cattle (Butler *et al.*, 1983; Price & Webb, 1988). Conversely, progesterone reduces mean gonadotrophin concentrations and LH pulse frequency (sheep: Karsch *et al.*, 1977,1978; Hauger, Karsch & Foster, 1977; Goodman & Karsch, 1980; cattle: Ireland & Roche, 1982b; Imakawa *et al.*, 1986; Price & Webb, 1988). During the luteal phase of the oestrous cycle, in the presence of elevated progesterone concentrations, differences between gonadotrophin concentrations in immunised and control animals were restricted to differences in mean concentrations alone. In the absence of progesterone, during the follicular phase of the oestrous cycle, LH pulse frequency was elevated and pulse amplitude was decreased in control animals as oestradiol concentrations rose. Similarly mean FSH concentrations fell in response to the increased oestradiol concentrations in these animals. However, in immunised animals, although LH pulse frequency was increased following luteolysis, LH pulse amplitude was not reduced and mean LH concentrations rose accordingly. FSH

concentrations also rose in immunised animals. These data are consistent with a selective loss of responsiveness to oestradiol negative feedback.

However, in spite of the markedly increased peripheral concentrations of both LH and FSH, no change in ovarian oestradiol production or ovulation rate was observed. This may reflect either an alteration in ovarian sensitivity, or a reduction in the bioactivity of the observed immunoactive gonadotrophins. Indeed, there are reports available in the literature which detail alterations in the bioactivity of plasma LH and FSH in several reproductive states and in several species. Of particular interest are the observations that gonadotrophins released in response to GnRH are preferentially enriched in bioactive forms of FSH and LH, and that oestradiol treatment of ovariectomised animals increases the proportion of bioactive gonadotrophin released by the pituitary gland (Tesone, Ladenheim, Cheb-Terrab, Chiauzzi, Solano, Podesta & Charreau, 1986; Veldhuis, Johnson & Dufau, 1987; Kessel, Dahl, Kazer, Liu, Rivier, Vale, Hsueh & Yen, 1988; Padmanabhan, Kelch, Sonstein, Foster & Beitins, 1988a; Padmanabhan, Lang, Sonstein, Kelch & Beitins, 1988b; Fauser, Soto, Czekala & Hsueh, 1989; Padmanabhan, Ebling, Sonstein, Fenner, Kelch, Foster & Beitins, 1989). It is therefore not inconceivable that the putative loss of the ability of the pituitary gland to respond to oestradiol in immunised animals may have resulted in an increase in basal, immunoactive, but biologically inactive, gonadotrophin secretion whilst the release of preferentially bioactive-enriched gonadotrophin in response to endogenous GnRH pulses remained sufficient to ensure normal ovarian function.

Several authors have also reported that ovariectomy results in a change in the metabolic clearance rate of the gonadotrophins in a wide range of species including sheep (Montgomery *et al.*, 1984; Fry *et al.*, 1987), monkeys (Peckham & Knobil, 1976), rats (Weick, 1977) and

bullfrogs (McCreery & Licht, 1983), an effect which may be reversed by the administration of exogenous oestradiol (Peckham & Knobil, 1976). It is therefore tempting to speculate that the large differences in peripheral basal gonadotrophin concentrations observed in immunised heifers may be due, in part, to a similar gross increase in the half lives of the gonadotrophins resulting from the presumed loss of sensitivity to oestradiol. This theory clearly merits further study, but may also explain why the changes in peripheral gonadotrophin concentrations were not reflected in any change in pituitary gonadotrophin content. Preliminary results also suggest that no difference in the level of gonadotrophin gene expression exists between immunised and control heifers (A. Turzillo, A.S. Law, R. Webb & J.R. McNeilly; unpublished results). However, if the major differences between the rapidly-cleared bioactive and more persistent inactive forms of the gonadotrophins involve differential glycosylation of the mature protein, then differences in pituitary gland gonadotrophin concentration and messenger RNA are not essential pre-requisites for elevated peripheral immunoactive gonadotrophin concentrations.

This study has confirmed the observation that immunisation against a relatively crude ovarian follicular fluid protein may result in increases in LH as well as FSH (Price, 1987). Furthermore, we have extended the initial observations and demonstrated that this phenomenon most probably involves a selective loss of pituitary gland responsiveness to oestradiol. This therefore implies that, in the normal heifer, the pituitary gland sensitivity to oestradiol is regulated by ovarian protein factors. This may in turn explain the loss of pituitary gland sensitivity to steroids in long term ovariectomised animals (Karsch, Legan, Hauger & Foster, 1977; Webb, Baxter, Preece, Land & Springbett, 1985). The physiological significance of such a regulatory system is unclear. However, it may

serve to "protect" the gonadotrophin negative feedback control system from the undue influence of non-ovarian steroids by the obligate involvement of an ovary specific product. Any lack of gonadotrophic support, due to high concentrations of non-ovarian steroids would result in a lack of ovarian protein secretion and hence a reduction in the ability of pituitary gland to respond to the steroid milieu. Consequently gonadotrophin concentrations would rise to allow normal ovarian function. Alternatively, such a protein may act to subtly regulate the isoforms of gonadotrophins secreted to optimise follicular development in an as yet undefined manner. However, these theories must remain mere speculation pending further study.

In conclusion, ovulation rate in heifers was not increased in response to immunisation against ovarian follicular proteins of porcine or ovine origin. In contrast, immunisation markedly elevated gonadotrophin secretion. The observed changes in the patterns of gonadotrophin secretion appear to involve a selective loss of pituitary gland responsiveness to oestradiol. The role of follicular fluid proteins in the functioning of the ovarian-pituitary/hypothalamus negative feedback system is more complicated than previously thought and clearly merits much further study.

CHAPTER 4

The effect of administration of a partially purified bovine follicular fluid preparation on FSH secretion and ovarian function

4.1. Introduction

Inhibin activity has been identified in the follicular fluid of many species, including cattle (de Jong & Sharpe, 1976), pigs (Schwartz & Channing, 1977) and sheep (Scott *et al.*, 1980). Indeed, follicular fluid from a variety of species has been used as a concentrated source of inhibin. In particular, bovine follicular fluid has been used to suppress FSH concentrations in intact and ovariectomised ewes (McNeilly, 1984,1985; Wallace & McNeilly, 1986; Clarke *et al.*, 1986; Findlay, Robertson & Clarke, 1987) and ovariectomised heifers (Ireland, Curato & Wilson, 1983; Beard *et al.*, 1988,1989,1990).

Although the role of inhibin in the control of FSH secretion in the sheep has been described (Martin *et al.*, 1986,1988), the situation in cattle is less clear. Some authors report that bovine follicular fluid suppresses FSH concentrations in intact heifers (Quirk & Fortune, 1986), but others have failed to demonstrate any such reduction (Johnson & Smith, 1985). Work involving ovariectomy and steroid hormone implants has suggested that combinations of oestradiol and progesterone alone are sufficient to account for cyclic changes in gonadotrophins in cattle (Price & Webb, 1988), particularly during the luteal phase. However, immunisation against follicular fluid proteins results in a marked disturbance in the

patterns of gonadotrophin secretion indicating that such proteins do indeed play some role in this negative feedback (see Chapter 3). However, further investigations have previously been hampered by the lack of a sufficiently sensitive assay for FSH and problems associated with the production of the large quantities of inhibin required for detailed studies.

Furthermore, many previous studies in both sheep and cattle have employed large doses of crude follicular fluid preparations which contained a considerable number of other proteins which must be assumed to play some role in reproductive function. These treatment regimes subsequently resulted in a gross disturbance of ovarian function. It therefore seemed necessary to delineate the response of cattle to lower, possibly more physiological doses of purer preparations.

The increase in sensitivity brought about by the modification of an existing specific radioimmunoassay for bovine FSH (see Chapter 2) allowed us to examine the effects of direct administration of low doses of follicular fluid proteins on FSH secretion and ovarian function in the intact heifer.

4.2. Materials and Methods

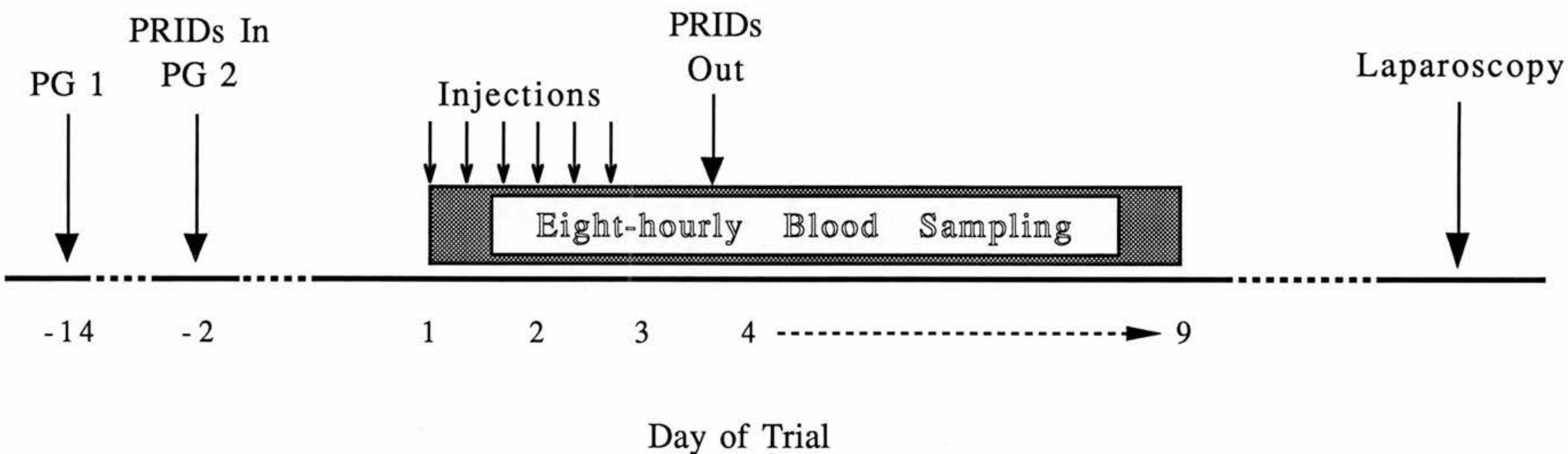
4.2.1. Animals

Eighteen Hereford x Friesan heifers were used in this study. Husbandry conditions were as described in Chapter 2.

4.2.2. Preparation of partially purified follicular fluid (ppff) fractions.

A pooled fraction of bovine follicular fluid was prepared by affinity chromatography on Red Sepharose as described in Chapter 2. Measurement of the inhibin-like activity of this bovine ppff fraction, as assessed by an *in vitro* bioassay based on the suppression of basal FSH secretion by cultured ovine pituitary cells confirmed that the

Figure 4.1 Experimental Protocol



purification process had concentrated the inhibin entity approximately 10-fold (138 u./mg protein vs. 1225 u./mg protein; Follicular fluid vs. ppff).

4.2.3. Protocol

The experimental protocol is detailed in Figure 4.1. Oestrous activity in all animals was synchronised using two i.m. injections of a potent prostaglandin $F_{2\alpha}$ analogue, cloprostenol (Estrumate: I.C.I. Ltd.) given twelve days apart. At the time of the second injection, all animals were fitted with a progesterone releasing intra-vaginal device (PRID: CEVA UK Ltd., Watford, UK), with the oestradiol capsule removed, and were assigned at random to one of three experimental groups. The use of PRIDs was designed firstly to reduce the between animal variation in progesterone concentrations and secondly to ensure that each animal was exposed to progesterone for a period similar to the luteal phase of the normal oestrous cycle. Blood samples (10 ml) were taken by direct venepuncture at eight hourly intervals (07.00 hrs, 15.00 hrs and 23.00 hrs) into heparinised tubes for nine days commencing three days after PRID insertion. Immediately prior to all blood samples on the first and second days of sampling, animals received an i.v. injection containing 0 mg (Control Group; n=6), 14 mg (Group 1; n=6) or 28 mg (Group 2; n=6) ppff protein in 10 mls physiological saline. The PRIDs were removed 24 hrs after the last injection and heats were detected with the aid of a commercial heat detection device (Kamar Tags: Kamar Inc., Steamboat Springs, Colorado). Ovulation rate was measured by sub-lumbar laparoscopy, as described in Chapter 2, during the luteal phase of the subsequent oestrous cycle.

4.2.4. Hormone Assays

4.2.4.1. FSH Assay

All samples in this study were assayed in a single assay. The sensitivity of the assay, defined as the dose of standard required to significantly suppress binding of radiolabelled tracer to antibody was 0.75 ng/tube (3 ng/ml), and the within assay coefficient of variation calculated over the whole assay was 11.62%.

4.2.4.2. Oestradiol Assay

All samples were assayed in a single assay, with a mean extraction efficiency of 71.95% ± 0.014 (sem). The sensitivity of the assay was 1.24 pg/tube and the within assay coefficient of variation calculated over the whole assay was 15.6%.

4.2.4.3 Progesterone Assay

All samples were assayed in a single assay. The sensitivity of the assay was 7.8 pg/tube (0.156 ng/ml) and the within assay coefficient of variation calculated over the whole assay was 10.29%.

4.2.5. Statistical analysis

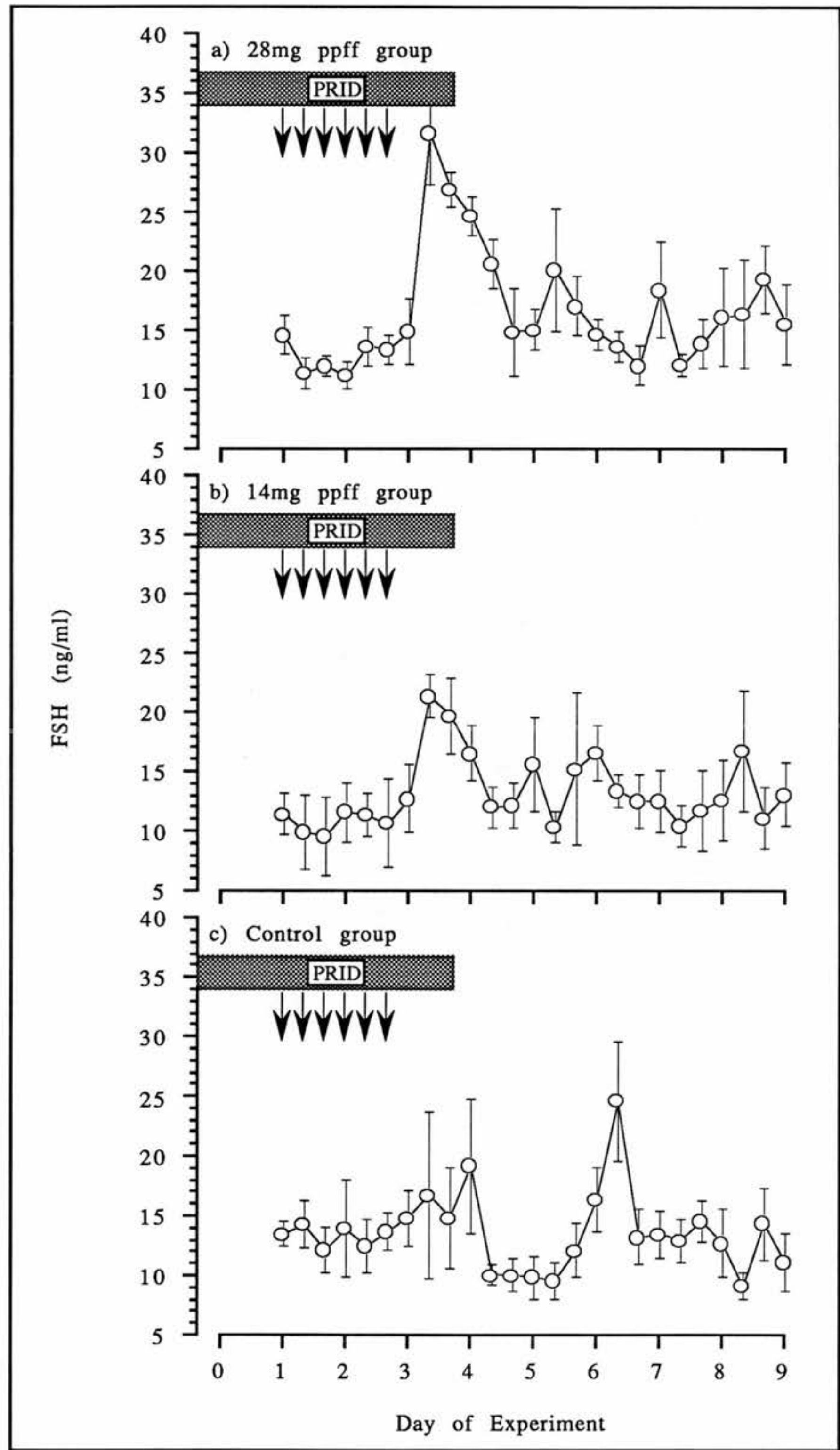
The FSH data are presented as arithmetic means (± sem). Comparisons between treatment groups and phases were carried out by analysis of variance, allowing for repeated measurements. Mean progesterone and oestradiol concentrations were compared by Student's t-test.

4.3. Results

4.3.1. Oestrus and Ovulation rate

Following PRID removal, all animals exhibited behavioural oestrus within four days and subsequently ovulated. However, no incidence of increased ovulation rate was observed in any animal, although one animal in the 28 mg group had what appeared to be a partially luteinised

Figure 4.2 Mean FSH profiles from animals treated with 28 mg (a) or 14 mg (b) bovine ppff proteins or saline (c). The error bars represent the standard error of each mean point. The arrows indicate the timing of injections. The period of PRID treatment is signified by the shaded bar.



large follicle on one ovary at laparoscopy in addition to a single normal corpus luteum.

4.3.2. FSH concentrations

The profile of FSH concentrations in each of the three treatment groups is shown in Figure 4.2. There was a trend for FSH concentrations to fall during both the pre-treatment and treatment period in all animals, including those in the control group, presumably due to endogenous ovarian events being synchronised in all the animals by the combination of prostaglandin and PRID treatment. However, although this reached statistical significance within each treatment group ($p < 0.05$) when compared to the pretreatment concentrations, there was no significant difference between the treatment and control groups during the treatment period. Following cessation of the ppff administration, FSH concentrations "rebounded" in a dose-dependent manner, reaching significantly higher levels than controls in both experimental groups ($p < 0.01$), and then rapidly returned to control levels. FSH concentrations in control animals remained relatively static throughout this period then tended to fall immediately after PRID removal. No significant differences were observed between groups at any other time points during the experiment.

4.3.3. Oestradiol concentrations

The oestradiol concentrations in each treatment group are shown in Table 4.1. There was no significant difference in oestradiol concentrations between groups at any point pre-, during or post- ppff administration.

Table 4.1 Mean oestradiol concentration (pg ± sem) before, during and after the treatment period in control animals and animals treated with 14 or 28 mg bovine ppff proteins. Within rows, different superscripts indicate significant differences ($p < 0.05$). Differences between treatment groups within treatment phases were not significant ($p > 0.05$)

	Oestradiol concentrations (pg/ml)		
	Pre-Treatment	During Treatment	Post-Treatment
Control	5.83 ± 0.37^{a}	6.58 ± 0.46^{ab}	8.27 ± 0.76^{b}
14 mg ppff	6.05 ± 0.44^{xy}	5.28 ± 0.45^{x}	6.96 ± 0.59^{y}
28 mg ppff	5.56 ± 0.57	7.11 ± 1.80	5.99 ± 0.66

4.3.4. Progesterone concentrations

There was no significant difference in progesterone concentrations between groups at any time point. Mean progesterone concentrations (± sem) during PRID treatment were 3.64 ± 0.33, 3.56 ± 0.28 and 4.68 ± 0.69 ng/ml for control, 14 mg and 28 mg groups respectively. Progesterone concentrations fell rapidly in all animals immediately following PRID removal. The period of PRID treatment during which progesterone concentrations were elevated (> 3.0 ng/ml) is represented by the shaded area in Figure 4.2.

4.4. Discussion

The i.v. administration of a partially purified inhibin-enriched fraction of steroid-free bovine follicular fluid failed to suppress plasma FSH concentrations below those observed in saline-treated controls. Likewise, the treatment failed to influence the length of time from PRID removal to oestrus or subsequent ovulation rate, despite eliciting a significant, dose-dependent rebound of plasma FSH concentrations following cessation of injections.

Although levels of FSH in both ppff-treated groups during the treatment period were significantly lower than pretreatment means ($p < 0.05$), at no point was there a significant difference between controls and treated animals. This suggests that any effect of treatment was being masked by endogenous negative feedback which was presumably synchronised in all animals by the PG/PRID treatment. This is consistent with the observations of other workers. Quirk & Fortune (1986) failed to suppress FSH in intact heifers with 10 mls charcoal-treated bovine follicular fluid given twice daily for three days, but twice this dose reportedly suppressed FSH by nearly 30%. Johnson & Smith (1985) similarly failed to suppress FSH secretion in heifers with three-times daily 8 ml injections of a similar bFF preparation. The doses of ppff used in this study were approximately equivalent to 2.5 and 5 mls whole follicular fluid three times daily in terms of inhibin bio-activity. One possible explanation may be that the frequency of blood sampling was too low to demonstrate short-term changes in FSH secretion. However this is unlikely since Quirk & Fortune (1986) took blood samples every 6 hours and still failed to demonstrate any suppression of FSH. In addition, studies in ovariectomised heifers with more frequent blood sampling regimes showed a continuous suppression of peripheral FSH concentrations throughout the entire treatment period (Ireland, Curato & Wilson, 1983). There are reports of a developing refractoriness to follicular fluid treatment in sheep (Miller, Critser & Ginther, 1982; McNeilly, 1984) and monkeys (Channing *et al.*, 1982) which has been attributed to a reduction in oestradiol ovarian negative-feedback or a developing immune response to repeated administration. However, there were no between-treatment differences in oestradiol concentrations observed during this study and the short time scale of the experiment make an immune response unlikely. In addition, no evidence of antibody production was

obtained by Ouchterlony analysis of serum samples (data not shown). The lack of suppression of FSH in this study is therefore unlikely to involve similar phenomena. Seemingly the most likely explanation for this failure is that the bovine pituitary is relatively insensitive to inhibin as suggested by others (Johnson & Smith, 1985; Price & Webb, 1988), particularly to the low doses used in this experiment.

It is therefore difficult to account for the "rebound" hypersecretion of FSH concentrations following treatment in the absence of any prior suppression. Clearly, the sudden removal of the exogenous source of ovarian proteins at the end of treatment rendered the animals temporarily deficient in some component of the pituitary-ovarian negative feedback system. Some authors have suggested that the rebound phenomenon following follicular fluid treatment is due to a lack of endogenous inhibin and oestradiol resulting from a suppression of follicular development (Miller, Critser & Ginther, 1982; Wallace & McNeilly, 1985; Henderson *et al.*, 1986; Wallace, Martin & McNeilly, 1988). However, in this study there was no lack of oestradiol production, nor was there any effect on the length of time to oestrus, or on ovulation (rate) following treatment, suggesting that follicular function was not impaired. It is possible that the proposed suppressive action of the ppff may have been specific to the production of ovarian proteins through an auto-regulatory mechanism, resulting in a deficiency of endogenous follicular protein(s) following treatment. Further studies would be required to demonstrate such a phenomenon.

Alternatively, the mode of action of the ppff treatment may be via a direct effect at the level of the pituitary gland. Recent reports have shown that inhibin acts to selectively suppress the intra-pituitary levels of mRNA encoding the β-subunit of FSH immediately following treatment of ovariectomised sheep (Mercer *et al.*, 1987) and heifers (Beard *et al.*,

1989), but there is no data available for the later post-treatment period when peripheral FSH concentrations may be expected to be rising. It is also possible that the situation in the ovariectomised heifer may differ from that in the intact animal given that no "rebound" of FSH concentrations is observed in ovariectomised heifers (Ireland, Curato & Wilson, 1983; Beard *et al.*, 1990). It has been suggested that the effect of follicular fluid treatment may be to cause a "build up" of unsecreted FSH in the pituitary gland which is available for release upon cessation of treatment (Miller, Critser & Ginther, 1982; Wallace & McNeilly, 1985; Wallace, Martin & McNeilly, 1988). However, the available evidence suggests that the effect of follicular fluid treatment is on the synthesis, rather than the release of FSH in that follicular fluid treatment does not influence pituitary gland FSH content (Beard *et al.*, 1989). In addition, the lack of any measurable suppression of peripheral FSH concentrations during this study argues against any such diversion of FSH from release to storage.

It is possible that the ppff-treatment acted to desensitise the pituitary gland, rendering the animal incapable of responding to its endogenous negative feedback following withdrawal of the exogenous source. This effect could also be involved in the refractoriness to follicular fluid treatment described in sheep (Miller, Critser & Ginther, 1982; McNeilly, 1984). Identification of an inhibin receptor and examination of the effect of treatment on the sensitivity of the pituitary gland to steroid negative feedback would allow this eventuality to be further investigated.

Price & Webb (1988) have questioned the role of inhibin in the control of plasma FSH concentrations in cattle, particularly during the luteal phase; the lack of suppression of FSH levels below those in control animals would tend to support this conclusion. However, the significant dose-dependent rebound following treatment clearly indicates a

physiological action of ovarian proteins in some component of gonadotrophin secretion, particularly in view of the still elevated progesterone concentrations in the animals at this time. The role of inhibin in the intact heifer therefore remains unresolved.

There was no incidence of increased ovulation rate in any treatment group despite rebound levels of FSH in excess of twice those seen in controls in certain individuals. This is in stark contrast to results from studies in sheep where consistent increases in ovulation rate are observed following similar treatments (McNeilly, 1985; Wallace & McNeilly, 1985; McNeilly & Wallace, 1987; Henderson *et al.*, 1986). This suggests that either the timing of the FSH rebound in relation to the removal of the PRID was inappropriate, or that the cow does not primarily regulate its ovulation rate through negative feedback control of FSH concentrations. Work in this laboratory has previously shown that cattle immunised against partially purified follicular fluid preparations from various species may display multiple ovulations with levels of FSH and LH within the normal range (Price *et al.*, 1987). Similar results have also been reported for sheep (Henderson *et al.*, 1984b; Al-Obaidi *et al.*, 1987a). Conversely, a separate group of heifers immunised against similar follicular fluid preparations failed to increase their ovulation rate despite highly significant increases in gonadotrophin concentrations (see Chapter 3) further suggesting that gonadotrophin concentrations *per se* are not the primary controllers of ovulation rate in this species.

In conclusion, the doses of partially purified steroid-free bovine follicular fluid utilised in this study were incapable of suppressing luteal phase concentrations of FSH in the presence of a competent endogenous negative feedback system. Although treatment resulted in a significant dose-related rebound of FSH following withdrawal, ovarian function was not altered by treatment. The role of inhibin in the normal bovine oestrous cycle remains to be determined.

CHAPTER 5

The effect of administration of low doses of partially purified bovine, ovine or porcine follicular fluid preparations on LH and FSH secretion and ovarian function

5.1. Introduction

In addition to the well-documented effects of bovine follicular fluid on FSH secretion, Wallace & McNeilly, (1986) have also demonstrated an increase in LH pulse amplitude during treatment of ewes with bovine follicular fluid. This they attributed to a decrease in oestradiol secretion due to an inhibin-induced reduction of peripheral FSH concentrations, although oestradiol concentrations were not reported. Other authors have also reported effects of follicular fluid treatment on LH secretion in long-term ovariectomised ewes (Cummins *et al.*, 1983; Findlay, Gill & Doughton, 1985), although the doses of follicular fluid required to elicit any effect were high and have been dismissed as pharmacological (Martin, Taylor & McNeilly, 1987). In the previous chapter, we demonstrated that changes in FSH secretion may be induced with no change in ovarian function and oestradiol concentrations. Furthermore, in Chapter 3, it was shown that immunisation against a follicular fluid preparation induced profound effects on both LH and FSH secretion. It was therefore of interest to investigate the effect of direct administration of ppff proteins on LH secretion. In addition, we extended our observations of the effects of bovine ppff proteins on FSH secretion (see Chapter 4) by investigating

the effects of similar ppff proteins from pig and sheep, two species with largely differing ovulation rates

5.2. Materials and Methods

5.2.1. Animals

A total of twenty-eight Hereford x Friesan heifers were used in the two studies. Husbandry conditions were as described in Chapter 2.

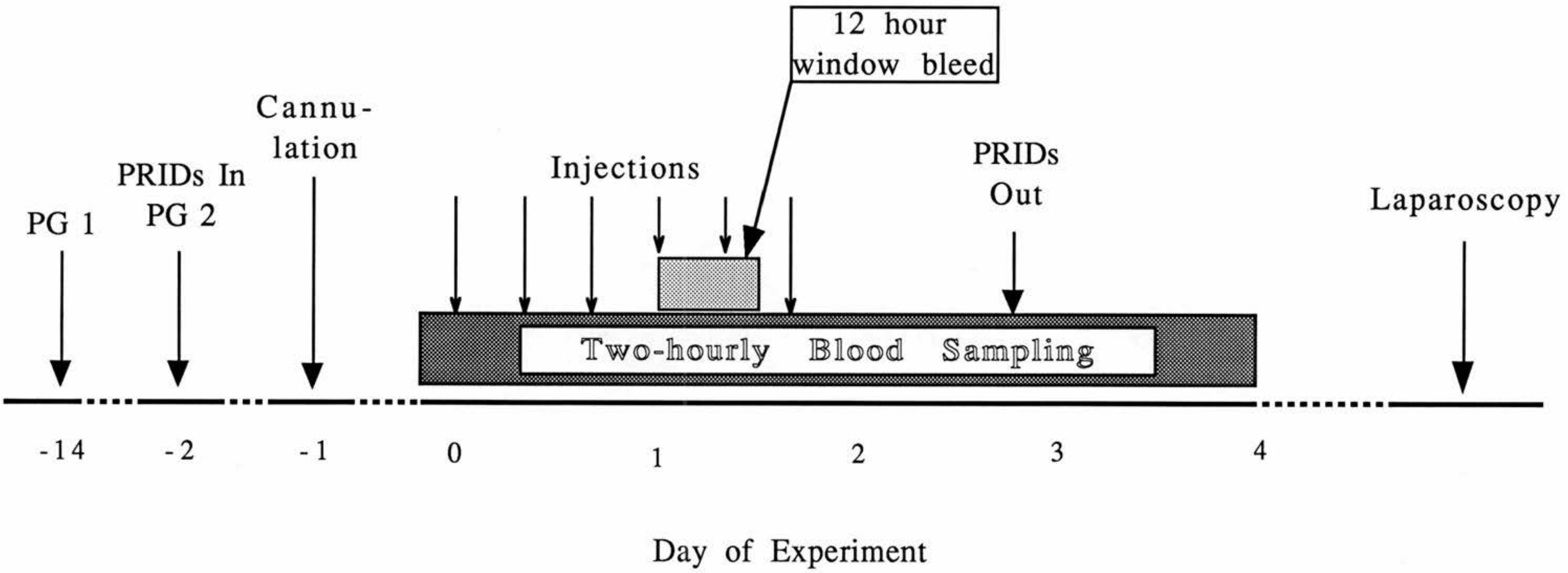
5.2.2. Preparation of partially purified follicular fluid fractions

Pooled fractions of bovine, ovine and porcine follicular fluid were prepared by affinity chromatography on Red Sepharose as described in Chapter 2.

5.2.3. Protocol

The experimental protocol is detailed in Figure 5.1. Oestrous activity was synchronised in twenty-eight animals with two i.m. injections of a potent prostaglandin $F_{2\alpha}$ analogue (Estrumate) given twelve days apart. At the time of the second injection, all animals were fitted with a progesterone releasing device (PRID) with the oestradiol capsule removed to mimic the normal luteal phase whilst reducing the between animal variation in progesterone concentrations. The following day all animals were fitted with an indwelling jugular venous catheter (Intranule; Vygon, Ecouen, France). Two days after the insertion of PRIDs the animals were assigned at random to one of four experimental groups and each received a series of six 10 ml i.v. injections of saline either alone (Controls; n=7) or containing 14 mg bovine ppff (Bovine group; n=7), 14 mg ovine ppff (Ovine group; n=7) or 14 mg porcine ppff (Porcine group; n=7) at eight hourly intervals via the indwelling cannula. Blood samples were also withdrawn via the cannula into heparinised tubes at two-hourly intervals for FSH analysis starting four hours prior to the initial injection and continuing for four days. In addition, intensive blood

Figure 5.1 Experimental Protocol



samples were taken into heparinised tubes every ten minutes for twelve hours on the second day of treatment for analysis of LH pulsatile secretion. Samples (20 ml) were also taken every three hours throughout the intensive blood sampling period for oestradiol assay. PRIDs were removed from all animals 36 hours after the final ppff injection at which time animals were fitted with a commercial heat detection device to aid the detection of oestrus (Kamar tags). Ovulation rate was determined by sub-lumbar laparoscopy approximately eight days after observed oestrus.

5.2.4. Hormone Assays

5.2.4.1. FSH Assay

Samples from this study were measured in four assays. The mean sensitivity of the assays was 0.9 ng/tube (3.6 ng/ml). The between assay coefficient of variation based on samples assayed at two doses at multiple points throughout the assays was 11.96% and the within assay coefficient of variation calculated over the whole assay was 14.26%.

5.2.4.2. LH Assay

The mean sensitivity of the LH assays was 0.05 ng/tube (0.5 ng/ml). Samples were assayed in five assays with a mean between assay coefficient of variation of 9.86%. The mean within assay coefficient of variation was 9.11%.

5.2.4.3. Oestradiol Assay

All samples were assayed for oestradiol in a single assay with a mean extraction efficiency of 43.67%. The intra-assay coefficient of variation of the assay was 13.36% and the sensitivity was 0.5 pg/tube.

5.2.4.4. Progesterone Assay

Samples were assayed in a single assay and the within assay coefficient of variation calculated over the whole assay was 4.66%. The sensitivity of the assay was 7.8 pg/tube (0.156 ng/ml).

5.2.5. Statistical analysis

The FSH data is presented as arithmetic means (± sem). Comparisons between treatment groups and phases were carried out by analysis of variance. Between group comparisons of mean LH pulse amplitude were by Student's t-test. Progesterone and oestradiol concentrations were compared by analysis of variance.

5.3. Results

5.3.1. Oestrus and ovulation rate

Following PRID removal, all animals showed behavioural oestrus within three days. Subsequent laparoscopy revealed that all animals had apparently ovulated normally. No animal had more than one fresh corpus luteum, and there was no evidence of any ovarian abnormality in any treatment group.

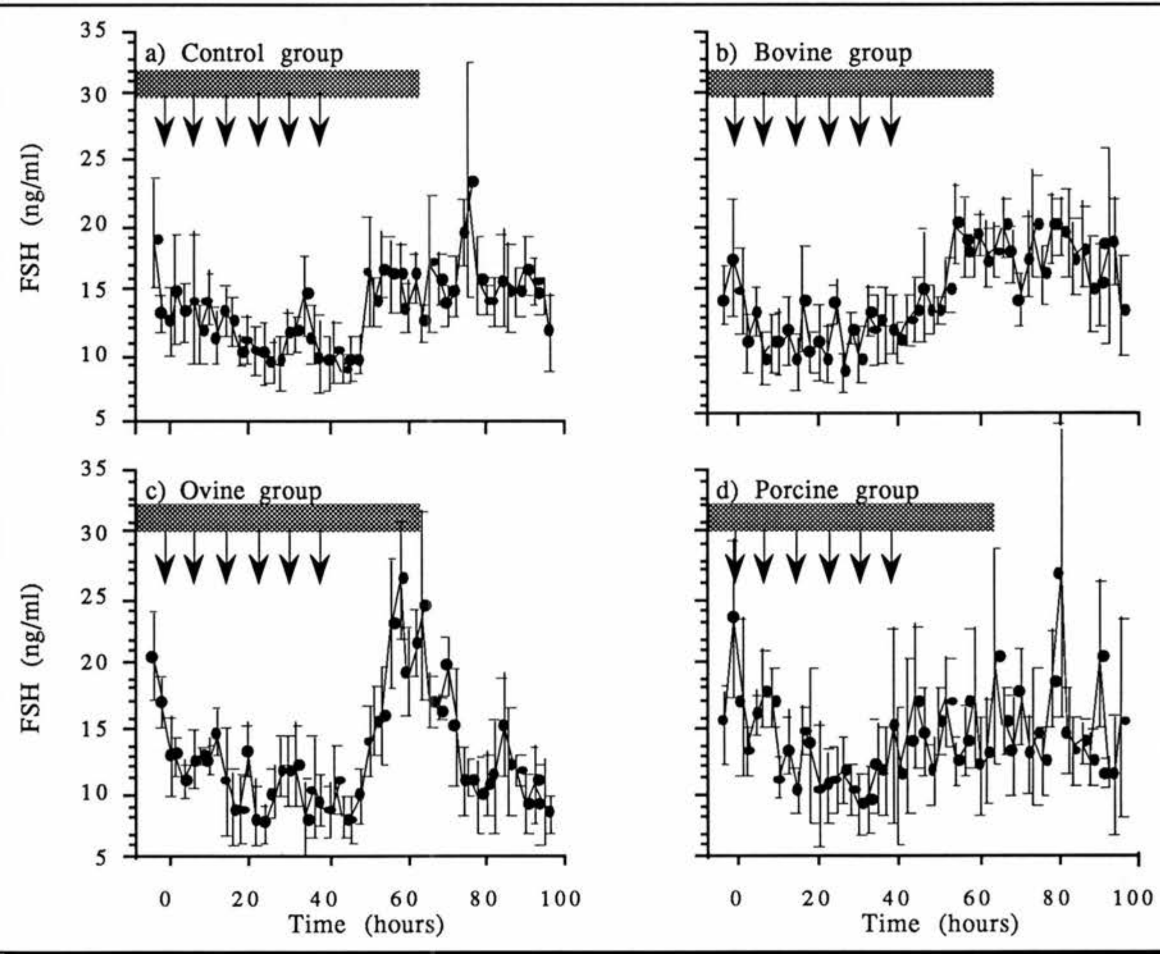
5.3.2. Oestradiol concentrations

Oestradiol concentrations in ppff treated animals, as determined in samples taken throughout the intensive blood sampling period, were not significantly different in any group from concentrations observed in control animals. Mean oestradiol concentrations for each group are shown in Table 5.1.

Table 5.1 Mean oestradiol concentrations (pg/ml ± sem) in animals treated with saline (control group) or porcine, bovine or ovine ppff proteins. Differences between groups were not significant ($p > 0.05$).

Control group	Porcine group	Bovine group	Ovine group
5.7 ± 0.49	6.2 ± 0.90	4.4 ± 0.44	5.4 ± 0.37

Figure 5.2 Mean FSH profiles from animals treated with saline (a) or 14 mg bovine (b), ovine (c) or porcine ppff proteins. The error bars represent the standard error of each point. The arrows indicate the timing of injections. The period of PRID treatment is signified by the shaded bar.



5.3.3. Progesterone concentrations

Mean progesterone concentrations were similar in all groups during the intensive blood sampling period (Control group 3.90 ± 0.43 ng/ml; Porcine group 4.09 ± 0.34 ng/ml; Ovine group 4.05 ± 0.38 ng/ml; Bovine group 3.90 ± 0.42 ng/ml). The period of PRID treatment is shown in Figure 5.2 by the shaded bar.

5.3.4. FSH concentrations

The profile of FSH concentrations in all groups is shown in Figure 5.2. There was again a tendency for FSH concentrations to fall in all groups during the treatment period in a similar fashion to that observed in the previous experiment. However this failed to reach statistical significance and at no time were there any significant differences between groups. Following cessation of injections, FSH concentrations in the ovine and bovine treated animals, but not the porcine ppff treated animals or the controls, rebounded to levels significantly ($p < 0.05$) above those seen during treatment, but rapidly returned back to pre-treatment levels. There was a tendency for the post-treatment peak concentrations in the bovine and ovine group to be higher than those observed in the control and porcine groups but this was not statistically significant.

5.3.5. LH concentrations and pulsatile secretion

Patterns of LH secretion are shown in Table 5.2. Treatment did not affect basal LH secretion or the frequency of pulses in any group, although LH pulse amplitude was significantly increased in animals treated with either the bovine ($p < 0.01$) or ovine ppff ($p < 0.001$). Representative LH profiles from animals in each group are shown in Figure 5.3.

Figure 5.3 Representative LH profiles from individual animals treated with saline (a) or 14 mg bovine (b), ovine (c) or porcine (d) ppff proteins.

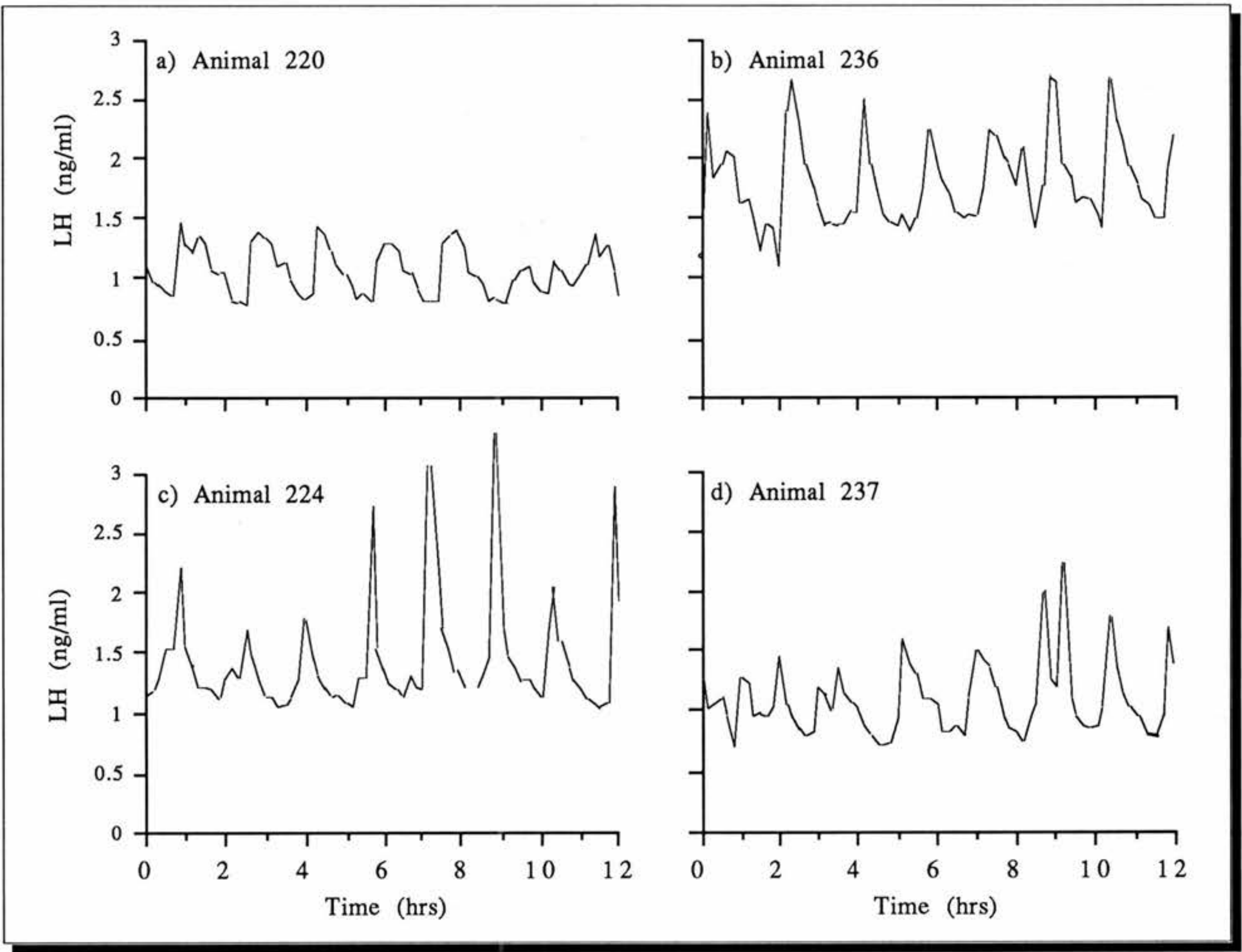


Table 5.2 Mean LH concentration, pulse frequency and pulse amplitude (± sem) in control, bovine, ovine and porcine ppff-treated groups. Within columns, different superscripts indicate significant differences ($p < 0.01$).

Group	Mean LH (ng/ml)	Pulse Frequency (pulses per hour)	Pulse amplitude (ng/ml)
Control	1.22 ± 0.13	0.58 ± 0.04	0.58^a ± 0.06
Bovine	1.43 ± 0.11	0.63 ± 0.03	0.86^b ± 0.07
Ovine	1.34 ± 0.11	0.57 ± 0.06	0.98^c ± 0.07
Porcine	1.33 ± 0.11	0.57 ± 0.06	0.67^a ± 0.05

5.4. Discussion

The administration of either bovine or ovine ppff to intact heifers resulted in a significant increase in FSH secretion following the cessation of treatment. LH pulse amplitude was also significantly increased during treatment with either ovine or bovine ppff. Similar treatment with porcine ppff had no effect on any parameter of gonadotrophin secretion. The observed changes in LH and FSH secretion in the animals treated with bovine or ovine ppff occurred in the absence of any alteration in oestradiol concentrations or subsequent ovarian function following treatment.

These results clearly indicate a species difference in the activity of components of follicular fluid in this experimental model. Price (1987) has previously demonstrated similar differential effects of porcine ppff in an *in vivo* system; porcine ppff stimulated whilst ovine ppff inhibited ovulation rate in a mouse ovulation rate bioassay. Clearly porcine follicular fluid contains inhibin which is bioactive in rats (Schwartz & Channing, 1977) and monkeys (Channing *et al.*, 1982), although the responsiveness to porcine follicular fluid in the latter species was rapidly

lost due to a developing immune response. No immune response was detected in any animal by Ouchterlony analysis of serum samples in this study. Whether the observed differences between follicular fluid preparations in this experiment were due to a lower activity of porcine inhibin in the heifer or to a reduced Red Sepharose affinity for porcine inhibin remains to be determined. Alternatively the effects observed in this and previous studies in this laboratory may be due to a factor other than inhibin which may be absent from porcine follicular fluid, or sufficiently different in the pig as to prevent its co-purification with inhibin from porcine follicular fluid. The pig is a polytocous species with a considerably higher natural ovulation rate than either cattle or sheep. This species difference in follicular fluid protein activity may be a reflection of the species differences in ovulation rate. The remainder of this discussion will concentrate on the effects of bovine and ovine ppff treatment.

The failure of ppff treatment to suppress FSH concentrations below those observed in control animals whilst still eliciting a rebound following cessation of treatment confirms previous observations using bovine ppff preparations in this laboratory (see Chapter 4). Johnson and Smith (1985) were similarly unable to suppress FSH concentrations in luteal-phase heifers with 8 ml injections of steroid-free follicular fluid every 12 hrs, but observed a similar rebound following treatment. Quirk and Fortune (1986) also reported a failure to suppress FSH concentrations in heifers treated with 10 mls follicular fluid twice daily.

The doses of follicular fluid proteins used in this experiment were estimated to be equivalent in inhibin content to approximately 3 mls follicular fluid and were administered every eight hours. It would appear that although inhibin is able to suppress peripheral FSH levels in ovariectomised ewes (Cummins *et al.*, 1983) and heifers (Ireland, Curato &

Wilson, 1983; Beard *et al.*, 1988,1989,1990) and in intact sheep (Miller, Critser & Ginther, 1982; McNeilly, 1985, Wallace & McNeilly, 1986), it is unable to do so in the presence of a functional ovarian negative feedback system in intact heifers. However, FSH concentrations are clearly capable of further suppression as FSH levels fell in control animals following PRID removal in the previous study (Chapter 4). A similar reduction in FSH concentrations has been observed in cyclic animals during the follicular phase and is presumably linked to the increase in oestradiol secretion from the developing pre-ovulatory follicle (Peters, 1985; Law & Webb; unpublished observations; Chapter 3). Together, these results support the conclusions of Price and Webb (1988) who questioned the importance of inhibin in the negative feedback regulation of FSH secretion in the heifer. Nevertheless, some component of follicular fluid is clearly involved in this negative feedback since cessation of treatment results in a significant rebound of FSH concentrations similar to that observed in previous studies. Price and Webb (1988) were able to account for the normal oestrous cycle negative feedback control of gonadotophin secretion through changes in steroids alone using steroid-implanted, recently ovariectomised heifers, particularly during the luteal phase, although other workers using long-term ovariectomised animals were unable to do so. In addition, Webb *et al.*, (1985b) have demonstrated the involvement of an ovarian component other than oestradiol in the negative feedback regulation of gonadotrophin secretion in the anoestrous ewe which became apparent after the removal of oestradiol implants from long-term ovariectomised animals. These studies, taken together, suggest the involvement of an ovarian factor in the long-term control of gonadotrophin secretion. However, the nature of the rebound response observed in this study was intrinsically short-term, suggesting that the mechanisms involved may be different. It is possible that the

continuous release of steroid hormone from the implants used in the above studies results in an artificial pattern of negative feedback when compared to the normal pulsatile or variable secretion observed in the intact animal. Martin *et al.* (1988) demonstrated that a component of follicular fluid, which they suggested was inhibin, was synergistic with oestradiol in controlling peripheral FSH concentrations in the ewe. Possibly there exists in follicular fluid a factor (perhaps inhibin) which potentiates the effectiveness of oestradiol in the negative feedback regulation of FSH in cattle in similar fashion. Administration of exogenous follicular fluid proteins may possibly cause either a down-regulation of the pituitary receptors for this factor, rendering the animal temporarily unable to respond to the endogenous factor following cessation of treatment, or reduce the endogenous ovarian production of the factor by auto-regulation with the same net result. The nature of this putative factor, and its mode of action require further study.

The lack of effect of ppff treatment on the length of time between PRID removal and oestrus is consistent with our previous observations (Chapter 4) and again suggests that the effects of ppff treatment are not mediated at the ovarian level. This is further supported by our inability to demonstrate any differences in peripheral oestradiol concentrations between groups in both this and the previous study (Chapter 4). Price (1987) has previously demonstrated the inability of a similar ovine ppff preparation to suppress follicular oestradiol production *in vitro*. Nevertheless, in spite of the lack of any follicular suppression during treatment, there was no incidence of any super-ovulatory response to the increased FSH concentrations immediately following the cessation of treatment. This implies that ovulation rate may not be principally controlled through FSH concentrations in this species. However, the superovulatory response of cattle to exogenous FSH-containing

preparations is antagonised by increasing LH content (Chupin, Combarnous & Procureur, 1984, Donaldson, Ward & Glenn, 1986) and the increased LH secretion observed during treatment may have been sufficient to prevent any increased FSH-induced follicular development.

The effects of ppff treatment on LH secretion are noteworthy. McNeilly (1984) reported that bovine follicular fluid failed to influence LH secretion in Welsh mountain ewes. However, Wallace & McNeilly (1986) later reported an increase in LH pulse amplitude following bFF treatment of Damline ewes, which they attributed to a reduction in oestradiol secretion. Although it is well documented that oestradiol suppresses LH pulse amplitude in sheep and cattle (Goodman & Karsch, 1980; Rawlings, Jeffcoate & Rieger, 1984; Price & Webb, 1988), in this study, oestradiol concentrations did not differ between groups, and there was no correlation between oestradiol concentrations and LH pulse amplitude in individual animals. Coupled with the failure of treatment to retard follicular development, these data suggest the influence of ovarian proteins in the control of LH pulse amplitude at a locus distal to the ovary. Although there is documented evidence for a role of follicular fluid proteins in the suppression of LH secretion (Webb *et al.*, 1985b; Nagesh Babu, Bona-Gallo, & Gallo, 1986; van Dieten, de Koning & van Rees, 1989) there are no such reports of follicular fluid proteins acting to increase LH pulse amplitude. However, Knight (1990) recently reported an increase in GnRH-induced LH secretion from ovine pituitary cells cultured *in vitro* and challenged with GnRH after incubation with bovine follicular fluid.

The physiological significance of these data is unclear. Given that the peripheral concentrations of FSH, LH and oestradiol measured represent the net balance of a complex set of negative and positive influences, it is possible that the observed increase in LH pulse amplitude is due to a

decrease in ovarian responsiveness to LH, and that this compensatory secretion of LH restored oestradiol secretion such that no differences in oestradiol concentrations were observed between groups. However, this seems unlikely for two reasons. Firstly, both in this experiment and the previous study there was no increase in the length of time from PRID removal to oestrus suggesting that follicular growth was not affected by treatment. Secondly treatment with similar ppff preparations does not reduce oestradiol production by ovarian follicles cultured *in vitro* (Price 1987). It would appear that the increase in LH pulse amplitude is due to a physiological action of follicular fluid proteins at the hypothalamic or pituitary level.

Recently McNeilly *et al.* (1990) reported that LH pulses were responsible for reducing the number of large follicles present in the ovaries of chronically GnRH agonist-treated, FSH-infused ewes, suggesting that the mechanism of selection of the dominant ovulatory follicle is mediated through LH pulses in some way. It is also of interest that although oestradiol is responsible for reducing LH pulse amplitude (Goodman & Karsch, 1980; Rawlings, Jeffcoate & Rieger, 1984; Price & Webb, 1988), the follicular phase of the bovine oestrous cycle is reportedly characterised by an increase in both oestradiol and LH pulse amplitude (Schallenberger *et al.*, 1984; Walters & Schallenberger, 1984; Schallenberger, Schöndorfer & Walters, 1985). If LH pulses are indeed important in the process of selection and/or dominance then any factor produced by a large dominant follicle which resulted in increased LH pulse amplitude would also be acting to reinforce the dominance of that large follicle.

In conclusion, we have confirmed our previous observations on the inability of low doses of partially purified inhibin-enriched follicular fluid fractions to suppress FSH concentrations in intact cyclic heifers.

Despite the lack of suppression of FSH secretion during treatment, administration of bovine or ovine, but not porcine, ppff resulted in a rebound of peripheral FSH concentrations. No treatment suppressed follicular growth as measured by delay in return to oestrus following PRID removal or peripheral oestradiol concentrations. In addition the presence of a factor in bovine and ovine ppff which acts to increase LH pulse amplitude was demonstrated. The identity and physiological significance of this factor remain to be determined, but it may be involved in the reinforcement of the dominance of the large pre-ovulatory follicle during the follicular phase of the oestrous cycle.

Chapter 6

The effect of administration of steroid-free bovine follicular fluid on patterns of follicular development

6.1. Introduction

Follicular development in the heifer is characterised by the periodical emergence of "waves" of follicular growth which can be monitored using ultrasound (Savio *et al.*, 1988; Sirois & Fortune, 1988; Savio, Boland & Roche, 1990). Each wave involves the initiation of the growth of a group of follicles, one of which becomes dominant and continues to grow, whilst the other subordinate follicles generally regress. Under the appropriate hormonal conditions, i.e. following luteolysis, a dominant follicle will continue its development through the full pre-ovulatory stages and will subsequently ovulate.

The administration of bovine follicular fluid around the time of prostaglandin injection results in a marked delay in the onset of oestrus in both sheep and cattle (Miller, Critser, Rowe & Ginther, 1979; Miller, Critser & Ginther, 1982; McNeilly, 1984,1985; Wallace & McNeilly, 1985,1986; Johnson & Smith, 1985; Henderson *et al.*, 1986; Quirk & Fortune, 1986). This phenomenon appears to involve a suppression of follicular development, as luteolysis occurs normally (Miller *et al.*, 1979; Johnson & Smith, 1985; Quirk & Fortune, 1986) and peripheral oestradiol concentrations are suppressed in treated animals (Johnson & Smith, 1985; Quirk & Fortune, 1986).

In our previous studies we have failed to influence ovarian function either by immunisation against or by the administration of follicular fluid proteins (Chapters 3 to 5). It was therefore of interest to us to characterise the ovarian response to follicular fluid treatment prior to a further investigation of this phenomenon as described in Chapter 7.

6.2. *Materials and methods.*

6.2.1. Animals

Nine Hereford x Friesan heifers were used in the study under the husbandry conditions described in Chapter 2.

6.2.2. Experimental protocol

Oestrous activity was synchronised in all animals by means of two i.m. injections of cloprostenol (Estrumate) given twelve days apart. At the time of the second injection, animals were assigned at random to one of three experimental groups and each subsequently received 6 injections of either 20 mls saline (Controls; n=3), 10 mls bFF (Group 1; n=3) or 20 mls bFF (Group 2; n=3) at eight-hourly intervals. Animals were observed every eight hours for a total of eleven days for signs of behavioural oestrus. The detection of oestrus was facilitated by the use of a commercial heat detection device (Kamar Tags). In addition, the ovaries of all animals were examined daily throughout the eleven day experimental period by real-time ultrasonography. The resulting ultrasound images were recorded on videotape (Kodak plc.) for later analysis.

6.2.3. Analysis of patterns of follicular development.

The size of an individual follicle was estimated by the use of a computerised calliper device. Each antral follicle visible within an ovary was measured in the vertical and horizontal planes and the mean of the two measurements recorded. The system allowed the measurement of

follicles with an antrum > 4 mm in diameter. However, it was difficult to accurately follow the development of follicles < 5 mm in diameter and so the smaller follicles have been excluded from the analysis of the patterns of follicular growth.

The number of follicles > 4 mm in diameter and the number of those follicles still growing was recorded for each animal. Follicles were retrospectively determined to be growing if they had not yet reached their maximum recorded size. The mean diameter of all follicles > 4 mm was calculated daily for each animal, as was the diameter of the largest follicle and the diameter of the largest *growing* follicle on an individual pair of ovaries. Comparisons between groups were performed by ANOVA.

6.3. Results

6.3.1. Oestrus

One control animal was not observed in oestrus throughout the duration of the experiment. The remaining two control animals displayed oestrus 52 and 72 hrs following the second PG injection respectively. Oestrus was uniformly and significantly ($p < 0.005$) delayed in all animals treated with either 10 mls (174.67 ± 2.67 hrs) or 20 mls bFF (182.67 ± 7.05 hrs).

6.3.2. Patterns of follicular development.

There was no difference in the patterns of follicular development between animals treated with either 10 mls or 20 mls steroid-free bFF. Consequently the results for these two treatment groups were pooled. However, examination of the ovarian activity in the single control animal that failed to display oestrus revealed the presence of a persistent, presumably cystic, follicle throughout the experiment (Figure 6.1). The data from this animal was therefore discarded from the analysis.

Figure 6.1 Abnormal patterns of follicular development observed in one control heifer (11002) following prostaglandin injection on Day 1. The closed circles represent the growth of the ovulatory follicle. Other follicles are represented by open circles.

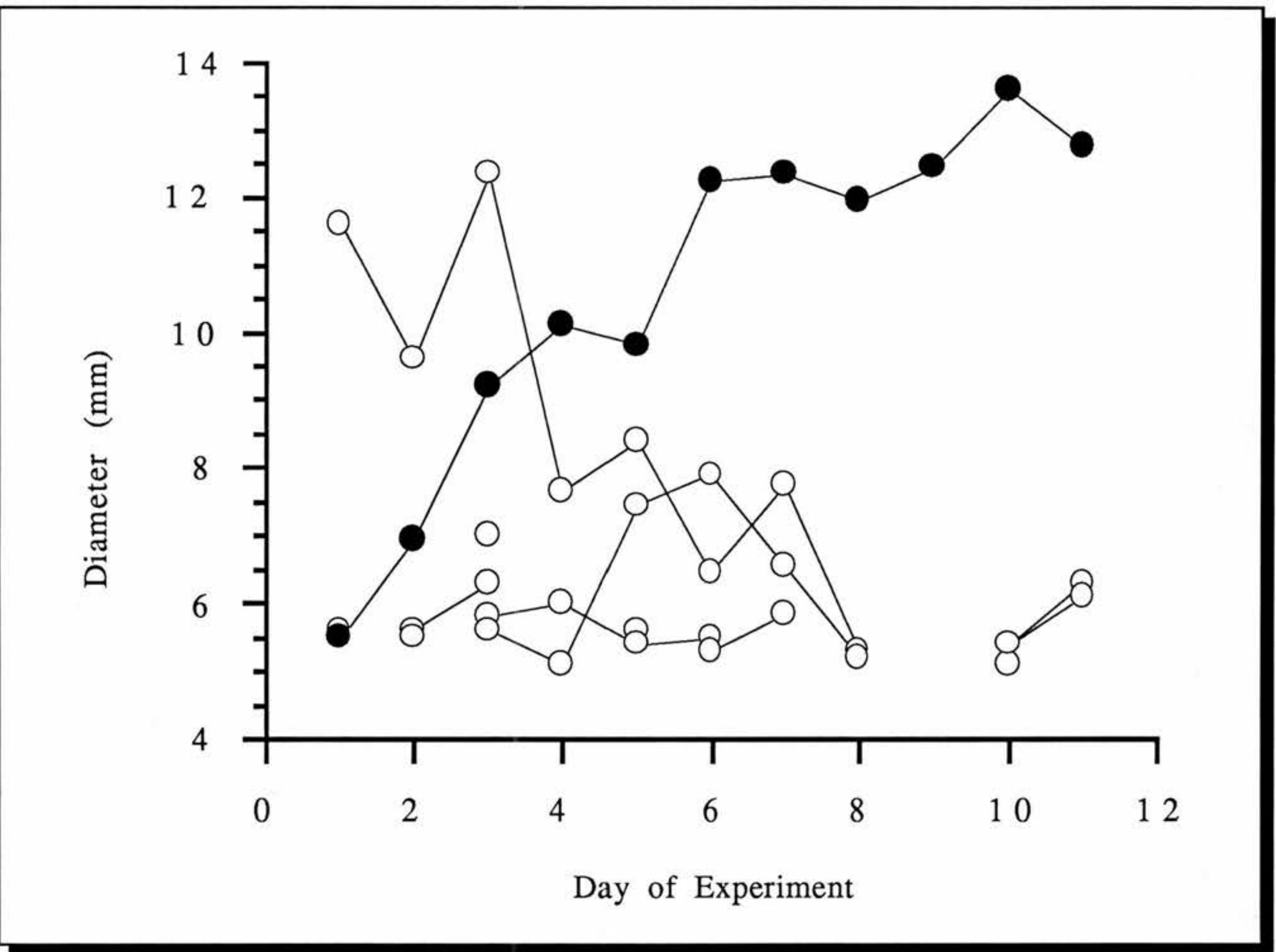
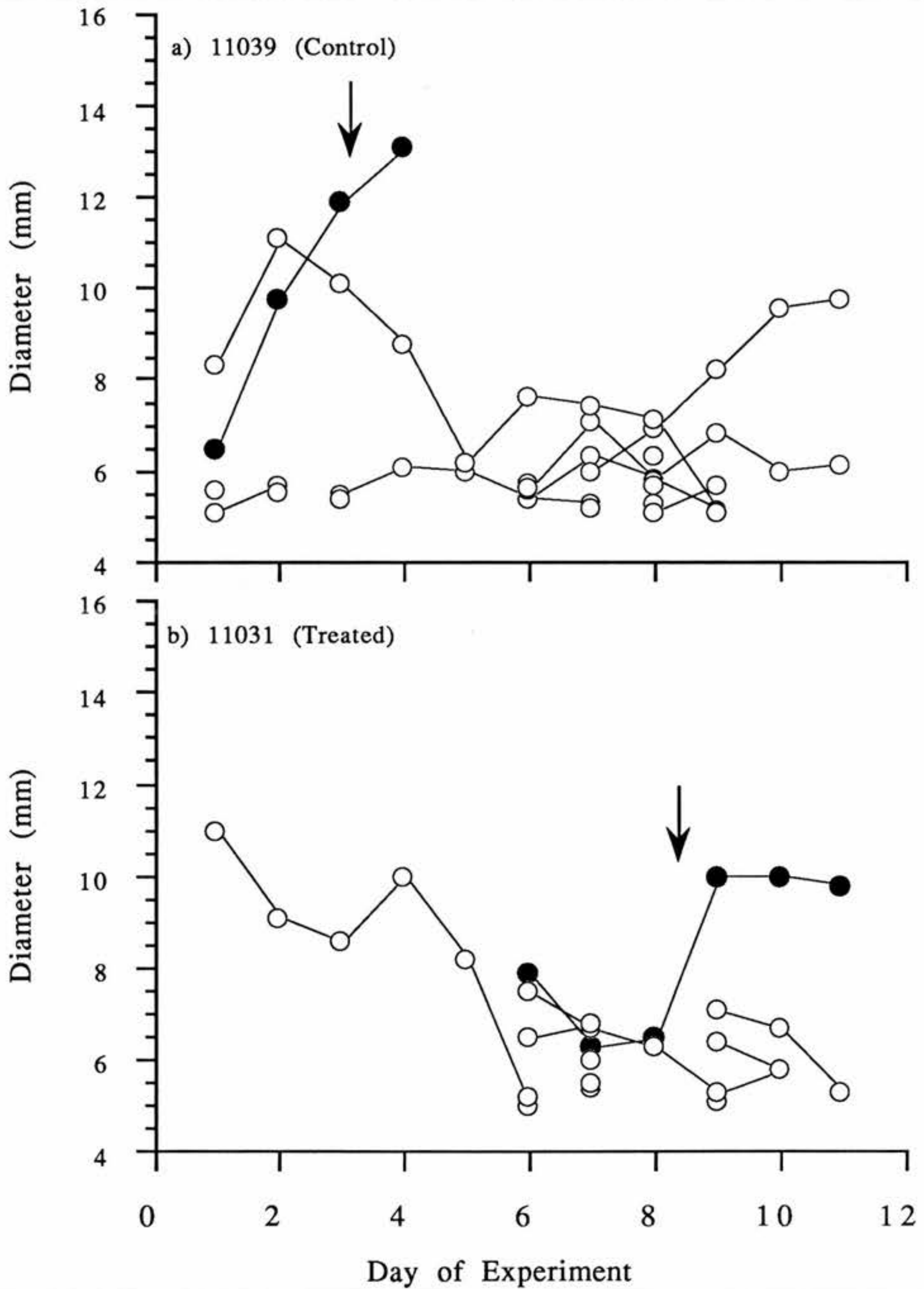


Figure 6.2

Patterns of follicular development in representative animals treated with saline (11039) or steroid-free bFF (11031;20 ml per injection) immediately following prostaglandin injection on Day 1. The arrows indicate the time of onset of oestrus in each animal. The growth of the ovulatory follicle in each animal is represented by the black circles.



Unfortunately, the loss of this control animal from the experiment tended to obscure any statistical differences between treatment groups.

Typical patterns of follicular development observed in control and treated animals are shown in Figure 6.2. In control animals, a single follicle emerged to dominance from a pool of growing follicles present within the ovary at the start of the experiment. The other growing follicles subsequently regressed whilst the dominant follicle continued to increase in size beyond the time of observed oestrus and eventually ovulated. Immediately following ovulation, a new wave of follicles began to grow, from which a new dominant follicle emerged. In bFF-treated animals, all follicles present at the commencement of treatment began to regress within three days and none ovulated. A new wave of follicles became detectable in treated animals 3-5 days after the end of treatment. One of these follicles eventually became dominant, continuing to grow after all other follicles had begun to regress. In five of the six treated animals this follicle ovulated; the remaining animal had still not ovulated by the end of the study. The day of emergence of the ovulatory follicle was significantly delayed ($p < 0.001$) in treated animals compared to controls.

Figure 6.3 shows the effect of treatment on the mean number of follicles > 4 mm and the mean number of growing follicles > 4 mm. Although no significant differences could be demonstrated between treated and control animals, there was a tendency for there to be fewer antral follicles and for fewer follicles to be growing in treated animals during the treatment and immediate post-treatment periods. The mean number of antral follicles declined steadily in both control and treated animals from Day 6 to the end of the study. There was a reduction in the number of growing follicles in control animals around Day 4 and again, commencing from a peak in numbers at Day 8, and continuing to the end

Figure 6.3

The effect of treatment with saline (n = 2; ○) or steroid-free bovine follicular fluid (n = 6; ●) following prostaglandin injection on the number of follicles and the number of growing follicles.

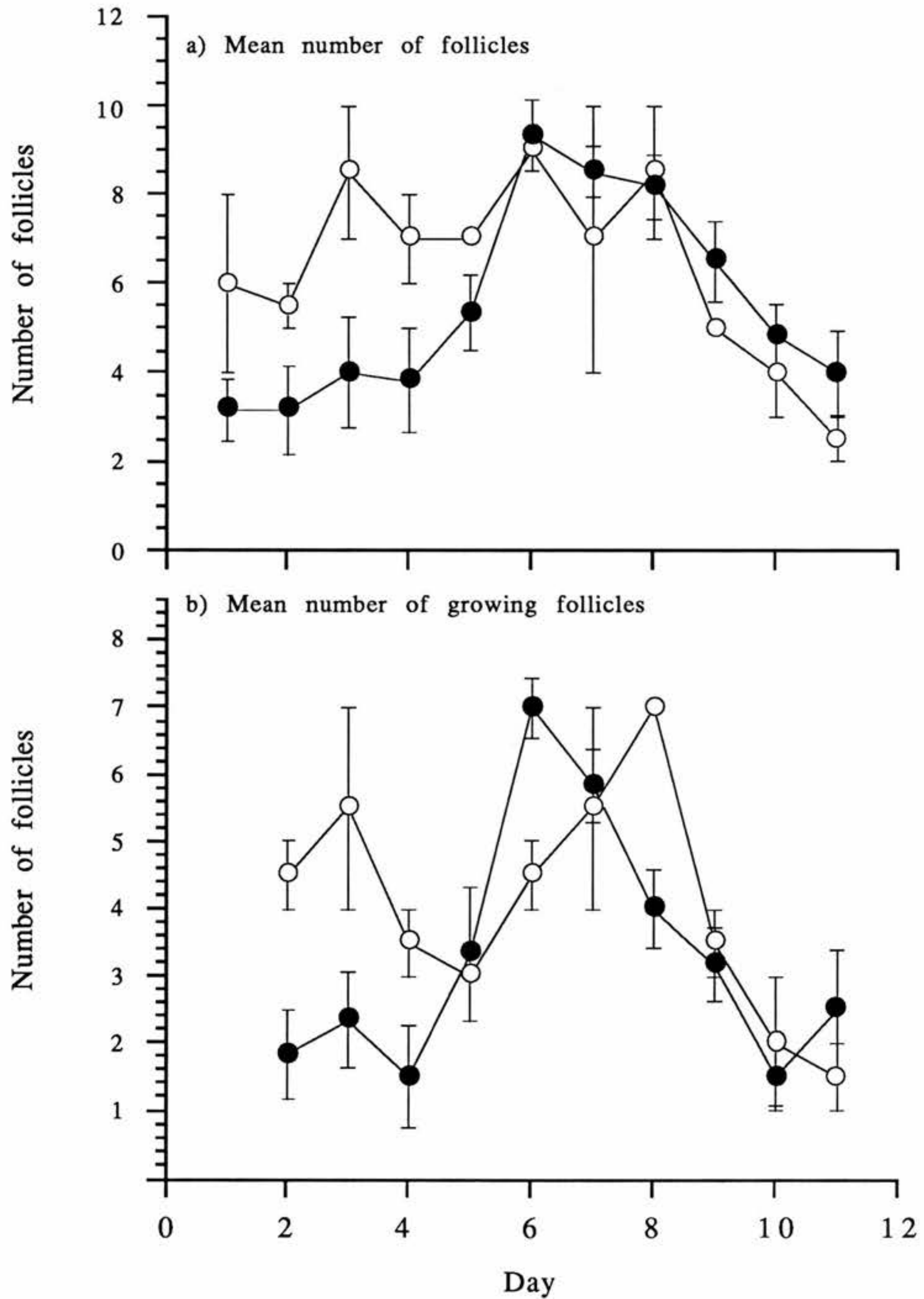
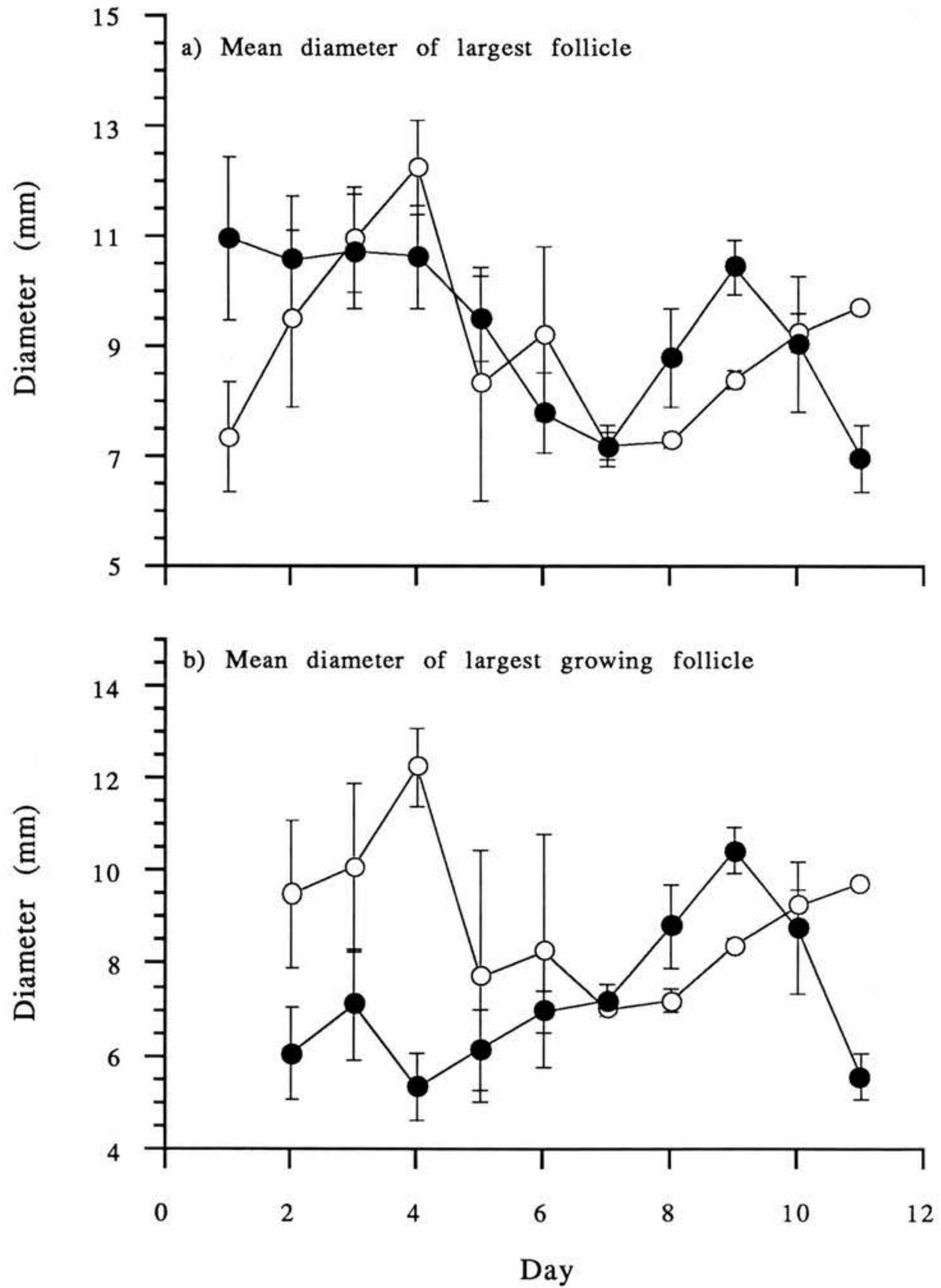


Figure 6.4 The effect of treatment with saline (○; n = 2) or steroid-free bovine follicular fluid (●; n = 6) on the diameters of the largest follicle and the largest growing follicle



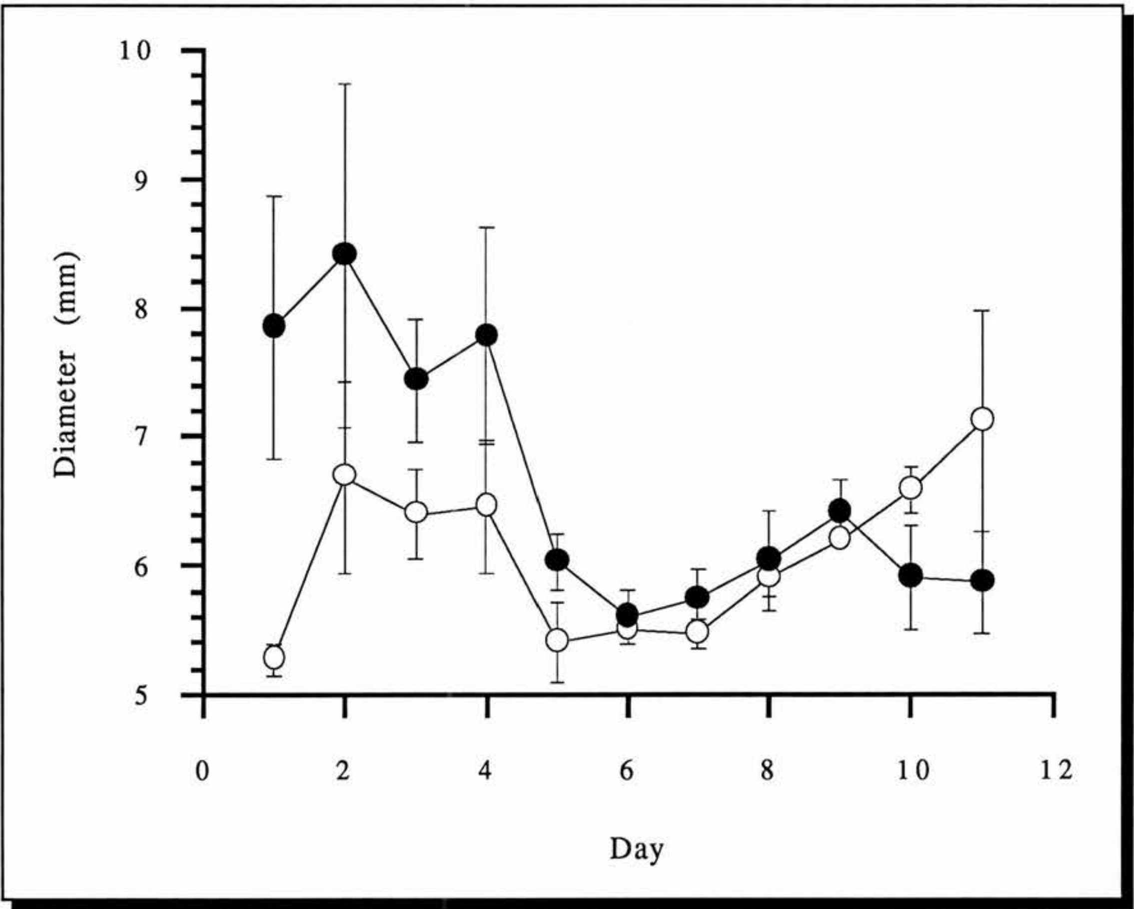
of the study. A similar fall in the number of growing follicles was observed in treated animals between Days 6 and 10.

The effect of treatment on the diameter of the largest follicle and the largest growing follicle are depicted in Figure 6.4. There was a decline in the diameter of the largest follicle in treated animals between Day 1 and Day 7. However, in control animals, the diameter of the largest follicle continued to increase to Day 4, at which point it decreased sharply. Mean largest follicle diameter subsequently increased in treated animals to Day 9, whereupon it fell away. The diameter of the largest follicle in control animals continued to rise to the end of the study. The mean diameter of the largest growing follicle followed a similar pattern in control animals to the mean size of the overall largest follicle in these individuals. This was also the case in treated animals during the latter period of the experiment (i.e. after Day 7). However, during the early portion of the experiment (before Day 7), the largest growing follicle was smaller than the overall largest follicle in treated animals.

Figure 6.5 shows the effect of treatment on the overall mean follicular diameter. The mean diameter of all follicles prior to the start of treatment was smaller in control animals than that seen in treated animals . The mean size of all follicles in treated animals declined from the start of treatment to a nadir at Day 6 whereas the mean size increased in control animals to Day 4 and then declined rapidly. Follicular diameter increased steadily in both treated and control animals to Day 9, at which point it declined in treated animals but continued to rise in control animals to the end of the study.

Significant negative correlations were found between the number of follicles and the mean diameter of those follicles ($r = -0.533$; $p < 0.001$), the number of growing follicles and the mean diameter of all follicles ($r = -0.440$; $p < 0.001$) and the number of growing follicles and the

Figure 6.5 The effect of treatment with saline (○; n = 2) or steroid-free bovine follicular fluid (●; n = 6) on the mean diameter of all follicles.



diameter of the largest follicle ($r = -0.243$; $p < 0.05$). There was no significant correlation between the number of follicles and the diameter of either the largest follicle ($r = -0.176$) or the largest growing follicle ($r = -0.191$) or between the number of growing follicles and the diameter of the largest growing follicle ($r = -0.168$; all $p > 0.05$). However, significant positive correlations were observed between the mean diameter of all follicles and the diameter of the largest follicle ($r = -0.653$; $p < 0.001$), the number of follicles and the diameter of the largest follicle on the subsequent day ($r = -0.317$; $p < 0.005$) and the number of growing follicles and the diameter of the largest follicle on the subsequent day ($r = -0.287$; $p < 0.005$).

6.4. Discussion

Treatment with steroid-free bFF significantly delayed the onset of oestrus following prostaglandin injection when compared to saline treated controls as previously demonstrated (Miller *et al.*, 1979; Johnson & Smith, 1985; Quirk & Fortune, 1986). The delay in oestrus was associated with the regression of all antral follicles present on the ovaries at the start of treatment and the subsequent delayed emergence of the ovulatory follicle.

The development of waves of follicular growth observed in this experiment were similar to those previously reported (Savio *et al.*, 1988; Sirois & Fortune, 1988; Ginther, Kastelic & Knopf, 1989; Ginther, Knopf & Kastelic, 1989; Savio, Boland & Roche, 1990). However, follicular fluid treatment disrupted the normal patterns of follicular development. Other authors have recently reported that similar follicular fluid treatment causes the rapid cessation of growth of developing follicles regardless of the stage of the oestrous cycle at which treatment was commenced (Kastelic, Ko & Ginther, 1990). They also reported that the onset of growth of the immediate post-treatment wave of follicles was first evident

approximately three days following the cessation of treatment. A similar delay was observed in this study. However, these authors observed that the regression of the dominant follicle did not commence until five days after the start of treatment. In this study, regression of all follicles had begun within only two days of the start of treatment. The reasons for this difference are not clear, although Kastelic, Ko & Ginther (1990) commenced their treatments following ovulation when a new wave of follicular growth is actively developing. Animals in this study were treated following prostaglandin injection during the luteal phase of the oestrous cycle, when follicular growth may have already peaked in some or all animals. The follicles may therefore have been more susceptible to the effects of treatment.

The patterns of mean follicular diameter and mean numbers of follicles are greatly influenced by the emergence of the dominant follicle. As the dominant follicle establishes its dominance, the number of other follicles steadily reduces. As the non-dominant follicles are generally smaller than the dominant follicle, this fall in numbers results in a gradual elevation of the overall mean follicular diameter and a highly significant negative correlation between the number of follicles and the mean diameter of all follicles. In addition, as the dominant follicle emerges and increases in size, the number of growing follicles is reduced. This explains the significant negative correlation between the number of growing follicles and the diameter of the largest follicle.

The significant positive correlation between the number of follicles and the diameter of the largest follicle on the subsequent day is, at first sight, less easily explained. As the dominant follicle emerges, the number of follicles growing/present is reduced whilst the diameter of the largest follicle is increasing, suggesting that the correlation between these parameters should be negative. On the day prior to ovulation, the number

of follicles is low. The following day, the dominant follicle has ovulated and, therefore, the diameter of the largest growing follicle is low. A new wave of follicles subsequently develops, resulting in a steady increase in the number of follicles. The diameter of the largest growing follicle also increases. Therefore, the correlation between the number of follicles and the diameter of the largest growing follicle on the subsequent day is positive.

The influence of the presence or absence of a dominant follicle on the characteristics of the ovarian follicular population suggests that the dominant follicle may be inhibiting the growth of other follicles. The disturbance of the patterns of follicular development by the injection of steroid-free bFF further suggests that the factor responsible for the inhibition of follicular growth is contained in follicular fluid. It is tempting to speculate on the hormonal consequences of follicular fluid treatment of heifers. Indeed, other authors have done so, occasionally in the total absence of any hormonal measurements (Kastelic, Ko & Ginther 1990). However, these aspects will be considered in detail in Chapter 7.

CHAPTER 7

An investigation of the importance of inhibin in the bovine follicular fluid-induced delay in return to oestrus following prostaglandin treatment

7.1. Introduction

The administration of steroid-free bFF to cattle or sheep around the time of progesterone withdrawal results in a marked increase in the length of time to the return of oestrus due to a suppression of follicular development (Miller *et al.*, 1979; Miller, Critser & Ginther, 1982; McNeilly, 1984,1985; Wallace & McNeilly, 1985,1986; Johnson & Smith, 1985; Henderson *et al.*, 1986; Quirk & Fortune, 1986; Chapter 6). In sheep, this follicular suppression is associated with, and has been attributed to, a decrease in peripheral concentrations of FSH (Miller, Critser & Ginther, 1982; McNeilly, 1984; Wallace & McNeilly, 1985, 1986; Henderson *et al.*, 1986; Hunter, Hindle, McLeod & McNeilly, 1988). Since the majority of the FSH-suppressing activity in bFF is attributable to its inhibin content (Muttukrishna & Knight, 1990), this delay in return to oestrus has been assumed to be due to the indirect action of inhibin on follicular growth. However, although Quirk & Fortune (1986) demonstrated a small decrease in peripheral FSH concentrations in bFF treated heifers, other workers have been unable to demonstrate any such suppression, in spite of a distinct delay in oestrus (Johnson & Smith, 1985). Furthermore, we have failed to suppress either FSH or follicular development using partially purified follicular fluid fractions (Chapters 4 and 5).

In addition, several studies using PMSG treated hypophysectomised ewes as experimental models have suggested that follicular fluid proteins may act at the level of the ovary to suppress follicular growth directly (Cahill, 1984; Cahill *et al.*, 1985; Larson *et al.*, 1987). In the light of these observations, we investigated the importance of inhibin in the bFF-induced delay in return to oestrus following prostaglandin treatment in heifers.

7.2. *Materials and Methods*

7.2.1. *Animals*

Eighteen Hereford x Friesan heifers were used in the study under the husbandry conditions described in Chapter 2.

7.2.2. *Preparation of follicular fluid*

Follicular fluid for both pilot and main experiments was obtained from bovine ovaries obtained at slaughter as detailed in Chapter 2.

7.2.3. *Pilot Experiment*

7.2.3.1. *Protocol*

Oestrous activity in all animals was synchronised with two i.m injections of a potent prostaglandin $F_{2\alpha}$ analogue (Estrumate) given twelve days apart. At the time of second PG injection (Day 0), the animals were assigned at random to one of four experimental groups and received i.v. injections of either 10 ml saline (Control group; n=6) or 2.5 ml (n=6), 5 ml (n=6) or 10 ml (n=6) whole bFF every eight hours for two days. Animals were observed every eight hours throughout the experiment for signs of oestrus; oestrus detection was aided by the use of a commercial heat detection device (Kamar Tags).

7.2.4. *Main Experiment*

7.2.4.1. *Immunoaffinity extraction of follicular fluid*

Immunoaffinity gels were prepared and follicular fluids processed as described in Chapter 2

7.2.4.2. *Characterisation of follicular fluid pools*

Electrophoretic analysis of the fraction of bFF retained on the anti-inhibin column revealed the presence of multiple molecular weight bands of staining (see Plate 7.1). These corresponded well to the multiple molecular weight species of inhibin initially described by Miyamoto *et al.* (1986), as well as the free monomeric α subunits known to be present in bFF (Knight *et al.*, 1989; Robertson *et al.*, 1989; Sugino *et al.*, 1989), confirming that the antibody was removing these components. The major band of intense staining corresponded to the 58 kDa molecular weight form of inhibin confirming that inhibin, not the free α subunit was the major extract. Estimation of the inhibin activity by radioimmunoassay (McNeilly *et al.*, 1989) demonstrated that at least 85% of the inhibin activity was removed by the chromatography process. A subsequent bioassay utilising the suppression of basal FSH release from ovine pituitary cells in culture as a measure of inhibin activity (Tsonis, McNeilly & Baird, 1986) demonstrated that the "inhibin-free" follicular fluid fraction contained less than 5% of the activity of an untreated follicular fluid pool (see Figure 7.1).

7.2.4.3. *Protocol*

Oestrous activity in all animals was synchronised with two i.m injections of a potent prostaglandin $F_{2\alpha}$ analogue (Estrumate) twelve days apart. At the time of second PG injection (Day 0), the animals were assigned at random to one of three experimental groups and received i.v. injections of either saline (Control group; n=6), whole bFF (FF group; n=6)

Plate 7.1

Electrophoretic analysis of the fraction of follicular fluid retained on the inhibin affinity column (Lane A). Molecular weight markers were run in Lane B. The molecular weights of the individual markers are shown to the right.

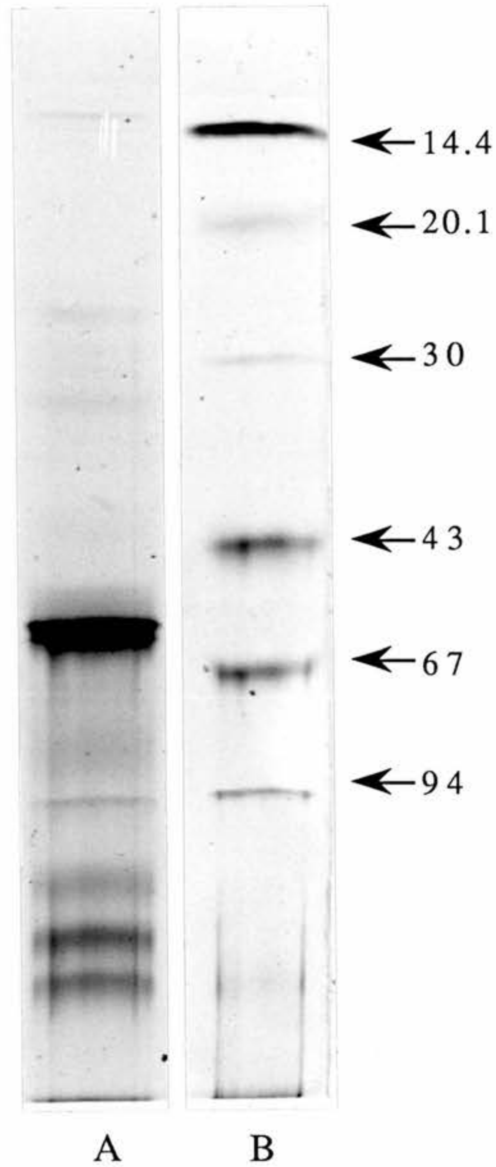
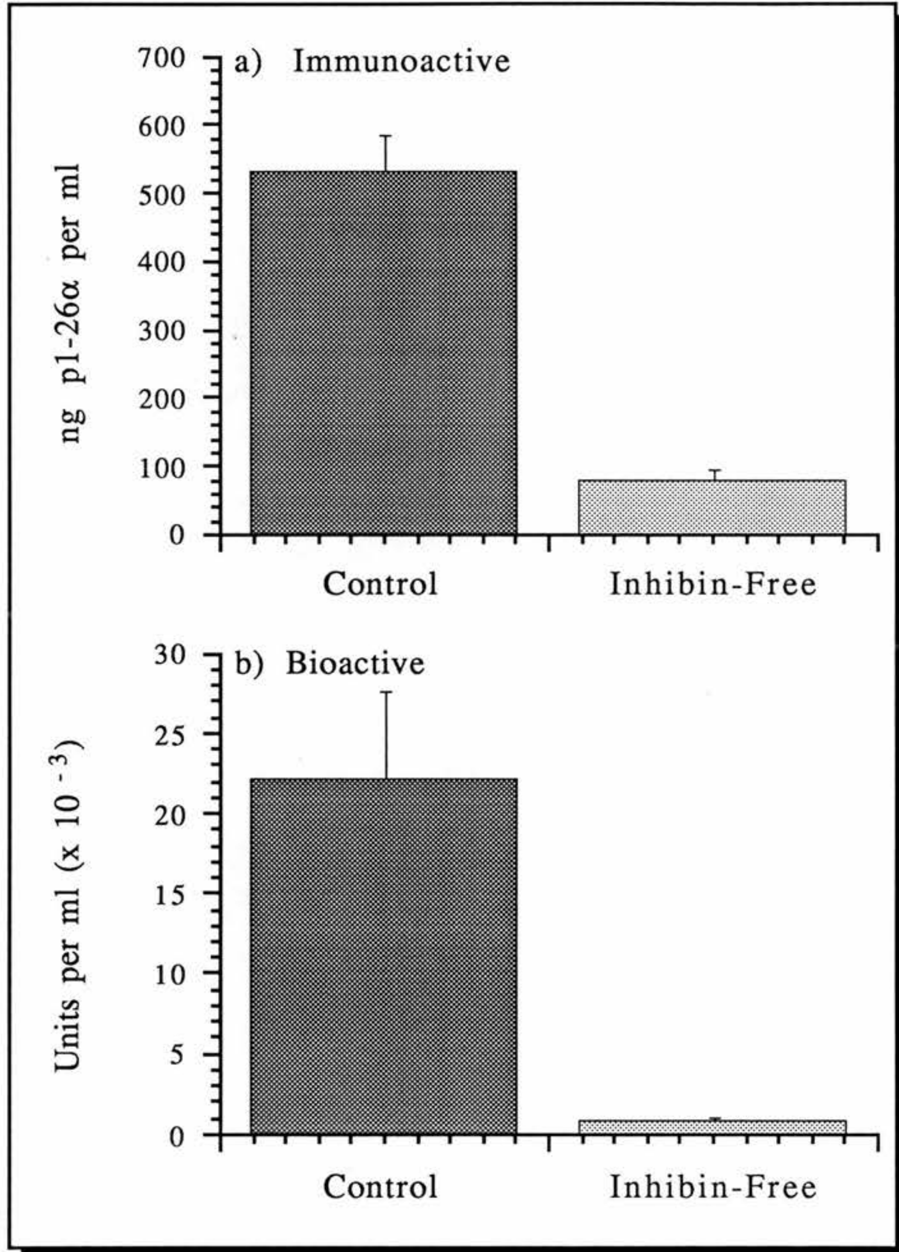


Figure 7.1

The effect of immunoaffinity extraction of follicular fluid on immunoactive (a) and bioactive (b) inhibin content (mean ± sem).



or bFF, the inhibin content of which had been greatly reduced (-INH group; n=6). Each follicular fluid injection contained the protein equivalent of 20 mls untreated follicular fluid diluted to a volume of 40 mls in saline. The injections were repeated every eight hours for two days, a total of six injections per animal. The inhibin content of each injection of "inhibin-free" follicular fluid was equivalent to less than 1 ml whole follicular fluid by bioassay or 2.8 ml by immunoassay. Blood samples were taken every eight hours, initially immediately before each injection and subsequently for a further 9 days for hormone analysis. Animals were observed every eight hours throughout the experiment for signs of oestrus; oestrus detection was aided by the use of a commercial heat detection device (Kamar Tags: Kamar Inc., Steamboat Springs, Colorado). In addition, the ovaries of all animals were examined using real time ultrasonography on Day 2 to examine the effects of treatment on the size of the largest follicle on a pair of ovaries.

7.2.4.4. Hormone Assays

7.2.4.4.1. FSH Assay

FSH was determined using a modification of the homologous double antibody radioimmunoassay system described by Bolt and Rollins (1983) as described in Chapter 2. All samples were measured in a single assay. The sensitivity of the assay, taken to be the dose of hormone required to significantly suppress the binding of labelled hormone below that observed in zero standards was 0.75 ng/tube (3 ng/ml) and the within assay coefficient of variation calculated over the whole assay was 16.21%.

7.2.4.4.2. Progesterone Assay

Progesterone was assayed directly in a single assay. The sensitivity of the assay was 7.8 pg/tube (0.16 ng/ml) and the within assay coefficient of variation was 13.16%.

7.2.4.4.3. *Oestradiol Assay*

Oestradiol was assayed in pooled daily samples. All samples were assayed in a single assay with a mean extraction efficiency of 58.05 ± 0.013%. The sensitivity of the assay was 1.2 pg/tube and the within assay coefficient of variation was 13.87%.

7.2.5. *Statistical analysis*

Comparison of hormone profiles between groups was carried out by analysis of variance. Between group comparisons of follicle diameter were performed using Student's t-test and length of time to return of oestrus was analysed by means of the Mann-Whitney test.

7.3. ***Results***

7.3.1. *Pilot Experiment*

7.3.1.1. *Oestrus*

All control animals exhibited behavioural oestrus within four days of the second PG injection with a mean interval to oestrus of 74.7 hrs (± 6.67 hrs; sem). Oestrus was not significantly delayed ($p > 0.05$) in animals treated with 2.5 ml (113.33 hrs ± 27.51 hrs; sem) or 5 ml bFF (138.67 ± 22.12 hrs; sem) per injection, although the delay in oestrus approached significance ($p = 0.0547$) in the higher dose group. Oestrus was uniformly and significantly ($p < 0.005$) delayed in all animals treated with 10 ml bFF per injection (190.67 hrs ± 5.72 hrs; sem).

7.3.2. *Main Experiment*

7.3.2.1. *Oestrus.*

All control animals exhibited behavioural oestrus within four days of the second PG injection with a mean interval to oestrus of 68 hrs (± 8.26 hrs; sem). Oestrus was uniformly and significantly ($p < 0.001$) delayed in both treatment groups to 186.4 ± 4.66 hrs (FF group; ± sem)

and 190.7 ± 5.72 hrs (-INH group; ± sem) after PG. There was no difference ($p > 0.05$) between the two treatment groups.

7.3.2.2. Follicle Size

The mean diameter of the largest follicle on a pair of ovaries after one day of treatment was not significantly different ($p > 0.05$) between the two follicular fluid treatment groups (7.54 ± 0.40 mm vs. 8.47 ± 0.88 mm; -INH vs. FF groups). However, the mean diameter of the largest follicle in the saline treated control group animals was significantly greater ($p < 0.01$) than that seen in the combined treatment groups (10.94 ± 0.90 mm vs. 8.05 ± 0.51 mm; Controls vs. All treated animals).

7.3.2.3. Progesterone Concentrations

The pattern of daily peripheral progesterone concentrations in each group is shown in Figure 7.2. Progesterone concentrations fell rapidly to below minimum detectable levels in all animals following the second PG injection. A steady and sustained increase was observed in control animals, commencing on Day 6 and continuing to the end of the sampling period. Progesterone remained below minimum detectable levels in all animals in both treatment groups. Samples taken from treated animals on Day 16 revealed that progesterone levels had risen significantly above minimum detectable concentrations by this time. (1.54 ng/ml ± 0.09; mean ± sem).

7.3.2.4. Oestradiol concentrations.

The pattern of daily oestradiol concentrations in each group is shown in Figure 7.3. Following PG injection at time 0, oestradiol concentrations rose rapidly in control animals, reaching a peak at around the time of onset of oestrus in these animals. Following oestrus, oestradiol fell rapidly to basal levels and then subsequently rose to reach a second, smaller peak level at around day 8-9. In treated animals, oestradiol concentrations were

Figure 7.2

Mean daily progesterone concentrations in heifers treated with saline (Control group), steroid-free bovine follicular fluid (FF group) or steroid and inhibin-free bovine follicular fluid (-INH group) following prostaglandin injection.

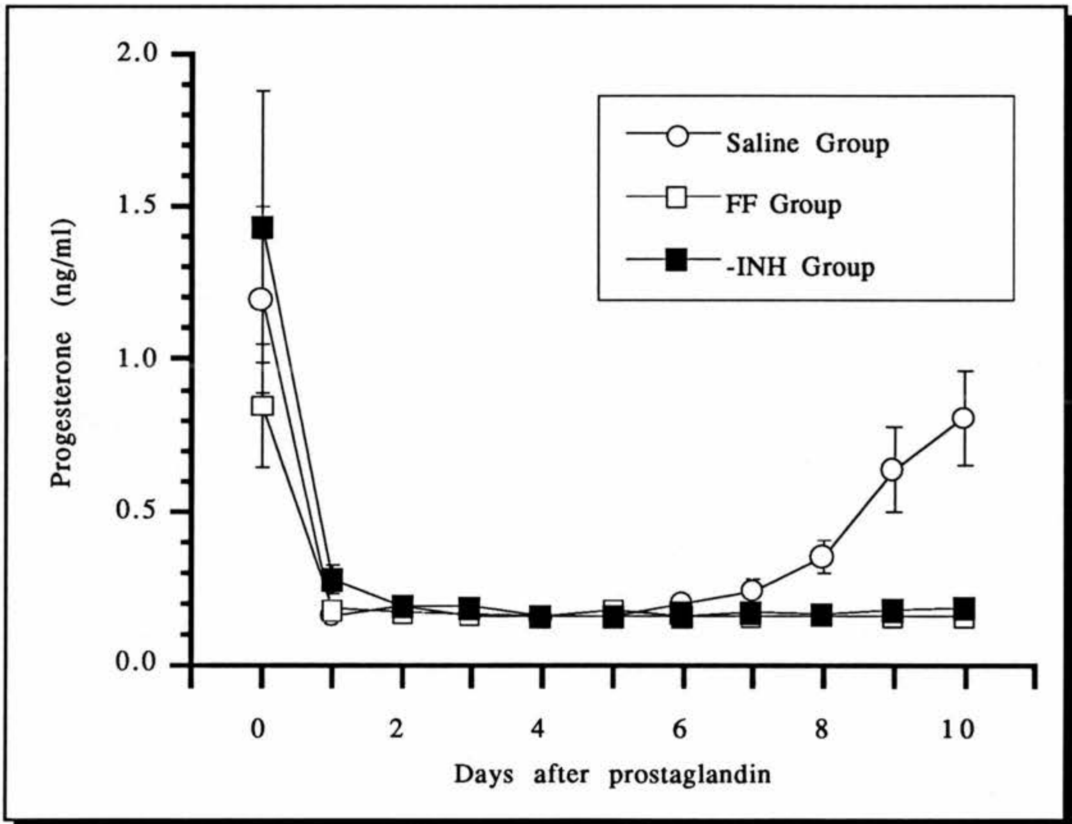


Figure 7.3

Mean daily oestradiol concentrations in heifers treated with saline (Control group), steroid-free bovine follicular fluid (FF group) or steroid and inhibin-free bovine follicular fluid (-INH group) following prostaglandin injection at day 0.

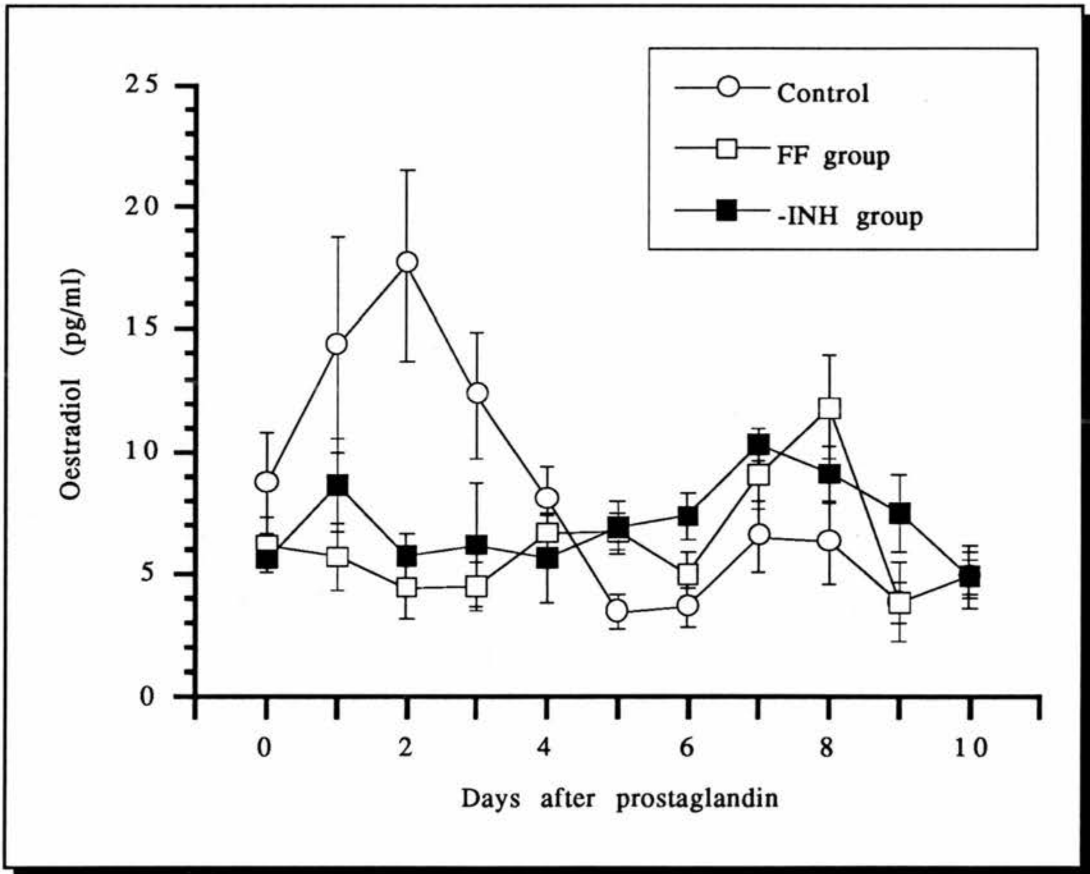
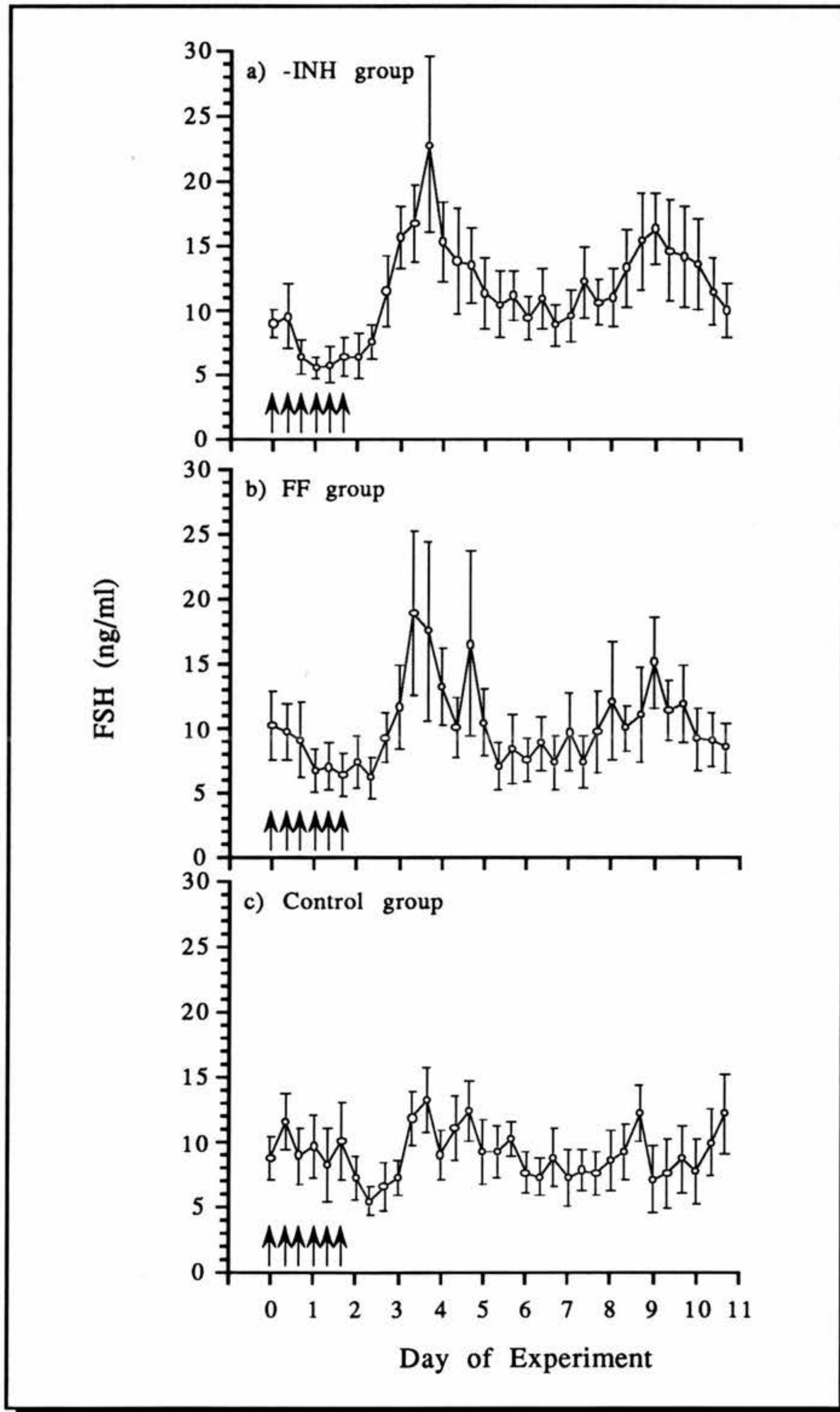


Figure 7.4
Mean FSH concentrations in blood samples taken every eight hours from heifers treated with steroid and inhibin-free bovine follicular fluid (-INH group), steroid-free bovine follicular fluid (FF group) or saline (Control group) following prostaglandin injection on Day 0. The arrows indicate the timing of each follicular fluid/saline injection.



initially suppressed below those seen in controls, but rose to a peak at around day 8-9, again coincident with the onset of oestrus in these animals. The amplitude of the pre-ovulatory peak of oestradiol in treated animals did not differ between treatment, but was significantly ($p < 0.05$) smaller than that seen in saline treated controls.

7.3.2.5. FSH concentrations

The pattern of FSH secretion in each group is shown in Figure 7.4. There was a tendency for FSH concentrations to fall throughout the experimental period in all groups including the controls. However, at no point during the treatment period were concentrations in treatment groups significantly different from those observed in control animals. Following the cessation of injections, FSH concentrations rebounded in both experimental groups reaching levels significantly higher than both treatment period values ($p < 0.01$) and control values ($p < 0.05$) and then returned back to levels similar to those seen in controls. There was no significant difference in FSH profiles between the -INH and the FF groups at any time point during the experiment.

7.4. Discussion

These results demonstrate that treatment of heifers with steroid-free bFF delays the onset of oestrus following prostaglandin injection as previously reported (Miller *et al.*, 1979; Johnson & Smith, 1985; Quirk & Fortune, 1986) and that the delay in oestrus is not dependent on the inhibin content of the follicular fluid. Furthermore, they also show that the delay in oestrus is not due to a failure of luteolysis, an observation previously confirmed in both cattle and sheep (Miller *et al.*, 1979; Johnson & Smith, 1985; Quirk & Fortune, 1986). Rather it involves a suppression of follicular development, as evidenced by the reduced peripheral oestradiol concentrations reported in this and previous

reports (Johnson & Smith, 1985; Quirk & Fortune, 1986), and confirmed by the ultrasound data. A similar ultrasound-monitored suppression of follicular development by bFF at various times throughout the oestrous cycle has also recently been reported (Kastelic, Ko & Ginther, 1990)

It has been suggested that the bFF-induced suppression of follicular development is due to an inhibin-mediated suppression of FSH secretion in both sheep (Miller, Critser & Ginther, 1982; McNeilly, 1984; Wallace & McNeilly, 1985, 1986; Henderson *et al.*, 1986; Hunter *et al.*, 1988) and cattle (Quirk & Fortune, 1986). In addition, McNeilly (1985) demonstrated that the bFF-induced delay in the onset of oestrus in the ewe could be prevented by the infusion of exogenous FSH, albeit using supra-physiological levels, thus confirming the FSH dependent nature of the phenomenon. However, in both the present study and that of Johnson & Smith (1985), no such suppression of FSH secretion was observed. In addition, no difference in response to treatment with either intact, or "inhibin-free" follicular fluid was observed in this study. Although follicular fluid contains at least one other protein with FSH-suppressing activity, namely follistatin (Robertson *et al.*, 1987; Ueno *et al.*, 1987), its potency is considerably less than that of inhibin (Robertson *et al.*, 1990), and co-incubation with specific anti-inhibin antisera completely abolishes the FSH-suppressive action of bFF in ovine pituitary cell cultures (Muttukrishna & Knight, 1990). Removal of the majority of the inhibin from follicular fluid would therefore be expected to remove the major part of any FSH-suppressing activity. The ability of the inhibin-free bFF to delay oestrus in this experiment would therefore suggest that the action of bFF in delaying oestrus is not related to its inhibin content. A recent report has also demonstrated that removal of 92% of the inhibin activity from bFF does not reduce its ability to suppress follicular development and delay oestrus in sheep (Campbell, Picton, Mann,

McNeilly & Baird, 1990). However, the possibility remains that the inhibin remaining in the follicular fluid was sufficient to cause the observed effects. Immunoaffinity extraction of the bFF removed more than 85% of the inhibin in this study; the remaining activity was therefore equivalent to less than 3 ml whole bFF inhibin content per injection. However, since Quirk & Fortune (1986) have previously failed to delay oestrus in half their animals using a dose of 10 ml bFF twice a day, and we have observed that doses of 2.5 ml and 5 ml bFF three times per day are unable to consistently delay oestrus, this seems unlikely, particularly in view of the fact that bovine pituitary cells are unresponsive to inhibin-containing preparations *in vitro* (Knight *et al.* 1990; A.S.Law & R.Webb, unpublished observations). The weight of evidence therefore suggests that the nature of the principal action of bFF is most probably a reduction in the ovarian sensitivity to gonadotrophins.

Indeed, evidence to support a direct ovarian action of bFF has already been documented in sheep; several groups using hypophysectomised ewes as experimental models have demonstrated that bFF will suppress follicular development in the face of controlled PMSG treatment (Cahill, 1984; Cahill *et al.*, 1985; Larson *et al.*, 1987). Furthermore, Fry, Clarke & Cahill (1987), using a similar model, have suggested that changes in gonadotrophin concentrations are not involved in the phenomenon of compensatory ovarian hypertrophy following unilateral ovariectomy. Therefore it would appear that ovarian follicular proteins are capable of acting to suppress the development of other follicles in some manner.

The nature of the protein responsible for this action is unclear. There are many proteins in follicular fluid, several of which have been characterised as having effects on the development and function of ovarian cells both *in vivo* and *in vitro*. Sato described the presence in bovine and porcine follicular fluid of a protein factor which was

produced by the granulosa cells, and which prevented compensatory ovarian hypertrophy in mice (Sato & Ishibashi, 1977, 1978; Sato, Miyamoto, Ishibashi & Iritani, 1978). Similarly the ovary containing the pre-ovulatory follicle secretes a protein named follicle-regulatory protein (FRP; diZerega, Goebelsmann & Nakamura, 1982) which is reportedly produced by the granulosa cells (diZerega *et al.*, 1983b; Tonetta *et al.*, 1988) and which reduces the responsiveness of other follicles to gonadotrophins (diZerega, Goebelsmann & Nakamura, 1982; diZerega *et al.*, 1983d). Injection of porcine FRP suppressed follicular development in cyclic monkeys (diZerega & Wilks, 1984) and guinea pigs (Fujimori *et al.*, 1987). Other authors have also reported the presence in porcine and bovine follicular fluid, of several factors which inhibit the binding of FSH to its receptor (Sluss, Fletcher & Reichert, 1983; Sluss & Reichert, 1984a; Sluss *et al.*, 1987; Sluss *et al.*, 1989), although this activity may be the result of bacterial action (Sluss & Reichert, 1984). Any one or more of these follicular fluid components may be responsible for the observed effects of bFF treatment. The exact nature of the active principle awaits further research.

Of additional interest is the observation of a 'rebound' hypersecretion of FSH in the absence of any prior suppression. The similarity of the rebound between intact and inhibin-stripped bFF-treated groups suggests that the rebound is not due to an action of inhibin. Campbell *et al.* (1990) have confirmed that this rebound of FSH is not inhibin mediated and concluded that it was due to the reduced ovarian negative feedback resulting from the bFF-induced suppression of follicular development. However, if this were the case, then the increase in FSH secretion would be coincident with the onset of treatment with inhibin-free bFF, as the effect of bFF treatment on follicular function is rapid (Baird, Campbell & McNeilly, 1990; Campbell *et al.*, 1990; Chapter 6; this study). The timing of

the FSH rebound in relation to the cessation of treatment is suggestive of the action of a bFF protein other than inhibin in the negative feedback regulation of FSH. The maintenance of FSH concentrations in the face of reduced ovarian oestradiol production during the treatment period would further suggest that the action of the putative bFF factor is to increase the sensitivity of the pituitary gland to steroid negative feedback. These observations also warrant further investigation.

The timing of the development of the ovulatory follicle in the bFF-treated groups in relation to the development of the first non-ovulatory wave of follicles in saline treated controls is also of interest. Waves of follicles develop regularly throughout the oestrous cycle (Sirois & Fortune, 1988; Savio *et al.*, 1988; Savio, Boland & Roche, 1990), with only the dominant follicle of the final wave ovulating. The process of ovulation presumably removes the factors responsible for the dominance of the ovulatory follicle and allows the initiation of the first non-ovulatory wave. The cessation of bFF treatment in this study was approximately coincident with the timing of oestrus and ovulation in the saline-treated control animals. The coincidental rise in oestradiol secretion in all treatment groups may therefore reflect a similar withdrawal of follicular suppression in all groups, suggesting that the factor(s) responsible for bFF-induced suppression of follicular development and follicular dominance are the same.

Interestingly, the size of the pre-ovulatory peak of oestradiol was lower in both bFF-treated groups than in saline treated controls. However, it was clearly sufficient to elicit oestrous behaviour and trigger the endocrine events leading to luteinisation. The reasons for this reduced oestradiol secretion are not immediately apparent. Possibly the prolonged 'follicular phase' caused by the bFF treatment following the fall in progesterone at luteolysis resulted in an increase in the sensitivity

of the hypothalamus/pituitary gland to oestradiol, and hence a premature initiation of the gonadotrophin surge, thus preventing the normal rise in oestradiol concentrations. Progesterone treatment has previously been demonstrated to delay the onset of the pre-ovulatory gonadotrophin surge in GnRH treated anoestrous ewes (McLeod, Haresign & Lamming, 1982). Alternatively, the reduced oestradiol secretion may be due to a direct action of the prior bFF treatment on the developing follicle. The dominant follicle of the primate ovarian cycle exerts effects on non-dominant follicles which remain even after ablation of the dominant follicle (diZerega & Hodgen, 1980). Possibly such effects remained following bFF treatment in this study.

In conclusion, the action of bFF in suppressing follicular development is a direct effect, and is not mediated via reduced FSH concentrations. The activity is not related to the inhibin content of follicular fluid. Furthermore, the hypersecretion of FSH observed following bFF treatment is likewise not due to inhibin, and is not dependent on a prior suppression of FSH. The patterns of oestradiol and FSH concentrations in relation to the timing of the bFF treatment are suggestive of a potentiating effect of non-inhibin bFF proteins on the negative feedback effects of oestradiol. These results further suggest that the mechanism of follicular dominance does not involve a selective reduction in FSH concentrations.

Chapter 8

General Discussion

In summary, we have demonstrated the possible existence of several novel protein components of follicular fluid which appear to be involved in the negative and positive feedback regulation of gonadotrophin secretion in the heifer. Immunisation against protein fractions of porcine or ovine follicular fluid resulted in a marked increase in both LH and FSH secretion, apparently through a disruption of oestradiol-mediated negative feedback. The i.v. administration of follicular fluid proteins from ovine or bovine, but not porcine follicular fluid resulted in an increase in the amplitude of LH pulses during treatment in the luteal phase of the oestrous cycle. This follicular fluid treatment failed to suppress FSH concentrations, although a dose-dependent hypersecretion of FSH was observed following the cessation of treatment. Similarly, treatment failed to disrupt follicular development and no change in ovulation rate was observed, in spite of a doubling of FSH concentrations in individual animals during the period of hypersecretion. However, the i.v. administration of entire steroid-free bovine follicular fluid clearly did disrupt the normal pattern of follicular development. This latter effect was not due to an action, direct or indirect, of inhibin.

In Chapter 3 we confirmed previous observations from this laboratory in that ovulation rate was unaffected by immunisation against a partially purified fraction of porcine follicular fluid (Price, 1987). This was in spite of marked changes in basal gonadotrophin concentrations. Another previous study in which heifers were immunised against a similar fraction of ovine follicular fluid suggested that ovulation rate could be

increased by such treatment and that gonadotrophin concentrations were increased only transiently (Price *et al.*, 1987). In our study, immunisation against a partially purified ovine follicular fluid preparation resulted in a similar response, in terms of ovulation rate and gonadotrophin secretion, to that observed in animals immunised against a porcine follicular fluid preparation. The reason for the differences between our study and that of Price *et al.*, (1987) is not clear.

In the study described in Chapter 3, differences between immunised and control animals during the luteal phase of the oestrous cycle were restricted to changes in mean concentrations, although LH pulse amplitude was significantly greater in immunised animals during the follicular phase. There was also a tendency for FSH concentrations to rise between the luteal and follicular phases in immunised animals whereas FSH concentrations were reduced during the follicular phase in control animals. These responses were all similar to those observed in previous studies (Price, 1987). Also in Chapter 3, no differences in oestradiol concentrations were observed. These data suggest that the immunised animals are unable to respond correctly to some component of negative feedback, (possibly oestradiol) and imply that some protein component of follicular fluid is necessary for steroid-mediated negative feedback to be fully effective.

It is of interest as to why the immunised animals did not respond to the greatly elevated gonadotrophin concentrations with similarly elevated oestradiol concentrations. If we are correct in our assumption that the changes in immunoassayable LH are due to a lack of sensitivity of the hypothalamus/pituitary gland to negative feedback then the logical conclusion must be that the immunoassayable LH in the peripheral serum of immunised heifers does not represent fully bioactive LH. Changes in the bioactivity of LH have been reported previously in other species and

associated with changes in the molecular form and degree of glycosylation of the LH molecule (Tesone *et al.*, 1986; Veldhuis, Johnson & Dufau, 1987; Kessel *et al.*, 1988; Padmanabhan *et al.*, 1988a,b,1989; Fauser *et al.*, 1989). Furthermore, the degree of LH glycosylation is known to be controlled by oestradiol (Peckham & Knobil, 1976; Padmanabhan *et al.*, 1988b). Changes in the clearance rate of both LH and FSH have also been reported following ovariectomy in several species (Peckham & Knobil, 1976; Weick, 1977; McCreery & Licht, 1983; Montgomery *et al.*, 1984; Fry *et al.*, 1987). Therefore, if our hypothesis is correct, and oestradiol negative feedback is follicular protein-dependent and disrupted by immunisation, then the changes in basal LH concentrations in the absence of changes in apparent endogenous bioactivity may be explained.

It is also of interest that the administration of exogenous follicular fluid proteins does not cause the reverse effect in intact heifers. In fact the administration of follicular fluid proteins of bovine or ovine origin failed to influence FSH secretion during treatment, resulted in a "rebound" hypersecretion of FSH following treatment (Chapters 4 and 5) and led to increased LH pulse amplitude during the luteal phase of the oestrous cycle (Chapter 5). The administration of porcine follicular fluid proteins which had elicited such a major response in Chapter 3 failed to influence any parameter of gonadotrophin secretion (Chapter 5). It is possible that the intact heifer is already responding maximally to its endogenous follicular proteins. Alternatively, since proteins must differ sufficiently from their endogenous homologue to be antigenic, the proteins responsible for the immune response observed in Chapter 3 may be so different from the bovine follicular proteins as to not be biologically active. However, since the administration of bovine follicular fluid proteins in Chapters 4 and 5 also failed to cause the reverse effects, the former explanation seems to be the most plausible.

A further point of note is that we were unable to suppress peripheral FSH in intact heifers with any treatment, even when administering 20 mls bFF three times per day (Chapter 7). It would appear that the bovine pituitary is unresponsive to exogenous inhibin-containing preparations in the presence of an intact endogenous negative-feedback system. It may be significant that the vast majority of reports detailing the effects of follicular fluid administration on FSH secretion in the heifer have been performed using ovariectomised heifers (e.g. Ireland, Curato & Wilson, 1983; Kiracofe *et al.*, 1983; Beard *et al.*, 1988,1989,1990). However, changes in LH pulse amplitude were observed during the administration of bovine and ovine follicular proteins in Chapter 5, despite the absence of any changes in oestradiol. This lack of any changes in oestradiol concentrations suggests that the observed effects do not involve any change in ovarian function and thereby imply that the follicular fluid proteins are directly affecting pituitary function.

Treatment of intact heifers with ovine or bovine follicular protein preparations also resulted in a "rebound" on FSH concentrations despite the absence of any prior suppression. This phenomenon could be inhibin-related as the dose of inhibin activity used in Chapter 5 was similar to that used in the inhibin-free follicular fluid treatment group in Chapter 7. However, since the "rebound" observed in Chapter 4 was dose-dependent, and the size of the "rebound" observed in Chapter 7 was similar in both treatment groups and therefore inhibin-independent, this seems unlikely. It is tempting to speculate that the factor responsible for this "rebound" may be similar to the oestradiol negative-feedback potentiating factor inferred from the results of Chapter 3. Administration of exogenous proteins may cause a down-regulation of either endogenous synthesis or pituitary gland sensitivity to the postulated factor. The sudden withdrawal of the exogenous source may therefore render the

animal temporarily unable to respond to its endogenous follicular proteins and result in the temporary loss of oestradiol negative-feedback control and a "rebound" hypersecretion of FSH.

In Chapter 6 the follicular fluid-induced delay in oestrus was demonstrated to be similar to the phenomenon of follicular dominance, implying that the active principle responsible for enforcing dominance is contained in follicular fluid. However, in Chapter 7 we demonstrated that the effect is a direct effect and is not mediated through inhibin-induced changes in FSH concentrations.

In conclusion, therefore, we have shown that the effects of follicular fluid treatment are not solely due to its inhibin content. Other factors exist which appear to have actions at the level of either the pituitary gland or the ovary to influence steroid negative feedback, LH pulse amplitude and follicular development. In addition, the principle level of the control of ovulation rate does not appear to involve subtle changes in gonadotrophin concentrations in this species. The factor in follicular fluid responsible for the suppression of follicular development following the injection of steroid-free follicular fluid therefore seems to be a likely candidate for this important role and warrants much further investigation. Its purification and isolation may finally provide a reliable means for controlling ovulation rate and inducing twinning in this important domestic species.

Bibliography

Adams, N.R. & Atkinson, S. (1986). Low amounts of oestradiol-17β reduce the ovulation rate of Merino ewes. *Proc. Aust. Soc. Reprod. Biol.* **18**, Abstr. 85.

Adashi, E.Y., Resnick, C.E., Brodie, A.M.H., Svoboda, M.E. & Van Wyk, J.J. (1985c). Somatomedin-C mediated potentiation of follicle-stimulating hormone induced aromatase activity of cultured rat granulosa cells. *Endocrinology* **117**, 2313-2320.

Adashi, E.Y., Resnick, C.E., D'Ercole, J., Svoboda, M.E., & Van Wyk, J.J. (1985d). Insulin-like growth factors as intraovarian regulators of granulosa cell growth and function. *Endocr. Rev.* **6**, 400-420.

Adashi, E.Y., Resnick, C.E., Hernandez, E.R., Hurwitz, A. & Rosenfeld, R.G. (1990). Follicle-stimulating hormone inhibits the constitutive release of insulin-like growth factor binding proteins by cultured rat ovarian granulosa cells. *Endocrinology* **126**, 1305-1307.

Adashi, E.Y., Resnick, C.E., Hernandez, E.R., May, J.V., Knecht, M., Svoboda, M.E. & Van Wyk, J.J. (1988). Insulin-like growth factor-1 as an amplifier of follicle-stimulating hormone action: studies on mechanism(s) and site(s) of action in cultured rat granulosa cells. *Endocrinology* **122**, 1583-1591.

Adashi, E.Y., Resnick, C.E., Svoboda, M.E. & Van Wyk, J.J. (1984). A novel role for somatomedin-C in the cytodifferentiation of the ovarian granulosa cell. *Endocrinology* **115**, 1227-1229.

Adashi, E.Y., Resnick, C.E., Svoboda, M.E. & Van Wyk, J.J. (1985a). Somatomedin-C enhances induction of luteinizing hormone receptors by follicle-stimulating hormone in cultured rat granulosa cells. *Endocrinology* **116**, 2369-2375.

Adashi, E.Y., Resnick, C.E., Svoboda, M.E. & Van Wyk, J.J. (1985b). Somatomedin-C synergizes with follicle-stimulating hormone in the acquisition of progestin biosynthetic capacity by cultured rat granulosa cells. *Endocrinology* **116**, 2135-2142.

Akbar, A.M., Reichert, L.E., Dunn, T.G., Kaltenbach, C.C. & Niswender, G.D. (1974). Serum levels of follicle-stimulating hormone during the bovine estrous cycle. *J. Anim. Sci.* **39**, 360-365.

Alfuraiji, M.M., Broadbent, P.J., Hutchinson, J.S.M., Dolman, D.F. & Atkinson, T. (1989). Effect of time of administration of monoclonal anti-PMSG on superovulatory response and embryo quality in cattle. *Theriogenology* **31**, 165. (Abstr.).

Almeida, A.P. (1987). Seasonal variations in the superovulatory responses to PMSG in dairy cows. *Theriogenology* **27**, 204. (Abstr.).

Al-Obaidi, S.A.R., Bindon, B.M., Findlay, J.K., Hillard, M.A. & O'Shea, T. (1987b). Plasma follicle stimulating hormone in Merino ewes immunized with an inhibin-enriched fraction from bovine follicular fluid. *Anim. Reprod. Sci.* **14**, 39-51.

Al-Obaidi, S.A.R., Bindon, B.M., Hillard, M.A. & O'Shea, T. (1987a). Reproductive characteristics of lambs actively immunized early in life with inhibin-enriched preparations from follicular fluid in cows. *J. Reprod. Fert.* **81**, 403-414.

Al-Obaidi, S.A.R., Bindon, B.M., Hillard, M.A., O'Shea, T. & Piper, L. (1986). Suppression of ovine plasma FSH by bovine follicular fluid: neutralization by plasma from ewes immunized against an inhibin-enriched preparation from bovine follicular fluid. *J. Endocr.* **111**, 1-5.

Amann, R.P. (1989). Treatment of sperm to predetermine sex. *Theriogenology* **31**, 49-60.

Amsterdam, A., May, J.V. & Schomberg, D.W. (1988). Synergistic effect of insulin and follicle-stimulating hormone on biochemical and morphological differentiation of porcine granulosa cells in vitro. *Biol. Reprod.* **39**, 379-390.

Andersen, M.M., Krøll, J., Byskov, A.G. & Faber, M. (1976). Protein composition in the fluid of individual bovine follicles. *J. Reprod. Fert.* **48**, 109-118.

Anderson, G.B. (1978). Methods for producing twins in cattle. *Theriogenology* **9**, 3-16.

Anderson, G.B. (1987). Identification of embryonic sex by detection of H-Y antigen. *Theriogenology* **27**, 81-97.

Anderson, R.R. & McShan, W.H. (1966). Luteinizing hormone levels in pig, cow and rat blood plasma during the estrous cycle. *Endocrinology* **78**, 976-982.

Anderson, S.T., Bindon, B.M., Hillard, M.A., Munro, R.K. & O'Shea, T. (1990b). Effect of immunization with inhibin preparations on plasma gonadotrophins of prepubertal lambs. *Proc. Aust. Soc. Reprod. Biol.* **22**, Abstr. 133.

Anderson, S.T., Gilchrist, R.B., Hinch, G.N., Johnston, T.J., Munro, R.K. & O'Shea, T. (1990a). Embryo recovery from ewes immunized with inhibin preparations. *Proc. Aust. Soc. Reprod. Biol.* **22**, Abstr. 18.

Apte, B.V. & Sheth, A.R. (1982). Levels of inhibin in rat placenta throughout gestation. *Ind. J. Exp. Biol.* **20**, 282-284.

Armstrong, D.T. & Black, D.L. (1966). Influence of luteinizing hormone on corpus luteum metabolism and progesterone biosynthesis throughout the bovine estrous cycle. *Endocrinology* **78**, 937-944.

Armstrong, D.T. & Papkoff, H. (1976). Stimulation of aromatization of exogenous and endogenous androgens in ovaries of hypophysectomized rats *in vivo* by follicle-stimulating hormone. *Endocrinology* **99**, 1144-1151.

Asdell, S.A., de Alba, J. & Roberts, S.J. (1949). Studies on the estrous cycle of dairy cattle: Cycle length, size of corpus luteum and endometrial changes. *Cornell Vet.* **39**, 389-402.

Ashworth, C.J. (1985). Maternal factors affecting early pregnancy in sheep. Ph.D. Thesis, University of Edinburgh.

Aten, R.F., Ireland, J.J., Weems, C.W. & Behrman, H.R. (1987). Presence of gonadotrophin-releasing hormone-like proteins in bovine and ovine ovaries. *Endocrinology* **120**, 1727-1733.

Bagnell, C.A., Greenwood, F.C. & Bryant-Greenwood, G.D. (1984). A paracrine role for follicular relaxin. In *"Gonadal proteins and peptides and their biological significance"*. Eds M.R.Sairam & L.E.Atkinson., World Scientific Publ. Co., Singapore. pp. 299-308.

Baird, D.T., Campbell, B.K. & McNeilly, A.S. (1990). Ovine follicular fluid suppresses the ovarian secretion of androgens, oestradiol and inhibin. *J. Endocr.* **127**, 23-32.

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., Ralph, M.M., Seamark, R.F., Amato, F. & Bindon, B.M. (1982). Pre-ovulatory follicular activity and estrogen secretion of high (Booroola) and low fecundity Merino ewes. *Proc. Aust. Soc. Reprod. Biol.* **14**, Abstr. 83.

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

Bak, A., Greve, T. & Schmidt, M. (1989). Effect of superovulation on reproduction. *Theriogenology* **31**, 169. (Abstr.).

Bak, A., Greve, T., Schmidt, M. & Liboriussen, T. (1989). Effect of superovulation on milk production. *Theriogenology* **31**, 170. (Abstr.).

Baker, H.W.G., Bremner, W.J., Burger, H.G., de Kretser, D.M., Dulmanis, A., Eddie, L.W., Hudson, B., Keogh, E.J., Lee, V.W.K. & Rennie, G.C. (1976). Testicular control of follicle-stimulating hormone secretion. *Rec. Prog. Horm. Res.* **32**, 429-469.

Baranao, J.L.S. & Hammond, J.M. (1984). Comparative effects of insulin and insulin-like growth factors on DNA synthesis and differentiation of porcine granulosa cells. *Biochem. Biophys, Res. Comm.* **124**, 484-490.

Barnes, M.A., Kazmer, G.W., Bierley, S.T., Richardson, M.E. & Dickey, J.F. (1980). Follicle stimulating hormone and estradiol-17β in dairy cows treated with progesterone-releasing intravaginal devices. *J. Dairy Sci.* **63**, 161-165.

Battin, D.A. & diZerega, G.S. (1985a). Effect of human menopausal gonadotropin and follicle regulatory protein(s) on 3β-hydroxy-steroid dehydrogenase in human granulosa cells. *J. Clin. Endocr. Metab.* **60**, 1116-1119.

Battin, D.A. & diZerega, G.S. (1985b). The effect of follicular fluid protein(s) on gonadotropin-modulated secretion of progesterone in porcine granulosa cell cultures. *Am. J. Obstet. Gynecol.* **153**, 432-438.

Beard, A.J., Castillo, R.J., Glencross, R.G., McLeod, B.J. & Knight, P.G. (1988). Highly purified 32kDa bovine inhibin is biologically active in prepubertal ovariectomized heifers. *J. Endocrinol.* **117** (Suppl.), Abstr. 134.

Beard, A.J., Castillo, R.J., McLeod, B.J., Glencross, R.G. & Knight, P.G. (1990). Comparison of the effects of crude and highly purified bovine inhibin (M_r 32 000) on plasma concentrations of FSH and LH in chronically ovariectomized prepubertal heifers. *J. Endocr.* **125**, 21-30.

Beard, A.J., Savva, D., Glencross, R.G., McLeod, B.J. & Knight, P.G. (1989). Treatment of ovariectomized heifers with bovine follicular fluid specifically suppresses pituitary levels of FSH-β mRNA. *J. Molec. Endocrinol.* **3**, 85-91.

Beck, T.W. & Convey, E.M. (1976). Serum LH in heifers after ovariectomy. *J. Anim. Sci.* **43**, 274 (Abstr).

Beck, T.W. & Convey, E.M. (1977). Estradiol control of serum luteinizing hormone concentrations in the bovine. *J. Anim. Sci.* **45**, 1096-1101.

Beck, T.W., Smith, V.G., Seguin, B.E. & Convey, E.M. (1976). Bovine serum LH, GH and prolactin following chronic implantation of ovarian steroids and subsequent ovariectomy. *J. Anim. Sci.* **42**, 461-468.

Beckers, J.F. (1987). Isolation and use of a porcine FSH to improve the quality of superovulation in cattle. *Theriogenology* **27**, 213. (Abstr.).

Bendell, J.J., Lobb, D.K., Chuma, A., Gysler, M. & Dorrington, J.H. (1988). Bovine thecal cells secrete factor(s) that promote granulosa cell proliferation. *Biol. Reprod.* **38**, 790-797.

Bicsak, T.A., Cajander, S.B., Vale, W. & Hsueh, A.J.W. (1988). Inhibin: Studies of stored and secreted forms by biosynthetic labeling and immunodetection in cultured rat granulosa cells. *Endocrinology* **122**, 741-748.

Bicsak, T.A., Tucker, E.M., Cappel, S., Vaughan, J., Rivier, J., Vale, W. & Hsueh, A.J.W. (1986). Hormonal regulation of granulosa cell inhibin biosynthesis. *Endocrinology* **119**, 2711-2719.

Bindon, B.M. (1984). Reproductive biology of the Booroola Merino sheep. *Aust. J. Biol. Sci.* **37**, 163-89.

Bindon, B.M., Piper, L.R., Cheers, M.A., Curtis, Y.M., Nethery, R.D. & Holland, E.J. (1982). Ovulation rate of cattle selected for twinning. *Proc. Aust. Soc. Reprod. Biol.* **14**, Abstr. 99.

Bjersing, L. & Carstensen, H. (1967). Biosynthesis of steroids by granulosa cells of the porcine ovary *in vitro*. *J. Reprod. Fert.* **14**, 101-111.

Blanc, M.R., Cahoreau, C., Courot, M., Dacheux, J.L., Hochereau-de-Reviers, M.Th. & Pisselet, Cl. (1978). Plasma follicle stimulating hormone (FSH) and luteinizing hormone (LH) suppression in the cryptorchid ram by a non-steroid factor (inhibin) from ram rete testis fluid. *Int. J. Androl. Suppl.* **2**, 139-146.

Bogovich, K. & Richards, J.S. (1982). Androgen biosynthesis in developing ovarian follicles: Evidence that luteinizing hormone regulates thecal 17α-hydroxylase and C_{17-20}-lyase activities. *Endocrinology* **111**, 1201-1208.

Boland, M.P., Nancarrow, C.D., Hoskinson, R.M., Murray, J.D., Scaramuzzi, R.J., Radford, H.M., Avenell, J.A. & Bindon, B.M. (1985). Superovulatory response in cows following immunization against testosterone and treatment with bovine follicular fluid and PMSG. *Theriogenology* **23**, 180 (Abstr.).

Bolt, D.J. & Rollins, R. (1983). Development and application of a radioimmunoassay for bovine follicle-stimulating hormone. *J. Anim. Sci.* **56**, 146-154.

Bondioli, K.R., Ellis, S.B. & Pryor, J.H. (1989). The use of male-specific chromosomal DNA fragments to determine the sex of bovine preimplantation embryos. *Theriogenology* **31**, 95-104.

Brambell, F.W.R. (1927). The development and morphology of the gonads of the mouse - Part I. The morphogenesis of the indifferent gonad and of the ovary. *Proc. Roy. Soc. London B* **101**, 391-409.

Bramley, T.A., Stirling, D., Swanston, I.A., Menzies, G.S., McNeilly, A.S. & Baird, D.T. (1987). Specific binding sites for gonadotrophin-releasing hormone, LH/chorionic gonadotrophin, low-density lipoprotein, prolactin and FSH in homogenates of human corpus luteum. II: Concentrations throughout the luteal phase of the menstrual cycle and early pregnancy. *J. Endocr.* **113**, 317-327.

Brand, A. & de Jong, W.H.R. (1973). Qualitative and quantitative micromorphological investigations of the tertiary follicle population during the oestrous cycle in sheep. *J. Reprod. Fert.* **33**, 431-439.

Bryan, K.A., Hammond, J.M., Canning, S., Moudshein, J., Carbaugh, D.E., Clark, A.M. & Hagen, D.R. (1989). Reproductive and growth responses of gilts to exogenous porcine pituitary growth hormone. *J. Anim Sci.* **67**, 196-205.

Buckler, H.M., Healy, D.L. & Burger, H.G. (1989). Purified FSH stimulates production of inhibin by the human ovary. *J. Endocr.* **122**, 279-285.

Burger, H.G. & Igarashi, M. (1988). Inhibin: Definition and nomenclature, including related substances. *J. Clin. Endo. Metab.* **66**, 885-886.

Buscaglia, M., Fuller, J., Mazzola, T., Bacigalupi, K., Shimasaki, S., Ui, M., Castillo, F., Schroeder, R. & Ling, N. (1989). A new intra-ovarian function for follistatin: The inhibition of oocyte meiosis. *Proc. Endocr. Soc. USA* **71**, Abstr. 883.

Butler, W.R., Katz, L.S., Arriola, J., Milvae, R.A. & Foote, R.H. (1983). On the negative feedback regulation of gonadotropins in castrate and intact cattle with comparison of two FSH radioimmunoassays. *J. Anim. Sci.* **56**, 919-929.

Butler, W.R., Malven, P.V., Willett, L.B. & Bolt, D.J. (1972). Patterns of pituitary release and cranial output of LH and prolaction in ovariectomized ewes. *Endocrinology* **91**, 793-801.

Butterwick, R.F., Rowlinson, P., Weekes, T.E.C., Parker, D.S. & Armstrong, D.G. (1988). The effect of long-term daily administration of bovine somatotropin on the performance of dairy heifers during their first lactation. *Anim. Prod.* **46**, 483-527.

Byskov, A.G. (1978). Follicular atresia. In "*The Vertebrate Ovary*." Ed. R.E.Jones. Plenum Press, New York. pp 533-562.

Cady, R.A. & Van Vleck, L.D. (1978). Factors affecting twinning and effects of twinning in holstein dairy cattle. *J. Anim. Sci.* **46**, 950-956.

Cahill, L.P. (1984). Folliculogenesis and ovulation rate in sheep. In "*Reproduction in sheep*", Eds. D.R.Lindsay & D.T.Pearce., Cambridge University Press, pp. 92-98.

Cahill, L.P., Driancourt, M.-A., Chamley, W.A. & Findlay, J.K. (1985). Role of intrafollicular regulators and FSH in growth and development of large antral follicles in sheep. *J. Reprod. Fert.* **75**, 599-607.

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

Cahoreau, C., Blanc, M.R., Dacheux, J.L., Pisselet, Cl. & Courot, M. (1979). Inhibin activity in ram rete testis fluid: Depression of plasma FSH and LH in the castrated and cryptorchid ram. *J. Reprod. Fert. Suppl.* **26**, 97-116.

Callesen, H., Bak, A., Greve, T., Avery, B., Gotfredsen, P., Holm, P., Hyttel, P., Pedersen, J.O., Schmidt, M., Smith, S. & Svanborg, N. (1989). Use of PMSG antisera in superovulated dairy heifers. *Theriogenology* **31**, 179. (Abstr.).

Campbell, B.K. (1989). Inhibin, oestradiol and progesterone production by ovine granulosa cells *in vitro*. *J. Reprod. Fert. Abstr. Series* **3**, Abstr. 37.

Campbell, B.K., McNeilly, A.S. & Baird, D.T. (1989). Episodic ovarian inhibin secretion is not due to LH pulses in anoestrous ewes. *J. Endocrinol.* **123**, 173-179.

Campbell, B.K., Picton, H.M., Mann, G.E., McNeilly, A.S. & Baird, D.T. (1990). Ovine follicular fluid (oFF) acts directly on the ovary to suppress follicular growth and ovarian hormone secretion. *Proc. Aust. Soc. Repr. Biol.* **22**, Abstr. 35.

Campbell, C.S. & Schwartz, N.B. (1977). Steroid feedback regulation of luteinizing hormone and follicle-stimulating hormone secretion rates in male and female rats. *J. Tox. Env. Hlth.* **3**, 61-95.

Carson, R.S. & McMaster, J.W. (1988). Ovarian inhibin content in immature female rats following hypophysectomy: Control by exogenous gonadotrophins. *J. Endocr.* **119**, 185-189.

Casida, L.E., Chapman, A.B. & Rupel, I.W. (1935). Ovarian development in calves. *J. Agric. Res.* **50**, 953-960.

Casida, L.E., Meyer, R.K., McShan, W.H. & Wisnicky, W. (1943). Effects of pituitary gonadotropins on the ovaries and induction of superfecundity in cattle. *Amer. J. Vet. Res.* **4**, 76-94.

Channing, C.P. (1975). Follicle stimulating hormone stimulation of ^{125}I-human chorionic gonadotropin binding in porcine granulosa cell cultures (38780). *Proc. Soc. Exp. Biol. Med.* **149**, 238-241.

Channing, C.P. (1979). Follicular non-steroidal regulators. In "*Ovarian follicular and corpus luteum function*". Eds. C.P.Channing, J.Marsh & W.A.Sadler. Plenum Press, New York, pp. 327-343.

Channing, C.P., Anderson, L.D., Hoover, D.J., Kolena, J., Osteen, K.G., Pomerantz, S.H. & Tanabe, K. (1982). The role of nonsteroidal regulators in control of oocyte and follicular maturation. *Rec. Prog. Horm. Res.* **38**, 331-400.

Channing, C.P., Gagliano, P., Hoover, D.J., Tanabe, K., Batta, S.K., Sulewski, J. & Lebech, P. (1981). Relationship between human follicular fluid inhibin F activity and steroid content. *J. Clin. Endocr. Metab.* **52**, 1193-1198.

Channing, C.P., Tanabe, K., Hahn, D., Phillips, A. & Carraher, R. (1984). Inhibin activity and steroid hormone levels in ovarian extracts and ovarian vein plasma of female monkeys during postnatal development. *Fertil. Steril.* **42**, 453-511.

Channing, C.P., Tanabe, K., Turner, C.K. & Hodgen, G.D. (1982). Antisera to porcine follicular fluid in monkeys: Neutralization of human and pig inhibin activity *in vivo* and *in vitro*. *J. Clin. Endocr. Metab.* **55**, 481-486.

Chappel, S.C. (1979). Cyclic fluctuations in ovarian FSH-inhibiting material in golden hamsters. *Biol. Reprod.* **21**, 447-453.

Chari, S., Duraiswami, S. & Franchimont, P. (1976). A convenient and rapid bioassay for inhibin. *Horm. Res.* **7**, 129-137.

Chari, S., Hopkinson, C.R.N., Daume, E. & Sturm, G. (1979). Purification of "inhibin" from human ovarian follicular fluid. *Acta Endocr.* **90**, 157-166.

Chenault, J.R., Thatcher, W.W., Kalra, P.S., Abrams, R.M. & Wilcox, C.J. (1975). Transitory changes in plasma progestin, estradiol and luteinizing hormone approaching ovulation in the bovine. *J. Dairy Sci.* **58**, 709-717.

Chicz, R.M., Nakamura, R.M., Goebelsmann, U., Campeau, J.D., Tonetta, S.A., Frederick, J.J. & diZerega, G.S. (1985). Follicle regulatory protein noncompetitively inhibits microsomal 3β-hydroxysteroid dehydrogenase activity. *J. steroid Biochem.* **23**, 663-668.

Childs, G., Ellison, D., Foster, L. & Ramaley, J.A. (1981). Postnatal maturation of gonadotropes in the male rat pituitary. *Endocrinology* **109**, 1683-1692.

Choudary, J.B., Gier, H.T. & Marion, G.B (1968). Cyclic changes in bovine vesicular follicles. *J. Anim. Sci.* **27**, 468-471.

Christian, R.E. & Casida, L.E. (1948). The effects of progesterone in altering the estrus cycle of the cow. *J. Anim. Sci.* **7**, 540.

Chupin, D., Cognié, Y., Combarnous, Y., Procureur, R. & Saumande, J. (1987). Effect of purified LH and FSH on ovulation in the cow and ewe. In *"Follicular growth and ovulation rate in farm animals"*. Eds J.F.Roche & D.O'Callaghan. Martinus NijHoff, Dordrecht. pp. 63-71.

Chupin, D., Combarnous, Y. & Procureur, R. (1984). Antagonistic effect of LH on FSH-induced superovulation in cattle. *Theriogenology* **21**, 229. (Abstr.).

Chupin, D., Combarnous, Y. & Procureur, R. (1985). Different effect of LH on FSH-induced superovulation in two breeds of cattle. *Theriogenology* **23**, 184 (Abstr.).

Chupin, D., Huy, N.N., Azan, M., Mauléon, P. & Ortavant, R. (1976). Induction hormonale de naissances gémellaires: Principales conséquences sur les performances zootechniques. *Ann. Zootech.* **25**, 79-94.

Clarke, I.J., Findlay, J.K., Cummins, J.T. & Ewens, W.J. (1986). Effects of ovine follicular fluid on plasma LH and FSH secretion in ovariectomized ewes to indicate the site of action of inhibin. *J. Reprod. Fert.* **77**, 575-585.

Clarke, I.J., Funder, J.W. & Findlay, J.K. (1982). Relationship between pituitary nuclear oestrogen receptors and the release of LH, FSH and prolactin in the ewe. *J. Reprod. Fert.* **64**, 355-362.

Cognie, Y. (1988). Applications of immunological techniques to enhance reproductive performance in the ewe. *11th Int. Congr. Anim. Reprod. A.I., Dublin.* pp. 192-200.

Convey, E.M., Beck, T.W., Neitzel, R.R., Bostwick, E.F. & Hafs, H.D. (1977). Negative feedback control of bovine serum luteinizing hormone (LH) concentration from completion of the preovulatory surge until resumption of luteal function. *J. Anim. Sci.* **46**, 792-796.

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

Cox, R.I., Hoskinson, R.M., Scaramuzzi, R.J., Wilson, P.A. & George, J.M. (1982). Fecundity of domestic livestock. *U.S. Patent 4,331,657.*

Cox, N.M., Stuart, M.J., Althen, T.G., Bennet, W.A. & Miller, H.W. (1987). Enhancement of ovulation rate in gilts by increasing dietary energy and administering insulin during follicular growth. *J. Anim. Sci.* **64**, 507-516.

Craig, J.F. (1930). "Fleming's Veterinary Obstetrics", 4th Ed., Ballière, Tindall and Cox, London p52.

Crawford, R.J., Hammond, V.E., Evans, B.A., Coghlan, J.P., Haralambidis, J., Hudson, B., Penschow, J.D., Richards, R.I. & Tregear, G.W. (1987). α-inhibin gene expression occurs in the ovine adrenal cortex, and is regulated by adrenocorticotropin. *Molec. Endocr.* **1**, 699-706.

Critser, J.K., Miller, K.F., Gunsett, F.C. & Ginther, O.J. (1983). Seasonal LH profile in ovariectomized cattle. *Theriogenology* **19**, 181-191.

Croker, K.P., Cox, R.I., Johnson, T.J. & Wilson, P.A. (1982). The immunization of Merino sheep in Western Australia against steroids as a means of increasing fecundity. *Proc. Aust. Soc. Reprod. Biol.* **14**, Abstr. 96.

Croze, F. & Franchimont, P. (1984a). An in-vitro model for the study of inhibin production by rat ovarian cells. *J. Reprod. Fert.* **72**, 249-260.

Croze, F. & Franchimont, P. (1984b). Biological determination of inhibin in rat ovarian-cell culture medium. *J. Reprod. Fert.* **72**, 237-248.

Cuevas, P., Ying, S.-Y., Ling, N., Ueno, N., Esch, F. & Guillemin, R. (1987). Immunohistochemical detection of inhibin in the gonad. *Biochem. Biophys. Res. Comm.* **142**, 23-30.

Culler, M.D. & Negro-Vilar, A. (1988). Passive immunoneutralization of endogenous inhibin: sex-related differences in the role of inhibin during development. *Molec. Cell. Endocr.* **58**, 263-273.

Culler, M.D. & Negro-Vilar, A. (1989). Endogenous inhibin suppresses only basal follicle-stimulating hormone secretion but suppresses all parameters of pulsatile luteinizing hormone secretion in the diestrous female rat. *Endocrinology* **124**, 2944-2953.

Culler, M.D. & Negro-Vilar, A. (1990). Destruction of testicular Leydig cells reveals a role of endogenous inhibin in regulating follicle-stimulating hormone secretion in the adult male rat. *Molec. Cell. Endocr.* **70**, 89-98.

Cummins, L.J., O'Shea, T., Al-Obaidi, S.A.R., Bindon, B.M. & Findlay, J.K. (1986). Increase in ovulation rate after immunization of Merino ewes with a fraction of bovine follicular fluid containing inhibin activity. *J. Reprod. Fert.* **77**, 365-372.

Cummins, L.J., O'Shea, T., Bindon, B.M., Lee, V.W.K. & Findlay, J.K. (1983). Ovarian inhibin content and sensitivity to inhibin in Booroola and control strain Merino ewes. *J. Reprod. Fert.* **67**, 1-7.

D'Occhio, M.J., Gifford, D.R., Cox, R.I., Weatherly, T. & Setchell, B.P. (1986). Ovarian responses of beef heifers to a bovine serum albumin-testosterone based immunogen. *Proc. Aust. Soc. Reprod. Biol.* **18**, Abstr. 38.

D'Occhio, M.J., Gifford, D.R., Hoskinson, R.M., Weatherley, T. & Setchell, B.P. (1989). Evidence for stimulation of ovarian activity and possible facilitation of puberty in heifers by strategic active immunization against androstenedione. *Proc. Aust. Soc. Reprod. Biol.* **21**, Abstr. 81.

D'Occhio, M.J., Kinder, J.E. & Schanbacher, B.D. (1982). Patterns of LH secretion in castrated bulls (steers) during intravenous infusion of androgenic and estrogenic steroids: Pituitary response to exogenous luteinizing hormone-releasing hormone. *Biol. Reprod.* **26**, 249-257.

Danforth, D.R., Sinosich, M.J., Anderson, T.L., Cheng, C.Y., Bardin, C.W. & Hodgen, G.D. (1987). Identification of a gonadotropin surge-inhibiting factor (GnSIF) in follicular fluid and its differentiation from inhibin. *Biol. Reprod.* **37**, 1075-1082.

Daniel, S.A.J. & Armstrong, D.T. (1983). Involvement of estrogens in the regulation of granulosa cell aromatase activity. *Can. J. Physiol. Pharmacol.* **61**, 507-511.

Darga N.C. & Reichert, L.E. (1978). Some properties of the interaction of follicle stimulating hormone with bovine granulosa cells and its inhibition by follicular fluid. *Biol. Reprod.* **19**, 235-241.

Davies, R.V., Main, S.J. & Setchell, B.P. (1978). Inhibin: Evidence for its existence, methods of bioassay and nature of the active material. *Int. J. Androl. Suppl.* **2**, 102-112.

Davies, R.V., Main, S.J., Young, M.G.W.L. & Setchell, B.P. (1976). Bioassay of inhibin-like activity in rete testis fluid and its partial purification. *J. Endocr.* **68**, 26P (Abstr.).

Davis, L.F., Brien, F.D., Findlay, J.K. & Cumming, I.A. (1981). Interactions between dietary protein, ovulation rate and follicle stimulating hormone level in the ewe. *Anim. Reprod. Sci.* **4**, 19-28.

Davis, M.E., Harvey, W.R., Bishop, M.D. & Gearheart, W.W. (1989). Use of embryo transfer to induce twinning in beef cattle: Embryo survival rate, gestation length, birth weight and weaning weight of calves. *J. Anim. Sci.* **67**, 301-310.

Davis, S.R., Dench, F., Nikolaidis, I., Clements, J.A., Forage, R.G., Krozowski, Z. & Burger, H.G. (1986). Inhibin A-subunit gene expression in the ovaries of immature female rats is stimulated by pregnant mare serum gonadotrophin. *Biochem. Biophys. Res. Comm.* **138**, 1191-1195.

Davis, S.R., Krozowski, Z., McLachlan, R.I. & Burger, H.G. (1987). Inhibin gene expression in the human corpus luteum. *J. Endocr.* **115**, R21-R23.

Day, M.L., Imakawa, K., Garcia-Winder, M., Zalesky, D.D., Schanbacher, B.D., Kittok, R.J. & Kinder, J.E. (1984). Endocrine mechanisms of puberty in heifers: Estradiol negative feedback regulation of luteinizing hormone secretion. *Biol. Reprod.* **31**, 332-341.

Day, M.L., Imakawa, K., Pennel, P.L., Zalesky, D.D., Clutter, A.C., Kittok, R.J. & Kinder, J.E. (1986). Influence of season and estradiol on secretion of luteinizing hormone in ovariectomised cows. *Biol. Reprod.* **35**, 549-553.

de Jong, F.H. (1988). Inhibin. *Physiol. Rev.* **68**, 555-607.

de Jong, F.H. & Sharpe, R.M. (1976). Evidence for inhibin-like activity in bovine follicular fluid. *Nature (Lond.)* **263**, 71-72.

de Jong, F.H., Welschen, R., Hermans, W.P., Smith, S.D. & van der Molen, H.J. (1978). Effects of testicular and ovarian inhibin-like activity, using *in vitro* and *in vivo* systems. *Int. J. Androl. Suppl.* **2**, 125-138.

de Jong, F.H., Welschen, R., Hermans, W.P., Smith, S.D. & van der Molen, H.J. (1979). Effects of factors from ovarian follicular fluid and Sertoli cell culture medium on in-vivo and in-vitro release of pituitary gonadotrophins in the rat: an evaluation of systems for the assay of inhibin. *J. Reprod. Fert. Suppl.* **26**, 47-59.

de Kretser, D.M. & Robertson, D.M. (1989). The isolation and physiology of inhibin and related proteins. *Biol. Reprod.* **40**, 33-47.

de Rose, E.P. & Wilton, J.W. (1988). Development of twinning in beef cattle: Aspects of productivity and profitability. *3rd World Congress on Sheep and Beef Cattle Breeding, Paris.* Vol 1, pp 204-206.

Dekel, N., Sherizly, I., Phillips, D.M., Nimrod, A., Zilberstein, M. & Naor, Z. (1985). Characterization of the maturational changes induced by a GnRH analogue in the rat ovarian follicle. *J. Reprod. Fert.* **75**, 461-466.

Desjardins, C. & Hafs, H.D. (1968). Levels of pituitary FSH and LH in heifers from birth through puberty. *J. Anim. Sci.* **27**, 472-477.

Desjardins, C. & Hafs, H.D. (1969). Maturation of bovine female genitalia from birth through puberty. *J. Anim. Sci.* **28**, 502-507.

Dickerson, G.E. (1978). Animal size and efficiency: Basic concepts. *Anim. Prod.* **27**, 367-379.

Dieleman, S.J. & Bevers, M.M. (1987). Effects of monoclonal antibody against PMSG administered shortly after the preovulatory LH surge on time and number of ovulations in PMSG/PG-treated cows. *J. Reprod. Fert.* **81**, 533-542.

Dieleman, S.J., Bevers, M.M. & Gielen, J.Th. (1987). Increase in the number of ovulations in PMSG/PG-treated cows by administration of monoclonal anti-PMSG shortly after the endogenous LH peak. *Theriogenology* **27**, 222. (Abstr.).

Dierschke, D.J., Battacharya, A.N., Atkinson, L.E. & Knobil, E. (1970). Circhoral oscillations of plasma LH levels in the ovariectomized Rhesus monkey. *Endocrinology* **87**, 850-853.

Diskin, M.G., McEvoy, T.G., Hickey, B.C. & Sreenan, J.M. (1987). More twins in the beef herd: Impact on beef output and financial returns. *Farm and food research (An foras talúntais)* October 1987, 4-6.

diZerega, G.S., Campeau, J.D., Nakamura, R.N., Ujita, E.L., Lobo, R. & Marrs, R.P. (1983c). Activity of a human follicular fluid protein(s) in spontaneous and induced ovarian cycles. *J. Clin. Endocr. Metab.* **57**, 838-846.

diZerega, G.S., Campeau, J.D., Ujita, E.L., Kling, D.R., Marrs, R.P., Lobo, R. & Nakamura, R.M. (1983a). The possible role for a follicular protein in the intraovarian regulation of folliculogenesis. *Sem. Reprod. Endocr.* **1**, 309-320.

diZerega, G.S., Goebelsmann, U. & Nakamura, R.M. (1982). Identification of protein(s) secreted by the preovulatory ovary which suppresses the follicle response to gonadotropins. *J. Clin. Endocr. Metab.* **54**, 1091-1096.

diZerega, G.S. & Hodgen, G.D. (1980). The primate ovarian cycle: Suppression of human menopausal gonadotropin-induced follicular growth in the presence of the dominant follicle. *J. Clin. Endocr. Metab.* **50**, 819-825.

diZerega, G.S. & Hodgen, G.D. (1982). The interovarian progesterone gradient: A spatial and temporal regulator of folliculogenesis in the primate ovarian cycle. *J. Clin. Endocr. Metab.* **54**, 495-499.

diZerega, G.S., Marrs, R.P., Campeau, J.D. & Kling, O.R. (1983b). Human granulosa cell secretion of protein(s) which suppress follicular response to gonadotropins. *J. Clin. Endocr. Metab.* **56**, 147-155.

diZerega, G.S., Marrs, R.P., Roche, P.C., Campeau, J.D. & Kling, O.R. (1983d). Identification of proteins in pooled human follicular fluid which suppress follicular response to gonadotropins. *J. Clin. Endocr. Metab.* **56**, 35-41.

diZerega, G.S., Marut, E.L., Turner, C.K. & Hodgen, G.D. (1980). Asymmetrical ovarian function during recruitment and selection of the dominant follicle in the menstrual cycle of the Rhesus monkey. *J. Clin. Endocr. Metab.* **51**, 698-701.

diZerega, G.S., Tonetta, S.A. & Westhof, G. (1987). A postulated role for naturally occurring aromatase inhibitors in follicle selection. *J. steroid Biochem.* **27**, 375-383.

diZerega, G.S., Turner, C.K., Stouffer, R.L., Anderson, L.D., Channing, C.P. & Hodgen, G.D. (1981). Suppression of follicle-stimulating hormone-dependent folliculogenesis during the primate ovarian cycle. *J. Clin. Endocr. Metab.* **52**, 451-456.

diZerega, G.S. & Wilks, J.W. (1984). Inhibition of the primate ovarian cycle by a porcine follicular fluid protein(s). *Fertil. Steril.* **41**, 635-638.

Dobson, H. (1978). Plasma gonadotrophins and oestradiol during oestrus in the cow. *J. Reprod. Fert.* **52**, 51-53.

Dobson, H. & Dean, P.D.G. (1974). Radioimmunoassay of oestrone, oestradiol-17α and -17β in bovine plasma during the oestrous cycle and last stages of pregnancy. *J. Endocrinol.* **61**, 479-486.

Dodson, W.C. & Schomberg, D.W. (1987). The effect of transforming growth factor-β on follicle-stimulating hormone-induced differentiation of cultured rat granulosa cells. *Endocrinology* **120**, 512-516.

Donaldson, L.E., Bassett, J.M. & Thorburn, G.D. (1970). Peripheral plasma progesterone concentration of cows during puberty, oestrous cycles, pregnancy and lactation and the effects of undernutrition or exogenous oxytocin on progesterone concentration. *J. Endocr.* **48**, 599-614.

Donaldson. L. E. & Hansel, W. (1968). Cystic corpora lutea and normal and cystic Graafian follicles in the cow. *Australian Vet. J.* **44**, 304-308.

Donaldson, L.E. & Ward, D.N. (1985). Superovulation in cattle: Dose response to FSH-W with and without LH contamination. *Theriogenology* **23**, 189 (Abstr.).

Donaldson, L.E. & Ward, D.N. (1987). LH effects on superovulation and fertilization rates. *Theriogenology* **27**, 225. (Abstr.).

Donaldson, L.E., Ward, D.N. & Glenn, S.D. (1986). Use of porcine follicle stimulating hormone after chromatographic purification in superovulation of cattle. *Theriogenology* **25**, 747-757.

Dorrington, J.H. & Armstrong, D.T. (1979). Effects of FSH on gonadal functions. *Rec. Prog. Horm. Res.* **35**, 301-333.

Dorrington, J.H., Bendell, J.J., Chuma, A. & Lobb, D.K. (1987). Actions of growth factors in the follicle. *J. steroid. Biochem.* **27**, 405-411.

Dorrington, J.H., Moon, Y.S. & Armstrong, D.T. (1975). Estradiol-17β biosynthesis in cultured granulosa cells from hypophysectomized immature rats; Stimulation by follicle-stimulating hormone. *Endocrinology* **97**, 1328-1331.

Downing, J.A. & Scaramuzzi, R.J. (1991). Nutrient effects on ovulation rate, ovarian function and the secretion of gonadotrophic and metabolic hormones. *J. Reprod. Fert. Suppl.* **43**, 209-227.

Downing, J.A., Scaramuzzi, R.J. & Joss, J. (1989). Glucose infusion for five days in the late luteal phase will increase ovulation rate in the ewe. *Proc. Aust. Soc. Reprod. Biol.* **21**, Abstr. 71.

Driancourt, M.-A. (1987). Ovarian features contributing to the variability of PMSG-induced ovulation rate in sheep. *J. Reprod. Fert.* **80**, 207-212.

Driancourt, M.A., Cahill, L.P. & Bindon, B.M. (1985). Ovarian follicular populations and preovulatory enlargement in Booroola and control Merino ewes. *J. Reprod. Fert.* **73**, 93-107.

Driancourt, M.A., Fry, R.C., Clarke, I.J. & Cahill, L.P. (1979). Follicular growth and regression during the 8 days after hypophysectomy in sheep. *J. Reprod. Fert.* **79**, 635-641.

Drouin, J., Lagacé, L. & Labrie, F. (1976). Estradiol-induced increase of the LH responsiveness to LH releasing hormone (LHRH) in rat anterior pituitary cells in culture. *Endocrinology* **99**, 1477-1481.

Dufour, J., Cahill, L.P. & Mauléon, P. (1979). Short- and long-term effects of hypophysectomy and unilateral ovariectomy on ovarian follicular populations in sheep. *J. Reprod. Fert.* **57**, 301-309.

Dufour, J.J., Whitmore, H.L., Ginther, O.J. & Casida, L.E. (1972). Identification of the ovulating follicle by its size on different days of the estrous cycle in heifers. *J. Anim. Sci.* **34**, 85-87.

Dunn, H.O., McEntee, K., Hall, C.E., Johnson, R.H. & Stone, W.H. (1979). Cytogenetic and reproductive studies of bulls born co-twin with freemartins. *J. Reprod. Fert.* **57**, 21-30.

Echternkamp, S.E., Gregory, K.E., Dickerson, G.., Cundiff, L.V., Koch, R.M. & Van Vleck, L.D. (1990b). Twinning in cattle: II. Genetic and environmental effects on ovulation rate in puberal heifers and postpartum cows and the effects of ovulation rate on embryonic survival. *J. Anim. Sci.* **68**, 1877-1888.

Echternkamp, S.E., Spicer, L.J., Gregory, K.E., Canning, S.F. & Hammond, J.M. (1990a). Concentrations of insulin-like growth factor-1 in blood and ovarian follicular fluid of cattle selected for twins. *Biol. Reprod.* **43**, 8-14.

Eddie, L.W., Baker, H.W.G., Higginson, R.E. & Hudson, B. (1979). A bioassay for inhibin using pituitary cell cultures. *J. Endocr.* **81**, 49-60.

Eddie, L.W., Baker, H.W.G., Higginson, R.E., Hudson, B., Keogh, E.J., de Kretser, D.M. & Burger, H.G. (1977). An in vitro bioassay for inhibin. *Proc. Endocr. Soc. USA* **59**, Abstr 268.

Edwards, R.G. (1974). Follicular fluid. *J. Reprod. Fert.* **37**, 189-219.

Elgin, R.G., Busby, W.H. & Clemmons, D.R. (1987). An insulin-like growth factor (IGF) binding protein enhances the biologic response to IGF-1. *Proc. Natl. Acad. Sci. USA* **84**, 3254-3258.

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

Erb, R.E. & Morrison, R.A. (1959). Effects of twinning on reproductive efficiency in a herd of Holstein-Friesan cattle. *J. Dairy Sci.* **42**, 512-519.

Erickson, B.H. (1966). Development and senescence of the postnatal bovine ovary. *J. Anim. Sci.* **25**, 800-805.

Erickson, G.F., Hofeditz, C., Unger, M., Allen, W.R. & Dulbecco, R. (1985). A monoclonal antibody to a mammary cell line recognizes two distinct subtypes of ovarian granulosa cells. *Endocrinology* **117**, 1490-1499.

Erickson, G.F. & Hsueh, A.J.W. (1978a). Stimulation of aromatase activity by follicle stimulating hormone in rat granulosa cells *in vivo* and *in vitro*. *Endocrinology* **102**, 1275-1282.

Erickson, G.F. & Hsueh, A.J.W. (1978b). Secretion of "inhibin" by rat granulosa cells in vitro. *Endocrinology* **103**, 1960-1963.

Erickson, G.F., Magoffin, D.A., Dyer, C.A. & Hofeditz, C. (1985). The ovarian androgen producing cells: A review of structure/function relationships. *Endocr. Rev.* **6**, 371-399.

Erickson, G.F. & Ryan, K.J. (1976). Stimulation of testosterone production in isolated rabbit thecal tissue by LH/FSH, dibutyryl cyclic AMP, $PGF_{2\alpha}$ and PGE_2. *Endocrinology* **99**, 452-458.

Erickson, G.F., Wang, C., Casper, R., Mattson, G. & Hofeditz, C. (1982). Studies on the mechanism of LH receptor control by FSH. *Molec. Cell. Endocr.* **27**, 17-30.

Esch, F.S., Shimasaki, S., Cooksey, K., Mercado, M., Mason, A.J., Ying, S.-Y., Ueno, N. & Ling, N. (1987). Complementary deoxyribonucleic acid (cDNA) cloning and DNA sequence analysis of rat ovarian inhibins. *Molec. Endocr.* **1**, 388-396.

Esch, F.S., Shimasaki, S., Mercado, M., Cooksey, K., Ling, N., Ying, S., Ueno, N. & Guillemin, R. (1987). Structural characterization of follistatin: A novel follicle-stimulating hormone release-inhibiting polypeptide from the gonad. *Molec. Endocr.* **1**, 849-855.

Eshkol, A. & Lunenfeld, B. (1972). Gonadotropic regulation of ovarian development in mice during infancy. In "*Gonadotropins*", Eds. B.B.Saxena, C.G.Beling & H.M.Gandy, John Wiley & Sons, Inc., New York. pp. 335-346.

Eto, Y., Tsuji, T., Takezawa, M., Takano, S., Yokogawa, Y. & Shibai, H. (1987). Purification and characterization of erythroid differentiation factor (EDF) isolated from human leukemia cell line THP-1. *Biochem. Biophys. Res. Comm.* **142**, 1095-1103.

Everett, J.W. (1988). Pituitary and hypothalamus: Perspectives and overview. In "*The Physiology of Reproduction*". Eds. E.Knobil & J.Neill, Raven Press Ltd., New York. pp. 1143-1159.

Falck, B. (1959). Site of production of oestrogen in rat ovary as studied in micro-transplants. *Acta. Physiol. Scand.* **47**, Suppl. 163.

Falconer, J., Bindon, B.M., Piper, L.R. & Hillard, M.A. (1989). Effects of presence of the F gene on insulin-like growth factor 1 (IGF-1) and its binding protein in follicular fluid from Booroola Merino ewes. *Proc. Aust. Soc. Reprod. Biol.* **21**, Abstr. 138.

Farnworth, P.G., Robertson, D.M., de Kretser, D.M., Findlay, J.K. & Burger, H.G. (1989). Interactions of purified gonadal proteins and steroids on pituitary gonadotrophin synthesis and secretion. *Proc. Aust. Soc. Reprod. Biol.* **21**, Abstr. 74.

Farookhi, R & Desjardins, J. (1986). Luteinizing hormone receptor induction in dispersed granulosa cells requires estrogen. *Molec. Cell. Endocr.* **47**, 13-24.

Fauser, B.C.J.M., Soto, D., Czekala, N.M. & Hsueh, A.J.W. (1989). Granulosa cell aromatase bioassay: Changes of bioactive FSH levels in the female. *J. steroid Biochem.* **33**, 721-726.

Fevold, H.L., Hisaw, F.L. & Leonard, S.L. (1931). The gonad stimulating and the luteinizing hormones of the anterior lobe of the hypophesis. *Am. J. Physiol.* **97**, 291-301.

Findlay, J.K., Clarke, I.J., Quigg, H., Katsahambas, S., Juhola, P., de Blasiis, M. & Doughton, B. (1988a). Inhibin in the sheep ovarian cycle. *Proc. Aust. Soc. Reprod. Biol.* **20**, Abstr. 29.

Findlay, J.K., Clarke, I.J. & Robertson, D.M. (1990). Inhibin concentrations in ovarian and jugular venous plasma and the relationship of inhibin with follicle-stimulating hormone and luteinizing hormone during the ovine estrous cycle. *Endocrinology* **126**, 528-535.

Findlay, J.K., Doughton, B., Robertson, D.M. & Forage, R.G. (1989a). Effects of immunization against recombinant bovine inhibin α subunit on circulating concentrations of gonadotrophins. *J. Endocr.* **120**, 59-65.

Findlay, J.K., Gill, T.W. & Doughton, B.W. (1985). Influence of season and sex on the inhibitory effect of ovine follicular fluid on plasma gonadotrophins in gonadectomized sheep. *J. Reprod. Fert.* **73**, 329-335.

Findlay, J.K., Robertson, D.M. & Clarke, I.J. (1987). Influence of dose and route of administration of bovine follicular fluid and the suppressive effect of purified bovine inhibin (M_r 31 000) on plasma FSH concentrations in ovariectomized ewes. *J. Reprod. Fert.* **80**, 455-461.

Findlay, J.K., Tsonis, C.G., Doughton, B., Brown, R.W., Bertram, K.C., Braid, G.H., Hudson, G.C., Tierney, M.L., Goss, N.H. & Forage, R.G. (1989b). Immunisation against the amino-terminal peptide (α_N) of the $alpha_{43}$ subunit of inhibin impairs fertility in sheep. *Endocrinology* **124**, 3122-3124.

Findlay, J.K., Tsonis, C.G., Doughton, B., Pearson, M., Borchers, C., Hungerford, J., Greenwood, P.E. & Forage, R.G. (1989c). The amino-terminal peptide (αN) of the alpha 43 kDa subunit of inhibin (α43) influences fertility in sheep. *Proc. Aust. Soc. Reprod. Biol.* **21**, Abstr. 134

Findlay, J.K., Xiao, S. & Shukovski, L. (1990). Role of inhibin-related peptides as intragonadal regulators. *Reprod. Fertil. Dev.* **2**, 205-218.

Fitzgerald, J.A., Ruggles, A.J. & Hansel, W. (1985). Increased ovulation rate of adult ewes treated with anti-bovine LH antiserum during the normal breeding season. *J. Anim. Sci.* **60**, 749-754.

Fletcher, P.W., Dias, J.A., Sanzo, M.A. & Reichert, L.E (1982). Inhibition of FSH action on granulosa cells by low molecular weight components of follicular fluid. *Molec. Cell. Endocr.* **25**, 303-315.

Forage, R.G., Brown, R.W., Oliver, K.J., Atrache, B.T., Devine, P.L., Hudson, G.C., Goss, N.H., Bertram, K. C., Tolstoshev, P., Robertson, D.M., de Kretser, D.M., Doughton, B, Burger, H.G. & Findlay, J.K. (1987). Immunization against an inhibin subunit produced by recombinant DNA techniques results in increased ovulation rate in sheep. *J. Endocr.* **114**, R1-R4.

Forage, R.G., Ring. J.M., Brown, R.W., McInerney, B.V., Cobon, G.S., Gregson, R.P., Robertson, D.M., Morgan, F.J., Hearn, M.T.W, Findlay, J.K., Wettenhal, R.E.H., Burger, H.G. & de Kretser, D.M (1986). Cloning and sequence analysis of cDNA species coding for the two subunits of inhibin from bovine follicular fluid. *Proc. Natl. Acad. Sci. USA* **83**, 3091-3095.

Forrest, D.W., Fleeger, J.L., Long, C.R., Sorenson, A.M. & Harms, P.G. (1980). Effect of exogenous prolactin on peripheral luteinizing levels in ovariectomized cows. *Biol. Reprod.* **22**, 197-201.

Fortune, J.E., Sirois, J., Turzillo, A.M. & Lavoir, M. (1991). Follicle selection in domestic ruminants. *J. Reprod. Fert. Suppl.* **43**, 187-198.

Foster, J.P., Lamming, G.E. & Peters, A.R. (1980). Short-term relationships between plasma LH, FSH and progesterone concentrations in post-partum dairy cows and the effect of GnRH injection. *J. Reprod. Fert.* **59**, 321-327.

Fowler, P.A., Messinis, I.E. & Templeton, A.A. (1990). Inhibition of LHRF-induced LH and FSH release by gonadotrophin surge-attenuating factor (GnSAF) from human follicular fluid. *J. Reprod. Fert.* **90**, 587-594.

Franchimont, P. (1972). Human gonadotrophin secretion. *J. Roy. Coll. Phycns., Lond.* **6**, 283-298.

Franchimont, P. & Channing, C.P. (1981). "Intragonadal regulation of reproduction". Academic Press, New York.

Franchimont, P., Chari, S., Hagelstein, M.T. & Duraiswami, S. (1975). Existence of a follicle-stimulating hormone inhibiting factor 'inhibin' in bull seminal plasma. *Nature* **257**, 402-404.

Franchimont, P., Demoulin, A., Verstraelen-Proyard, J., Hazee-Hagelstein, M.T. & Tunbridge, W.M.G. (1979). Identification in human seminal fluid of an inhibin-like factor which selectively regulates FSH secretion. *J. Reprod. Fert. Suppl.* **26**, 123-133.

Franchimont, P., Demoulin, A., Verstraelen-Proyard, J., Hazee-Hagelstein, M.T., Walton, J.S. & Waites, G.M.H. (1978). Nature and mechanisms of action of inhibin: Perspective in regulation of male fertility. *Int. J. Androl. Suppl.* **2**, 69-79.

Franchimont, P., Hazee-Hagelstein, M.T., Charlet-Renard, Ch. & Jaspar, J.M. (1986). Effect of mouse epidermal growth factor on DNA and protein synthesis, progesterone and inhibin production by bovine granulosa cells in culture. *Acta Endocr.* **111**, 122-127.

Franchimont, P., Verstraelen-Proyard, J., Hazee-Hagelstein, M.T., Renard, Ch., Demoulin, A., Bourguignon, J.P & Hustin, J. (1979). Inhibin: From concept to reality. *Vitam. Horm.* **37**, 243-302.

Fraser, H.M., Robertson, D.M. & de Kretser, D.M. (1989). Immunoreactive inhibin concentrations in serum throughout the menstrual cycle of the macaque: Suppression of inhibin during the luteal phae after treatment with an LHRH antagonist. *J. Endocr.* **121**, R9-R12.

Frebling, J., Gillard, P. & Menissier, F. (1982). Preliminary results of natural twinning ability in a selected sample of Charolais and Maine-Anjou cows and their heifer progeny. *2nd W.Congr. Genet. Appl. Livest., Madrid.* Vol. VIII, 351-355.

Fredericks, C.M., Lundquist, L.E., Mathur, R.S., Ashton, S.H. & Landgrebe, S.C. (1983). Effects of vasoactive intestinal polypeptide upon ovarian steroids, ovum transport and fertility in the rabbit. *Biol. Reprod.* **28**, 1052-1060.

Fry, R.C., Cahill, L.P., Cummins, J.T., Bindon, B.M., Piper, L.R. & Clarke, I.J. (1987). The half-life of follicle-stimulating hormone in ovary-intact and ovariectomised Booroola and control Merino ewes. *J. Reprod. Fert.* **81**, 611-615.

Fry, R.C., Clarke, I.J. & Cahill, L.P. (1987). Changes in gonadotrophin concentrations are not necessarily involved in ovarian compensation after unilateral ovariectomy in sheep. *J. Reprod. Fert.* **79**, 45-48.

Fujii, T., Hoover, D.J. & Channing, C.P. (1983). Changes in inhibin activity, and progesterone, oestrogen and androstenedione concentrations, in rat follicular fluid throughout the oestrous cycle. *J. Reprod. Fert.* **69**, 307-314.

Fujimori, K., Nakamura, R.M., Tonetta, S.A. & diZerega, G.S. (1987). Cessation of transition-phase follicle growth in the guinea pig by follicle-regulatory protein. *Biol. Reprod.* **37**, 812-822.

Fujimori, K., Rodgers, K.E., Nakamura, R.M., Katt, E., Yanagihara, D. & diZerega, G.S. (1988). Localization of follicle regulatory protein in the porcine ovary. *J. Histochem. Cytochem.* **36**, 589-595.

Fukada, M., Miyamoto, K., Hasegawa, Y, Nomura, M., Igarashi, M., Kangawa, K. & Matsuo, H. (1986). Isolation of bovine follicular fluid inhibin of about 32kDa. *Mol. Cell. Endocrinol.* **44**, 55-60.

Fukuoka, M., Yasuda, K., Taii, S., Takakura, K. & Mori, T. (1989). Interleukin-1 stimulates growth and inhibits progesterone secretion in cultures of porcine granulosa cells. *Endocrinology* **124**, 884-890.

Gay, V.L. & Sheth, N.A. (1972). Evidence for a periodic release of LH in castrated male and female rats. *Endocrinology* **90**, 158-162.

Gilmore, L.O. (1949). The inheritance of functional causes of reproductive inefficiency: A review. *J. Dairy Sci.* **32**, 71-91.

Ginther, O.J., Kastelic, J.P. & Knopf, L. (1989). Composition and characteristics of follicular waves during the bovine estrous cycle. *Anim. Reprod. Sci.* **20**, 187-200.

Ginther, O.J., Knopf, L. & Kastelic, J.P. (1989). Temporal associates among ovarian events in cattle during oestrous cycles with two and three follicular waves. *J. Reprod. Fert.* **87**, 223-230.

Glencross, R.G., Munro, I.B., Senior, B.E. & Pope, G.S. (1973). Concentrations of oestradiol-17β, oestrone and progesterone in jugular venous plasma of cows during the oestrous cycle and in early pregnancy. *Acta Endocrinol.* **73**, 374-384.

Goddard, C., Houston, B. & Gray, C. (1987). Monoclonal antibody to chicken growth hormone. *J. Endocr.* **112** Suppl., Abstr. 125.

Goding, J.R., Blockey, M.A. de B., Brown, J.M., Catt, K.J. & Cumming, I.A. (1970). The rôle of oestrogen in the control of the oestrous cycle in the ewe. *J. Reprod. Fert.* **21**, 368-369.

Goding, J.R., Catt, K.R., Brown, J.M., Kaltenbach, C.C., Cumming, I.A. & Mole, B.J. (1969). Radioimmunoassay for ovine luteinizing hormone. Secretion of luteinizing hormone during estrus and following estrogen administration in the sheep. *Endocrinology* **85**, 133-142.

Goldenberg, R.L., Vaitukaitis, J.L. & Ross, G.T. (1972). Estrogen and follicle stimulating hormone interactions on follicle growth in rats. *Endocrinology* **90**, 1492-1498.

Gong, J.G., Bramley, T.A. & Webb, R. (1990). The effect of bovine somatotropin (BST) on ovarian folliculogenesis and ovulation rate in heifers. *J. Reprod. Fert. Abstract Series* **6**, Abstr. 9.

Gonzalez, A., Lussier, J.G., Carruthers, T.D., Muphy, B.D. & Mapletoft, R.J. (1990). Superovulation of beef heifers with follitropin: A new FSH preparation containing reduced LH activity. *Theriogenology* **33**, 519-529.

Goodman, R.L. & Karsch, F.J. (1980). Pulsatile secretion of luteinizing hormone: Differential suppression by ovarian steroids. *Endocrinology* **107**, 1286-1290.

Goodman, R.L., Legan, S.J., Ryan, K.D., Foster, D.L. & Karsch, F.J. (1980). Two effects of estradiol that normally contribute to the control of tonic LH secretion in the ewe. *Biol. Reprod.* **23**, 415-422.

Goodman, R.L., Pickover, S.M. & Karsch, F.J. (1981). Ovarian feedback control of follicle-stimulating hormone in the ewe: Evidence for selective suppression. *Endocrinology* **108**, 772-777.

Gordon, I., Boland, M.P., McGovern, H. & Lynn, G. (1987). Effect of season on superovulatory responses and embryo quality in Holstein cattle in Saudi Arabia. *Theriogenology* **27**, 231. (Abstr.).

Gordon, I., Williams, G. & Edwards, J. (1962). The use of serum gonadotrophin (P.M.S.) in the induction of twin-pregnancy in the cow. *J. Agric. Sci.* **59**, 143-198.

Gordon, W.L., Liu, W-K., Akiyama, K., Tsuda, R., Hara, M., Schmid, K. & Ward, D.N. (1987). Beta-microseminoprotein (β-MSP) is not an inhibin. *Biol. Reprod.* **36**, 829-835.

Gore-Langton, R.E. & Armstrong, D.T. (1988). Follicular steroidogenesis and its control. In "*The Physiology of Reproduction*". Eds. E. Knobil & J. Neill., Raven Press Ltd., New York. pp. 331-385.

Gottschall, P.E., Uehara, A., Hoffmann, S.T. & Arimura, A. (1987). Interleukin-1 inhibits follicle stimulating hormone-induced differentiation in rat granulosa cells in vitro. *Biochem. Biophys. Res. Comm.* **149**, 502-509.

Grasso, F., Guilbault, L.A., Roy, G.L., Matton, P. & Lussier, J.G. (1989). The influence of the presence of a dominant follicle at the time of initiation of a superovulatory treatment on superovulatory responses in cattle. *Theriogenology* **31**, 199. (Abstr).

Green, J.B.A. & Smith, J.C. (1990). Graded changes in dose of *Xenopus* activin A homologue elicit stepwise transitions in embryonic cell fate. *Nature* **347**, 391-394.

Greenwald, G.S. & Terranova, P.F. (1981). Induction of superovulation in the cyclic hamster by a single injection of antiluteinizing hormone serum. *Endocrinology* **108**, 1903-1908.

Greenwald, G.S. & Terranova, P.F. (1983). "Factors regulating ovarian function". Raven Press, New York.

Greenwood, F.C., Hunter, W.M. & Glover, J.S. (1963). The preparation of ^{131}I-labelled human growth hormone of high specific radioactvity. *Biochem. J.* **89**, 114-123.

Greep, R.O., van Dyke, H.B. & Chow, B.F. (1942). Gonadotropins of the swine pituitary. I. Various biological effects of purified thylakentrin (FSH) and pure metakentrin (ICSH). *Endocrinology* **30**, 635-649.

Gregory, K.E., Echternkamp, S.E., Dickerson, G.E., Cundiff, L.V., Koch, R.M. & Van Vleck, L.D. (1990). Twinning in cattle: I. Foundation animals and genetic and environmental effects on twinning rate. *J. Anim. Sci.* **68**, 1867-1876.

Griffin, J.L. & Randel, R.D. (1978). Reproductive studies of Brahman cattle. II. Luteinizing hormone patterns in ovariectomized Brahman and Hereford cows before and after injection of gonadotropin releasing hormone. *Theriogenology* **9**, 437-446.

Grootenhuis, A.J., Steenbergen, J., Timmerman, M.A., Dorsman, A.N.R.D., Schaaper, W.M.M., Meloen, R.H. & de Jong, F.H. (1989). Inhibin and activin-like activity in fluids from male and female gonads: different molecular weight forms and bioactivity/immunoactivity ratios. *J. Endocr.* **122**, 293-301.

Guerra-Martinez, P., Anderson, G.B. & Dickerson, G.E. (1987). Effects of twin calves on performance and efficiency in beef production. *J. Anim. Sci.* **65** Suppl. 1, 205 (Abstr.).

Guilbault, L.A., Lussier, J.G., Grasso, F. & Roy, G.L. (1989). Concentrations of progesterone and FSH in superovulated heifers pretreated with FSH-P at the beginning of the estrous cycle. *Theriogenology* **31**, 200. (Abstr.).

Guthrie, H.D., Bolt, D.J., Kiracofe, G.H. & Miller, K.F. (1986). Effect of charcoal-extracted porcine follicular fluid (pFF) and porcine follicle-stimulating hormone (USDA-pFSH-B1) on recruitment of medium follicles in gilts. *Biol. Reprod.* **34** Suppl. 1, Abstr. 71.

Hackett, A.J. & Hafs, H.D. (1969). Pituitary and hypothalamic endocrine changes during the bovine estrous cycle. *J. Anim. Sci.* **28**, 531-536.

Hamada, T., Watanabe, G., Kokuho, T., Taya, K., Sasamoto, S., Hasegawa, Y., Miyamoto, K. & Igarashi, M. (1989). Radioimmunoassay of inhibin in various mammals. *J. Endocr.* **122**, 697-704.

Hammond, J. (1927). "The physiology of reproduction in the cow". Cambridge University Press.

Hammond, J. (1946). The induction of ovulation in domestic animals. In "*The problem of fertility*" Ed. E.T.Engle, Princeton University Press, pp. 60-65.

Hamori, D. (1975). The hereditary-pathological and breeding hygienic problems of twinning in cattle. *Acta Agron. Acad. Sci. Hung., Tomus* **24**, 11-17.

Hansel, W. & Echternkamp, S.E. (1972). Control of ovarian function in domestic animals. *Am. Zool.* **12**, 225-243.

Hansel, W. & Snook, R.B. (1970). Pituitary ovarian relationships in the cow. *J. Dairy Sci.* **53**, 945-961.

Hansel, W. & Trimberger, G.W. (1952). The effect of progesterone on ovulation time in dairy heifers. *J. Dairy Sci.* **35**, 65-70.

Hansen, P.J., Kamwanja, L.A. & Hauser, E.R. (1982). The effect of photoperiod on serum concentrations of luteinizing and follicle stimulating hormones in prepubertal heifers following ovariectomy and estradiol injection. *Theriogenology* **18**, 551-559.

Hasegawa, Y., Miyamoto, K., Abe, Y., Nakamura, T., Sugino, H., Eto, Y., Shibai, H., Igarashi, M. (1988). Induction of follicle stimulating hormone receptor by erythroid differentiation factor on rat granulosa cell. *Biochem. Biophys. Res. Comm.* **156**, 668-674.

Hasegawa, Y., Miyamoto, K., Iwamura, S. & Igarashi, M. (1988). Changes in serum concentrations of inhibin in cyclic pigs. *J. Endocr.* **118**, 211-219.

Hasler, J.F., McCauley, A.D., Schermerhorn, E.C. & Foote, R.H. (1983). Superovulatory responses of Holstein cows. *Theriogenology* **19**, 83-99.

Hauger, R.L., Karsch, F.J. & Foster, D.L. (1977). A new concept for control of the estrous cycle of the ewe based on the temporal relationships between luteinizing hormone, estradiol and progesterone in peripheral serum and evidence that progesterone inhibits tonic LH secretion. *Endocrinology* **101**, 807-817.

Hausler, C.L. & Malven, P.V. (1976). Interaction of progesterone, GnRH and estradiol in the control of LH release in castrate heifers. *J. Anim. Sci.* **42**, 1239-1243.

Henderson, K.M. & Franchimont, P. (1981). Regulation of inhibin production of bovine ovarian cells *in vitro*. *J. Reprod. Fert.* **63**, 431-442.

Henderson, K.M. & Franchimont, P. (1983). Inhibin production by bovine ovarian tissues *in vitro* and its regulation by androgens. *J. Reprod. Fert.* **67**, 291-298.

Henderson, K.M., Franchimont, P., Charlet-Renard, Ch. & McNatty, K.P. (1984a). Effect of follicular atresia on inhibin production by bovine granulosa cells *in vitro* and inhibin concentrations in the follicular fluid. *J. Reprod. Fert.* **72**, 1-8.

Henderson, K.M., Franchimont, P., Lecomte-Yerna, M.J., Hudson, N. & Ball, K. (1984b). Increase in ovulation rate after active immunisation of sheep with inhibin partially purified from bovine follicular fluid. *J. Endocr.* **102**, 305-309.

Henderson, K.M., Prisk, M.D., Hudson, N, Ball, K., McNatty, K.P., Lun, S., Heath, D.A., Kieboom, L.E. & McDiarmid, J. (1986). Use of bovine follicular fluid to increase ovulation rate or prevent ovulation in sheep. *J. Reprod. Fert.* **76**, 623-635.

Hendy, C.R.C. & Bowman, J.C. (1970). Twinning in cattle. *Anim. Breeding Abstr.* **38**, 22-37.

Henricks, D.M., Dickey, J.F. & Hill, J.R. (1971). Plasma estrogen and progesterone levels in cows prior to and during estrus. *Endocrinology*, **89**, 1350-1355.

Henricks, D.M., Dickey, J.F. & Niswender, G.D. (1970). Serum luteinizing hormone and plasma progesterone levels during the oestrous cycle and early pregnancy in cows. *Biol. Reprod.* **2**, 346-351.

Hermans, W.P., van Leeuwen, E.C.M., Debets, M.H.M., Sander, H.J. & de Jong, F.H. (1982). Estimation of inhibin-like activity in spent medium from rat ovarian granulosa cells during long-term culture. *Molec. Cell. Endocr.* **27**, 277-290.

Herrier, A., Farries, E. & Niemann, H. (1990). A trial to stimulate insulin like growth factor 1 levels to improve superovulatory response in dairy cows. *Theriogenology* **33**, 248 (Abstr.).

Hillard, M.A., Bindon, B.M., King, B., O'Shea, T., Andrews, C.A. & Hinch, G.N. (1990). Superovulation of cows immunized against native ovine inhibin. *Proc. Aust. Soc. Reprod. Biol.* **22**, Abstr. 134.

Hillier, S.G., van Hall, E.V., van den Boogaard, A.J.M., de Zwart, F.A. & Keyzer, R. (1982). Activation and modulation of the granulosa cell aromatase system: Experimental studies with rat and human ovaries. In "*Follicular maturation and ovulation*". Eds. R.Rolland, E.V.van Hall, S.G.Hillier, K.P.McNatty & J.Schoemaker, Excerpta Medica Int. Congress Series 560, Amsterdam. pp. 51-70.

Hobson, W.C. & Hansel, W. (1972). Plasma LH levels after ovariectomy, corpus luteum removal and estradiol administration in cattle. *Endocrinology* **91**, 185-190.

Hochberg, Z., Weiss, J. & Richman, R.A. (1981). Inhibin-like activity in extracts of rabbit placentae. *Placenta* **2**, 259-264.

Holland, E.J., Bindon, B.M., Piper, L.R., Thimonier, J., Cornish, K.A. & Radford, H.M. (1981). Endoscopy in cattle: Techniques for ovarian examination by the paralumbar and mid-ventral routes. *Anim. Reprod. Sci.* **4**, 127-135.

Holly, J.M.P., Eden, J.A., Alaghband-Zadeh, J., Carter, G.D., Jemmot, R.C., Chard, T. & Wass, J.A.H. (1989). Insulin-like growth factor binding proteins in human follicular fluid (FF) from normal dominant and cohort follicles. *J. Endocr.* **123**, Suppl., Abstr. 101.

Holmberg, E.A., Campeau, J.D., Devereaux, D.L., Ono, T. & diZerega, G.S. (1986a). Comparison of isolelectric focusing in sephadex vs immobiline flatbeds for the recovery of follicle regulatory protein from porcine follicular fluid. *Prep. Biochem.* **16**, 275-295.

Holmberg, E.A., Campeau, J.D., Devereaux, D.L., Ono, T. & diZerega, G.S. (1986b). The use of isolectric focusing in immobiline flatbeds for the recovery of follicle regulatory protein from porcine follicular fluid. *Protein Biol. Fluid.* **34**, 911-915.

Hopkinson, C.R.N., Fritze, E., Chari, S., Sturm, G. & Hirschäuser, C. (1977). Interaction between testosterone and an inhibin preparation in male rats. *IRCS Med. Sci.* **5**, 83.

Hoskinson, R.M., Hinks, N.T. & Scaramuzzi, R.J. (1982). Effects of PMSG on ovulation rate in the oestrone-immunized ewe. *Proc. Aust. Soc. Reprod. Biol.* **14**, Abstr. 93.

Hoskinson, R.M., Scaramuzzi, R.J., Downing, J.A., Hinks, N.T. & Turnbull, K.E. (1982). Observations of oestrus and ovulation in ewes actively immunized against progesterone. *Proc. Aust. Soc. Reprod. Biol.* **14**, Abstr. 92.

Hsueh, A.J.W., Dahl, K.D., Vaughan, J., Tucker, E., Rivier, J., Bardin, C.W. & Vale, W. (1987). Heterodimers and homodimers of inhibin subunits have different paracrine action in the modulation of luteinizing hormone-stimulated androgen biosynthesis. *Proc. Natl. Acad. Sci. USA* **84**, 5082-5086.

Hsueh, A.J.W., Erickson, G.F. & Yen, S.S.C. (1979). The sensitizing effect of estrogens and catechol estrogen on cultured pituitary cells to luteinizing hormone-releasing hormone: Its antagonism by progestins. *Endocrinology* **104**, 807-813.

Hsueh, A.J.W., Welsh, T.H. & Jones, P.B.C. (1981). Inhibition of ovarian and testicular steroidogenesis by epidermal growth factor. *Endocrinology* **108**, 2002-2004.

Hudson, B., Baker, H.W.G., Eddie, L.W., Higginson, R.E., Burger, H.G., de Kretser, D.M., Dobos, M. & Lee, V.W.K. (1979). Bioassays for inhibin: A critical review. *J. Reprod. Fert. Suppl.* **26**, 17-29.

Hudson, N., McNatty, K.P., Ball, K., Gibb, M., Heath, D.A., Lun., S., Kieboom, L. & Henderson, K.M. (1985). Influence of follicle stimulating hormone on ovarian follicular development and ovulation rate in Romney ewes. *Proc. NZ Soc. Anim. Prod.* **45**, 51-54.

Hunter, M.G., Hindle, J.E., McLeod, B.J. & McNeilly, A.S. (1988). Treatment with bovine follicular fluid suppresses follicular development in gonadotrophin-releasing hormone treated anoestrous ewes. *J. Endocr.* **119**, 95-100.

Hunter, W.M., Nars, P.W. & Rutherford, F.J. (1975). Preparation and behaviour of ^{125}I-labelled radioligands for phenolic and neutral steroids. In "*Steroid immunoassay*". Eds. E.H.D.Cameron, S.G.Hillier & K.Griffiths, Alpha Omega, Cardiff. pp. 141-152.

Hutchinson, L.A., Findlay, J.K., de Vos, F.L. & Robertson, D.M. (1987). Effects of bovine inhibin, transforming growth factor-β and bovine activin-A on granulosa cell differentiation. *Biochem. Biophys. Res. Comm.* **146**, 1405-1412.

Imakawa, K., Day, M.L., Zalesky, D.D., Garcia-Winder, M., Kittock, R.J. & Kinder, J.E. (1986). Regulation of pulsatile LH secretion by ovarian steroids in the heifer. *J. Anim. Sci.* **63**, 162-168.

Ireland, J.J. (1987). Control of follicular growth and development. *J. Reprod. Fert. Suppl.* **34**, 39-54.

Ireland, J.J., Coulson, P.B. & Murphree, R.L. (1979). Follicular development during four stages of the estrous cycle of beef cattle. *J. Anim. Sci.* **49**, 1261-1269.

Ireland, J.J., Curato, A.D. & Wilson, J. (1983). Effect of charcoal-treated bovine follicular fluid on secretion of LH and FSH in ovariectomised heifers. *J. Anim. Sci.* **57**, 1512-1516.

Ireland, J.J. & Richards, J.S. (1978b). A previously undescribed role for luteinizing hormone (LH:hCG) on follicular cell differentiation. *Endocrinology* **102**, 1458-1465.

Ireland, J.J. & Richards, J.S. (1978a). Acute effects of estradiol and follicle-stimulating hormone on specific binding of human [^{125}I]iodo-follicle-stimulating hormone to rat ovarian granulosa cells *in vivo* and *in vitro*. *Endocrinology* **102**, 876-883.

Ireland, J.J. & Roche, J.F. (1982a). Development of antral follicles in cattle after prostaglandin-induced luteolysis: Changes in serum hormones, steroids in follicular fluid, and gonadotropin receptors. *Endocrinology* **111**, 2077-2086.

Ireland, J.J. & Roche, J.F. (1982b). Effect of progesterone on basal LH and episodic LH and FSH secretion in heifers. *J. Reprod. Fert.* **64**, 295-302.

Ireland, J.J. & Roche, J.F. (1983a). Development of nonovulatory antral follicles in heifers: Changes in steroids in follicular fluid and receptors for gonadotropins. *Endocrinology* **112**, 150-156.

Ireland, J.J. & Roche, J.F. (1983b). Growth and differentiation of large antral follicles after spontaneous luteolysis in heifers: Changes in concentrations of hormones in follicular fluid and specific binding of gonadotropins to follicles. *J. Anim. Sci.* **57**, 157-167.

Ireland, J.J. & Roche, J.F. (1987). Hypotheses regarding development of dominant follicles during a bovine estrous cycle. In "*Follicular growth and ovulation rate in farm animals*" Eds. J.F.Roche & D.O'Callaghan. Martinus Nijhoff, Lancaster. pp. 1-18.

Jansen, E.H.J.M., Steenbergen, J., de Jong, F.H. & van der Molen, H.J. (1981). The use of affinity matrices in the purification of inhibin from bovine follicular fluid. *Molec. Cell. Endocr.* **21**, 109-117.

Johansson, J., Sheth, A., Cederlund, E. & Jörnvall, H. (1984). Analysis of an inhibin preparation reveals apparent identity between a peptide with inhibin-like activity and a sperm-coating antigen. *FEBS Lett.* **176**, 21-26.

Johnson, L.A., Flook, J.P. & Hawk, H.W. (1989). Altered sex ratios in offspring after surgical insemination of flow-sorted populations of X and Y chromosome-bearing sperm. *Biol. Reprod.* **40**, Suppl 1., 162 (Abstr. 344).

Johnson, M.R., Turman, E.J. & Stephens, D.F. (1975). Gonadotropin induced multiple births in beef cows following estrus synchronization. *J. Anim. Sci.* **41**, 1394-1399.

Johnson, S.K. & Smith, M.F. (1985). Effects of charcoal-extracted, bovine follicular fluid on gonadotropin concentrations, the onset of estrus and luteal function in heifers. *J. Anim. Sci.* **61**, 203-209.

Johnson, S.K., Smith, M.F. & Elmore, R.G. (1985). Effect of unilateral ovariectomy and injection of bovine follicular fluid on gonadotrophin secretion and compensatory ovarian hypertrophy in prepubertal heifers. *J. Anim. Sci.* **60**, 1055-1060.

Kanchev, L.N. & Dobson, H. (1976). Plasma concentration of androstenedione during the bovine oestrous cycle. *J. Endocr.* **71**, 351-354.

Kanchev, L.N., Dobson, H., Ward, W.R. & Fitzpatrick, R.J. (1976). Concentration of steroids in bovine peripheral plasma during the oestrous cycle and the effect of betamethasone treatment. *J. Reprod. Fert.* **48**, 341-345.

Karg, H., Aust, D. & Böhm, S. (1967). Versuche zur bestimmung des Luteinisierungshormons (LH) im blut von kühen unter berücksichtigung des zyklus. *Zuchthyg.* **2**, 55-62.

Karsch, F.J., Foster, D.L., Bittman, E.L. & Goodman, R.L. (1983). A role for estradiol in enhancing luteinizing hormone pulse frequency during the follicular phase of the estrous cycle of sheep. *Endocrinology* **113**, 1333-1339.

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

Karsch, F.J., Legan, S.J., Ryan, K.D. & Foster, D.L. (1978). The feedback effects of ovarian steroids on gonadotrophin secretion. In "*Control of ovulation*". Eds. D.B.Crighton, N.B.Haynes, G.R.Foxcroft & G.E.Lamming, Butterworths, London, pp. 29-48.

Karsch, F.J., Legan, S.J., 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.

Karsch, F.J., Weick, R.F., Hotchkiss, J., Dierschke, D.J. & Knobil, E. (1973). An analysis of the negative feedback control of gonadotrophin secretion utilizing chronic implantation of ovarian steroids in ovariectomised Rhesus monkeys. *Endocrinology* **93**, 478-486.

Kasson, B.G., Meidan, R., Davoren, J.B. & Hsueh, A.J.W. (1985). Identification of subpopulations of rat granulosa cells: Sedimentation properties and hormonal responsiveness. *Endocrinology* **117**, 1027-1034.

Kastelic, J.P., Ko, J.C.H. & Ginther, O.J. (1990). Suppression of dominant and subordinate ovarian follicles by a proteinaceous fraction of follicular fluid in heifers. *Theriogenology* **34**, 499-509.

Katayama, T., Shiota, K. & Takahashi, M. (1990). Activin A increases the number of follicle-stimulating hormone cells in anterior pituitary cultures. *Molec. Cell. Endocr.* **69**, 179-185.

Katt, E., Fujimori, K., Yanagihara, D., Campeau, J., Numazaki, M., Holst, P., Tonetta, S., Rodgers, K., Westhof, G., Mishell, D., Horenstein, J. & diZerega, G.S. (1988). Determination of follicle regulatory protein levels in urine during the normal menstrual cycle using an enzyme-linked immunosorbent assay. *J. Clin. Endocrinol. Metab.* **66**, 1213-1219.

Kellas, L.M., van Lennep, E.W. & Amoroso, E.C. (1958). Ovaries of some fœtal and prepubertal giraffes (*Giraffa camelopardalis* (Linnæus)). *Nature* **181**, 487-488.

Kennedy, R.I. & Rawlings, N.C. (1984). Administration of constant low doses of androgens to steers by silastic implant: Suppression of gonadotropins and peripheral conversion of androgens. *J. Androl.* **5**, 87-92.

Keogh, E.J., Lee, V.W.K., Rennie, G.C., Burger, H.G., Hudson, B. & de Kretser, D.M. (1976). Selective suppression of FSH by testicular extracts. *Endocrinology* **98**, 997-1004.

Kerr, C.M., Matthaei, K.I., Bradley, M.P. & Reed, K.C. (1990). Rapid, accurate sexing of livestock embryos. *Proc. 4th W. Cong. Genet. Appl. Livest. Prod.* Vol XVI, pp. 334-343.

Kesner, J.S., Convey, E.M. & Anderson, C.R. (1981). Evidence that estradiol induces the preovulatory LH surge in cattle by increasing pituitary sensitivity to LHRH and then increasing LHRH release. *Endocrinology* **108**, 1386-1391.

Kesner, J.S., Padmanabhan, V. & Convey, E.M. (1982). Estradiol induces and progesterone inhibits the preovulatory surges of luteinizing hormone and follicle-stimulating hormone in heifers. *Biol. Reprod.* **26**, 571-578.

Kesner, J.S., Wilson, R.C., Kaufman, J.-M., Hotchkiss, J., Chen, Y., Yamamoto, H., Pardo, R.R. & Knobil, E. (1987). Unexpected responses of the hypothalamic gonadotropin-releasing hormone "pulse generator" to physiological estradiol inputs in the absence of the ovary. *Proc. Natl. Acad. Sci. USA* **84**, 8745-8749.

Kessel, B., Dahl, K.D., Kazer, R.R., Liu, C.-H., Rivier, J., Vale, W., Hsueh, A.J.W. & Yen, S.S.C. (1988). The dependency of bioactive follicle-stimulating hormone secretion on gonadotropin-releasing hormone in hypogonadal and cycling women. *J. Clin. Endocr. Metab.* **66**, 361-366.

Khan, S.A., Schmidt, K., Hallin, P., Di Pauli, R., De Geyter, Ch. & Nieschlag, E. (1988). Human testis cytosol and ovarian follicular fluid contains high amounts of interleukin-1-like factor(s). *Molec. Cell. Endocr.* **58**, 221-230.

Kim, H.N., Rorie, R.W., Youngs, C.R., White, K.L. & Godke, R.A. (1987). The use of anti-PMSG antibodies with PMSG for superovulating beef cattle. *Theriogenology* **27**, 243. (Abstr.).

Kinder, J.E., Garcia-Winder, M., Imakawa, K., Day, M.L., Zalesky, D.D., D'Occhio, M.L., Kittok, R.J. & Schanbacher, B.D. (1983). Influence of different estrogen doses on concentrations of serum LH in acute and chronic ovariectomized cows. *J. Anim. Sci.* **57**, Suppl. 1, p350. (Abstr. 525).

Kiracofe, G.H., Ramirez-Godinez, J.A., McGowan, R.D. & Bolt, D.J. (1983). Reduction of serum FSH in ovariectomized heifers with bovine follicular fluid. *J. Anim. Sci.* **57**, Suppl. 1, p350. (Abstr. 526).

Kirkwood, R.N., Thacker, P.A., Gooneratne, A.D., Guedo, B.L. & Laarveld, B. (1988). The influence of exogenous growth hormone on ovulation rate in gilts. *Can. J. Anim. Sci.* **68**, 1097-1103.

Klein, R., Robertson, D.M., Shukovski, L., Findlay, J.K. & de Kretser, D.M. (1990). The measurement of FSH suppressing protein secretion by bovine granulosa cells in culture. *Proc. Aust. Soc. Repr. Biol.* **22**, Abstr. 36.

Kling, O.R., Roche, P.C., Campeau, J.D., Nishamura, K., Nakamura, R.M. & diZerega, G.S. (1984). Identification of a porcine follicular fluid fraction which suppresses follicular response to gonadotrophins. *Biol. Reprod.* **30**, 564-572.

Knecht, M. & Catt, K. (1983). Epidermal growth factor and gonadotropin-releasing hormone inhibit cyclic AMP-dependent luteinizing hormone receptor formation in ovarian granulosa cells. *J. Cell. Biochem.* **21**, 209-217.

Knight, P.G., Beard, A.J., Wrathall, J.H.M. & Castillo, R.J. (1989). Evidence that the bovine ovary secretes large amounts of monomeric inhibin α subunit and its isolation from bovine follicular fluid. *J. Molec. Endocr.* **2**, 189-200.

Knight, P.G. & Castillo, R.J. (1988). Effects of bovine follicular fluid on gonadotrophin secretion in intact and chronically ovariectomized ewes before and after desensitization of pituitary gonadotrophs to gonadotrophin-releasing hormone. *J. Endocr.* **117**, 431-439.

Knight, P.G., Lacey, M., Peter, J.L.T. & Whitehead, S.A. (1990). Demonstration of a nonsteroidal factor in human follicular fluid that attenuates the self-priming action of GnRH on pituitary gonadotropes. *Biol. Reprod.* **42**, 613-618.

Knight, T.W., Oldham, C.M. & Lindsay, D.R. (1975). Studies in ovine infertility in agricultural regions in Western Australia: The influence of a supplement of lupins (*Lupinus angustifolius* cv. Uniwhite) at joining on the reproductive performance of ewes. *Aust. J. Agric. Res.* **26**, 567-575.

Kolena, J. & Šeböková, E. (1986). Porcine follicular fluid containing water-soluble LH/hCG receptor. *Arch. Int. de Phys. et de Biochim.* **94**, 261-270.

Kolena, J., Šeböková, E. & Horkovics-Kováts, Š. (1986). LH/hCG receptor in pig follicular fluid. *Endocr. Exp.* **20**, 339-348.

Kovacs, A. & Foote, R.H. (1989). Chromosome preparation from bovine spermatazoa. *Theriogenology* **31**, 213. (Abstr.).

Krarup, T., Pedersen, T. & Faber, M. (1969). Regulation of oocyte growth in the mouse ovary. *Nature* **224**, 187-188.

Krohn, P.L. (1967). Factors influencing the number of oocytes in the ovary. *Arch. Anat. Micros.* **56**, 151-159.

Lagacé, L., Massicotte, J. & Labrie, F. (1980). Acute stimulatory effects of progesterone on luteinizing hormone and follicle-stimulating hormone release in rat anterior pituitary cells in culture. *Endocrinology* **106**, 684-689.

Land, R.B., Morris, B.A., Baxter, G., Fordyce, M. & Forster, J. (1982). Improvement of sheep fecundity by treatment with antisera to gonadal steroids. *J. Reprod. Fert.* **66**, 625-634.

Land, R.B. & Scaramuzzi, R.J. (1979). A note on the ovulation rate of sheep following treatment with clomiphene citrate. *Anim. Prod.* **28**, 131-134.

Lane, C.E. (1935). The follicular apparatus of the ovary of the immature rat and some of the factors which influence it. *Anat. Rec.* **61**, 141-153.

LaPolt, P.S., Soto, D., Su, J.-G., Campen, C.A., Vaughan, J., Vale, W. & Hsueh, A.J.W. (1989). Activin stimulation of inhibin secretion and messenger RNA levels in cultured granulosa cells. *Molec. Endocr.* **3**, 1666-1673.

Larson, G.H., Mallory, D.S., Lewis, P.E., Dailey, R.A. & Inskeep, E.K. (1987). Effect of bovine follicular fluid (BFF) on follicular development following pituitary stalk-transection in ewes. *J. Anim. Sci.* **65**, (Suppl.1), Abstr. 440.

Laster, D.B., Glimp, H.A. & Gregory, K.E. (1972). Age and weight at puberty and conception in different breeds and breed-crosses of beef heifers. *J. Anim. Sci.* **34**, 1031-1036.

Ledwitz-Rigby, F. (1987). Local regulation of granulosa cell maturation. *J. steroid Biochem.* **27**, 385-391.

Ledwitz-Rigby, F., Stetson, M. & Channing, C.P. (1973). Follicular fluid (FFl) inhibition of LH stimulation of cyclic-AMP levels in porcine granulosa cells (GC). *Biol. Reprod.* **9**, 94 (Abstr. 85).

Lee, V.W.K. (1983). PMSG treated immature female rat- A model system for studying control of inhibin secretion. In "*Factors regulating ovarian function*". Eds G.S.Greenwald & P.F.Terranova, Raven Press, New York. pp. 157-161.

Lee, V.W.K., Colvin, N., Quigg, H., Atley, L., McMaster, J., Leversha, L. & Burger, H.G. (1987). A rapid, sensitive and reliable assay for inhibin bioactivity. *Aust. J. Biol. Sci.* **40**, 105-113.

Lee, V.W.K., Keogh, E.J., de Kretser, D.M. & Hudson, B. (1974). Selective suppression of FSH by testis extracts. *IRCS Libr. Compend.* **2**, 1406.

Lee, V.W.K., McMaster, J., Quigg, H., Findlay, J. & Leversha, L. (1981). Ovarian and peripheral blood inhibin concentrations increase with gonadotropin treatment in immature rats. *Endocrinology* **108**, 2403-2405.

Lee, V.W.K., McMaster, J., Quigg, H. & Leversha, L. (1982). Ovarian and circulating inhibin levels in immature female rats treated with gonadotropin and after castration. *Endocrinology* **111**, 1849-1854.

Leonard, M., Kirszenbaum, M., Cotinot, C., Chesné, P., Heyman, Y., Stinnakre, M.G., Bishop, C., Delouis, C., Vaiman, M. & Fellous, M. (1987). Sexing bovine embryos using Y chromosome specific DNA probe. *Theriogenology* **27**, 248. (Abstr.).

Leung, P.C.K. & Armstrong, D.T. (1979). Estrogen treatment of immature rats inhibits ovarian androgen production *in vitro*. *Endocrinology* **104**, 1411-1417.

Leung, P.C.K. & Armstrong, D.T. (1980). Interactions of steroids and gonadotropins in the control of steroidogenesis in the ovarian follicle. *Ann. Rev. Physiol.* **42**, 71-82.

Leung, P.C.K., Goff, A.K., Kennedy, T.G. & Armstrong, D.T. (1978). An intraovarian inhibitory action of estrogen on androgen production in vivo. *Biol. Reprod.* **19**, 641-647.

Leversha, L.J., Robertson, D.M., de Vos, F.L., Morgan, F.J., Hearn, M.T.W., Wettenhall, R.E.H., Findlay, J.K., Burger, H.G. & de Kretser, D.M. (1987). Isolation of inhibin from ovine follicular fluid. *J. Endocr.* **113**, 213-221.

Li, C.H., Hammonds, G., Ramasharma, K. & Chung, D. (1985). Human seminal α inhibins: Isolation, characterization, and structure. *Proc. Natl. Acad. Sci. USA* **82**, 4041-4044.

Liao, T.-H. & Pierce, J.G. (1970). The presence of a common type of subunit in bovine thyroid-stimulating and luteinizing hormone. *J. Biol. Chem.* **245**, 3275-3281.

Lilja, H. & Jeppsson, J.-O. (1985). Amino acid sequence of the predominant basic protein in human seminal plasma. *FEBS Lett.* **182**, 181-184.

Ling, N., Ying, S.-Y., Ueno, N., Esch, F., Denoroy, L. & Guillemin, R. (1985). Isolation and partial characterization of a M_r 32,000 protein with inhibin activity from porcine follicular fluid. *Proc. Natl. Acad. Sci. USA* **82**, 7217-7221.

Ling, N., Ying, S.-Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M. & Guillemin, R. (1986a). A homodimer of the β-subunits of inhibin A stimulates the secretion of pituitary follicle stimulating hormone. *Biochem. Biophys. Res. Comm.* **138**, 1129-1137.

Ling, N., Ying, S.-Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M. & Guillemin, R. (1986b). Pituitary FSH is released by a heterodimer of the β subunits from the two forms of inhibin. *Nature* **321**, 779-782.

Lino, J., Baranao, S. & Hammond, J.M. (1985). Multihormone regulation of steroidogenesis in cultured porcine granulosa cells: Studies in serum-free medium. *Endocrinology* **116**, 2143-2151.

Litch, S.J. & Condon, W.A. (1988). Interaction of hCG and lutalyse on steroidogenesis of bovine luteal cells. *Molec. Cell. Endocrinol.* **57**, 81-85.

Lobb, D.K., Skinner, M.K. & Dorrington, J.H. (1988). Rat thecal/interstitial cells produce a mitogenic activity that promotes the growth of granulosa cells. *Molec. Cell. Endocrinol.* **55**, 209-217.

Lobel, B.L. & Levy, E. (1968). Enzymic correlates of development, secretory function and regression of follicles and corpora lutea in the bovine ovary. I. Growth and maturation of follicles. *Acta Endocrinol. (Suppl.)* **132**, 7-33.

Lowry, O.H., Rosebrough, H.F., Farr, H.L. & Randall, R.F. (1951). Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **143**, 265-275.

Luck, M.R., Rodgers, R.J. & Findlay, J.K. (1989). Expression of inhibin by bovine granulosa cells during spontaneous luteinisation in culture. *J. Reprod. Fert. Abstr. Series* **3**, Abstr. 36.

Magoffin, D.A. & Erickson, G.F. (1981). Mechanism by which 17β-estradiol inhibits ovarian androgen production in the rat. *Endocrinology* **108**, 962-967.

Magoffin, D.A. & Erickson, G.F. (1982). Direct inhibitory effect of estrogen on LH-stimulated androgen synthesis by ovarian cells cultured in defined medium. *Molec. Cell. Endocr.* **28**, 81-89.

Maijala, K. & Syväjärvi, J. (1977). On the possibility of developing multiparous cattle by selection. *Z. Tierzüchtg. Züchtgsbiol.* **94**, 136-150.

Makris, A. & Ryan, K.J. (1980). The source of follicular androgens in the hamster follicle. *Steroids* **35**, 53-64.

Mann, G.E., McNeilly, A.S. & Baird, D.T. (1989). Source of ovarian inhibin secretion during the oestrous cycle of the sheep. *J. Endocrinol.* **123**, 181-188.

Manns, J.G. & Niswender, G.D. (1983). FSH receptors in the bovine corpus luteum. *J. Anim. Sci.* **57**, Suppl. 1, p354 (Abstr. 536).

Manns, J.G., Niswender, G.D. & Braden, T. (1984). FSH receptors in the bovine corpus luteum. *Theriogenology* **22**, 321-328.

Maraček, I., Tokoš, M. & Halagan, J. (1977). Tertiary follicles in heifers treated with melengestrol acetate. *Endocr. Exp.* **11**, 249-262.

Marder, M.L., Channing, C.P. & Schwartz, N.B. (1977). Suppression of serum follicle stimulating hormone in intact and acutely ovariectomized rats by porcine follicular fluid. *Endocrinology* **101**, 1639-1642.

Marion, G.B. & Gier, H.T. (1971). Ovarian and uterine embryogenesis and morphology of the non-pregnant female mammal. *J. Anim. Sci.* **32**, Suppl. 1, 24-47.

Marion, G.B., Smith, V.R., Wiley, T.E. & Barrett, G.R. (1950a). The effect of sterile copulation on the time of ovulation in dairy heifers. *J. Dairy Sci.* **33**, 391 (Abstr. P61).

Marion, G.B., Smith, V.R., Wiley, T.E. & Barrett, G.R. (1950b). The effect of sterile copulation on time of ovulation in dairy heifers. *J. Dairy Sci.* **33**, 885-889.

Marshall, F.H.A. (1937). On the change over in the oestrous cycle in animals after transference over the equator, with further observations on the incidence of the breeding seasons and the factors controlling sexual periodicity. *Proc. Roy. Soc. B* **122**, 413-428.

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

Martensz, N.D. & Scaramuzzi, R.J. (1979). Plasma concentrations of luteinizing hormone, follicle-stimulating hormone and progesterone during the breeding season in ewes immunized against androstenedione or testosterone. *J. Endocr.* **81**, 249-259.

Martensz, N.D., Scaramuzzi, R.J. & Van Look, P.F.A. (1979). Plasma concentrations of luteinizing hormone and follicle-stimulating hormone during anoestrus in ewes actively immunized against oestradiol-17β, oestrone or testosterone. *J. Endocr.* **81**, 261-269.

Martin, G.B., Price, C.A., Thiery, J-C. & Webb, R. (1988). Interactions between inhibin, oestradiol and progesterone in the control of gonadotrophin secretion in the ewe. *J. Reprod. Fert.* **82**, 319-328.

Martin, G.B., Scaramuzzi, R.J. & Henstridge, J.D. (1983). Effects of oestradiol, progesterone and androstenedione on the pulsatile secretion of luteinizing hormone in ovariectomized ewes during spring and autumn. *J. Endocr.* **96**, 181-193.

Martin, G.B., Taylor, P.L. & McNeilly, A.S. (1987). Effect of small doses of bovine follicular fluid on the tonic secretion of gonadotrophins in the ewe. *J. Endocr.* **114**, 73-79.

Martin, G.B., Wallace, J.M., Taylor, P.L., Fraser, H.M., Tsonis, C.G. & McNeilly, A.S. (1986). The roles of inhibin and gonadotrophin-releasing hormone in the control of gonadotrophin secretion in the ewe. *J. Endocr.* **111**, 287-296.

Martin, T.E., Henricks, D.M., Hill, J.R. & Rawlings, N.C. (1978). Active immunization of the cow against oestradiol-17β. *J. Reprod. Fert.* **53**, 173-178.

Maruo, T., Hayashi, M., Matsuo, H., Yamamoto, T., Okada, H. & Mochizuki, M. (1987). The role of thyroid hormone as a biological amplifier of the actions of follicle-stimulating hormone in the functional differentiation of cultured porcine granulosa cells. *Endocrinology* **121**, 1233-1241.

Mason, A.J., Hayflick, J.S., Ling, N., Esch, F., Ueno, N., Ying, S.-Y., Guillemin, R., Niall, H. & Seeburg, P.H. (1985). Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor β. *Nature* **318**, 659-663.

Mason, A.J., Niall, H.D. & Seeburg, P.H. (1986). Structure of two human ovarian inhibins. *Biochem. Biophys. Res. Comm.* **135**, 957-964.

Massey, J.M. & Oden, A.J. (1984). No seasonal effect on embryo donor performance in the southwest region of the USA. *Theriogenology* **21**, 196-217.

Matamoros, I.A., Cox, N.M. & Moore, A.B. (1990). Exogenous insulin and additional energy affect follicular distribution, follicular steroid concentrations, and granulosa cell human chorionic gonadotropin binding in swine. *Biol. Reprod.* **43**, 1-7.

Matton, P., Adelakoun, V., Couture, Y. & Dufour, J.J. (1981). Growth and replacement of the bovine ovarian follicles during the estrous cycle. *J. Anim. Sci.* **52**, 813-820.

Mechling, E.A. & Carter, R.C. (1964). Selection for twinning in a grade Aberdeen-Angus herd. *J. Hered.* **55**, 73-75.

Mercer, J.E., Clements, J.A., Funder, J.W. & Clarke, I.J. (1987). Rapid and specific lowering of pituitary FSHβ mRNA levels by inhibin. *Molec. Cell. Endocr.* **53**, 251-254.

Merchenthaler, I., Culler, M.D., Petrusz, P. & Negro-Vilar, A. (1987). Immunocytochemical localization of inhibin in rat and human reproductive tissues. *Molec. Cell. Endocr.* **54**, 239-243.

Mercier, E. & Salisbury, G.W. (1947). Seasonal variations in hours of daylight associated with fertility of cattle under natural breeding conditions. *J. Dairy Sci.* **30**, 747-755.

Meunier, H., Cajander, S.B., Roberts, V.J., Rivier, C., Sawchenko, P.E., Hsueh, A.J.W. & Vale, W. (1988). Rapid changes in the expression of inhibin α-, βA-, and βB-subunits in ovarian cell types during the rat estrous cycle. *Molec. Endocr.* **2**, 1352-1363.

Midgley, A.R. & Sadler, W.A. (1979). "Ovarian follicular development and function". Raven Press, New York.

Miller, K.F., Critser, J.K. & Ginther, O.J. (1982). Inhibition and subsequent rebound of FSH secretion following treatment with bovine follicular fluid in the ewe. *Theriogenology* **18**, 45-53.

Miller, K.F., Critser, J.K., Rowe, R.F. & Ginther, O.J. (1979). Ovarian effects of bovine follicular fluid treatment in sheep and cattle. *Biol. Reprod.* **21**, 537-544.

Miller, K.F., Wesson, J.A. & Ginther, O.J. (1979). Changes in concentrations of circulating gonadotropins following administration of equine follicular fluid to ovariectomized mares. *Biol. Reprod.* **21**, 867-872.

Miller, W.L., Knight, M.M., Grimek, H.J. & Gorski, J. (1977). Estrogen regulation of follicle stimulating hormone in cell cultures of sheep pituitaries. *Endocrinology* **100**, 1306-1316.

Miyamoto, K., Hasegawa, Y., Fukada, M. & Igarashi, M. (1986). Demonstration of high molecular weight forms of inhibin in bovine follicular fluid (bFF) by using monoclonal antibodies to bFF 32K inhibin. *Biochem. Biophys. Res. Comm.* **136**, 1103-1109.

Miyamoto, K., Hasegawa, Y., Fukada, M., Nomura, M., Igarashi, M., Kangawa, K. & Matsuo, H. (1985). Isolation of porcine follicular fluid inhibin of 32K Daltons. *Biochem. Biophys. Res. Comm.* **129**, 396-403.

Monniaux, D., Chupin, D. & Saumande, J. (1983). Superovulatory responses of cattle. *Theriogenology* **19**, 55-81.

Montgomery, G.W., Crosbie, S.F., Martin, G.B. & Pelletier, J. (1984). Changes in the clearance rate of immunoreactive LH after ovariectomy in Ile-de France ewes. In "*Reproduction in sheep*". Eds. D.R.Lindsay & D.T.Pearce. Cambridge University Press, pp.23-25.

Montz, F.J., Ujita, E.L., Campeau, J.D. & diZerega, G.S. (1984). Inhibition of luteinizing hormone/human chorionic gonadotropin binding to porcine granulosa cells by a follicular fluid protein(s). *Am. J. Obstet. Gynecol.* **148**, 436-441.

Moon, Y.S., Dorrington, J.H. & Armstrong, D.T. (1975). Stimulatory action of follicle-stimulating hormone on estradiol-17β secretion by hypophysectomized rat ovaries in organ culture. *Endocrinology* **97**, 244-247.

Moor, R.M., Kruip, Th.A.M. & Green, D. (1984). Intraovarian control of folliculogenesis: Limits to superovulation. *Theriogenology* **21**, 103-116.

Morris, C.A. (1984). A review of the genetics and reproductive physiology of dizygotic twinning in cattle. *Anim. Breeding. Abstr.* **52**, 803-819.

Morris, C.A. (1990). Theoretical and realised responses to selection for reproductive rate. *Proc. 4th W. Congr. Genet. Appl. Livest. Prod., Edinburgh.* Vol. XVI, pp. 309-318.

Morris, C.A. & Day, A.M. (1986). Potential for genetic twinning in cattle. *Proc. 3rd W. Cong. Genet. Appl. Livest. Prod. Lincoln, Nebraska.* Vol XI, pp. 14-29.

Moss, G.E., Crowder, M.E. & Nett, T.M. (1981). GnRH-receptor interaction. VI. Effect of progesterone and estradiol on hypophyseal receptors for GnRH, and serum and hypophyseal concentrations of gonadotropins in ovariectomized ewes. *Biol. Reprod.* **25**, 938-944.

Mottram, J.C. & Cramer, W. (1923). On the general effects of exposure to radium on metabolism and tumour growth in the rat and the special effects on testis and pituitary. *Q. J. Exp. Phys.* **13**, 209-229.

Murata, M., Eto, Y., Shibai, H., Sakai, M. & Muramatsu, M. (1988). Erythroid differentiation factor is encoded by the same mRNA as that of the inhibin β_A chain. *Proc. Natl. Acad. Sci. USA* **85**, 2434-2438.

Murphy, B.D., Mapletoft, R.J., Manns, J. & Humphrey, W.D. (1984). Variability in gonadotrophin preparations as a factor in the superovulatory response. *Theriogenology* **21**, 117-125.

Murphy, B.E.P. (1969). Protein binding and the assay of nonantigenic hormones. *Rec. Prog. Horm. Res.* **25**, 563-.

Murray, J.F., Downing, J.A., Evans, G., Findlay, J.K. & Scaramuzzi, R.J. (1989). Epidermal growth factor (EGF) inhibits inhibin secretion in the follicular phase of the oestrous cycle. *Proc. Aust. Soc. Reprod. Biol.* **21**, Abstr. 136.

Murray, J.F., Downing, J.A., Evans, G. & Scaramuzzi, R.J. (1990). TGFα acts directly on the ovarian follicle. *Proc. Aust. Soc. Reprod. Biol.* **22**, Abstr. 101.

Murthy, H.M.S., Ramasharma, K. & Moudgal, N.R. (1979). Studies on purification of sheep testicular inhibin. *J. Reprod. Fert. Suppl.* **26**, 61-70.

Muttukrishna, S. & Knight, P.G. (1990). Effects of crude and highly purified bovine inhibin (M_r32 000 form) on gonadotrophin production by ovine pituitary cells *in vitro*: inhibin enhances gonadotrophin-releasing hormone-induced release of LH. *J. Endocr.* **127**, 149-159.

McCarthy, M.S. & Swanson, L.V. (1976). Serum LH concentration following castration, steroid hormone and gonadotrophin releasing hormone treatment in the male bovine. *J. Anim. Sci.* **43**, 151-158.

McCracken, J.A., Uno, A., Goding, J.R., Ichikawa, Y. & Baird, D.T. (1969). The in-vivo effects of sheep pituitary gonadotrophins on the secretion of steroids by the auto-transplanted ovary of the ewe. *J. Endocrinol.* **45**, 425-440.

McCreery, B.R. & Licht, P. (1983). Effects of gonadectomy on polymorphism in stored and circulating gonadotropins in the bullfrog, Rana catesbeiana. I. Clearance profiles. *Biol. Reprod.* **29**, 637-645.

McCullagh, D.R. (1932). Dual endocrine activity of the testes. *Science* **76**, 19-20.

McCullagh, D.R. & Schneider, I. (1940). The effect of a non-androgenic testis extract on the estrous cycle in rats. *Endocrinology* **27**, 899-902.

McCullagh, D.R. & Walsh, E.L. (1934). Further studies concerning testicular function. *Proc. Soc. Exp. Biol. Med.* **31**, 678-679.

McLachlan, R.I., Healy, D.L., Robertson. D.M., Burger, H.G. & de Kretser, D.M. (1986). The human placenta: A novel source of inhibin. *Biochem. Biophys. Res. Comm.* **140**, 485-490.

McLeod, B.J., Haresign, W. & Lamming, G.E. (1982). Response of seasonally anoestrous ewes to small-dose multiple injections of Gn-RH with and without progesterone pretreatment. *J. Reprod. Fert.* **65**, 223-230.

McLeod, B.J. & McNeilly, A.S. (1987). Suppression of plasma FSH concentrations with bovine follicular fluid blocks ovulation in GnRH-treated seasonally anoestrous ewes. *J. Reprod. Fert.* **81**, 187-194.

McMillan, W.H. & Hall, D.R.H. (1990). Superovulation in the ewe: Effects of stage of cycle and PMSG in oFSH (ovagen) treated ewes. *Proc. Aust. Soc. Reprod. Biol.* **22**, Abstr. 38.

McNatty, K.P., Heath, D.A., Hudson, N. & Clarke, I.J. (1990). Effect of long-term hypophysectomy on ovarian follicle populations and gonadotrophin-induced adenosine cyclic 3',5'-monophosphate output by follicles from Booroola ewes with or without the F gene. *J. Reprod. Fert.* **90**, 515-522.

McNatty, K.P. & Henderson, K.M. (1987). Gonadotrophins, fecundity genes and ovarian follicular function. *J. steroid. Biochem.* **27**, 365-373.

McNatty, K.P., Henderson, K.M., Lun, S., Heath, D.A., Ball, K., Hudson, N.L., Fannin, J., Gibb, M., Kieboom, L.E. & Smith, P. (1985). Ovarian activity in Booroola x Romney ewes which have a major gene influencing their ovulation rate. *J. Reprod. Fert.* **73**, 109-120.

McNatty, K.P., Hudson, N., Henderson, K.M., Gibb, M., Morrison, L., Ball, K. & Smith, P. (1987). Differences in gonadotrophin concentrations and pituitary responsiveness to GnRH between Booroola ewes which were homozygous (FF), heterozygous (F+) and non-carriers (++) of a major gene influencing their ovulation rate. *J. Reprod. Fert.* **80**, 577-588.

McNeilly, A.S. (1984). Changes in FSH and the pulsatile secretion of LH during the delay in oestrus induced by treatment of ewes with bovine follicular fluid. *J. Reprod. Fert.* **72**, 165-172.

McNeilly, A.S. (1985). Effects of changes in FSH induced by bovine follicular fluid and FSH infusion in the preovulatory phase on subsequent ovulation rate and corpus luteum function in the ewe. *J. Reprod. Fert.* **74**, 661-668.

McNeilly, A.S. & Baird, D.T. (1989). Episodic secretion of inhibin into the ovarian vein during the follicular phase of the oestrous cycle in the ewe. *J. Endocr.* **122**, 287-292.

McNeilly, A.S. & Fraser, H.M. (1987). Effect of gonadotrophin-releasing hormone agonist-induced suppression of LH and FSH on follicle growth and corpus luteum function in the ewe. *J. Endocr.* **115**, 273-282.

McNeilly, A.S., Jonassen, J.A. & Fraser, H.M. (1986). Suppression of follicular development after chronic LHRH immunoneutralization in the ewe. *J. Reprod. Fert.* **76**, 481-490.

McNeilly, A.S., Picton, H.M., Campbell, B.K. & Baird, D.T. (1991). Gonadotrophic control of follicle growth in the ewe. *J. Reprod. Fert. Suppl.* **43**, 177-186

McNeilly, A.S., Swanston, I.A., Crow, W., Tsonis, C. & Baird, D.T. (1989). Changes in the plasma concentrations of inhibin throughout the normal sheep oestrous cycle and after the infusion of FSH. *J. Endocr.* **120**, 295-305.

McNeilly, A.S. & Wallace, J.M. (1987). The effect of follicular fluid on ovulation rate in the ewe. In "*Follicular growth and ovulation rate in farm animals*". Eds. J.F.Roche & D.O'Callaghan, Martinus Nijhoff, Dordrecht. pp. 119-127.

McNeilly, J.R., Fordyce, M., Land, R.B., Martin, G.B., Springbett, A.J. & Webb, R. (1988). Changes in the feedback control of gonadotrophin secretion in ewes from lines selected for testis size in the ram lamb. *J. Reprod. Fert.* **84**, 213-221.

Nagesh Babu, G., Bona-Gallo, A. & Gallo, R.V. (1986). Interaction between estradiol and a non-steroidal factor in porcine follicular fluid in regulating LH pulse amplitude between the mornings of diestrus 2 and proestrus in the rat. *Neuroendocrinology* **44**, 8-14.

Nakamura, Y., Kato, H. & Terranova, P.F. (1990). Interleukin-1α increases thecal progesterone production of preovulatory follicles in cyclic hamsters. *Biol. Reprod.* **43**, 169-173.

Nalbandov, A. & Casida, L.E. (1942). Ovulation and its relation to estrus in cows. *J. Anim. Sci.* **1**, 189-198.

Nandini, S.G., Lipner, H. & Moudgal, N.R. (1976). A model system for studying inhibin. *Endocrinology* **98**, 1460-1465.

Naor, Z., Childs, G.V., Leifer, A.M., Clayton, R.N., Amsterdam, A. & Catt, K.J. (1982). Gonadotropin-releasing hormone binding and activation of enriched population of pituitary gonadotrophs. *Molec. Cell. Endocr.* **25**, 85-97.

Nelson, W.O. (1934). Effect of oestrin and gonadotropic hormone injections upon hypophysis of the adult rat. *Proc. Soc. Exp. Biol. Med.* **32**, 452-454.

Nelson, W.O. & Gallagher, T.F. (1935). Studies on the anterior hypophysis. IV. The effect of male hormone preparations upon the anterior hypophyses of gonadectomized male and female rats. *Anat. Rec.* **64**, 129-145.

Nottle, M.B., Seamark, R.F. & Setchell, B.P. (1988). Supplementation with lupin grain increases FSH in ovariectomised ewes. *J. Reprod. Fert. Abstract Series* **1**, Abstr. 51.

O, W.-S., Robertson, D.M. & de Kretser, D.M. (1989). Inhibin as an oocyte meiotic inhibitor. *Molec. Cell. Endocr.* **62**, 307-311.

O'Shea, T., Al-Obaidi, S.A.R., Hillard, M.A., Bindon, B.M., Cummins, L.J. & Findlay, J.K. (1984). Increased ovulation rate in Merino ewes and advancement of puberty in Merino lambs immunized with a preparation enriched in inhibin. In "*Reproduction in sheep*". Eds. D.R.Lindsay & D.T.Pearce, Cambridge University Press, pp. 335-337.

O'Shea, T., Anderson, S.T., Bindon. B.M., Hillard, M.A. & Sinosich, M.J. (1990). Ovulation rates in Merino ewes immunized with synthetic inhibin peptides. *Proc. Aust. Soc. Reprod. Biol.* **22**, Abstr. 96.

O'Shea, T., Bindon, B.M., Hillard, M.A., Piper, L.R., Findlay, J.K. & Miyamoto, K. (1989). Increase in ovulation rate in Merino ewes after active immunization with inhibin preparations obtained by immunoaffinity chromatography. *Reprod. Fertil. Dev.* **1**, 347-355.

O'Shea, T., Cummins, L.J., Bindon, B.M. & Findlay, J.K. (1982). Increased ovulation rate in ewes vaccinated with an inhibin-enriched fraction from bovine follicular fluid. *Proc. Aust. Soc. Reprod. Biol.* **14**, Abstr. 85.

Ohno, S. (1969). The problem of the bovine freemartin. *J. Reprod. Fert. Suppl.* **7**, 53-60.

Ono, T., Campeau, J.D., Holmberg, E.A., Nakamura, R.M., Ujita, E.L., Devereaux, D.L., Tonetta, S.A., DeVinna, R., Ugalde, M. & diZerega, G.S. (1986). Biochemical and physiologic characterization of follicle regulatory protein: A paracrine regulator of folliculogenesis. *Am. J. Obstet. Gynecol.* **154**, 709-716.

Ouchterlony, Ö & Nilsson, L.-Å. (1978). Immunodiffusion and immunoelectrophoresis. In "*Handbook of experimental immunology*". Ed. D.M.Weir, Blackwell Scientific Publications, Oxford, pp. 19.1-19.44.

Padmanabhan, V. & Convey, E.M. (1981). Progesterone inhibits the ability of estradiol to increase basal and luteinizing hormone-releasing hormone-induced luteinizing hormone release from bovine pituitary cells in culture: neither progesterone nor estradiol affects follicle-stimulating hormone release. *Endocrinology* **109**, 1091-1096.

Padmanabhan, V., Convey, E.M., Roche, J.F. & Ireland, J.J. (1984). Changes in inhibin-like bioactivity in ovulatory and atretic follicles and utero-ovarian venous blood after prostaglandin-induced luteolysis in heifers. *Endocrinology* **115**, 1332-1340.

Padmanabhan, V., Ebling, F.J.P., Sonstein, J., Fenner, D.E., Kelch, R.P., Foster, D.L. & Beitins, I.Z. (1989). Bioactive follicle-stimulating hormone release in nutritionally growth-retarded ovariectomized lambs: Regulation by nutritional repletion. *Endocrinology* **125**, 2517-2526.

Padmanabhan, V., Kelch, R.P., Sonstein, J., Foster, C.L. & Beitins, I.Z. (1988a). Bioactive follicle-stimulating hormone responses to intravenous gonadotropin-releasing hormone in boys with idiopathic hypogonadotropic hypogonadism. *J. Clin. Endocr. Metab.* **67**, 793-800.

Padmanabhan, V., Lang, L.L., Sonstein, J., Kelch, R.P. & Beitins, I.Z. (1988b). Modulation of serum follicle-stimulating hormone bioactivity and isoform distribution by estrogenic steroids in normal women and in gonadal dysgenesis. *J. Clin. Endocr. Metab.* **67**, 465-473.

Page, R.D., Jordan, J.E. & Johnson, S.K. (1989). Superovulation of Holstein heifers under heat stress with FSH-P or follitropin. *Theriogenology* **31**, 236-237.

Pant, H.C. & Rawlings, N.C. (1973). The effect of active immunization against oestradiol-17β on plasma gonadotrophin concentrations in sheep. *J. Reprod. Fert.* **35**, 610-611 (Abstr.).

Parfet, J.R., Smith, C.A., Cook, D.L., Skyer, D.M., Youngquist, R.S. & Garverick, H.A. (1989). Secretory patterns of LH and FSH and follicular growth following administration of $PGF_{2\alpha}$ during the early luteal phase in cattle. *Theriogenology* **31**, 513-524.

Parlow, A.F. (1961). Bio-assay of pituitary luteinizing hormone by depletion of ovarian ascorbic acid. In "*Human pituitary gonadotrophins*". Ed. A.Albert., C.C.Thomas, Springfield, Illinois., pp. 300-310.

Pathiraja, N. (1982). Physiological basis of genetic variation in ovulation rate. *PhD. Thesis*, University of Edinburgh.

Pathiraja, N., Carr, W.R., Fordyce, M., Forster, J., Land, R.B. & Morris, B.A. (1984). Concentration of gonadotrophins in the plasma of sheep given gonadal steroid antisera to raise ovulation rate. *J. Reprod. Fert.* **72**, 93-100.

Payne, R.W. & Runser, R.H. (1958). The influence of estrogen and androgen on the ovarian response of hypophysectomized immature rats to gonadotropins. *Endocrinology* **62**, 313-321.

Peckham, W.D. & Knobil, E. (1976). The effects of ovariectomy, estrogen replacement, and neuraminidase treatment on the properties of the adenohypophyseal glycoprotein hormones of the rhesus monkey. *Endocrinology* **98**, 1054-1060.

Peel, C.J. & Bauman, D.E. (1987). Somatotropin and lactation. *J. Dairy Sci.* **70**, 474-486.

Perry, J.S. & Rowlands, I.W. (1962). The ovarian cycle in vertebrates. In "*The ovary*". Ed. S.Zuckerman, A.M.Mandl & P.Eckstein. Vol. I. pp. 275-309.

Peters, A.R. (1984). Effect of exogenous oestradiol-17β on gonadotrophin secretion in post-partum beef cows. *J. Reprod. Fert.* **72**, 473-478.

Peters, A.R. (1985). Studies of hormone patterns during the oestrous cycle of beef cows. *Reprod. Nutr. Dévelop.* **25**, 919-927.

Peters, H. (1969). The development of the mouse ovary from birth to maturity. *Acta Endocr.* **62**, 98-116.

Peters, H. (1978). Folliculogenesis in mammals. In "*The vertebrate ovary*". Ed. R.E.Jones., Plenum Press, New York. pp 121-144.

Peters, H. (1979). Some aspects of early follicular development. In "*Ovarian follicular development and function*". Eds. A.R.Midgley & W.A.Sadler. Raven Press, New York. pp 1-13.

Peters, H., Byskov, A.G. & Faber, M. (1973). Intraovarian regulation of follicle growth in the immature mouse. In "*The development and maturation of the ovary and its functions*". Ed. H.Peters. Excerpta Medica, Amsterdam. pp. 20-23.

Peters, H. & Levy, E. (1966). Cell dynamics of the ovarian cycle. *J. Reprod. Fert.* **11**, 227-236.

Petr, J., Míka, J. & Jílek, F. (1990). The effect of PMSG priming on subsequent superovulatory response in dairy cattle. *Theriogenology* **33**, 1151-1155.

Philipon, P. & Terqui, M. (1987). Increase of prolificacy in sheep immunized against androstenedione. In "*Follicular growth and ovulation rat in farm animals*". Eds. J.F.Roche & D.O'Callaghan, Martinus Nijhoff, Dordrecht. pp. 129-133.

Phipps, R.H., Weller, R.F., Austin, A.R., Craven, N. & Peel, C.J. (1988). A preliminary report on a prolonged release formulation of bovine somatotrophin with particular reference to animal health. *Vet. Rec.* **122**, 512-513.

Picton, H.M., Tsonis, C.G. & McNeilly, A.S. (1990). FSH causes a time-dependent stimulation of preovulatory follicle growth in the absence of pulsatile LH secretion in ewes chronically treated with gonadotrophin-releasing hormone agonist. *J. Endocr.* **126**, 297-307.

Pierce, J.G. (1971). Eli Lilly Lecture: The subunits of pituitary thyrotropin - Their relationship to other glycoprotein hormones. *Endocrinology* **89**, 1331-1344.

Pierce, J.G., Liao, T.-H., Carlsen, R.B. & Reimo, T. (1971). Comparisons betweeen the α chain of bovine thyrotropin and the CI chain of luteinizing hormone. Compositions of tryptic peptides, cyanogen bromide fragments, and carbohydrate moieties. *J. Biol. Chem.* **246**, 866-872.

Pierce, J.G. & Parsons, T.F. (1981). Glycoprotein hormones: Structure and function. *Ann. Rev. Biochem.* **50**, 465-495.

Pierson, R.A. & Ginther, O.J. (1984). Ultrasonography of the bovine ovary. *Theriogenology* **21**, 495-504.

Pierson, R.A. & Ginther, O.J. (1986). Ovarian follicular populations during early pregnancy in heifers. *Theriogenology* **26**, 649-659.

Pierson, R.A. & Ginther, O.J. (1987a). Follicular populations during the estrous cycle in heifers. I. Influence of day. *Anim. Reprod. Sci.* **14**, 165-176.

Pierson, R.A. & Ginther, O.J. (1987b). Reliability of diagnostic ultrasonography for identification and measurement of follicles and detecting the corpus luteum in heifers. *Theriogenology* **28**, 929-936.

Pierson, R.A. & Ginther, O.J. (1987c). Follicular populations during the estrous cycle in heifers. II. Influence of right and left sides and intraovarian effect of the corpus luteum. *Anim. Reprod. Sci.* **14**, 177-186.

Pierson, R.A. & Ginther, O.J. (1987d). Intraovarian effect of the corpus luteum on ovarian follicles during early pregnancy in heifers. *Anim. Reprod. Sci.* **15**, 53-60.

Pierson, R.A. & Ginther, O.J. (1988). Follicular population during the estrous cycle in heifers. III. Time of selection of the ovulatory follicle. *Anim. Reprod. Sci.* **16**, 81-95.

Poretsky, L., Grigorescu, F., Siebel, M., Moses, A.C. & Flier, J.S. (1985). Distribution and characterization of insulin and insulin-like growth factor 1 receptors in normal human ovary. *J. Clin. Endocr. Metab.* **61**, 728-734.

Price, C.A. (1987). Endocrine control of ovulation rate in the cow. Ph.D. Thesis, University of Edinburgh.

Price, C.A., Morris, B.A., O'Shea. T. & Webb, R. (1987). Active immunisation of cattle against partly purified follicular fluid from sheep. *J. Reprod. Fert.* **81**, 161-168.

Price, C.A., Morris, B.A. & Webb, R. (1987). Reproductive and endocrine effects of active immunisation against a testosterone conjugate in the heifer. *J. Reprod. Fert.* **81**, 149-160.

Price, C.A. & Webb, R. (1988). Steroid control of gonadotrophin secretion and ovarian function in heifers. *Endocrinology* **122**, 2222-2231.

Price, C.A. & Webb, R. (1989). Ovarian response to hCG treatment during the oestrous cycle in heifers. *J. Reprod. Fert.* **86**, 303-308.

Quirk, S.M. & Fortune, J.E. (1986). Plasma concentrations of gonadotrophins, preovulatory follicular development and luteal function associated with bovine follicular fluid-induced delay of oestrus in heifers. *J. Reprod. Fert.* **76**, 609-621.

Quirk, S.M., Hickey, G.J. & Fortune, J.E. (1986). Growth and regression of ovarian follicles during the follicular phase of the oestrous cycle in heifers undergoing spontaneous and PGF-2α-induced luteolysis. *J. Reprod. Fert.* **77**, 211-219.

Radford, H.M., Avenell, J.A. & Panaretto, B.A. (1987). Some effects of epidermal growth factor on reproductive function in Merino sheep. *J. Reprod. Fert.* **80**, 113-118.

Radford, H.M., Nancarrow, C.D. & Mattner, P.E. (1978). Ovarian function in suckling and non-suckling beef cows post partum. *J. Reprod. Fert.* **54**, 49-56.

Radford, H.M., Panaretto, B.A., Avenell, J.A. & Turnbull, K.E. (1987). Effect of mouse epidermal growth factor on plasma concentrations of FSH, LH and progesterone and on oestrus, ovulation and ovulation rate in Merino ewes. *J. Reprod. Fert.* 80, 383-393.

Rahe, C.H., Owens, R.E., Fleeger, J.L., Newton, H.J. & Harms, P.G. (1980). Pattern of plasma luteinizing hormone in the cyclic cow: Dependence upon the period of the cycle. *Endocrinology* **107**, 498-503.

Rajakoski, E. (1960). The ovarian follicular system in sexually mature heifers with special reference to seasonal, cyclical and left-right variations. *Acta Endocrinol. Suppl.* **52**, 1-68.

Rakha, A.M. & Robertson, H.A. (1965). Changes in levels of follicle stimulating hormone and luteinizing hormone in the bovine piuitary gland at ovulation. *J. Endocrinol.* **31**, 245-249.

Rall, W.F. & Leibo, S.P. (1987). Production of sexed bovine pregnancies by cytogenetic analysis of cultured demi-embryos. *Theriogenology* **27**, 269. (Abstr.).

Ramasharma, K., Murthy, H.M.S. & Moudgal, N.R. (1979). A rapid bioassay for measuring inhibin activity. *Biol. Reprod.* **20**, 831-835.

Ramasharma, K., Sairam, M.R., Seidah, N.G., Chrétien, M., Manjunath, P., Schiller, P.W. (1984). Isolation, structure, and synthesis of a human seminal plasma peptide with inhibin-like activity. *Science* **223**, 1199-1202.

Rawlings, N.C., Kennedy, S.W. & Henricks, D.M. (1978). Effect of active immunization of the cyclic ewe against oestradiol-17β. *J. Endocr.* **76**, 11-19.

Rawlings, N.C., Jeffcoate, I.A. & Rieger, D.L. (1984). The influence of estradiol-17β and progesterone on peripheral serum concentrations of luteinizing hormone and follicle stimulating hormone in the ovariectomized ewe. *Theriogenology* **22**, 473-488.

Reece, R.P. & Turner, C.W. (1938). The functional activity of the right and left bovine ovary. *J. Dairy Sci.* **21**, 37-39.

Reichert, L.E., Andersen, T.T., Branca, A.A., Fletcher, P.W. & Sluss, P.M. (1984). FSH binding inhibitors in follicular fluid. In "*Gonadal proteins and peptides and their biological significance*". Eds. M.R.Sairam & L.E.Atkinson, World Scientific Publishing Co. Ltd., Singapore, pp. 153-160.

Reid, J.P., Wilton, J.W. & Walton, J.S. (1986). Comparative productivity of cows after receiving two embryos at transfer. *Can. J. Anim. Sci.* **66**, 373-380.

Richards, J.S. (1979). Hormonal control of ovarian follicular development: A 1978 perspective. *Rec. Prog. Horm. Res.* **35**, 343-368.

Richards, J.S. (1980). Maturation of ovarian follicles: Actions and interactions of pituitary and ovarian hormones on follicular cell differentiation. *Physiol. Rev.* **60**, 51-89.

Richards, J.S., Ireland, J.J., Rao, M.C., Bernath, G.A., Midgley, A.R. & Reichert, L.E. (1976). Ovarian follicular development in the rat: Hormone receptor regulation by estradiol, follicle stimulating hormone and luteinizing hormone. *Endocrinology* **99**, 1562-1570.

Richards, J.S. & Kersey, K.A. (1979). Changes in theca and granulosa cell function in antral follicles developing during pregnancy in the rat: Gonadotropin receptors, cyclic AMP and estradiol-17β. *Biol. Reprod.* **21**, 1185-1201.

Richards, J.S. & Midgley, A.R. (1976). Protein hormone action: A key to understanding ovarian follicular and luteal cell development. *Biol. Reprod.* **14**, 82-94.

Rieger, D., Desaulnier, D. & Goff, A.K. (1988). Ovulatory response and embryo yield in superovulated Holstein heifers given a priming dose of FSH-P at day 2 of the estrous cycle. *Theriogenology* **30**, 695-699.

Rieger, D., Walton, J.S., Goodwin, M.L. & Johnson, W.H. (1990). The effect of co-treatment with recombinant bovine somatotrophin (rBST) on the superovulatory response in Holstein heifers. *Theriogenology* **33**, 306 (Abstr.).

Rivier, C., Cajander, S., Vaughan, J., Hsueh, A.J.W. & Vale, W. (1988). Age-dependent changes in physiological action, content, and immunostaining of inhibin in male rats. *Endocrinology* **123**, 120-126.

Rivier, C., Rivier, J. & Vale, W. (1986). Inhibin-mediated feedback control of follicle-stimulating hormone secretion in the female rat. *Science* **234**, 205-208.

Rivier, J., Spiess, J., McClintock, R., Vaughan, J. & Vale, W. (1985). Purification and partial characterization of inhibin from porcine follicular fluid. *Biochem. Biophys. Res. Comm.* **133**, 120-127.

Robertson, D.M. (1990). The measurement of inhibin. *Reprod. Fertil. Dev.* **2**, 101-105.

Robertson, D.M., de Vos, F.L., Foulds, L.M., McLachlan, R.I., Burger, H.G., Morgan, F.J., Hearn, M.T.W. & de Kretser, D.M. (1986). Isolation of a 31KDa form of inhibin from bovine follicular fluid. *Molec. Cell. Endocr.* **44**, 271-277.

Robertson, D.M., Farnworth, P.G., Clarke, L., Jacobsen, J., Cahir, N.F., Burger, H.G. & de Kretser, D.M. (1990). Effects of bovine 35 kDa FSH-suppressing protein on FSH and LH in rat pituitary cells *in vitro*: comparison with bovine 31kDa inhibin. *J. Endocr.* **124**, 417-423.

Robertson, D.M., Foulds, L.M., Leversha, L., Morgan, F.J., Hearn, M.T.W., Burger, H.G., Wettenhall, R.E.H & de Kretser, D.M. (1985). Isolation of inhibin from bovine follicular fluid. *Biochem. Biophys. Res. Comm.* **126**, 220-226.

Robertson, D.M., Giacometti, M., Foulds, L.M., Lahnstein, J., Goss, N.H., Hearn, M.T.W. & de Kretser, D.M. (1989). Isolation of inhibin α-subunit precursor proteins from bovine follicular fluid. *Endocrinology* **125**, 2141-2149.

Robertson, D.M., Hayward, S., Irby, D., Jacobsen, J., Clarke, L., McLachlan, R.I. & de Kretser, D.M. (1988). Radioimmunoassay of rat serum inhibin: Changes after PMSG stimulation and gonadectomy. *Molec. Cell. Endocr.* **58**, 1-8.

Robertson, D.M., Klein, R., de Vos, F.L., McLachlan, R.I., Wettenhall, R.E.H., Hearn, M.T.W., Burger, H.G. & de Kretser, D.M. (1987). The isolation of polypeptides with FSH suppressing activity from bovine follicular fluid which are structurally different to inhibin. *Biochem. Biophys. Res. Comm.* **149**, 744-749.

Robertson, H.A. (1972). Sequential changes in plasma progesterone in the cow during the estrous cycle, pregnancy, at parturition, and postpartum. *Can. J. Anim. Sci.* **52**, 645-658.

Roche, J.F. (1978). Control of oestrus in cattle using progesterone coils. *Anim. Reprod. Sci.* **1**, 145-154.

Roche, J.F. & Ireland, J.J. (1981a). Effect of exogenous progesterone on time of occurence of the LH surge in heifers. *J. Anim. Sci.* **52**, 580-586.

Roche, J.F. & Ireland, J.J. (1981b). The differential effect of progesterone on concentrations of luteinizing hormone and follicle-stimulating hormone in heifers. *Endocrinology* **108**, 568-572.

Roche, J.F. & O'Callaghan, D. (1987). "Follicular growth and ovulation rate in farm animals". Martinus Nijhoff, Dordrecht.

Rodgers, R.J., Stuchberry, S.J. & Findlay, J.K. (1989). Inhibin mRNAs in ovine and bovine ovarian follicles and corpora lutea throughout the estrous cycle and gestation. *Molec. Cell. Endocr.* **62**, 95-101.

Rokukawa, S., Inoue, K, Miyamato, K., Kurosumi, K. & Igarashi, M. (1986). Immunohistochemical localization of inhibin in porcine and bovine ovaries. *Arch. histol. Jap.* **49**, 603-611.

Rolland, R., van Hall, E.V., Hillier, S.G., McNatty, K.P. & Schoemaker, J. (1982). "Follicular maturation and ovulation". Excerpta Medica Int. Congress Series 560, Amsterdam.

Rubin, D. (1941). The question of an aqueous hormone from the testicle. *Endocrinology* **29**, 281-287.

Rutledge, J.J. (1975). Twinning in cattle. *J. Anim. Sci.* **40**, 803-815.

Ryan, R.J., Keutmann, H.T., Charlesworth, M.C., McCormick, D.J., Milius, R.P., Calvo, F.O. & Vutyavanich, T. (1987). Structure-function relationships of gonadotropins. *Rec. Prog. Horm. Res.* **43**, 383-429.

Sato, E. & Ishibashi, T. (1977). Inhibition of compensatory ovarian hypertrophy in the mouse by the administration of the non-dialysable fration of bovine follicular fluid. *Jap. J. Zootech. Sci.* **48**, 782-783.

Sato, E. & Ishibashi, T. (1978). Partial purification of the gonadotropin inhibiting substance found in bovine follicular fluid. *Jap. J. Zootech. Sci.* **49**, 313-318.

Sato, E., Miyamoto, H., Ishibashi, T. & Iritani, A. (1978). Identification, purification and immunohistochemical detection of the inhibitor from porcine follicular fluid to compensatory ovarian hypertrophy in mice. *J. Reprod. Fert.* **54**, 263-267.

Saumande, J. & Chupin, D. (1987). The search for a reference method to test the effectiveness of anti-PMSG in superovulatory treatment in cattle. *Theriogenology* **27**, 274. (Abstr.).

Savio, J.D., Boland, M.P. & Roche, J.F. (1990). Development of dominant follicles and length of ovarian cycles in post-partum dairy cows. *J. Reprod. Fert.* **88**, 581-591.

Savio, J.D., Keenan, L., Boland, M.P. & Roche, J.F. (1988). Pattern of growth of dominant follicles during the oestrous cycle of heifers. *J. Reprod. Fert.* **83**, 663-671.

Scaramuzzi, R.J. (1979). Antibodies to androgens, and ovulation in the ewe. *J. steroid Biochem.* **11**, 957-961.

Scaramuzzi, R.J. & Baird, D.T. (1977). Pulsatile release of luteinizing hormone and the secretion of ovarian steroids in sheep during anestrus. *Endocrinology* **101**, 1801-1806.

Scaramuzzi, R.J., Baird, D.T., Clarke, I.J., Martensz, N.D. & Van Look, P.F.A (1980). Ovarian morphology and the concentration of steroids during the oestrous cycle of sheep actively immunized against androstenedione. *J. Reprod. Fert.* **58**, 27-35.

Scaramuzzi, R.J., Davidson, W.G. & Van Look, P.F.A. (1977). Increasing ovulation rate in sheep by active immunisation against an ovarian steroid androstenedione. *Nature* **269**, 817-818.

Scaramuzzi, R.J. & Hoskinson, R.M. (1984). Active immunization against steroid hormones for increasing fecundity. In "*Immunological aspects of reproduction in mammals*". Ed. D.B.Crighton., Butterworths, London. pp. 445-474.

Scaramuzzi, R.J., Hoskinson, R.M., Radford, H.M., Hinks, N.T. & Turnbull, K.E. (1984). Ovarian responses in the steroid-immune ewe. *10th Int. Cong. Anim. Repr. A.I.* Vol. 4., section VIII pp. 7-13.

Scaramuzzi, R.J. & Martensz, N.D. (1975). The effects of active immunisation against androstenedione on luteinizing hormone levels in the ewe. In "*Immunization with hormones in reproduction research*". Ed. E. Nieschalg, North-Holland Publishing Co., Amsterdam. pp. 141-152.

Scaramuzzi, R.J., Martensz, N.D. & Van Look, P.F.A. (1980). Ovarian morphology and the concentration of steroids, and of gonadotrophins during the breeding season in ewes actively immunized against oestradiol-17β or oestrone. *J. Reprod. Fert.* **59**, 303-310.

Scaramuzzi, R.J., Martin, G.B., Hoskinson, R.M., Downing, J.A., Gow, C.B., Turnbull, K.E. & Hinks, N.T. (1982). Studies of the periovulatory period in Merino ewes immunized against androstenedione or oestrone. *Proc. Aust. Soc. Reprod. Biol.* **14**, Abstr. 94.

Scaramuzzi, R.J., Murray, J.F., Campbell, B.K., Downing, J.A., Evans, G. & Panaretto, B.A. (1988). Epidermal growth factor (EGF) acts directly on the ovary in the ewe. *J. Reprod. Fert. Abstract Series No.* **1**, Abstr. 52.

Scaramuzzi, R.J., Tillson, S.A., Thorneycroft, I.H. & Caldwell, B.V. (1971). Action of exogenous progesterone and estrogen on behavioural estrus and luteinizing hormone levels in the ovariectomized ewe. *Endocrinology* **88**, 1184-1189.

Scaramuzzi, R.J., Turnbull, K.E. & Nancarrow, C.D. (1980). Growth of Graafian follicles in cows following luteolysis induced by the prostaglandin $F_{2\alpha}$ analogue, cloprostenol. *Aust. J. Biol. Sci.* **33**, 63-69.

Schallenberger, E. & Peterson, A.J. (1982). Effect of ovariectomy on tonic gonadotropin secretion in cyclic and postpartum dairy cows. *J. Reprod. Fert.* **64**, 47-52.

Schallenberger, E., Rampp, J. & Walters, D.L. (1983). Pulsatile progesterone secretion occurs during midgestation in the cow even though pulsatile LH secretion is abolished. *J. Anim. Sci.* **57**, Suppl. 1, p 371 (Abstr. 577).

Schallenberger, E., Schams, D., Bullerman, B. & Walters, D.L. (1984). Pulsatile secretion of gonadotrophins, ovarian steroids and ovarian oxytocin during prostaglandin-induced regression of the corpus luteum in the cow. *J. Reprod. Fert.* **71**, 493-501.

Schallenberger, E., Schöndorfer, A.M. & Walters, D.L. (1985). Gonadotrophins and ovarian steroids in cattle. I. Pulsatile changes of concentrations in the jugular vein throughout the oestrous cycle. *Acta Endocrinol.* **108**, 312-321.

Schams, D. & Karg, H. (1969). Radioimmunologische LH-bestimmung im blutserum vom rind unter besonderer berücksichtigung des brunstzyklus. *Acta Endocrinol.* **61**, 96-103.

Schams, D. & Schallenberger, E. (1976). Heterologous radioimmunoassay for bovine follicle-stimulating hormone and its application during the oestrous cycle in cattle. *Acta Endocr.* **81**, 461-473.

Schams, D., Schallenberger, E., Hoffmann, B. & Karg, H. (1977). The oestrous cycle of the cow: Hormonal parameters and time relationships concerning oestrus, ovulation and electrical resistance of the vaginal mucus. *Acta Endocrinol.* **86**, 180-192.

Schmidt, G., Jörgensen, J., Kannisto, P., Liedberg, F., Ottesen, B. & Owman, Ch. (1990). Vasoactive intestinal polypeptide in the PMSG-primed immature rat ovary and its effect on ovulation in the isolated rat ovary perfused *in vitro*. *J. Reprod. Fert.* **90**, 465-472.

Schneyer, A.L., Reichert, L.E., Franke, M., Ryan, R.J. & Sluss, P.M. (1988). Follicle-stimulating hormone (FSH) immunoactivity in porcine follicular fluid is not pituitary FSH. *Endocrinology* **123**, 487-491.

Schoenemann, H.M., Humphrey, W.D., Crowder, M.E., Nett, T.M. & Reeves, J.J. (1985). Pituitary luteinizing hormone-releasing hormone receptors in ovariectomized cows after challenge with ovarian steroids. *Biol. Reprod.* **32**, 574-583.

Schreiber, J.R. & diZerega, G.S. (1986). Porcine follicular fluid protein(s) inhibit rat ovary granulosa cell steroidogenesis. *Am. J. Obstet. Gynecol.* **155**, 1281-1288.

Schwall, R.H., Nikolics, K., Szonyi, E., Gorman, C. & Mason, A.J. (1988). Recombinant expression and characterization of human activin A. *Molec. Endocr.* **2**, 1237-1242.

Schwartz, N.B. & Channing, C.P. (1977). Evidence for ovarian "inhibin": Suppression of the secondary rise in serum follicle stimulating hormone levels in proestrous rats by injection of porcine follicular fluid. *Proc. Natl. Acad. Sci. USA* **74**, 5721-5724.

Scott, R.S., Burger, H.G. & Quigg, H. (1980). A simple and rapid *in vitro* bioassay for inhibin. *Endocrinology* **107**, 1536-1542.

Scott, R.S., Quigg, H., Trounson, A., Tsonis, C. & Findlay, J.K. (1980). Inhibin in ovine follicular fluid. *Proc. Endocr. Soc. Aust.* **23**, Abstr. 67.

Seidah, N.G., Arbatti, N.J., Rochemont, J., Sheth, A.R. & Chrétien, M. (1984). Complete amino acid sequence of human seminal plasma β-inhibin. Prediction of post Gln-Arg cleavage as a maturation site. *FEBS Lett.* **175**, 349-355.

Setchell, B.P., Davies, R.V. & Main, S.J. (1977). Inhibin. In "*The testis*". Eds. A.D.Johnson & W.R.Gomes. Vol. IV. Academic Press, New York. pp. 189-238.

Setchell, B.P. & Jacks, F. (1974). Inhibin-like activity in rete testis fluid. *J. Endocr.* **62**, 675-676.

Setchell, B.P. & Sirinathsinghji, D.J. (1972). Antigonadotrophic activity in rete testis fluid, a possible 'inhibin'. *J. Endocrinol.* **53**, lx-lxi.

Shanbhag, S.A., Sheth, A.R., Nanivadekar, S.A. & Sheth, N.A. (1984). Studies on inhibin-like peptide in gastric juice and serum of patients with duodenal ulcers. *J. Endocr.* **103**, 389-393.

Shaw, G., Jorgensen, G.I., Tweedale, R., Tennison, M. & Waters, M.J. (1985). Effect of epidermal growth factor on reproductive function of ewes. *J. Endocr.* **107**, 429-436.

Shea, B.F., Janzen, R.E. & McDermand, D.P. (1984). Seasonal variation in response to stimulation and related embryo transfer procedures in Alberta over a nine year period. *Theriogenology* **21**, 186-195.

Shemesh, M., Ayalon, N. & Lindner, H.R. (1972). Oestradiol levels in the peripheral blood of cows during the oestrous cycle. *J. Endocr.* **55**, 73-78.

Shemesh, M. & Hansel, W. (1974). Measurement of bovine plasma testosterone by radioimmunoassay (RIA) and by a rapid competetive protein binding (CPB) assay. *J. Anim. Sci.* **39**, 720-724.

Shemesh, M., Lindner, H.R. & Ayalon, N. (1971). Competetive protein-binding assay of progesterone in bovine jugular venous plasma during the oestrous cycle. *J. Reprod. Fert.* **26**, 167-174.

Sherman, B.M. (1976). The menopausal transition: Hormonal evidence for independent ovarian control of pituitary FSH secretion. *Univ. Mich. Med. Cent. J.* **42**, 33-37.

Sheth, A.R., Joshi, L.R., Moodbidri, S.B. & Rao, S.S. (1979). Characterization of a gonadal factor involved in the control of FSH secretion. *J. Reprod. Fert. Suppl.* **26**, 71-85.

Sheth, N.A., Vaze, A.Y. & Sheth, A.R. (1982). A peptide in gastric secretion with inhibin-like properties. *Clin. Endocr.* **17**, 157-163.

Shimasaki, S., Koga, M., Buscaglia, M.L., Simmons, D.M., Bicsak, T.A. & Ling, N. (1989). Follistatin gene expression in the ovary and extragonadal tissues. *Molec. Endocr.* **3**, 651-659.

Shimasaki, S., Koga, M., Esch, F., Cooksey, K., Mercado, M., Koba, A., Ueno, N., Ying, S.-Y., Ling, N. & Guillemin, R. (1988). Primary structure of the human follistatin precursor and its genomic organization. *Proc. Natl. Acad. Sci. USA* **85**, 4218-4222.

Shimasaki, S., Shimonaka, M., Ui, M., Inouye, S., Shibata, F. & Ling, N. (1990). Structural characterization of a follicle-stimulating hormone action inhibitor in porcine ovarian follicular fluid: Its identification as the insulin-like growth factor-binding protein. *J. Biol. Chem.* **265**, 2198-2202.

Short, R.E. & Bellows, R.A. (1971). Relationships among weight gains, age at puberty and reproductive performance in heifers. *J. Anim. Sci.* **32**, 127-131.

Short, R.E., Howland, B.E., Randel, R.D., Christensen, D.S. & Bellows, R.A. (1973). Induced LH release in spayed cows. *J. Anim. Sci.* **37**, 551-557.

Short, R.E., Randel, R.D., Staigmiller, R.B. & Bellows, R.A. (1979). Factors affecting estrogen-induced LH release in the cow. *Biol. Reprod.* **21**, 683-689.

Short, R.V. (1962). Steroids present in the follicular fluid of the cow. *J. Endocr.* **23**, 401-411.

Shukovski, L. & Findlay, J.K. (1989). Activin inhibits oxytocin and progesterone production by bovine granulosa cells. *Proc. Aust. Soc. Reprod. Biol.* **21**, Abstr. 135.

Sirois, J. & Fortune, J.E. (1988). Ovarian follicular dynamics during the estrous cycle in heifers monitored by real-time ultrasonography. *Biol. Reprod.* **39**, 308-317.

Skinner, M.K. & Osteen, K.G. (1988). Developmental and hormonal regulation of bovine granulosa cell function in the preovulatory follicle. *Endocrinology* **123**, 1668-1675.

Sluss, P.M., Branca, A.A., Ford, J.J., Krishnan, K.A. & Reichert, L.E. (1989). Purification, measurement, and tissue distribution of a dansyl-derivatized glycopeptide from low-molecular weight follicle-simulating hormone-inhibitor-containing fractions of porcine follicular fluid. *Biol. Reprod.* **40**, 407-415.

Sluss, P.M., Fletcher, P.W. & Reichert, L.E. (1983). Inhibition of ^{125}I-human follicle-stimulating hormone binding to receptor by a low molecular weight fraction of bovine follicular fluid: Inhibitor concentration is related to biochemical parameters of follicular development. *Biol. Reprod.* **29**, 1105-1113.

Sluss, P.M. & Reichert, L.E. (1984a). Porcine follicular fluid contains several low molecular weight inhibitors of follicle-stimulating hormone binding to receptor. *Biol. Reprod.* **30**, 1091-1104.

Sluss, P.M. & Reichert, L.E. (1984b). Secretion of an inhibitor of follicle stimulating hormone binding to receptor by the bacteria Serratia, including a strain isolated from porcine follicular fluid. *Biol. Reprod.* **31**, 520-530.

Sluss, P.M., Schneyer, A.L., Franke, M.A. & Reichert, L.E. (1987). Porcine follicular fluid contains both follicle-stimulating hormone agonist and antagonist activities. *Endocrinology* **120**, 1477-1481.

Smeaton, T.C. & Robertson, H.A. (1971). Studies on the growth and atresia of Graafian follicles in the ovary of the sheep. *J. Reprod. Fert.* **25**, 243-252.

Smith, J.C., Price, B.M.J., Van Nimmen, K. & Huylebroeck, D. (1990). Identification of a potent *Xenopus* mesoderm-inducing factor as a homologue of activin A. *Nature* **345**, 729-731.

Smith, J.F., Cox, R.I., McGowan, L.T. & Wilson, P.A. (1982). Immunization of sheep against oestrone to offset the effects of coumestrol. *Proc. Aust. Soc. Reprod. Biol.* **14**, Abstr. 95.

Smith, J.F., Fairclough, R.J., Payne, E. & Peterson, A.J. (1975). Plasma hormone levels in the cow. I. Changes in progesterone and oestrogen during the normal oestrous cycle. *NZ J. Agr. Res.* **18**, 123-129.

Smith, J.F., Payne, E., Tervit, H.R., McGowan, L.T., Fairclough, R., Kilgour, R. & Goold, P.G. (1981). The effect of suckling upon the endocrine changes associated with anoestrous in identical twin dairy cows. *J. Reprod. Fert. Suppl.* **30**, 241-249.

Smith, K.B., Millar, M.R., McNeilly, A.S., Illingworth, P.J., Fraser, H.M. & Baird, D.T. (1990). Immunocytochemical localisation of inhibin in the human corpus luteum. *J. Reprod. Fert. Abstr. Series* **5**, p37, Abstr 64.

Smith, P.E. (1926a). Ablation and transplantation of the hypophysis in the rat. *Anat. Rec.* **32**, 221 (Abstr. 46).

Smith, P.E. (1926b). Hastening development of female genital system by daily homoplastic pituitary transplants. *Proc. Soc. Exp. Biol.* **24**, 131-132.

Smith, S.P., Pollak, E.J. & Anderson, G.B. (1982). Maternal influences on growth of twin and single calves. *J. Anim. Sci.* **55**, 533-542.

Snook, R.B., Saatman, R.R. & Hansel, W. (1971). Serum progesterone and luteinizing hormone levels during the bovine estrous cycle. *Endocrinology* **88**, 678-686.

Spicer, L.J., Echternkamp, S.E., Canning, S.F. & Hammond, J.M. (1988). Relationship between concentrations of immunoreactive insulin-like growth factor-1 in follicular fluid and various biochemical markers of differentiation in bovine antral follicles. *Biol. Reprod.* **39**, 573-580.

Sreenan, J.M. (1984). Steroid immunization in cows: Potential for increasing ovulation and twinning rates. *10th Int. Cong. Anim. Repr. A.I.* Vol. 4., section VIII pp. 22-27.

Sreenan, J.M., Diskin, M.G., Morris, D., Tait, A. & Kilpatrick, M. (1983). Effect of immunisation against gonadal steroids on anti-body titres, oestrous and ovulation responses. *An Foras Talúntais Annual Research Report*, Dublin, pp. 83-84.

Sreenan, J.M. & Gosling, J.P. (1977). The effect of cycle stage and plasma progesterone level on the induction of multiple ovulations in heifers. *J. Reprod. Fert.* **50**, 367-369.

Sreenan, J.M., Morris, D., Tait, A. & Diskin, M.G. (1987). Manipulation of the immune system to increase ovulation rate in the cow. In "*Follicular growth and ovulation rate in farm animals.*" Eds. J.F.Roche & D.O'Callaghan, Martinus Nijhoff, Dordrecht. pp. 73-86.

Staigmiller, R.B., England, B.G., Webb, R., Short, R.E. & Bellows, R.A. (1982). Estrogen secretion and gonadotropin binding by individual bovine follicles during estrus. *J. Anim. Sci.* **55**, 1473-1482.

Staigmiller, R.B., Short, R.E. & Bellows, R.A. (1979). Induction of LH surges with 17β estradiol in prepuberal beef heifers: An age-dependent response. *Theriogenology* **11**, 453-457.

Steinberger, A. & Steinberger, E. (1976). Secretion of an FSH-inhibiting factor by cultured Sertoli cells. *Endocrinology* **99**, 918-921.

Stewart, A.G., Milborrow, H.M., Ring, J.M., Crowther, C.E. & Forage, R.G. (1986). Human inhibin genes: Genomic characterisation and sequencing. *FEBS Lett.* **206**, 329-334.

Stumpf, T.T., Day, M.L., Wolfe, P.L., Wolfe, M.W., Clutter, A.C., Kittok, R.J. & Kinder, J.E. (1988). Feedback of 17β-Estradiol on secretion of luteinizing hormone during different seasons of the year. *J. Anim. Sci.* **66**, 447-451.

Sugino, H., Nakamura, T., Hasegawa Y., Miyamoto, K., Abe, Y., Igarashi, M., Eto, Y., Shibai, H. & Titani, K. (1988a). Erythroid differentiation factor can modulate follicular granulosa cell functions. *Biochem. Biophys. Res. Comm.* **153**, 281-288.

Sugino, H., Nakamura, T., Hasegawa Y., Miyamoto, K., Igarashi, M., Eto, Y., Shibai, H. & Titani, K. (1988b). Identification of a specific receptor for erythroid differentiation factor on follicular granulosa cell. *J. Biol. Chem.* **263**, 15249-15252.

Sugino, K., Nakamura, T., Takio, K., Titani, K., Miyamoto, K., Hasegawa, Y., Igarashi, M. & Sugino, H. (1989). Inhibin alpha-subunit monomer is present in bovine follicular fluid. *Biochem. Biophys. Res. Comm.* **159**, 1323-1329.

Suzuki, T., Miyamoto, K., Hasegawa, Y., Abe, Y., Ui, M., Ibuki, Y. & Igarashi, M. (1987). Regulation of inhibin production by rat granulosa cells. *Molec. Cell. Endocr.* **54**, 185-195.

Swanson, L.V. & McCarthy, S.K. (1978). Estradiol treatment and luteinizing hormone (LH) response of prepubertal Holstein heifers. *Biol. Reprod.* **18**, 475-480.

Swanson, L.V., Hafs, H.D. & Morrow, D.A. (1972). Ovarian characteristics and serum LH, prolactin, progesterone and glucocorticoid from first estrus to breeding size in Holstein heifers. *J. Anim. Sci.* **34**, 284-293.

Teleni, E., Rowe, J.B. & Croker, K.P. (1984). Ovulation rates in ewes: The role of energy-yielding substrates. In "*Reproduction in Sheep*", Eds. D.R.Lindsay & D.T.Pearce., Cambridge University Press, pp. 277-278.

Terranova, P.F. & Greenwald, G.S. (1981). Increased ovulation rate in the cyclic guinea-pig after a single injection of an antiserum to LH. *J. Reprod. Fert.* **61**, 37-42.

Tesone, M., Ladenheim, R.G., Cheb-Terrab, R., Chiauzzi, V., Solano, A., Podesta, E. & Charreau, E.H. (1986). Comparison between bioactive and immunoactive luteinizing hormone (LH) in ovariectomized streptozotocin-induced diabetic rats: Response to LH-releasing hormone. *Endocrinology* **119**, 2412-2416.

Thakur, A.N., Vaze, A.Y., Dattatreyamurty, B., Arbatti, N.J. & Sheth, A.R. (1978). Isolation & characterization of inhibin from human seminal plasma. *Ind. J. Exp. Biol.* **16**, 854-856.

Thomas, G.B., Martin, G.B. & Pearce, D.T. (1982). Luteinizing hormone secretion in the anoestrous ewe: Manipulation by antisera to oestradiol and by exogenous oestrogen. *Proc. Aust. Soc. Reprod. Biol.* **14**, Abstr. 90.

Thompson, D.L., Voelkel, S.A., Reville-Moroz, S.I., Godke, R.A. & Derrick, D.J. (1984). Testosterone effects on gonadotrophin response to GnRH: Cows and pony mares. *J. Anim. Sci.* **58**, 409-415.

Thorrel, J.I. & Johansson, B.G. (1971). Enzymatic iodination of polypeptides with ^{125}I to high specific activity. *Biochim. Biophys. Acta* **251**, 363-369.

Tonetta, S.A. & diZerega, G.S. (1986). Paracrine regulation of follicular maturation in primates. *Clin. Endocr. Metab.* **15**, 135-156.

Tonetta, S.A. & diZerega, G.S. (1990). Local regulatory factors controlling folliculogenesis in pigs. *J. Reprod. Fert. Suppl.* **40**, 151-161.

Tonetta, S.A., Yanagihara, D.L., DeVinna, R.S. & diZerega, G.S. (1988). Secretion of follicle-regulatory protein by porcine granulosa cells. *Biol. Reprod.* **38**, 1001-1005.

Törnell, J., Carlsson, B. & Hillensjö, T. (1988). Vasoactive intestinal peptide stimulates oocyte maturation, steroidogenesis, and cyclic adenosine 3', 5'-monophosphate production in isolated preovulatory rat follicles. *Biol. Reprod.* **39**, 213-220.

Totey, S.M., Singh, G. & Talwar, G.P. (1989). Superovulatory response in lactating *Bos indicus* x *Bos taurus* cows primed with FSH-P at the beginning of the estrous cycle. *Theriogenology* **31**, 268. (Abstr.).

Touati, K., van der Zwalmen, P., Ectors, F.J., Beckers, J.F. & Ectors, F. (1989). Low dose of FSH early in estrous cycle enhances superovulatory response in heifers. *Theriogenology* **31**, 269. (Abstr.).

Trimberger, G.W. (1941). Menstruation frequency and its relation to conception in dairy cattle. *J. Dairy Sci.* **24**, 819-823.

Trimberger, G.W. (1948). Breeding efficiency in dairy cattle from artificial insemination at various intervals before and after ovulation. *Nebraska Agric. Exp. Sta. Res. Bull.* **153**.

Tsang, B.K., Armstrong, D.T. & Whitfield, J.F. (1980). Steroid biosynthesis by isolated human ovarian follicular cells *in vitro*. *J. Clin. Endocr. Metab.* **51**, 1407-1411.

Tsang, B.K., Moon, Y.S., Simpson, C.W. & Armstrong, D.T. (1979). Androgen biosynthesis in human ovarian follicles: Cellular source, gonadotropic control, and adenosine 3',5'-monophasphate mediation. *J. Clin. Endocr. Metab.* **48**, 153-158.

Tsonis, C.G., Baird, D.T., Campbell, B.K., Downing, J.A. & Scaramuzzi, R.J. (1988b). Secretion of bioactive inhibin by the ovary of the Booroola Merino ewe with or without a copy of the fecundity (F) gene. *J. Endocr.* **119**, R5-R9.

Tsonis, C.G., Baird, D.T., Campbell, B.K., Leask, R. & Scaramuzzi, R.J. (1988a). The sheep corpus luteum secretes inhibin. *J. Endocr.* **116**, R3-R5.

Tsonis, C.G., Hillier, S.G. & Baird, D.T. (1987). Production of inhibin bioactivity by human granulosa-lutein cells: Stimulation by LH and testosterone in vitro. *Endocrinology* **112**, R11-R14.

Tsonis, C.G., McNeilly, A.S. & Baird, D.T. (1986). Measurement of exogenous and endogenous inhibin in sheep serum using a new and extremely sensitive bioassay for inhibin based on inhibition of ovine pituitary FSH secretion *in vitro*. *J. Endocr.* **110**, 341-352.

Tsonis, C.G., McNeilly, A.S. & Baird, D.T. (1988). Inhibin secretion by the sheep ovary during the luteal and follicular phases of the oestrous cycle and following stimulation with FSH. *J. Endocr.* **117**, 283-291.

Tsonis, C.G., Quigg, H., Lee, V.W.K., Leversha, L., Trounson, A.O. & Findlay, J.K. (1983). Inhibin in individual follicles in relation to diameter and atresia. *J. Reprod. Fert.* **67**, 83-90.

Tsonis, C.G., Sharp, P.J. & McNeilly, A.S. (1988). Inhibin bioactivity and pituitary cell mitogenic activity from cultured ovarian granulosa and thecal/stromal cells. *J. Endocr.* **116**, 293-299.

Tucker, H.A (1982). Seasonality in cattle. *Theriogenology* **17**, 53-59.

Turman, E.J., Laster, D.B., Renbarger, R.E. & Stephens, D.F. (1971). Multiple births in beef cows treated with equine gonadotropin (PMS) and chorionic gonadotropin (HCG). *J. Anim. Sci.* **32**, 962-967.

Turner, H.N. (1978). Selection for reproduction rate in Australian Merino sheep: Direct response. *Aust. J. Agric. Res.* **29**, 327-350.

Ueno, N, Ling, N., Ying, S.-Y., Esch, F, Shimasaki, S. & Guillemin, R. (1987). Isolation and partial characterization of follistatin: A single-chain M_r 35,000 monomeric protein that inhibits the release of follicle-stimulating hormone. *Proc. Natl. Acad. Sci. USA* **84**, 8282-8286.

Ursely, J. & Leymarie, P. (1979). Varying response to luteinizing hormone of two luteal cell types isolated from bovine corpus luteum. *J. Endocr.* **83**, 303-310.

Vale, W., Rivier, J., Vaughan, J., McClintock, R., Corrigan, A., Woo, W., Karr, D. & Spiess, J. (1986). Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. *Nature* **321**, 776-779.

van den Eijnden-Van Raaij, A.J.M., van Zoelent, E.J.J., van Nimmen, K., Koster, C.H., Snoek, G.T., Durston, A.J. & Huylebroeck, D. (1990). Activin-like factor from a *Xenopus laevis* cell line resposible for mesoderm induction. *Nature* **345**, 732-734.

van Dieten, J.A.M.J., de Koning, J. & van Rees, G.P. (1989). Regulation by ovarian factors of the LHRH-induced LH response in pituitary glands in situ or grafted under the kidney capsule in intact and ovariectomized rats. *J. Endocrinol.* **123**, 41-45.

Van Look, P.F.A., Clarke, I.J., Davidson, W.G. & Scaramuzzi, R.J. (1978). Ovulation and lambing rates in ewes actively immunized against androstenedione. *J. Reprod. Fert.* **53**, 129-130.

Vaze, A.Y., Thakur, A.N. & Sheth, A.R. (1979). Development of a radioimmunoassay for human seminal plasma inhibin. *J. Reprod. Fert. Suppl.* **26**, 135-146.

Veldhuis, J.D., Johnson, M.L. & Dufau, M.L. (1987). Preferential release of bioactive luteinizing hormone in response to endogenous and low dose exogenous gonadotropin-releasing hormone pulses in man. *J. Clin. Endocr. Metab.* **64**, 1275-1282.

Veldhuis, J.D., Weiss, J., Mauras, N., Rogol, A.D., Evans, W.S. & Johnson, M.L. (1986). Appraising endocrine pulse signals at low circulating hormone concentrations: use of regional coefficients of variation in the experimental series to analyze pulsatile luteinizing hormone release. *Pediatric research* **20**, 632-637.

Vidgoff, B. & Vehrs, H. (1940). Studies on the inhibitory hormone of the testes. IV. Effect on the pituitary, thyroid and adrenal glands of the adult male rat. *Endocrinology* **26**, 656-661.

Vincent, C.K. & Mills, A.C. (1972). Gonadotropin levels for multiple births in beef cattle. *J. Anim. Sci.* **34**, 77-81.

Wallace, J.M., Martin, G.B. & McNeilly, A.S. (1988). Changes in the secretion of LH pulses, FSH and prolactin during the preovulatory phase of the oestrous cycle of the ewe and the influence of treatment with bovine follicular fluid during the luteal phase. *J. Endocr.* **116**, 123-135.

Wallace, J.M. & McNeilly, A.S. (1985). Increase in ovulation rate after treatment of ewes with bovine follicular fluid in the luteal phase of the oestrous cycle. *J. Reprod. Fert.* **73**, 505-515.

Wallace, J.M. & McNeilly, A.S. (1986). Changes in FSH and the pulsatile secretion of LH during treatment of ewes with bovine follicular fluid throughout the luteal phase of the oestrous cycle. *J. Endocr.* **111**, 317-327.

Walters, D.L. & Schallenberger, E. (1984). Pulsatile secretion of gonadotrophins, ovarian steroids and ovarian oxytocin during the periovulatory phase of the oestrous cycle in the cow. *J. Reprod. Fert.* **71**, 503-512.

Walters, D.L., Schams, D. & Schallenberger, E. (1984). Pulsatile secretion of gonadotrophins, ovarian steroids and ovarian oxytocin during the luteal phase of the oestrous cycle in the cow. *J. Reprod. Fert.* **71**, 479-491.

Walton, J.S. (1985). Effect of immunization against androstenedione on ovarian function in dairy cows and prepubertal gilts. *Anim. Reprod. Sci.* **8**, 349-364.

Wang, C., Hsueh, A.J.W. & Erickson, G.F. (1981). LH stimulation of estrogen secretion by cultured rat granulosa cells. *Molec. Cell. Endocr.* **24**, 17-28.

Wang, H., Wu, M., Xu, K., Hagele, W.C. & Mapletoft, R.J. (1987). Control of superovulation in the cow with a PMSG antiserum. *Theriogenology* **27**, 291. (Abstr.).

Wang, Q.F., Farnworth, P.G., Burger, H.G. & Findlay, J.K. (1990). Effect of inhibin on activators of protein kinase-C and calcium-mobilizing agents which stimulate secretion of gonadotropins *in vitro*: Implication of a postgonadotropin-releasing hormone receptor effect of inhibin on gonadotropin release. *Endocrinology* **126**, 3210-3217.

Wang, Q.-F., Farnworth, P.G., Robertson, D.M. & Findlay, J.K. (1989a). Acute inhibitory effect of follicle-suppressing protein (FSP) on GnRH-stimulated gonadotropin secretion and its mechanism of action in cultured rat anterior pituitary cells. *Proc. Endocr. Soc. Aust.* **32**, Abstr. 103.

Wang, Q.-F., Farnworth, P.G., Robertson, D.M. & Findlay, J.K. (1989b). Chronic inhibitory effect of follicle-stimulating hormone suppressing protein (FSP) on GnRH-stimulated FSH secretion and synthesis in cultured rat anterior pituitary cells. *Proc. Aust. Soc. Reprod. Biol.* **21**, Abstr. 75.

Webb, R. (1987). Increasing ovulation rate and lambing rate in sheep by treatment with a steroid enzyme inhibitor. *J. Reprod. Fert.* **79**, 231-240.

Webb, R., Baxter, G., McBride, D., Nordblom, G.D. & Shaw, M.P.K. (1985a). The measurement of testosterone and oestradiol-17β using iodinated tracers and incorporating an affinity chromatography extraction procedure. *J. steroid Biochem.* **23**, 1043-1051.

Webb, R., Baxter, G., Preece, R.D., Land, R.B. & Springbett, A.J. (1985b). Control of gonadotrophin release in Scottish Blackface ewes during seasonal anoestrus. *J. Reprod. Fert.* **73**, 369-378.

Webb, R. & Bellows, R.A. (1980). Relationship between gonadotropin binding and steroidogensis in individual bovine follicles. *Biol. Reprod.* **22** Suppl. 1, p45A, Abstr. 50.

Webb, R. & England, B.G. (1982a). Identification of the ovulatory follicle in the ewe: Associated changes in folliular size, thecal and granulosa cell luteinizing hormone receptors, antral fluid steroids, and circulating hormones during the preovulatory period. *Endocrinology* **110**, 873-881.

Webb, R. & England, B.G. (1982b). 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. & Gauld, I.K. (1985). Folliculogenesis in sheep: Control of ovulation rate. In "*Genetics of reproduction in sheep*". Eds. R.B.Land & D.W.Robinson, Butterworths, London. pp. 261-274.

Webb, R., Land, R.B., Pathiraja, N. & Morris, B.A. (1984). Passive immunization against steroid hormones in the female. In "*Immunological aspects of reproduction in mammals.*" Ed. D.B.Crighton, Butterworths, London. pp. 475-499.

Weber, A.F., Morgan, B.B. & McNutt, S.H. (1948). A histological study of metrorrhagia in the virgin heifer. *Amer. J. Anat.* **83**, 309-327.

Weick, R.F. (1977). A comparison of the disappearance rates of luteinizing hormone from intact and ovariectomized rats. *Endocrinology* **101**, 157-161.

Welschen, R., Dullaart, J. & de Jong, F.H. (1978). Interrelationships between circulating levels of estradiol-17β, progesterone, FSH and LH immediately after unilateral ovariectomy in the cyclic rat. *Biol. Reprod.* **18**, 421-427.

West, J.D., West, K.M. & Aitken, R.J. (1989). Detection of Y-bearing spermatazoa by DNA-DNA in situ hybridisation. *Molec. Reprod. Develop.* **1**, 201-207.

Westhof, G., Westhof, K.F., Ahmad, N. & diZerega, G.S. (1988). Steroidal secretion and morphology of single porcine follicles exposed to follicle regulatory protein (FRP) in vitro: Does FRP induce atresia? *J. Reprod. Fert. Abstract Series No.* **1**, Abstr. 39.

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

Whitaker, D.A., Smith, E.J., Kelly, J.M. & Hodgson-Jones, L.S. (1988). Health, welfare and fertility implications of the use of bovine somatotrophin in dairy cattle. *Vet. Rec.* **122**, 503-505.

Whitehead, S.A. (1990). A gonadotrophin surge attenuating factor. *J. Endocr.* **126**, 1-4.

Wilson, P.A., Cox, R.I., Wong, M.S.F. & Paull, D.R. (1986). Improved prolificacy in Merino ewes immunized against a combination of androgens and oestrogens. *Proc. Aust. Soc. Reprod. Biol.* **18**, Abstr. 41.

Wiltbank, J.N., Gregory, K.E., Swiger, L.A., Ingalls, J.E., Rothlisberger, J.A. & Koch, R.M. (1966). Effects of heterosis on age and weight at puberty in beef heifers. *J. Anim. Sci.* **25**, 744-751.

Wiltbank, J.N., Kassons, C.W. & Ingalls, J.E. (1969). Puberty in crossbred and straightbred beef heifers on two levels of feed. *J. Anim. Sci.* **29**, 602-605.

Wise, T.H., Caton, D., Thatcher, W.W., Rami Lehrer, A. & Fields, M.J. (1982). Androstenedione, dehydroepiandrostenedione and testosterone in ovarian vein plasma and androstenedione in peripheral arterial plasma during the bovine oestrous cycle. *J. Reprod. Fert.* **66**, 513-518.

Wise, T. & Schanbacher, B.D. (1983). Reproductive effects of immunizing heifers against androstenedione and oestradiol-17β. *J. Reprod. Fert.* **69**, 605-612.

Woodruff, T.K., Meunier, H., Jones, P.C.B., Hsueh, A.J.W. & Mayo, K.E. (1987). Rat inhibin: Molecular cloning of α- and β-subunit complementary deoxyribonucleic acids and expression in the ovary. *Molec. Endocr.* **1**, 561-568.

Wrathall, J.H.M., McLeod, B.J., Glencross, R.G., Beard, A.J. & Knight, P.G. (1990). Inhibin immunoneutralization by antibodies raised against synthetic peptide sequences of inhibin α subunit: effects on gonadotrophin concentrations and ovulation rate in sheep. *J. Endocr.* **124**, 167-176.

Xiao, S., Findlay , J.K. & Robertson, D.M. (1990). The effect of bovine activin and follicle-stimulating hormone (FSH) suppressing protein/follistatin on FSH-induced differentiation of rat granulosa cells in vitro. *Molec. Cell. Endocr.* **69**, 1-8.

Xu, K.P., Picard, L., King, W.A. & Goff, A.K. (1989). Sexing and bisecting bovine embryos produced by in vitro maturation and fertilization. *Theriogenology* **31**, 274. (Abstr.).

Yamashiro, D., Li, C.H., Ramasharma, K. & Sairam, M.R. (1984). Synthesis and biological activity of human inhibin-like peptide-(1-31). *Proc. Natl. Acad. Sci. USA* **81**, 5399-5402.

Yarney, T.A., Sairam, M.R., Bhargavi, G.N., Downey, B.R. & Srikandakumar, A. (1990). Gonadotrophin-binding components in porcine follicular fluid. *J. Endocr.* **124**, 485-494.

Ying, S.-Y. (1988). Inhibins, activins, and follistatins: Gonadal proteins modulating the secretion of follicle-stimulating hormone. *Endocr. Rev.* **9**, 267-293.

Ying, S.-Y. (1989). Inhibins, activins and follistatins. *J. steroid. Biochem.* **33**, 705-713.

Ying, S.-Y., Becker, A., Ling, N., Ueno, N. & Guillemin, R. (1986). Inhibin and beta type transforming growth factor (TGFβ) have opposite modulating effects on the follicle stimulating hormone (FSH)-induced aromatase activity of cultured rat granulosa cells. *Biochem. Biophys. Res. Comm.* **136**, 969-975.

Ying, S.-Y., Becker, A., Swanson, G., Tan, P., Ling, N., Esch, F., Ueno, N., Shimasaki, S. & Guillemin, R. (1987). Follistatin specifically inhibits pituitary follicle stimulating hormone release in vitro. *Biochem. Biophys. Res. Comm.* **149**, 133-139.

Ying, S.-Y. & Guillemin, R. (1981). Gonadocrinins: Novel peptides in ovarian follicular fluid stimulating gonadotropin secretion. In "*Intragonadal regulation of reproduction*". Eds P.Franchimont & C.P.Channing, Academic Press, London. pp. 157-166.

Zeleznik, A.J. (1981). Premature elevation of systemic estradiol reduces serum levels of follicle-stimulating hormone and lengthens the follicular phase of the menstrual cycle in Rhesus monkeys. *Endocrinology* **109**, 352-355.

Zeleznik, A.J., Midgley, A.R. & Reichert, L.E. (1974). Granulosa cell maturation in the rat: Increased binding of human chorionic gonadotropin following treatment with follicle-stimulating hormone *in vivo*. *Endocrinology* **95**, 818-825.

Zhang, Z., Carson, R.S., Herington, A.C., Lee, V.W.K. & Burger, H.G. (1987a). Follicle-stimulating hormone and somatomedin-C stimulate inhibin production by rat granulosa cells *in vitro*. *Endocrinology* **120**, 1633-1638.

Zhang, Z., Findlay, J.K., Carson, R.S., Herington, A.C. & Burger, H.G. (1988b). Transforming growth factor β enhances basal and FSH-stimulated inhibin production by rat granulosa cells in vitro. *Molec. Cell. Endocr.* **58**, 161-166.

Zhang, Z., Herington, A.C., Carson, R.S., Findlay, J.K. & Burger, H.G. (1987b). Direct inhibition of rat granulosa cell inhibin production by epidermal growth factor in vitro. *Molec. Cell. Endocr.* **54**, 213-220.

Zhang, Z., Lee, V.W.K., Carson, R.S. & Burger, H.G. (1988a). Selective control of rat granulosa cell inhibin production by FSH and LH in vitro. *Molec. Cell. Endocr.* **56**, 35-40.

Zhuang, L.-Z., Adashi, E.Y. & Hsueh, A.J.W. (1982). Direct enhancement of gonadotropin-stimulated ovarian estrogen biosynthesis by estrogen and clomiphene citrate. *Endocrinology* **110**, 2219-2221.

Ziecik, A.J., Esbenshade, K.L. & Britt, J.H. (1988). Absence of specific follicle stimulating hormone receptors in porcine corpora lutea. *Theriogenology* **29**, 525-533.

Zuckerman, S. (1953). The breeding seasons of mammals in captivity. *Proc. Zool. Soc. Lond.* **122**, 827-950.

Zupp, B.A. (1926). The cyclic secretory phenomena of estrus in the cow. *Vet. Pract. Bull. Iowa State Coll.* **8**, 123-152.