THE ROLE OF GROWTH HORMONE, INSULIN-LIKE GROWTH FACTOR-I AND INSULIN IN THE CONTROL OF OVARIAN FOLLICULAR GROWTH AND DEVELOPMENT IN THE HEIFER

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ABSTRACT

Recent experimental evidence has suggested that the control of ovarian folliculogenesis and ovulation rate in cattle cannot be accounted for solely in terms of changes in pituitary gonadotrophins, gonadal steroids and/or proteins. The aim of this project was to investigate the role of growth hormone (GH), insulin-like growth factor-I (IGF-I) and insulin in the control of ovarian follicular growth and development in heifers.

Daily treatment of heifers with 25mg recombinant bovine somatotropin (BST), for a period of 2 oestrous cycles, significantly increased the population of small ovarian follicles (2-5mm in diameter) although ovulation rate was unaltered. While peripheral concentrations of GH, IGF-I and insulin were significantly increased, there was no effect of BST on either circulating concentrations of oestradiol, progesterone, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) or numbers of FSH and LH binding sites in granulosa and thecal cells.

A further study using real-time ultrasound demonstrated that in our population of heifers the majority (9/12) have 3 waves of follicular growth and development per oestrous cycle, whilst the remainder displayed a 2-wave pattern. The growth of each dominant follicle was always associated with a marked reduction in the number and growth of the subordinate follicles. Whilst there was no effect of BST on the numbers of follicular waves, large follicles (>10mm) and medium-sized follicles (5-10mm), BST treatment significantly increased the number of small follicles throughout the treatment period. However, the inhibitory effect of the dominant follicle on subordinate follicles was not affected by BST treatment. Treatment of heifers with a single dose of BST (320mg in a

sustained-release formulation) demonstrated that the increase in the number of small follicles was temporally correlated with changes in peripheral IGF-I and insulin concentrations.

To investigate if the increase in the number of small follicles induced by BST treatment could enhance superovulatory responses, heifers were pretreated with a single dose of BST (320mg) 5 days before a superovulatory treatment with PMSG. The results from two experiments demonstrated that pre-treatment with BST significantly increased both ovulation rate and number of embryos recovered. Interestingly, this treatment also appeared to reduce the between-animal variability in the response.

As an initial step to determine the mechanism(s) underlying the effect of BST on small follicle population, the effects of physiological concentrations of BST, IGF-I and insulin, together with their interactions with FSH and LH, on bovine granulosa cell proliferation were investigated *in vitro* using a serum-free culture system. BST at doses up to 1.0 μg/ml, given alone or in combination with FSH or LH, did not affect the incorporation of ^{3}H-thymidine into granulosa cells from small or medium-sized follicles but inhibited the proliferation of granulosa cells from large follicles. Both IGF-I and insulin produced a dose-dependent stimulation in the proliferation of granulosa cells from all three size categories of follicles. A synergistic interaction between either FSH or LH with both IGF-I and insulin was observed in granulosa cells from small follicles, while both FSH and LH inhibited the stimulatory effect of IGF-I and insulin on granulosa cells from medium-sized and large follicles.

In conclusion, these results demonstrate that BST treatment can

significantly increase the population of small ovarian follicles in heifers, possibly through increased peripheral concentrations of IGF-I and/or insulin although a possible direct effect of BST at the ovarian level remains to be investigated. This effect appears to result from an enhancement in the process of small follicle recruitment via a mechanism other than that involved in the inhibitory effect of the dominant follicle. Furthermore, small follicles induced by BST treatment are functionally healthy as they can be stimulated to ovulate by PMSG treatment. This may provide a valuable approach to improve superovulatory responses and increase the efficiency of embryo production in cattle. In addition, the effects of metabolic-related hormones on ovarian function demonstrated in these studies may provide a new avenue to study the relationships between nutrition and reproduction.

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 designing and execution of the experiments contained in this thesis and during the preparation of this thesis.

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INTRODUCTION

The integrated role of pituitary gonadotrophins, ovarian steroids and, more recently, follicular proteins in the control of ovarian follicular growth and development in the domestic species has been intensively studied by reproductive physiologists. This has led to the progress in improving the prolificacy of sheep by the use of immunization against various gonadal steroids and/or proteins. Unfortunately, similar attempts to increase fecundity in cattle have met with limited success. It appears that there may be differences between sheep and cattle in the mechanism controlling reproductive function. The fundamental processes involved in the control of ovarian follicular growth and development in cattle need to be studied further in order to obtain better understanding of the mechanism(s) controlling ovulation rate in this species.

It is well established that nutrition has a profound modulatory effect on reproductive function, although the underlying mechanism is not well understood. Recent experimental evidence suggests that circulating metabolic hormones such as growth hormone (GH) and insulin, and locally-produced ovarian substances such as insulin-like growth factors (IGFs), may play an important role in the control of folliculogenesis. Study of these factors in cattle may facilitate a better understanding of the mechanism(s) controlling follicular recruitment, selection and dominance, and thus help to reliably control reproductive performance in this species.

The aim of this project was to examine the effects of GH, insulin and IGF-I on ovarian follicular growth and development in heifers, and to study their interactions with other regulators of follicular growth and ovarian function such as gonadotrophins and gonadal steroids.

CHAPTER 1

Literature review: The control of ovarian follicular growth and development in the cow

1.1. General reproductive physiology of the cow

The ovary of the female cow is well developed at birth (Hammond, 1927), as formation of the pool of primordial ovarian follicles starts around Day 130 of the foetal life and is completed by the time of birth (Erickson, 1966a; Peters, Byskov, Himmelstein-Braw and Faber, 1975; Peters, 1978). Follicular growth from this non-proliferating pool is initiated as soon as primordial follicles are formed during foetal development (Peters, 1978). Antral follicles are present in ovaries during the prepubertal period (Casida, Chapman and Rupel, 1935; Perry and Rowlands, 1962; Desjardins and Hafs, 1969). However, the follicles undergoing growth during foetal life and before puberty regress at an early stage of development.

The cow generally reaches puberty at an age of 9 - 15 months, with possible variation between individuals ranging from 5 to 24 months (Hammond, 1927; Moran, Quirke and Roche, 1989). Despite extensive studies, the control of the onset of puberty in heifers is still not fully understood (for reviews see Kinder, Day and Kittok, 1987; Moran *et al.*, 1989). Breed, environment and particularly nutritional condition appear to have a profound effect on the attainment of puberty (Wiltbank, Gregory, Swiger, Ingalls, Rothlisberger and Koch, 1966; Wiltbank, Kassons and Ingalls, 1969; Short and Bellows, 1971; Laster, Glimp and

Gregory, 1972), as individual animals can reach puberty as early as 5 month of age if they reach a certain body size (bodyweight) (Hammond, 1927; Craig, 1930). After the attainment of sexual maturity, except during pregnancy and the early postpartum period, the cow undergoes regular cyclic ovarian activity (the oestrous cycle), which normally produces a single ovulatory follicle.

Although some differences in the length of the oestrous cycle, behavioural oestrous activity, endocrine patterns and fertility between different seasons have been observed (Hammond, 1927; Mercier and Salisbury, 1947; Hansen, Kamwanja and Hauser, 1982; Critser, Miller, Gunsett and Ginther, 1983; Critser, Linstrom, Hinshelwood and Hauser, 1987a; Critser, Block, Folkman and Hauser, 1987b), the domestic cow is generally agreed to be an aseasonal species. The major anoestrous period in the cow is during pregnancy and the immediate postpartum period. The length of pregnancy is approximately 285 days and the anoestrous period following parturition ranges from 30 to 80 days (Hammond, 1927; Morrow, Roberts and McEntee, 1969).

1.2. The oestrous cycle

1.2.1. Temporal events

The mean length of the oestrous cycle is about 20 days for heifers and 21 days for cows, with variation observed both between and within individual animals ranging from 17 to 24 days (Hafez and Sugie, 1963; Robinson, 1977; Robinson and Shelton, 1991). Each oestrous cycle consists of a short duration of sexual receptivity (oestrus) and a much longer

period of behavioural quiescence. For convenience and practice, the oestrous cycle in the cow, as in other farm species, is usually divided into 2 stages: the luteal phase and the follicular phase, with the day of oestrus designated as Day 0 of the oestrous cycle (Hunter, 1980). Unless otherwise specified, this temporal definition will be used throughout this thesis when referring to the oestrous cycle. The duration of the luteal phase is approximately 17 days and is characterised by the presence of a functional corpus luteum (CL) in the ovary. Following luteolysis at the end of the luteal phase, the follicular phase begins. It is characterised by the regression of the CL and the rapid development of usually a single preovulatory follicle. The follicular phase lasts for 3 to 4 days and culminates in oestrus followed by ovulation.

There is a large variation in the duration of oestrus (means, 15 to 18 hours; ranges, 6 to 30 hours) (Hammond, 1927; Gomes, 1978). Oestrus duration tends to be shorter during the winter season and the peak of lactation (Gomes, 1978), and is shorter in heifers than in cows (Trimberger, 1948). During oestrus, many animals will display some specific sexual behaviour such as restlessness, arching the back and raising the hindquarters, mounting other cows, standing when mounted and discharging of mucus from the vulva. Such oestrous behaviour is commonly used in farm practice to identify animals in oestrus and to decide the time for artificial insemination, although the intensity of oestrous behaviour may vary markedly between individual animals (Hammond, 1927).

Ovulation normally occurs 24 to 48 hours after the onset of oestrus (Hansel and Trimberger, 1952; Quirk, Hickey and Fortune, 1986) or 10 to 12 hours following the end of oestrus (Henricks, Dickey and Niswender,

1970; Schams, Schallenberger, Hoffmann and Karg, 1977). Although the cow is normally considered to be a spontaneous ovulator, it has been reported that the presence of a bull, particularly the act of mating, may accelerate or even induce ovulation (Marion, Smith, Wiley and Barrett, 1950; Hunter, 1980).

1.2.2. Endocrinology of the oestrous cycle

The development of radioimmunoassays and competitive protein binding techniques has enabled serum concentrations of steroids and gonadotrophins to be accurately measured during the oestrous cycle (Hansel and Echternkamp, 1972; Chenault, Thatcher, Kalra, Abrams and Wilcox, 1975; Ireland and Roche, 1982a; Peters, 1985; reviewed by Hansel, Concannon and Lukaszewska, 1973; Hunter, 1976; Ireland, 1987). Here the principal endocrine changes during the oestrous cycle will be described in relation to the temporal events of the cycle described above. Although changes in the different hormones will be described separately for the sake of clarity, it is emphasized that changes in these hormones are closely related to one another. The physiological role of these hormones in the control of folliculogenesis will be dealt with in Section 1.4.

Luteinizing hormone (LH) concentrations remain low, rarely exceeding 2.0 - 3.0 ng/ml, throughout the luteal phase of the cycle (Hunter, 1980; Walters, Schams and Schallenberger, 1984; Price, Morris and Webb, 1987a). Following luteolysis, LH concentrations begin to increase due to an increase in pulse frequency of secretion (see below), although the rise is not significant until serum progesterone levels fall below 1 ng/ml (Hansel *et al.*, 1973). At about the same time as the onset of oestrus (Swanson and Hafs, 1971), there is a sudden increase (the

preovulatory surge) in LH concentrations which reach a peak as high as 50 - 60 ng/ml (Black and Hansel, 1972; Walters and Schallenberger, 1984). This LH surge lasts for 6 to 8 hours (Snook, Saatman and Hansel, 1971), and LH concentrations then decline sharply to those of the luteal phase. Studies using frequent sampling have shown that the secretion of LH occurs in a pulsatile pattern and the pulse characteristics vary markedly between different stages of the cycle (Rahe, Owens, Fleeger, Newton and Harms, 1980). During the luteal phase, LH pulses occur at a frequency of one pulse every 3 to 4 hours with an amplitude of 0.7 - 7.0 ng/ml (Walters *et al.*, 1984). Following luteolysis and during the early stage of the follicular phase, LH pulse frequency increases to about one pulse every 40 to 60 minutes while the pulse amplitude either slightly decreases (Rahe *et al.*, 1980), increases (Schallenberger, Schondorfer and Walters, 1985) or remains unchanged (Peters, 1985). Throughout the whole period of the preovulatory surge, both LH pulse frequency and pulse amplitude are greatly increased. After the LH surge, LH pulses are abolished for about 12 hours, before returning to pre-surge profiles and, subsequently, developing the luteal phase pattern of secretion (Walters and Schallenberger, 1984; Schallenberger *et al.*, 1985).

Unfortunately, due to the lack of sufficiently specific, sensitive and reliable radioimmunoassays for bovine follicle-stimulating hormone (FSH), reports concerning changes in FSH concentrations during the oestrous cycle are often conflicting. Most reports show that FSH concentrations during the luteal phase are relatively low with some fluctuation between days (Walters *et al.*, 1984). Some authors have reported that a distinct peak of FSH can be detected on Days 5 - 8 and Days 13 - 16 of the cycle (Schams *et al.*, 1977; Ireland and Roche, 1982a; Ireland, 1987). FSH concentrations around the time of luteolysis change little,

though there is a tendency for basal FSH levels to decline slightly (Butler, Katz, Arriola, Milvae and Foote, 1983; Parfet, Smith, Cook, Skyer, Youngquist and Garverick, 1989). Coincident with the preovulatory LH surge, there is also a surge of FSH (Walters and Schallenberger, 1984; Schallenberger *et al.*, 1985). A secondary surge of FSH is also observed, starting 4 to 12 hours after the end of preovulatory LH surge and reaching a peak within 24 hours (Dobson, 1978; Walters and Schallenberger, 1984). Following this secondary FSH surge, FSH concentrations return to luteal phase levels. Some authors have found the secretion of FSH to occur in a pulsatile manner (Ireland and Roche, 1982a; Walters *et al.*, 1984; Walters and Schallenberger, 1984; Schallenberger, Schams, Bullerman and Walters, 1984; Schallenberger *et al.*, 1985), whilst others have failed to identify such a pattern (Peters, 1985). FSH pulses have been reported with a frequency of one pulse every 2 hours during the luteal phase and with an amplitude of around 17.0 ng/ml (Walters *et al.*, 1984). There is little change in the pulsatile characteristics of FSH secretion around luteolysis, though FSH pulse frequency possibly increases during the preovulatory LH surge. Following the LH surge, when LH pulses are abolished (see above), FSH secretion continues with approximately the same frequency as before the surge (Walters and Schallenberger, 1984). However, during the secondary FSH surge, FSH pulse amplitude may be increased (Dobson, 1978; Walters and Schallenberger, 1984).

Peripheral progesterone concentrations are very low (below 0.5 ng/ml) on the day of oestrus and following ovulation (Glencross, Munro, Senior and Pope, 1973), but increase steadily from about Days 3 - 4 of the oestrous cycle to reach a peak on Days 10 -14, thereafter remaining at a plateau until the end of the luteal phase (Donaldson, Bassett and

Thorburn, 1970; Henricks, Dickey and Hill, 1971; Smith, Fairclough, Payne and Peterson, 1975). Following luteolysis, progesterone concentrations decline precipitously, falling below 1.0 ng/ml within 24 to 36 hours (Schallenberger *et al.*, 1984), and remaining low until Days 3 - 4 of the subsequent cycle (Henricks *et al.*, 1970; Robertson, 1972). A small rise in ovarian progesterone secretion during the preovulatory LH surge has been detected, but the physiological significance of this rise is unclear (Walters and Schallenberger, 1984). While ill-defined, pulses of progesterone secretion have been detected both in peripheral and vena cava plasma samples during the luteal phase, with a pulse frequency of one pulse every 2 hours (Walters *et al.*, 1984).

During the luteal phase, circulating oestradiol concentrations are low, although an elevation in oestradiol concentration around Days 4 - 6 and around Days 12 - 15 of the oestrous cycle have been detected by some workers (Shemesh, Ayalon and Linder, 1972; Dobson and Dean, 1974; Ireland and Roche, 1987). The elevation in oestradiol seems to be coincident with the rise in FSH concentrations (see above). Following the onset of luteolysis, oestradiol concentrations rise steadily to reach a peak at about the time of oestrus (Shemesh *et al.*, 1972; Hansel and Echternkamp, 1972; Dobson and Dean, 1974; Walters and Schallenberger, 1984), but fall rapidly during and following the preovulatory LH surge. Pulses of oestradiol secretion can be detected in vena cava blood samples, but have been difficult to detect in peripheral samples (Walters and Schallenberger, 1984; Walters *et al.*, 1984; Schallenberger *et al.*, 1984). The pulse frequency of oestradiol during the luteal phase and around luteolysis is similar to that of LH (Walters *et al.*, 1984). During the early follicular phase, oestradiol pulse frequency increases, but changes in pulse amplitude are difficult to detect (Schallenberger *et al.*, 1984). Just

before the onset of oestrus, oestradiol pulse amplitude increases, but there is little change in the pulse frequency (Walters and Schallenberger, 1984).

The changes in the circulating concentrations of other hormones such as testosterone, androstenedione, oxytocin, prostaglandin, prolactin and inhibin throughout the bovine oestrous cycle have also been studied (Wise, Caton, Thatcher, Rami Lehrer and Fields, 1982; Schams, 1983; Kindahl, Edqvist, Bane and Granstrom, 1976; Swanson, Hafs and Morrow, 1972; Hasegawa, Miyamoto, Igarashi, Yanaka, Sasaki and Iwamura, 1987). However, compared to LH, FSH, progesterone and oestradiol profiles, the secretion profiles of these hormones are often less defined and inconclusive, and will not be described here in detail.

1.3. Ovarian follicular growth and development

1.3.1. Folliculogenesis

Most of the current understanding of folliculogenesis is derived from studies carried out in mice or rats. Hence, many references in this section are not related to cattle, although the data from cattle or sheep are used wherever possible. Folliculogenesis is the process whereby primordial follicles develop through various stages of maturity, culminating in either atresia or ovulation. The formation of primordial follicles in the cow is completed by the time of birth, so the number of primordial follicles present in the ovaries for an individual cow (approximately 75,000 - 160,000; Erickson, 1966a) is fixed at birth. The origins of primordial follicles and the factors affecting their formation

have been reviewed by Rajakoski (1960) and Peters (1978) and will not be described here. A primordial follicle consists of an oocyte (arrested at the diplotene stage of the first meiotic division) surrounded by a single layer of follicular cells (granulosa cells) and a basal lamina (Rajakoski, 1960; Peters, 1978). These primordial follicles constitute a non-proliferating pool of follicles in the ovaries. The initiation of follicular growth from this non-proliferating pool is continuous throughout the reproductive life of the female, starting during foetal life as soon as primordial follicles are formed, and continuing until the pool of primordial follicles is exhausted. The follicles generated from the non-proliferating pool form a "cohort" of growing follicles in the ovaries (Peters *et al.*, 1975). Once the development of a primordial follicle begins, it is a continuous process until the follicle either ovulates or becomes atretic (Peters and Levy, 1966; Peters, 1969). However, the factors responsible for the initiation of the growth of primordial follicles are unknown (Cahill and Mauleon, 1980; Cahill, 1981; Cahill, 1984; Webb and Gauld, 1985).

The first stage of folliculogenesis (preantral development) is characterised by the significant enlargement of the primary oocyte with the formation of the zona pellucida, proliferation of the granulosa cells leading to an increase in granulosa cell layers, and the development of a thecal cell layer outside the basal lamina (Peters, 1979; Johnson and Everitt, 1988). Only the thecal cell layer is in direct contact with the vascular system. The granulosa cells and oocyte (which communicate with each other via gap junctions that penetrate the zona pellucida) have access to the vascular system only through the basal lamina (Johnson and Everitt, 1988). At this stage of development granulosa cells acquire FSH receptors (Hsueh, Adashi, Jones and Welsh Jr., 1984; Monniaux, Mariana and Gibson, 1984; Webb and Gauld, 1985) while thecal cells acquire LH

receptors (Hsueh *et al.*, 1984; Ireland, 1987). The majority of growing follicles will become atretic at this stage of development and only a small proportion of follicles survive and continue to develop further (Brand and de Jong, 1973).

Based on studies in primates, the following terms have been proposed by Goodman and Hodgen (1983) to describe the further development of preantral follicles: (1) **recruitment**- a group of follicles from the "cohort" of growing follicles acquire the ability to respond to pituitary gonadotrophins and thereafter require gonadotrophins for continued growth; (2) **selection**- a process whereby only a few of the recruited follicles are selected to continue to develop further, resulting eventually in the establishment of the dominant follicle(s) (the number is dependent on the species) and (3) **dominance**- the mechanism(s) whereby the dominant follicle(s) undergoes rapid development in an environment which suppresses the growth and development of other follicles.

Following follicle recruitment, both granulosa cells and thecal cells replicate rapidly and the thecal cells differentiate into two separate zones, the theca interna (which has an increased vascular supply) and the less vascular theca externa (Johnson and Everitt, 1988). The formation of a follicular antrum, filled with follicular fluid secreted by the granulosa cells (Baird and McNeilly, 1981; Cahill 1984; Johnson and Everitt, 1988), marks the next stage of folliculogenesis (development of antral follicles). The oocyte is now surrounded by a mass of granulosa cells (corona radiata) and this complex is attached to the antrum wall (granulosa cells) by a thin stalk of granulosa cells (cumulus oophorus) (Erickson, 1986). Such antral follicles are termed "Graafian follicles" and

they enter a stage of relatively rapid development. It has been estimated that in sheep it takes approximately six months for follicles to develop from the primordial follicle stage to large preovulatory follicles (Cahill and Mauleon, 1980). However, it takes only about 22 days for an early antral stage follicle in cattle to develop into the preovulatory follicle (Scaramuzzi, Turnbull and Nancarrow, 1980). Antral follicle size increases as a result of the proliferation of granulosa cells and enlargement of the antrum (Eshkol and Lunenfeld, 1972; Richards, 1980). Some follicles are selected to undergo further growth resulting in the establishment of the dominant follicle(s), whilst other follicles, recruited at about the same time, become atretic and regress. In the cow normally only a single follicle will be selected to become dominant (Ireland, 1987). The further development of the dominant follicle involves a series of morphological and functional changes including enlargement in size, production of steroids (especially oestradiol) by rapidly proliferating granulosa cells and thecal cells (Ireland and Roche, 1982a; Kruip and Dieleman, 1985; Webb and Bellows, 1980; Webb and England, 1982; Staigmiller, England, Webb, Short and Bellows, 1982) and the formation of functional LH receptors on the granulosa cells (Webb and England, 1982; Merz, Hauser and England, 1981; Webb and Bellows, 1980; Staigmiller *et al.*, 1982). If this stage of follicle development does not coincide with the onset of luteal regression, the dominant follicle itself then becomes atretic and regresses (Ireland and Roche, 1987). However, if luteolysis occurs, the dominant follicle advances to the final stages of development and maturation (preovulatory development).

Preovulatory development of the dominant follicle following luteolysis involves a further enlargement in follicle size, an increase in the number of LH receptors on the granulosa cells, increased production

of oestradiol (which will eventually trigger the onset of the preovulatory LH surge and oestrus), and increased vascularization of the follicle (Ireland, 1987; Johnson and Everitt, 1988). Following the preovulatory LH surge, the oocyte (which has until this stage remained in the diplotene stage of the first meiotic division), resumes meiosis to become the secondary oocyte (Thibault, Szollosi and Gerard, 1987). Ovulation then occurs with the release of the secondary oocyte surrounded by cumulus oophorus, and the follicular remnants hypertrophy to become the highly vascular CL (Johnson and Everitt, 1988).

1.3.2. The pattern of ovarian follicular growth and development during the oestrous cycle

As development of the ovulatory follicles is closely related to the endocrine events of the oestrous cycle (see above), follicular growth and development during the bovine oestrous cycle has been extensively studied in order to better understand the control of folliculogenesis (reviewed by Spicer and Echternkamp, 1986; Ireland and Roche, 1987; Fortune, Sirois and Quirk, 1988). Early studies, using the histological examination of ovaries obtained from cows slaughtered on a predetermined day of the oestrous cycle, demonstrated that the diameter of the largest dominant follicle present on a pair of ovaries was greater on the day immediately before oestrus than on any other day of the oestrous cycle (Hammond, 1927). Therefore it was concluded that follicles grow throughout the oestrous cycle, resulting in the development of a dominant preovulatory follicle. Subsequent observations, involving a greater number of cows, confirmed previous findings regarding the dominant ovulatory follicle, but showed that a second large dominant follicle could be detected around Day 10 - 12 of the oestrous cycle

(Rajakoski, 1960). These results led to the proposal that there were two waves of follicular growth and development, with the first wave starting on Day 1 of the cycle resulting in the development of a large dominant follicle at the mid-luteal phase, and a second wave beginning on about Day 13 of the oestrous cycle and resulting in a large dominant follicle which ovulated at the following oestrus. Several other reports using similar approachs either supported the proposal by Rajakoski (1960) (Hackett and Hafs, 1969; Swanson *et al.*, 1972; Ireland, Coulson and Murphree, 1979) or detected no such wave pattern of follicular development and concluded that follicular growth and development during the oestrous cycle was continuous and independent of the stage of the cycle in cattle (Choudary, Gier and Marion, 1968; Donaldson and Hansel, 1968; Lobel and Levy, 1968; Marion and Gier, 1971).

The disadvantages of the above studies, which obtain data at only a single time point from a large number of animals and rely entirely on statistical analysis to interpret the data, led to the development of other techniques in an attempt to follow the development of individual follicles throughout the oestrous cycle within an animal. Initially, the two largest follicles were marked with carbon or India ink at laparotomy on given days of the oestrous cycle in sheep (Smeaton and Robertson, 1971). Repeat observations of the same follicle at laparotomy demonstrated that the ovulatory follicle did not become the largest follicle until 2 - 3 days before oestrus (sheep: Smeaton and Robertson, 1971; cattle: Dufour, Whitemore, Ginther and Casida, 1972; Matton, Adelakoun, Couture and Dufour, 1981). Furthermore, the majority (nearly 90%) of the second largest follicles on Days 3, 8, 13 of the oestrous cycle failed to become the largest follicle over the next 5 days, but underwent regression concurrently with the development of the largest follicle (Matton *et al.*,

1981). These results supported the hypothesis that follicular growth and development during the bovine oestrous cycle occurs in a wave pattern (Rajakoski, 1960). However, while the "ink marking" technique allows the study of the developmental fate of individual follicles over a certain period of time, it is unable to follow shorter term (daily) changes in the growth of individual follicles.

While the majority of the early studies concluded that there were two distinct waves of follicular growth and development during the bovine oestrous cycle, some investigators found a 3-wave pattern. Smeaton and Robertson (1971) demonstrated that there were 3 waves of follicular growth during the oestrous cycle of the sheep. Ireland and Roche (1987) hypothesized that three, rather than two, waves of follicular growth occurred during the oestrous cycle in cattle, on the basis of experiments which identified the presence of one dominant "oestrogen-active" follicle during three different periods of the oestrous cycle (Ireland and Roche, 1983a,b) and detected an increase in oestradiol concentrations in blood samples obtained from peripheral and/or ovarian veins during approximately the same three periods (Ireland, Fogwell, Oxender, Ames and Cowley, 1985; Fogwell, Cowley, Wortman, Ames and Ireland, 1985). The differences in the conclusions generated from the above studies may well have been due to the techniques used, as none of them could reliably follow the growth pattern of individual follicles throughout the oestrous cycle on a daily basis. The development of real-time B-mode ultrasound, especially the introduction of the intra-rectal transducer for use in large animals (Palmer and Driancourt, 1980), has made the detailed study of ovarian follicular dynamics possible. Initial studies (Pierson and Ginther, 1984, 1986, 1987a,b,c,d, 1988; Quirk *et al.*, 1986) in cattle demonstrated that this technique could be used

successfully to monitor the antral ovarian follicles. Furthermore, the growth pattern of individual follicles larger than 5mm in diameter could be followed, since individual follicles could be identified from day to day by their positions relative to other structures such as follicles and the CL.

Following these initial studies, detailed daily monitoring of ovarian follicular dynamics throughout the oestrous cycle in heifers has been carried out by several groups (Sirois and Fortune, 1988; Savio, Keenan, Boland and Roche, 1988; Knopf, Kastelic, Schallenberger and Ginther, 1989). The results confirmed previous hypotheses that follicular growth and development during the bovine oestrous cycle occurs in waves, rather than being continuous and independent of the stage of the cycle. Each wave of follicular growth and development is characterised by the simultaneous growth of a group of antral follicles, one of which is selected to become dominant while other cohort follicles regress. The dominant follicle grows steadily and normally reaches a diameter greater than 10mm, and remains at maximum size for 2 - 3 days before regression. A new wave of follicular growth and development then starts. If luteolysis occurs during the growth phase or early plateau stage of a dominant follicle, this dominant follicle then undergoes rapid preovulatory development and eventually ovulates.

There is still conflict regarding the number of follicular waves during an oestrous cycle in heifers however. Sirois and Fortune (1988) and Savio *et al.* (1988) found that the majority of heifers had 3 waves per oestrous cycle, with the first, second and third waves starting on approximately Day 2, Day 10 and Day 16 of the oestrous cycle respectively, while Knopf *et al.* (1989) reported that 9 out of 10 of the heifers in their study displayed a 2-wave pattern, with the first and second waves

identified on Day 1 and Day 10 of the cycle respectively. It has been suggested that the length of oestrous cycle with a 2-wave pattern tended to be shorter than cycles with 3 waves of follicular growth, and that the onset of luteolysis occurred earlier in heifers with 2 waves per cycle (Knopf *et al.*, 1989; Sirois and Fortune, 1988; Ginther, Knopf and Kastelic, 1989a). On the basis of these results and reports that the wave pattern of follicular growth and development can also be detected during early pregnancy (Ginther, Knopf and Kastelic, 1989b; Driancourt, Thatcher, Terqui and Andrieu, 1991a) and during the postpartum period (Rajamahendran and Taylor, 1990; Boland, Murphy and Roche, 1990; Savio, Boland and Roche, 1990a), it has been proposed (Robinson and Shelton, 1991) that the wave pattern of follicular growth and its continuous turnover are an intrinsic characteristic of follicular growth and development in the cow. As the fate of a dominant follicle may be determined by whether luteolysis occurs or not (see above), the number of follicular waves during an oestrous cycle may be coincident with the length of the cycle and time of luteolysis, and hence a different number of waves (2, 3, 4 waves) can be detected within the same study (Sirois and Fortune, 1988). This proposal has been supported by reports that artificial extension of the length of the oestrous cycle by the administration of exogenous progesterone increased the number of follicular waves within the cycle (Sirois and Fortune, 1989; Fortune, Sirois, Turzillo and Lavoir, 1991). Moreover, continued periodic turnover of follicular waves could be detected in progesterone-treated heifers (Bergfelt, Kastelic and Ginther, 1991). However, the control of this regular turnover in the waves of follicular growth and development in the cow is still poorly understood (review: Ireland, 1987; Mariana, Monniaux, Driancourt and Mauleon, 1991), although it has been suggested

that FSH may be involved in the initiation of follicular waves (Turzillo and Fortune, 1990; Adams, Matteri, Kastelic, Ko and Ginther, 1992).

1.4. The control of ovarian follicular growth and development

As described in Section 1.3., ovarian follicular growth and development is an extremely complicated and highly coordinated physiological process. The development of a primordial follicle to the preovulatory stage may result in a 400- to 600-fold increase in diameter and a more than 100-fold increase in granulosa cell numbers (Ireland, 1987; Mariana *et al.*, 1991). Furthermore, in most mammals including cattle, only a small percentage of primordial follicles will ever enter the growing "cohort" of antral follicles, while most follicles are destined to become atretic at different early stages of development. Moreover, only a few follicles recruited from the growing "cohort" will be selected to become the dominant follicle(s) and then undergo the final stages of rapid preovulatory development culminating in ovulation (Ireland, 1987). It has been estimated that during the lifespan of a female animal only about 0.1% of the primordial follicles ever develop to ovulation (Byskov, 1978; Erickson, 1986; Ireland, 1987). However, despite extensive studies, the precise mechanism(s) involved in the regulation of folliculogenesis are still not well understood.

As summarized in Figure 1.1., the roles of the pituitary gonadotrophins (FSH and LH), ovarian steroids (oestrogen, progesterone and androgen) and, more recently, follicular proteins (such as inhibin) in the control of folliculogenesis has been relatively well established

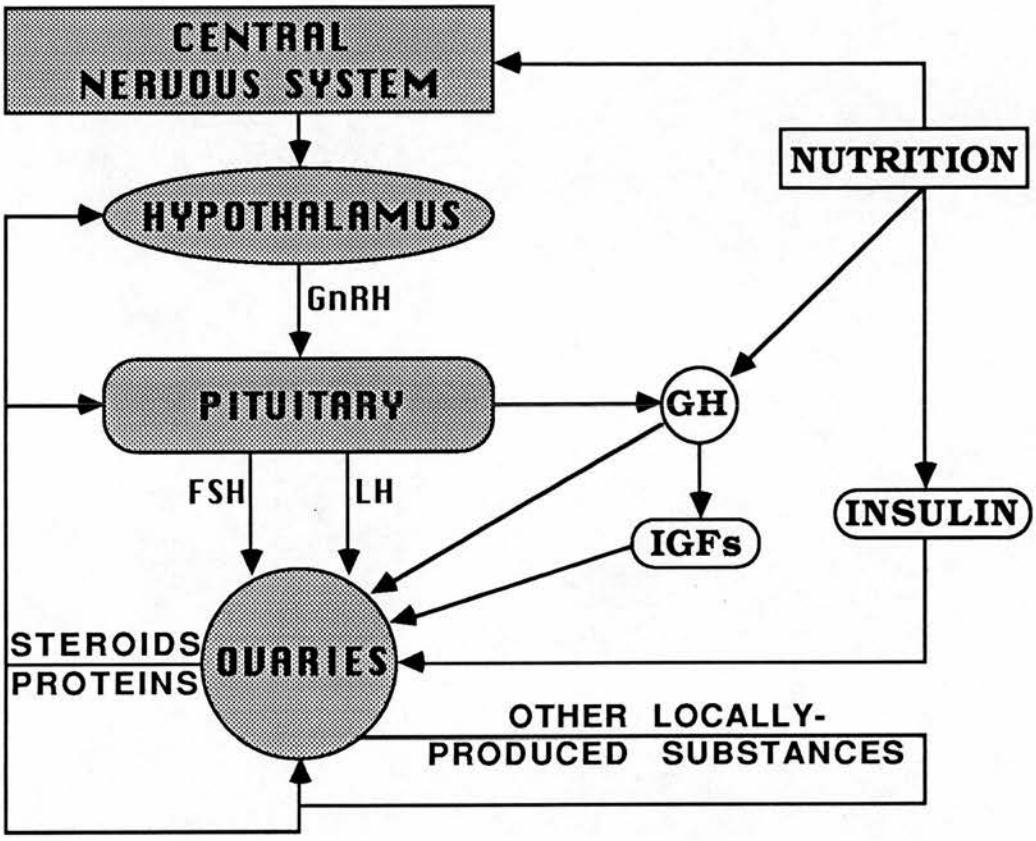


Figure 1.1. A diagrammatic representation of the possible mechanisms controlling ovarian follicular growth and development in cattle. The pathways on the left side (highlighted by a hatched background) are those involved in the classical control of the hypothalamic-pituitary-ovarian axis which have been well established to have a primary role in the control of ovarian function. Other pathways (on the right side) represent mechanisms which have been proposed more recently to play an important modulatory role in the control of folliculogenesis.

(Richards, 1979; Hsueh, Bicsak, Jia, Dahl, Fauser, Galway, Czekala, Pavlou, Papkoff, Keene and Boime, 1989). The regulation of gonadotrophin-releasing hormone (GnRH) secretion from the hypothalamus, and pituitary gonadotrophin secretion, by the central nervous system has been well reviewed recently by Ganong (1991) and will not be considered here. Gonadotrophins provide the primary drive for the recruitment of growing antral follicles and, at least in part, control the selection and further development of the dominant follicle (see above). However, in the last two decades, experimental evidence has been accumulating steadily to suggest that mechanism(s) other than gonadotrophins may also be involved in the regulation of folliculogenesis (Mariana *et al.*, 1991). Firstly, as has been pointed out above, the initiation of primordial follicle growth from the non-proliferating pool is not dependent on gonadotrophins, although gonadotrophins can increase the rate of this advancement (sheep: Cahill, 1981; Cahill, 1984). The stimulus for the initiation of primordial follicle growth is unknown (Cahill, 1981; Cahill, 1984; Webb and Gauld, 1985; Mariana *et al.*, 1991), although the rate of growth initiation appears to be partly dependent on the size of the primordial follicle pool (Krohn, 1967; Krarup, Pederson and Faber, 1969). Furthermore, it has been reported that factors in follicular fluid may inhibit growth initiation of the primordial follicles in mice (Peters, Byskov and Faber, 1973).

Secondly, the preantral stage of folliculogenesis and the early antral stage of development are not solely dependent on gonadotrophins. During the early preantral stage (before the formation of the thecal layer and the establishment of the follicular vascular system), follicles have extremely poor access to circulating gonadotrophins (Johnson and Everitt, 1988). Dufour, Cahill and Mauleon (1979) demonstrated that

preantral follicular development was unaffected up to ten weeks after hypophysectomy in sheep. Antral follicles up to 2mm in diameter were present in the ovaries of hypophysectomised ewes (Driancourt, Fry, Clarke and Cahill, 1979; Dufour *et al.*, 1979; McNatty, Heath, Hudson and Clarke, 1990). In sheep, blockage of gonadotrophin secretion by immunization against GnRH (McNeilly, Jonassen and Fraser, 1986) or by chronic GnRH agonist administration (McNeilly and Fraser, 1987; Picton, Tsonis and McNeilly, 1990) had no effect on preantral and early antral follicular development.

Thirdly, the process of dominance of the ovulatory follicle(s) remains poorly understood, and the role of gonadotrophins in this process is still uncertain, especially in the cow (Ireland, 1987). Dominance has been proposed to be the result of feedback interactions between ovarian steroids and inhibin from the ovulatory follicle(s) and gonadotrophins at both the hypothalamo-pituitary and ovarian level (reviewed by Webb and Morris, 1988). However, the fates of the dominant follicle(s) and the fate of other follicles recruited at similar time are completely different, despite the fact that they are exposed to the same gonadotrophin environment. This suggests the possible existence of intraovarian modulatory mechanisms, which could directly affect folliculogenesis or modulate the secretion and/or actions of gonadotrophins (Hsueh *et al.*, 1984; Tonetta and diZerega, 1989). Furthermore, attempts to alter ovulation rate by immunization against gonadal steroids and/or inhibin in the cow have suggested that changes in ovulation rate were not consistently correlated to changes in gonadotrophin secretion (Price, 1987). It has become increasingly accepted that the dominant follicle could inhibit the growth and development of subordinate follicles in cattle (Staigmiller and England,

1982; Ireland, 1987; Pierson and Ginther, 1987b; Kastelic, Ko and Ginther, 1990; Fortune *et al.*, 1991; Ko, Kastelic, Del Campo and Ginther, 1991), although the exact nature of this action remains poorly understood.

Finally, the influence of nutrition on the reproductive performance of female animals has long been recognized (see Downing and Scaramuzzi, 1991), but is still not well understood. Nutrition and energy balance may affect reproductive function by modulating gonadotrophin secretion (Davis, Brien, Findlay and Cumming, 1981) or via metabolic hormones such as growth hormone (GH), insulin and insulin-like growth factors (IGFs) (Spicer, Tucker and Adams, 1990; Downing and Scaramuzzi, 1991), all of which have been demonstrated to have a role in the regulation of ovarian function (Jia, Kalmijn and Hsueh, 1986; Poretsky and Kalin, 1987; Adashi, Resnick, D'Ercole, Svoboda and Van Wyk, 1985a).

The roles of the pituitary gonadotrophins, locally-produced ovarian substances (steroids and proteins), nutrition and metabolic hormones, and various growth factors in the process of folliculogenesis will be briefly summarised here.

1.4.1. Gonadotrophins

The gonadotrophins, FSH and LH, are synthesised and secreted by the anterior pituitary gland under the control of GnRH, a decapeptide hormone secreted from the hypothalamus (Pierce and Parsons, 1981; Ryan, Keutmann, Charlesworth, McCormick, Millius, Calvo and Vutyavanich, 1987; Everett, 1988). FSH and LH are glycoproteins (Reichert, 1962; Reichert and Jiang, 1965), composed of two subunits (α

and β) where the α subunit is common to both hormones while the β subunits are hormone-specific (Butt, 1975).

Gonadotrophins have long been recognized to be the primary regulators of ovarian function (Smith, 1926a,b; Casida, Meyer, McShan and Wisnicky, 1943). Both FSH and LH are able to regulate the production of ovarian steroids (see 1.4.2. for the description of steroidogenesis). Infusion of FSH increases peripheral oestradiol concentrations in ewes (Hudson, McNatty, Ball, Gibb, Heath, Lun, Kieboom and Henderson, 1985). Pulsatile secretion of oestradiol in the ovarian vein has been shown to be regulated by LH, as most LH pulses are followed by an oestradiol pulse approximately 5 to 30 minutes later (sheep: Baird, Swanston and Scaramuzzi, 1976; Scaramuzzi and Baird, 1977; Baird and McNeilly, 1981; cow: Walters and Schallenberger, 1984) and a single injection of exogenous LH induces an increase in oestradiol concentration in sheep (Baird and McNeilly, 1981; Campbell, McNeilly and Baird, 1989). Exogenous LH administration increases luteal progesterone production both *in vivo* and *in vitro* in cattle (Armstrong and Black, 1966; Donaldson, Hansel and Van Vleck, 1965; Hoffman, Schams, Bopp, Ender, Gimenez and Karg, 1974; Litch and Condon, 1988), although other investigators fail to confirm this effect of LH in sheep (Baird *et al.*, 1976). It has also been implied that FSH may be involved in the regulation of luteal progesterone production in cattle (Schallenberger, Rampp and Walters, 1983; Walters *et al.*, 1984) and FSH receptors have been identified in CL (cow: Manns, Niswender and Braden, 1984; women: Bramley, Stirling, Swanston, Menzies, McNeilly and Baird, 1987a).

Gonadotrophins also play an important role in the control of folliculogenesis (Ireland, 1987; Johnson and Everitt, 1988). As has been

pointed out above, although gonadotrophins may not initiate follicular growth from the non-proliferating pool of the primordial follicles, they can increase the rate of primordial follicle growth initiation (Cahill, 1981; Cahill, 1984). During preantral development, FSH activates oestradiol production (see 1.4.2. for the description of steroidogenesis) in those follicles which have acquired FSH receptors in their granulosa cells and LH receptors in their thecal cells, and increases FSH receptors in granulosa cells and LH receptors in thecal cells (Hsueh *et al.*, 1984; Baird and McNeilly, 1981; Ireland, 1987). FSH also stimulates the formation of gap junctions between granulosa cells and increases both the number and size of the gap junctions (rabbit: Albertini and Anderson, 1974; rat: Burghardt and Matheson, 1982). As the antral follicles grow larger, they become increasingly dependent on the support of both FSH and LH for further development (see Mariana *et al.*, 1991). In sheep immunized against GnRH or treated chronically with GnRH agonist (McNeilly *et al.*, 1986; McNeilly and Fraser, 1987; Picton *et al.*, 1990), no antral follicles larger than 2mm were found in the ovaries. However, follicular growth to preovulatory size could be restored by FSH infusion alone (Picton *et al.*, 1990), although immuno-neutralization of basal LH secretion during FSH infusion blocked this follicular development (McNeilly, Picton, Campbell and Baird, 1991). All large antral follicles regressed rapidly following the cessation of FSH infusion (Picton *et al.*, 1990) or following hypophysectomy (Dufour *et al.*, 1979; Driancourt *et al.*, 1979; McNatty *et al.*, 1990). The major roles of gonadotrophins at this stage are to stimulate the proliferation of granulosa cells and the differentiation of thecal cells (mouse: Eshkol and Lunenfeld, 1972; human: McNatty, Smith, Osathanondh and Ryan, 1979; review: Richards, 1980), to increase the production of ovarian steroids, especially oestradiol (rat: Dorrington, Moon and

Armstrong, 1975; Moon, Dorrington and Armstrong, 1975; Armstrong and Papkoff, 1976; Erickson and Hsueh, 1978; review: Dorrington and Armstrong, 1979), to stimulate the production of inhibin-related proteins (review; Findlay, Xiao and Shukovski, 1990) and to induce functional LH receptors on the granulosa cells (rat: Zeleznik, Midgley and Reichert, 1974; Richards, Ireland, Rao, Bernath, Midgley and Reichert, 1976; Adashi, Resnick, Svoboda and Van Wyk, 1985c; human: Erickson, Wang, Casper, Mattson and Hofeditz, 1982; pig: Channing, 1975; Amsterdam, May and Schomberg, 1988). In rats, FSH can also stimulate the production of its own receptor on granulosa cells (Richards *et al.*, 1976; Ireland and Richards, 1978a) and LH enhances the FSH-induced LH receptor formation (Ireland and Richards, 1978b) in granulosa cells and stimulates oestrogen production (Wang, Hsueh and Erickson, 1981). As the production of oestradiol and formation of functional LH receptors in the granulosa cells are two important characteristics acquired by the dominant follicle (Ireland, 1987), gonadotrophins may therefore play an important role in the selection of the dominant follicle. LH supports the final stages of follicular development and maturation following luteolysis and increases production of oestradiol by granulosa cells (Staigmiller *et al.*, 1982; Baird and McNeilly, 1981; Johnson and Everitt, 1988). The preovulatory LH surge stimulates the luteinization of the granulosa cells (Johnson and Everitt, 1988), switches oestradiol production in the granulosa cells to progesterone synthesis (Dieleman, Bevers, Poortman and Van Tol, 1983a; Dieleman, Kruip, Fontijne, de Jong and Van Der Weyden, 1983b), induces the resumption of oocyte meiosis (Thibault *et al.*, 1987) and triggers ovulation and the formation of the CL (Johnson and Everitt, 1988; Robinson and Shelton, 1991).

Recent research has suggested that gonadotrophins also regulate

the production of ovarian proteins such as inhibin and its related proteins which may be potential intraovarian regulators of folliculogenesis (Findlay *et al.*, 1990; also see 1.4.2. and 1.4.3). Administration of pregnant mare serum gonadotrophin (PMSG) or FSH *in vivo* increases peripheral inhibin concentration (rat: Lee, McMaster, Quigg, Findlay and Leversha, 1981; Carson and McMaster, 1988; sheep: McNeilly, Swanston, Crow, Tsonis and Baird, 1989) and increases ovarian inhibin gene expression in rats (Davis, Burger, Robertson, Farnworth, Carson and Krozowski, 1988). FSH stimulates the secretion of inhibin by rat granulosa cells *in vitro* with this effect being enhanced by low doses of LH (Bicsak, Tucker, Cappel, Vaughan, Rivier, Vale and Hseuh, 1986; Zhiwen, Carson and Burger, 1988). Higher doses of LH inhibit FSH-induced inhibin production both *in vitro* (Zhiwen *et al.*, 1988) and *in vivo* (Lee, 1983). Gonadotrophins may also be involved in the regulation of ovarian production of inhibin-related proteins such as follistatin and activin in the rat (Shimasaki, Koga, Buscaglia, Simmons, Bicsak and Ling, 1989; Findlay *et al.*, 1990).

Although it is still not well understood, gonadotrophins have also been suggested to play a role in the regulation of the local production of many other potential intraovarian regulators of folliculogenesis, including growth factors (Ireland, 1987; Mariana *et al.*, 1991; also see 1.4.4 and 1.4.6). The roles of various growth factors in folliculogenesis will be discussed in Section 1.4.6.

1.4.2. Ovarian steroids and proteins

The major ovarian steroids produced in the cow are progesterone, androstenedione, testosterone and oestradiol (Short, 1962). The process of

ovarian steroidogenesis and its control has been well reviewed by Gore-Langton and Armstrong (1988). Briefly, it involves both granulosa and thecal cells and both FSH and LH ("two-cell-two-gonadotrophin concept) (Erickson, 1986) as originally proposed by Falck (1959). When LH and FSH/LH bind to their receptors in the thecal and granulosa cells respectively, they activate the adenylate cyclase-cyclic adenosine monophosphate (cAMP) response system (see Ireland, 1987), resulting in the activation of enzymes responsible for steroidogenesis. Both granulosa cells and thecal cells can produce progesterone from cholesterol by the action of a cytochrome P_{450}-linked enzyme complex, cholesterol side-chain cleavage enzyme (P_{450scc}) (see Ireland, 1987). Thecal cells possess the 17α-hydroxylase and 17-20 desmolase enzyme system necessary for the production of androgens (Bjersing and Carstensen, 1967), but granulosa cells do not. Hence, progesterone is converted into androgens in the thecal cells. Granulosa cells possess aromatase ($P_{450arom}$) which converts androgens into oestradiol. Thus, the androgens produced by the theca are transferred across the basal lamina into granulosa cells and then converted into oestradiol (Erickson, Magoffin, Dyer and Hofeditz, 1985).

Apart from being involved in the control of the gonadotrophin secretion (see 1.4.3.), ovarian steroids exert direct actions on folliculogenesis. Oestradiol stimulates the proliferation of granulosa cells (rat: Goldenberg, Vaitukaitis and Ross, 1972), and in cooperation with FSH increases the number of FSH receptors in granulosa cells (rat: Richards *et al.*, 1976; Ireland and Richards, 1978a; review: Hsueh *et al.*, 1984) and the number of LH receptors in thecal cells (sheep: Baird and McNeilly, 1981; Hsueh *et al.*, 1984), enhances the induction of aromatase activity (rat: Zhuang, Adashi and Hsueh, 1982; Daniel and Armstrong, 1983) and induces

the formation of LH receptors in the granulosa cells (pig: Channing and Kammerman, 1974; sheep: Baird and McNeilly, 1981; review: Ireland, 1987). Oestradiol inhibits progesterone synthesis (cattle: Williams and Marsh, 1978; Fortune and Hansel, 1979; Henderson, McNatty, Smith, Gibb, O'Keeffe, Lun, Heath and Prisk, 1987) and reduces androgen production by the thecal cells (rat: Leung, Goff, Kennedy and Armstrong, 1978; Magoffin and Erickson, 1982). These inhibitory actions of oestradiol may represent a mechanism whereby selected follicles protect themselves from atresia, as an increase in androgen to oestrogen ratio has been associated with follicular atresia (cattle: Ireland and Roche, 1983a) and exogenous androgen administration induces atresia (rat: Payne and Runser, 1958; cattle: Maracek, Tokos and Halagan, 1977). Generally the intraovarian actions of progesterone suppress folliculogenesis, although some evidence (Hunter, Southee, McLeod and Haresign, 1986) has suggested that progesterone may have a stimulatory effect on the development of GnRH-induced preovulatory follicles in anoestrous ewes. Progesterone inhibits the induction of aromatase activity (Fortune and Vincent, 1983; Chan and Tan, 1986) and LH receptors (Schreiber, Nakamura, Truscello and Erickson, 1985) by FSH. Progesterone also decreases 17α-hydroxylase/17-20 desmolase activity (Johnson, 1987), which is responsible for the conversion of progesterone into androgen. Androgens are involved in both follicular development and atresia (Erickson, 1986). They can stimulate both the activation of aromatase enzyme activity (Daniel and Armstrong, 1980; Hillier and DeZwart, 1981) and progesterone synthesis in the granulosa cells (Haney and Schomberg, 1978; Nimrod and Pearlsman, 1984).

Ovarian follicular fluid contains numerous compounds which are either transudates from the blood (Edwards, 1974) or produced locally

(Ireland, 1987). Some of these locally-produced proteins have been suggested to be potential intragonadal modulators of folliculogenesis (Ireland, 1987; Mariana *et al.*, 1991). Treatment with follicular fluid in cattle and sheep can suppress ovarian follicular development (Miller, Crister, Rowe and Ginther, 1979; Johnson and Smith, 1985; Wallace and McNeilly, 1985, 1986; Henderson, Prisk, Hudson, Ball, McNatty, Lun, Heath, Kieboom and McDiarmid, 1986; Hunter, Hindle, McLeod and McNeilly, 1988). Among follicular proteins, inhibin and its related proteins (follistatin, activin and α-N peptide) have been studied most. The amino acid sequences of inhibin and activin have been determined (Ling, Ying, Ueno, Esch, Denoroy and Guillemin, 1985; Esch, Shimasaki, Mercado, Cooksey, Ling, Ying, Ueno and Guillemin, 1987; Vale, Rivier, Vaughan, McClintock, Corrigan, Woo, Karr and Spiess, 1986). Initially, they were considered to be involved in the regulation of gonadotrophin secretion (see 1.4.3.). However their involvement in the intraovarian regulation of folliculogenesis has received increasing attention recently (reviewed by Findlay *et al.*, 1990).

Porcine inhibin suppresses FSH-induced oestrogen production by rat granulosa cells *in vitro* (Ying, Becher, Ling, Ueno and Guillemin, 1986a), though this effect was not confirmed by other investigators using bovine inhibin preparations (Hutchinson, Findlay, de Vos and Robertson, 1987; Sugino, Nakamura, Hasegawa, Miyamoto, Abe, Igarashi, Eto, Shibai and Titani, 1988a). In the study of Sugino *et al.*, (1988a), production of progesterone by granulosa cells was slightly suppressed. Inhibin enhances LH-induced androgen production by rat thecal cells (Hsueh, Dahl, Vaughan, Tucker, Rivier, Bardin and Vale, 1987) and inhibits the maturation of rat oocytes *in vitro* (O, Robertson and de Kretser, 1989). Messenger ribonucleic acid (mRNA) for follistatin (also named FSH-

suppressing protein, FSP) has recently been identified in bovine ovarian tissues (Shukovski, Zhang, Michel and Findlay, 1992). FSP stimulates FSH-induced progesterone production by rat granulosa cells, while inhibiting FSH-induced aromatase activity and inhibin production (Xiao, Findlay and Robertson, 1990). It can either stimulate (O *et al.*, 1989) or inhibit (Buscaglia, Fuller, Mazzola, Bacigalupi, Shimasaki, Ui, Simmons, Castillo, Schroeder and Ling, 1989) oocyte maturation *in vitro*. Activin increases both basal (Hutchinson *et al.*, 1987; Xiao *et al.*, 1990) and FSH-stimulated (Xiao *et al.*, 1990) inhibin production and induces FSH receptors (Hasegawa, Miyamoto, Abe, Nakamura, Sugino, Eto, Shibai and Igarashi, 1988) in undifferentiated rat granulosa cells. In addition, binding sites for activin have been detected in granulosa cells and are induced in response to FSH stimulation (Sugino, Nakamura, Hasegawa, Miyamoto, Igarashi, Eto, Shibai and Titani, 1988b). Activin has also been shown to stimulate FSH-induced aromatase activity and progesterone production (Xiao *et al.*, 1990) and the induction of LH receptors (Sugino *et al.*, 1988a). On the other hand, activin inhibits progesterone and oxytocin production by differentiated bovine granulosa cells (Shukovski and Findlay, 1989). Finally, immunization against a recombinant form of bovine α-N peptide reduced the number of lambs born per ewe mated, even with an increase in the number of CL (Findlay, Tsonis, Doughton, Brown, Bertram, Braid, Hudson, Tierney, Goss and Forage, 1989a; Findlay *et al.*, 1990). As the number of eggs recovered from the oviducts of the immunized ewes was significantly reduced, and the CL of the immunized ewes had the appearance of luteinized unruptured follicles, it was concluded that the decrease in lambs born was probably the result of either an ovulation failure (Findlay, Robertson, Clarke, Klein, Doughton, Xiao, Russell and Shukovski, 1992) or due to the luteinization of ovulatory follicles

(Channing, 1979).

In summary, both ovarian steroids and follicular proteins are involved in the intraovarian control of folliculogenesis. Oestradiol and activin seem to promote follicular growth and differentiation, and prevent the premature luteinization of follicles, while FSP, androgen and possibly progesterone act in the opposite direction and α-N peptide may be involved in follicular luteinization and ovulation (Ireland, 1987; Findlay *et al.*, 1990). However, much more research is required before the physiological roles of these factors can be definitely understood.

1.4.3. Interactions between gonadotrophins and ovarian steroids and proteins

The interaction between the pituitary gland and the gonads has long been recognized (Smith, 1926a,b; McCullagh, 1932). Pituitary gonadotrophins, through binding to their specific receptors in ovaries, regulate the production of ovarian steroids and proteins (see 1.4.1). During the last two decades it has become evident that these ovarian steroids and proteins, apart from being involved in the intraovarian control of folliculogenesis (see 1.4.2), play an important role in the regulation of gonadotrophin synthesis and secretion through feedback endocrine mechanisms (positive and negative) (McNeilly, 1988; Baird, Campbell, Mann and McNeilly, 1991; Taya, Kaneko, Watanabe and Sasamoto, 1991). This part of the review will summarise briefly the extensive studies in this area, which have employed a wide variety of approaches including temporal hormone changes during the oestrous cycle, hormonal consequences following ovariectomy, the administration

of (or immunization against) exogenous ovarian steroid and protein preparations, and cell culture experiments.

The possible involvement of ovarian substances in the control of gonadotrophin secretion is indicated by observations that there is a temporal relationship between changes in circulating ovarian steroid and pituitary gonadotrophin concentrations during the oestrous cycle (sheep: Hauger, Karsch and Foster, 1977; see 1.2.2) and that peripheral concentrations of FSH and LH increase following castration (cattle: Hobson and Hansel, 1972; Convey, Beck, Neitzel, Bostwick and Hafs, 1977). Subsequently the roles of ovarian steroids and proteins in the feedback control of gonadotrophin secretion have been investigated extensively.

Administration of exogenous oestradiol has been demonstrated to induce a surge-like increase in LH and FSH concentrations (cows: Hobson and Hansel, 1972; Short, Randel, Staigmiller and Bellows, 1979; prepubertal heifers: Swanson and McCarthy, 1978; cyclic heifers: Peters, 1984; ovariectomised heifers: Butler *et al.*, 1983), resulting from an increase in pituitary sensitivity to GnRH and/or an increase in GnRH secretion from the hypothalamus (rat: Drouin, Lagace and Labrie, 1976; cattle: Kesner, Convey and Anderson, 1981). Oestradiol has also been reported to increase the secretion of FSH from cultured rat pituitaries (Farnworth, Robertson, de Kretser, Findlay and Burger, 1989), to increase mean LH concentration (cattle: Critser *et al.*, 1983) and LH pulse amplitude (cattle: Kinder, Garcia-Winder, Imakawa, Day, Zalesky, D'Occhio, Kittok and Schanbacher, 1983; Stumpf, Day, Wolfe, Wolfe, Clutter, Kittok and Kinder, 1988) with a decrease in pulse frequency (Kinder *et al.*, 1983). In contrast, other investigators show that oestradiol suppresses basal and mean FSH and LH (sheep: Clarke, Funder and Findlay, 1982; Rawlings,

Jeffcoate and Rieger, 1984; cattle; Butler *et al.*, 1983; Price and Webb, 1988) with an increase in LH pulse frequency (sheep: Karsch, Foster, Bittman and Goodman, 1983) and a decrease in LH pulse amplitude (sheep: Goodman and Karsch, 1980; cattle: Price and Webb, 1988). The ability of oestradiol to reduce the secretion of FSH by ovine pituitary cells in culture has also been reported (Tsonis, McNeilly and Baird, 1986). Moreover, both active and passive immunization against either oestradiol-17β or oestrone lead to elevated plasma LH concentrations (sheep: Pant and Rawlings, 1973; Rawlings, Kennedy and Henricks, 1978; Scaramuzzi, Martensz and Van Look, 1980; Pathiraja, Carr, Fordyce, Forster, Land and Morris, 1984; review: Webb, Land, Pathiraja and Morris, 1984) as a result of an increase in LH pulse frequency (sheep: Martensz, Scaramuzzi and Van Look, 1979; Thomas, Martin and Pearce, 1982; Webb *et al.*, 1984). Peripheral FSH concentrations in some of the immunized animals from these studies were also found to be elevated (Pant and Rawlings, 1973; Martensz *et al.*, 1979; Scaramuzzi *et al.*, 1980; Pathiraja *et al.*, 1984; Webb *et al.*, 1984).

The role of progesterone in the feedback control of gonadotrophin secretion antagonised that of oestradiol, as exogenous progesterone was capable of blocking the oestradiol-induced gonadotrophin surge in cattle (Hobson and Hansel, 1972; Barnes, Kazmer, Bierley, Richardson and Dickey, 1980; Roche and Ireland, 1981a,b; Schoenemann, Humphrey, Crowder, Nett and Reeves, 1985) and high concentrations of endogenous progesterone suppressed the gonadotrophin surge (cattle: Hausler and Malven, 1976; Short *et al.*, 1979). While not affecting the oestradiol-induced increase in pituitary GnRH receptor numbers (Schoenemann *et al.*, 1985), progesterone suppressed both the increase in pituitary cell responsiveness to GnRH (rat: Hsueh, Erickson and Yen, 1979; cattle:

Padmanabhan and Convey, 1981) and the increase in pituitary gonadotrophin concentrations (Schoenemann *et al.*, 1985) following oestradiol administration. However, no effect of progesterone alone could be demonstrated in these studies. In ovariectomised cattle, progesterone reduced tonic LH secretion (Price and Webb, 1988), LH pulse frequency (Ireland and Roche, 1982b; Price and Webb, 1988) and mean FSH concentrations (Price and Webb, 1988), although other investigators (Barnes *et al.*, 1980; Schoenemann *et al.*, 1985) found no effect of progesterone treatment on LH or FSH. There appeared to be a synergy between progesterone and oestradiol which will be discussed later. Ireland and Roche (1982b) also reported that removal of progesterone from the peripheral circulation, by the withdrawal of progesterone-releasing intravaginal device or the induction of luteolysis, resulted in an increase in both LH and FSH pulse amplitude and LH pulse frequency. Furthermore, immunization of ewes against progesterone resulted in an increase in LH pulse frequency (Thomas, Oldham, Hoskinson and Scaramuzzi, 1984), although the effect of immunization against progesterone on FSH has not been reported.

There are few reports concerning the effects of androgens on the feedback control of gonadotrophin secretion. The majority of reports conclude that there is little role of androgens, as all the parameters of LH and FSH secretion are not affected by androgens (McCarthy and Swanson, 1976; D'Occhio, Kinder and Schanbacher, 1982; Butler *et al.*, 1983). However, some authors have reported that androgens reduced mean plasma FSH (Kennedy and Rawlings, 1984) and testosterone decreased the sensitivity of cultured pituitary cells to GnRH (Drouin *et al.*, 1976). Testosterone had also been reported to reduce the LH response to GnRH, although it has no effect on the FSH response and basal gonadotrophin

secretion (Thompson, Voelkel, Reville-Moroz, Godke and Derrick, 1984). Furthermore, immunization against androgens has been shown to alter the secretion of gonadotrophins. Immunization against androstenedione or testosterone increased the LH pulse frequency and therefore plasma LH concentrations (sheep: Martensz, Baird, Scaramuzzi and Van Look, 1976; Martensz and Scaramuzzi, 1979; Scaramuzzi, 1979; review: Webb *et al.*, 1984; cattle: Price *et al.*, 1987a) though other authors failed to alter LH in cattle immunized against androstenedione (D'Occhio, Gifford, Hoskinson, Weatherly and Setchell, 1989). However, immunization against androgens either had no effect or reduced FSH concentrations (Martensz and Scaramuzzi, 1979; Scaramuzzi, 1979; Webb *et al.*, 1984; Price *et al.*, 1987; D'Occhio *et al.*, 1989).

Although the role of androgens is less clear, both oestradiol and progesterone play an important role in the feedback control of gonadotrophin secretion. Furthermore, these two ovarian steroids seem to act in concert as neither steroid alone can totally account for the cyclic changes in LH concentrations. When oestradiol and progesterone, at their respective physiological concentrations, were combined they suppressed LH concentrations in ovariectomized sheep to those observed in normal physiological states (Goodman, Legan, Ryan, Foster and Karsch, 1980; Karsch, Legan, Ryan and Foster, 1980; Martin, Scaramuzzi and Henstridge, 1983; Rawlings *et al.*, 1984). Similar results have been reported for cows (Beck, Smith, Seguin and Convey, 1976; Price and Webb, 1988). Price and Webb (1988) also showed that the combination of oestradiol and progesterone in physiological concentrations were sufficient to maintain normal levels of FSH in short-term ovariectomized heifers. However, other investigators have reported that treatment of ovariectomized animals with ovarian steroids fails to suppress FSH concentrations to

levels observed in intact controls (cattle: Roche and Ireland, 1981b; rat: Campbell and Schwartz, 1977; sheep: Goodman, Pickover and Karsch, 1981) and have suggested that other ovarian substances may also participate in the feedback control of gonadotrophin secretion.

One of the best studied non-steroid ovarian factors which may regulate gonadotrophin secretion is inhibin and its related proteins. Indeed, inhibin has been defined as "a glycoprotein which inhibits pituitary gonadotrophin production and/or secretion, preferentially that of FSH" (Burger and Igarashi, 1988). Analysis of peripheral FSH and inhibin concentrations in rats suggested that they were inversely related (Hamada, Watanabe, Kokuho, Taya, Sasamoto, Hasegawa, Miyamoto and Igarashi, 1989), although this observation was not confirmed in sheep (McNeilly and Baird, 1989). Administration of steroid-free follicular fluid preparations suppressed peripheral FSH concentrations in both ovariectomized (Cummins, O'Shea, Bindon, Lee and Findlay, 1983; Findlay, Gill and Doughton, 1985; Martin, Taylor and McNeilly, 1987; Knight and Castillo, 1988) and intact sheep (Miller, Critser and Ginther, 1982; McNeilly, 1984; Martin *et al.*, 1987; Wallace, Martin and McNeilly, 1988; Baird, Campbell and McNeilly, 1990). While replacement of ovarian steroids alone failed to suppress FSH concentrations in ovariectomized ewes to within the normal range (Goodman *et al.*, 1981), a combination of steroids and follicular fluid was capable of reducing FSH concentrations to physiological levels (Martin, Price, Thiery and Webb, 1988). Pituitary FSH production and FSH β-subunit gene expression were also reduced by follicular fluid treatment in ovariectomized sheep (Martin, Wallace, Taylor, Fraser, Tsonis and McNeilly, 1986; Mercer, Clements, Funder and Clarke, 1987) and heifers (Ireland, Curato and Wilson, 1983; Kiracofe, Ramirez-Godinez, McGowan and Bolt, 1983; Beard, Castillo, McLeod,

Glencross and Knight, 1990; Beard, Savva, Glencross, McLeod and Knight, 1989). Follicular fluid treatment prevented the transient rise in FSH concentrations following unilateral ovariectomy in heifers (Johnson, Smith and Elmore, 1985). Similar suppression of FSH secretion was observed when highly purified inhibin was used (Beard *et al.*, 1990), an effect attributed to a reduction in pituitary FSH-β gene expression (Beard *et al.*, 1989). The role of inhibin in the control of FSH secretion in intact cattle is less certain (Knight, 1991). While Quirk and Fortune (1986) reported that follicular fluid treatment of intact cattle suppressed peripheral FSH concentrations, Johnson and Smith (1985) were unable to do so. Moreover, a recent study has questioned the importance of inhibin in the control of FSH release in intact heifers (Law, Baxter, Logue, O'Shea and Webb, 1992), as the previously reported effects of steroid-free follicular fluid could still be demonstrated even when inhibin was removed from the follicular fluid using affinity chromatography. Similar results have recently been demonstrated in inhibin-immunized heifers (Wood, Glencross, Bleach, Lovell, Beard and Knight, 1991) and in sheep (Campbell, Picton, Mann, McNeilly and Baird, 1991). Immunisation against inhibin increased basal FSH concentrations in sheep (Findlay, Doughton, Robertson and Forage, 1989b; Wrathall, McLeod, Glencross, Beard and Knight, 1990), whilst others observed either a transient increase (O'Shea, Bindon, Hillard, Piper, Findlay and Miyamoto, 1989) or no changes at all (O'Shea, Anderson, Bindon, Hillard and Sinosich, 1990). Moreover, the changes in FSH concentrations following immunization against partially purified follicular fluid was extremely variable between animals (Al-Obaidi, Bindon, Findlay, Hillard and O'Shea, 1987). Peripheral LH concentrations in ovariectomized ewes have also been reduced by bovine follicular fluid administration (Findlay *et al.*, 1985; Clarke, Findlay,

Cummins and Ewens, 1986).

Other inhibin-related proteins such as FSP and activin may also participate in the regulation of FSH secretion. FSP suppresses pituitary FSH secretion (Robertson, Klein, de Vos, McLachlan, Wettenhall, Hearn, Burger and de Krester, 1987; Ying, Becker, Swanson, Tan, Ling, Esch, Ueno, Shimasaki and Guillemin, 1987; Wang, Farnworth, Robertson and Findlay, 1989), while activin stimulated pituitary FSH release (Ling, Ying, Ueno, Shimasaki, Esch, Hotta and Guillemin, 1986a,b; Schwall, Nikolics, Szonyi, Gorman and Mason, 1988) *in vitro*. Although it has been reported that treatment of immature female rats with recombinant human activin-A increased serum FSH concentrations *in vivo* (Schwall, Schmelzer, Matsuyama and Mason, 1989), the physiological significance of these inhibin-related proteins remains inconclusive as there is little information available regarding their concentrations in the circulation due to the lack of reliable assay systems. Peripheral concentrations of FSP during the oestrous cycle in ewes have been measured and there was no apparent relationship between FSP and either FSH or LH (Findlay *et al.*, 1992). One *in vivo* study (Woodruff, Lyon, Hansen, Price and Mather, 1990) has demonstrated that administration of activin into the ovarian bursa of rats reduced the proportion of follicles greater than 350μm and decreased thymidine incorporation into granulosa cells.

1.4.4. Other locally-produced ovarian substances

As the concept of intraovarian regulation of ovarian function has gained increasing acceptance, considerable effort has been put into identifying locally-produced ovarian substances which may have a role in the control of folliculogenesis (Ireland, 1987; Findlay *et al.*, 1990;

Tonetta and diZerega, 1989). Apart from ovarian steroids, inhibin-related proteins (see above) and various growth factors (see 1.4.6), many putative local regulators of ovarian function have been identified in follicular fluid. These factors include: an inhibitor of compensatory ovarian hypertrophy (Sato and Ishibashi, 1978; Sato, Miyamoto, Ishibashi and Iritani, 1978), follicular regulatory protein (FRP) (diZerega, Marrs, Campeau and Kling, 1983; Tonetta, Yanagihara, DeVinna and diZerega, 1988), FSH-binding inhibitor (Sanzo and Reichert, 1982; Sluss and Reichert, 1984), LH-binding inhibitor (Sanzo and Reichert, 1982; Kumari, Kumar, Duraiswami and Roy, 1984), luteinization inhibitor (Hammond, 1981), gonadotrophin surge inhibiting factor (Littman and Hodgen, 1984), gonadocrinin (Ying, Ling, Bohlen and Guillemin, 1981) or GnRH-like ovarian hormone (Aten, Ireland, Weems and Behrman, 1987), oocyte maturation inhibitor (Hillensjo, Pomerantz, Schwartz-Kripner, Anderson and Channing, 1980; Miller and Behrman, 1986) and gonadotrophin binding factor (Yarney, Sairam, Bhargavi, Downey and Srikandakumar, 1990). Most of these identified factors, as their names suggested, are possibly involved in the regulation of ovarian function by modulating the effects of gonadotrophins on ovaries. FRP has been shown to antagonise the actions of FSH on aromatase activity, progesterone secretion (human: Battin and diZerega, 1985, rat: Schreiber and diZerega, 1986) and LH receptor induction (Montz, Ujita, Campeau and diZerega, 1984) in granulosa cells *in vitro*, disrupt folliculogenesis and ovarian cyclicity in monkeys (diZerega and Wilks, 1984) and suppress follicular growth in cyclic guinea pigs (Fujimori, Nakamura, Tonetta and diZerega, 1987) *in vivo*. However, the cellular origin, control of production and structural identity of these factors has not yet been established. Furthermore, evidence for the involvement of these factors in the

control of ovarian function is largely preliminary, and their physiological significance and mode of action remain to be determined.

1.4.5. Nutrition, growth hormone and insulin

The influence of nutrition on the reproductive performance of the female has long been recognized (Youatt, 1837; see Robinson, 1990). Early observations were mainly restricted to the effect of nutrition on lambing rate. It was first noted (Youatt, 1837) that ewes maintained in good body condition or on good quality pasture had a higher twinning rate. Heape (1899) observed that heavier ewes produced more lambs than did lighter ewes of the same breed. In the early part of this century, numerous reports showed that "flushing", ie. short periods of improved nutrition before and during mating, could increase the proportion of twin lambing, especially for ewes with a poor or moderate body condition (Marshall, 1904, 1905, 1908; Marshall and Potts, 1921; Nichols, 1924, 1926; Underwood and Shier, 1941). Flushing has also been shown to be associated with an increase in ovulation rate (Coop, 1966; Morley, White, Kenney and Davis, 1978). Following these early observations, the interaction between nutrition and ovulation rate was studied extensively (Killeen, 1967; Allison, 1968; Edey, 1968; Gunn, Doney and Russel, 1969; Cumming, 1977; Morley *et al.*, 1978; Kelly, Thompson, Hawker, Crosbie and McEwan, 1983), demonstrating a positive relationship between the liveweight of ewes and ovulation rate.

Nutrition also affects the ovarian follicular population. Flushing of ewes on a diet of hay increased the total number of follicles (Bellows, Pope, Meyer, Chapman and Casida, 1963). The feeding of ewes with a sub-maintenance ration was reported to alter the distribution of follicle sizes

(Allen and Lamming, 1961) and flushing of these ewes increased the number of follicles <2mm and 2-3mm in diameter. Haresign (1981) reported that ewes maintained at a higher plane of nutrition had greater numbers of follicles 2-3mm in diameter at oestrus, but similar numbers of follicles <2mm and >3mm in diameter, when compared to ewes fed a maintenance ration. He suggested that "flushing" did not affect the number of small follicles, but increased ovulation rate by preventing atresia of 2-3mm follicles, since from 18 hours after oestrus onwards the number of follicles >3mm in diameter was greater in ewes on a higher plane of nutrition. This proposal has been supported by subsequent studies. Flushing of ewes with lupin grain did not alter the number of follicles >2mm in diameter at oestrus, but reduced the number of atretic follicles >2mm in diameter 48 hours after oestrus (Nottle, Setchell and Seamark, 1986). Ewes maintained in high body condition score had more follicles of >4mm, but a similar number of small follicles, compared to ewes with a low body condition score (Rhind and McNeilly, 1986). Furthermore, heavier ewes had more follicles >2mm in diameter on the surface of ovary and fewer of these follicles were atretic (Xu, McDonald and McCutcheon, 1989).

The influence of nutrition on reproduction has also been demonstrated by its effect on the onset of puberty (Kinder *et al.*, 1987; Moran *et al.*, 1989). As has been pointed out in Section 1.1, female cattle reach puberty at a wide range of ages. However, heifers attain puberty at a certain bodyweight or bodysize rather than at a given age (Sorenson, Hansel, Hough, Armstrong, McEntee and Bratton, 1959; Robinson, 1977). Heifers with lower average daily weight gains were of similar weight to, but much older than, heifers with higher average daily weight gains at the time of puberty, when reared on different planes of nutrition.

Likewise, Short and Bellows (1971) found that heifers reared on higher planes of nutrition were heavier, but younger, than nutritionally restricted animals at puberty. Similar results have been demonstrated for sheep (Foster, Yellon and Olster, 1985; Foster, Karsch, Olster, Ryan and Yellon, 1986). The role of nutrition *per se* has also been demonstrated by Gonzalez-Padilla, Wiltbank and Niswender (1975) who showed that 14 month old heifers of adequate liveweight could be maintained in a prepubertal state and suddenly induced to ovulate in a synchronized manner in response to an increase in nutritional intake. Similarly, cycling heifers became anoestrus when their nutrition was severely restricted (Imakawa, Day, Zalesky, Clutter, Kittok and Kinder, 1987).

While the effect of nutrition on the reproductive performance of female animals is well demonstrated, the underlying mechanism(s) remains unclear (Smith, 1985; 1988), although changes in energy and protein balance may be important (Lindsay, 1976; Smith, 1985; Nottle, Hynd, Seamark and Setchell, 1988). Lupin grain, a supplement high in both energy and protein, has been widely used since the early 1970s in the practice of "flushing" (Knight, Oldham, and Lindsay, 1975; Kleeman and Cutten, 1978; Nottle *et al.*, 1988; Teleni, Rowe, Croker, Murray and King, 1989), which increases ovulation rate within as little as 4-6 days after the start of feeding (Lindsay, 1976; Oldham and Lindsay, 1984; Nottle *et al.*, 1986; Stewart and Oldham, 1986). This short term model has been widely used in experiments to investigate possible mechanism(s) by which nutrition may influence ovarian function.

As gonadotrophins are the primary regulators of ovarian function (see above), the modulating effect of nutrition was first thought to act via increased gonadotrophin concentrations (Hammond, 1952). Giving grain

to ewes on a diet of hay increased pituitary FSH and LH content (Bellows *et al.*, 1963; Howland, Kirkpatrick, Pope and Casida, 1966), and ewes flushed with lupin grain had a higher mean plasma concentration of FSH (Brien, Baxter, Findlay and Cumming, 1976; Davis *et al.*,, 1981). This effect suggested a response at the level of the hypothalamo-pituitary axis (Nottle, Setchell and Seamark, 1987), since similar results were obtained in oestradiol-implanted ovariectomized ewes. Feeding ewes a high protein diet also led to increased ovulation rate and elevated FSH concentrations (Smith, 1988). However, other workers failed to observe an effect of nutrition on gonadotrophin secretion (Findlay and Cumming, 1976; Radford, Donegan and Scaramuzzi, 1980; Scaramuzzi and Radford, 1983; Ritar and Adams, 1988), and neither plasma FSH and LH concentrations nor LH pulse pattern were altered (see Downing and Scaramuzzi, 1991). These results suggested that "flushing" with lupin grain supplement increased ovulation rate by increasing ovarian sensitivity to gonadotrophins rather than by increasing gonadotrophin concentrations.

Whether the effects of nutrition mediate the onset of puberty by alteration of gonadotrophin secretion has also been investigated (Kinder *et al.*, 1987). When the growth rate of ewe lambs was retarded by restricted food intake, LH pulse frequency was reduced (Foster *et al.*, 1986) and preovulatory-like surges of LH secretion following oestradiol administration were suppressed (Foster and Olster, 1985). When restricted feeding was switched to feeding *ad libitum*, an increase in LH secretion occurred (Foster and Olster, 1985; Kinder *et al.*, 1987). Similar observations were reported for heifers (Day, Imakawa, Zalesky, Kittok and Kinder, 1986; Kinder *et al.*, 1987). Mean LH concentrations, LH pulse frequency and LH pulse amplitude were reduced by restricted feeding.

Moreover, the response of LH to administration of GnRH was also reduced by restricted feeding (Kinder *et al.*, 1987). Conversely, when heifers were fed an additive rich in the volatile fatty acid propionate, pituitary response to exogenous GnRH (Randel and Rhodes, 1980; Rutter, Randel, Schilling and Forrest, 1983), LH response to oestradiol administration (Randel, Rutter and Rhodes, 1982), and ovarian response to FSH and human chorionic gonadotrophin (hCG) (Bushmick, Randel, McCartor and Carroll, 1980) were all enhanced.

Alternatively, increased reproductive performance may involve changes in metabolic hormones such as GH, insulin and IGFs, which are associated with changes in body energy and protein balance induced by changes in nutrition (Pell and Bates, 1990; Beitz, 1985; Bass, Davis, Peterson, Gluckman, Butler and Gray, 1984; Waghorn, Flux and Ulyatt, 1987; Spicer *et al.*, 1990; Spicer, Crowe, Prendiville, Goulding and Enright, 1992). Each of these metabolic hormones has been shown to influence ovarian function either directly at the ovarian level and/or by modulating gonadotrophin actions on ovaries (see below). The increase in ovulation rate in ewes following flushing with lupin grain was accompanied by an elevation in peripheral concentrations of insulin and prolactin (Downing and Scaramuzzi, 1991). Moreover, direct infusion of glucose or a mixture of branched-chain amino acids into the peripheral circulation increased insulin and GH concentrations (Downing and Scaramuzzi, 1991).

The involvement of GH in the regulation of ovarian function in mammals has been suggested by several lines of observation. Circulating concentrations of GH increased rapidly during pubertal development (Ojeda and Jameson, 1977), and the delay in puberty which was associated

with GH deficiency could be restored by administration of exogenous GH both in humans and in rats (Sheikholislam and Stempfe, 1972; Ramaley and Phares, 1980). Furthermore, GH administered to hypophysectomized rats increased the steroidogenic responsiveness of granulosa cells to FSH *in vitro*, while suppression of endogenous GH secretion in intact rats (using an intra-hypothalamic implant of GH) reduced ovarian LH receptor content and steroidogenic response to hCG, with this latter effect being overridden by replacement of GH (Advis, White and Ojeda, 1981). Circulating GH levels were higher during the late follicular phase of the human menstrual cycle (Yen, Vela, Rankin and Littell, 1970; Genazzini, Lemarchand-Beraud, Aubert and Felber, 1975). Similarly, peripheral GH concentrations, and pituitary GH mRNA were increased during the follicular phase of the ovine oestrous cycle (Landefeld and Suttie, 1989). Specific binding sites for GH have been identified in ovarian granulosa cells (Hsueh *et al.*, 1984), and GH enhanced FSH-stimulated differentiation of cultured rat granulosa cells and increased *in vivo* ovarian secretion of immunoreactive IGF-I (Jia *et al.*, 1986; Davoren and Hsueh, 1986), a peptide proposed to be an important local regulator of ovarian function (Adashi *et al.*, 1985a). GH also stimulated IGF-I and progesterone secretion by porcine granulosa cells *in vitro* (Hsu and Hammond, 1987a).

Treatment of lactating dairy cows in early postpartum with recombinant bovine somatotropin (BST) to enhance milk production increased the incidence of twin-calving (Butterwick, Rowlinson, Weeks, Parker and Armstrong, 1988), although other studies have failed to observe such an increase (Peel and Bauman, 1987; Phipps, Weller, Austin, Craven and Peel, 1988; Whitaker, Smith, Kelly and Hodgson-Jones, 1988). Administration of BST did not alter ovulation rate in sheep (Davis, Smith and Gluckman, 1990), while treatment of gilts with porcine GH increased

ovulation rate although 45% of treated animals became anoestrus (Kirkwood, Thacker, Gooneratne, Guedo and Laarveld, 1988). Treatment with GH also reportedly improved ovulation induction in woman (Blumenfeld and Lunenfeld, 1989; Matson, Ibrahim, Morris, Sun and Lieberman, 1989; Homburg, West, Torresani and Jacobs, 1990) and enhanced the superovulatory response in cattle to some degree (Herrler, Farries and Niemann, 1990; Rieger, Walton, Goodwin and Johnson, 1991).

The idea that insulin may be involved in the regulation of ovarian physiology first arose from clinical observations that insulin-dependent diabetes due to insulin deficiency was always associated with ovarian hypofunction (Joslin, Root and White, 1925), whilst patients with insulin resistance exhibited features of ovarian hyperstimulation (Poretsky and Kalin, 1987). Subsequently, experiments were carried out to examine the relationship between insulin and ovarian function *in vitro* and *in vivo*. Insulin receptors have been identified in ovarian tissues from humans (Poretsky, Grigorescu, Seibel, Moses and Flier, 1985; Jarrett, Ballejo, Tsibris and Spellacy, 1985), pigs (Rein and Schomberg, 1982) and rats (Ladenheim, Tesone and Charreau, 1984). Insulin was present in human follicular fluid (Diamond, Webster, Carr, Wentz and Osteen, 1985) and its levels in follicular fluid correlated positively with follicular fluid progesterone concentrations. Insulin stimulated both proliferation and steroidogenesis of cultured bovine (Savion, Liu, Laherty and Gospodarowicz, 1981), porcine (Channing, Tsai and Sachs, 1976; Veldhuis, Kolp, Toaff, Strauss and Demers, 1983), rat (Davoren, Kasson, Li and Hsueh, 1986) and ovine (Webb and McBride, 1991) granulosa cells and porcine thecal cells (Barbieri, Makris and Ryan, 1983). Direct infusion of insulin to pigs *in vivo* stimulated follicular development (Matamoros, Cox and Moore, 1990) and increased ovulation rate without altering peripheral

gonadotrophin concentrations (Cox, Stuart, Althen, Bennet and Miller, 1987).

In summary, it is now well established and widely accepted that nutrition plays an important modulatory role in the control of ovarian function. However, whether it is acting through changes in gonadotrophins, metabolic hormones or other pathways awaits further investigation.

1.4.6. The role of growth factors in the control of ovarian function

As pointed out above, while gonadotrophins provide the primary drive for the further development of small antral follicles, folliculogenesis prior to this stage seems not to be solely dependent on gonadotrophins. Furthermore, the process of follicular dominance remains poorly understood (Ireland, 1987). The fact that the dominant follicle(s) can survive and continue to develop in an environment suppressive to the development of other follicles recruited at about the same time suggests the existence of some local modulatory mechanisms which could have direct effects on folliculogenesis or regulate the actions of gonadotrophins (Hsueh *et al.*, 1984; Tonetta and diZerega, 1989). In recent years, many potential local modulators of folliculogenesis have been identified from ovarian tissues and follicular fluids in several species (Ireland, 1987; Schams, 1989). They may act in an endocrine, paracrine and/or autocrine manner to regulate the actions of gonadotrophins and thus the development of dominant follicle(s) (see Mariana *et al.*, 1991). Among these potential regulators of folliculogenesis, growth factors have been shown to play an important part (Hammond, Hsu, Mondschein and Canning, 1988a; Carson, Zhang,

Hutchinson, Herington and Findlay, 1989). IGFs (Adashi *et al.*, 1985a), platelet-derived growth factor (Knecht and Catt, 1983) and transforming growth factor β (Dodson and Schomberg, 1987) have been shown to enhance various aspects of FSH action, whilst epidermal growth factor (Zhang, Herington, Carson, Findlay and Burger, 1987a), transforming growth factor α (Adashi, Resnick and Twardzik, 1987) and fibroblastic growth factor (Gospodarowicz, Cheng, Liu, Baird, Esch and Bohlen, 1985) inhibit FSH action, although the mechanisms underlying these effects of growth factors are still not clearly understood.

Three criteria have been proposed to define a substance as a local regulator (Adashi *et al.*, 1985a; Franchimont, 1986; Findlay *et al.*, 1990). Firstly, the substance should be demonstrated to be produced locally under the regulation of peripheral and/or local hormones; secondly, the target organ should be the same one which produces the substance and thirdly, the substance can alter the function of the target organ in a paracrine and/or autocrine manner. This part of the review will summarize briefly the experimental observations which support the role of growth factors in ovarian follicular growth and development.

1.4.6.1. Insulin-like growth factors

IGFs constitute a family of homologous polypeptides which have remarkable structural and functional similarity to insulin (Bradshaw and Niall, 1978). They can be divided into two distinct groups based on their physicochemical properties: a) basic IGFs (human IGF-I/somatomedin-C and rat IGF-I) and b) neutral IGFs [human IGF-II and its likely murine equivalent, multiplication-stimulating activity (MSA) or rat IGF-II] (Van Wyk, Svoboda and Underwood, 1980; Rubin, Mariz, Jacobs, Daughaday and Bradshaw, 1982; Rinderknecht and Humbel, 1978). Like insulin, IGFs

stimulate both the proliferation and differentiation of cells from very diverse origins (Clemmons and Van Wyk, 1981; Turo and Florini, 1982). They exert their biological functions through binding to receptors in membranes of target cells. Specific receptors have been characterized for both basic IGFs (type-I IGF receptor) and neutral IGFs (type-II IGF receptor) (Nissley, 1985). The type-I IGF receptor is composed of two α and two β subunits and binds IGF-I, IGF-II and insulin in decreasing order of affinity. The type-II IGF receptor is a single chain peptide that binds IGF-II with greater affinity than IGF-I and is incapable of binding insulin (Nissley, 1985).

Type-I, type-II IGF and insulin receptors have all been demonstrated to be present in ovaries (Davoren *et al.*, 1986; Sauerwein, Miyamoto, Gunther, Meyer and Schams, 1992), but the fact that the mitogenic activity of IGFs on granulosa cells was mimicked by insulin only at high concentrations was taken to imply that IGFs rather than insulin may be the physiological regulators of ovarian function (Adashi *et al.*, 1985a; Rein and Schomberg, 1982; Otani, Manro, Yukimur and Mochizuki, 1985). Indeed, the levels of insulin in follicular fluid were too low to activate the high affinity granulosa cell insulin receptor in pigs (Hammond, Yoshida, Veldhuis, Reshler and Knight, 1983). Moreover, no distinct differences in insulin receptor concentrations could be detected between small, medium and large follicles in pigs (Otani *et al.*, 1985). However, some recent *in vitro* observations have suggested that insulin probably acts via its own receptor to stimulate the proliferation of ovine granulosa cells (Webb and McBride, 1991).

The production of IGFs by the ovary has been supported by several lines of evidence. Firstly, measurable quantities of IGFs have been

demonstrated in follicular fluid of several species. A MSA-like substance was detected in porcine follicular fluid by Hammond *et al.* (1983). Further studies indicated that IGF-I was also present in porcine follicular fluid and that IGF-I levels were significantly greater in follicular fluid from preovulatory follicles than in either serum or follicular fluid from immature follicles (Hammond, Baranao, Skaleris, Knight, Romanus and Rechler, 1985). IGF-I concentrations in porcine follicular fluid were increased by gonadotrophins *in vivo* (Hammond, Hsu, Klindt, Tsang and Downey, 1988b). Both IGF-I and IGF-II were measurable in follicular fluid from women undergoing *in vitro* fertilization (IVF) (Ramasharma, Cabera and Li, 1986). Concentrations of IGF-I in bovine follicular fluid were within the concentration range shown to be effective in stimulating steroidogenesis and proliferation of bovine granulosa cells *in vitro* (Spicer, Echternkamp, Canning and Hammond, 1988).

Secondly, granulosa cells of rats and pigs cultured under serum-free conditions secreted immunoreactive IGF-I (Hammond *et al.*, 1985; Adashi *et al.*, 1985a). IGF-I production by porcine granulosa cells *in vitro* was under the control of GH, gonadotrophins, steroids and other growth factors, but FSH treatment had no significant effect on immunoreactive IGF-I/somatomadin-C accumulation in media from rat granulosa cells cultured under serum-free conditions (Davoren and Hsueh, 1986; Hsu and Hammond, 1987a,b; Mondschein and Hammond, 1988). *In vivo*, GH increased IGF-I concentrations in rat ovarian tissue extracts (Davoren and Hsueh, 1986). Finally, both IGF-I and IGF-II mRNA have been detected in rat and human ovary (Murphy, Bell and Friesen, 1987; Voutilainen and Miller, 1987).

Specific IGF binding sites have been detected in ovaries of several

species. A high affinity, low capacity binding site was identified in freshly obtained granulosa cells from prepubertal pigs using direct binding studies (Adashi *et al.*, 1985a). Displacement studies revealed a potency order of IGF-I > IGF-II > insulin, consistent with a type-I IGF receptor. Specific type-I IGF receptors have also been detected in swine granulosa cells cultured under serum-free conditions (Adashi *et al.*, 1985a). Using affinity cross-linking techniques, both type-I and type-II IGF receptors have been demonstrated in rat whole ovarian membranes and isolated rat granulosa cells (see Adashi *et al.*, 1985a). Furthermore, IGF binding sites in rat granulosa cells were regulated by FSH, through a cAMP-dependent mechanism, both *in vitro* and *in vivo* (Carson *et al.*, 1989). Recently, saturable, specific and high affinity IGF-I receptors were demonstrated in ovine granulosa cells and atresia of small follicles was accompanied by a decrease in IGF-I receptor number (Monget, Monniaux and Durand, 1989). In the human ovary, type-I IGF receptor has been identified in granulosa or granulosa/luteal cells from IVF patients using either a direct binding assay or immunocytochemical staining (Gates, Bayer, Seibel, Poretsky, Flier and Moses, 1987).

The actions of IGFs on granulosa cells have been extensively investigated using *in vitro* cell culture systems under serum-free conditions. Early studies were interested mainly in the mitogenic effect of IGFs on granulosa cells, as IGFs are known to be potent mitogens for tissues of diverse origin, and in most species, ovarian folliculogenesis is associated with marked increases in granulosa cell numbers. IGF-I (at concentrations as low as 1ng/ml) stimulated proliferation of bovine granulosa cells from small-sized (4-6 mm) follicles *in vitro* under serum-free conditions (Savion *et al.*, 1981) and no saturation of the effect was observed at a concentration of 60 ng/ml. Treatment of cultured porcine

granulosa cells from immature animals with IGF-I and IGF-II resulted in a dose-dependent increase of [^{3}H]-thymidine incorporation into cellular deoxyribonucleic acid (DNA) (Adashi *et al.*, 1985a), with IGF-I being more effective than either IGF-II or insulin. MSA stimulated replication of porcine granulosa cells from 1-2 mm follicles and increased granulosa cell ornithine decarboxylase activity, an enzyme linked to cell replication (Baranao and Hammond, 1984). In contrast, IGF-I at a dose of 50 ng/ml failed to stimulate replication of cultured rat granulosa cells either in the presence or absence of FSH, though it prevented the FSH-induced decrease in [^{3}H]-thymidine incorporation into cellular DNA (Adashi, Resnick, Svoboda and Van Wyk, 1985b).

IGFs also play a role in granulosa cell differentiation during follicular development. Indeed, almost all the biological markers of granulosa cell differentiation, including steroid synthesis, inhibin production, LH receptor induction, oxytocin secretion and aromatase activity, have been demonstrated to be under the influence of IGFs (Carson *et al.*, 1989). Treatment of cultured rat granulosa cells with highly purified IGF-I (40 ng/ml) was without effect on oestrogen secretion, as assessed by the conversion of androstenedione to oestrogen (Adashi *et al.*, 1985a). However, concurrent treatment with FSH produced a 4-fold increase in oestrogen accumulation. Similarly, IGF-I increased FSH-induced rat granulosa cell aromatase activity >5-fold (as assessed by the stereospecific generation of tritiated water from [^{3}H]-androstenedione substrate). $P_{450arom}$ activity in granulosa cells, which plays a key role in controlling oestradiol production, was increased by treatment with IGF-I (Erickson, Garzo and Magoffin, 1989). Whilst highly purified IGF-I alone had only a limited effect on cultured rat granulosa cell progestin biosynthesis, concurrent treatment with FSH resulted in a

dose- and time-dependent increment in the accumulation of progesterone and 20α-hydroxy-pregn-4-en-3-one which could not be accounted for by increases in cell number or DNA synthesis (Adashi *et al.*, 1985b). Furthermore, this synergistic interaction of IGF-I with FSH could be blocked by immunoneutralization with a specific monoclonal antibody against IGF-I. Similar results were reported for porcine and bovine granulosa cells (Veldhuis and Furlanetto, 1985; Schams, Koll and Li, 1987). In addition, a partially purified (>80%) IGF-I preparation exerted a potent, dose-dependent stimulatory effect on the production of pregnenolone, progesterone and 20α-hydroxy-pregn-4-en-3-one by cultured pig granulosa cells (Veldhuis and Furlanetto, 1985). These effects of IGF-I were inhibited by cycloheximide and actinomycin, but enhanced by oestradiol-17β, 8-bromo-cAMP and 25-hydroxy-cholesterol (Veldhuis and Furlanetto, 1985). Moreover, treatment with IGF-I enhanced granulosa cell low density lipoprotein and sterol metablism, and FSH-induced P_{450scc} activity (Veldhuis, Rodger, Dee and Simpson, 1986b; Veldhuis, Nestler and Strauss, 1987). The ability of IGF-II/MSA to stimulate progesterone biosynthesis by porcine and rat granulosa cells cultured under serum-free conditions, in synergism with FSH, has also been demonstrated (May and Schomberg, 1981).

The acquisition of LH receptors by granulosa cells is an essential prerequisite for the final maturation of follicles and subsequent follicular luteinization. FSH plays an important role in granulosa cell LH receptor induction through a cAMP-dependent pathway (Adashi *et al.*, 1985a). Although LH/hCG binding by rat granulosa cells cultured under serum-free conditions was not significantly affected by highly purified IGF-I, treatment with FSH produced a 3.8-fold increase in LH/hCG binding. This FSH effect was enhanced to approximately 6-fold by concurrent

treatment with IGF-I (Adashi *et al.*, 1985c). Furthermore, this enhancement was due to increased binding capacity rather than a change in binding affinity. Similar results were reported for LH/hCG binding in porcine granulosa cells (Veldhuis and Furlanetto, 1985), and a stimulatory effect of IGF-II/MSA on rat granulosa cell LH receptor induction has been reported by Davoren *et al.* (1986).

Functional differentiation of granulosa cells also results in the production of inhibin (Carson *et al.*, 1989). Since inhibin may have an important role in the regulation of pituitary gonadotrophin secretion in some species, factors which influence follicular inhibin production may be important in the control of follicular development. IGF-I alone stimulated secretion of inhibin by cultured rat granulosa cells in a dose-dependent fashion. The response to 300ng/ml FSH, a dose previously shown to give maximal stimulation of inhibin production, was further increased when IGF-I was added together with FSH (Zhang, Carson, Herington, Lee and Burger, 1987b).

The production of oxytocin increases during the luteinization of granulosa cells in ruminants (Schams, 1989), and treatment of cultured bovine granulosa cells with IGF-I resulted in a dose-dependent increment in oxytocin secretion (Schams *et al.*, 1987). More recently, IGF-I has been shown to increase oxytocin production and oxytocin mRNA levels in highly differentiated bovine granulosa cells obtained from follicles containing high follicular fluid concentrations of oestradiol (>40ng/ml) and high levels of LH receptors (Holtorf, Furuya, Ivell and McArdle, 1989). These results support a possible role for IGFs in the regulation of granulosa cell luteinization.

In summary, experimental evidence has emerged recently to

establish ovarian granulosa cells as a site for the production, reception and action of IGFs, thus satisfying the requirements for IGFs as local regulators of ovarian follicular growth and development. Indeed, the replication, differentiation, and luteinization of granulosa cells can all be modulated by IGFs. One recent report indicated that the stimulatory effects of porcine follicular fluid, FSH, oestradiol and GH on the progesterone production by cultured porcine granulosa cells could be inhibited by up to 50% using a specific monoclonal antibody against IGF-I (Mondschein, Canning, Miller and Hammond, 1989a). IGFs may also have a paracrine role in the ovaries, as IGF binding sites have been identified in the theca (Monget *et al.*, 1989), and IGF-I enhanced androgen production by cultured thecal cells (Hernandez, Resnick, Svoboda, Van Wyk, Payne and Adashi, 1988).

The mechanism(s) of IGF action remain uncertain although it has been proposed that IGFs exert their biological actions via binding to the α subunits of the type-I IGF receptor which are covalently linked to the β subunit possessing IGF-I dependent tyrosine kinase activity (see Hsueh *et al.*, 1989). However, the fact that both type-II IGF and insulin receptors are also present in the ovary (see Hammond, Mondschein, Samaras, Smith and Hagen, 1991) has raised the question of the relative importance of type-I IGF, type-II IGF, and insulin receptors. To make things more complicated, IGF-I, IGF-II and insulin can compete for each other's receptors (though with markedly different affinities) and IGF-II and insulin can mimic most of the effects of IGF-I on granulosa cells (Adashi *et al.*, 1985a) (although at a much higher concentrations). Some investigators have suggested that the effects of insulin on ovarian function were exerted through binding to type-I IGF receptor, as the effective concentrations of insulin were much higher than the binding

affinity of the insulin receptor but in line with the weak affinity of insulin for the type-I IGF receptor (Poretsky *et al.*, 1985). The role of IGF type-II receptors in the regulation of ovarian function, however, is less certain. A recent investigation using a specific antibody against rat type-II IGF receptor reported that the action of IGF-I and IGF-II on ovary was mediated via type-I IGF, but not type-II, receptors (Adashi, Resnick and Rosenfeld, 1990). Moreover, type-II IGF receptor has been demonstrated only in the rat ovary (Davoren *et al.*, 1986) and bovine luteal tissue (Sauerwein *et al.*, 1992) so far. It is likely that more definitive conclusions will require studies with species-specific antibodies raised against the different receptors.

The cellular mechanism(s) underlying the synergistic interaction of IGFs with FSH are still poorly understood, though enhancement of FSH-induced cAMP accumulation and stimulation of adenylate cyclase activity may play a part (Adashi *et al.*, 1985a). Concurrent treatment of cultured rat granulosa cells with 20ng/ml IGF-I and FSH produced dose- and time-dependent increments in accumulation of cAMP which was 8-fold greater than that induced by FSH alone (Adashi *et al.*, 1985a). Furthermore, most IGF-I effects could be augmented by cAMP analogues or adenylate cyclase activitors (see Adashi *et al.*, 1985a).

Attempts to understand the mechanism(s) underlying IGF action in ovarian folliculogenesis are further complicated by the reports that IGF binding proteins (IGF-BPs) are secreted together with IGFs by human granulosa cells (Suikkari, Jalkanen and Koistinen, 1989). IGF-BPs have also been found to be present in ovine, rat, porcine and bovine follicular fluids (Monget *et al.*, 1989; Jalkanen, Suikkari, Koistinen, Butzow, Rituos, Seppala and Ranta, 1989; Hammond *et al.*, 1991). Three IGF-BPs have now

been cloned and sequenced and were designated, in order of molecular size, IGF-BP-1, -2 and -3 (Hammond *et al.*, 1991), all of which have been shown to be secreted by the ovary (Seppala, Wahlstrom, Koskimies, Tenhunen, Rutanen, Koistinen, Huhtaniemi, Bohn and Stenman, 1984; Mondschein, Hammond and Etheston, 1989b; Giudice, Milki, Milkowski and Danasouri, 1991; Hamori, Blum, Torok, Stehle, Waibel, Cledon and Ranke, 1991; Riccarelli, Hernandez, Tedeschi, Botero, Rohan, Rosenfeld, Albiston, Herington and Adashi, 1992). While IGF-BPs are important in regulating the biological functions of IGFs in an autocrine or paracrine manner (Holly and Wass, 1989), little is known of their significance in the regulation of ovarian function. IGF-BP-3 was reported to inhibit the binding of FSH to its receptors in granulosa cells (Ui, Shimonaka, Shimasaki and Ling, 1989) and to be an anti-gonadotrophin (Riccarelli *et al.*, 1992). Other workers reported that IGF-BPs could enhance the biological actions of IGFs (Busby, Hossenlopp, Binoux and Clemmons, 1989; Blum, Jenne, Reppin, Kietzmann, Ranke and Bierich, 1989), although positive effects of IGF-BPs have yet to be demonstrated in the ovary. Nevetheless, these results suggest that IGF-BPs may be as important as IGFs themselves. Furthermore, most radioimmunoassays (RIA) and radioreceptorassays (RRA) for IGFs reported so far, in a variety of species, use antibodies directed against human IGF-I, which rely on the cross-immunoreactivity of IGFs between the different species. More accurate profiles of IGF biosynthesis in the ovary and its regulation must await the availability of homologous RIAs and RRAs.

1.4.6.2. Epidermal growth factor and transforming growth factor-α

Epidermal growth factor (EGF) is a single-chain polypeptide with 53 amino acids which was first isolated from mouse submaxillary glands

in 1960 and subsequently named for its capacity to induce proliferation of the basal layer of the skin (Carpenter, 1987). Transforming growth factor α (TGF-α) is a single 50-amino acid chain and its sequence is highly homologous to that of EGF. Although EGF and TGF-α are derived from unlinked genes, they bind to the same receptor in the cell membrane and display similar biological activities (Lee, Rose, Webb and Todaro, 1985).

Binding sites for EGF have been identified in granulosa cells and were shown to be regulated by gonadotrophins (Jones, Welsh and Hsueh, 1982; Feng, Knecht and Catt, 1987). EGF-like activity has been detected in rat, porcine and bovine follicular fluid and in the thecal-interstitial cell preparations using either RIA, RRA or bioassay (Skinner, Lobb and Dorrington, 1987b, see Hammond *et al.*, 1988a). Recently, mRNA for EGF was found to be present in the ovaries of mice (Rall, Scott and Bell, 1985). TGF-α mRNA was also identified in rat and bovine ovaries and immunoreactive TGF-α was detected in interstitial and thecal cells (Kudlow, Kobrin, Purchio, Twardzik, Hernandez, Asa and Adashi, 1988, Lobb and Dorrington, 1992).

EGF stimulated bovine granulosa cell proliferation and DNA synthesis *in vitro* (Skinner *et al.*, 1987a). Extracts from human and porcine theca, porcine follicular fluid and conditioned media of rat and chicken theca could stimulate the mitosis of granulosa cells (Carson *et al.*, 1989). The substance(s) exerting this effect was suggested to be an EGF or TGF-α like molecule. TGF-α has also been localized in bovine thecal cells by immunoperoxidase staining, using a monoclonal antibody against TGF-α which did not cross-react with EGF. Significantly, TGF-α staining was most intense in the theca from follicles (0.7 - 2.0mm diameter) showing rapid granulosa cell replication, whereas staining declined in large

preovulatory follicles (Lobb, Kobrin, Kudlow and Dorrington, 1989, Lobb and Dorington, 1992).

While EGF and TGF-α promoted granulosa cell proliferation, they generally inhibited granulosa cell differentiation. Thus, EGF inhibited FSH-induced granulosa cell aromatase activity, LH receptor induction and adenylate cyclase activity, although it slightly increased progesterone production (Carson *et al.*, 1989). Treatment of rat granulosa cells with EGF *in vitro* decreased basal as well as FSH-, 8-bromo-cAMP- and prostaglandin E_2-induced inhibin secretion (Zhang *et al.*, 1987a). FSH-induced inhibin production from cultured bovine granulosa cells was also inhibited by EGF (Franchimont, Hazee-Hagelstein, Charlet-Renard and Jaspar, 1986). Similarly, treatment of granulosa cells with TGF-α caused a dose-dependent decrease in FSH-stimulated aromatase activity (Adashi *et al.*, 1987).

The above results suggested that EGF and TGF-α may be produced in the theca and exert a paracrine regulatory effect on granulosa cells to regulate follicular growth and development. These factors were produced most abundantly in small follicles (Lobb *et al.*, 1989) and they stimulated the proliferation of granulosa cells, while inhibiting differentiation, indicating that EGF and TGF-α may be important in the early stages of folliculogenesis by preventing the premature differentiation of small follicles (Lobb and Dorrington, 1992). Interestingly, treatment of mice (Lintern-Moore, Moore, Panaretto and Robertson, 1981) and sheep (Shaw, Jorgensen, Tweedale, Tennison and Waters, 1985) with EGF in vivo resulted in an inhibition in ovarian follicular growth and development. In addition, an autocrine role of EGF and TGF-α in theca cannot be excluded, as binding sites for EGF/TGF-α were also present in theca (Feng

et al., 1987).

1.4.6.3. Transforming growth factor β

Transforming growth factor β (TGF-β) represents a family of homodimeric peptides which consist of two identical polypeptide chains (Massague, 1985). TGF-β subunits share sequence homology with the β-chain of inhibin and inhibin-related proteins such as activin (see 1.4.2). These molecules are believed to be derived from the same gene family. The TGF-β receptor contains two subunits and unlike most other growth factor receptors, it appears to have no tyrosine kinase activity (Sporn, Roberts, Wakefield and Associan, 1986).

TGF-β regulates pituitary gonadotrophin secretion (Ying, Becher, Ling, Ueno and Guillemin, 1986b), and its role in the intraovarian regulation of folliculogenesis has also been investigated. Using an *in vitro* culture system, TGF-β increased [^{3}H]-thymidine incorporation into rat granulosa cells, but inhibited [^{3}H]-thymidine incorporation into porcine granulosa cellular DNA (May, Frost and Schomberg, 1988). TGF-β suppressed the proliferation, while stimulating FSH-induced aromatase activity, of cultured bovine granulosa cells (Lobb and Dorrington, 1992). Whilst treatment of cultured rat granulosa cells with TGF-β alone was without effect on basal oestrogen production, FSH-stimulated accumulation of oestrogen and progesterone was enhanced dose-dependently by TGF-β (Adashi and Resnick, 1986). In contrast, TGF-β increased basal progesterone secretion in rat granulosa cells, but inhibited both basal and FSH-stimulated progesterone production by porcine granulosa cells (Dodson and Schomberg, 1987). Inhibin production by rat granulosa cells *in vitro* was significantly increased by TGF-β (1ng/ml) treatment and addition of FSH (1ng/ml) and TGF-β

together produced an increase in inhibin production which was greater than that stimulated by either hormone alone (Zhang, Findlay, Carson, Herington and Burger, 1988). TGF-β treatment alone had no effect on rat granulosa cell LH binding, but concurrent treatment with FSH produced an increase in LH binding (Dodson and Schomberg, 1987). Furthermore, the effect of TGF-β on FSH-stimulated LH receptor induction was dependent on the concentration of FSH. Whilst TGF-β enhanced LH receptor induction at low doses of FSH (5ng/ml), it inhibited at higher FSH doses (50ng/ml) (Knecht, Feng and Catt, 1987).

Besides modulating the proliferation and differentiation of granulosa cells, TGF-β has also been shown to regulate steroidogenesis in thecal cells. TGF-β treatment inhibited both basal and hCG-stimulated progesterone and androstenedione secretion, and hCG-stimulated testosterone production, by thecal cells obtained from large porcine follicles (Caubo, Devinna and Tonetta, 1989). In contrast, TGF-β treatment enhanced basal and hCG-induced oestradiol secretion by thecal cells (Caubo *et al.*, 1989).

TGF-β production by the ovary has been demonstrated. TGF-β has been identified in follicular fluid and in bovine thecal cells (Ying *et al.*, 1986a; Lobb and Dorrington, 1992), and thecal cells in culture secreted TGF-β as determined by both RRA and RIA (Skinner, Keskioja, Osteen and Moses, 1987a). More recently, TGF-β gene expression in the ovaries of rats was shown to be stimulated up to 4-fold by FSH (Hernandez, Twardzik, Purchio and Adashi, 1987).

Although some inconsistent results have been observed with regard to the potential effects of TGF-β on the regulation of ovarian function, it is possible that TGF-β produced by the theca regulates

granulosa cell proliferation and differentiation through a paracrine mechanism(s). Its autocrine regulatory effects have yet to be investigated.

1.4.6.4. Fibroblast growth factor

Fibroblast growth factors (FGF) are single-chain peptides named for their ability to stimulate the proliferation of fibroblasts. They are classified into two forms, basic and acidic, according to their isoelectric points (Gospodarowicz, Neufeld and Schweigerer, 1986). FGF can act as potent mitogens and morphogens for a wide range of mesoderm- and neuroectoderm-derived cells both *in vitro* and *in vivo*, controlling their proliferation and differentiation (Gospodarowicz *et al.*, 1986). Although the two forms of FGF have 55% amino acid sequence homology and share a common receptor, basic FGF (bFGF) has a greater biopotency than acidic FGF (Gospodarowicz and Ferrara, 1989).

FGF were originally identified in pituitary and brain extracts, but recent studies have indicated that they are also produced by ovaries (Neufeld, Ferrara, Schweigerer, Mitchell and Gospodarowicz, 1987). In fact, almost every cell type in the ovary has been demonstrated to produce bFGF. Early studies demonstrated an angiogenic factor present in rabbit luteinizing granulosa cells, luteal cells and bovine CL (Gospodarowicz and Thakral, 1978; Jakob, Jentzsch, Baruersberger and Oehme, 1977). Basic FGF was purified to homogeneity from bovine CL and accounted for all of the previously reported mitogenic and angiogenic activity of CL extracts (Gospodarowicz *et al.*, 1985). Similar results were obtained with rat follicles and CL, and with human follicular fluid. More recent studies demonstrated that cultured bovine granulosa cells express the gene encoding bFGF (Neufeld *et al.*, 1987). Moreover, bFGF derived

from this gene was bioactive as it stimulated the proliferation of cultured bovine granulosa cells and capillary endothelial cells. Thecal cells were also suggested to be a source of ovarian bFGF (Gospodarowiz and Ferrara, 1989).

Basic FGF is a potent mitogen and morphogen for ovarian granulosa cells. It stimulates proliferation of bovine granulosa cells cultured *in vitro*, and delays terminal differentiation of granulosa cells by preventing them from entering the G_0 phase of the cell cycle (Gospodarowicz and Bialecki, 1978). Furthermore, treatment of cultured granulosa cells with bFGF inhibits FSH-stimulated LH receptor induction and FSH-induced aromatase activity (Mondschein and Schomberg, 1981), but enhances progesterone secretion following stimulation by low doses of FSH (Baird and Hsueh, 1986). This suggests that bFGF may play an important role in the early stages of folliculogenesis and follicular morphogenesis (Gospodarowicz and Bialecki, 1978).

Basic FGF is considered to be important in the ovulation process (Gospodarowicz and Ferrara, 1989), based on its ability to modulate the secretion of plasminogen activitor (PA), collagenase and other proteolytic enzymes which are hypothesized to be responsible for the degradation of the follicular wall. Indeed, PA was produced by granulosa cells in a manner closely correlated with ovulation, and treatment of granulosa cells with preparations of bovine LH and cAMP *in vitro* increased extracellular PA concentration. Although no direct evidence is available for granulosa cells, bFGF can stimulate PA and collagenase production in vascular endothelial cells and tumor cells (Gospodarowiz and Ferrara, 1989). In addition, bFGF also decreased the production of a PA inhibitor (Gospodarowiz and Ferrara, 1989).

Finally, bFGF was proposed to play a major role in CL formation and in the early phase of CL development. Basic FGF was shown to have a mitogenic effect on CL-derived capillary endothelial cells and was associated with the perifollicular neo-vascularization which occured during folliculogenesis, and the rapid capillary invasion of the avascular granulosa cell layer (Gospodarowiz and Ferrara, 1989).

Hence, FGFs are produced by almost all the components of the ovary, and may act as autocrine and/or paracrine regulators of granulosa proliferation and differentiation, as well as playing an important role in the regulation of theca morphogenesis, ovulation, and CL formation and development.

1.4.6.5. Platelet-derived growth factor

Platelet-derived growth factor (PDGF) was originally isolated from human platelets and subsequently was found to be synthesized and secreted by many other cell types (Ross, 1989). PDGF is a potent mitogen for mesenchymal/ connective-tissue forming cells (Deuel and Huang, 1983), so its potential roles in the regulation of ovarian follicular growth and development have been investigated. Only a relatively crude platelet extract could stimulate ornithine decarboxylase activity, a marker of cell proliferation, in cultured porcine granulosa cells (Baranao and Hammond, 1985; Mondschein and Schomberg, 1984). This effect may be a result of contamination with TGF-β or EGF (TGF-α)-like activity which are also present in platelets (see Hammond *et al.*, 1988a; Assoian, Roberts, Wakefield, Anzano and Sporn, 1984; Ross, 1989), as addition of purified PDGF was without effect (Knecht and Catt, 1983). It is also possible that PDGF did not stimulate porcine granulosa cell ornithine decarboxylase activity or that its action required the presence of other factors in

platelet extracts. Indeed, platelets also contain a molecule that induces multiplication of capillary endothelial cells (Ross, 1989). However, purified PDGF was shown to enhance FSH-induced progesterone secretion, LH receptor induction and adenylate cyclase activity in granulosa cells (Knecht and Catt, 1983; Mondschein and Schomberg, 1984). Aside from augmentation of FSH-induced granulosa cell differentiation, the role of PDGF in granulosa cell proliferation, as well as its sites of ovarian production and mechanism of action have yet to be determined.

1.4.6.6. Interleukin-1 and other cytokines

Increasing evidence has suggested that there is a bi-directional communication between the immune and the endocrine systems (Adashi, 1989). Cytokines (polypeptides synthesized and secreted by a variety of immune cells) participate in the regulation of the secretion of a number of hormones such as insulin, corticotropin-releasing factor, cortisol and thyroglobulin production (Hsueh *et al.*, 1989). Recent studies have demonstrated an emerging role for the cytokines in the regulation of ovarian function (Adashi, 1990). This is not unexpected, because all reproductive organs host a large population of immune cells (Seamark, Hadjisavas and Robertson, 1992), and macrophages (cells which produce a variety of cytokines) have been clearly established as a source of several growth factors such as TGF-α, TGF-β and bFGF (Rapolee, Mark, Banda and Werb, 1988). Interleukin-1 (IL-1), a cytokine derived from activated macrophages, stimulates proliferation of porcine granulosa cells obtained from small (1-2 mm) and medium (3-5 mm), but not large (8-11 mm) follicles (Fukuoka, Yasuda, Tsii, Takakura and Mori, 1989). IL-1 was reported to inhibit FSH-induced LH receptor induction, basal or LH-stimulated progesterone production and FSH-stimulated oestrogen

secretion in porcine and rat granulosa cells *in vitro* (Fukuoka, Mori, Tsii and Yasuda, 1988; Gottschall, Uehara, Hoffmann and Arimura, 1987; Gottschall, Katsuura, Hoffmann and Arimura, 1988). Another macrophage-derived monokine, tumor necrosis factor–α (TNF-α), has also been found to affect rat granulosa cell steroidogenesis *in vitro* (Roby and Terranova, 1988). TNF-α may be important in follicular atresia and luteolysis, as the TNF-α gene was expressed in granulosa cells of atretic follicles, and TNF-α was derived from activated macrophages of regressing (but not young) CL (Adashi, 1989). An (as yet) unidentified macrophage factor was found to stimulate progesterone production by luteinized murine granulosa cells (Kirsch, Vogel and Flickinger, 1983). Moreover, lymphokines (secreted from lymphocytes), including IL-2, have also been shown to regulate rat granulosa cell steroidogenesis (Gorospe and Kasson, 1988) and proliferation and secretion of progesterone in human granulosa-luteal cells (Wang, Robertson, Seamark and Norman, 1991) *in vitro*.

Although intraovarian production and secretion of cytokines still remains to be established, resident ovarian (extravascular) immune cells (including macrophages, lymphocytes and polymorphonuclear granulocytes) can be detected at distinct stages of the ovarian cycle (Hume, Halpin, Charlton and Gordon, 1984). IL-1-like activity was reported to be present in human follicular fluid (Khan, Schmidt, Hallin, Pauli, deGeyter and Nieschlag, 1988). Furthermore, resident ovarian immune cells are established as targets of steroidal signalling (Adashi, 1989). Indeed, IL-1 gene expression is progesterone-dependent (Polan, Carding and Loukides, 1988) and oestrogen receptor has been identified in certain peripheral T lymphocytes (Cohen, Danel, Cordier, Saez and Revillard, 1983).

Thus, although the evidence is limited at the present, cytokines are potential intraovarian regulators of ovarian function, though much has yet to be done to establish intraovarian production of cytokines and their regulation, mechanism(s) of action and their relationships to other modulators of ovarian function.

In summary, a large body of evidence has now emerged to support the concept that a variety of growth factors, including IGFs, EGF/TGF-α, TGF-β, FGF, PDGF and more recently cytokines, may play an important part in the regulation of ovarian folliculogenesis. These factors can modulate gonadotrophin actions on the ovary such as proliferation, differentiation (steroidogenesis, LH receptor induction and inhibin production) and luteinization (progesterone and oxytocin secretion) of granulosa cells, LH receptor induction and steroidogenesis of thecal cells, and may play a role in ovulation and the formation and development of the CL. Locally-produced growth factors, together with other intraovarian regulators, may establish a unique microenvironment for each individual follicle, thus ensuring the continued development of the dominant follicle(s) while other follicles undergo atresia. Indeed, intraovarian factors produced by the dominant follicle(s) may suppress the development of other follicles and induce atresia. Although growth factors may act in an autocrine and/or paracrine fashion (see Hammond *et al.*, 1988a), the mechanism(s) of action of most of these growth factors in the ovary still remains uncertain at this stage. Much work remains to be done, particularly with regard to intraovarian production and regulation of growth factors, their relative importance to other regulators of ovarian function, and relationships between different growth factors. Studies of the intraovarian regulation of ovarian folliculogenesis may facilitate a

more complete understanding of the mechanism(s) controlling ovarian follicular growth and development, especially the processes of follicular dominance and follicular atresia, and thereby lead to more reliable control of reproductive performance in female animals.

1.5. The induction of multiple ovulation

The incidence of spontaneous twinning in cattle is normally very low (approximately 3.0%; Nielen, Schukken, Scholl, Wilbrink and Brand, 1989). Although twin-calvings have been reported to be associated with some disadvantages such as higher incidence of retained placentae (Turman, Laster, Renbarger and Stephens, 1971; Johnson, Turman and Stephens, 1975), increased calving difficulties (Cady and Van Vleck, 1978; Diskin, McEvoy, Hickey and Sreenan, 1987), low calf bodyweight (Vincent and Mills, 1972; de Rose and Wilton, 1988) and the high occurrence of freemartins (over 90%) in females of mixed sex twins (Erb and Morrison, 1959; Dunn, McEntee, Hall, Johnson and Stone, 1979), an increased incidence of twin-calving is financially beneficial, as the total weight of calf produced and weaned per cow is significantly increased (Vincent and Mills, 1972; Smith, Pollak and Anderson, 1982; de Rose and Wilton, 1988; Gregory, Echternkamp, Dickerson, Cundiff, Koch and Van Vleck, 1990). It has been estimated that a twin-calving rate of 40% would increase gross margins by up to 20% (Diskin *et al.*, 1987). Furthermore, it has been indicated that the disadvantages (see above) associated with twinning could be overcome if animals were managed and fed using programmes specially developed for twin-bearing cattle (Lamond, 1974; Morris, 1984). Therefore, over the years, several approaches have been employed in an attempt to induce twinning or multiple ovulations in cattle.

1.5.1. Genetic selection

Using conventional genetic selection methods, several herds of cattle selected for twinning have been established (Bindon, Piper, Cheers, Curtis, Nethery and Holland, 1982; Morris, 1984; Frebling, Gillard and Menissier, 1982; Hendy and Bowman, 1970; Mechling and Carter, 1964, Gregory *et al.*, 1990). However, the heritability of twinning in cattle is very low, ranging from 0.006 to 0.06 (Bar-Anan and Bowman, 1974; Morris and Day, 1986; Gregory *et al.*, 1990), and therefore progress by selection is very slow.

1.5.2. Gonadotrophin treatment

The ability of gonadotrophin preparations to increase the number of ovulations in cattle has long been recognized (Folley and Malpress, 1945; Hammond, 1949). However, reliable induction of twin ovulations by treating cows with exogenous gonadotrophin is still not possible, as the treatment usually increases ovulation rate in excess of that required for twinning (i.e. superovulation) (Hafez, Jainudeen and Lindsay, 1965; Smith, Sitton and Vincent, 1973). Moreover, the response to a given dose of gonadotrophin is extremely variable both between and within individuals (Monniaux, Chupin and Saumande, 1983; Moor, Kruip and Green, 1984). The ovulatory response to exogeneous gonadotrophin treatment may depend on the gonadotrophin preparations utilised (Murphy, Mapletoft, Manns and Humphrey, 1984), the stage of the oestrous cycle when treatment is given (Sreenan and Gosling, 1977; Hasler, McCauley, Schermerhorn and Foote, 1983; Price and Webb, 1989), the presence or otherwise of a dominant follicle (Guilbault, Grasso, Lussier, Rouillier and Matton, 1991) and ovarian status at the time of

treatment (Monniaux *et al.*, 1983). Therefore, it is not surprising that none of the numerous attempts using different gonadotrophin treatment regimes to date has been able to overcome the high variability in the response, a major limitation to superovulation in cattle (Moor *et al.*, 1984; Bellows, Staigmiller, Wilson, Phelps and Darling, 1991). This problem must be resolved before gonadotrophin treatment can be reliably and practically used to induce twin ovulation, otherwise this technique will remain simply as a means of improving the efficiency of embryo transfer and as an aid to the genetic selection.

1.5.3. Endocrine manipulation

Since endogenous gonadotrophin secretion is controlled by gonadal feedback systems (1.4.3), and gonadotrophin treatment can increase ovulation rate (see above), many investigations have manipulated these feedback control systems in an attempt to increase endogenous gonadotrophin secretion, and hence, ovulation rate (see Webb and Morris, 1988). These investigations include the manipulation of both ovarian steroid and protein feedback systems.

Interference with ovarian steroid negative feedback by active immunization against androstenedione in sheep increases ovulation and lambing rates (Scaramuzzi, Davidson and Van Look, 1977; Cox, Hoskinson, Scaramuzzi, Wilson and George, 1982) and has been marketed as a commercially patented technique (Cox *et al.*, 1982). In addition, active immunization against other ovarian steroids (Scaramuzzi, 1979; Pathiraja *et al.*, 1984; Webb *et al.*, 1984; Scaramuzzi and Hoskinson, 1984), reduction of ovarian steroid synthesis (Land and Scaramuzzi, 1979; Webb, 1987), administration of follicular fluid (McNeilly, 1985; Henderson *et al.*, 1986;

McNeilly and Wallace, 1987) or immunization against inhibin preparations (O'Shea, Al-Obaidi, Hillard, Bindon, Cummins and Findlay, 1984; O'Shea *et al.*, 1990) have all been demonstrated to increase ovulation and lambing rates in sheep, although results were not consistent. Similar approaches in cattle have met with much less success. Immunization against ovarian steroids increased ovulation rate, but resulted in a high frequency of abnormal ovarian function (Walton, 1985; Sreenan, 1984; Webb *et al.*, 1984; Price, Morris and Webb, 1987a). Similarly, immunization against partially purified inhibin-enriched follicular fluid increased ovulation rate in heifers (Price, 1987; Price, Morris, O'shea and Webb, 1987b), but results were highly variable with some animals becoming anoestrus. More recently, immunization against synthetic inhibin preparations have been demonstrated to more consistently increase ovulation rate in heifers (Morris, McDermott and Sreenan, 1991; Glencross, Bleach, McLeod, Beard and Knight, 1992).

Thus, although more successful in sheep, endocrine manipulation is not yet a reliable technique for increasing ovulation rate in cattle.

1.5.4. Embryo transfer

Since other techniques for the reliable induction of twinning in cattle are not available, embryo transfer remains the only proven approach (Guerra-Martinez, Anderson and Dickerson, 1987; Diskin *et al.*, 1987; Davis, Harvey, Bishop and Gearheart, 1989). This approach is economically beneficial (Vincent and Mills, 1972; Diskin *et al.*, 1987; Dickerson, 1978), although there are still some technical difficulties in the practical application of this technique at the farm level.

1.6. Summary

Ovarian follicular growth and development is an extremely complicated and highly coordinated physiological event which requires very precise regulation. While the hypothalamo-pituitary-ovarian axis is central to the control of folliculogenesis by providing the primary drive for the development of antral follicles, many other factors, including metabolic hormones and some locally-produced ovarian substances, may play important modulatory roles in this process, particularly during the development of preantral follicles and the selection of the dominant follicle(s). Approaches which have been proven successful in increasing ovulation rate in sheep have failed to achieve similar results in cattle, suggesting that the relative importance of specific mechanisms may vary between different species. Therefore, to achieve reliable and precise control of ovulation rate, more fundamental studies are required to elucidate the relative importance of, and inter-relationships between, the different mechanisms involved in the control of folliculogenesis in cattle. The work in this thesis was aimed at investigating one aspect of this control system, namely the role of GH and associated factors in the control of follicular growth and development.

CHAPTER 2

Materials and Methods

2.1. Experimental animals

All animals used in the *in vivo* studies in this thesis were Hereford cross Friesian heifers, approximately 2-3 years of age. They were raised from calves at the Institute's farms in Scotland. Animals were moved into the Large Animal Unit about two months before the start of each experiment to allow for adjustment to the experimental environment, for halter training and for observations on behavioural oestrus. All heifers were kept together in a large open shed with natural photoperiod. Heifers were fed hay (dry matter, DM: 874 g/kg; crude protein: 84 g/kgDM; metabolizable energy, ME: 8.4 MJ/kgDM; digestibility: 54.3%) twice daily, supplemented with concentrates, as in normal farm practice. Fresh water was provided *ad libitum*. The body weights of all the heifers, monitored once a week, did not change significantly throughout the experimental periods. Oestrus was determined by observation at four times daily for behavioural oestrus (standing heat), aided by a KAMAR heatmount detector (Kamar Inc., Steamboat Springs, CO, USA).

All experimental procedures were carried out in compliance with the Animal (Scientific Procedures) Act, 1986.

2.2. Surgical procedures

2.2.1. Blood sampling

Infrequent blood samples were collected by venepuncture from

the jugular vein. Blood was allowed to clot overnight at room temperature before centrifuging at 1000g for 30 minutes. Serum was then harvested and stored at -20°C until required for hormone assay.

Frequent blood samples (every 10 minutes for 8 hours; "window" bleeds) were taken via indwelling jugular cannulae. Cannulation was performed the day before sampling as follows. Heifers were restrained without general anaesthesia in a crush, the skin around the jugular vein was surgically prepared and given local anaesthesia (Lignol; Arnolds Veterinary Products Ltd., Essex, England). A 13 gauge cannula (Intranule, Vygon UK Ltd., Cirencester) was inserted into the vein through a small skin incision. The cannulae was then stoppered, secured in place by two sutures and flushed with sterile heparinised saline. This procedure did not cause any obvious discomfort or distress to the animals either during or after the cannulation. Blood samples were collected into tubes containing heparin and centrifuged within 30 minutes at 1000g for 30 minutes. Plasma was harvested and stored at -20°C until required for hormone assay. Animals were decannulated immediately after sampling was finished and any bleeding was stopped easily by applying gentle direct pressure.

2.2.2. Laparoscopy

Laparoscopy was used to assess ovulation rate following superovulation. The procedures were modified from those of Holland, Bindon, Piper, Thimonier, Cornish and Radford (1981). The heifers were fasted for 48 hours prior to laparoscopy, with water also being withheld during the last 24 hours. Animals were restrained in a crush which was tilted forwards such that the hindquarters of the heifers were higher

than the forequarters. Heifers were sedated with Rompun (Intervet, Bayer UK Ltd., Bury St. Edmunds), and the right sublumbar fossa region surgically prepared and given local anaesthesia (Lignol; Arnolds Veterinary Products Ltd., Essex, England). Two small skin incisions, one approximately 10 cm ventral and dorsal to the other, were made and trocars and cannulae (Richard Wolf UK Ltd., Mitcham) were inserted in a slightly posterior direction. The peritoneal cavity was inflated with medical grade nitrous oxide. The ovaries were examined directly using an endoscope and manipulator introduced through their respective cannulae. Numbers of corpora lutea (CL), visible follicular or other structures such as cysts were recorded, with representative photography taken. After observation, gas was vented and cannulae withdrawn. No sutures were required to close the incisions and a topical antibiotic (Terramycin Spray, Pfizer Ltd., Sandwich, Kent) was applied to the incision sites. Animals were given an intramuscular (i.m.) injection of long-acting penicillin (Duphapen, Duphar Veterinary Ltd., Southampton). No obvious complications were noted as a result of this surgical procedure for any of the 48 laparoscopies that were carried out.

2.3. Non-surgical procedures

2.3.1. Ultrasound examinations

The ovaries of heifers were examined using a real-time B-mode linear array ultrasound scanner (Aloka Echo Camera SSD-210 DX II) equipped with a 7.5 MHz intrarectal probe (UST-5511I-7.5) (Aloka Co. Ltd., Japan). A plastic rod was attached to the cable of the probe to enable the manipulation of the probe from outside the rectum. Animals were

naturally standing in the stanchion while examination was carried out. The routine procedures for ultrasound scanning were as follows: (1) faeces were removed from the rectum; (2) each ovary was located and held in position with one hand; (3) the probe was inserted into the rectum and placed directly on the top of the ovary held by hand; (4) the probe was manipulated from outside the rectum using the other hand to scan slowly over the top of the ovary several times; (5) ultrasonography for each ovary was recorded on videotape (Kodar plc., USA) for subsequent analysis. Videotapes were reviewed using a television monitor and the positions and sizes of all visible antral follicles (and CL if present) were drawn in a diagram for each ovary. Diameters of individual follicles were determined using a computerised calliper device, taking the mean of 2 estimates in the vertical and horizontal planes. The serial daily diagrams obtained were used to determine the growth pattern of individual follicles which were identified by their positions in relation to other follicles and/or CL.

2.3.2. Embryo recovery

Embryos were recovered non-surgically as described previously by Brand, Aarts, Zaayer and Oxender (1978) and Newcomb, Christie and Rowson (1978) with slight modification. The animals were restrained in a conventional crush, the rectum was emptied and epidural anaesthesia was induced with Lignol (Arnolds Veterinary Products Ltd., Essex, England). The vulva and its surrounding area was cleaned and disinfected. A cannula with a central insert was then introduced into the vagina and passed through the cervix into one of the uterine horns (guided by rectal manipulation). The central insert was withdrawn and a sterile PVC 3 lumen catheter was introduced into the uterine horn as far as possible

toward the utero-tubal junction. The cuff of the catheter was then inflated to secure the catheter inside the uterine horn. 50ml of ovum culture medium (Flow Laboratories, Scotland) was flushed using a syringe into the uterine horn and the medium was then recovered into a collection tube by gently manipulating the uterus. This flushing was repeated 5 or 6 times followed by 20ml of air to clear out the medium inside the uterine horn. The cuff was then flattened and the catheter removed. A fresh catheter was used to flush the other uterine horn as described above. After flushing, each heifer was given an i.m. injection of long-acting penicillin (Duphapen, Duphar Veterinary Ltd., Southampton).

The collection tubes were allowed to stand at room temperature for about 30 minutes and the medium was removed carefully using a syringe until approximately 10ml of medium remained. The medium was emptied into a dish which was then placed under a dissecting microscope to examine for eggs. All the eggs recovered were classified into the following three groups according to their morphologiesas as described by Seidel, Seidel and Bowen (1980): unfertilised eggs, transferable embryos and degenerate embryos.

2.4. Radioimmunoassays

All the assays were dispensed in 12 x 75mm plastic tubes except for progesterone and oestradiol assays where 12 x 75mm glass tubes (both obtained from LIP Equipment and Services Ltd., West Yorks, England) were used. All standard tubes were run in triplicate and unknown samples in duplicate.

2.4.1. Assay buffers

Assay buffers were freshly prepared for each assay, using double-distilled water (referred to as "water" below) purified by a Waters Milli-Q purification system (Millipore Corporation, Milford, MA, USA) as diluent. All buffers were filtered through filter paper prior to use to remove particulate matter.

(1) 0.5M phosphate buffer, pH 7.5 (phosphate buffer)

This buffer was prepared by adjusting the pH of 0.5M di-sodium hydrogen orthophosphate (BDH Chemicals Ltd., Poole, England) to 7.5 using 0.5M sodium dihydrogen orthophosphate (FSA Laboratory Supplies, Loughborough, England).

(2) 0.1% phosphate gelatin assay buffer (0.1% gelatin)

This buffer contained 9g of sodium chloride, 1g of swine skin gelatin (300 bloom), 100mg of thimerosal (all obtained from Sigma, Poole, Dorset, UK) and 100ml of phosphate buffer per litre.

(3) 0.1% phosphate BSA assay buffer (0.1% BSA)

This buffer was made up of 9g of sodium chloride, 1g of BSA (Boehringer Mannheim GmBH, Germany), 100ml of phosphate buffer and 100mg of thimerosal per litre.

(4) 0.5% phosphate BSA assay buffer (0.5% BSA)

This buffer contained 9g of sodium chloride, 5g of BSA, 100ml of phosphate buffer and 100mg of sodium azide (BDH Chemicals Ltd., Poole) per litre.

(5) Borate buffer, pH 7.8

3.1g of boric acid (FSA Laboratory Supplies, Loughborough), 2.0g of BSA and 1.0g of sodium azide were dissolved in 1000ml of water. The pH of the solution was then adjusted to 7.8 with 1M NaOH (FSA Laboratory Supplies).

(6) IGF-I assay buffer, pH 7.5

4.14g of NaH_2PO_4, 3.72g of EDTA (Fisons Scientific Equipment, Loughborough, England), 10ml of 2% sodium azide, 0.5ml of Tween 20 (Sigma) and 1g of BSA were dissolved in 1000ml of water. The pH of the solution was then adjusted to 7.5 with 2M NaOH.

2.4.2. Assay calculations

All assays were analysed on a Macintosh computer using the AssayZap software (Zaristow Software, Haddington, East Lothian, Scotland). Assay sensitivities, within- and between-assay coefficients of variation for each hormone, were calculated from all individual assays in this thesis and are described below. The ALLFIT program (de Lean, Munson and Rodbard, 1978) was used to analyse the displacement curves for parallelism.

2.4.3. Luteinising hormone (LH)

Concentrations of LH were determined by the double antibody RIA described by Price *et al.*, (1987a). The preparation used for iodination was USDA-bLH-I1 and the hormone preparation used as standard was USDA-bLH-B5. The anti-serum (R.B. Staigmiller, USDA) was affinity-purified

and diluted in assay buffer (0.1% BSA) to an initial dilution of 1:20,000. Standard solutions (ranging from 0.05 to 2.0 ng/tube) and samples (100µl) were made up to 500µl with assay buffer and incubated with 200µl antiserum for 48 hours at 4°C. Then 100µl [^{125}I]-labelled LH (≈12,000cpm) was added and tubes were incubated for a further 48 hours at 4°C. Bound hormone was separated by the addition of 100µl of normal rabbit serum (NRS; diluted 1:400) and 200µl of donkey anti-rabbit serum (DARS; diluted 1:45 incorporating 0.01M EDTA) (both obtained from Scottish Antibody Production Unit, Law Hospital, Carluke, Lanarkshire, Scotland). Tubes were incubated overnight at 4°C, then 1ml of cold assay buffer was added and tubes were centrifuged at 2000g for 30 minutes at 4°C. Supernatants were decanted and pellets counted on a gamma-counter. The sensitivity of the assay was 0.5 ng/ml, and the inter-assay and intra-assay coefficients of variation (CV) were 10.5% and 6.3% respectively.

2.4.4. Follicle-stimulating hormone (FSH)

FSH concentrations were measured by a radioimmunoassay routinely used in our laboratory (Bolt and Rollins, 1983; Law *et al.*, 1992). The preparation used for iodination was USDA-bFSH-BP3 and the hormone preparation used as standard was USDA-bFSH-B-1. The anti-serum was USDA-5-pool (bFSH-B antiserum) and was diluted in assay buffer (0.1% BSA) to an initial dilution of 1:10,000. Standard solutions (ranging from 0.5 to 32 ng/tube) and samples (250µl) were made up to 500µl with assay buffer and incubated with 200µl antiserum for 48 hours at 4°C. Then 100µl [^{125}I]-labelled bFSH (≈12,000cpm) was added and tubes were incubated for a further 48 hours at 4°C. Bound hormone was separated by the addition of 100µl of NRS (1:400) and 200µl of DARS (1:45 incorporating

0.01M EDTA). Tubes were incubated overnight at 4°C, then 1ml of cold assay buffer added and tubes centrifuged at 2000g for 30 minutes at 4°C. Supernatants were decanted and pellets counted on a gamma-counter. The sensitivity of the assay was 2.1 ng/ml. The inter-assay CV was 13.9% and the intra-assay CV was 8.3%.

2.4.5. Oestradiol

Peripheral serum oestradiol concentrations were measured by a RIA incorporating an affinity chromatography extraction procedure as described previously (Webb, Baxter, McBride, Nordblom and Shaw, 1985).

For extraction, 3ml serum samples were dispensed into 16 x 125mm screw-capped glass tubes followed by approximately 1500dpm [^{3}H]-oestradiol-17β (Amersham International plc, UK) in 10μl HPLC-grade absolute ethanol (FSA Laboratory Supplies). 7ml of water and 400μl Sepharose-linked ovine anti-oestradiol antiserum were then added and the tubes were mixed end-over-end at room temperature overnight. The samples, with the subsequent washing of 7ml water, were poured onto pre-prepared chromatography columns fitted with a sinter disc (Schott Glass, UK). Columns were then washed three times with 7ml water and residual water after the last washing was removed by applying gentle air pressure. Bound oestradiol was then eluted into glass tubes (16 x 125mm) with 3ml of 90% methanol. Methanol was evaporated in a Buchler vortex evaporator at 40°C and 1.8ml of 0.1% gelatin was then added to each tube. 500μl of each reconstituted sample was counted in a beta-counter for [^{3}H] activity to determine the extraction efficiency. Reconstituted samples (2 x 500μl) were taken for assay. The Sepharose-linked ovine anti-oestradiol was then recovered, recycled (washing with 3 cycles of 1 x 3ml

of 90% methanol and 3 x 7ml of water), made up to the original volumn with water and kept at 4°C for re-use. Chromatography columns were also washed and recycled (washing with 3 cycles of 1 x 3ml of 90% methanol and 3 x 7ml of water) ready for re-use.

The assay procedure was as follows. Standard solutions (ranging from 0.5 to 48 pg/tube in 500µl 0.1% gelatin) and reconstituted samples (500µl) were incubated with 200µl antiserum (R48; initial dilution 1:40,000) and 100µl [^{125}I]-labelled oestradiol (≈12,000cpm) for 3 hours at room temperature. 100µl of NRS (1:400) and 100µl of DARS (1:40 incorporating 0.01M EDTA) were then added and the tubes were incubated overnight at 4°C. Following the addition of 1ml of cold assay buffer, the tubes were centrifuged at 2000g for 30 minutes at 4°C. The supernatant was decanted and the pellet counted on a gamma-counter. The sensitivity of the assay was 1.2 pg/ml. The inter-assay CV was 10.3% and the intra-assay CV was 7.4%.

2.4.6. Progesterone

Concentrations of progesterone in peripheral serum were determined using a RIA described previously (Corrie, Hunter and Macpherson, 1981) and modified for a non-extraction procedure (Law *et al.*, 1992). 50µl of serum from ovariectomized cattle was added to the non-specific binding tubes and all the standard tubes. [^{125}I]-labelled progesterone (obtained from MRC Reproductive Biology Unit, Edinburgh) was diluted in assay buffer (0.1% gelatin) incorporating 1mg/ml 8-anilino-1-naphthalene sulfonic acid (Sigma, Poole). The final assay procedure was as follows. Standard solutions (ranging from 7.8 to 1000 pg/tube) and samples (50µl) were made up to 500µl with assay buffer and

incubated with 200µl antiserum (initial dilution 1:8,000) and 100µl [^{125}I]-labelled progesterone (≈12,000cpm) for 3 hours at room temperature. 100µl of NRS (1:300) and 100µl of DARS (1:35 incorporating 0.01M EDTA) were then added and the tubes were incubated overnight at 4°C. Following the addition of 1ml cold assay buffer, the tubes were centrifuged at 2000g for 30 minutes at 4°C. The supernatant was decanted and the pellet counted on a gamma-counter. The sensitivity of the assay was 0.15 ng/ml. The inter- and intra- assay CVs were 8.4% and 5.2% respectively.

2.4.7. Growth hormone (GH)

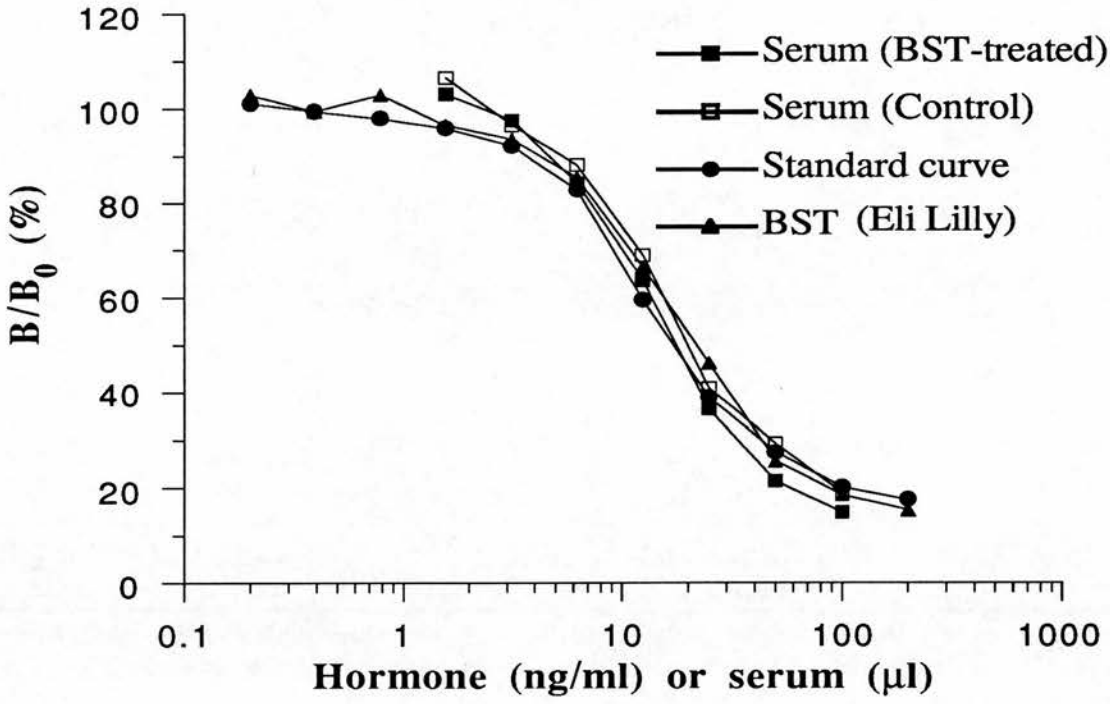
GH concentrations were determined by a modified form of the RIA of Hart, Flux, Andrews and McNeilly (1975), previously validated within the Institute (Lovendahl, Angus and Woolliams, 1991). Recombinant bovine somatotropin (American Cyanamid Company, Princeton, New Jersey, USA) was used as both standard and tracer. The guinea pig anti-bovine GH serum was used at an initial dilution of 1:10,000 in assay (borate) buffer. Various amounts of standard (ranging from 0.02 to 20 ng/tube) and samples were made up to 100µl with assay buffer, mixed with 50µl tracer (≈20,000cpm) and 50µl first antibody and incubated overnight at 4°C. Separation of bound and free tracer was achieved by addition of 100µl of donkey anti-guinea-pig SAC CEL (Immunodiagnostic Ltd., Boldon Business Park, Boldon, Tyne and Wear, UK; which consists of a second antibody covalently coupled to cellulose), followed by addition of 1ml water and centrifugation at 2000g for 30 minutes. Supernatants were aspirated and pellets counted. Displacement curves of recombinant bovine somatotropin (BST) (Somidobove, Eli Lilly & Co., IN, USA) solutions used for injection and sera from both BST-treated and control heifers

were parallel to the standard curve (Figure 2.1). The sensitivity of the assay was 1.2 ng/ml. The inter-assay CV was 9.9% and the intra-assay CV was 7.4%.

2.4.8. Insulin-like growth factor I (IGF-I)

Concentrations of IGF-I were measured by a RIA as described previously within our Institute by Armstrong, Duclos and Goddard (1990), with a modification of the sample extraction procedure which was validated for cattle. The serum samples were extracted as described by Enright, Chapin, Moseley, Zinn, Kamdar, Krabill and Tucker (1989) using a mixture of ethanol : acetone : acetic acid (60 : 30 : 10 by volume) (FSA Laboratory Supplies). Briefly, 100µl of sample was mixed with 400µl of extraction mixture and incubated for 30 minutes at room temperature, followed by centrifugation at 1800g for 30 minutes at 4°C. 250µl of supernatant was neutralized with 100µl 0.855M Tris (Sigma), then centrifuged for another 30 minutes and the supernatant kept for assay. The assay procedures were as follows. Various amounts of standard (ranging from 10 to 4000 pg/tube) and extracted samples (10µl) were made up to 200µl with assay buffer and incubated with 200µl antiserum (initial dilution 1:4000) for 24 hours at 4°C. 100µl of tracer (≈30,000cpm) was added, followed by incubation for a further 24 hours at 4°C. 100µl of DARS (diluted 1:20 in assay buffer) containing NRS (1:200) were then added and tubes were incubated for 3 hours at room temperature. Following addition of 1ml of 4% polyethylene glycol (mw: 8000; Sigma), incubation for 30 minutes at room temperature and centrifugation for 15 minutes at 1800g at 4°C, 100µl of 10% starch (BDH Chemicals Ltd.) was added and the tubes were centrifuged for another 15 minutes at 1800g. Supernatants were aspirated and pellets were counted in a gamma-

Figure 2.1. Comparison of a standard curve for growth hormone and displacement curves of recombinant bovine somatotropin used for injection (BST: Somidobove, Eli Lilly & Co., IN, USA) and serum obtained from both control and BST-treated heifers.

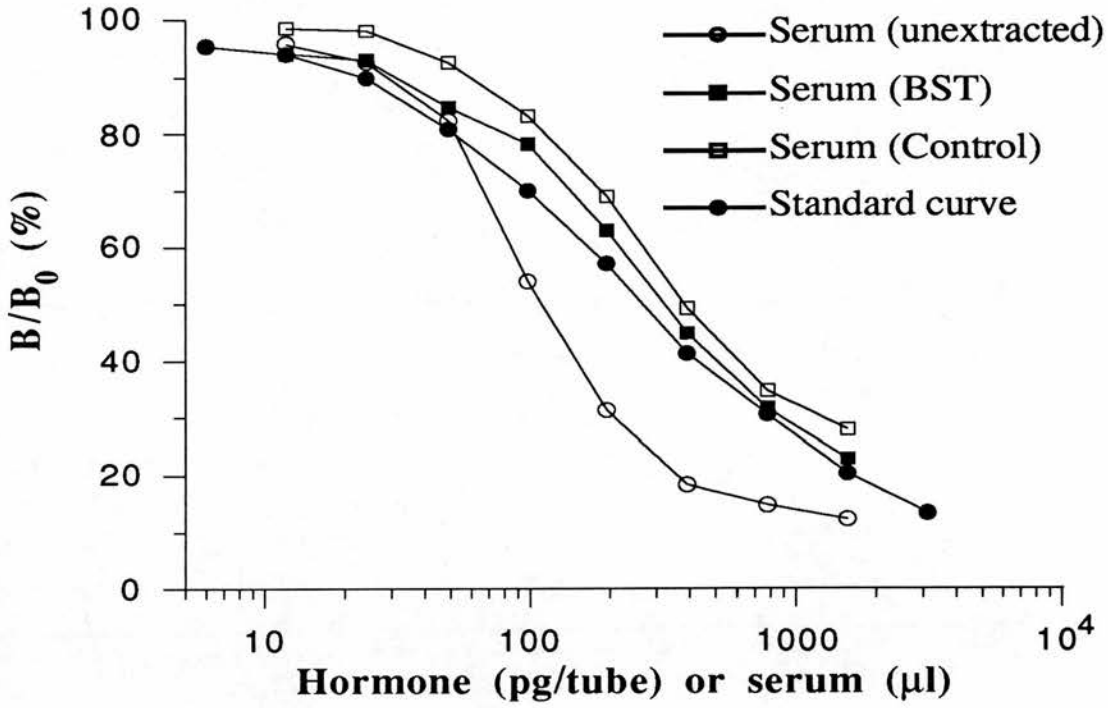

120
100
80
60
40
20
0
B/B0 (%)
0.1
1
10
100
1000
Hormone (ng/ml) or serum (µl)
Serum (BST-treated)
Serum (Control)
Standard curve
BST (Eli Lilly)

counter. While the dilution-response curve of unextracted serum was not parallel to the standard curve, displacement curves of extracted serum samples from both control and BST-treated heifers showed excellent parallelism with the standard curve (Figure 2.2). Recovery efficiency of the extraction, assessed by the recovery of IGF-I or [^{125}I]-labelled IGF-I added into serum samples, was 101.2 ± 3.8%. The sensitivity of the assay was 20.3 ng/ml. The inter-assay CV was 12.9% and the intra-assay CV was 5.7%.

2.4.9. Insulin

Insulin concentrations were determined using a RIA described previously by Osmond, Carr, Hinks, Land and Hill (1981) with modification and revalidation. Bovine insulin was obtained from Sigma and [^{125}I]-labelled bovine insulin from Amersham (UK). The first antibody was a guinea pig anti-bovine insulin (Miles Laboratories, Slough) and was used at a final dilution of 1:120,000. The assay procedures were as follows. Various amount of standard solutions (ranging from 5 to 5000 pg/tube) and samples (250µl) were made up to 500µl with assay buffer (0.5% BSA) and incubated with 200µl antiserum for 48 hours at 4°C. Then 100µl [^{125}I]-labelled insulin (≈12,000cpm) was added and tubes were incubated for a further 48 hours at 4°C. The bound and free fractions were separated by the addition of 100µl of normal guinea pig serum (1:200) and 200µl of sheep anti-guinea pig serum (1:25 incorporating 0.01M EDTA) (both obtained from Scottish Antibody Production Unit, Law Hospital, Carluke, Lanarkshire, Scotland). Tubes were incubated overnight at 4°C, 1ml of cold assay buffer added and tubes were centrifuged at 2000g for 30 minutes at 4°C. The supernatant was decanted and pellet counted on a gamma-counter.

Figure 2.2. Comparison of a standard curve for insulin-like growth factor I and dilution-response curves of unextracted bovine serum (unextracted; diluted 1:150) and extracted serum from control (Control) and BST-treated (BST) heifers.


100
80
60
40
20
0
B/B0 (%)
10
100
1000
10^4
Hormone (pg/tube) or serum (µl)
Serum (unextracted)
Serum (BST)
Serum (Control)
Standard curve

At a final dilution of 1:120,000, the antibody bound 38 to 50% of [^{125}I]-labelled bovine insulin with an ED_{50} of 165.2 ± 7.6 pg (n = 13 assays) bovine insulin/tube. Dilution-response curves of serum samples from both control and BST-treated heifers showed excellent parallelism with the standard curve (Figure 2.3). Recombinant human IGF-I (Eli Lilly) and IGF-II (Ciba Geigy), purified pituitary bovine GH (USDA-bGH-B-1) and BST at doses of up to 1600 ng/tube did not crossreact with the guinea pig anti-bovine insulin antibody (Figure 2.4). Bovine insulin added to serum samples was recovered with an efficiency of 98.4 ± 3.2 %. The minimum detectable insulin concentration was 0.08 ng/ml. The inter- and intra- assay CV were 9.3% and 5.8% respectively.

2.5. FSH and LH binding assays

FSH and LH binding to bovine granulosa and thecal cells and LH binding to CL were determined as described by Bramley, Stirling, Swanston, Menzies and Baird (1987b). The results were expressed as picograms of [^{125}I]-labelled hormone bound specifically per milligram of protein.

2.6. Culture of bovine granulosa cells

For *in vitro* studies, bovine granulosa cells were cultured using an ovine granulosa cell serum-free culture system developed in our laboratory (Webb and McBride, 1991) with slight modifications. All the reagents, unless otherwise specified, were obtained from Sigma (Poole, Dorset, UK). The detailed procedures were as follows.

Figure 2.3. Comparison of a standard curve for insulin and dilution-response curves of serum samples from both control (Control) and BST-treated (BST) heifers.

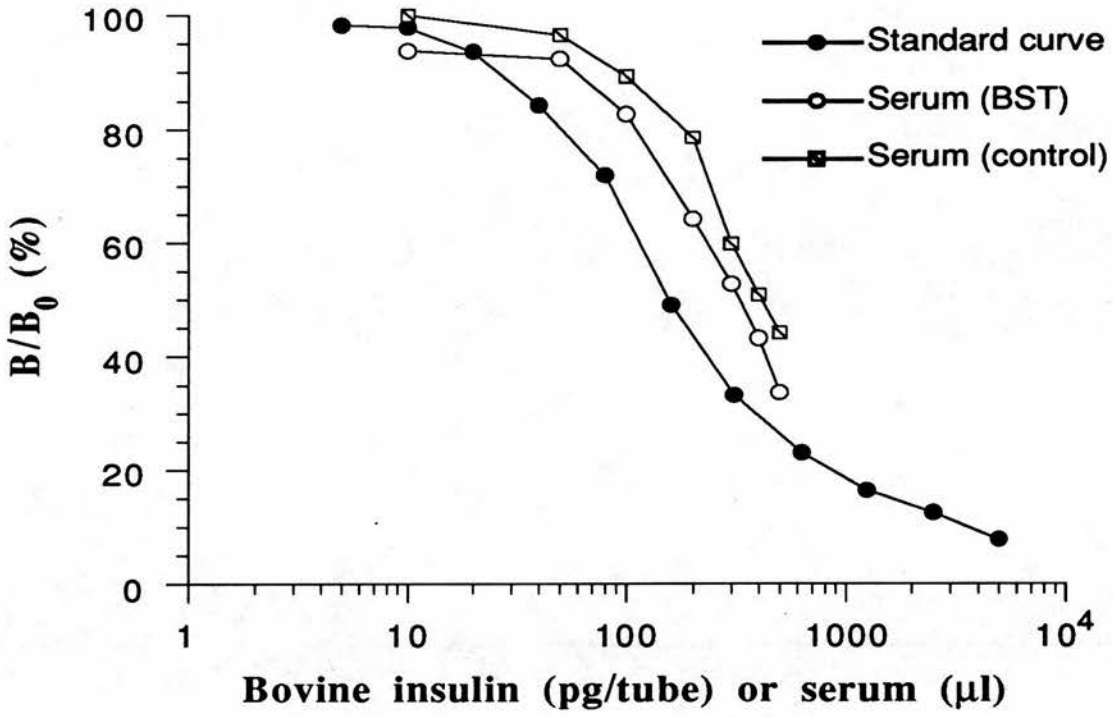
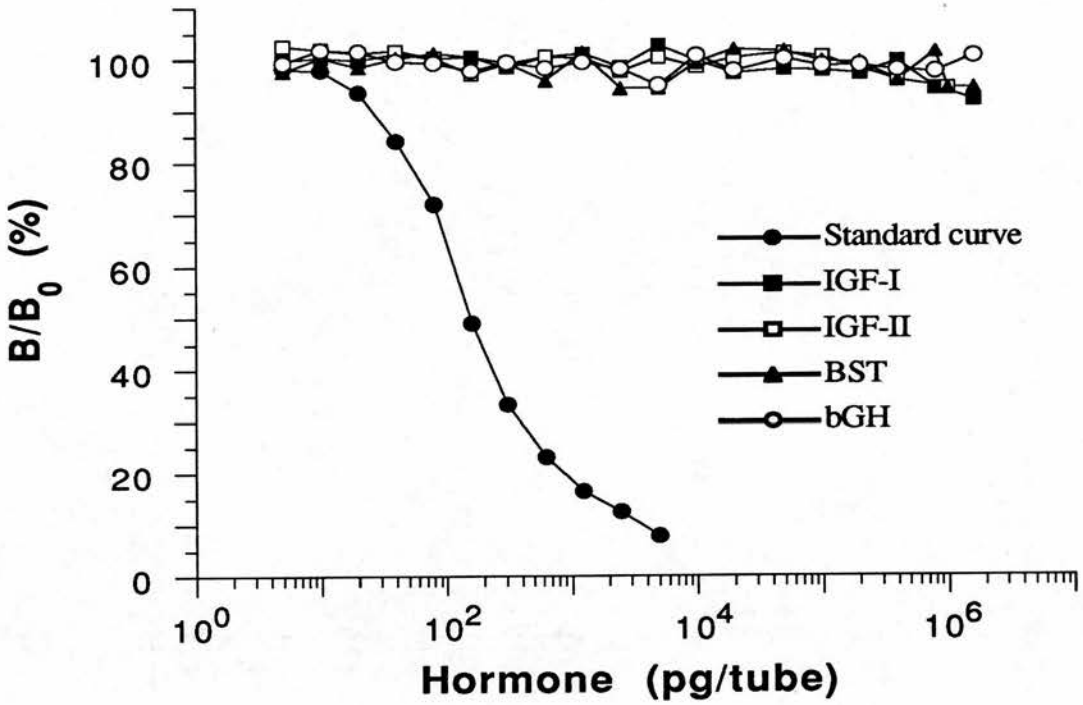

100
80
60
40
20
0
B/B0 (%)
1
10
100
1000
10^4
Bovine insulin (pg/tube) or serum (µl)
Standard curve
Serum (BST)
Serum (control)

Figure 2.4. Specificity of guinea pig anti-bovine insulin serum used in the insulin RIA. The standard curve was tested against recombinant human IGF-I (Eli Lilly & Co., USA) and IGF-II (Amersham, UK), recombinant bovine somatotropin (BST) and purified pituitary bovine growth hormone (bGH, USDA-bGH-B-1).


100
80
60
40
20
0
B/B0 (%)
10^0
10^2
10^4
10^6
Hormone (pg/tube)
Standard curve
IGF-I
IGF-II
BST
bGH

(1). Coating of plates: 500µl of donor calf serum (GIBCO Life Technologies Ltd., Scotland) was added to each well of the plates (48-well tissue culture clusters, Costar, MA, USA). The plates were then incubated overnight at 37°C in a humidified atmosphere of 2% CO_2 + 98% air.

(2). Preparation of culture medium: 100ml of Medium199 containing Earle's salts and 20mM Hepes without glutamine was supplemented with 2mM glutamine, 1mM sodium pyruvate, 1mM sodium bicarbonate and 1ml of penicillin/streptomycin solution. 70nM testosterone was added as a substrate for oestradiol synthesis.

(3). Preparation of granulosa cells: Bovine ovaries were collected from a local abattoir into a thermo-flask containing Medium199, 1% (w/v) BSA and 1% (v/v) penicillin/streptomycin (37°C) and transported to the laboratory within 1 hour of slaughter. The follicles were dissected out in supplemented medium and grouped according to their size (<5mm, 5-10mm and >10mm). After removal of as much stroma as possible, follicles were punctured with an 18-guage needle. The inner wall of the punctured follicle was then scraped gently to remove granulosa cells, leaving the basement membrane and theca shells intact. Granulosa cells were harvested by centrifuging the medium at 700g for 10 minutes. The cells were washed twice with medium and the number of cells was then estimated using a haemocytometer. Viability of cells was assessed by trypan blue exclusion (cell suspensions were diluted 1:1 with 0.4% trypan blue solution and incubated for 10-15 minutes: dead cells stain blue) and ranged from 55% to 72%.

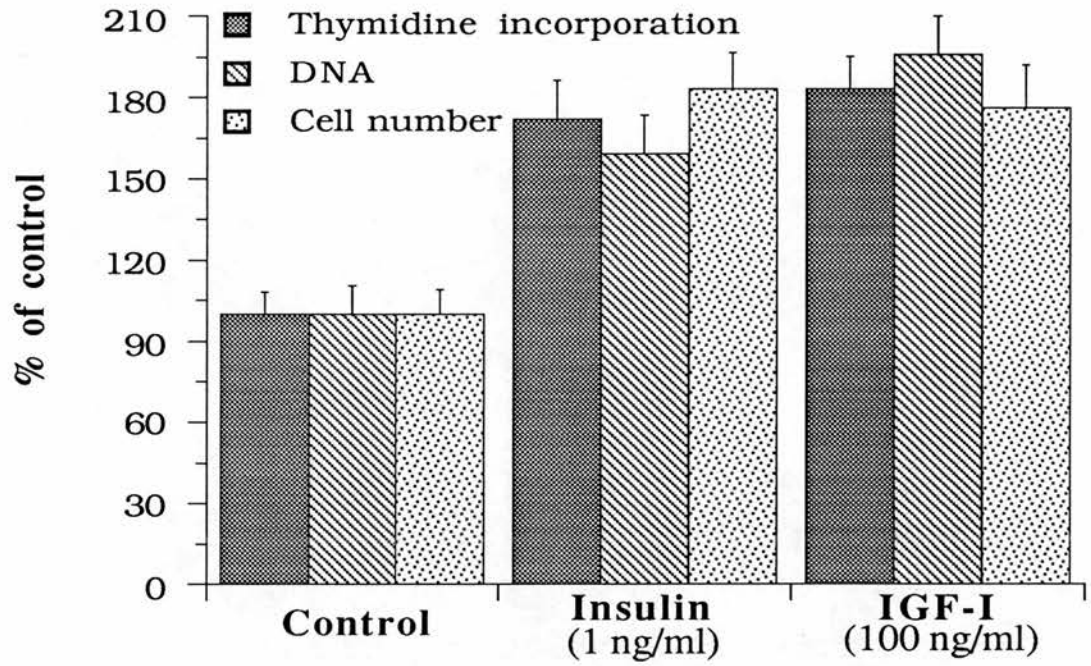
(4). Plating out: The donor calf serum was decanted from the precoated plates and the plates were washed with 1ml of Medium199. Granulosa cells were then plated out at a density of 5×10^5 cells/ml (250

µl/well). The plates were left for approximately 24 hours at 37°C in a humidified atmosphere of 2 % CO_2 : 98% air to allow the cells to adhere to the plate.

(5). Challenge tests: 24 hours after plating out, medium was removed and cells were washed once with 0.5ml of supplemented medium. The cells were then challenged with various hormones (as detailed in Chapter 8) followed by the addition of approximately 0.2µCi of [^{3}H]-thymidine (80 Ci/mMol, Amersham plc.).

(6). [^{3}H]-thymidine incorporation assay: The proliferation of granulosa cells was monitored by [^{3}H]-thymidine incorporation into the cells. Approximately 24 hours following the challenge test, the medium was removed. Cells were washed with 1ml of cold-phosphate buffered saline (Northumbria Biologicals Ltd., UK). 1ml of cold 10% trichloroacetic acid (TCA) (BDH Chemicals Ltd.) was then added to each well and the plates were left at room temperature for 30 minutes. TCA was then removed and cells were solubilised with 0.5ml of 0.5M NaOH per well and the plates were left overnight at room temperature. Cell suspensions were then pipetted into plastic scintillation vials followed by the addition of 50µl concentrated HCl and 4.5 ml of Optiphase X (FSA Laboratory Supplies). The samples were then counted in a beta-counter. To validate that [^{3}H]-thymidine incorporation was a marker for granulosa cell proliferation, it was compared with both cell number and deoxyribonucleic acid (DNA) contents (measured by the method of West, Scattar and Kumar; 1985). As shown in Figure 2.5, [^{3}H]-thymidine incorporation was highly correlated with both cell number and DNA concentration ([^{3}H]-thymidine incorporation versus cell number: $r = 0.71$, $p<0.01$, $n = 12$; [^{3}H]-thymidine incorporation versus DNA concentration: $r = 0.79$, $p<0.01$, $n = 12$).

Figure 2.5. Comparison of [^{3}H]-thymidine incorporation, DNA concentration and cell number in bovine granulosa cells in serum-free culture. The correlation between [^{3}H]-thymidine incorporation and both DNA concentrations and cell number was examined for control, IGF-I- and insulin-stimulated cultured granulosa cells (see text).


% of control
210
180
150
120
90
60
30
0
Thymidine incorporation
DNA
Cell number
Control
Insulin
(1 ng/ml)
IGF-I
(100 ng/ml)

2.7. Statistical analyses

All data with continuous variation are presented as arithmetic means ± s.e.m.. Depending on the type of data, statistical differences between treatment groups were tested using either Student's *t* test or ANOVA (allowing for repeated measurements if applicable). Data with discrete observations were analysed using the Chi square test. The pulsatile characteristics of LH secretion were analysed by the Hormone Pulse-Profile Analysis programme (Munro Zaristow Software, Scotland) using a regional coefficient of variation algorithm as described previously (Veldhuis, Weiss, Mauras, Rogol, Evans and Johnson, 1986a). The correlations between different parameters were determined by regression analysis. All statistical analyses were performed on a Macintosh computer using the Minitab statistical software (Pennsylvania State University, USA).

CHAPTER 3

The effect of recombinant bovine somatotropin on ovarian follicular populations and peripheral hormones in heifers

3.1. Introduction

The role of pituitary gonadotrophins in the control of ovarian folliculogenesis in cattle has been well established (Ireland, 1987). However, although exogenous gonadotrophin treatment can induce superovulation (Monniaux *et al.*, 1983) the physiological mechanisms controlling ovulation rate are still not fully understood. Attempts to increase ovulation rate by utilitization of both active and passive immunization against either gonadal steroids and/or partially-purified follicular fluid proteins has demonstrated that changes in ovulation rate were not consistently correlated with changes in the pattern of gonadotrophin secretion (see Webb and Morris, 1988). This, together with the fact that the fate of the dominant follicle(s) differs from other cohort antral follicles recruited at a similar time, despite being exposed to the same gonadotrophin environment, suggests that gonadotrophins may not be solely responsible for the control of folliculogenesis and that other mechanisms may play an important role.

The possible involvement of GH in the regulation of ovarian follicular growth and development in mammals has been suggested by several observations. Circulating concentrations of GH increase rapidly during pubertal development (Ojeda and Jameson, 1977). Delay in puberty has been associated with GH deficiency and this can be restored by

administration of exogenous GH, both in humans and in rats (Sheikholislam and Stempfe, 1972; Ramaley and Phares, 1980). Furthermore, GH administered to hypophysectomized rats increased the steroidogenic responsiveness of granulosa cells to FSH *in vitro*, while suppression of endogenous GH secretion in intact rats, using an intra-hypothalamic implant of GH, reduced ovarian LH receptor content and steroidogenic response to hCG, with these latter effects being overridden by replacement of GH (Advis *et al.*, 1981). More recently, GH has been shown to enhance FSH-stimulated differentiation of cultured rat granulosa cells and increase ovarian secretion of immunoreactive IGF-I *in vivo* (Jia *et al.*, 1986; Davoren and Hsueh, 1986), a peptide demonstrated to be a local regulator of ovarian function (Adashi *et al.*, 1985a). In another study, GH stimulated IGF-I and progesterone secretion from porcine granulosa cells *in vitro* (Hsu and Hammond, 1987a). Moreover, GH-dependent insulin has also been suggested to play a role in the regulation of ovarian physiology (Adashi *et al.*, 1985a; Poretsky and Kalin, 1987). Taken as a whole these observations support a role for GH in the regulation of ovarian function, though the exact mechanism of action remains to be elucidated.

A recent report that treatment of lactating dairy cows in early postpartum with recombinant bovine somatotropin (BST) increased the incidence of twin-calving (Butterwick *et al.*, 1988) raised the possibility that GH may also play a role in the control of follicular growth and development in cattle, although the underlying mechanism was not investigated. This was supported by a more recent report that natural twinning in cattle was associated with higher peripheral concentrations of IGF-I (Echternkamp, Spicer, Gregory, Canning and Hammond, 1990), although no data on GH levels were reported.

Hence the aims of this study were to investigate the possible effects of BST on ovarian folliculogenesis by measuring follicular populations and ovulation rate in mature heifers, and to investigate the underlying mechanism(s) by determining peripheral concentrations of GH, IGF-I, insulin, steroids and gonadotrophins during the treatment.

3.2. Materials and methods

3.2.1. Experimental procedures

The experimental protocol is shown in Figure 3.1. Briefly, oestrous cycles of 12 heifers were synchronized by two i.m. injections of 500 μg synthetic prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Estrumate, Coopers Animal Health Ltd., Crewe, Cheshire, UK), given 12 days apart. Heifers were divided randomly into two groups of 6 (BST group and control group). From Day 7 of the first oestrous cycle, animals in the BST group were injected subcutaneously each day (at 9:00am) with a sterile preparation of 25mg BST (Somidobove; Eli Lilly & Co.; 1 mg/ml in a vehicle containing 0.9% NaCl, 0.025M $NaHCO_3$ and 0.025M Na_2CO_3, pH 9.6), for a period of two oestrous cycles until slaughter. Control animals received 25ml of vehicle only. Blood samples were collected by jugular venepuncture three times a week prior to the daily treatment from Day 3 of the first oestrous cycle until the end of experiment. Serum was assayed for peripheral GH, IGF-I, insulin, FSH, LH, oestradiol and progesterone concentrations. Frequent serial blood samples were taken on Days 12 and 19 of the second oestrous cycle. Plasma was measured for GH, insulin, IGF-I, FSH and LH concentrations. At the end of treatment (approximately Day 7 of the third oestrous cycle) heifers were sent for slaughter and the ovaries collected.

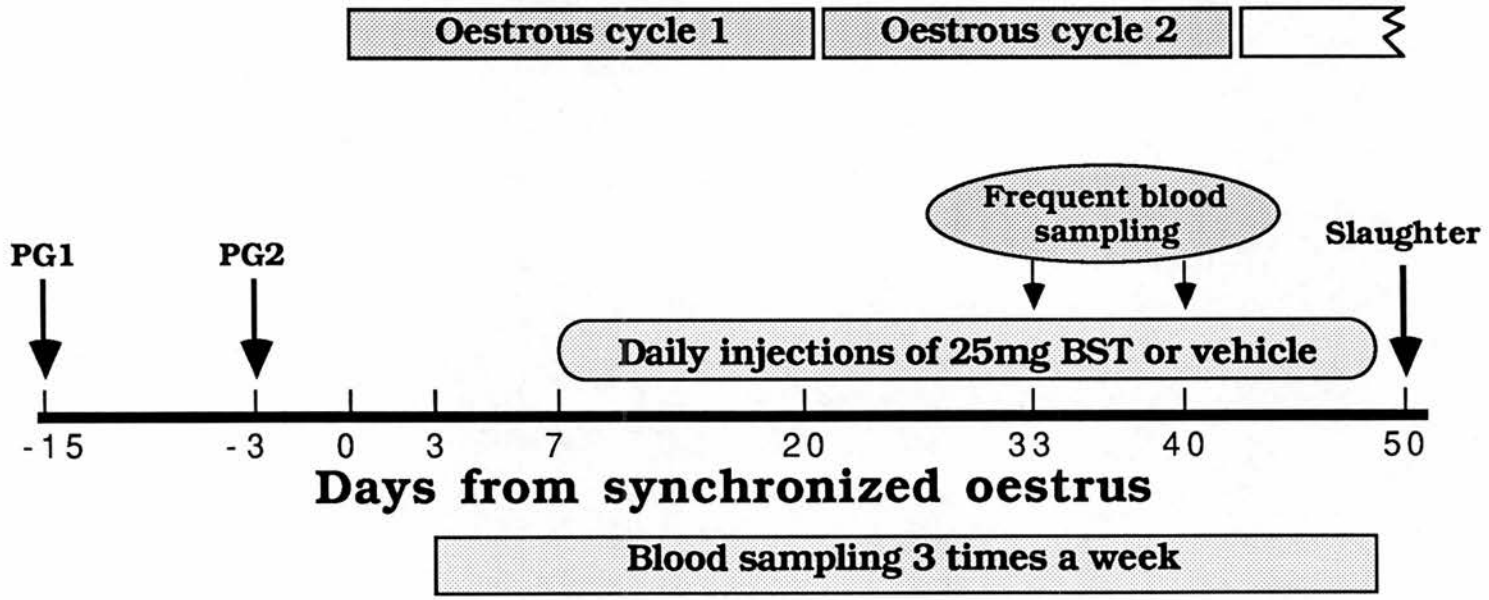


Figure 3.1. Experimental protocol

Ovulation rate was determined by counting the number of fresh CL in the pair of ovaries. All antral follicles ≥2mm in diameter were dissected and diameters measured using vernier calipers to assess antral follicle populations. Granulosa and thecal cells from the 3 largest follicles and CL, for each heifer, were collected into liquid nitrogen and stored at -80°C for FSH and LH binding measurements.

3.2.2. Statistical analyses

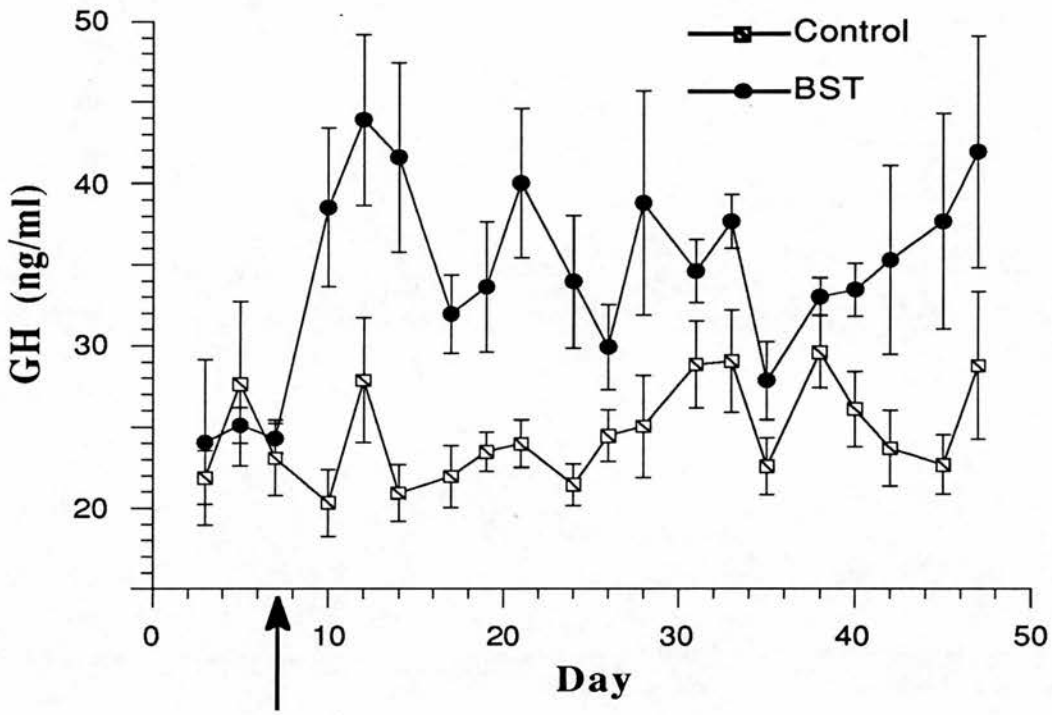
Temporal hormone data were plotted relative to BST treatment. Statistical differences between the two treatment groups in terms of follicular populations, length of the oestrous cycle, length of the luteal and follicular phases, LH pulse characteristics and FSH and LH binding data were tested by Student's *t* test. All temporal hormone data were analyzed separately before and after the start of BST treatment by split-plot ANOVA for repeated measures. The main effects included treatment, time and the interaction of treatment by time. Analysis of serum progesterone, oestradiol, FSH and LH data was also performed relative to the stage of the oestrous cycle. For this purpose the beginning of the luteal and follicular phases were defined as the first sample with a progesterone concentration above or below 1ng/ml respectively. Correlations between follicular populations and mean GH, IGF-I and insulin concentrations throughout the treatment period were determined by regression analysis.

3.3. Results

3.3.1. Peripheral GH concentrations

As shown in Figure 3.2, GH concentrations were not different

Figure 3.2. Mean (±sem) serum GH concentrations for the two treatment groups (n = 6) before and after the start of daily BST injection. Day 0 on the horizontal axis represents the day of oestrus after the second injection of prostaglandin. Blood samples were taken three times a week just prior to the daily subcutaneous BST injection, beginning on Day 3 of the first oestrous cycle with the daily BST injection starting on Day 7 as indicated by the arrow.


Control
BST
GH (ng/ml)
50
40
30
20
0
10
20
30
40
50
Day

between the two treatment groups before the start of daily BST injection (effect of treatment, $P>0.05$). Heifers treated with BST had higher peripheral GH concentrations throughout the treatment period compared to the controls after the start of BST injection (effect of treatment, $P<0.01$). Treatment by day interaction was not significant ($P>0.05$) either before or after BST treatment, whilst the effect of day was significant ($P<0.05$) after BST treatment. Since blood samples were taken immediately prior to the daily subcutaneous BST injections, the GH concentrations measured in the BST-treated heifers reflected GH levels approximately 24 hours after the previous BST injection.

GH concentrations in serial plasma samples, collected every 10 min for 8 h on Days 12 and 19 of the second oestrous cycle (with the daily BST injection given at the time of the 6th sampling), showed the acute response to BST injection (Figure 3.3). BST-treated heifers had significantly (effect of treatment, $P<0.01$) higher GH concentrations throughout the whole of the sampling period compared to control animals. The higher concentration of GH in the BST group before injection was due to the persistence of BST from the previous days treatment. There was a significant ($P<0.05$) effect of time, and treatment by time interaction after BST injection. There were no significant ($P>0.05$) differences in the GH response to BST between the mid-luteal and the follicular phases of the oestrous cycle.

3.3.2. Peripheral IGF-I concentrations

Mean (± s.e.m.) peripheral IGF-I concentrations for the heifers in the control group were maintained at a stable level throughout the experiment (Figure 3.4). Similar concentrations were measured in BST-

Figure 3.3. Mean (±sem) plasma GH concentrations measured in the serial blood samples from the two treatment groups (n = 6) before and after daily BST injection. Blood samples were taken every ten minutes for eight hours, with the BST injection given at the time of sixth sampling as indicated by the arrow, on Day 12 (luteal phase, upper panel) and Day 19 (follicular phase, lower panel) of the second oestrous cycle.

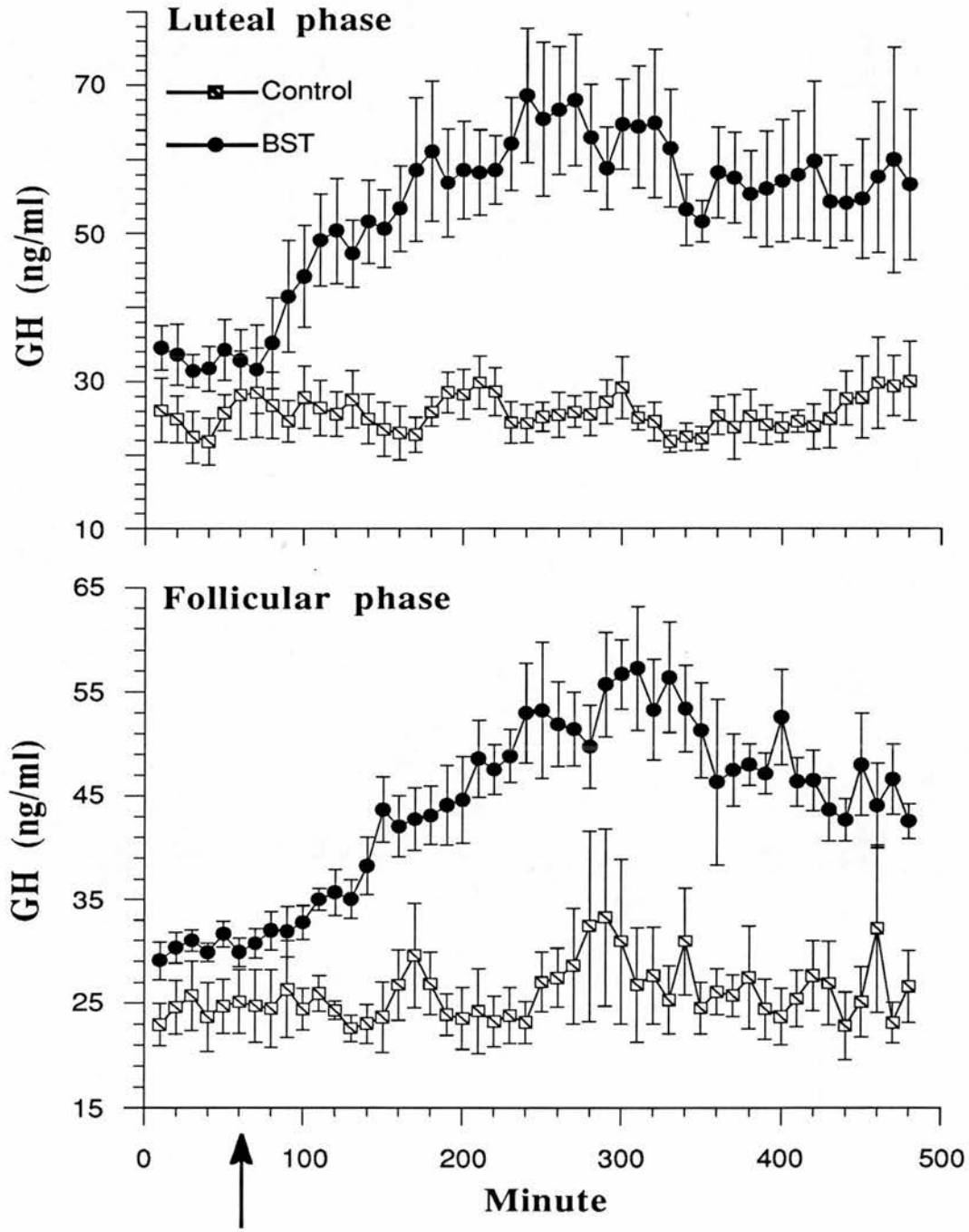
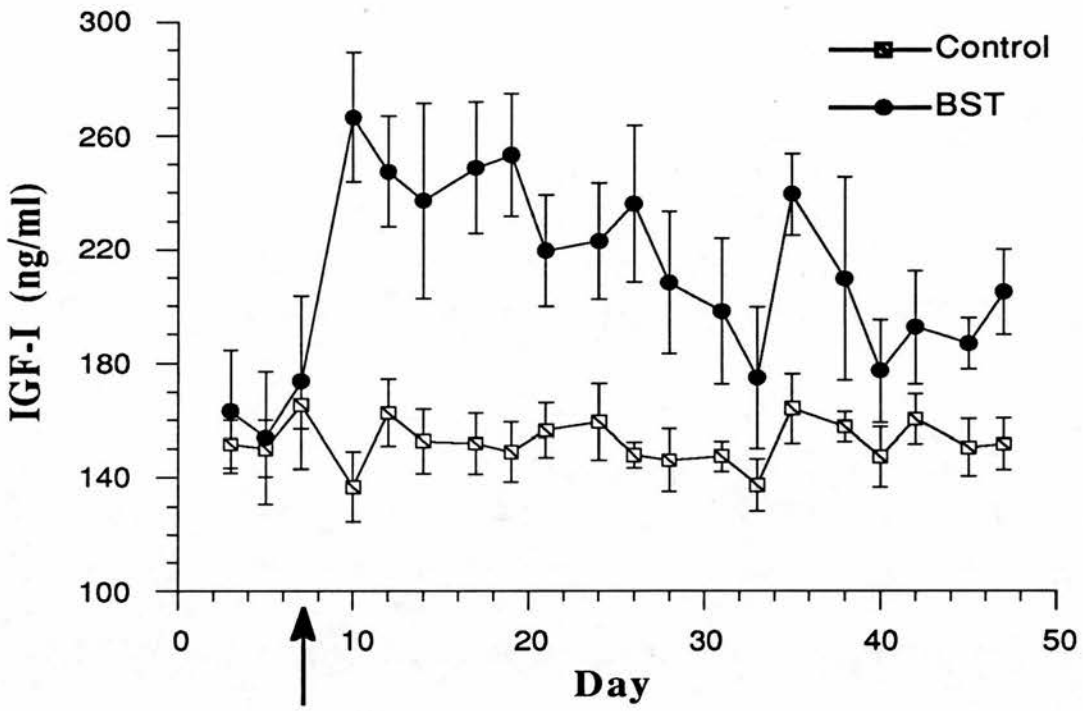

Luteal phase
Control
BST
GH (ng/ml)
70
50
30
10
Follicular phase
GH (ng/ml)
65
55
45
35
25
15
0
100
200
300
400
500
Minute

Figure 3.4. Mean (±sem) serum IGF-I concentrations for the two treatment groups (n = 6) before and after the start of daily BST treatment. Day 0 on the horizontal axis represents the day of oestrus after the second injection of prostaglandin. Blood samples were taken three times a week just prior to the daily subcutaneous BST injections, beginning on Day 3 of the first oestrous cycle, with daily BST injections starting on Day 7 as indicated by the arrow.


300
260
220
180
140
100
IGF-I (ng/ml)
0
10
20
30
40
50
Day
Control
BST

treated animals prior to the start of daily BST injection (effect of treatment, $P>0.05$). IGF-I concentrations in the BST-treated heifers increased within three days after the start of BST injection, and remained elevated compared to the control heifers throughout the treatment period (effect of treatment, $P<0.01$). Treatment by day interaction was not significant ($P>0.05$) either before or after BST treatment, while the effect of day was significant ($P<0.05$) after BST treatment. As for GH, peripheral IGF-I concentrations in BST-treated heifers reflected the elevated levels approximately 24 hours after the previous BST injection. Unlike GH, IGF-I did not show an acute response to subcutaneous BST injection (Figure 3.5) (effect of time, and treatment by time interaction, $P>0.05$). However, IGF-I concentrations for BST-treated heifers were significantly higher (effect of treatment, $P<0.01$) than those in the control animals throughout the whole of the sampling period. There were no significant ($P>0.05$) differences in the IGF-I response to BST between the mid-luteal and the follicular phases of the oestrous cycle.

3.3.3. Peripheral insulin concentrations

Peripheral insulin concentrations for heifers in the control group were maintained at a stable level throughout the experiment (Figure 3.6). Similar concentrations were measured in BST-treated animals prior to the start of daily BST injection (effect of treatment, $P>0.05$). Insulin concentrations in the BST-treated heifers increased within three days of the start of daily BST injection, and remained significantly elevated throughout the treatment period (effect of treatment, $P<0.01$) compared to those of the control heifers. Treatment by day interaction was not significant ($P>0.05$) either before or after BST treatment, while the effect of day was significant ($P<0.05$) after BST treatment. As for GH, peripheral

Figure 3.5. Mean (±sem) plasma IGF-I concentrations measured in the serial blood samples from the two treatment groups (n = 6) before and after daily BST injection. Blood samples were taken every ten minutes for eight hours, with the BST injection given at the time of sixth sampling as indicated by the arrow, on Day 12 (luteal phase, upper panel) and Day 19 (follicular phase, lower panel) of the second oestrous cycle.

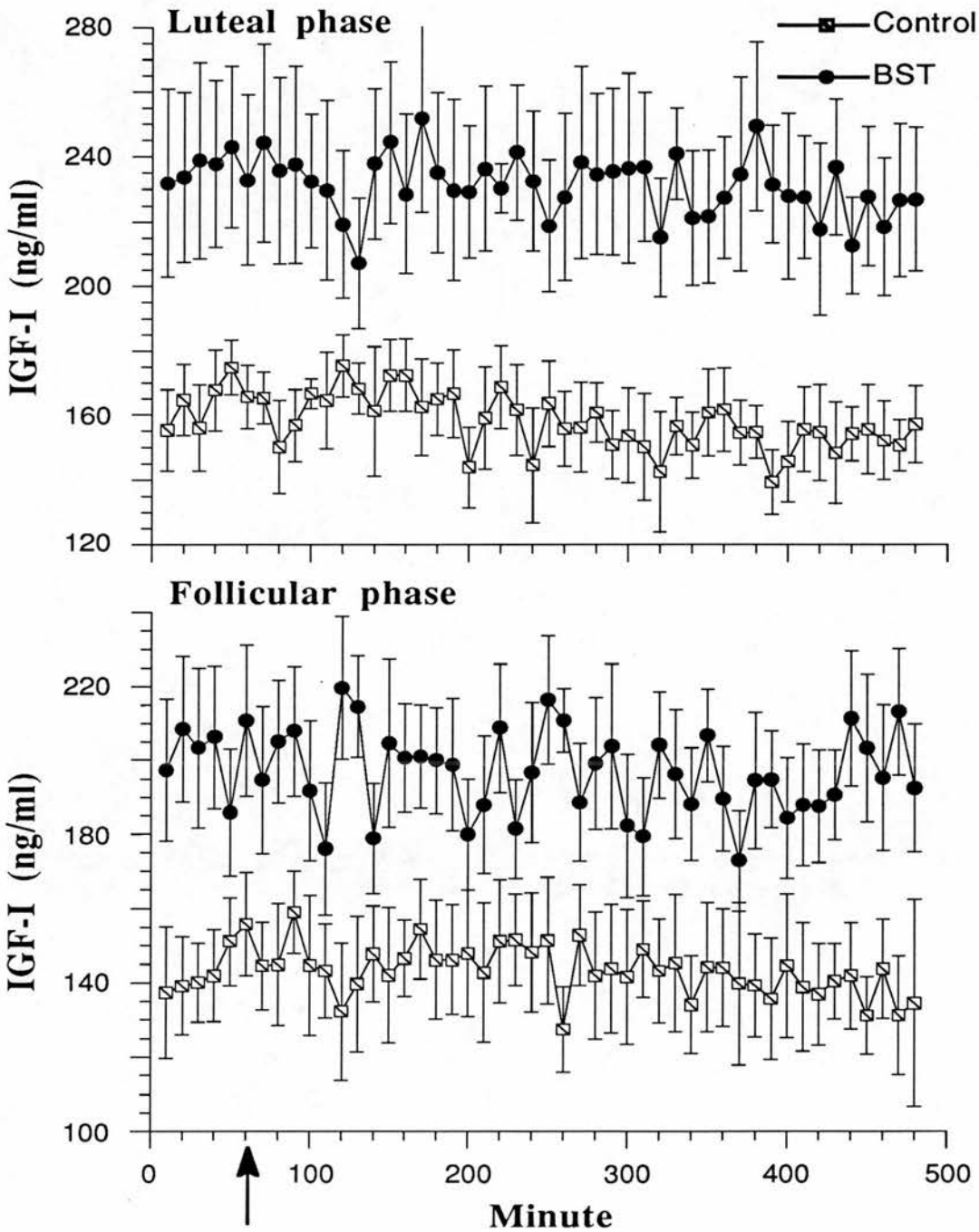
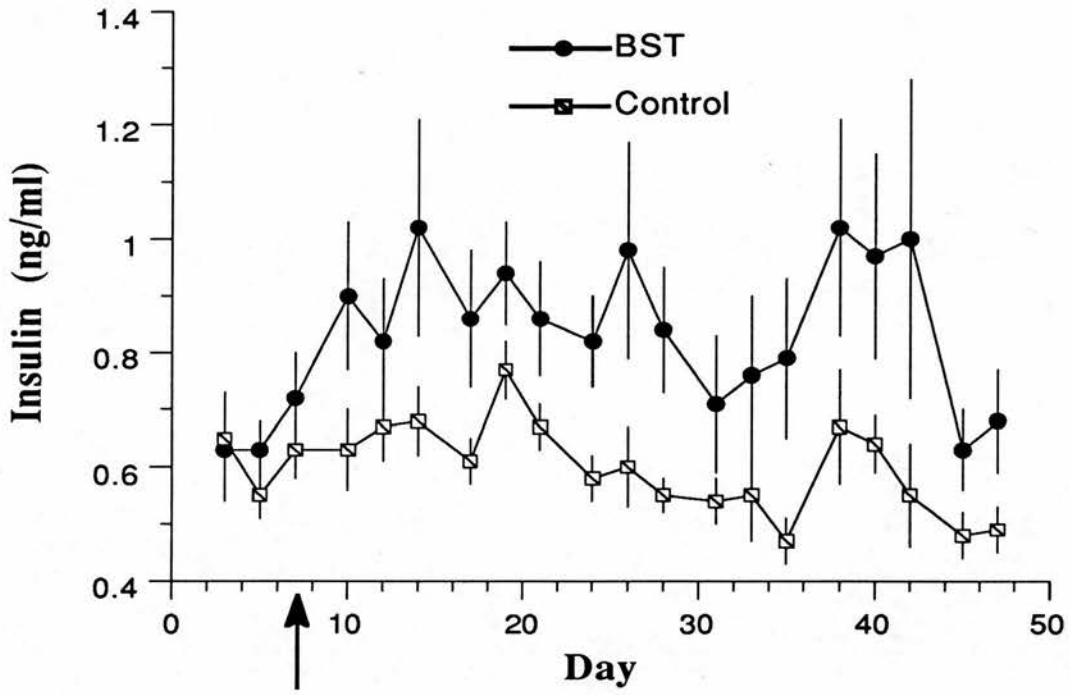

Luteal phase
Control
BST
IGF-I (ng/ml)
280
240
200
160
120
Follicular phase
IGF-I (ng/ml)
220
180
140
100
0
100
200
300
400
500
Minute

Figure 3.6. Mean (±sem) serum insulin concentrations for the two treatment groups (n = 6) before and after the start of daily BST treatment. Day 0 on the horizontal axis represents the day of oestrus after the second injection of prostaglandin. Blood samples were taken three times a week just prior to the daily subcutaneous BST injections, beginning on Day 3 of the first oestrous cycle, with the daily BST injection starting on Day 7 as indicated by the arrow.


BST
Control
Insulin (ng/ml)
1.4
1.2
1
0.8
0.6
0.4
0
10
20
30
40
50
Day

insulin concentrations measured in the BST-treated heifers reflected the elevated levels approximately 24 hours after the previous BST injection. Similar to IGF-I but in contrast to GH, insulin did not show an acute response to subcutaneous BST injection (Figure 3.7) (effect of time, and treatment by time interaction, $P>0.05$). However, insulin concentrations in BST-treated heifers were significantly (effect of treatment, $P<0.01$) higher than those in the control animals throughout the whole of the sampling period. There were no significant ($P>0.05$) differences in the insulin response to BST between mid-luteal and follicular phases of the oestrous cycle.

3.3.4. Peripheral oestradiol and progesterone concentrations

Heifers in both the BST and control groups displayed normal cycles of peripheral oestradiol and progesterone profiles during the experiment (Figure 3.8). Oestrus was always preceded by a rapid decrease in peripheral progesterone concentrations and an increase in peripheral oestradiol concentrations. However, there were no differences in oestradiol and progesterone concentrations between the two treatment groups either throughout the treatment period or during the luteal and follicular phases of the oestrous cycle (effect of treatment, $P>0.05$). The differences in progesterone and oestradiol concentrations between the first and second luteal and follicular phases following the start of BST treatment were not significant ($P>0.05$). Treatment by day interaction was not significant ($P>0.05$), while the effect of day was significant ($P<0.05$). Oestrous cycle length (21.6 ± 1.6 versus 20.8 ± 2.1 days), and duration of the luteal (14.6 ± 1.7 versus 14.9 ± 1.5 days) and follicular phases (6.7 ± 1.2 versus 6.1 ± 1.3 days) for the control and BST groups respectively were not significantly different ($P>0.05$).

Figure 3.7. Mean (±sem) plasma insulin concentrations measured in the serial blood samples from the two treatment groups (n = 6) before and after daily BST injection. Blood samples were taken every ten minutes for eight hours, with the BST injection given at the time of sixth sampling as indicated by the arrow, on Day 12 (luteal phase, upper panel) and Day 19 (follicular phase, lower panel) of the second oestrous cycle.

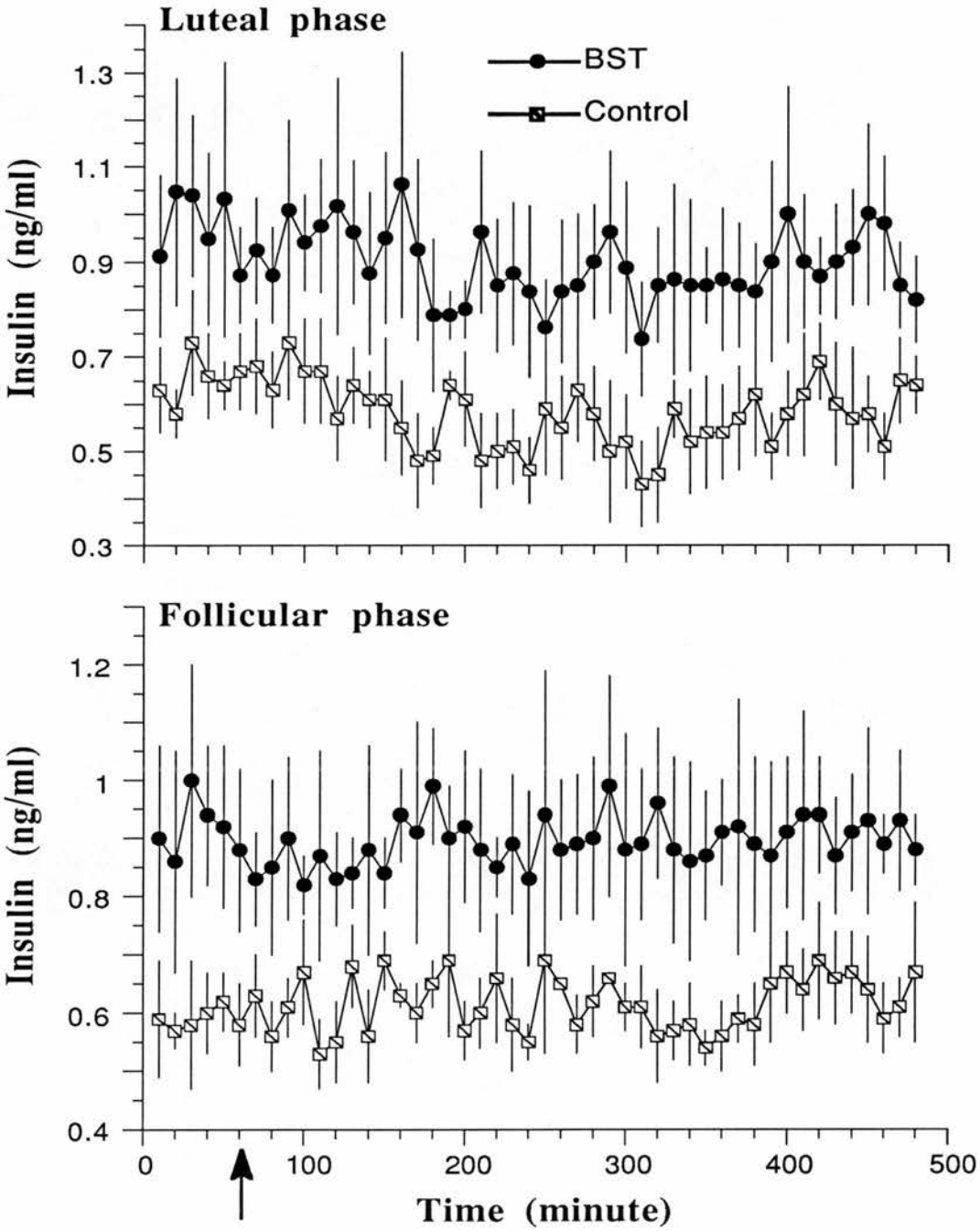
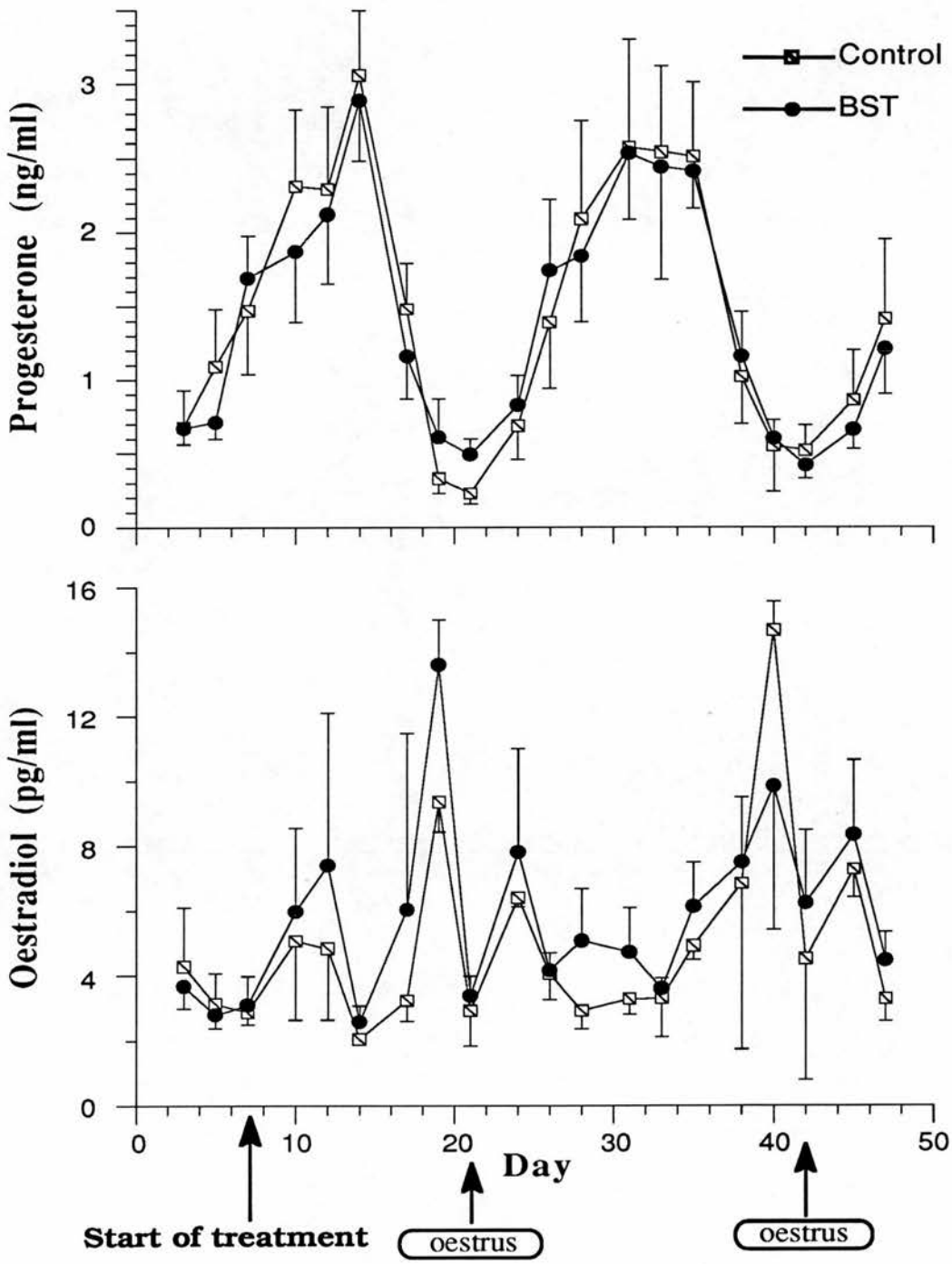

Luteal phase
BST
Control
Insulin (ng/ml)
1.3
1.1
0.9
0.7
0.5
0.3
Follicular phase
Insulin (ng/ml)
1.2
1
0.8
0.6
0.4
0
100
200
300
400
500
Time (minute)

Figure 3.8. Mean (+/-sem) serum progesterone (upper panel) and oestradiol (lower panel) concentrations for the two treatment groups (n = 6) before and after the start of daily BST treatment. Day 0 on the horizontal axis represents the day of oestrus after the second injection of prostaglandin. Mean day (n = 12) of subsequent oestrus is also shown. Blood samples were taken three times a week just prior to the daily subcutaneous BST injections, beginning on Day 3 of the first oestrous cycle, with the daily BST injection starting on Day 7.


Control
BST
Progesterone (ng/ml)
0
1
2
3
Oestradiol (pg/ml)
0
4
8
12
16
0
10
20
30
40
50
Day
Start of treatment
oestrus
oestrus

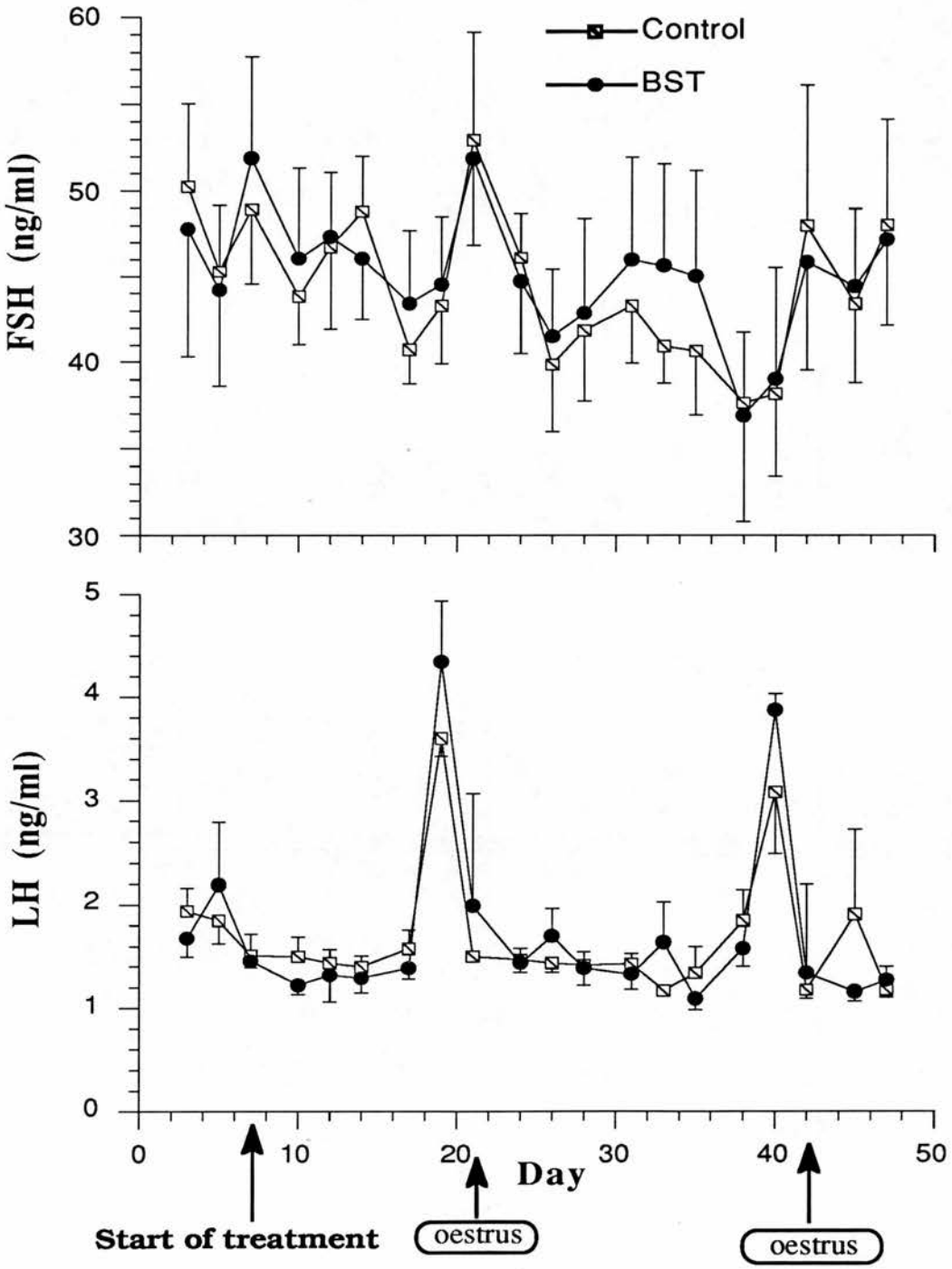
3.3.5. Peripheral FSH and LH concentrations

The preovulatory LH surge was detected in 1 control and 1 BST-treated heifer in the first oestrous cycle and 1 control and 2 BST-treated heifers in the second oestrous cycle following the start of BST treatment (Figure 3.9). However, peripheral FSH and LH concentrations were not affected by BST injection, either throughout the treatment period or during the luteal and follicular phases of the oestrous cycle (effect of treatment, $P>0.05$). The differences in FSH and LH concentrations between the first and second luteal and follicular phases following the start of BST treatment were not significant ($P>0.05$). There was no treatment by day interaction for any of the parameters tested, whilst the effect of day was significant ($P<0.05$) except for LH concentrations during the luteal phases of the oestrous cycle. There were no significant differences between the two experimental groups in mean FSH concentrations measured in serial plasma samples taken on Days 12 (45.47 ± 3.69 ng/ml for the control and 48.38 ± 7.25 ng/ml for the BST group) and 19 (41.33 ± 4.75 ng/ml for the control and 39.05 ± 5.24 ng/ml for the BST group) of the second oestrous cycle.

3.3.6. LH pulsatile characteristics

Mean LH concentrations for the control and BST-treated groups, during the mid-luteal phase (1.09 ± 0.17 and 1.25 ± 0.09 ng/ml respectively) and follicular phase (1.28 ± 0.11 and 1.44 ± 0.16 ng/ml respectively) of the oestrous cycle, were not significantly different ($P>0.05$). Similarly, basal LH concentrations in the control and BST groups were not different (0.94 ± 0.05 and 1.12 ± 0.09 ng/ml for the mid-luteal phase; 1.07 ± 0.14 and 1.27 ± 0.12 ng/ml for the follicular phase).

Figure 3.9. Mean (+/-sem) serum FSH (upper panel) and LH (lower panel) concentrations for the two treatment groups (n = 6) before and after the start of daily BST treatment. Day 0 in the horizontal axis represents the day of oestrus after the second injection of prostaglandin. Mean day (n = 12) of subsequent oestrus is also shown. Blood samples were taken three times a week just prior to the daily subcutaneous BST injections, beginning on Day 3 of the first oestrous cycle, with the daily BST injection starting on Day 7.


Control
BST
FSH (ng/ml)
LH (ng/ml)
Day
Start of treatment
oestrus
oestrus

Analysis of LH pulse profiles during the mid-luteal and follicular phases of the second oestrous cycle for the two experimental groups are shown in Table 3.1. A significantly ($P<0.01$) greater LH pulse frequency was observed during the follicular phase than during the mid-luteal phase of the oestrous cycle in both BST-treated and control heifers. However, there was no significant difference in LH pulse frequency nor pulse amplitude between the two treatment groups, either during the mid-luteal or follicular phases of the oestrous cycle.

3.3.7. FSH and LH binding to granulosa and thecal cells and LH binding to CL

As shown in Figure 3.10 and Figure 3.11, FSH and LH binding to thecal and granulosa cells and LH binding to CL did not differ between the two treatment groups ($P>0.05$).

3.3.8. Ovulation rate and follicle population

The effects of BST treatment on ovulation rate and antral follicle population are shown in Table 3.2. All heifers had a single ovulation, with or without BST treatment. However, the number of antral follicles ≥2mm in diameter was significantly greater in BST-treated heifers when compared to that of the control group ($P<0.001$). Only one heifer in the BST-treated group had a follicle number which overlapped with the number of follicles in the control group. When grouped according to follicular diameter, there was a significant difference between the two experimental groups in the number of follicles 2-5mm in diameter ($P<0.001$), but no difference in the numbers of large (>10mm in diameter) and medium-sized (5-10mm in diameter) follicles. The number of follicles

Table 3.1. **The effect of BST treatment on LH pulse frequency and amplitude (mean ± sem) during the mid-luteal and follicular phases of the second oestrous cycle**

Treatment	Luteal phase		Follicular phase	
	Pulse frequency (pulses/8hrs)	Pulse amplitude (ng/ml)	Pulse frequency (pulses/8hrs)	Pulse amplitude (ng/ml)
Control (n=6)	3.50 ± 0.51^a	0.42 ± 0.07	8.25 ± 1.12^b	0.54 ± 0.06
BST (n=6)	3.99 ± 0.82^a	0.35 ± 0.07	7.66 ± 0.83^b	0.48 ± 0.07

a versus *b:* significantly different ($P<0.01$)

Figure 3.10. Mean (±sem) specific FSH and LH binding to thecal (upper panel) and granulosa (lower panel) cells for the two treatment groups (n = 6 heifers per group). Granulosa and thecal cells were collected from the 3 largest follicles for each heifer at the end of experiment. The results were expressed as pg of [^{125}I]-labelled gonadotrophin specifically bound per mg of protein in the homogenized tissue preparations.

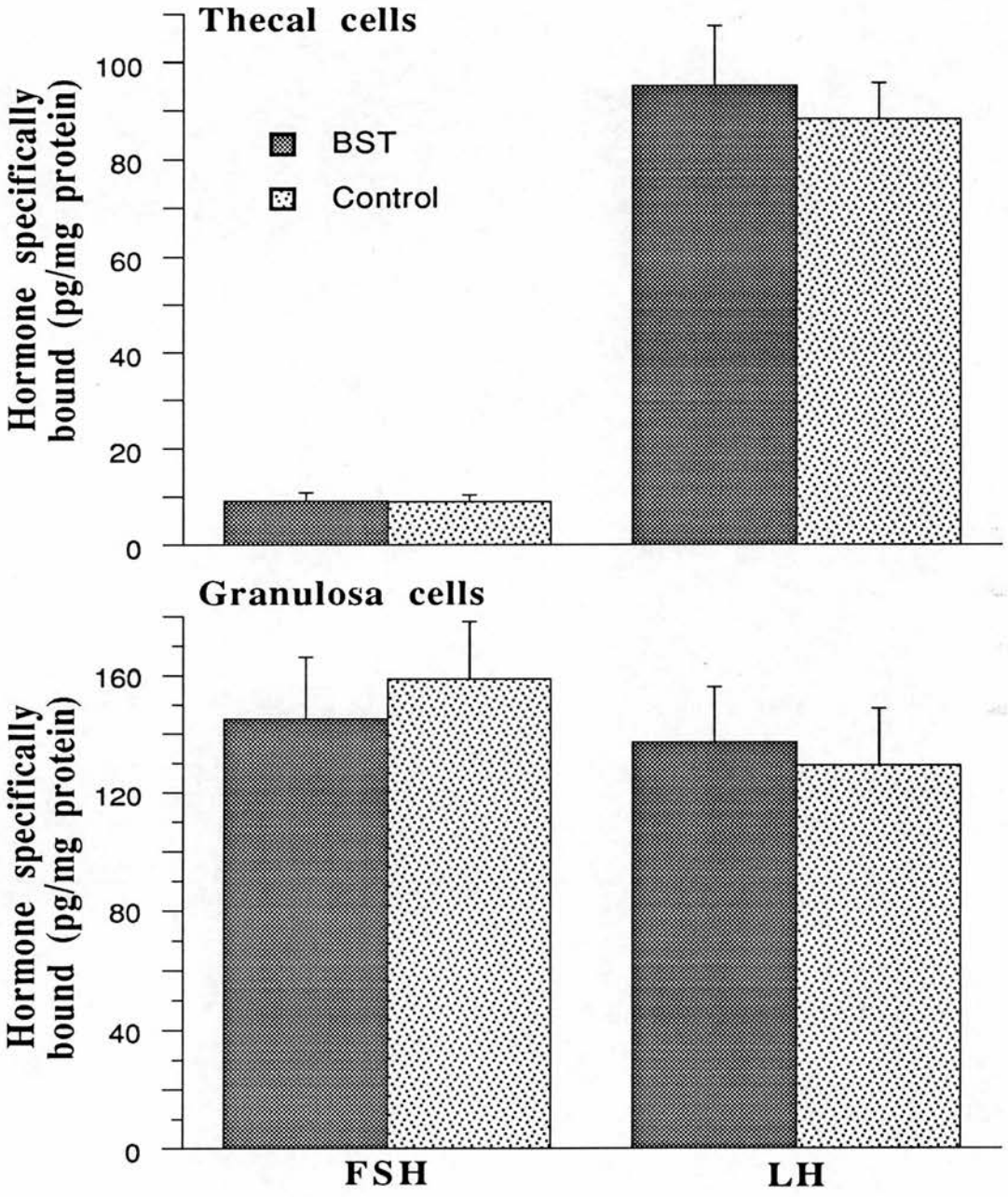

Thecal cells
Hormone specifically bound (pg/mg protein)
100
80
60
40
20
0
BST
Control
Granulosa cells
Hormone specifically bound (pg/mg protein)
160
120
80
40
0
FSH
LH

Figure 3.11. Mean (±sem) specific LH binding to CL for the two treatment groups. CL were collected at the end of experiment. The results were expressed as pg of tracer bound per mg of protein in the homogenized tissue preparations.

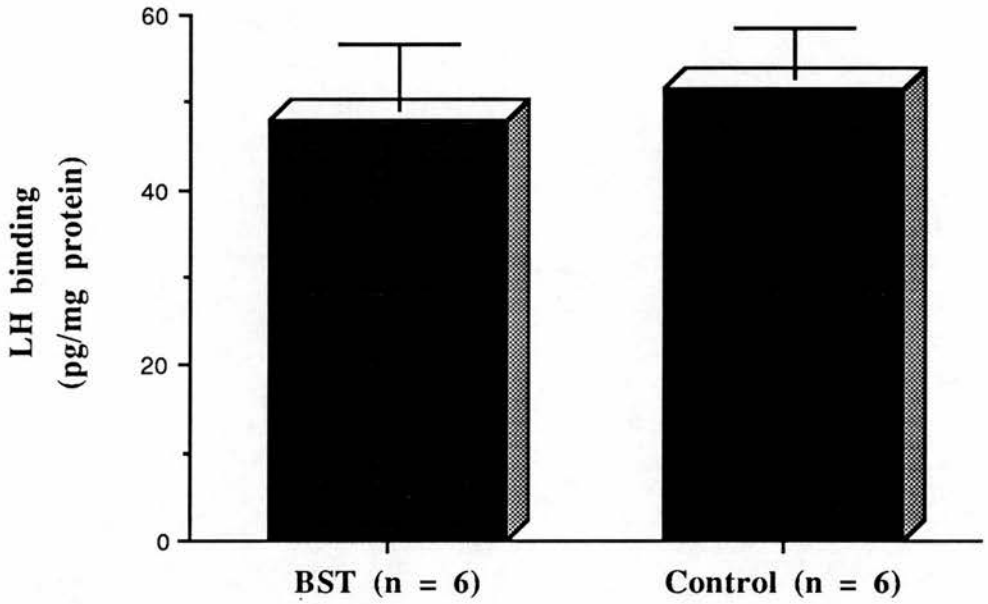

LH binding
(pg/mg protein)
60
40
20
0
BST (n = 6)
Control (n = 6)

Table 3.2. The effect of BST treatment on ovulation rate and populations of antral follicles ≥2mm (mean ± sem) in heifers

Treatment	Ovulation rate	Number of antral follicles			
		Total	>10mm diameter	5-10mm diameter	2-5mm diameter
Control (n=6)	1.0±0.0	33.2±3.2^a (26-45)*	0.8±0.2 (0-1)	6.5±1.0 (3-9)	25.8±2.7^a (18-36)
BST (n=6)	1.0±0.0	60.2±6.7^b (32-76)	0.8±0.2 (0-1)	6.8±1.4 (3-11)	52.5±6.5^b (28-72)

a versus *b:* significantly different (P<0.001);

*Numbers in brackets indicate the range of numbers of follicles within each group.

2-5mm in diameter was significantly correlated with mean GH concentration ($r = 0.63$, $P<0.05$), mean IGF-I concentration ($r = 0.73$, $P<0.01$) and mean insulin concentration ($r = 0.69$, $P<0.01$) throughout the treatment period.

3.4. Discussion

BST has been observed to increase twinning rates in lactating dairy cows (Butterwick *et al.*, 1988). Since this result was reported as an incidental observation from experiments designed to study the effect of BST treatment on lactation, the possible underlying mechanism(s) was not investigated. Nevertheless, it suggested that GH may be involved in the control of ovarian function in cattle. The present study has demonstrated for the first time that BST treatment has a significant effect on the small antral follicle population in mature heifers. As expected, BST treatment significantly increased peripheral GH, IGF-I and insulin concentrations throughout the treatment period. There was no effect on other characteristics of reproductive function, including the length of the oestrous cycle, the duration of the luteal and follicular phases, peripheral progesterone and oestradiol concentrations, mean FSH and LH concentrations, LH pulse frequency and pulse amplitude in either the mid-luteal or follicular phases of the second oestrous cycle, nor in the numbers of FSH and LH binding sites in granulosa and thecal cells and amount of LH binding to CL.

The GH concentrations for vehicle-treated heifers in this study appeared to be higher than most previous reports (McShane, Schillo, Estienne, Boling, Bradley and Hall, 1989; Plouzek and Trenkle, 1991). The reason for this is not known since concentrations in prepubertal cattle,

measured using the same assay (Lovendahl *et al.*, 1991), were lower and similar to reported data. Possible explanations may include breed and/or environmental differences or the relative potency of the BST used as the standard in the assay. Importantly, there were no significant differences between the groups prior to the start of BST treatment.

In the present study, all animals were slaughtered at approximately the same stage of the oestrous cycle to reduce variability. Although it has been suggested that the number of small antral follicles in cattle is extremely variable among individuals (Erickson, 1966b), the effect of BST treatment was so pronounced that differences between the two treatment groups were clearly demonstrated. Indeed, only one heifer in the BST-treated group had a follicle number which overlapped with the follicle numbers in the control group, whereas there were at least 6 more follicles in the other BST-treated animals than the highest number in the control group.

As reviewed by Gluckman, Breier and Davis (1987), peripheral IGF-I is secreted mainly by the liver, and GH plays an important role in the regulation of its secretion. In this current study, daily treatment of heifers with 25mg BST increased peripheral concentrations of IGF-I within 3 days. This increase was maintained throughout the whole treatment period. This is in agreement with previous studies in cattle and sheep (Peel and Bauman, 1987; Davis *et al.*, 1990). Since the increase in peripheral IGF-I concentrations was accompanied by a significant increase in the numbers of follicles 2-5mm in diameter, it is possible that BST treatment altered the pattern of ovarian folliculogenesis via the increase in peripheral IGF-I concentrations. Interestingly, the numbers of follicles 2-5mm in diameter correlated with mean IGF-I concentrations

(r = 0.73, P<0.01) throughout the treatment period. A recent report by Echternkamp *et al.* (1990) demonstrated that natural twinning in cyclic cattle was associated with increased concentrations of IGF-I, both in the peripheral circulation and in the follicular fluid of the two largest follicles. IGF-I has also been implicated in the regulation of bovine granulosa cell proliferation (Savion *et al.*, 1981) and luteinization (Schams *et al.*, 1987) and may act as an intraovarian regulator of granulosa cell growth and differentiation (Adashi *et al.*, 1985a).

BST treatment increased peripheral concentrations of GH by approximately 2-fold throughout the treatment period, so a direct effect of BST at the ovarian level cannot be excluded. Indeed, the numbers of follicles 2-5mm in diameter was also correlated with mean GH concentrations (r = 0.63, P<0.05) throughout the treatment period. GH has been shown to stimulate IGF-I and to a lesser extent progesterone production by porcine granulosa cells *in vitro* (Hsu and Hammond, 1987a) and both GH and IGF-I enhanced PMSG-stimulated progesterone and oestrogen production in rat granulosa cells (Hutchinson, Findlay and Herington, 1988). Progesterone production was increased approximately four-fold when GH was combined with FSH and oestradiol (Hsu and Hammond, 1987a). GH has also been shown to enhance FSH-stimulated differentiation of cultured rat granulosa cells and to increase ovarian concentrations of immunoreactive IGF-I (Jia *et al.*, 1986; Davoren and Hsueh, 1986). Therefore one possible mechanism could be that either GH and/or GH-stimulated ovarian IGF-I acts synergistically with FSH.

In contrast to the view that BST administration usually has no effect on serum insulin concentration in lactating animals (Vernon, 1990), BST treatment in this study significantly increased peripheral

insulin concentrations throughout the treatment period. Since insulin has also been suggested to play a role in the control of ovarian function (Savion *et al.*, 1981; Adashi *et al.*, 1985a; Poretsky and Kalin, 1987; Webb and McBride, 1991), insulin may also be involved in the effects of BST on small follicles. Indeed, like GH and IGF-I, mean insulin concentrations were significantly correlated with the number of small follicles ($r = 0.69$, $P<0.01$) throughout the treatment period. In a recent study, bovine GH when given together with insulin stimulated bovine granulosa cell proliferation and steroidogenesis *in vitro* (Langhout, Spicer and Geisert, 1991).

Although environmental factors such as nutrition are known to affect reproductive function (Butler, Everett and Coppock, 1981) and BST treatment in cattle can change energy balance and body condition (Soderholm, Otterbry, Ehle, Linn, Hensen and Annexstad, 1986), it is unlikely that the effects of BST seen in the present experiment were due to changes in feed intake and/or body condition since the body weights of all heifers did not change significantly throughout the experiment.

Despite the major effect of BST on follicle populations, gross ovarian function was not affected by BST treatment. In agreement with previous studies in lactating dairy cows (Schemm and Deaver, 1988; Schemm, Deaver, Griel and Muller, 1990), we have demonstrated that the length of the oestrous cycle, the duration of the luteal and follicular phases, peripheral oestradiol and FSH concentrations were not influenced by BST treatment. In contrast to previous observations which found significantly higher plasma progesterone concentrations in cows treated with BST (Schemm *et al.*, 1990; Gallo and Block, 1989), peripheral progesterone concentrations were unaffected by BST treatment in this

experiment. The reason for this difference is not clear, although the two previous studies were carried out in lactating dairy cows. It has been demonstrated that IGF-I treatment of both granulosa (Schams *et al.*, 1987) and luteal cells (McArdle and Holtorf, 1989) *in vitro*, and luteal cells *in vivo* (Sauerwein *et al.*, 1992), resulted in increased progesterone secretion. Moreover, the IGF-I gene is expressed, and IGF-I receptors have been identified in bovine luteal tissue (Einspanier, Miyamoto, Schams, Muller and Brem, 1990; Sauerwein *et al.*, 1992). However, a possible effect of peripheral IGF-I on CL function is less certain. In the present experiment, treatment of heifers with BST did not affect CL weight (data not shown) nor the number of LH binding sites in CL. The study by Schemm *et al.* (1990) indicated that LH pulse frequency during the early follicular phase of the first treatment oestrous cycle was increased by BST treatment, although not significantly ($p = 0.06$), while LH pulse amplitude was unchanged, and basal and mean concentrations of LH were significantly lower. However, by the third oestrous cycle, these treatment differences were no longer present. In contrast, no significant differences were observed for any of the characteristics of LH secretion in the present study, either during the mid-luteal or follicular phases of the oestrous cycle. In agreement with previous studies (Carson, Findlay, Burger and Trounson, 1979; Ireland and Roche, 1982a; Staigmiller *et al.*, 1982), this study showed that FSH binding in thecal cells was very low compared to binding in granulosa cells, whereas LH binding in granulosa cells was as high as binding to thecal cells. However, numbers of FSH and LH binding sites in granulosa and thecal cells were not influenced by BST treatment, suggesting that the effects of BST on ovarian folliculogenesis were not mediated through changes in circulating gonadotrophin concentrations or ovarian gonadotrophin

receptor levels.

Despite doubling the numbers of follicles 2-5mm in diameter, BST treatment failed to change ovulation rate, presumably due to the inhibitory action of the dominant follicle (Ireland, 1987). This is similar to results reported for ewes given BST during the breeding season (Davis *et al.*, 1990), although no data on follicle populations was presented. However, these results differ from those reported in cyclic gilts, in which treatment with porcine GH significantly increased ovulation rate and IGF-I concentrations (Kirkwood *et al.*, 1988), although 45% of the treated gilts were anovular. Surprisingly, in the study of Butterwick *et al.* (1988), treatment of lactating dairy cows with BST, starting from week 2 (but not from week 10 of lactation), did induce a high incidence of twinning. Hence, the length of treatment may account for the observed effect on twinning. However, in our study there was no increase in ovulation rate. The incidence of twin calvings in cattle is normally very low, being approximately 3% (Nielen *et al.*, 1989). Therefore, large numbers of animals would be required to demonstrate a statistically significant effect. Indeed, a large study with over 800 cattle demonstrated that treatment with BST doubled the twinning rate from 3.7% to 8.0% (Wilkinson and Tarrant, 1991). However, whether this effect was due to an increase in ovulation rate or an effect on establishment of twin pregnancies is not known. It has been reported that the incidence of twin calvings is even lower in heifers (0.8%) (Nielen *et al.*, 1989) and that BST treatment had no effect on conception rate or the number of calves born to heifers (Grings, deAvila, Eggert and Reeves, 1990). In the breed of heifers used in the present experiment, we have only observed one double ovulation in over 300 laparoscopies of control animals (R. Webb, unpublished data). Nevertheless, the increase in the number of small follicles by BST

treatment demonstrated in this study may be significant in future attempts to increase ovulation rate. Furthermore, this result was similar to the effects of priming cows with FSH early in the oestrous cycle (Monniaux *et al.* 1983), and may provide an alternative approach to improve superovulatory regimes.

In conclusion, although ovulation rate was not altered, treatment with BST significantly increased the population of antral follicles 2-5mm in diameter in mature heifers supporting a role of GH in the control of folliculogenesis in cattle. This effect does not appear to be mediated through changes in circulating gonadotrophin concentrations or ovarian gonadotrophin receptor levels. Whilst it is possible that BST may be acting via increased peripheral IGF-I and/or insulin concentrations, a direct effect of BST at the ovarian level cannot be excluded and the exact mechanism(s) remains to be elucidated.

CHAPTER 4

Study of the dynamics of ovarian follicular growth and development during the oestrous cycle using real-time ultrasound in heifers

4.1. Introduction

The application of real-time ultrasound in cattle has enabled the reliable determination of growth patterns for ovarian follicles >5mm in diameter during the oestrous cycle (Pierson and Ginther, 1984; Savio *et al.*, 1988; Sirois and Fortune, 1988; Knopf *et al.*, 1989; Boland *et al.*, 1990). These studies have demonstrated that the growth of follicles during the oestrous cycle in cattle occurs in a wave pattern with each wave involving the synchronous development of a cohort of follicles within which one follicle subsequently becomes dominant whilst other cohort follicles (subordinate follicles) regress. However, different investigators observe different numbers of waves of follicle growth during an oestrous cycle. Savio *et al.* (1988) and Sirois and Fortune (1988) found most animals in their studies had 3 waves per oestrous cycle, whilst Knopf *et al.* (1989) and Driancourt *et al.* (1991a) reported that the majority of heifers in their studies had a 2-wave pattern. It appears that ovarian follicular growth pattern during the oestrous cycle may differ between breeds and/or strains of cattle. Moreover, although it is generally accepted that the dominance of a single follicle during the process of folliculogenesis in cattle may be associated with inhibition of the growth of other cohort follicles (Ireland, 1987; Pierson and Ginther, 1987b, 1988; Kastelic *et al.*,

1990; Fortune *et al.*, 1991; Ko *et al.*, 1991), the precise timing of the growth of the dominant follicle in relation to the dynamics of subordinate follicles (particularly those <5mm in diameter) has not been investigated in detail.

As described in Chapter 3, BST treatment increased the population of ovarian follicles 2-5mm in diameter in mature heifers. We therefore determined whether real-time ultrasound scanning could be used to investigate the mechanism(s) involved in this BST effect. The objectives of this study were: (1) to establish whether the number and/or growth pattern of small follicles (<5mm in diameter) could be accurately determined by ultrasound scanning; (2) to examine the patterns of ovarian follicular growth and development during the oestrous cycle in our own population of heifers; and (3) to study the temporal relationships between the growth of the dominant follicle and the growth and regression of subordinate follicles.

4.2. Materials and Methods

12 heifers were used in this study under the husbandry conditions described in Chapter 2.

4.2.1. Validation of real-time ultrasound measurements

To validate the ultrasound procedures, the ovaries of 12 heifers were scanned (see Chapter 2) and the animals were then slaughtered and their ovaries collected. All follicles ≥2mm in diameter were dissected and divided into 3 size categories: <5mm, 5-10mm and >10mm in diameter. The numbers of follicles in the 3 size categories and the diameters of the

largest follicles in a pair of ovaries observed by ultrasound scanning and gross dissection were then compared.

The following definitions have been used (see also Ireland, 1987; Savio *et al.*, 1988) to describe the pattern of follicular growth and development. *(1) Follicular wave:* the synchronous recruitment and growth of a group of follicles from which usually a single follicle develops to a diameter of >10mm (*dominant follicle*) while other cohort follicles (*subordinate follicles*) regress; *(2) The onset of a follicular wave:* the first day a follicle, which subsequently becomes the dominant follicle, can be identified.

4.2.2. Experimental procedures

Oestrous cycles of heifers were synchronized using Estrumate as described in Chapter 3. Following the second injection of Estrumate, animals were scanned daily (see Chapter 2) for approximately 30 days. Blood samples were taken daily and serum harvested for progesterone measurement.

All follicles detected by ultrasound scanning were divided into 3 size categories according to their diameters: <5mm (small), 5-10mm (medium-sized) and >10mm (large).

4.2.3. Statistical analyses

Daily changes in diameter for all follicles that could be followed by ultrasound for at least 2 consecutive days were plotted for each animal throughout the oestrous cycle to determine the wave pattern of follicular growth and development. Daily changes in the numbers of small,

medium-sized and large follicles during each oestrous cycle were also determined. Temporal relationships between the dominant follicle and changes in the number of follicles for all the three size categories during each follicular wave were examined separately for heifers with 3 waves per cycle and heifers with 2 waves per cycle. To measure the lengths of the follicular and luteal phases of each oestrous cycle, the beginning of the luteal and follicular phases were defined by a progesterone concentration above or below 1ng/ml respectively. Differences in the length of the oestrous cycle and the duration of the luteal and follicular phases between heifers with 3 follicular waves per oestrous cycle and heifers with 2 waves were tested statistically by Student's *t* test. Correlations between different parameters were determined by regression analysis.

4.3. Results

4.3.1. Validation of real-time ultrasound measurements

A comparison between ultrasound measurements and gross dissection was made for the numbers of small, medium-sized and large follicles and diameter of the largest follicle in 12 heifers. The mean (± s.e.m.) diameter of the largest follicles was 12.1 ± 0.7mm after dissection versus 11.9 ± 0.7mm by ultrasound ($r = 0.89$, $p<0.01$). All large follicles present at dissection could be detected by ultrasound ($r = 1.0$, $p<0.01$). For the medium-sized follicles there was a highly significant correlation ($r = 0.92$, $p<0.01$) between the numbers of follicles detected by ultrasound and by gross dissection, with approximately 44% of follicles at dissection being detected by ultrasound scanning. For small follicles there was also

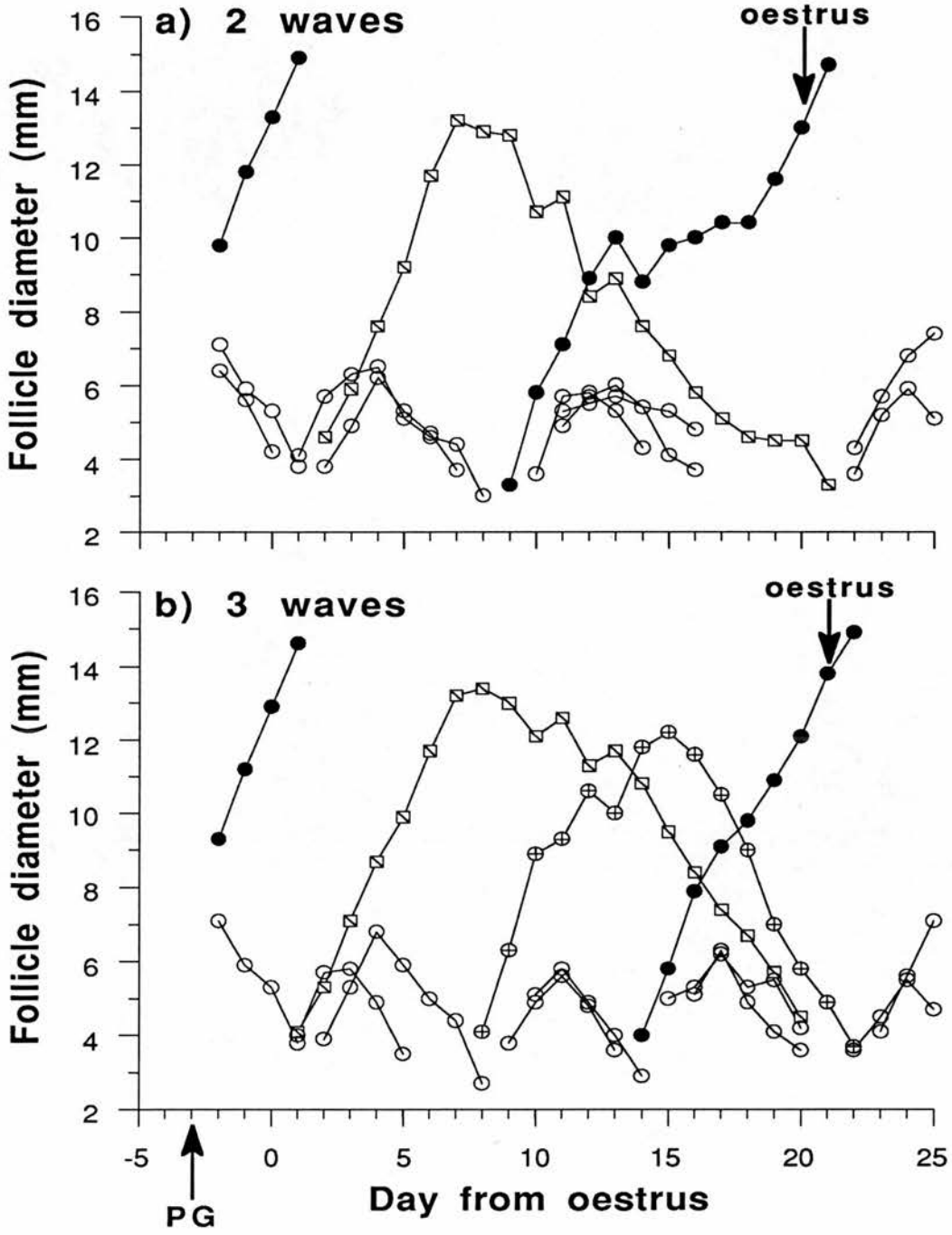
a highly significant correlation ($r = 0.94$, $p<0.01$) between the numbers of follicles detected by ultrasound and by gross dissection, with approximately 19% of follicles at dissection being detected by ultrasound.

4.3.2. Pattern of follicular growth during the oestrous cycle

The daily growth pattern (i.e. daily changes in the diameter of follicles) throughout the oestrous cycle for medium-sized and large follicles could be followed accurately. Follicles with a diameter as low as 2mm could also be detected by ultrasound scanning. However, although it was possible to assess the number of small follicles on a daily basis, the daily growth pattern of individual small follicles could not be followed accurately.

Nine out of 12 heifers (75%) displayed three waves of dominant follicle growth and development during the oestrous cycle whilst the remaining 3 heifers had two waves. The dynamics of follicular growth for one representative heifer with three waves per oestrous cycle and one representative heifer with two waves per cycle are shown in Figure 4.1. For the 3-wave cycles, mean (± s.e.m.) day of the initiation of the first, second and third waves was Days 1.7 ± 0.2, 9.1 ± 0.8 and 15.8 ± 1.1 of the oestrous cycle (Day 0 = day of oestrus) respectively, whilst in the 3 heifers with a 2-wave pattern, the first and second waves started on Days 1.3 ± 0.5 and 10.0 ± 1.0 respectively. Daily changes throughout the oestrous cycle in the mean numbers of follicles detected by ultrasound, for all the 3 size categories (small, medium-sized and large), also displayed a wave pattern. When analysed on an individual heifer basis, the number of waves per oestrous cycle for all three follicle size categories was the same as the number of dominant follicle waves, displaying either a 3-

Figure 4.1. Dynamics of ovarian follicular growth and development during the oestrous cycle for a representative heifer with 2 follicular waves (3 out of 12 animals) per oestrous cycle (upper panel) and a representative heifer with 3 follicular waves (9 out of 12 animals) (lower panel). Oestrous cycles of heifers were synchronized by two i.m. injections, given 12 days apart, of 0.5mg synthetic prostaglandin $F_{2\alpha}$ (PG). Following the 2nd PG injection, heifers were scanned daily for approximately 30 days. All the follicles with a development pattern which could be followed for at least 2 consecutive days are shown. Ovulation occurred approximately 24 hours following the detection of oestrus as indicated. Dark circles represent the dominant follicle that ovulated.


a) 2 waves
oestrus
b) 3 waves
oestrus
Follicle diameter (mm)
Follicle diameter (mm)
16
14
12
10
8
6
4
2
-5
0
5
10
15
20
25
PG
Day from oestrus

wave pattern (Figure 4.2) or a 2-wave pattern (Figure 4.3). In addition, wave patterns for the three size categories of follicles were highly synchronized during each wave of follicular growth, with an increase in the number of follicles occurring first in small follicles, followed by medium-sized follicles and finally large follicles (Figure 4.2; Figure 4.3 and Table 4.1).

There was no significant difference in the mean length of the oestrous cycles in heifers having 3 follicular waves (21.4 ± 0.9 days) when compared with heifers having 2 follicular waves (20.3 ± 0.6 days). However, based on peripheral progesterone concentrations, the durations of both the luteal and follicular phases of the oestrous cycle for heifers with 3 follicular waves (16.3 ± 0.6 and 4.8 ± 0.4 days respectively) were significantly different ($p<0.05$) from those of heifers with 2 follicular waves (13.9 ± 0.9 and 6.9 ± 0.9 days respectively).

4.3.3. Temporal relationship between the dominant follicle and subordinate follicles

As shown in Figure 4.2 and Figure 4.3, in all heifers (regardless of the number of follicular waves per oestrous cycle) the period when the dominant follicle grew and reached its maximum diameter always corresponded to the period of the highest number of large follicles, but associated with a marked reduction in the numbers of small and medium-sized follicles. The growth of the dominant follicle was also associated with an inhibition in the growth of other cohort follicles (Figure 4.1). Moreover, a new wave of follicular growth was initiated just after the start of regression of the dominant follicle from the previous wave (Figure 4.1 and Table 4.1).

Figure 4.2. Daily changes throughout the oestrous cycle in the mean (±s.e.m.) number of follicles: a) <5mm, b) 5-10mm and c) >10mm in diameter in heifers (n = 9) having 3 waves of dominant follicle growth and development. The periods (day; mean ± s.e.m.) when the dominant follicles were at their maximum size are shown by the hatched bars. Oestrous cycles of heifers were synchronized by two i.m. injections, given 12 days apart, of 0.5mg synthetic prostaglandin $F_{2\alpha}$ (PG). Following the 2nd PG injection, heifers were scanned daily for approximately 30 days. Ovulation occurred approximately 24 hours following the detection of oestrus as indicated.

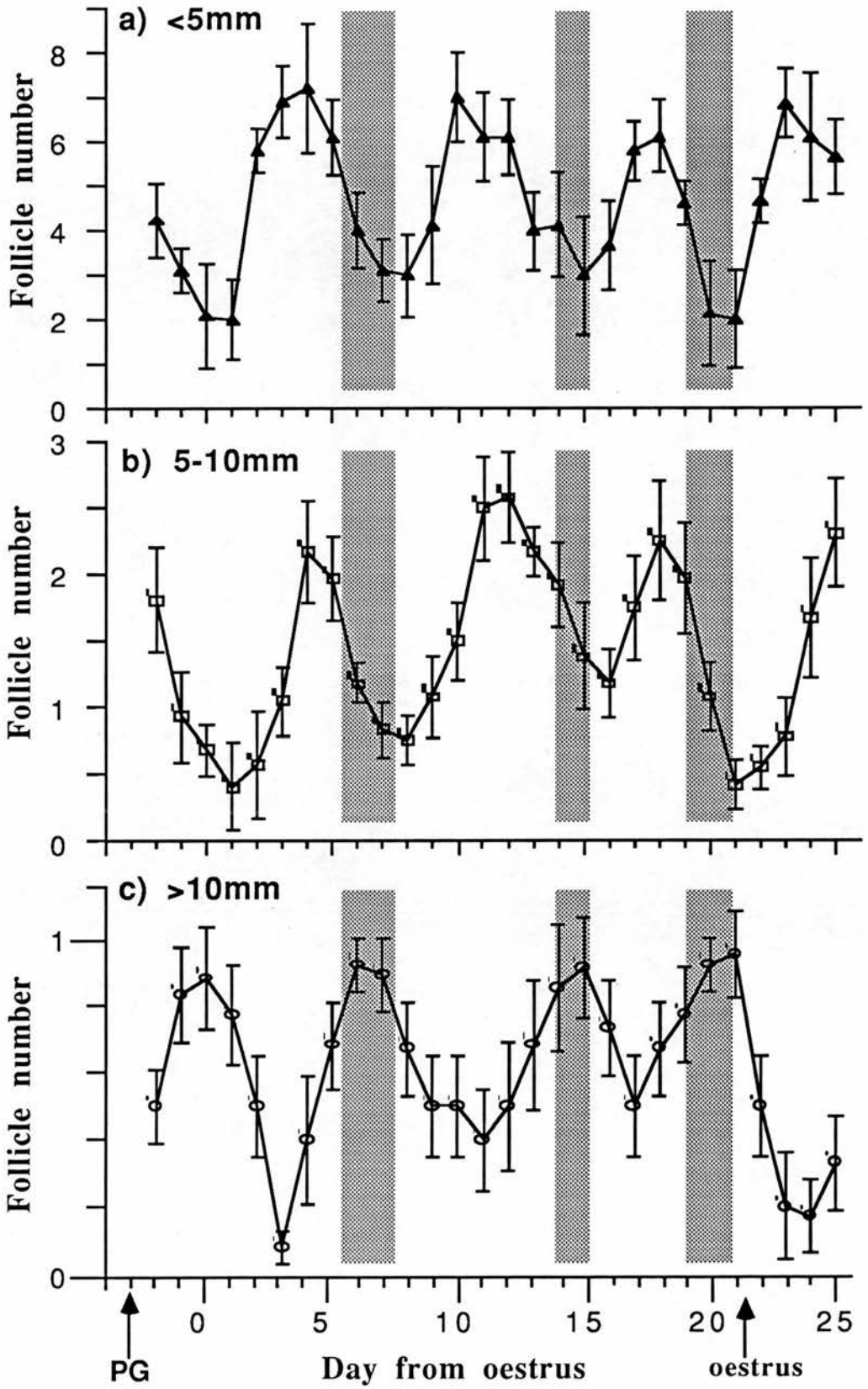

a) <5mm
b) 5-10mm
c) >10mm
Follicle number
Follicle number
Follicle number
0
2
4
6
8
0
1
2
3
0
1
0
5
10
15
20
25
PG
Day from oestrus
oestrus

Figure 4.3. Daily changes throughout the oestrous cycle in the mean (±s.e.m.) number of follicles: a) <5mm, b) 5-10mm and c) >10mm in diameter in heifers (n = 3) having 2 waves of dominant follicle growth and development. The periods (mean day ± s.e.m.) when the dominant follicles were at their maximum size are shown by the hatched bars. Oestrous cycles of heifers were synchronized by two i.m. injections, given 12 days apart, of 0.5mg synthetic prostaglandin $F_{2\alpha}$ (PG). Following the 2nd PG injection, heifers were scanned daily for approximately 30 days. Ovulation occurred approximately 24 hours following the detection of oestrus as indicated.

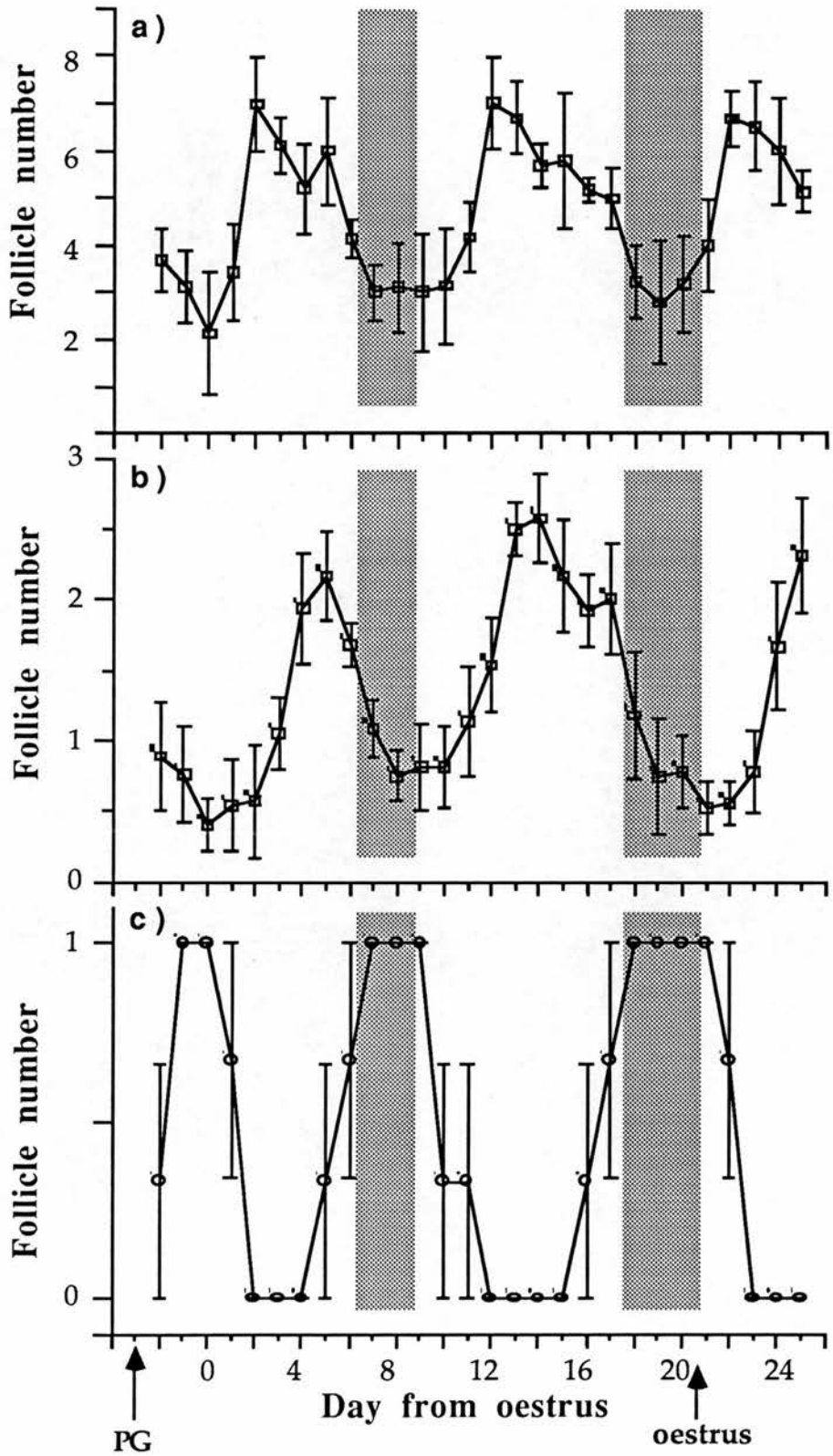

a)
b)
c)
Follicle number
Follicle number
Follicle number
8
6
4
2
3
2
1
0
1
0
0
4
8
12
16
20
24
Day from oestrus
PG
oestrus

Table 4.1. Temporal relationships between wave patterns for the number of ovarian follicles of different sizes during the oestrous cycle in heifers

Wave pattern		*Days with the peak number of follicles		
		<5mm diameter	5-10mm diameter	>10mm diameter
3 waves (n = 9)@	wave 1	2 - 5	3 - 5	6 - 9
	wave 2	10 - 12	11 - 13	14 - 15
	wave 3	16 - 18	17 - 19	20 - 22
2 waves (n = 3)	wave 1	2 - 5	3 - 5	6 - 10
	wave 2	11 - 13	11 - 15	15 - 21

*Day 0 = Day of oestrus; @n = number of heifers

4.4. Discussion

This study demonstrated firstly that the majority of heifers in our herd have 3 waves of follicular growth and development per oestrous cycle. Although the individual growth pattern for small follicles (<5mm in diameter) could not be accurately followed, their number could be assessed using real-time ultrasound. Furthermore, numbers of small follicles assessed by ultrasound scanning was highly correlated ($r = 0.94$) with follicle numbers observed at gross dissection. Secondly, during the oestrous cycle there was a wave pattern in the numbers of follicles in all three size categories. The turnover in the wave patterns for these three categories of follicles was closely related throughout the oestrous cycle, with the growth of the dominant follicle always being associated with a marked reduction in the number and/or growth of its subordinate follicles.

In agreement with previous studies (Quirk *et al.*, 1986; Pierson and Ginther, 1987a; Pieterse, Taverne, Kruip and Willemse, 1990; Driancourt *et al.*, 1991a), our results demonstrated that there was a highly significant correlation ($p<0.01$) between both the diameter of the largest follicles and the numbers of all three size classes of follicles measured by ultrasound and by gross dissection. All large follicles, approximately 44% of medium-sized follicles and 19% of small follicles present at dissection were detected by ultrasound. This is in agreement to the report by Pieterse *et al.* (1990) who found 34% of medium-sized follicles and 96% of large follicles at dissection could be detected by ultrasound. Surprisingly Pierson and Ginther (1987a) reported that nearly all follicles ≥2mm, identified after taking 2mm thick slices of ovaries, could be detected by ultrasound. The reason for the difference in these studies is not known.

In agreement with the previous studies (Savio *et al.*, 1988; Sirois and Fortune, 1988; Knopf *et al.*, 1989), our results confirmed that ovarian follicular growth and development during the oestrous cycle in cattle was not continuous or independent of the stage of the cycle (Choudray *et al.*, 1968; Donaldson and Hansel, 1968; Marion, Gier and Choudray, 1968; Dufour *et al.*, 1972), but occurred in a wave pattern (Rajakoski, 1960; Boland *et al.*, 1990). The majority of the heifers in this study displayed a 3-wave pattern per oestrous cycle, with the first, second and third waves beginning at approximately Days 2, 10 and 16 respectively, in agreement with the reports by Savio *et al.* (1988) and Sirois and Fortune (1988). In contrast, Knopf *et al.* (1989) reported that 9 out of 10 heifers in their study had 2 waves of follicular development during the inter-ovulatory interval. In our study 3 out of 12 heifers showed two waves of follicular development, with characteristics similar to those reported by Knopf *et al.* (1989). It has been suggested that the number of waves observed during an oestrous cycle may depend on the length of the cycle. Moreover, the regression of the CL occurred earlier in heifers with two waves (Knopf *et al.*, 1989; Sirois and Fortune, 1988; Ginther *et al.*, 1989a). No difference in oestrous cycle length between heifers with a 3-wave pattern and heifers with a 2-wave pattern was demonstrated in this study. However, although the number of heifers with a 2-wave pattern was small (n = 3), the duration of the luteal phase was shorter whilst the duration of the follicular phase was longer for heifers with 2 waves compared to heifers with 3 waves of follicular development. It is possible that the turnover in waves of follicular development in cattle is continuous and will only be interrupted when luteolysis and/or a LH surge occur to enable the dominant follicle to undergo preovulatory maturation and subsequently ovulate. This may explain why different

numbers of waves of follicular growth and development could be identified during the oestrous cycle in heifers in both this and previous studies (Savio *et al.*, 1988; Sirois and Fortune, 1988; Knopf *et al.*, 1989). Indeed, it has been shown that follicular growth and development also occurs in waves during the postpartum period (Rajamahendran and Taylor, 1990; Boland *et al.*, 1990) and in early pregnancy (Ginther *et al.*, 1989b; Driancourt *et al.*, 1991a) in cattle. Furthermore, it has also been reported that treatment with hCG during the early (Days 4-7) and late (Days 14-16) stages of the luteal phase (Price and Webb, 1989), or treatment with GnRH on Day 6 of the oestrous cycle (Rusbridge and Webb, 1991), can induce an additional CL in heifers, and that the first dominant follicle of the oestrous cycle in heifers can ovulate following induced luteolysis on Day 7 (Savio, Boland, Hynes, Mattiacci and Roche, 1990b). Moreover, continued periodic emergence of follicular waves has been detected in non-pregnant progesterone-treated heifers (Bergfelt *et al.*, 1991). Similar results have also been reported when the luteal phase of the oestrous cycle was artificially extended by progesterone treatment on Day 14 of the cycle, although the third follicular wave of the oestrous cycle in 3 out of 6 treated animals was considerably prolonged (Sirois and Fortune, 1989).

In this study we have demonstrated clearly that daily changes in the numbers of all follicles, not only medium-sized and large follicles but also small follicles, displayed a wave pattern similar to the turnover of follicular waves throughout the oestrous cycle. When analyzed on an individual heifer basis, the turnover in the wave patterns for these three categories of follicles was closely related, with increases in the numbers of follicles of <5mm, 5-10mm and >10mm in diameter occurring in sequence during each follicular wave throughout the oestrous cycle.

These data agree with the results of Driancourt *et al.* (1991) who reported a well-timed association between the development of the dominant follicle and a change in distribution of follicle numbers in small (<5mm), medium (6-8mm) and large (≥9mm) follicular size classes, but only for the first wave of follicular growth during the oestrous cycle. These results, in addition to confirming the wave pattern of follicular development during the oestrous cycle in cattle, support previous hypotheses regarding the process of folliculogenesis (diZerega, Marut, Turner and Hodgen, 1980; Goodman and Hodgen, 1983; Hodgen, Kenigsburg, Collins and Schenken, 1985; Ireland and Roche, 1987), which suggest that folliculogenesis involves three consecutive phases, i.e. follicle recruitment, follicle selection and follicle dominance.

Our results also demonstrated that the growth of the dominant follicle in each wave was consistently associated with a marked reduction in both the number and growth of medium-sized subordinate follicles. Growth of the dominant follicle was also associated with a decrease in the number of small subordinate follicles. However, whether this was due to an inhibition in follicle recruitment, to an inhibition in the growth of small follicles or to an increase in the atresia rate of these small follicles could not be assessed, as the growth pattern for small follicles could not be determined precisely. Inhibition of follicle growth and/or increase in atresia rate seems the more likely, as the recruitment of small follicles into the growing pool has been suggested to be a continuous process (Peters *et al.*, 1975; Ireland, 1987). Furthermore, it appears that regression of the dominant follicle is necessary for the initiation of the following wave of follicular growth during the oestrous cycle (see Table 4.1). These results provide direct evidence to support the concept that dominant follicles have an inhibitory effect on subordinate follicles in

both ovaries (Ireland, 1987; Pierson and Ginther, 1987b, 1988; Kastelic *et al.*, 1990).

In conclusion, this study demonstrates that, whilst the growth pattern of small follicles (<5mm in diameter) could not be followed individually, the number of small follicles could be assessed using real-time ultrasound. In addition, the growth of the dominant follicle was associated with a marked reduction in the number and/or growth of subordinate follicles.

CHAPTER 5

The effect of recombinant bovine somatotropin on dynamics of ovarian follicular growth and development in heifers

5.1. Introduction

Treatment of heifers with BST for a period of two oestrous cycle doubled the population of small ovarian follicles (2-5mm in diameter), as measured by gross dissection at slaughter (see Chapter 3). This effect did not appear to be mediated through changes in circulating gonadotrophin concentrations or in ovarian gonadotrophin receptor levels. The underlying mechanism(s), however, remains unclear, although peripheral IGF-I, insulin and GH concentrations were increased. Since gross dissection only allowed follicular populations to be measured at a single time point, monitoring the dynamic changes in ovarian follicular populations during BST treatment using daily real-time ultrasound scanning should lead to a better understanding of the underlying mechanism(s). Furthermore, this approach would also allow the determination of the temporal relationships, between the changes in the follicular population and the changes in peripheral GH, insulin and IGF-I concentrations following BST treatment, which may also throw some light on the mechanism(s) involved, and help to overcome the high variability in ovarian follicular populations between animals (Rajakoski, 1960; Erickson, 1966b) which may bias the results obtained using gross dissection.

We have demonstrated in Chapter 4 that not only large (>10mm)

and medium-sized follicles (5-10mm) (Boland *et al.*, 1990), but also small ovarian follicles (<5mm), can be assessed by real-time ultrasound scanning. The objectives of this study were to: (1) examine the effect of daily BST (25mg) injections on ovarian follicular dynamics; (2) using a single dose of BST (320mg in a sustained-release formulation), determine the temporal relationships between the changes in peripheral GH, insulin and IGF-I concentrations and the changes in the small follicle population.

5.2. Materials and Methods

24 heifers were used in two experiments of this study under the husbandry conditions described in Chapter 2.

5.2.1. Experimental procedures

Experiment I: The experimental protocol used is shown in Figure 5.1. Oestrous cycles of 12 heifers were synchronized as described in Chapter 3. Heifers were divided randomly into two groups of 6 (BST group and control group). From Day 7 (day of oestrus = Day 0) of the first oestrous cycle, all animals in the BST group were injected daily, subcutaneously, with a sterile preparation of 25 mg BST (Somidobove; Eli Lilly & Co., Indianapolis, USA; 1 mg/ml in a vehicle containing 0.15M NaCl, 0.025M $NaHCO_3$ and 0.025M Na_2CO_3, pH 9.6), for a period of two oestrous cycles (43 days). Control animals received 25ml of vehicle for the same period. Blood samples were collected by jugular venepuncture three times a week from Day 3 of the first oestrous cycle until the end of the experiment. Serum was stored at -20°C for the subsequent

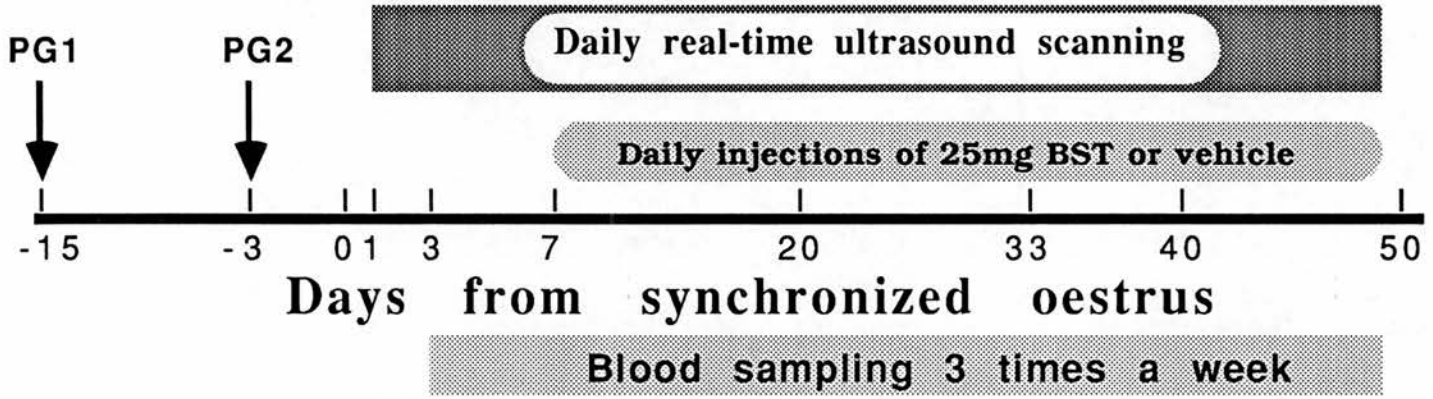


Figure 5.1. Protocol for Experiment I

measurement of peripheral GH, insulin, IGF-I and progesterone concentrations. All heifers were scanned daily from Day 1 of the first oestrous cycle until the end of the experiment. Data obtained from ultrasound scanning were analysed as described in Chapter 4.

Experiment II: The experimental protocol is detailed in Figure 5.2. Oestrous cycles of a second batch of 12 heifers were synchronized. Heifers were then divided randomly into two groups of 6. On Day 7 of the oestrous cycle, one group (BST group) was injected subcutaneously with 320mg Somidobove in a sustained release formulation (Eli Lilly & Co., Indianapolis, IN, USA) while the other group (control group) received 10ml of saline. All heifers were scanned daily from Day 6 of the oestrous cycle until 3 days after ovulation, as determined by ultrasound scanning. Blood samples were collected daily, by jugular venepuncture, throughout the experimental period. Serum was collected and stored at -20°C for the subsequent measurement of peripheral GH, insulin, IGF-I and progesterone concentrations.

5.2.2. Statistical analyses

Daily changes in diameter for all the follicles that could be followed by ultrasound for at least 2 consecutive days were plotted for each animal throughout each oestrous cycle, to determine the wave pattern of follicular development. Daily changes in follicle numbers for the three size categories of follicles (small, medium-sized and large) throughout the oestrous cycles were determined. Statistical differences in the numbers of follicles and temporal changes in hormone concentrations between the two treatment groups were analyzed separately before and after the start of BST treatment by split-plot ANOVA

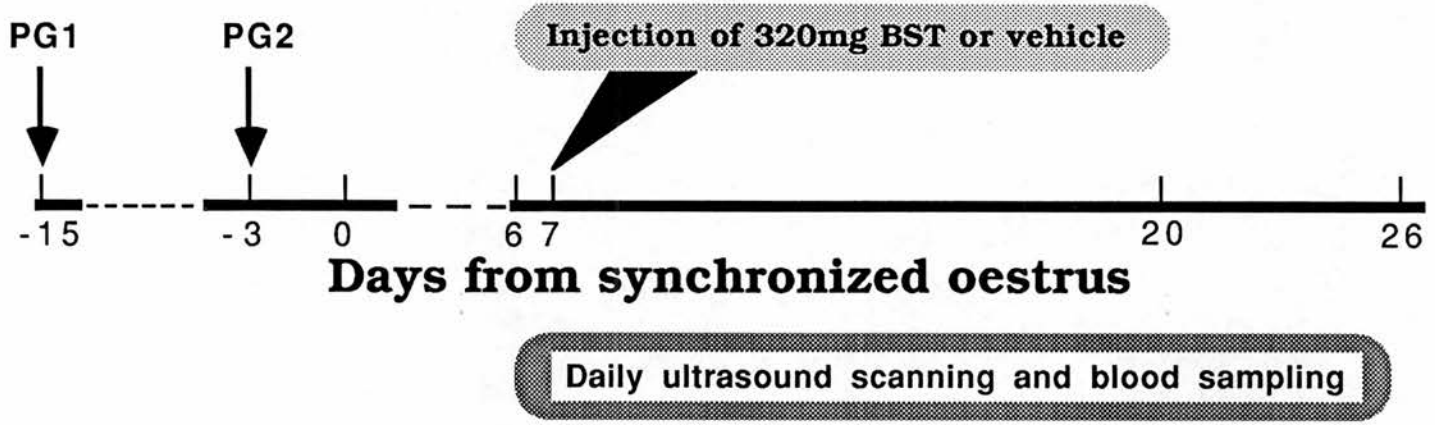


Figure 5.2. Protocol for Experiment II

for repeated measures. The main effects included treatment, time and the interaction of treatment by time. The effect of BST treatment on the number of follicular waves per oestrous cycle was analyzed by Chi square test. Correlations between mean follicle numbers and GH, insulin and IGF-I concentrations throughout the treatment periods were determined by regression analysis.

5.3. Results

5.3.1. Experiment I

5.3.1.1. Peripheral GH, insulin and IGF-I concentrations

The effects of daily treatment with 25mg BST on peripheral GH, insulin and IGF-I concentrations were similar to the results in Chapter 3. Before the start of BST treatment, peripheral GH, insulin and IGF-I concentrations were not different between the two treatment groups, being 22.15 ± 2.96, 0.59 ± 0.09 and 151.89 ± 8.76 ng/ml respectively for the control group and 23.43 ± 3.12, 0.64 ± 0.11 and 158.74 ± 9.43 ng/ml respectively for the BST group (effect of treatment and effect of treatment by day, $p>0.05$). Peripheral GH, insulin and peripheral IGF-I concentrations for the BST group increased within three days after the start of daily BST injection and were significantly (effect of treatment, $p<0.01$; effect of treatment by day, $p>0.05$) elevated (37.44 ± 2.31, 0.97 ± 0.17 and 231.08 ± 16.76 ng/ml respectively) throughout the treatment period compared to the control group (23.34 ± 1.86, 0.54 ± 0.08 and 153.87 ± 10.89 ng/ml respectively).

5.3.1.2. Patterns of follicular growth and development

2/6 of the BST-treated heifers and 1/6 of the control heifers ($p>0.05$) displayed a 2-wave pattern of follicular development during the oestrous cycle, whilst the remainder had 3 waves per oestrous cycle. Within each animal, the number of follicular waves per oestrous cycle was consistent over the two oestrous cycles for all heifers. Dynamics of follicular growth and development throughout the experimental period for a representative heifer with a 2-wave oestrous cycle and a representative heifer with a 3-wave cycle is shown in Figure 5.3. All characteristics of follicular dynamics during the oestrous cycle (the starting day of each wave, length of the oestrous cycle, wave pattern of changes in follicle numbers and the relationship between the dominant follicle and its subordinate follicles) were similar to those described in Chapter 4.

Whilst there was no difference between the two treatment groups before the start of daily BST injections (effect of treatment, $P>0.05$; effect of treatment by day, $P>0.05$), BST-treated heifers had significantly more small follicles throughout the treatment period (effect of treatment, $P<0.01$) (Figure 5.4). There was no interaction between treatment and day ($p>0.05$), but the effect of day for the number of small follicles was significant ($p<0.01$). There was no effect of BST treatment on the inhibitory effect of the dominant follicle on subordinate follicles (Figure 5.4). As shown in Figure 5.5, there was no effect of BST treatment on either the number or the wave pattern for medium sized and large follicles (effect of treatment; effect of treatment by day, $p>0.05$).

There was a significant correlation between the overall mean number of small follicles and mean GH ($r = 0.65$, $p<0.05$), IGF-I ($r = 0.78$,

Figure 5.3. Dynamics of ovarian follicular growth and development throughout the experiment period for a representative heifer with 2 follicular waves per cycle (upper panel) and a representative heifer with 3 follicular waves (lower panel) in Experiment I. Oestrous cycles of heifers were synchronized by two i.m. injections, given 12 days apart, of 0.5mg synthetic prostaglandin $F_{2\alpha}$ (PG). Day 0 on the horizontal axis represents the day of oestrus following the 2nd PG injection. Heifers were injected daily with 25mg BST or vehicle for a period of approximately two oestrous cycles, starting on Day 7 of the first synchronized oestrous cycle as indicated by the arrow. Animals were scanned daily from Day 1 of the cycle until the end of the experiment. All follicles with a growth pattern which could be followed for at least two consecutive days are plotted. The dark circles represent the growth pattern of the ovulatory dominant follicles which normally ovulated approximately 24 hours following the detection of oestrus as indicated.

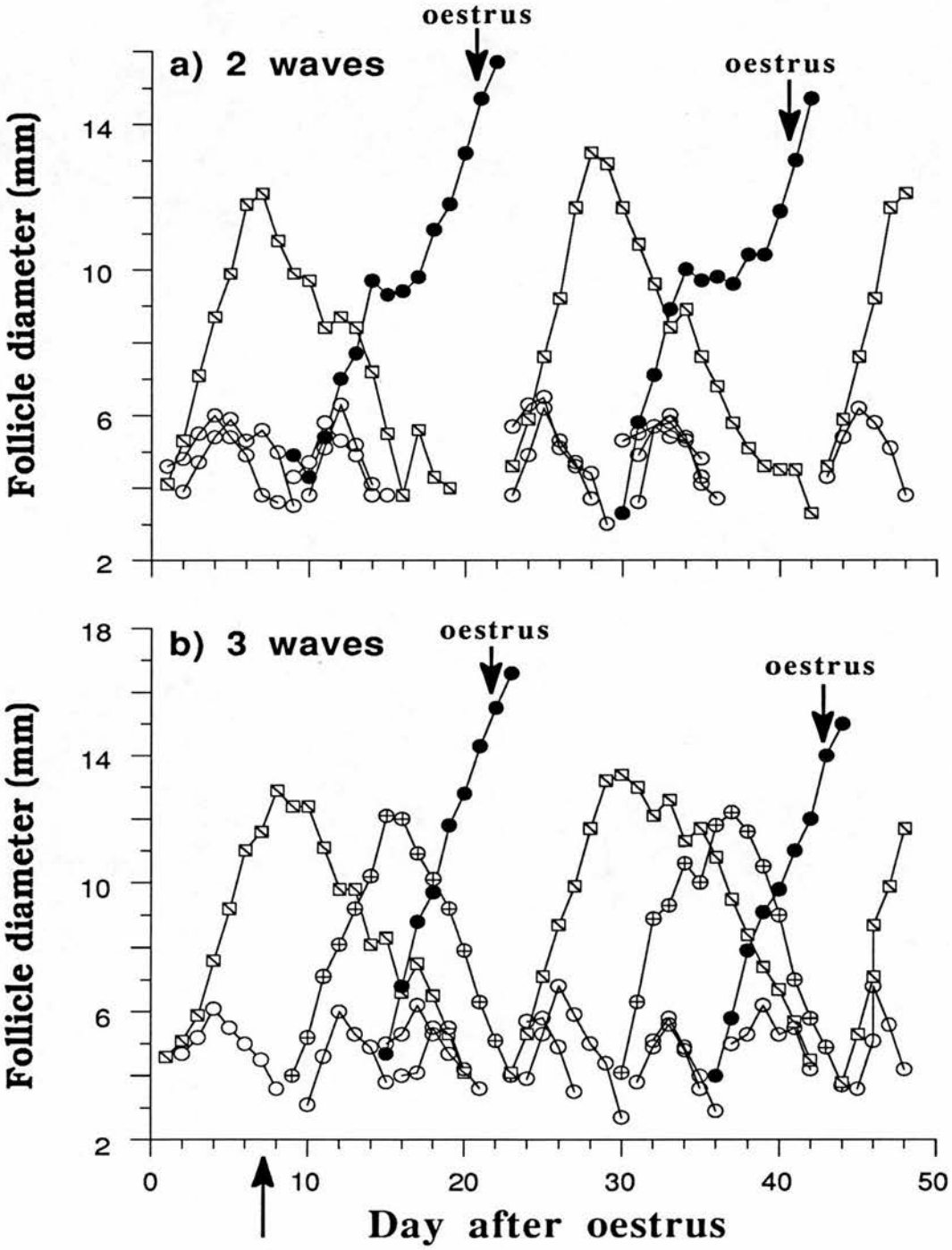

a) 2 waves
oestrus
oestrus
Follicle diameter (mm)
14
10
6
2
b) 3 waves
oestrus
oestrus
Follicle diameter (mm)
18
14
10
6
2
0
10
20
30
40
50
Day after oestrus

Figure 5.4. Mean (±s.e.m.) number of follicles <5mm in diameter before and after the start of daily injections of either BST or vehicle (n = 6 per group). All animals with either 2 or 3 waves of dominant follicles have been included in the figure. Heifers were treated as described in Figure 5.3. The arrow on the horizontal axis indicates the start of daily BST or vehicle injection. The period (mean day ± s.e.m., n = 12 as there was no difference in this parameter between the two groups) when the dominant follicles were at their maximum diameter is shown by the hatched boxes.

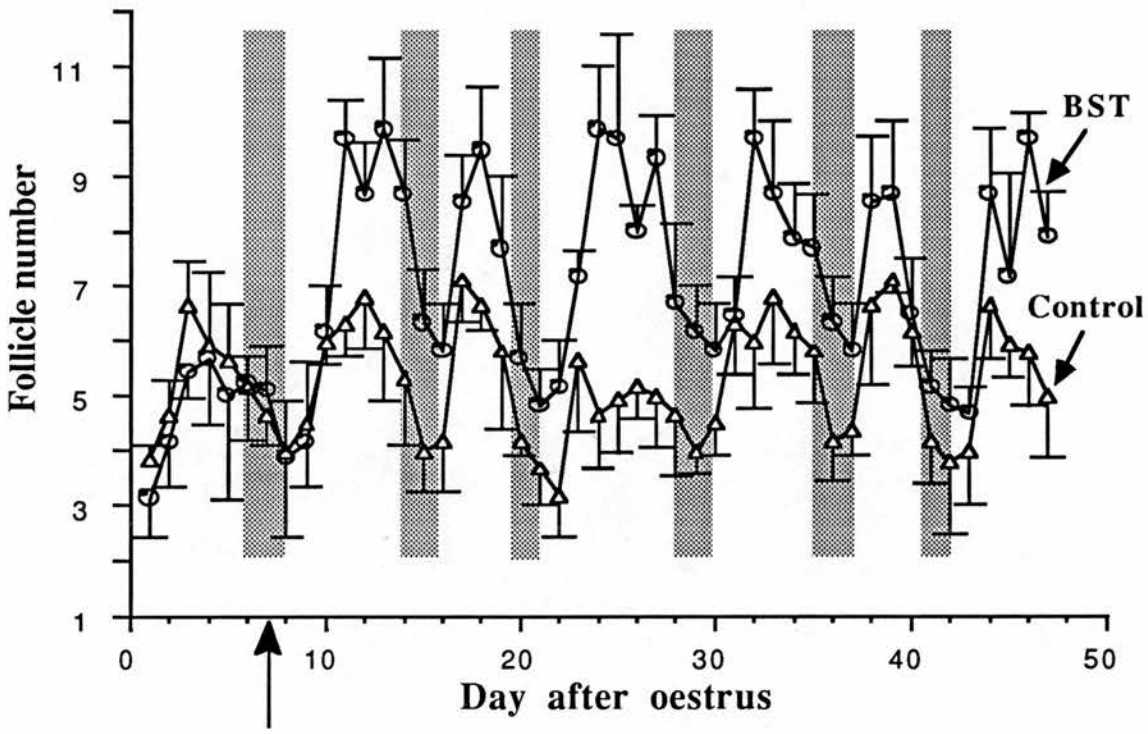
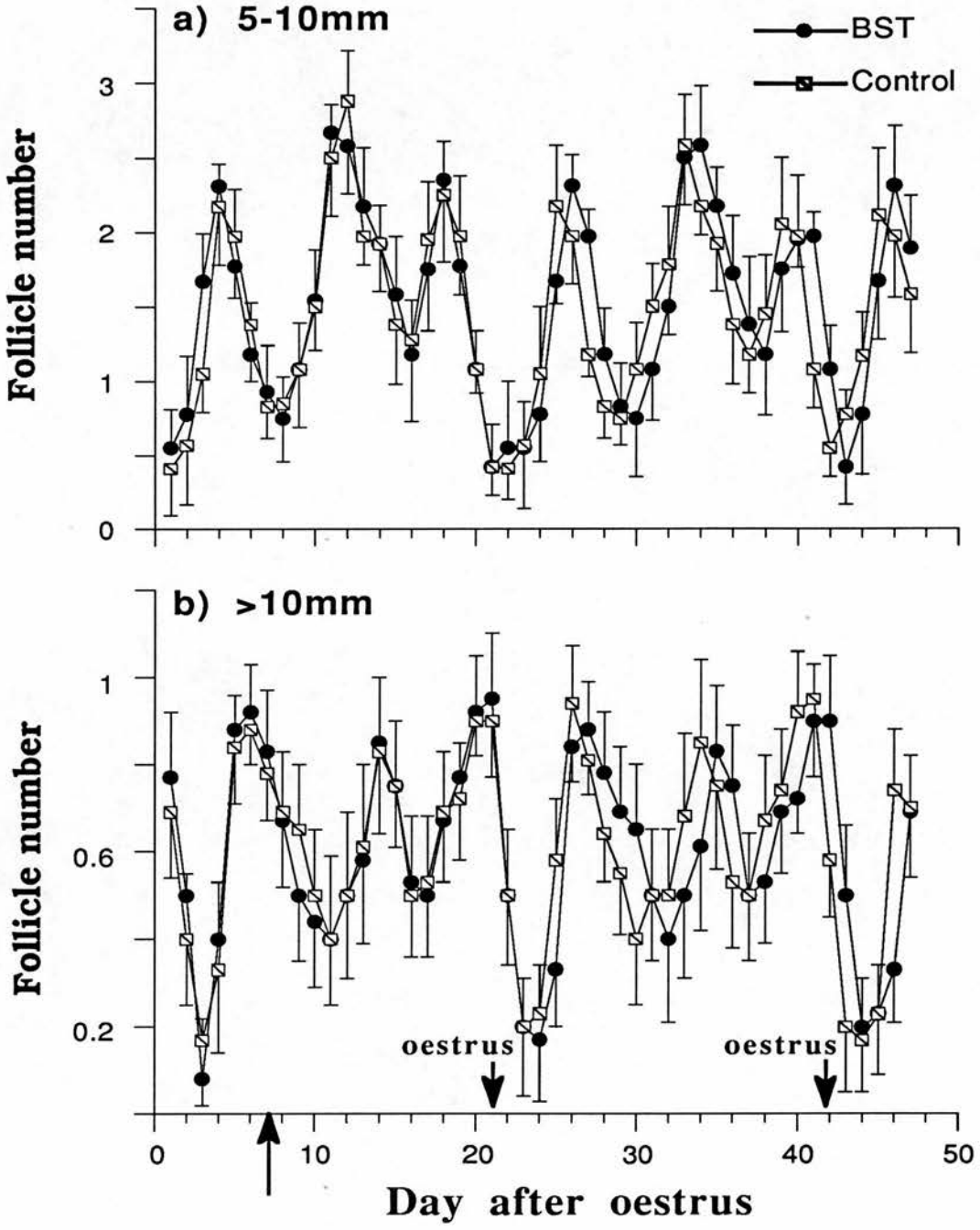

Follicle number
11
9
7
5
3
1
0
10
20
30
40
50
Day after oestrus
BST
Control

Figure 5.5. The effect of daily BST injection on the number and wave pattern for follicles 5-10mm (upper panel) and >10mm (lower panel) in diameter throughout the experiment period (n = 6 heifers per group). Heifers were treated as described in Figure 5.3. The arrow on the horizontal axis indicates the start of daily BST or vehicle injections.


a) 5-10mm
BST
Control
Follicle number
3
2
1
0
b) >10mm
Follicle number
1
0.6
0.2
oestrus
oestrus
0
10
20
30
40
50
Day after oestrus

$p<0.01$) and insulin ($r = 0.64$, $p<0.05$) concentrations throughout the experimental period.

5.3.2. Experiment II

5.3.2.1. Temporal relationships between GH, IGF-I and insulin and numbers of small follicles

The changes in peripheral GH, insulin and IGF-I concentrations and numbers of follicles <5mm in diameter following a single treatment with a sustained-release formulation of BST (320mg) are shown in Figure 5.6. Peripheral GH in BST-treated heifers increased within 24 hours after BST injection, followed within 24 hours by a significant increase in both IGF-I and insulin concentrations. The levels of all three hormones remained significantly elevated (effect of treatment, $p<0.01$) for approximately 7-9 days when compared to control animals. There was a significant effect of day ($p<0.05$) and of treatment by day ($p<0.01$). The numbers of small follicles increased within 24 hours of the rise in IGF-I and insulin concentrations and remained elevated throughout the period when peripheral IGF-I and insulin concentrations were increased (effect of treatment, $p<0.01$; effect of day, $p<0.05$; effect of treatment by day, $p<0.01$). There was a significant correlation between numbers of small follicles and mean peripheral GH ($r = 0.68$, $P<0.01$), IGF-I ($r = 0.79$, $P<0.01$) and insulin ($r = 0.73$, $p<0.01$) concentrations throughout the experimental period.

Similar to Experiment I, all other parameters of ovarian follicular dynamics were unchanged by BST treatment. There was no effect of BST treatment on peripheral progesterone profiles (Figure 5.7).

Figure 5.6. Daily mean (±s.e.m.) peripheral GH concentrations (a), peripheral IGF-I concentrations (b), peripheral insulin concentrations (c) and number of follicles <5mm in diameter (d) in control and BST-treated heifers (n = 6 heifers per group) throughout Experiment II. Heifers were given a single injection, as shown by the arrow, of either BST (320mg in a sustained release formulation) or saline (10ml) on Day 7 of the synchronized oestrous cycle. Day 0 on the horizontal axis represents the day of oestrus following synchronisation with prostaglandin $F_{2\alpha}$ as described in Figure 5.3.

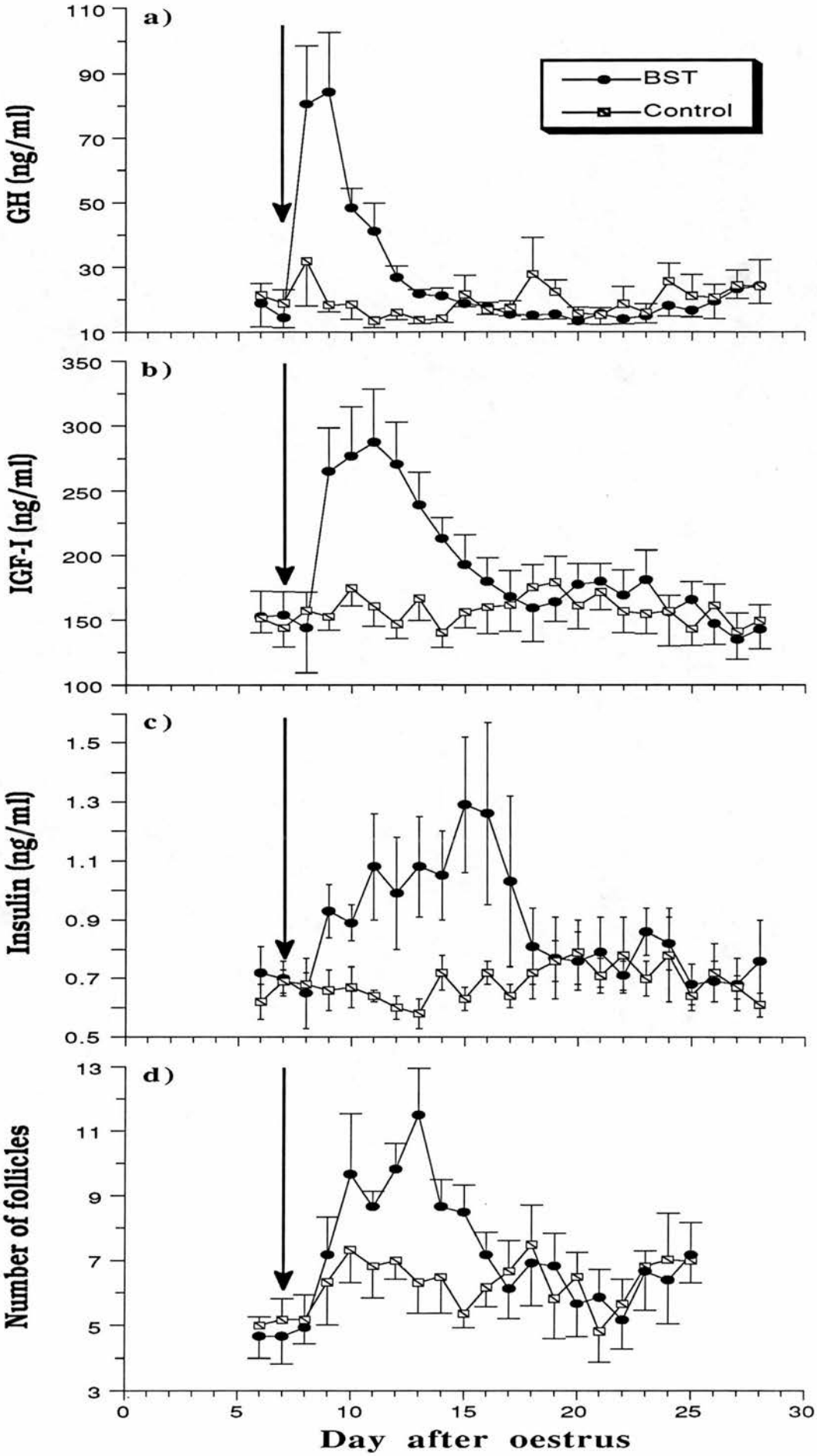
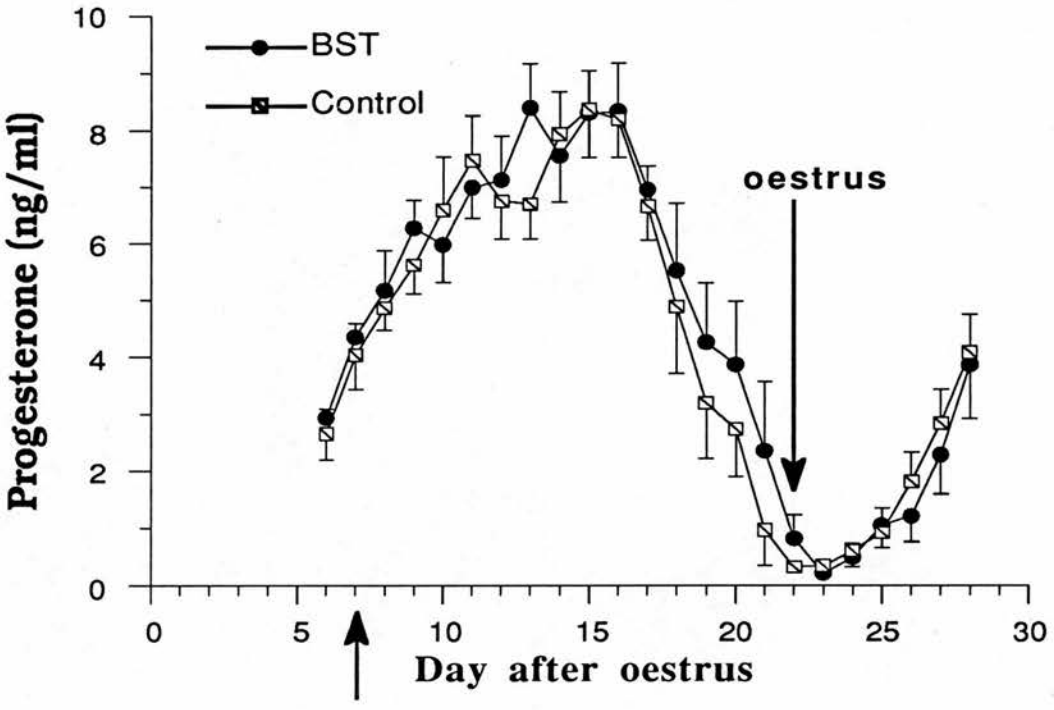

a)
b)
c)
d)
BST
Control
GH (ng/ml)
IGF-I (ng/ml)
Insulin (ng/ml)
Number of follicles
Day after oestrus
110
90
70
50
30
10
350
300
250
200
150
100
1.5
1.3
1.1
0.9
0.7
0.5
13
11
9
7
5
3
0
5
10
15
20
25
30

Figure 5.7. The effect of a single dose of BST (320mg in a sustained-release formulation) on peripheral progesterone concentrations (n = 6 heifers per group). Animals were treated as described in Figure 5.6. The arrow on the horizontal axis indicates the day of BST or vehicle injection.


BST
Control
oestrus
Progesterone (ng/ml)
Day after oestrus
0
2
4
6
8
10
0
5
10
15
20
25
30

5.4. Discussion

This study, using real-time ultrasonography, has demonstrated that BST treatment could increase the number of small ovarian follicles within approximately 3 days following the start of treatment and that the changes in the number of small follicles was temporally correlated with peripheral IGF-I and insulin concentrations. Moreover, BST treatment significantly increased the number of small follicles at all stages of the oestrous cycle, although the dynamics of follicular growth and the inhibitory effect of the dominant follicle on its subordinate follicles were not altered by BST treatment.

The results from this study, using real-time ultrasound, have confirmed our previous finding using gross dissection (see Chapter 3) that BST treatment can double the number of small follicles in mature heifers. The study has also extended this finding in that the effect of BST could be detected within three days after the start of BST treatment. This temporal relationship was further confirmed using a single injection of BST in a sustained-release formulation. Moreover, changes in the numbers of small follicles were temporally correlated with changes in peripheral IGF-I and insulin concentrations. Within 24 hours following a single dose of BST injection, peripheral GH concentrations were increased, followed within 24 hours by a rise in peripheral IGF-I and insulin concentrations, and within 48 hours by a significant increase in the number of follicles <5mm in diameter. Both IGF-I and insulin have been implicated in the regulation of ovarian function (Adashi *et al.*, 1985a; Savion *et al.*, 1981; Poretsky and Kalin, 1987; Langhout *et al.*, 1991). Similar to the results in Chapter 3, the number of small follicles was significantly correlated with not only peripheral IGF-I and insulin

concentrations, but also GH concentrations. However, the increase in the number of small follicles remained throughout the period when peripheral IGF-I and insulin concentrations were elevated, even when peripheral GH concentrations had returned to control levels. These results suggest that the effect of BST on small follicles is mediated through increased peripheral IGF-I and/or insulin concentrations.

The increase in numbers of small follicles was maintained throughout the BST treatment period, even during the period when the dominant follicle was at its maximum diameter and the growth of the subordinate follicles was suppressed. Furthermore, neither the wave dynamics of follicular growth and development during the oestrous cycle nor the inhibitory effect of the dominant follicle on its subordinate follicles were altered by BST treatment. These results suggest that the mechanism(s) by which BST increased the number of small ovarian follicles are different from the mechanism(s) by which the dominant follicle suppresses its subordinate follicles. As has been discussed in Chapter 4, the dominant follicle most likely suppresses its subordinate follicles by inhibiting the growth and/or increasing the atresia rate of these follicles, rather than by inhibiting follicle recruitment, since recruitment of small follicles into the growing pool is thought to be a continuous process (Peters *et al.*, 1975; Ireland, 1987). Hence, our data suggest that the increase in the number of small follicles induced by BST treatment results from an enhancement in the recruitment of these small follicles, although this will require further investigation.

In agreement with the previous study using daily BST injection (see Chapter 3), a single dose of 320mg BST in a sustained-release formulation did not affect peripheral progesterone concentrations. This

contrasts with studies of Gallo and Block (1989) and Schemm *et al.* (1990) who found significantly higher plasma progesterone concentrations in cows treated with BST. The reasons for these differences are not known although these two previous studies were carried out in lactating dairy cows.

In conclusion, the results from this study suggest that the increase in the number of small follicles induced by BST treatment in heifers seems to result from an enhancement in the recruitment of these follicles, possibly through increased peripheral IGF-I and/or insulin concentrations (though a direct effect of BST at the ovarian level cannot be excluded). Furthermore, BST did not affect the wave pattern of follicular growth, nor the inhibitory action of the dominant follicle on its subordinate follicles. Thus, the effect of BST on small follicle numbers does not appear to be mediated through the mechanism(s) by which the dominant follicle inhibits its subordinate follicles.

CHAPTER 6

The effect of recombinant bovine somatotropin on the superovulatory response to PMSG in heifers

6.1. Introduction

Currently, one of the major problems associated with superovulation in cattle is the large variability in response, both between and within individual animals (Monniaux *et al.*, 1983; Rieger, Desaulniers and Goff, 1988). This has limited the efficiency of embryo transfer because of the lack of a predictable and reliable supply of embryos (Gordon, 1982). To obtain a large (but predictable) number of viable embryos, the two key determinants are the administration of appropriate hormones and the number of gonadotrophin-responsive follicles present in ovaries at the time of gonadotrophin stimulation (Moor *et al.*, 1984; Murphy *et al.*, 1984). Attempts to improve the superovulatory response by using different gonadotrophin preparations and treatment regimens, and/or pretreatment of animals with low doses of gonadotrophin, have achieved some success in increasing ovulation rate and number of transferable embryos recovered (Monniaux *et al.*, 1983; Moor *et al.*, 1984; Murphy *et al.*, 1984; Rieger *et al.*, 1988; Price, 1991). However, none of the superovulatory regimens currently used in cattle can control between-animal differences in the population of gonadotrophin-responsive follicles at the time of stimulation, and therefore this primary source of high variability in response during superovulation remains (Moor *et al.*, 1984; Bellows *et al.*, 1991).

Treatment with BST increases the number of antral follicles 2-5mm in diameter in mature heifers (see Chapter 3 and 5). It has been suggested that the population of healthy ovarian follicles >1.7mm in diameter present at the time of gonodotrophin treatment may play a very important part in determining the subsequent superovulatory response in cattle (Monniaux *et al.*, 1983). Thus, if the small follicles induced by BST treatment are functionally healthy, this increase in small antral follicle numbers by BST treatment may enhance the superovulatory response. When follicular populations were not investigated, co-treatment with GH has been shown to improve (although not consistently) the response of ovulation induction in women undergoing IVF treatment (Blumenfeld and Lunenfeld, 1989; Matson *et al.*, 1989; Homburg *et al.*, 1990) and increase the superovulatory response in cattle to some limited extent (Herrler *et al.*, 1990; Rieger *et al.*, 1991).

As demonstrated in Chapter 5, a single injection of 320mg BST (in a sustained-release formulation) on Day 7 of the oestrous cycle in heifers induced, within approximately 3 days of the treatment, an increase in the number of small antral follicles which was maintained for about 8 days. Using this experimental model, the aim of this study was to investigate whether the superovulatory response to PMSG stimulation in heifers could be enhanced and/or improved by pretreatment with BST.

6.2. Materials and Methods

24 heifers were used in this study under the husbandry conditions described in Chapter 2.

6.2.1. Experimental procedures

The experimental protocol used is shown in Figure 6.1. The timing of the treatment was based on the results shown in Figure 5.6 (Chapter 5). Briefly, oestrous cycles of the heifers were synchronized using Estrumate as described in the previous Chapters. Heifers were then divided into four treatment groups of 6 animals in a randomized block design: (1) control; (2) BST (320mg); (3) PMSG (2000iu); (4) BST (320mg) + PMSG (2000iu). On Day 7 of the oestrous cycle (Day 0 = Day of oestrus), animals in Groups (2) and (4) were injected subcutaneously with 320mg BST (Somidobove; Eli Lilly & Co., Indianapolis, USA) in a sustained-release formulation, whilst heifers in Groups (1) and (3) received 10ml of saline. Five days later, heifers in Groups (3) and (4) were treated with a single dose of 2000iu PMSG (Folligon; Intervet U.K. Ltd., Cambridge, England) to induce superovulation, whilst heifers in Groups (1) and (2) received vehicle only. On Day 14 of the oestrous cycle, all heifers were injected with 1mg of Estrumate (Coopers Animal Health Ltd., Crewe, Cheshire, England). Animals in Groups 3 and 4 were artificially inseminated (2 straws of frozen semen) twice daily during oestrus. Embryos were recovered non-surgically (see Chapter 2) on Days 6 - 8 of the following oestrous cycle and classified either as unfertilised eggs, degenerate embryos or transferable embryos as described in Chapter 2. All heifers underwent laparoscopy on Day 9 (see Chapter 2) to assess ovulation rate. Heifers were scanned daily from the day before BST injection until the day of oestrus to monitor dynamics of ovarian follicular growth and development. Blood samples were collected daily throughout the experimental period for progesterone, oestradiol, GH, and IGF-I measurements.

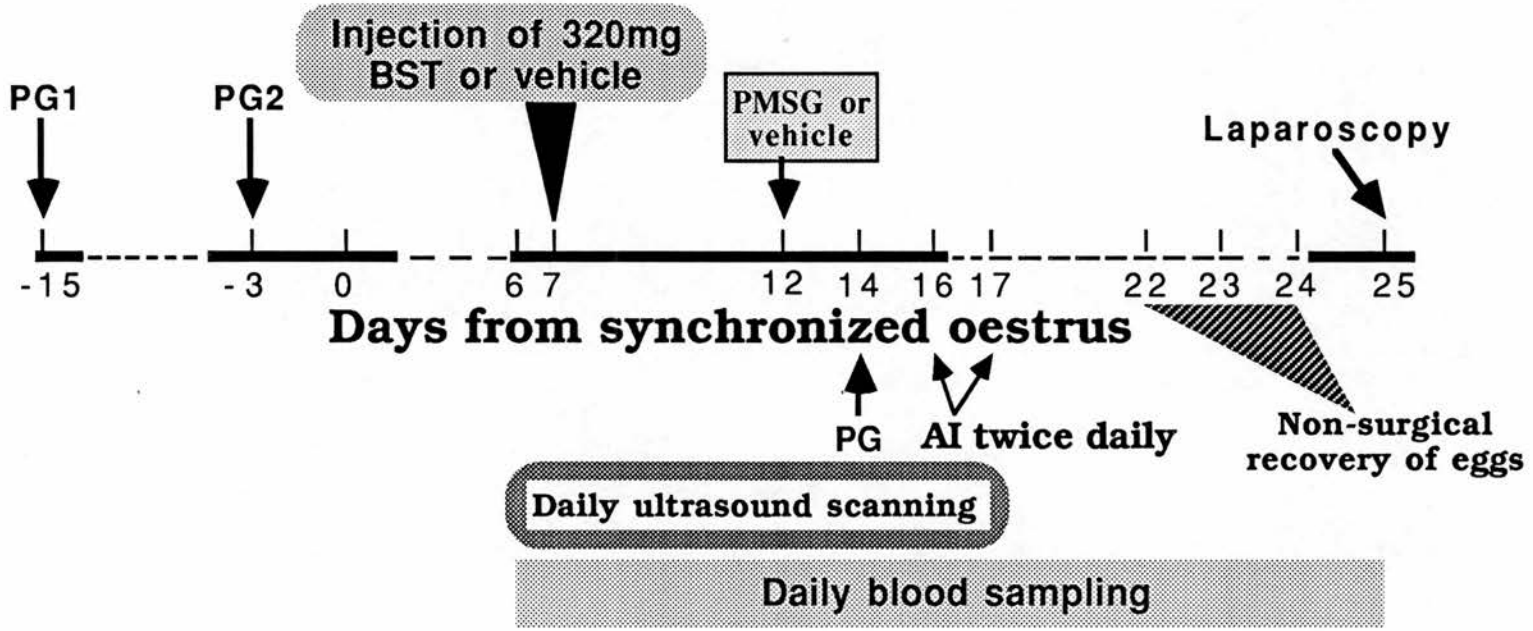


Figure 6.1. Experimental protocol

6.2.2. Statistical analyses

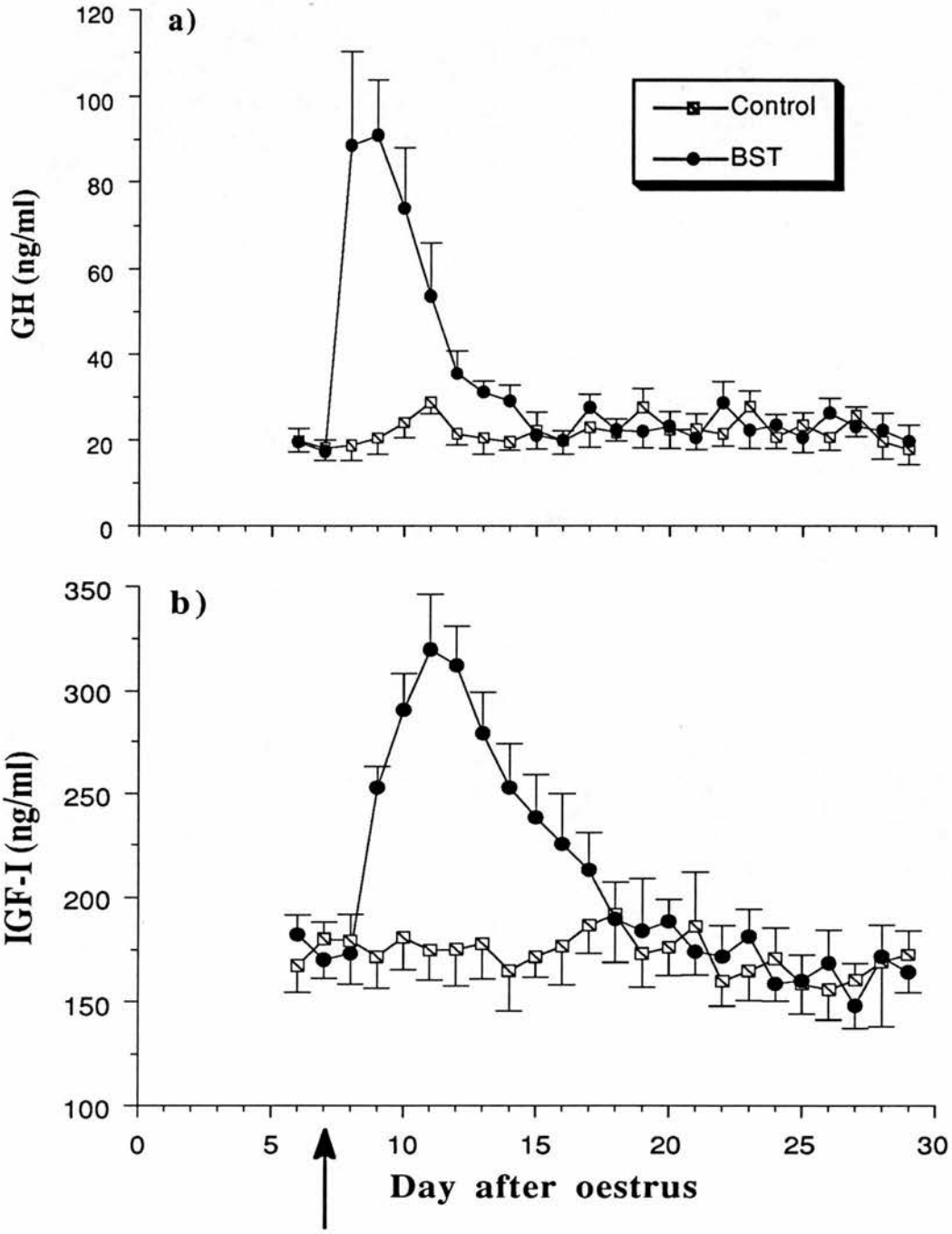
All follicles detected by ultrasound were divided into three size groups: <5mm (small), 5-10mm (medium-sized) and >10mm (large) in diameter. The daily changes in the populations of follicles in all three size groups were determined throughout the experiment. Statistical differences in follicle numbers, number of ovulations, number of eggs recovered, number of transferable embryos and temporal hormone data between the treatment groups were analysed by ANOVA (with repeat measurements, if applicable). The percentage of transferable embryos was calculated as the number of transferable embryos divided by the number of fertilized eggs. Differences between groups in the percentage of transferable embryos and the numbers of heifers with a poor response were analysed by Chi square test. A poor response was defined as an ovulation rate of ≤3 and/or the development of either follicular cysts or cystic ovaries (see also Saumande, Chupin, Mariana, Ortavant and Mauleon, 1978). Correlations between two parameters were determined by regression analysis.

6.3. Results

6.3.1. Peripheral profiles of GH and IGF-I following BST treatment

The profiles of peripheral GH and IGF-I before and after a single injection of BST are shown in Figure 6.2. There were no differences in the profiles of GH or IGF-I between the control and PMSG groups and between the BST and BST + PMSG groups. Peripheral concentrations of GH for the BST-treated groups increased within 24 hours after the start of BST

Figure 6.2. Mean (+/-s.e.m.) serum GH (a, upper panel) and IGF-I (b, lower panel) concentrations throughout the experimental period for four treatment groups (n = 6). Animals were treated as described in Figure 6.1. As there were no differences in the profiles of GH and IGF-I both between the control and PMSG groups and between the BST and PMSG + BST groups, the data were pooled respectively (n = 12) and shown as "Control" and "BST" respectively in the figure. The arrow on the horizontal axis represents the day of BST or vehicle injection.


a)
GH (ng/ml)
120
100
80
60
40
20
0
Control
BST
b)
IGF-I (ng/ml)
350
300
250
200
150
100
0
5
10
15
20
25
30
Day after oestrus

injection and remained significantly higher (effect of treatment, $p<0.01$) for about 7 days when compared to the control and PMSG groups. The increase in GH concentrations was followed about 24 hours later by an increase in peripheral IGF-I levels and this increase was maintained for about 8 days (effect of treatment, $p<0.01$).

6.3.2. Ovulation rate

Ovulation rate (taken as the number of CL at laparoscopy, see Figure 6.3) for all the treatment groups is shown in Table 6.1. There was no effect on ovulation rate of BST treatment alone. However, heifers pretreated with BST had a significantly greater (effect of treatment, $p<0.01$) ovulation rate following superovulatory treatment with 2000iu of PMSG when compared to the PMSG group. One heifer from the BST + PMSG group and three out of six heifers in the PMSG group ($p<0.05$) had a poor response (see 6.2.2 for definition).

6.3.3. Number of eggs recovered and transferable embryos

The BST + PMSG group had a greater total number of eggs recovered ($p<0.01$) when compared to the PMSG group (Table 6.2). The number of transferable embryos was not different between the two treatment groups ($p>0.05$), whilst the number of unfertilised eggs was greater ($p<0.05$) and the percentage of transferable embryos was lower ($p<0.01$) in the BST + PMSG group compared to PMSG group (Table 6.2).

6.3.4. Peripheral oestradiol concentrations

Profiles of peripheral oestradiol concentrations following PMSG treatment are shown in Figure 6.4. There was no significant difference between the control and BST groups. Both PMSG-treated groups had

Figure 6.3. A representative photograph (taken during laparoscopy) showing both ovaries with multiple corpora lutea from heifers which have been treated with PMSG to induce superovulation.

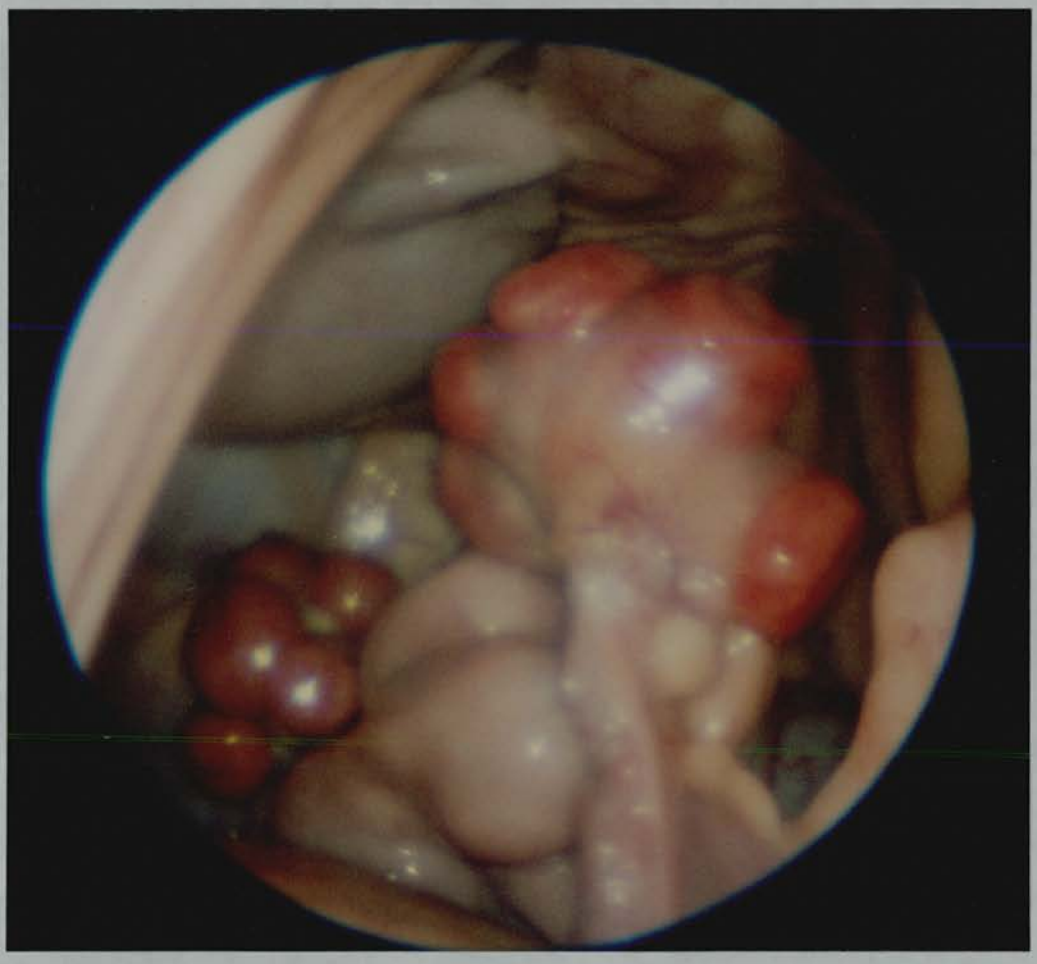


Table 6.1. Effect of pretreatment with BST on the superovulatory response to 2000iu PMSG stimulation (n = 6)

Treatment	Number of ovulations	Number of heifers with a poor response*
Control	1.0 ± 0.0^{a}	0
BST	1.0 ± 0.0^{a}	0
PMSG	12.5 ± 4.1^{b}	3^{d}
BST + PMSG	23.2 ± 3.4^{c}	1^{e}

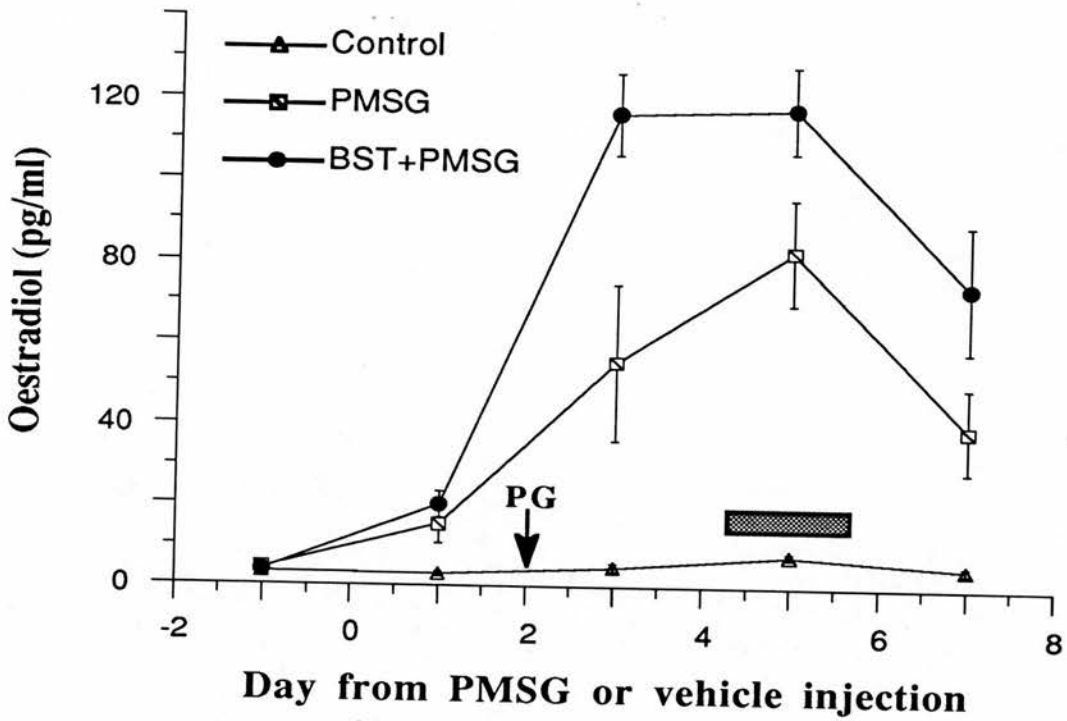
Values in the same column with different superscripts differ significantly;
*Defined as either ≤3 ovulations and/or cystic ovaries.

Table 6.2. Effect of BST pretreatment on numbers of eggs recovered following superovulation with 2000iu PMSG (n = 6)

Treatment	Total	Unfertilised	Transferable	Percentage of transferable embryos (%)
PMSG	3.8 ± 1.2^{a}	0.8 ± 0.2^{c}	2.7 ± 1.2	90.0 ± 11.2^{e}
BST+PMSG	7.4 ± 1.6^{b}	2.0 ± 0.9^{d}	2.6 ± 1.1	48.2 ± 7.8^{f}

Values in the same column with different superscripts differ significantly.

Figure 6.4. Mean (±s.e.m.) serum oestradiol concentrations for the 4 treatment groups (n = 6) from 2 days before PMSG or vehicle injection until 2 days after oestrus was detected. Serum samples from individual heifers were pooled every 2 days for the measurement of oestradiol concentration throughout this period. Animals were treated as described in Figure 6.1. The data from the control and BST groups were pooled (n = 12) and shown as "Control" in the figure since there was no difference in oestradiol profiles between these two groups. The hatched box represents the period when oestrus was detected.


Control
PMSG
BST+PMSG
120
80
40
0
PG
-2
0
2
4
6
8
Oestradiol (pg/ml)
Day from PMSG or vehicle injection

significantly higher concentrations of oestradiol ($p<0.01$) compared to the control and BST groups. Oestradiol levels in the BST + PMSG group were higher than those of the PMSG group ($p<0.01$). Mean concentrations of oestradiol were positively correlated with ovulation rate ($r = 0.74$, $p<0.01$) and the number of unfertilised eggs ($r = 0.64$, $p<0.05$), but negatively correlated with the percentage of transferable embryos ($r = -0.63$, $p<0.05$).

6.3.5. Peripheral concentrations of progesterone

There were no significant differences in circulating levels of progesterone between the control and BST groups (Figure 6.5). Peripheral progesterone concentrations in both PMSG-treated groups were higher than those of the control and BST groups ($p<0.01$) from 2 days after oestrus. The levels in the BST + PMSG group were significantly higher than those of the PMSG group ($p<0.01$). The overall mean concentrations of progesterone were positively correlated with the ovulation rate ($r = 0.78$, $p<0.01$), but negatively correlated with the percentage of transferable embryos ($r = -0.69$, $p<0.01$).

6.3.6. Dynamics of follicular growth and development

Analysis of the growth patterns for individual follicles demonstrated that most of the antral follicles already present in the ovaries at the time of PMSG treatment could be induced to undergo continued growth until ovulation. In contrast, most follicles recruited following PMSG injection could only develop up to a medium size (5-10mm in diameter) on the day of oestrus. The diameter of the ovulatory follicles ranged from 8.7 to 15.3 mm. The daily changes in the numbers of follicles of the three size classes detected by real-time ultrasound are shown in

Figure 6.5. Mean (±s.e.m.) serum progesterone concentrations for the 4 treatment groups (n = 6) from the day of prostaglandin $F_{2\alpha}$ (PG) injection until the day of laparoscopy. Animals were treated as described in Figure 6.1. The data from the control and BST groups were pooled (n = 12) and shown as "Control" in the figure since there was no difference in progesterone profiles between these two groups. The hatched box represents the period when oestrus was detected.

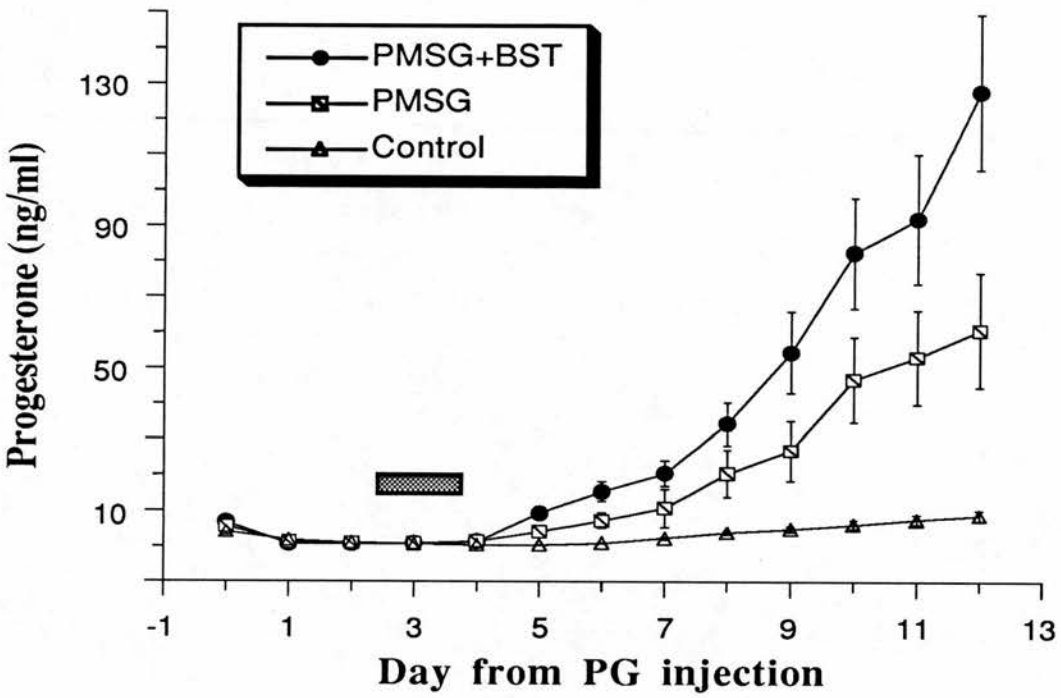
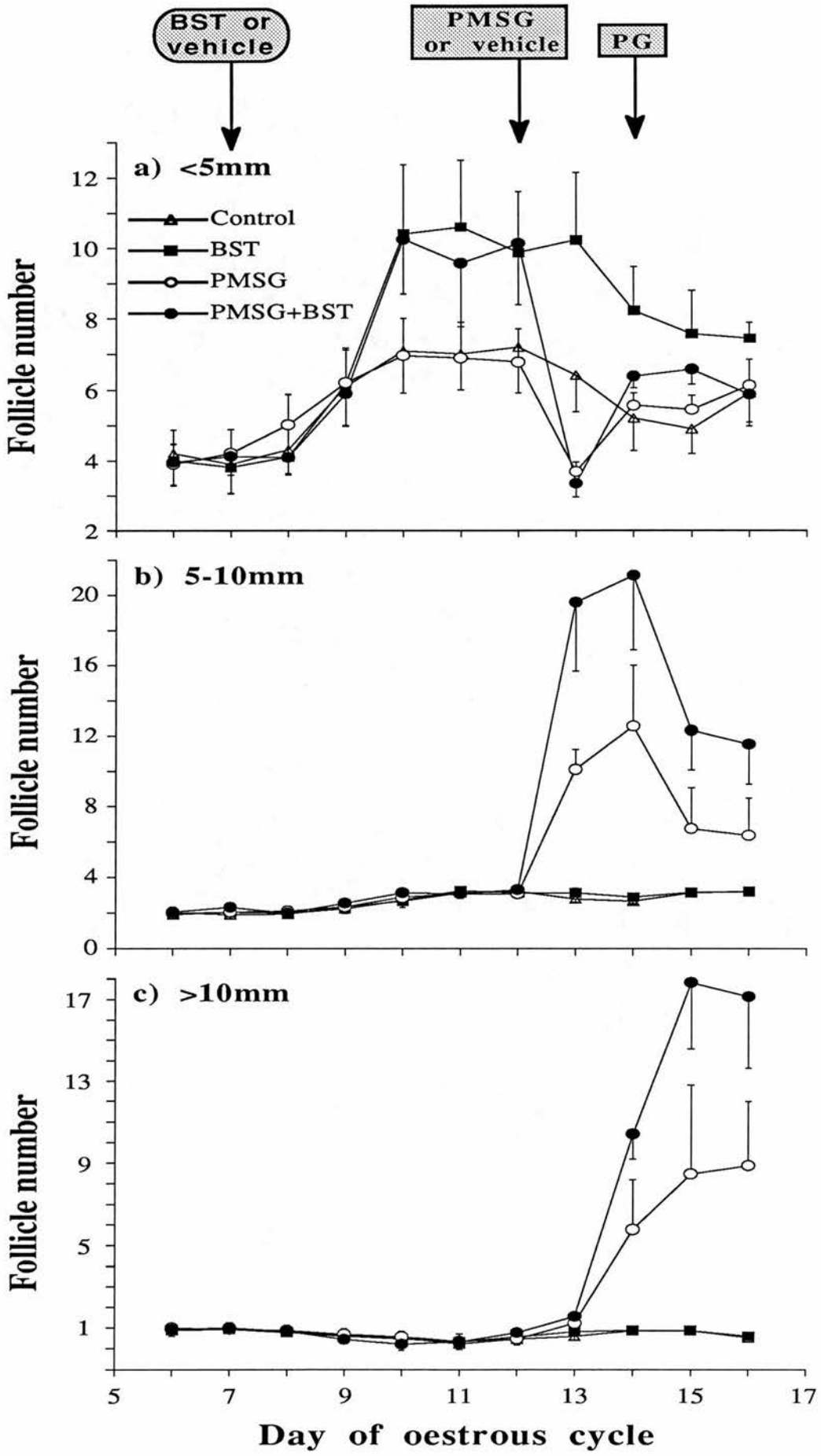

PMSG+BST
PMSG
Control
130
90
50
10
Progesterone (ng/ml)
-1
1
3
5
7
9
11
13
Day from PG injection

Figure 6.6 for all four treatment groups, from the day before BST injection until the day of oestrus. Approximately 72 hours following BST injection, the number of small follicles for the two BST-treated groups started to increase (effect of treatment, $p<0.01$). Numbers of small follicles in both PMSG-treated groups decreased significantly about 24 hours following PMSG treatment before showing a second increase. While the number of small follicles decreased, the number of medium-sized follicles showed a significant increase which was then followed by an increase in the number of large follicles. On the day of oestrus, the number of medium-sized follicles ($p<0.05$) and the number of large follicles ($p<0.01$) were significantly higher in the BST + PMSG group than in the PMSG group. Both the number of small follicles on the day of PMSG treatment ($r = 0.64$, $p<0.05$) and the total number of medium-sized and large follicles on the day of oestrus ($r = 0.69$, $p<0.01$) were positively correlated with the subsequent ovulation rate.

6.4. Discussion

This study has demonstrated that pretreatment with BST significantly enhances the superovulatory response to PMSG in heifers as indicated by the increases in ovulation rate, the total number of eggs recovered and peripheral progesterone concentrations. The enhancement in the superovulatory response was further demonstrated by a greater follicular growth and development during the period from PMSG injection to oestrus, as assessed by concentrations of peripheral oestradiol concentrations and by real-time ultrasound monitoring. In addition, the percentage of heifers with a poor response was reduced by BST pretreatment.

Figure 6.6. Mean (+/-s.e.m.) number of follicles detected by ultrasound scanning for the four treatment groups (n = 6) from the day before BST injection until the day of oestrus: a) <5mm; b) 5-10mm and c) >10mm in diameter. The day of BST or vehicle, PMSG or vehicle and PG injections is indicated by the arrows on the top of the figure.


BST or vehicle
PMSG or vehicle
PG
a) <5mm
Control
BST
PMSG
PMSG+BST
Follicle number
12
10
8
6
4
2
b) 5-10mm
Follicle number
20
16
12
8
4
0
c) >10mm
Follicle number
17
13
9
5
1
5
7
9
11
13
15
17
Day of oestrous cycle

In agreement with previous reports that co-treatment with GH could improve the response to ovulation induction in women (Blumenfeld and Lunenfeld, 1989; Matson *et al.*, 1989; Homburg *et al.*, 1990) and increased the superovulatory response in cattle to a limited extent (Herrler *et al.*, 1990; Rieger *et al.*, 1991), this study demonstrated that BST pretreatment significantly increased both ovulation rate and total numbers of eggs recovered following superovulation with 2000iu PMSG. These increases seemed to result from an increase in the number of small antral follicles induced by BST treatment, as real-time ultrasound scanning demonstrated that most of the antral follicles present in the ovaries at the time of PMSG treatment could be induced to undergo continued growth until ovulation. Moreover, the number of small follicles on the day of PMSG treatment was significantly correlated with the subsequent ovulation rate ($r = 0.64$, $p<0.05$). It has been proposed that exogenous gonadotrophin induces superovulation by rescuing antral follicles from atresia (Moor *et al.*, 1984) and that only the healthy antral follicles >1.7mm in diameter present in the ovaries at the time of gonadotrophin treatment can respond to gonadotrophin and develop to ovulate (Monniaux *et al.*, 1983). As further evidence to support the interpretation that the effect of BST pretreatment on the superovulatory response seen in this study reflected an increase in the number of small antral follicles induced by BST treatment, the presence of a dominant follicle has been associated with both a reduction in the number of small follicles (see Chapter 4) and a decrease in the superovulatory response in cattle (Pierson and Ginther, 1988; Lussier and Carruthers, 1989; Guilbault *et al.*, 1991; Huhtinen, Rainio, Aalto, Bredbacka and Maki-Tanila, 1992).

In addition, BST treatment induced an elevation in peripheral GH, IGF-I and insulin concentrations (Chapter 5, Figure 6.2). As all three

hormones have been shown to enhance the ovarian response to gonadotrophins (Jia *et al.*, 1986; Adashi *et al.*, 1985a; Poretsky and Kalin, 1987), it is possible that the increased concentrations of GH, IGF-I or insulin, acting separately or in synergy with each other and/or with PMSG, could also be involved in the enhancement of the superovulatory response. Indeed, the elevation in peripheral GH and IGF-I concentrations (Figure 6.2), and presumably in insulin concentrations (see Chapter 5), induced by BST treatment was maintained during the period from PMSG injection to oestrus.

Although the number of ovulations and total number of eggs recovered were significantly increased by BST pretreatment, the number of transferable embryos was unaffected as a result of a greater number of unfertilized eggs and a lower percentage (higher number of degenerate embryos) of transferable embryos. The reasons for these adverse results are uncertain. Peripheral oestradiol concentrations, the number of ovulations and peripheral progesterone concentrations following treatment with 2000iu PMSG but without BST pretreatment in this study were within the range reported by previous studies (Lemon and Saumande, 1972; Schams, Menzer, Schallenberger, Hoffman, Hahn and Hahn, 1978; Saumande, 1980). However, these parameters in the BST-pretreated heifers were about 2 - 2.5 fold of those reported previously. Therefore it is possible that the response to PMSG in heifers pretreated with BST was excessive, resulting in peripheral oestradiol concentrations which were detrimental to fertilization and/or subsequent development of early embryos, and in an environment of peripheral progesterone detrimental to development of early embryos (Booth, Newcomb, Strange, Rowson and Sacher, 1975; Boland, Crosby and Gordon, 1978; Monniaux *et al.*, 1983; Tamboura, Chupin and Saumande, 1985; Frydman, Foreman,

Belaisch-Allart, Hazout, Rainhorn, Fries and Testart, 1987; Forman, Fries, Testart, Belaisch-Allart, Hazout and Frydman, 1988). Interestingly, in this current study, mean concentrations of oestradiol were positively correlated with the number of unfertilized eggs ($r = 0.64$, $p<0.05$), but negatively correlated with the percentage of transferable embryos ($r = -0.63$, $p<0.05$), whereas mean concentrations of progesterone were negatively correlated with the percentage of transferable embryos ($r = -0.69$, $p<0.01$).

In the study of Rieger *et al.* (1991), co-treatment of heifers with BST and FSH had a limited effect on the ovulatory response, but did not change the number of embryos recovered. Peripheral IGF-I and insulin concentrations and the number of small antral follicles were only increased approximately 48 and 72 hours respectively following BST treatment (Figure 6.2, see Chapter 5), suggesting that the timing of BST treatment may be important. The ultrasound observations in this study suggested that only antral follicles already present in the ovaries at the time of PMSG administration were induced to ovulate, whereas most follicles recruited from the non-proliferating pool by PMSG treatment only developed up to a medium size at the time of oestrus, and did not ovulate. These observations are in agreement with the previous proposals of Monniaux *et al.* (1983). A preliminary study where dairy cows were given a subcutaneous depot of 640mg BST 6 days before superovulation was induced with 2500iu PMSG (Herrler *et al.*, 1990) demonstrated that although the number of ovulations was unaffected, the number of embryos collected was increased.

Our data also demonstrated that the percentage of heifers with a poor response, defined as an ovulation rate of ≤ 3 and/or the development

of either follicular cysts or cystic ovaries, was significantly ($p<0.05$) reduced by BST pretreatment. Similarly, in the study of Rieger *et al.* (1991) 21.1% of the heifers in control group had a response of 5 or fewer ovulations, with none in the group co-treated with BST. In fact in our study, when the heifers with a poor response were excluded, the difference in ovulation rate between the two superovulation groups failed to reach statistical significance. Therefore, BST treatment may be particularly beneficial in reducing the variability in response among individual animals. However, this prospect needs to be investigated further using more animals per group.

In conclusion, BST pretreatment enhanced the superovulatory response of heifers to 2000iu PMSG and reduced between animal variability in response. However, to achieve an increase in the number of transferable embryos, the optimal dose of PMSG needs to be established.

CHAPTER 7

Further study of the effect of recombinant bovine somatotropin on superovulatory response to PMSG in heifers

7.1. Introduction

BST pretreatment significantly increased ovulation rate and the total number of eggs recovered following superovulation with 2000iu PMSG in heifers (see Chapter 6). However, the number of transferable embryos was not increased, as significantly fewer eggs were fertilized and a lower percentage of embryos were transferable in BST pretreated heifers. As discussed in Chapter 6, the dose of PMSG (2000iu) used may have been too large, inducing very high concentrations of oestradiol and progesterone, and thus creating an adverse environment for the fertilization of eggs and/or development of early embryos (Booth *et al.*, 1975; Boland *et al.*, 1978; Frydman *et al.*, 1987; Forman *et al.*, 1988). It has been reported that the superovulatory response to PMSG is dose-dependent in heifers (Saumande *et al.*, 1978). Therefore, reducing the dose of PMSG may overcome these adverse effects and produce a better response in term of numbers of transferable embryos.

Using the same experimental model as that described in Chapter 6, the aims of this study were (1) to confirm the effect of BST pretreatment on the superovulatory response to 2000iu PMSG, and (2) to determine if the adverse effects seen in the previous study with 2000iu of PMSG could be overcome by reducing the dose of PMSG to 1000iu.

7.2. Materials and Methods

24 heifers were used in this study under the husbandry conditions described in Chapter 2.

7.2.1. Experimental procedures

The experimental protocol was exactly the same as that described in Chapter 6 (see Figure 6.1). Heifers were divided into four treatment groups of 6 in a randomized block design: (1) PMSG (1000iu); (2) PMSG (1000iu) + BST (320mg); (3) PMSG (2000iu); (4) PMSG (2000iu) + BST (320mg). On Day 7 of the oestrous cycle (Day 0 = Day of oestrus), animals in Groups (2) and (4) were injected subcutaneously with 320mg BST (Somidobove; Eli Lilly & Co., Indianapolis, USA) in a sustained-release formulation, while heifers in Groups (1) and (3) received 10ml saline. Five days later, heifers in all Groups were treated with a single dose of either 1000iu or 2000iu PMSG (Folligon, Intervet U.K. Ltd., Cambridge, England), followed 48 hours later by 1mg of Estrumate (Coopers Animal Health Ltd., Crewe, Cheshire, England), to induce superovulation. All animals were artificially inseminated twice daily during oestrus. Eggs were recovered non-surgically (see Chapter 2) on Days 6-8 of the following cycle and classified either as unfertilised eggs, degenerate embryos or transferable embryos as described in Chapter 2. All heifers underwent laparoscopy on Day 9 to assess ovulation rate by counting the number of CL. Heifers were scanned daily from the day before BST injection until the day of the last insemination, to monitor the dynamics of ovarian follicular growth and development. Blood samples were collected daily throughout the experimental period for progesterone, oestradiol, GH, and IGF-I measurements.

7.2.2. Statistical analyses

Data obtained from ultrasound scanning were analysed as described in Chapter 6. Statistical differences between the treatment groups in the number of ovulations, total number of eggs recovered, number of transferable embryos and temporal hormone data were analyzed by ANOVA (with repeated measurements, if applicable). The percentage of transferable embryos was calculated as the number of transferable embryos divided by the number of fertilized eggs. Differences between groups in the percentage of transferable embryos and the number of heifers with a poor response were analysed by Chi-square test. A poor response was defined as an ovulation rate of ≤3 and/or developing either follicular cysts or cystic ovaries (see also Saumande *et al.*, 1978). Correlations between two parameters were determined by regression analysis.

7.3. Results

7.3.1. Profiles of peripheral GH and IGF-I

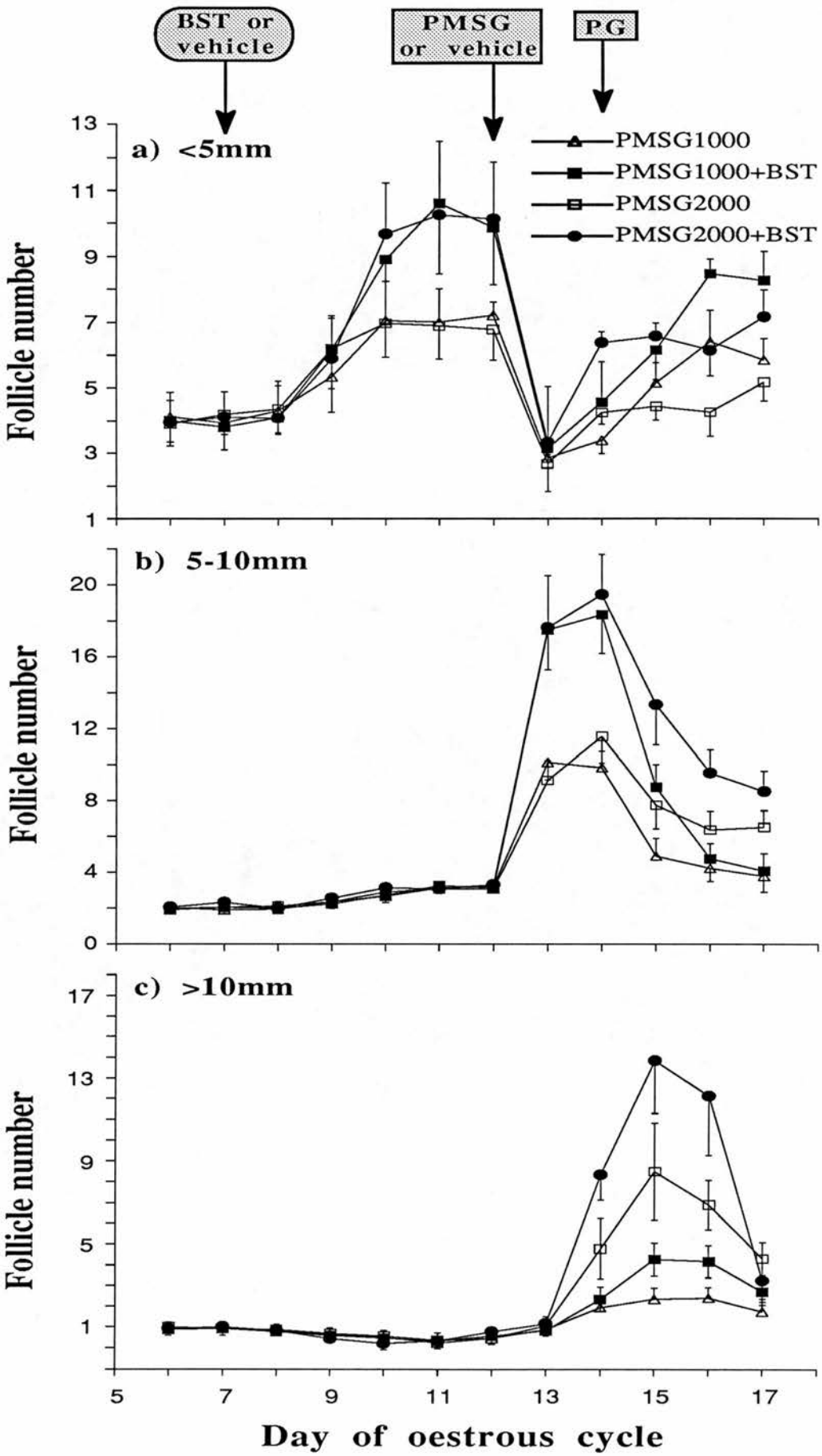
The profiles of peripheral GH and IGF-I before and after a single injection of BST were similar to those described in Chapter 6 (see Figure 6.2). There were no differences in GH and IGF-I profiles both between the PMSG (1000iu) and PMSG (2000iu) groups and between the PMSG (1000iu) + BST and PMSG (2000iu) + BST groups. GH and IGF-I concentrations for the two groups treated with PMSG alone were maintained at a stable level throughout the experimental period. In the two groups pretreated with BST, GH and IGF-I concentrations increased significantly within 24 and 48 hours respectively of the BST injections. The elevation in both GH and

IGF-I following BST treatment were maintained for approximately 8 days.

7.3.2. Dynamics of follicular growth

Analysis of the growth patterns for individual follicles demonstrated that most of the antral follicles already present in the ovaries at the time of PMSG treatment could be induced to undergo further growth. While most of these growing follicles in heifers given 2000iu PMSG could continue to develop up to ovulation, a large proportion of these growing follicles in heifers treated with 1000iu PMSG started to regress 2 or 3 days after PMSG injection. Most follicles recruited following PMSG injection could only develop up to a medium size (5-10mm in diameter) on the day of oestrus. The daily changes in the numbers of follicles of the three size classes detected by real-time ultrasound are shown in Figure 7.1 for all four treatment groups, from the day before BST injection until the day of oestrus. Approximately 72 hours following BST injection, the number of small follicles for the two BST-treated groups started to increase (effect of treatment, $p<0.01$). Numbers of small follicles in all four groups decreased significantly about 24 hours following PMSG treatment before showing a second increase. While the number of small follicles decreased, the number of medium-sized follicles showed a significant increase which was then followed by an increase in the number of large follicles. There were significant effects of both BST ($p<0.01$) and dose of PMSG ($p<0.01$) on the number of medium-sized follicles and the number of large follicles on the day of oestrus. Both the number of small follicles on the day of PMSG treatment ($r = 0.69$, $p<0.01$) and total number of medium-sized and large follicles on the day of oestrus ($r = 0.73$, $p<0.01$) were positively correlated with the subsequent ovulation rate.

Figure 7.1. Mean (+/-s.e.m.) number of follicles detected by ultrasound scanning for the four treatment groups (n = 6) from the day before BST injection until the day of oestrus: a) <5mm; b) 5-10mm and c) >10mm in diameter. Heifers were injected with a single dose of BST or vehicle on Day 7 of the oestrous cycle. On Day 12 of the cycle, animals were treated with either 1000iu or 2000iu PMSG, followed 48 hours later by 1mg synthetic prostaglandin $F_{2\alpha}$ (PG). The time of BST or vehicle, PMSG or vehicle and PG injections is indicated by the arrows on the top of the figure.


BST or vehicle
PMSG or vehicle
PG
a) <5mm
b) 5-10mm
c) >10mm
PMSG1000
PMSG1000+BST
PMSG2000
PMSG2000+BST
Follicle number
Follicle number
Follicle number
Day of oestrous cycle

7.3.3. Ovulation rate

Ovulation rates for all four treatment groups are shown in Table 7.1. Both the effect of BST pretreatment and effect of PMSG dose on the number of ovulations were statistically significant ($p<0.01$). In the two groups treated with 2000iu PMSG, none of the heifers from the PMSG + BST group, and 3 out of the 6 heifers in the group treated with PMSG alone had a poor response ($p<0.01$). However, none of the heifers in the BST-pretreated group and only 1 of 6 in the group given PMSG alone had a poor response when treated with 1000iu PMSG ($p>0.05$).

7.3.4. Number of eggs recovered and transferable embryos

As shown in Table 7.2, the results for the two groups treated with 2000iu PMSG were similar to those presented in Chapter 6. The total number of eggs recovered was significantly ($p<0.01$) increased by BST pretreatment, but the number of transferable embryos was unaffected. The number of unfertilized eggs was significantly higher ($p<0.05$) following BST-pretreatment while the percentage of transferable embryos was significantly lower ($p<0.05$). In contrast, both the total number of eggs recovered and number of transferable embryos were significantly ($p<0.05$) increased by BST pretreatment in the group treated with 1000iu PMSG, whilst there was no difference in the number of unfertilized eggs and percentage of transferable embryos between the two treatment groups.

7.3.5. Peripheral oestradiol concentrations

Profiles of peripheral oestradiol concentrations following PMSG

Table 7.1. **Effect of pretreatment with BST on superovulatory responses (mean ± s.e.m.) to different doses of PMSG (n = 6)**

Treatment	Number of ovulations	Number of heifers with a poor response*
PMSG(1000iu)	2.5 ± 0.6[a]	1
PMSG(1000iu) + BST	4.5 ± 0.7[b]	0
PMSG(2000iu)	10.6 ± 2.7[c]	3[e]
PMSG(2000iu) + BST	17.2 ± 2.8[d]	0[f]

Values in the same column with different superscripts differ significantly;
*Defined as either ≤3 ovulations and/or cystic ovaries.

Table 7.2. **Effect of BST pretreatment on numbers (mean ± s.e.m.) of eggs recovered following treatment with different doses of PMSG (n = 6)**

Treatment	Total	Unfertilised	Transferable	Percentage of transferable embryos (%)
PMSG(1000iu)	1.3 ± 0.3^{a}	0	1.3 ± 0.3^{k}	100^{x}
PMSG(1000iu)+BST	2.1 ± 0.4^{b}	0	2.1 ± 0.4^{l}	100^{x}
PMSG(2000iu)	4.1 ± 1.3^{c}	0.8 ± 0.2^{e}	$2.9 \pm 1.0^{l;m}$	90.6 ± 12.6^{x}
PMSG(2000iu)+BST	7.1 ± 1.4^{d}	1.8 ± 0.7^{f}	3.6 ± 1.1^{m}	67.9 ± 8.3^{z}

Values in the same column with different superscripts differ significantly.

treatment are shown in Figure 7.2. There was a significant effect ($p<0.01$) of both BST pretreatment and PMSG dose on the concentrations of serum oestradiol. Once again, mean concentrations of oestradiol were positively correlated with the ovulation rate ($r = 0.76$, $p<0.01$) for all four treatment groups. In the two groups treated with 2000iu PMSG, mean oestradiol concentrations were positively correlated with the number of unfertilised eggs ($r = 0.69$, $p<0.01$), but negatively correlated with the percentage of transferable embryos ($r = -0.64$, $p<0.05$). In contrast, no such correlation was demonstrated in heifers treated with 1000iu PMSG.

7.3.6. Peripheral concentrations of progesterone

There were no significant differences in peripheral progesterone concentrations among the four groups before insemination (Figure 7.3). However, for both doses of PMSG, peripheral progesterone concentrations in the BST-pretreated group were significantly higher ($p<0.01$) than animals treated with PMSG alone from 2 days after oestrus. The effect of dose of PMSG on peripheral progesterone concentrations was significant ($p<0.01$). Mean concentrations of progesterone were positively correlated with ovulation rate ($r = 0.81$, $p<0.01$) for all the four treatment groups, but negatively correlated with the percentage of transferable embryos ($r = -0.66$, $p<0.05$) only in the two groups treated with 2000iu PMSG.

7.4. Discussion

This experiment confirmed the results of the previous study (see Chapter 6) showing that pretreatment of heifers with BST significantly increased ovulation rate and total number of eggs recovered following

Figure 7.2. Mean (±s.e.m.) serum oestradiol concentrations for the four treatment groups (n = 6) from 2 days before PMSG injection until 2 days after oestrus. Serum samples from individual animals were pooled every 2 days for the measurement of oestradiol throughout this period. Heifers were treated as described in Figure 7.1. The arrow indicates the day of PG injection and the period when oestrus was detected is indicated by the hatched bar.

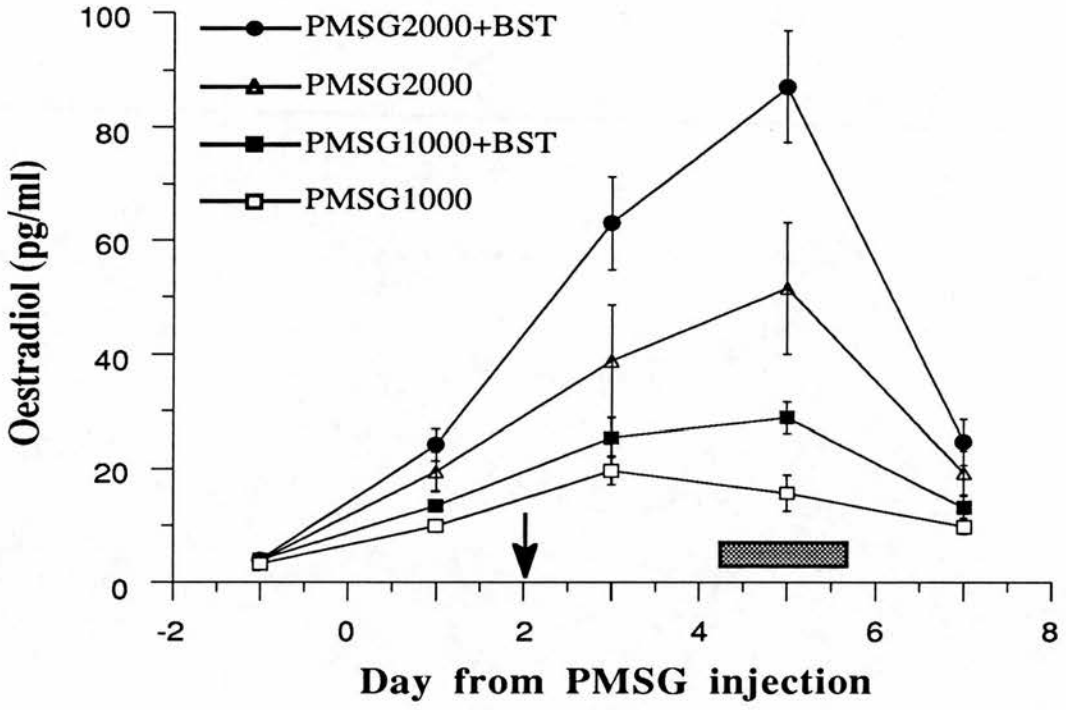
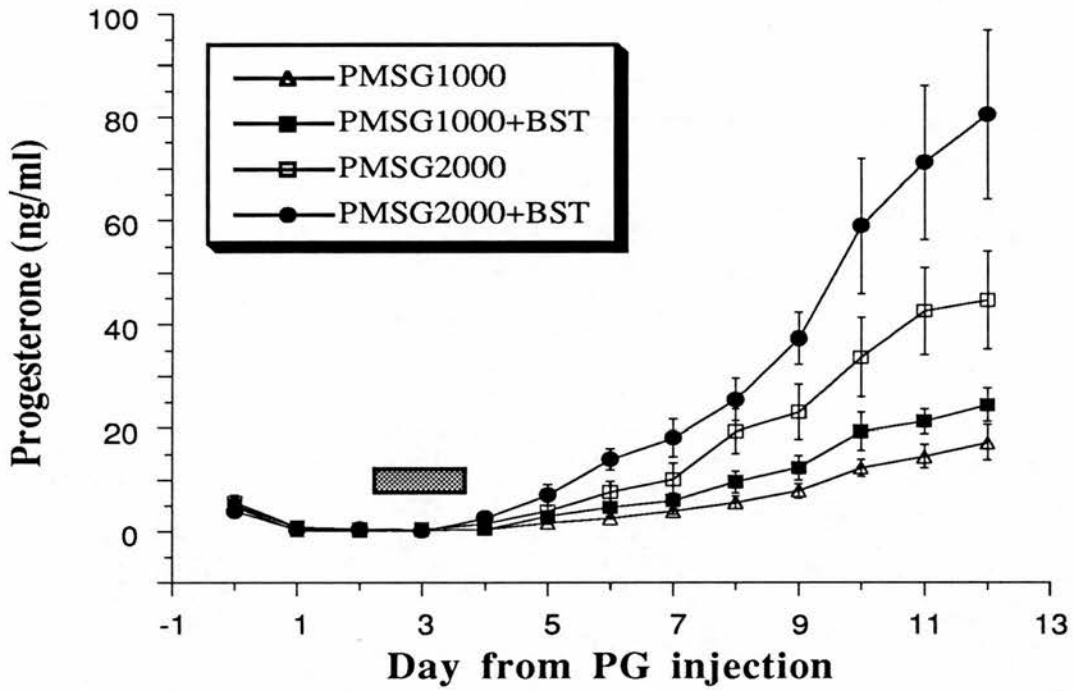

PMSG2000+BST
PMSG2000
PMSG1000+BST
PMSG1000
Oestradiol (pg/ml)
100
80
60
40
20
0
-2
0
2
4
6
8
Day from PMSG injection

Figure 7.3. Mean (±s.e.m.) serum progesterone concentrations for the four treatment groups (n = 6) from the day of prostaglandin $F_{2\alpha}$ (PG) injection until the day of laparoscopy. Heifers were injected with a single dose of BST or vehicle on Day 7 of the oestrous cycle. On Day 12 of the cycle, animals were treated with either 1000iu or 2000iu PMSG, followed 48 hours later by 1mg synthetic prostaglandin $F_{2\alpha}$ (PG). Laparoscopy was performed on Day 9 of the following oestrous cycle. The hatched bar represents the period when oestrus was detected.


PMSG1000
PMSG1000+BST
PMSG2000
PMSG2000+BST
Progesterone (ng/ml)
100
80
60
40
20
0
-1
1
3
5
7
9
11
13
Day from PG injection

superovulation with 2000iu PMSG. In addition, peripheral oestradiol concentrations after PMSG treatment, dynamics of follicular growth and development during BST and PMSG treatment and peripheral progesterone concentrations following oestrus were similar to those observed in the previous study (Chapter 6) in heifers treated with 2000iu PMSG. When 1000iu PMSG was used to induce superovulation following BST pretreatment, ovulation rate, the total number of eggs recovered and the number of transferable embryos were significantly increased compared to PMSG treatment alone. Peripheral concentrations of oestradiol and progesterone were significantly higher in BST-pretreated heifers, compared to animals treated with 1000iu PMSG alone.

As shown in Chapter 6, BST pretreatment significantly increased the number of ovulations, total number of eggs recovered and peripheral concentrations of oestradiol and progesterone following treatment with 2000iu PMSG in this current study, but did not alter the number of transferable embryos. A significant increase in the number of unfertilized eggs and a lower percentage of transferable embryos was again observed for animals treated with BST and 2000iu PMSG. As discussed in Chapter 6, the combination of BST and 2000iu PMSG elicited a supra-maximal response, producing a detrimental environment for the fertilization of eggs and/or development of early embryos, perhaps due to the very high concentrations of oestradiol following PMSG treatment, and/or the high levels of progesterone following oestrus (Booth *et al.*, 1975; Boland *et al.*, 1978; Frydman *et al.*, 1987; Forman *et al.*, 1988). This was further supported by the observations that concentrations of oestradiol following PMSG treatment (and progesterone levels following oestrus) were dependent on the dose of PMSG, and that mean oestradiol concentration was positively correlated with the number of unfertilized

eggs, but negatively correlated with the percentage of transferable embryos, whereas mean progesterone concentration was negatively correlated with the percentage of transferable embryos in heifers treated with 2000iu PMSG. It has been reported that, although the superovulatory response is increased with increasing dose of PMSG, the variability in response between individual animals is also increased (Saumande *et al.*, 1978). Interestingly, these adverse effects were not observed for heifers treated with BST and 1000iu PMSG. These animals had a significantly higher number of transferable embryos, compared to heifers treated with 1000iu PMSG alone.

The effect of BST treatment on the population of ovarian follicles was similar to that described in Chapters 5 and 6. Before PMSG injection, the number of follicles of all three sizes was not different both between the PMSG (1000iu) and PMSG (2000iu) groups and between the PMSG (1000iu) + BST and PMSG (2000iu) + BST groups (see Figure 7.1). Ultrasound scanning showed that most of these follicles were induced to grow further following PMSG treatment, again demonstrating that the number of follicles present at the ovaries at the time of gonadotrophin treatment is important in determining the superovulatory response (Monniaux *et al.*, 1983; Chapter 6). However, significantly more PMSG-induced growing follicles ceased to grow at the medium size and started to regress in heifers treated with 1000iu PMSG than animals given 2000iu PMSG (see Figure 7.1). Therefore, the dose of gonadotrophin is also important in determining the subsequent ovulation rate (Saumande *et al.*, 1978). Further studies will be required to determine the optimal combinations of BST and gonadotrophin preparations.

The percentage of heifers with a poor response was reduced by BST pretreatment, confirming observations in the previous study (Chapter 6).

This may be of particular benefit in improving the efficiency of embryo transfer in cattle, as one of the major limitations is still the large variability in the response of individual animals (see Boland, Goulding and Roche, 1991).

In conclusion, BST pretreatment enhanced the superovulatory response to PMSG in heifers and reduced the between-animal variability in the response. This may provide a new approach in attempts to improve superovulation regimes in cattle. However, optimal combinations of BST and PMSG (or other gonadotrophin preparations such as purified or recombinant FSH; Boland *et al.*, 1991; Bellows *et al.*, 1991) need to be established to optimise the number of transferable embryos recovered. Moreover, before a practical superovulatory regime incorporating BST pretreatment can be developed, relatively large scale field investigations need to be carried out.

CHAPTER 8

In vitro investigations into the possible mechanisms by which recombinant bovine somatotropin affects ovarian follicular populations in heifers

8.1. Introduction

As detailed in Chapters 3 and 5, treatment with BST significantly increased the population of small ovarian follicles (2-5mm in diameter) in mature heifers, possibly through an enhancement in the recruitment of these small follicles. This effect of BST does not appear to be mediated via changes in either circulating gonadotrophin concentrations or numbers of ovarian gonadotrophin binding sites. The underlying mechanism(s), however, remain(s) unclear (Chapter 3). BST treatment significantly increased peripheral concentrations of IGF-I and insulin as well as GH (Chapters 3 and 5). Furthermore, the number of small follicles correlated numerically (Chapters 3 and 5) with peripheral GH, IGF-I and insulin concentrations, and temporally (Chapter 5) with peripheral IGF-I and insulin concentrations during BST treatment. As the involvement of IGFs and insulin in the regulation of ovarian function has been suggested (Adashi *et al.*, 1985a; Poretsky and Kalin, 1987), it is possible that the BST effect on follicular populations is mediated by changes in IGF-I and insulin, though a direct effect of BST cannot be excluded (Jia *et al.*, 1986; Langhout *et al.*, 1991; Chapter 3).

To further assess the relative importance of GH, IGF-I and insulin

in the effect of BST on the small follicle population and to examine possible mechanisms underlying BST action, this study investigated (1) the effects of BST, IGF-I and insulin on the proliferation of cultured bovine granulosa cells and (2) the interactions of BST, IGF-I and insulin with both FSH and LH, at concentrations within the range measured in the previous *in vivo* studies (Chapters 3 and 5).

8.2. Materials and Methods

Bovine granulosa cells, obtained from follicles of three sizes (<5mm, small; 5-10mm, medium-sized; >10mm, large), were cultured in a serum-free culture system (Webb and McBride, 1991). The proliferation of granulosa cells was assessed by the incorporation of [^{3}H]-thymidine, as described in Chapter 2. Validation of this procedure, compared to measurements of both cell number and deoxyribonucleic acid (DNA) content (West *et al.*, 1985), is shown in Figure 2.5.

8.2.1. Treatment of bovine granulosa cells

(1) Dose response studies: Cultured bovine granulosa cells were challenged, as described in Chapter 2, with BST (1 to 1000 ng/ml; Somidobove, Eli Lilly, IN, USA), IGF-I (10 to 3000 ng/ml; recombinant human insulin-like growth factor-I, Eli Lilly) or bovine insulin (0.5 to 1000 ng/ml; Sigma, Poole, Dorset).

(2) Interaction of BST, IGF-I and insulin with FSH or LH: The doses used in these studies were as follows: BST (30 ng/ml); IGF-I (100 ng/ml); insulin (1 ng/ml); FSH (50 ng/ml; USDA-bFSH-B-1); LH (5 ng/ml; USDA-bLH-B5).

Each experiment was repeated three times and each treatment was performed in quadruplicate.

8.2.2. Statistical Analyses

The amount of [^{3}H]-thymidine (cpm) incorporated into the cultured cells was expressed for each treatment as a percentage of controls (treated with supplemented medium only; see Chapter 2). Experimental data are presented as mean ± s.e.m. of three experiments in quadruplicate. Statistical differences were analysed by ANOVA (allowing for repeated measurements, where applicable).

8.3. Results

8.3.1. Effects of BST

As shown in Figure 8.1, BST treatment inhibited [^{3}H]-thymidine incorporation into granulosa cells from large follicles in a dose-dependent fashion by up to 50%, whilst there was no significant effect of BST on the incorporation of [^{3}H]-thymidine into granulosa cells obtained from small and medium-sized follicles.

Treatment with FSH (50 ng/ml) or LH (5 ng/ml) alone inhibited [^{3}H]-thymidine incorporation into granulosa cells from all three sizes of follicles (Figure 8.2). When BST (30 ng/ml) was administered in combination with FSH or LH, no interaction between BST and either FSH or LH on the incorporation of [^{3}H]-thymidine into cultured cells was observed for granulosa cells obtained from all three size classes of follicles (Figure 8.2).

Figure 8.1. The effect (mean±s.e.m: n = 3 experiments; 4 replicates per treatment) of BST on the incorporation of [^{3}H]-thymidine into bovine granulosa cells obtained from three sizes of follicles. Granulosa cells were cultured under serum-free condition as described in Chapter 2.

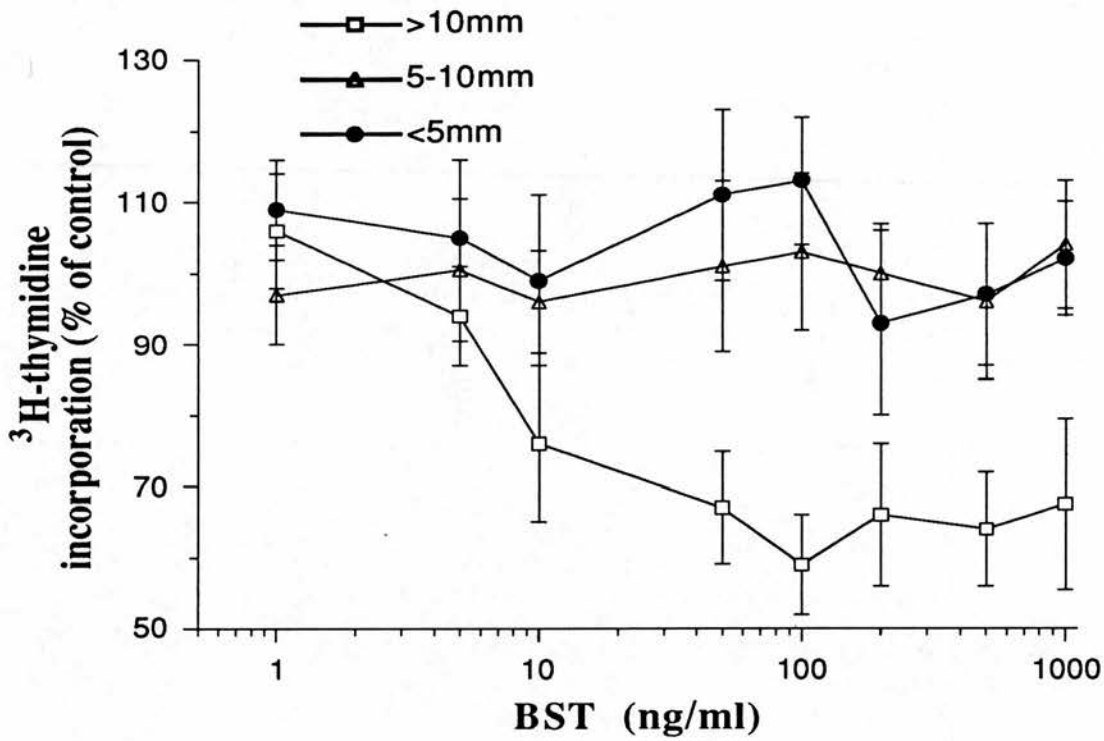
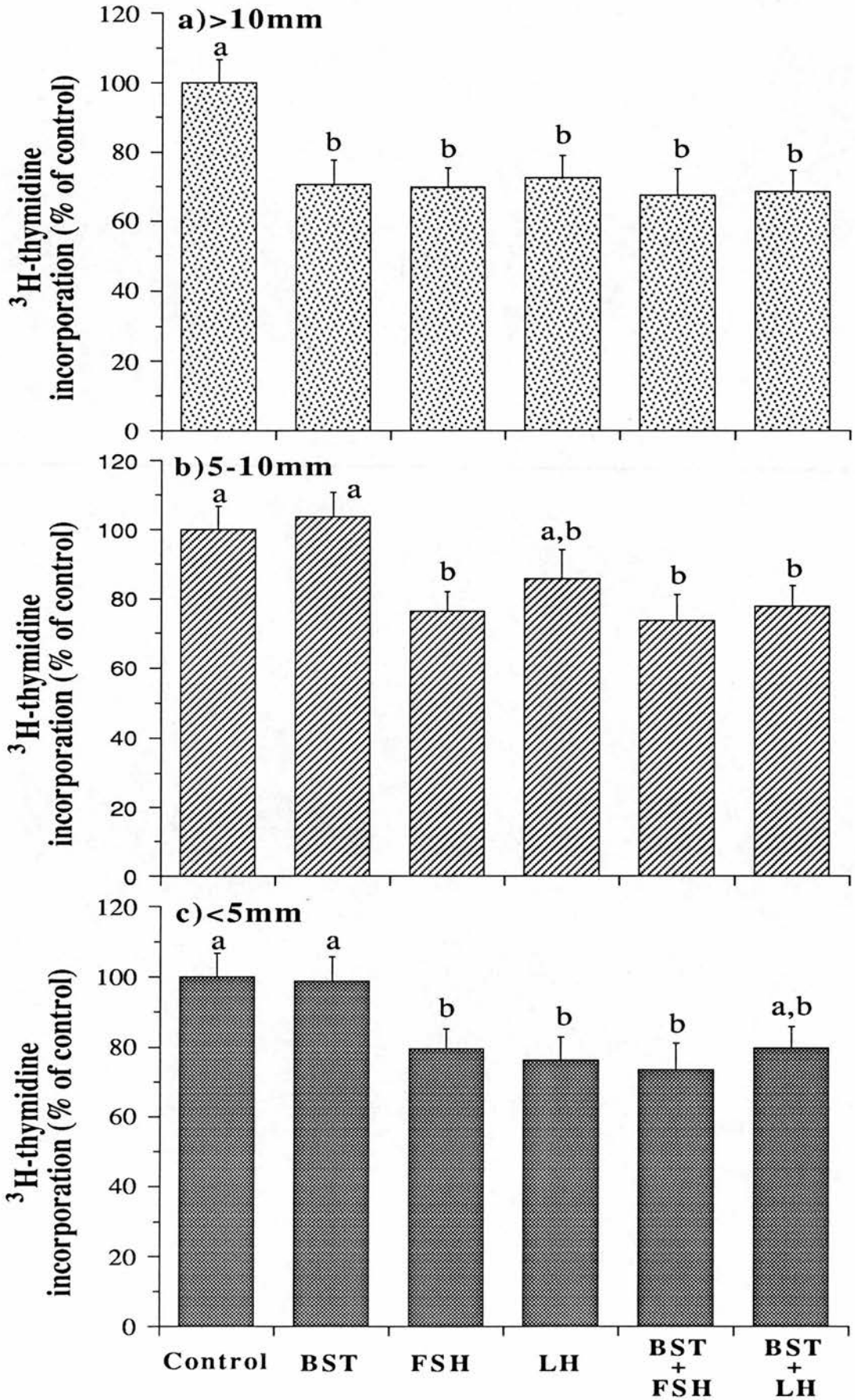

>10mm
5-10mm
<5mm
130
110
90
70
50
^{3}H-thymidine incorporation (% of control)
1
10
100
1000
BST (ng/ml)

Figure 8.2. Interactions (mean±s.e.m: n = 3 experiments; 4 replicates per treatment) between BST (30 ng/ml) and FSH (50 ng/ml) or LH (5 ng/ml) on incorporation of [^{3}H]-thymidine into bovine granulosa cells obtained from three sizes of follicles (a, >10mm; b, 5-10mm; c, <5mm in diameter). Granulosa cells were cultured under serum-free condition as described in Chapter 2. For each panel of the figure, the columns indicated by different letters differed significantly ($p<0.05$; ANOVA).


a)>10mm
b)5-10mm
c)<5mm
^{3}H-thymidine incorporation (% of control)
120
100
80
60
40
20
0
a
b
a,b
Control
BST
FSH
LH
BST + FSH
BST + LH

8.3.2. Effects of IGF-I

Treatment with IGF-I stimulated [^{3}H]-thymidine incorporation into granulosa cells obtained from all three size categories of follicles in a dose-dependent manner (Figure 8.3). This effect continued to increase up to the highest dose tested (3000 ng/ml).

IGF-I (100 ng/ml) acted in synergy with both FSH (50 ng/ml) and LH (5 ng/ml) to stimulate [^{3}H]-thymidine incorporation into granulosa cells from small follicles, but no such interaction between IGF-I and either FSH or LH was observed for granulosa cells obtained from medium-sized and large follicles (Figure 8.4). The inhibitory effect of both FSH and LH alone was similar to that shown in Figure 8.2.

8.3.3. Effects of insulin

Insulin stimulated incorporation of [^{3}H]-thymidine into granulosa cells from small and medium-sized follicles in a dose-dependent manner (Figure 8.5). However, whilst low doses of insulin (≤50ng/ml) stimulated [^{3}H]-thymidine incorporation in granulosa cells obtained from large follicles (Figure 8.5) in a dose-dependent manner, higher doses of insulin (>50ng/ml) caused a reduction in [^{3}H]-thymidine incorporation into cultured cells.

Similar to results described for IGF-I, insulin (1 ng/ml) also acted in synergy with both FSH (50 ng/ml) and LH (5 ng/ml) to stimulate [^{3}H]-thymidine incorporation into granulosa cells from small follicles. No such interaction between insulin and either FSH or LH was observed in granulosa cells obtained from medium-sized and large follicles (Figure 8.6). The inhibitory effects of both FSH and LH alone on the proliferation

Figure 8.3. The effect (mean±s.e.m: n = 3 experiments; 4 replicates per treatment) of IGF-I on incorporation of [^{3}H]-thymidine into bovine granulosa cells obtained from three sizes of follicles. Granulosa cells were cultured under serum-free condition as described in Chapter 2.

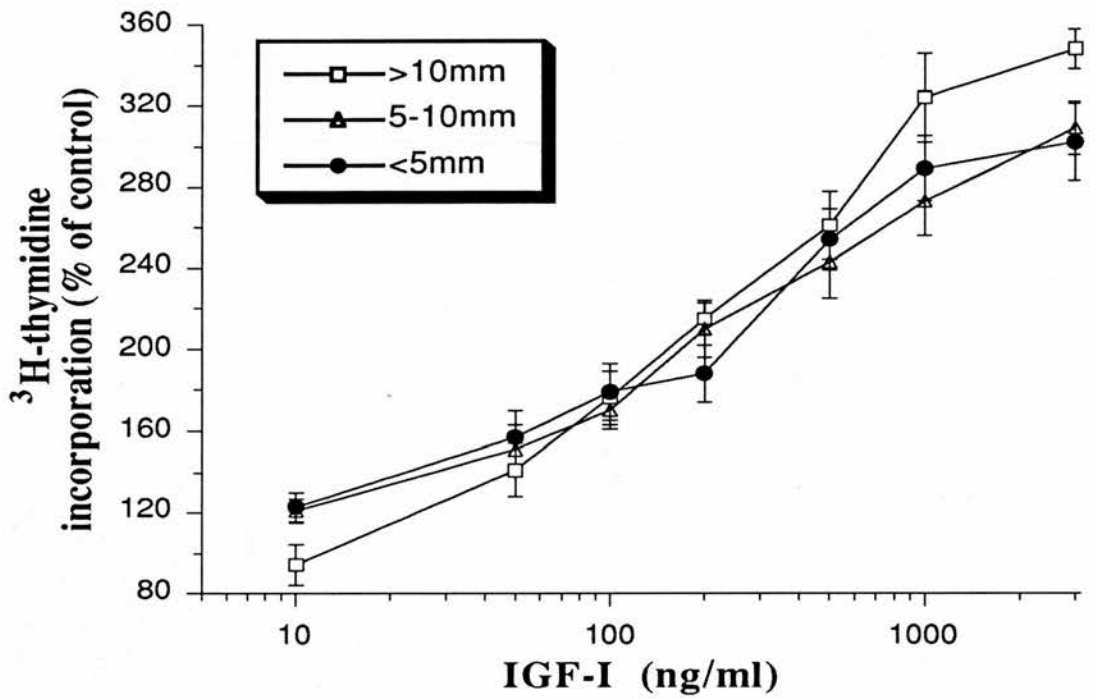

>10mm
5-10mm
<5mm
360
320
280
240
200
160
120
80
10
100
1000
IGF-I (ng/ml)
3H-thymidine incorporation (% of control)

Figure 8.4. Interactions (mean±s.e.m: n = 3 experiments; 4 replicates per treatment) between IGF-I (100 ng/ml) and FSH (50 ng/ml) or LH (5 ng/ml) on the incorporation of [^{3}H]-thymidine into bovine granulosa cells obtained from three sizes of follicles (a, >10mm; b, 5-10mm; c, <5mm in diameter). Granulosa cells were cultured under serum-free condition as described in Chapter 2. For each panel of the figure, the columns indicated by different letters differed significantly ($p<0.05$; ANOVA).

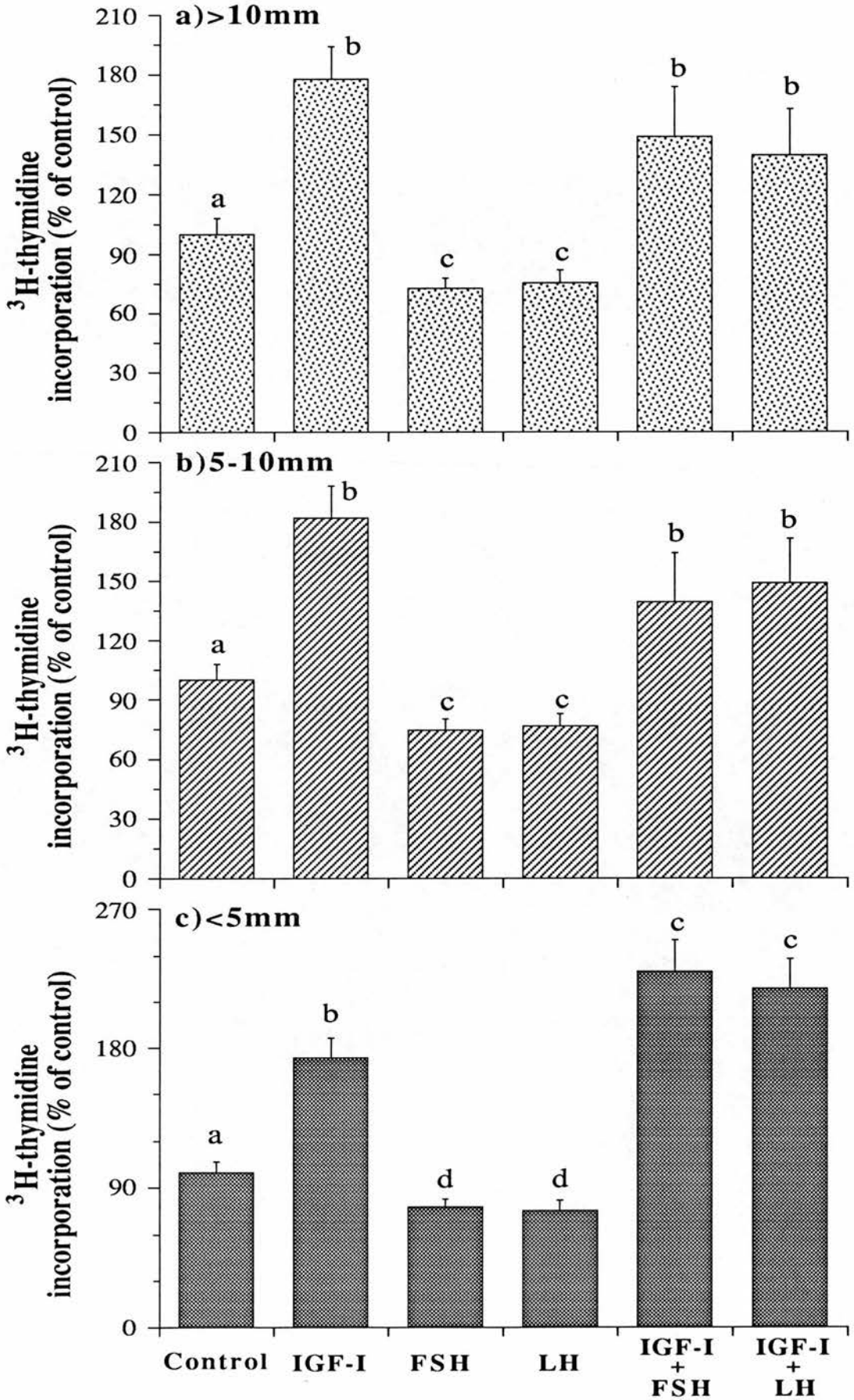

a)>10mm
b)5-10mm
c)<5mm
^{3}H-thymidine incorporation (% of control)
210
180
150
120
90
60
30
0
270
a
b
c
d
Control
IGF-I
FSH
LH
IGF-I + FSH
IGF-I + LH

Figure 8.5. The effect (mean ± s.e.m: n = 3 experiments; 4 replicates per treatment) of bovine insulin on the incorporation of [^{3}H]-thymidine into bovine granulosa cells obtained from three sizes of follicles. Granulosa cells were cultured under serum-free condition as described in Chapter 2.

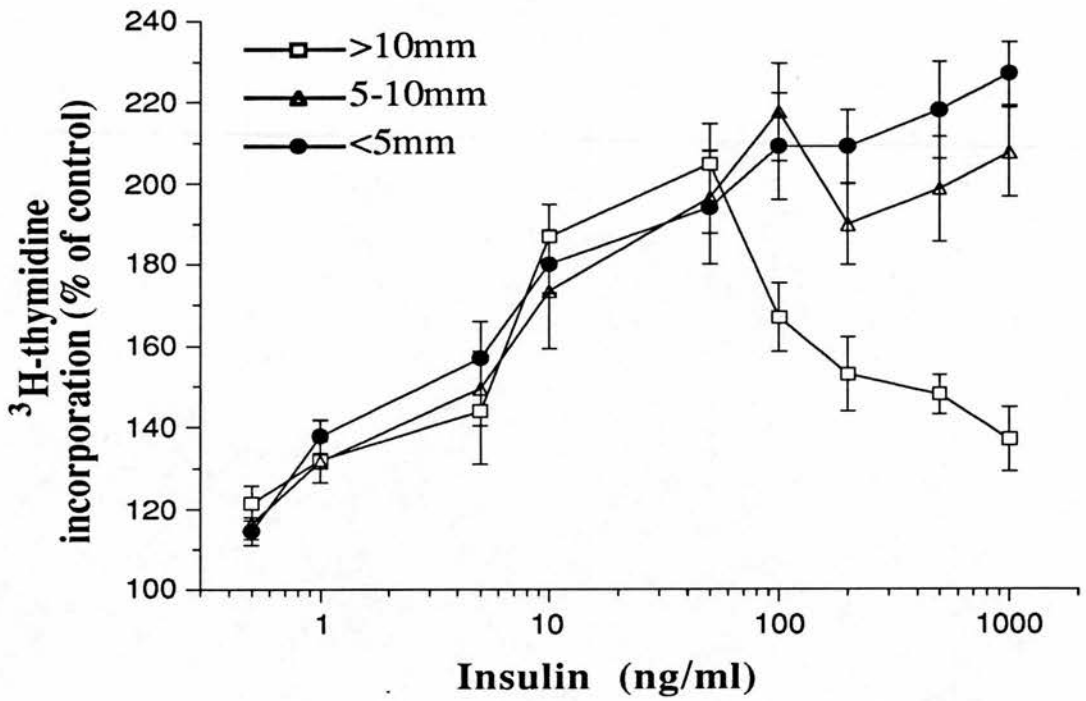
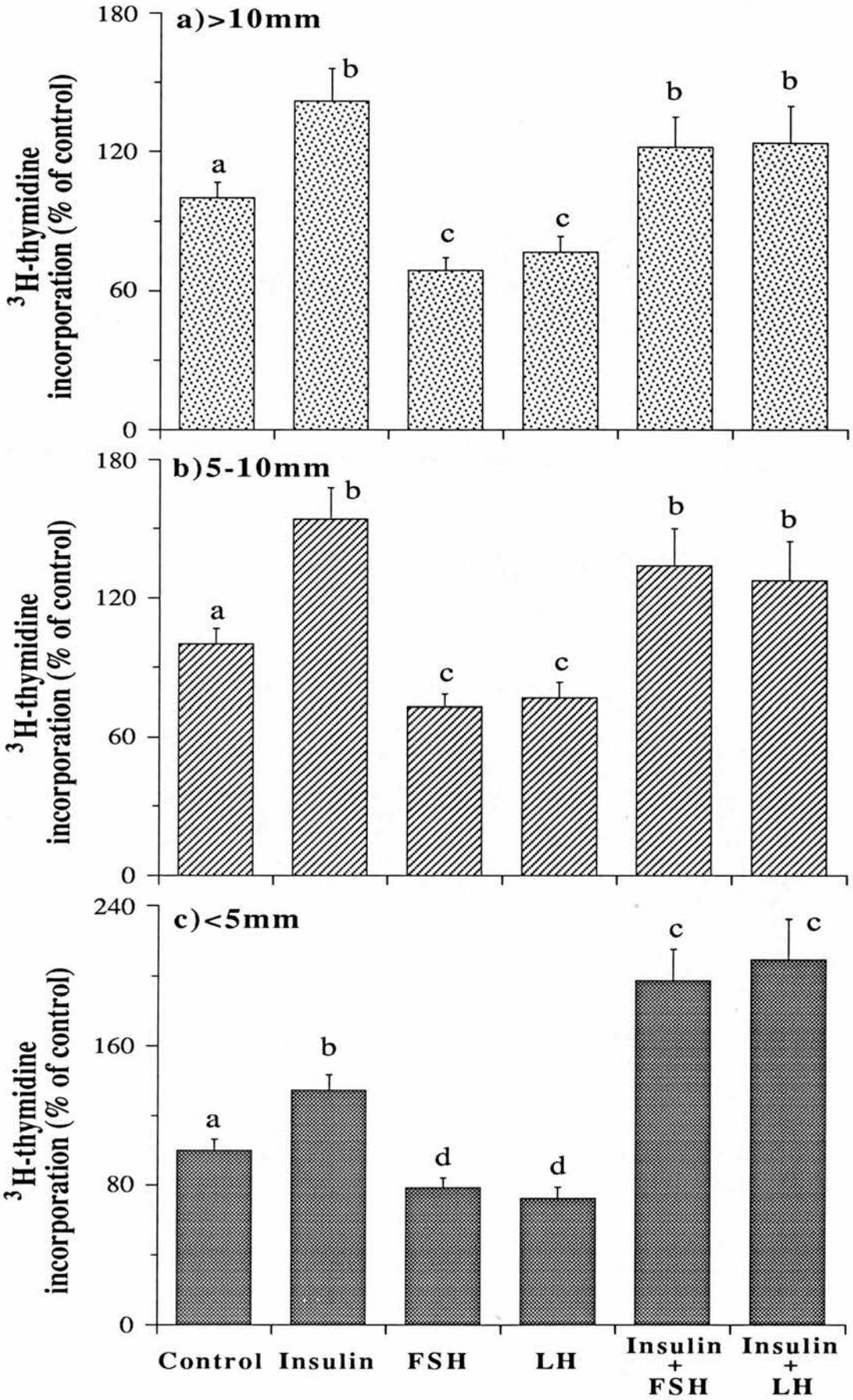

>10mm
5-10mm
<5mm
^{3}H-thymidine incorporation (% of control)
240
220
200
180
160
140
120
100
1
10
100
1000
Insulin (ng/ml)

Figure 8.6. Interactions (mean±s.e.m: n = 3 experiments; 4 replicates per treatment) between insulin (1 ng/ml) and FSH (50 ng/ml) or LH (5 ng/ml) on the incorporation of $[^3H]$-thymidine into bovine granulosa cells obtained from three sizes of follicles (a, >10mm; b, 5-10mm; c, <5mm in diameter). Granulosa cells were cultured under serum-free condition as described in Chapter 2. For each panel of the figure, the columns indicated by different letters differed significantly ($p<0.05$; ANOVA).


a)>10mm
b)5-10mm
c)<5mm
^{3}H-thymidine incorporation (% of control)
180
120
60
0
240
160
80
a
b
c
d
Control
Insulin
FSH
LH
Insulin + FSH
Insulin + LH

of granulosa cells from all three sizes of follicles was again observed (Figure 8.6).

8.4. Discussion

Doses of all hormones used in this study (including BST, IGF-I, insulin, FSH and LH) were within the range of concentrations measured in the *in vivo* studies of this thesis (see Chapters 3 and 5). Hence, these *in vitro* data presumably reflect physiological changes and relate directly to the mechanism(s) underlying the effects of BST treatment on the ovarian follicular population described in the earlier Chapters of this thesis.

Both IGF-I and insulin, at doses within the physiological range, significantly stimulated the proliferation of bovine granulosa cells cultured in serum-free medium. These results, apart from further confirming the effects of BST demonstrated in this thesis, also supported conclusions drawn from Chapters 3 and 5 that increased peripheral IGF-I and/or insulin concentrations following BST treatment may play an important role in the effect of BST on ovarian follicular population. Furthermore, these results are in line with the concept that IGF-I (see Adashi *et al.*, 1985a; Hammond *et al.*, 1991) and insulin (see Poretsky and Kalin, 1987) may play important modulatory roles in the control of ovarian function. In agreement with previous studies (Hammond *et al.*, 1991; Savion *et al.*, 1981; Saumande, 1991), synergistic interactions between FSH and both IGF-I and insulin on the proliferation of bovine granulosa cells were observed. Moreover, LH also acted in synergy with both IGF-I and insulin to stimulate the proliferation of granulosa cells in our study. Interestingly, synergistic interactions between

gonadotrophins and both IGF-I and insulin were only observed for granulosa cells from small follicles, but not with cells from medium-sized and large follicles. As doses of FSH and LH were also within the concentrations achievable in our *in vivo* studies, the BST effect on the small follicle population (Chapter 3, 5) could be mediated via IGF-I and/or insulin acting in synergy with FSH and/or LH.

Although no effect of BST on the proliferation of bovine granulosa cells obtained from small and medium-sized follicles was demonstrated in this study, a direct effect of BST cannot be excluded. Indeed, GH receptors have been shown to be present in the bovine ovary (Tanner and Hauser, 1989). Moreover, GH was shown to enhance steroidogenesis of rat (Jia *et al.*, 1986) and porcine (in the presence of insulin; Hsu and Hammond, 1987a) granulosa cells, and stimulated both proliferation and progesterone secretion of bovine granulosa cells in the presence of insulin (Langhout *et al.*, 1991) *in vitro*. Although a monoclonal antibody against IGF-I only partially neutralized the stimulatory effects of GH (Mondschein *et al.*, 1989a), it was proposed that the effects of GH were mediated via stimulation of ovarian IGF-I production (Davoren and Hsueh, 1986; Bryan, Hammond, Canning, Mondschein, Carbaugh, Clark and Hagen, 1989; Mondschein *et al.*, 1989a) rather than via a direct effect of GH. As peripheral IGF-I concentrations only increased approximately 48 hours following BST treatment (Chapter 5), the lack of effect of BST on the proliferation of granulosa cells from small and medium-sized follicles in this study may be due to the fact that granulosa cells were challenged with BST for only 24 hours (see Chapter 2).

BST treatment inhibited the proliferation of granulosa cells from large follicles in this study, presumably because these granulosa cells

were more differentiated. Indeed, mitosis of granulosa cells has been shown to be inversely related to their differentiation (Carson *et al.*, 1989), and our preliminary studies indicated that BST treatment could increase progesterone, and possibly oestradiol-17β, secretion by bovine granulosa cells obtained from large follicles (data not shown). However, it is uncertain whether the inhibitory effect of BST on the proliferation of granulosa cells from large follicles seen in this *in vitro* study is related to the *in vivo* observation that BST treatment had no effect on the population of large follicles or ovulation rate (Chapters 3 and 5) despite inducing an increase in the number of small follicles. Nevertheless, a direct effect of BST at the ovarian level such as the stimulation of local IGF-I production observed in rats (Davoren and Hsueh, 1986) and pigs (Bryan *et al.*, 1989), remains to be demonstrated in cattle.

In agreement with this study, numerous previous reports have shown that insulin has a profound effect on cultured granulosa cells, both in cattle (Savion *et al.*, 1981; Saumande, 1991; Langhout *et al.*, 1991) and in other species (Channing *et al.*, 1976; May and Schomberg, 1981; Davoren and Hsueh, 1984; Otani *et al.*, 1985; Maruo, Hayashi, Matsuo, Ueda, Morikawa and Mochizuki, 1988). However, the doses of insulin used in most of these previous studies were in the microgram range. Furthermore, the effective doses of insulin required for stimulation of granulosa cell functions were found to be much higher than those of IGF-I, and were outside the range of physiological concentrations (Adashi *et al.*, 1985a; Saumande, 1991). Since insulin can bind to type-I IGF receptors with a lower affinity (Adashi *et al.*, 1985a), it was proposed that the effects of insulin on granulosa cells were mediated via crossreaction with type-I IGF receptors (Davoren *et al.*, 1986; Maruo *et al.*, 1988). Indeed, it has been suggested that any effect of insulin achieved at a dose higher than 50

ng/ml should be considered to be acting through type-I IGF receptors (Kahn, Baird, Flier, Grunfeld, Harmon, Harrison, Karlsson, Kasuga, King, Lang, Podskalny and Van Obberghen, 1981). In contrast, the effective dose of insulin required to stimulate the proliferation of bovine granulosa cells was as low as 0.5 ng/ml in this study, suggesting that this effect of insulin was possibly mediated by insulin acting through its own receptor, rather than by crossreacting with type-I IGF receptors. This is in agreement with Webb and McBride (1991) who have demonstrated that insulin stimulated the proliferation of cultured ovine granulosa cells at nanogram levels. Interestingly, previous work suggesting that the effects of insulin on granulosa cells were mediated via crossreaction with type-I IGF receptors (Davoren *et al.*, 1986; Maruo *et al.*, 1988) was carried out with granulosa cells from rats or pigs. Therefore, between-species differences may exist with respect to the physiological importance of insulin in the control of ovarian function. However, although insulin receptors have been demonstrated in ovarian granulosa cells from humans (Poretsky *et al.*, 1985; Jarrett *et al.*, 1985) and pigs (Rein and Schomberg, 1982), they have only been demonstrated in bovine luteal tissue (Sauerwein et al., 1992) and their presence in bovine granulosa cells has yet to be established.

In conclusion, this study demonstrated that IGF-I and/or insulin, acting alone or in synergy with gonadotrophins, may play an important role in the increase in small ovarian follicle numbers induced by BST treatment in heifers. However, a direct effect of BST at the ovarian level cannot yet be excluded. The results from this study also provide a basis to further elucidate the mechanism(s) underlying the effect of BST on ovarian function.

CHAPTER 9

General discussion

The work in this thesis has demonstrated that hormones classically thought to be involved in the regulation of metabolic processes and growth, such as GH, insulin and IGF-I, also have profound effects on ovarian follicular growth and development in cattle.

Studies of the dynamics of ovarian follicular growth and development in our population of heifers using real-time ultrasound (Chapter 4 and Chapter 5) confirmed the wave pattern of follicular growth and development in cattle (Boland *et al.*, 1990). The results also supported the proposal that ovarian folliculogenesis involves three consecutive processes: follicle recruitment, follicle selection and follicle dominance (Goodman and Hodgen, 1980; Ireland, 1987). Furthermore, it was demonstrated that the growth and development of the largest follicle was consistently associated with a marked inhibition in the growth and development of small and medium-sized follicles, in line with previous hypotheses (Ireland and Roche, 1987; Ireland, 1987) that the "dominant" follicle in cattle can inhibit the development of "subordinate" follicles. Follicle dominance seems to be particularly important in the control of ovulation rate in cattle, in contrast with other species such as sheep where the follicle "dominance" phenomenon is more difficult to demonstrate (Driancourt, Webb and Fry, 1991b). Interestingly, although BST treatment significantly enhanced the recruitment of small follicles (2-5mm in diameter) it did not alter ovulation rate (Chapters 3 and 5),

presumably due to the inhibitory action of the dominant follicle on subordinate follicles as the wave pattern of follicular growth and development was unaffected (Chapter 5). Though the mechanism of follicle dominance in cattle is still not well understood, it is unlikely to be due to inhibin (Law, 1991) as originally proposed. Moreover, follicular "dominance" does not appear to be solely an intraovarian process, as the development of a single dominant follicle inhibits the growth and development of subordinate follicles in both ovaries. Whilst the dominant follicle may inhibit subordinate follicles systemically, it may also enhance its own development through intraovarian mechanisms, ensuring its survival and continued development in an environment which is suppressive to the growth and development of other (subordinate) follicles. Further understanding of the mechanism(s) or factor(s) responsible for follicle dominance should enable better control of oestrous cycle synchronization and ovulation rate in cattle.

Daily treatment of heifers with 25mg of BST for a period of two oestrous cycles significantly increased the population of small ovarian follicles, as measured by gross dissection (Chapter 3) or real-time ultrasound scanning (Chapter 5). Whilst this effect of BST did not appear to be mediated through changes in either circulating gonadotrophin concentrations or in numbers of ovarian gonadotrophin binding sites, BST treatment significantly increased peripheral concentrations of IGF-I, insulin and GH, supporting a role for these metabolic hormones in the control of ovarian function (Adashi *et al.*, 1985a: Poretsky and Kalin, 1987; Jia *et al.*, 1986). The effects of BST treatment on the small follicle population and peripheral IGF-I, insulin and GH concentrations were confirmed, using a single dose of BST, in Chapter 5. In addition, investigation of the dynamics of follicular growth and development

during BST treatment (Chapter 5) and study of the temporal relationships between changes in follicle number and changes in peripheral GH, IGF-I and insulin concentrations following a single dose of BST treatment (Chapter 5), suggested that BST treatment increased small follicle numbers by enhancing the recruitment of small follicles, possibly via increased peripheral concentrations of IGF-I and/or insulin. Moreover, the mechanism(s) by which BST enhanced the recruitment of small follicles appeared to differ from those involved in follicle "dominance". Experimental evidence from sheep has indicated that the early stage of follicular growth and development (up to a size of approximately 2mm in diameter) was unaffected when gonadotrophic support from the pituitary gland was removed by either hypophysectomy (Dufour *et al.*, 1979) or chronic treatment with (or immunization against) GnRH agonist (McNeilly and Fraser, 1987; McNeilly *et al.*, 1986). However, the factors which are important in the control of folliculogenesis during this gonadotrophin-independent stage are still unclear, although it has been suggested that locally-produced ovarian substances such as various growth factors (Mariana *et al.*, 1991) may play a role. Whilst direct evidence for a gonadotrophin-independent stage of folliculogenesis has yet to be shown in cattle, the results from this thesis suggest that the metabolic hormones may be involved in early folliculogenesis.

The *in vitro* studies described in Chapter 8 demonstrated that both IGF-I and insulin significantly stimulated the proliferation of cultured bovine granulosa cells obtained from all sizes of follicles (<5mm, small; 5-10mm, medium-sized; >10mm, large). BST had no effect on the proliferation of granulosa cells from small and medium-sized follicles but inhibited the growth of granulosa cells from large follicles. Interestingly, synergistic interactions between gonadotrophins (FSH and

LH) and both IGF-I and insulin were observed only in granulosa cells obtained from small follicles. It is uncertain whether these *in vitro* results were related to the *in vivo* observation that BST treatment only had an effect on the population of small follicles, but not medium-sized and large follicles (Chapters 3 and 5). However, since the doses for all hormones (BST, IGF-I, insulin, FSH and LH) used in these *in vitro* studies were within the range of concentrations measured in the *in vivo* studies (Chapters 3 and 5), these *in vitro* results may be of physiological significance. They further suggested that BST treatment enhanced the recruitment of small follicles by promoting the growth of these follicles via increased peripheral concentrations of IGF-I and/or insulin, which may act alone or in synergy with gonadotrophins. Therefore, increased concentrations of gonadotrophins were not required to stimulate the growth of small follicles in *in vivo* experiments (see Chapter 3). However, a direct effect of BST at the ovarian level (such as the stimulation of intraovarian IGF-I production) cannot yet be excluded and the mechanism(s) underlying the effect of BST on ovarian folliculogenesis in cattle remain to be elucidated.

The superovulation studies (Chapters 6 and 7) demonstrated that pretreatment of heifers with BST significantly enhanced the superovulatory response to PMSG. The ultrasound data showed that most follicles which eventually ovulate came from antral follicle populations already present in the ovaries at the time of PMSG administration rather than from those recruited following PMSG injection. Hence, the enhanced superovulatory response following PMSG treatment apparently reflected the increase in the population of small follicles induced by BST pretreatment, which were capable of responding to subsequent PMSG stimulation and developing through to ovulation. Other workers have

demonstrated that only healthy antral follicles of >1.7mm in diameter can respond to PMSG (Monniaux *et al.*, 1983) and that the numbers of gonadotrophin-responsive follicles present in ovaries at the time of gonadotrophin stimulation play an important role in determining the subsequent superovulatory response (Moor *et al.*, 1984). However, Rieger *et al.* (1991) observed only a limited effect of BST in their study where BST was given at the same time as FSH. Since the number of small follicles was only increased approximately 3 to 4 days following BST treatment (Chapter 5), it seems that the timing of BST treatment in relation to gonadotrophin treatment is important. Furthermore, BST pretreatment reduced the variability in response between individual heifers (Chapters 6 and 7). Since one of the major limitations in superovulation of cattle has been the large variability in response, both between and within individual animals (Boland *et al.*, 1991), BST pretreatment may provide a new approach to improve superovulation regimes in cattle.

Collectively, the results in this thesis have demonstrated that BST treatment can significantly increase the numbers of small ovarian follicles in heifers, possibly through increased peripheral concentrations of IGF-I and/or insulin which may act in synergy with gonadotrophins. However, a direct effect of BST at the ovarian level cannot yet be excluded. This effect of BST appears to result from an enhancement in the recruitment of small follicles via mechanisms other than those involved in the inhibitory actions of the dominant follicle on subordinate follicles. Furthermore, small follicles induced by BST treatment appear to be functionally healthy, and can be stimulated to develop and ovulate by PMSG treatment. This may provide a valuable approach to improve superovulation regimens, and increase the efficiency of embryo production in cattle. Moreover, the effects of

hormones, classically thought to be related to nutrition and metabolism, on ovarian function demonstrated in this thesis may provide a useful avenue in which to study the well-known but little understood inter-relationships between nutrition and reproduction.

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