# EFFECTS OF GONADOTROPHIN-RELEASING HORMONE AND BOVINE SOMATOTROPHIN ON HORMONE PROFILES AND OVARIAN FUNCTION IN POSTPARTUM BEEF COWS

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**Chapter 4. Investigation of the role and mode of action of bST in the control of ovarian function in postpartum beef cows**

# Abstract

Reproductive efficiency in beef cows is limited by the length of the postpartum anoestrous period which in turn is controlled by pituitary gonadotrophins and gonadal steroids and/or proteins. The first aim of this study was to elucidate the role of LH pulses in the development of ovarian activity in postpartum beef cows, specifically to determine whether or not the normal, relatively slow process of follicular development could be accelarated in cows in low body condition (BC) with pulses of gonadotrophin-releasing hormone (GnRH). The second aim was to determine the role of the metabolic hormones and in particular growth hormone (GH) in ovarian follicle development and function.

Treatment of cows in low BC with pulsed intravenous infusions of 2 μg every 2 hours from 5 to 7 weeks postpartum induced ovulation in 10 out of 12 cows. Saline infusions resulted in ovulation in only 1 of 12 cows in high BC and 1 of 11 cows in low BC groups. Plasma concentrations of GH, IGF-I and insulin were not affected by GnRH treatment, but cows in high BC had higher IGF-I and lower GH levels than cows in low BC. Gonadotrophin profiles, luteinizing hormone (LH) pulse frequency and LH pulse amplitude were not affected by either GnRH treatment or BC. Numbers of small (3-7.9 mm diameter) and large (≥ 8 mm diameter) follicles present at week 7 postpartum and numbers of LH receptors in theca and granulosa tissue were not affected by GnRH treatment or BC. The number of granulosa cells present in large follicles at week 7 postpartum was also unaffected. Intrafollicular concentrations of oestradiol, testosterone and IGF-I in large follicles at 7 weeks postpartum were not significantly affected by GnRH treatment or BC but there was a marked trend towards higher concentrations of oestradiol in cows in high BC compared with cows in low BC.

In Experiment 2, depot injections of 320 mg bovine somatotrophin (bST) (each designed to release 23 mg/day for 14 days) administered at weeks 2, 4, 6 and 8 postpartum induced ovulation in 4 out of 17 cows while 0 out of 15 ovulated in the control group ($p= 0.10$). While peripheral concentrations of GH and IGF-I were significantly increased throughout the postpartum period, there was no effect of bST on circulating concentrations of insulin, glucose, follicle-stimulating hormone (FSH), LH, LH pulse frequency or LH pulse amplitude. Numbers of small (3-7.9 mm diameter) and large (≥ 8 mm diameter) follicles and numbers of LH and FSH receptors in both theca and granulosa tissue at week 9 and numbers of granulosa cells present in large follicles was also unaffected. bST treatment significantly enhanced intrafollicular concentrations of oestradiol and IGF-I in large follicles (≥ 8 mm diameter) at 9 weeks postpartum but no difference in testosterone concentrations was observed.

It is concluded that infusion of exogenous GnRH pulses enhanced the process of follicular development in cows in low BC. While it was not possible to identify causal relationships between BC, plasma GH and IGF-I concentrations and intrafollicular oestradiol concentrations, bST treatment was found to increase oestradiol production in large follicles. This effect was not mediated through changes in gonadotrophin profiles or receptors, indicating that GH has a more direct role in the control of ovarian function. It is suggested that this effect may be mediated through changes in intrafollicular IGF-I concentrations.

# 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. I 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.

**Luis Pedro Mota Pinto de Andrade**

# Publications Arising from the Thesis

**Poster presentations**

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| | |
|---|---|
| ACTH- | Adrenocorticotropic hormone |
| BC - | Body condition |
| bFF - | bovine follicular fluid |
| BSA - | Bovine serum albumin |
| bST - | bovine somatotrophin |
| CL - | Corpus luteum |
| DARS - | Donkey anti-rabbit serum |
| DASS - | Donkey anti-sheep serum |
| DF - | Dominant follicle |
| $E_2$ - | Oestradiol |
| EGF - | Epidermal growth factor |
| EOP - | Endogenous opioid peptides |
| FGF - | Fibroblast growth factor |
| FSH - | Follicle-stimulating hormone |
| FSP - | FSH suppressing protein |
| GH - | Growth hormone |
| GnRH - | Gonadotrophin-releasing hormone |
| hCG - | human chorionic gonadotrophin |
| IGF-I - | Insulin-like growth factor I |
| IGFBP - | Insulin growth factor binding protein |
| LH - | Luteinizing hormone |
| LW - | Live weight |
| ME - | Metabolizable energy |
| MJ - | Mega joules |
| NEFA - | Non esterified fatty acids |
| NRS - | Normal rabbit serum |
| NSS - | Normal sheep serum |
| PA - | Plasminogen activator |
| PDGF - | Platelet-derived growth factor |
| PEG - | Polyethylene glycol |
| PG - | Prostaglandin |
| PMSG - | Pregnant mare serum gonadotrophin |
| TGF - | Transforming growth factor |
| TIC - | Theca interstitial cells |

# CHAPTER 1

## Introduction

One of the major constraints on economic efficiency of beef cows is reproductive efficiency, and in particular the length of the postpartum anoestrous period. Normally a cow has to conceive by day 85 postpartum and maintain the pregnancy, in order to produce a calf each year. However the duration of the postpartum anoestrous period is extremely variable and frequently exceeds 80 days (Peters and Riley, 1982).

Genetic, management and environmental factors including season, breed, age or parity, dystocia, presence of a bull, suckling and nutrition, may prevent the resumption of normal ovarian activity postpartum (Short, Bellows, Staigmiller, Berardinelli and Custer, 1990) but nutrient intake, weight change, body condition at calving and suckling seem to be the most important factors in determining the length of the postpartum anestrous period (Richards, Spitzer and Warner, 1986).

The effect of nutrition on postpartum reproductive activity depends partly on nutritional state before and after calving, although deficiencies already present at calving as estimated by precalving body condition score (BCS) are more important than those that occur after calving (Short and Adams, 1988).

While associations between nutritional state and endogenous metabolic profiles and between nutritional state and reproductive activity have each been well documented (Downing and Scaramuzzi, 1991; Rutter, Snopek and Manns, 1989), the mechanisms through which nutritional state affects reproductive activity remain poorly understood and require further investigation.

Circulating metabolic hormones such as growth hormone (GH) and insulin, growth factors such as IGF-I and blood metabolites such as glucose may play a role in

mediating the effects of nutrition on reproductive activity. Restriction of feed intake in ruminants increases circulating concentrations of GH (Breier, Bass, Buttler and Gluckman, 1986; Foster, Ebling, Micka, Vannerson, Bucholtz, Wood, Suttie and Fenner, 1989), while depressing IGF-I (Breier, *et al.*, 1986; McGuire, Vicini, Bauman and Veenhuizen, 1992; Rutter and Manns, 1989) and insulin (Butler, Everett and Coppock, 1981; Lucy, Beck, Staples, Head, De La Sota and Thatcher, 1992). Feed restriction also results in mobilization of body energy reserves and decreased circulating glucose concentrations (Rutter and Manns, 1987; 1988).

In most investigations of the mechanisms through which nutrition affects reproductive performance, the effects of body condition and level of feed intake on gonadotrophin production, metabolic hormone and metabolite profiles as well as on reproductive activity have been confounded. The effects of body condition *per se*, on these factors requires further study.

One mechanism by which energy restriction may affect reproductive activity is suppression of LH pulse frequency which may be a determinant of the rate of growth of ovarian follicles to the preovulatory stage (Richards, Wetteman and Schoenemann, 1989a). Reductions in LH pulse frequency have been reported in association with both low energy intake (Echternkamp, Ferrel and Rone, 1982; Terqui, Chupin, Gauthier, Perez, Pelot and Mauleon, 1982) and low body condition (Wright, Rhind, White, Smith, McMillen and Prado, 1990). However, in contrast to these results Rhind, Bramley, Wright and McMillen (1992) reported no difference in gonadotrophin profiles with body condition. While gonadotrophins are vital to ovarian follicle development, the equivocal nature of these results suggests that the effects of body condition on follicle development may not be mediated through differences in gonadotrophin profiles alone. If the importance of LH pulses in mediating the effects of body condition on reproductive performance is to be understood, there is a need to elucidate the role of LH pulses in ovarian function.

The studies of Murphy, Boland and Roche (1990) and of Savio, Boland, Hynes and Roche (1990) indicate that large ovarian follicles are detectable as early as 7-15 days postpartum, although the first ovulation does not occur until approximately 36 days postpartum. This indicates that failure to ovulate earlier is not attributable to a lack of large, "dominant" follicles; however, the physiological state of these follicles in animals in different nutritional states is unknown. Clearly, the presence of large follicles is not the only prerequisite for ovulation; an appropriate pattern of LH pulses may also be one of the keys to final maturation and ovulation.

One possible effect of frequent LH pulses may be to stimulate development of the biochemical pathways required for steroid synthesis. Prado, Rhind, Wright, Russel, McMillen, Smith and McNeilly (1990) showed that low body condition at calving in beef cows results in the growth of large follicles which have a reduced oestrogen biosynthetic activity *in vitro*. It was suggested that the aromatase systems in the large follicles was poorly developed since they had a reduced ability to convert testosterone (which was present in large amounts) to oestradiol.

The oestrogenic capacity of the follicles is dependent on their ability to respond to gonadotrophins (Gore-Langton and Armstrong, 1988), which in turn depends on the presence of adequate concentrations of FSH receptors on granulosa cells and LH receptors in thecal tissue. The results of Rhind, *et al.* (1992) suggests that the delay in the development of oestrogenicity of follicles which is associated with low levels of body condition is not attributable to a deficiency in FSH or LH receptor concentration in either granulosa or thecal tissue at 9 weeks postpartum. However the capacity to synthesize oestradiol could also be affected by the number and size of granulosa cells present in large follicles. This has not been investigated with respect to effects of nutritional state or time postpartum.

It has been suggested that nutritional modulation of reproduction may be exerted not only at the hypothalamic/pituitary level (Procknor, Forrest, McArthur and

Harms, 1986) but also at the gonadal level (Cox, Stuart, Althen, Bennet and Miller, 1987; Harrison and Randel, 1986). Body condition probably affects ovarian function through metabolic hormones profiles. These could act either through altered gonadotrophin profiles, altered tissue metabolism, or through a direct effect of the metabolic hormones on ovarian function. In addition to their metabolic effects, recent evidence suggests that GH, IGF-I and insulin may act directly on the ovary to change reproductive function independently of energy balance (Adashi, Resnick, D' Ercole, Svoboda and Van Wick, 1985).

Downing and Scaramuzzi (1991) demonstrated that increases in ovulation rate in sheep, rather than being related to changes in gonadotrophin concentrations, correlate with increases in plasma insulin concentrations after infusion of glucose or branched-chain amino acids. This measurement probably represents an index of the availability to tissues of oxidizable metabolic fuels, such as glucose and nonesterefied fatty acids. However, the relative importance, with respect to ovarian function, of insulin concentrations and associated differences in nutrient availability is unclear.

Recent studies have shown that GH treatment *in vivo* can increase the number of small follicles in mature heifers (Gong, Bramley and Webb, 1991; Gong, Bramley and Webb, 1993), and increase the incidence of twin calving in lactating dairy cows in early postpartum (Butterwick, Rowlinson, Weekes, Parker and Armstrong, 1988). Studies *in vitro* have shown that it also stimulates follicle growth, oocyte maturation and ovarian oestradiol production in the rabbit ovary (Yoshimura, Nakamura, Koyama, Iwashita, Adachi and Takeda, 1993). GH treatment has been shown to elevate the IGF-I levels in the circulation by stimulating the production of IGF-I in the liver (D'Ercole, Stiles and Underwood, 1984), but it is not yet known if the effects of GH on follicular development are exerted directly on the ovary, or mediated by inducing the hepatic production of IGF-I or through changes in availability of metabolic fuels. The effects of GH on gonadotrophin profiles, follicular size,

steroidogenicity, gonadotrophin receptors and numbers of granulosa cells in postpartum cows are not known.

Study of these factors in postpartum beef cows may improve understanding of the mechanism(s) controlling follicular development and reduce the postpartum anoestrous period.

The aim of the first experiment was to elucidate the role of LH pulses in the development of ovarian activity in postpartum beef cows and specifically to determine whether or not the normal, relatively slow, process of follicular development could be accelerated in cows in low body condition by the infusion of pulses of GnRH. A further objective was to determine the underlying effect of GnRH pulse infusion on associated gonadotrophin profiles, ovarian follicle growth and function.

The aim of the second experiment was to determine the effect of GH on ovarian follicle development and function and, thus, to elucidate the possible role of this hormone in mediating the effects of body condition on reproductive performance.

# CHAPTER 2

## Literature Review

### 2.1- Factors affecting the length of postpartum anoestrus in the beef cow

The duration of the postpartum interval is dependent on many different factors which do not operate independently of each other. However, for the purpose of this review, the effects of each of many factors are considered separately.

#### 2.1.1- Nutrition

Nutritional management in the pre- and postpartum period is one of the most important factors limiting or controlling the timing of the resumption of ovarian activity in beef cattle (Rutter and Randel, 1984).

##### 2.1.1.1- Effect of level of Feed Intake

###### 2.1.1.1.1- Energy

Effects of dietary energy content have been most commonly studied (Short and Adams, 1988), although effects of energy intake cannot usually be completely separated from other components of feed intake such as protein, minerals and vitamins. When energy requirements are not met, it is unusual for other nutrients to be present in adequate amounts (Maas, 1987).

Energy restriction during the late prepartum period extends the interval to first postpartum oestrus, and decreases the probability of a high percentage of cows

exhibiting oestrus at an early stage of the postpartum period (Boyd, 1977; Perry, Corah, Cochran, Beal, Stevenson, Minton, Simms and Brethour, 1991; Whisnant, Kiser, Thompson and Hall, 1985; Wiltbank, Rowden, Ingalls and Zimmerman, 1964), and this effect is even stronger in females calving at a young age (Bellows and Short, 1978; Hansen and Hauser, 1983).

After parturition, low levels of energy intake decrease the percentage of cows that ovulate by the end of a finite breeding season, and low conception rates have also been reported (Imakawa, Day, Garcia-Winder, Zalesky, Kittok, Schanbacher and Kinder, 1986; Johnson, Wagner and Ray, 1987; Wiltbank, Rowden, Ingalls and Zimmerman, 1962). However, other authors have reported no effect of level of postpartum nutrition on the length of the anoestrous period (King and MacLeod, 1982; Moore and da Rocha, 1983; Richards, *et al.*, 1986). This may be a function of comfounding factors such as timing and length of feed restriction, body condition and age of cow (Short and Adams, 1988). Some experiments indicate that increasing dietary energy intake for periods of approximately four weeks in suckled postpartum beef cows shortens the anovulatory period (Connor, Houghton, Lemenager, Malven, Parfet and Moss, 1990). However, others have found that longer periods (7 and 12 weeks) are necessary for the cows to respond to high energy diets (Terqui, *et al.*, 1982; Wiltbank, *et al.*, 1962) and some reports showed that similar increases in energy intake failed to reduce the postpartum interval at all (Holness, Hopley and Hale, 1978; Wetteman, Hill, Boyd, Spitzer, Forrest and Beal, 1986).

#### 2.1.1.1.2- Protein

Recent work has shown that low protein intakes initiated, prior to and maintained following parturition, lengthen the postpartum interval and decrease the number of animals that show oestrus and conceive (Harrison and Randel, 1986; Nolan, Bull, Sasser, Ruber, Panlasagui, Schoeneman and Reeves, 1989; Sasser, Willians, Bull, Ruder and Falk, 1988).

#### 2.1.1.2- Body Condition

Body condition at calving, which is highly correlated with the amount of fat stored in mature beef cows, reflects prepartum levels of feeding (Wright and Russel, 1984), and is an important factor with respect to re-establishment of cyclic ovarian activity in the beef cow (Dunn and Kaltenbach, 1980; Richards, *et al.*, 1986; Selk, Wetteman, Lusby, Oltjin, Mobley, Rasby and Garmendia, 1988; Wright, Rhind, Russel, White, McBean and McMillen, 1987). Some reports suggest that the key to an early return to oestrus in beef cows is adequate prepartum levels of nutrition, which results in cows calving in moderate to good body condition (Richards, *et al.*, 1986; Spitzer, 1986). More recently, a negative association between body condition score at calving and the length of the anoestrous period has been reported. For each unit increase in body condition score at calving the duration of the postpartum anoestrous period was reduced by between 43 and 86 days (Wright, *et al.*, 1987; Wright, Rhind and Whyte, 1992a).

#### 2.1.1.3- Body condition and feed intake interaction

There is clear evidence that body condition and feeding level interact to influence the duration of the postpartum anoestrous period.

##### 2.1.1.3.1- Prepartum

There appears to be no experimental evidence of an interaction between intake and body condition during the prepartum period.

##### 2.1.1.3.2-Postpartum

The results of Richards, *et al.* (1986), indicate that, if cows calve in moderate to good body condition, postpartum nutrient intake has little or no influence on postpartum interval to oestrus, and this is also supported by King and MacLeod (1984), Moore and da Rocha (1983) and Wright *et al.* (1987). Furthermore, Wright, *et*

*al.* (1992a) showed that level and pattern of feeding postpartum has little effect on the duration of the postpartum anoestrous period when cows calve at a body condition of approximately 2.25. However, Rutter and Randel (1984) found that cows that calved in good body condition and were fed low postpartum energy diets had a longer postpartum interval than those fed on a high energy diet.

Cows in moderate to good condition at the time of calving can lose some liveweight postpartum without increasing the length of the postpartum interval (Holness, *et al.*, 1978; Humphrey, Kaltenbach, Dunn, Koritnik and Niswender, 1983; Moore and da Rocha, 1983; Richards, *et al.*, 1986) but, if the weight loss after calving is severe, even cows calving in good body condition score may show delayed cyclicity (Wiltbank, *et al.*, 1962).

In summary, provision of appropriate levels of intake during both the pre-and postpartum periods is essential to maximize reproductive performance and minimize the negative influence of lactation and suckling (Connor, *et al.*, 1990). It seems that body condition at calving is more important than postpartum food intake in determining the length of the postpartum anoestrous period, but there is also some evidence that the effect of food intake is more important in thin cows.

### 2.1.2- Suckling

Suckling has an inhibitory effect on the return to oestrus (Carruthers and Hafs, 1980; Oxenreider, 1968; Randel, 1981). This may lead to an extended calving interval in individual cows and an extended herd calving season, both of which increase the cost of beef production and are undesirable (Allen, Nilsen-Hamilton and Hamilton, 1981; Randel, 1981).

The postpartum interval has been shown to be about one month longer in suckled cows than in milked cows (Boyd, 1977). Several reports suggest that the intensity of mammary stimulation is a major determinant of the length of the postpartum anoestrous interval in suckling cows (Short, Bellows, Moody and

Howland, 1972; Wetteman, Turman, Wyatt and Totusek, 1978). Restriction of the suckling frequency to once daily results in a shortening of the postpartum interval without depressing cow-calf performance (Randel, 1981; Reeves and Gaskin, 1981). However, the duration of the postpartum acyclic period was not significantly affected by restricting calves to twice daily suckling compared with suckling *ad libitum* (Lamming, Wathes and Peters, 1981). Double or multiple suckling (two or more calves per cow) has been reported to increase the postpartum acyclic period (Bellows, Short, Urick and Pahoush, 1974; Wetteman, *et al.*, 1978).

If suckling has an inhibitory effect on the return to oestrus, temporary or permanent calf removal and removal of the suckling stimulus should shorten the acyclic period. However, while a reduction in the calving to conception interval, following temporary calf removal or weaning, has been reported by several authors (Dunn, Smith, Garverick and Foley, 1985; Houghton, Lemenager, Horstman, Hendrix, Wesley and Moss, 1986; Laster, Glimp and Gregory, 1973; Randel, 1981), results have been inconsistent. The duration of the acyclic period seems to be connected with the level of nutrition, (Bellin, Hinshelwood, Hauser and Ax, 1984; Rutter and Randel, 1984) and indeed, temporary calf removal or weaning, shortened the interval to first oestrus only in cows receiving a high plane of nutrition or with good body condition (Connor, *et al.*, 1990; Holness, *et al.*, 1978).

### 2.1.3- Season

Cattle are not generally considered to be seasonal breeders. However numerous reports have described seasonal variations in reproductive traits (Hansen, 1985; Horta, Vasques, Leitão, Robalo Silva and Vaz Portugal, 1989; King and MacLeod, 1984; Mascarenhas, Rios Vasques, Horta, Robalo Silva and Vaz Portugal, 1986; Peters and Lamming, 1984) probably as a result of changes in photoperiod. Intervals to first ovulation and first oestrus were observed to be longer for cows calving in autumn or winter than in spring (Boyd, 1977; Hansen, 1985; King and MacLeod, 1984; Peters

and Riley, 1982). Effects of season may be mediated through changes in photoperiod alone or through factors such as temperature and nutrition which also vary seasonally (Mascarenhas, *et al.*, 1986; Stahringer, Neuendorff and Randel, 1990). However, Gauthier, Couland, Varo and Thimonier (1984) showed that oestrous cycles were significantly longer in December when the photoperiod was shortest, even when similar feeding regimes were applied.

Manipulation of the photoperiod has been shown to modify reproductive activity. Light supplementation decreased the interval from calving to conception in suckled beef cows through reductions in the interval to first oestrus, fertility, or both (Deas, 1971; Hansen and Hauser, 1984; Rhodes, Forrest and Randel, 1979).

Seasonal differences in the initiation of postpartum ovarian activity in suckled beef cows are also dependent on interactions with other factors, such as nutritional status, management, breed, age and body condition (Hansen, 1985; King and MacLeod, 1984). Differences between winter and summer calving cows in interval to first oestrus were greater for suckled cows than for nonsuckled and greater for cows with a genetic capacity for high milk production than for those with a low production capacity (Hansen and Hauser, 1983). Furthermore, cows fed diets low in energy were influenced by season more than cows fed high energy diets and primiparous animals were more susceptible to seasonal variations than multiparous animals (Hansen and Hauser, 1983).

In summary there is clear evidence of decreased reproductive function in cattle during the winter, and some of the effects have been shown to be due to photoperiod and not to seasonal changes in management or nutritional state.

### 2.1.4- Male effect

The results of several studies indicate that introduction of vasectomized 'teaser', penis-deviated or intact bulls to the female herd at the onset of calving provides a practical option for reducing postpartum anoestrous intervals (Gifford,

D'Occhio, Sharpe, Weatherly, Pittar and Reeve, 1989; Naasz and Miller, 1990; Zalesky, Day, Garcia-Winder, Imakawa, Kittok, D'Occhio and Kinder, 1984). There is a pheromone in bull urine that hastens the onset of puberty in heifers (Izard and Vandenbergh, 1982). The response of cows to bull exposure (proportion of cows responding and time to respond) differs with time of first exposure (number of days postpartum) (Izard and Vandenbergh, 1982) and with age of cow (Azzam, Werth, Kinder and Nielsen, 1991).

In some experiments, exposure of heifers to mature bulls failed to hasten onset of puberty (Roberson, Ansotegui, Berardinelli, Whitman and McInerney, 1987). Nutrition has been suggested as one factor that influences responses to bull exposure (Terqui, 1985); only cows receiving a high plane of nutrition or in good body condition, when exposed to the bull had a shortened postpartum anoestrus (Alberio, Schiersmann, Carou and Mestre, 1987; Terqui, 1985).

It seems that responses to bull exposure, *per se*, are not enough to explain differences in intervals of postpartum anoestrous; a combination of other factors including nutrition, body condition, time postpartum and age also affect the response.

### 2.1.5- Age

In first-calf heifers, the duration of the postpartum anoestrous period is approximately four weeks longer than in mature cows (Short and Adams, 1988; Wiltbank, 1978). This effect of age on the duration of the postpartum anoestrous may be a function of the combined demands of lactation and growth in heifers still growing after their first pregnancy (Sawyer and Carrick, 1984).

### 2.1.6- Uterine involution

It has been reported that cows with delayed uterine and cervical involution after parturition have longer intervals to first conception (Kiracofe, 1980). Kindahl, Odensvik, Aiumlamai and Fredericksson (1992) also showed that resumption of

ovarian activity is positively correlated with uterine involution. Furthermore, retained placenta can induce higher incidence of uterine infections, which may delay folliculogenesis and suppress the rate of follicular growth in dairy cows in the early postpartum period (Peter and Bosu, 1988).

### 2.1.7- Milk production and breed

Dairy breeds that are milked have shorter postpartum intervals than suckled beef breeds but, when dairy cows are suckled, they have longer postpartum intervals than beef cows (Short, *et al.*, 1990). Studies have shown that when managed comparably, dairy genotypes have longer postpartum intervals than beef genotypes and that these effects are more pronounced at first parity and at lower dietary intakes (Hansen, Baik, Rutledge and Hauser, 1982; Kropp, Stephens, Holloway, Whiteman, Knori and Totusek, 1973). Differences in beef breeds have also been reported with animals with lower dietary intakes having a longer postpartum intervals (Bellows and Short, 1978; Dunn, Ingalls, Zimmerman and Wiltbank, 1969). Boyd (1977) and Peters (1984) suggest that it is difficult to separate the effects of milk yield from other factors particularly nutritional status.

It is not known how genotype affects postpartum intervals. Short, *et al.* (1990) concluded that these effects may be due to true physiological differences between breeds and/or confounding effects such as differences in amount of milk produced or appetite and feed intake.

## 2.2- Ovarian follicle growth and development

### 2.2.1- Follicle development during the oestrous cycle

Oestrous behaviour and other aspects of reproductive activity depend on the pattern of ovarian follicular development during the postpartum period. This is a continuous process occuring over one or more oestrous cycles (Hansel and Convey, 1983; Spicer and Echternkamp, 1986). Ovarian follicles grow, regress and are

replaced by others during the oestrous cycle (Matton, Adela Koun, Couture and Dufour, 1981) and before cycles recommence after parturition

Several hypotheses concerning the pattern of follicular development in cattle were generated following the earlier studies of Rajakoski (1960). Measurements of the dynamics of ovarian follicular development were initially limited to observations of ovaries following surgery (using india ink to monitor change) (Matton, *et al.*, 1981), and following slaughter or ovariectomy (Ireland and Roche, 1983b). Before the development of ultrasound technology, Dufour, Whitmore, Ginther and Casida (1972) and Marion, Gier and Choudray (1968) suggested that the turnover of ovarian follicles > 5mm was continuous and independent of the phase of the cycle. With the advent of real-time β ultrasonography it is now possible to visualize the numbers of antral follicles of each size and how they change during the oestrous cycle in large animal species.

The technology has been used in cattle (Savio, Keenan, Boland and Roche, 1988) to measure and count follicles > 2mm during a normal oestrous cycle and early pregnancy. Sirois and Fortune (1988) indicated that it was possible to trace the development of individual follicles > 5mm throughout the oestrous cycle in heifers by ultrasonography.

Rajakoski (1960) suggested that there are 2 waves of follicular growth in heifers. Support for the two-wave model was provided by Pierson and Ginther (1984), Pierson and Ginther (1986), Pierson and Ginther (1987) and Pierson and Ginther (1988) who measured and counted follicles > 2mm by ultrasonography (each day of the cycle without following individual follicles from day to day), and also by Knopf, Kastelic, Schallenberger and Ginther (1989) who have reported that in heifers 80% of the interovulatory intervals had 2 waves of follicle development.

In the two-wave pattern the first wave (Wave 1) begins at approximately Day 0 (day of ovulation). A dominant follicle of wave 1 reaches approximately 16 mm in diameter on day 7, remains static for approximately 6 days and then regresses. The

second wave (Wave 2) is first identified on approximately Day 9. A dominant follicle emerges which becomes the ovulatory follicle. For both waves, subordinate follicles stop growing approximately 3 days after the first detection of a wave.

Recently other authors, using ultrasonography to monitor sequential daily growth of individual follicles in heifers, suggested that there are 3 or 4 periods of growth of different dominant follicles (Ireland, 1987; Ireland and Roche, 1983b; Savio, *et al.*, 1988). Knopf, *et al.* (1989) also identified heifers with 3 follicle waves. The first wave was similar to the first wave in the two-wave interval. The second wave, which also contained a dominant anovulatory follicle, began on approximately the same day (Day 10) as in the two-wave intervals, and the third (ovulatory) wave was first detected on days 16 or 18 for the respective interovulatory intervals.

Knopf, *et al.* (1989) concluded, on the basis of results of earlier studies, that there are dominant anovulatory and dominant ovulatory follicles during the bovine oestrous cycle and that it remained uncertain whether the wave pattern was bimodal or trimodal. It seems that heifers can have both two wave and three wave interovulatory intervals. In heifers with three follicular waves per cycle, the growth rate of the dominant non-ovulatory follicle in the first wave is not different from the growth rate of the dominant ovulatory follicle in the third wave (Sirois and Fortune, 1988). Ginther, Kastelic and Knopf (1989) suggest that in the formation of waves there is a consistent temporal relationship between emergence of the ovulatory wave and onset of regression of the dominant follicle of the anovulatory wave (length of interval from regression of the anovulatory wave to appearance of an ovulatory follicle approximately 3 days). It has been suggested by some authors 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 occurs earlier in heifers with 2 waves per cycle (Ginther, *et al.*, 1989; Knopf, *et al.*, 1989; Sirois and Fortune, 1988).

It has been suggested by Pierson and Ginther (1987) that there are differences in follicular activity in right and left ovaries. They have demonstrated a higher

incidence of dominant follicles and ovulation in the right ovary but recently Ginther, *et al.* (1989) with a larger number of observations found no significant differences between the ovaries.

### 2.2.2-Follicle steroidogenesis during the oestrous cycle

One limitation of ultrasound measurements of follicle development is that they do not reveal the physiological state of the follicle. This can be deduced from its steroid content. Steroid hormone concentrations in follicular fluid vary considerably with stage of the cycle and with size and physiological status of the follicle (Fortune and Hansel, 1985).

Higher concentrations of testosterone are present in the fluid of small follicles in the follicular and late luteal stage than in the early luteal stage (Kruip and Dielemans, 1985). Concentrations of oestradiol in fluid of large follicles (≥ 6 mm) are high or increase slightly during the 3 to 5 d before the gonadotrophin surges, and then decline after the LH surge (Ireland and Roche, 1982; Ireland and Roche, 1983b; Staigmillar, England, Webb and Bellows, 1982).

Changes in follicle size and steroid content with stage of the oestrous cycle depend partly on numbers of receptors for gonadotrophic hormones and changes in LH pulse frequency (Hansel and Convey, 1983). Oestradiol production by bovine follicles occurs primarily in granulosa cells through the androgen aromatizing system which is under the influence of FSH (Hillier, Reichert Jr. and van Hall, 1981; Merz, Hauser and England, 1981). LH receptors in the membrana granulosa are necessary, in addition to FSH receptors, for a high rate of production of oestradiol (Hillier, *et al.*, 1981; Merz, *et al.*, 1981).

Small follicles (2-5 mm) have receptors for LH in the theca interna and will produce androgens. Due to the small numbers of LH receptors in the granulosa they are not able to aromatize the androgens into oestrogen and consequently small follicles

are androgen-dominated (Kruip and Dielemans, 1985). Until the preovulatory stage, large follicles exhibit increasing numbers of LH receptors with increasing size in both the theca and granulosa layer; they can therefore produce more oestradiol than testosterone (Ireland and Roche, 1982), so that follicles > 11 mm diameter are oestrogen-dominated (Kruip and Dielemans, 1985). Staigmillar, *et al.* (1982) reported that the largest follicle removed from heifers during oestrus produces 500 to 1,000 times more oestradiol *in vitro* than smaller follicles. Ireland, Fogwell, Oxender, Ames and Cowley (1984) suggested that, in the cow, potentially ovulatory follicles capable of producing significant quantities of oestradiol are present during most of the oestrous cycle.

Degeneration of follicles involves a decrease in numbers of receptors for LH in the granulosa (Merz, *et al.*, 1981) and Kruip and Dielemans (1985) reported that this is a gradual process. Consequently, there is practically no difference in oestradiol concentrations in healthy follicles compared to follicles starting to undergo atresia (degeneration) (Merz, *et al.*, 1981).

### 2.2.3- Follicle development and steroidogenesis during the postpartum anoestrous period

Data on follicular dynamics in the early postpartum period show that postpartum beef cows, like dairy cows, resume follicular growth and development of dominant follicles at 7 - 15 days after parturition (Savio, *et al.*, 1990). It has been reported that as early as five days postpartum it is possible to detect ovarian follicles 5-10 mm in diameter (Webb, Lamming, Haynes and Foxcroft, 1980) and this is possibly the result of the increase in FSH levels soon after calving (Driancourt, 1991).

The results of Spicer, Leung, Convey, Gunther, Short and Tucker (1986a) and Murphy, Boland and Roche (1990) show that appearance of large follicles occurs long before the first postpartum ovulation in cattle and, according to Roche, Crowe and Boland (1992), the longer postpartum anoestrous period in beef cows is due to

failure to ovulate of the majority of first dominant follicles (DF) (classified according size), rather than a delay in development of DF. Spicer, *et al.* (1986a) reported a four-fold increase in numbers of medium-sized follicles (4.0 - 7.9 mm diameter) between days 7 and 42 to 56 after parturition in anovulatory cows, with no changes in numbers of small (1.0 - 3.9 mm) or large (≥ 8 mm) follicles. These authors suggested that an increase in the numbers of medium follicles may provide a pool from which ovulatory follicles are selected and that this may be one of the necessary steps leading to the first postpartum ovulation. Whether or not a large pool of follicles is necessary for resumption of ovulatory cycles after parturition in cattle is not known. However, it was reported that the decline in uterine size postpartum was associated with increased numbers of medium follicles; thus some factor related to uterine regression may directly or indirectly be involved (Schirar and Martinet, 1982).

The steroidogenic capabilities of small or medium follicles do not change during the anovulatory period in postpartum cows but there is a very large increase in oestradiol production in large follicles with length of the postpartum period which may be necessary for the return to cyclicity (Spicer, Leung, Convey, Gunther, Tucker and Short, 1984). It has also been reported that ovarian activity during postpartum anoestrus was not limited by the number of LH and FSH receptors since no significant differences were found for gonadotrophin receptors in large or small follicles (Braden, Manns, Cernak, Nett and Niswender, 1986; Spicer, Convey, Leung, Short and Tucker, 1986b).

### 2.2.4-Effect of nutritional status on follicular development, populations and function

#### 2.2.4.1- Effect of feed intake

Restriction of feed intake in beef heifers reduces the maximum diameter and persistence of the dominant follicles and increases the proportion of oestrous cycles with three dominant follicles rather than two when compared with heifers gaining or

maintaining weight (Murphy, Enright, Crowe, McConnell, Spicer, Boland and Roche, 1991). In postpartum beef cows with a low intake combined with poor body condition, the growth of follicles to larger size (≥ 10 mm diameter) was decreased or absent (Perry, *et al.*, 1991). Similar results have been reported in cows receiving low-energy diets (Wiltbank, *et al.*, 1964), and in states of negative energy balance (Lucy, Staples, Thatcher, Lough, Michel and Brede, 1989).

Restriction of dietary energy interferes with follicular maturation; Henricks, Rone, Ferrel and Echternkamp (1986) have shown that follicles do not mature to the same extent and secrete lower levels of oestradiol-17β at low energy intakes which in turn results in a longer anoestrous period. However, the mechanisms by which dietary energy intake affects follicular growth and steroid production are not clear.

#### 2.2.4.2- Effect of body condition

Prado, *et al.* (1990) has shown that, at five weeks postpartum, the number of large follicles present in cows in high and low levels of body conditon were similar and there was no difference in their steroidogenic capacity. However, at nine weeks postpartum, the same authors have found that a higher proportion of the large follicles were highly oestrogenic and potentially ovulatory in cows in high body condition rather than cows in low body condition. These results were based on small numbers of animals.

## 2.3- Metabolite and hormone profiles

The effects of nutritional state on the ovary may be mediated through changes in metabolite and metabolic hormone profiles acting directly on the ovary or through effects on hypothalamic and pituitary function. There is a wide range of possible mediators.

### 2.3.1- Blood Metabolites

#### 2.3.1.1- Glucose

Plasma glucose concentrations change according to body condition, which is a reflection of the nutritional status of the animal (Easdon, Chesworth, Aboul-Ela and Henderson, 1985).

Glucose is the main source of energy utilized by the nervous system and this, is directly involved in the control of reproduction (Randel, 1990; Richards, *et al.*, 1989b; Short and Adams, 1988). These authors have suggested that plasma glucose concentration is the specific mediator for the effects of energy intake on reproduction.

Links have been identified between glucose and gonadotrophin secretion. Foster, Ebling, Vannerson, Bucholtz, Wood, Micka, Suttie and Vennvliet (1988) found LH pulsatility to be increased in food-restricted ewe lambs, following the infusion of either glucose or a mixture of amino acids. However, in other work in which sheep were treated with glucose for 5 days, they had an increased ovulation rate but unaltered LH concentrations and lower FSH concentrations (Downing and Scaramuzzi, 1991).

Richards, Wettemann and Schoenemann (1989a) have shown that reduced nutrient intake is associated with a reduction in plasma glucose concentrations in cows which results in serum concentrations of LH which are insufficient to stimulate normal cyclic ovarian function. Furthermore, in cattle McClure, Nancarrow and Radford (1978) found that a glucose metabolic inhibitor (2-deoxy-D-Glucose) blocked oestrus and ovulation. On the other hand, McCaughey, Rutter and Manns (1988) have shown

that increasing glucose availability in adequately fed cows does not alter the pulsatile patterns of LH or GnRH-stimulated LH release. When glucose clearance was increased by infusion of phlorizin, the LH pulse amplitude was depressed during the follicular phase of the oestrous cycle and during the postpartum period of beef cows although the GnRH-induced release of LH was unaffected (Rutter and Manns, 1987; Rutter and Manns, 1988a). Further studies are required in order to establish the role of glucose in reproductive activity.

#### 2.3.1.2- Nonesterified Fatty Acids (NEFA)

Work done in beef cows has shown that non-esterified fatty acid concentrations are a useful index of either energy intake or energy status (Russel and Wright, 1983). In response to negative energy balance, animals mobilize adipose fat to form nonesterified fatty acids (NEFA) (Hart, Bines, Morant and Ridley, 1978; Hart, Bines, Balch and Cowie, 1975; Waghorn, Flux and Ulyatt, 1987).

More recently, the results from Canfield and Buttler (1991) showed that when the reproductive activity of lactating dairy cows is impaired, the levels of NEFA are high. Since nutrititional factors affect gonadotrophin secretion, Estienne, Schillo, Hileman, Green, Hayes and Boling (1990) suggested that NEFA may inhibit LH release during periods of inadequate nutrition and evaluated this effect on peripubertal lambs after ovariectomy. After infusion of lipids for 8 h. there was a rise in the circulating concentrations of NEFA, but no effect on the pattern of LH.

Schillo (1992) concluded that NEFA can influence neuroendocrine function but do not act act as modulators of LH secretion, at least in sheep.

#### 2.3.1.3- Ketones

Animals that are in negative energy balance mobilize adipose fat in the form of NEFA and therefore generate ketones (Brockman and Laarved, 1986).

Rutter and Manns (1987; 1988a) have shown that beef cows treated with phlorizin generate ketones which could be used by neurons to sustain pulsatile LH release such that changes in circulating concentrations could affect reproductive activity.

In summary it seems that different energy substrates could be used by the GnRH pulse generator. According the hypothesis of Schillo (1992), during times of adequate dietary energy, propionate is utilized for production of glucose which is the major energy substrate used by the brain. During negative energy balance, NEFA are mobilized by the liver, and ketones are synthesized and these can be used as energy substrates for the central nervous system. Ebling, Wood, Karsch, Vannerson, Suttie, Bucholtz, Schall and Foster (1990) also suggested that amino acids, such as aspartate or glutamate, can also be mobilized and oxidized by the brain and/or metabolized to form glucose and/or ketones.

Changes in concentrations of these substances may thus affect hypothalamic activity and gonadotrophin production directly. However, they may also act through changes in metabolic hormones.

### 2.3.2- Metabolic Hormones

Changes in reproductive performance may involve changes in metabolic hormones such as insulin, GH and IGFs which are associated with changes in energy metabolism induced by changes in nutrition (Bass, Spencer and Hodgkinson, 1992; Pell and Bates, 1990; Spicer, Tucker and Adams, 1990; Waghorn, *et al.*, 1987). These metabolic hormones have been shown to influence ovarian function either directly at the ovarian level and/or by modulating gonadotrophin actions on ovaries.

#### 2.3.2.1- Insulin

Nutritional status influences metabolism and plasma insulin profiles and so it is possible that insulin may serve as a nutritional signal and may influence LH secretion.

Periods of low nutrition are associated with a decrease in insulin secretion in sheep (Bassett, 1974) and heifers (McCann and Hansel, 1986) and low levels of nutrition are associated with longer postpartum periods.

The observation that increases in ovulation rate in sheep, arise from improved nutritional status of the animal, suggests that the 'flushing' effect on ovulation rate may be mediated by the associated increase in insulin (Robinson, 1990), even although this effect is not always accompanied by increases in the plasma gonadotrophins.

The idea that insulin may be involved in the regulation of ovarian physiology originated from observations that humans with insulin-dependent diabetes due to insulin deficiency, exhibit ovarian hypofunction and that patients with insulin resistance show ovarian hyperstimulation (Poretsky and Kalim, 1987). Experiments have shown that insulin stimulates ovine granulosa cell proliferation (Webb and McBride, 1991), and stimulates bovine granulosa cell proliferation and differentiation (Langhout, Spicer and Geisert, 1991; Savion, Lui, Laherty and Gospodarowicz, 1981). Indeed, increases in plasma insulin concentrations induced by the infusion of glucose or branched-chain amino acids into sheep were associated with an increase in ovulation rate (Downing and Scaramuzzi, 1991) and in pigs infusion of insulin results in stimulation of follicular development (Matamoros, Cox and Moore, 1991), and an enhanced ovulation rate (Cox, *et al.*, 1987).

In heifers, infused with insulin, the pattern of LH during the oestrous cycle was not affected (Harrison and Randel, 1986) but, in swine, exogenous insulin has been reported to alter LH secretion patterns (Jones, Bennett, Althen and Cox, 1983).

Thus, although changes in nutritional status are associated with changes in circulating concentrations of insulin, the role of insulin in the control of LH secretion is equivocal.

#### 2.3.2.2- Growth hormone

Several reports suggests that growth hormone (GH) is involved in the regulation of reproductive activity; in both humans (Goodman, Grunbach and Kaplan, 1968) and in rats (Advis, Smith White and Ojeda, 1981), puberty is delayed in somatotrophin (ST)-deficient individuals but the problem can be corrected with ST replacement. The observations that bovine somatotrophin (bST) increases twinning rates (Butterwick, *et al.*, 1988) and number of small ovarian follicles (Lucy, De La Sota, Staples and Thatcher, 1993) in lactating dairy cows and that, in heifers treated with bST, there is an increase in the population of antral follicles, without changes in circulating gonadotrophin concentrations (Gong, *et al.*, 1991; Gong, *et al.*, 1993), indicate that GH may be involved in the control of ovarian function in cattle. Furthermore, Yoshimura, *et al.* (1993) has shown that GH acts on the rabbit ovary to stimulate ovarian oestradiol production *in vitro* and Barreca, Artini, Del Monte, Ponzani, Pasquini, Cariola, Volpe, Genazzani, Giordano and Minuto (1993) showed that GH treatment increases oestradiol production *in vivo* and *in vitro* in human follicles.

There is increasing evidence that exogenous bST influences concentrations and activities of other hormones in tissues and thereby affects nutrient availability and utilization (McDowell, 1991). GH has been shown to elevate the IGF-I levels in the circulation by stimulating the production of IGF-I in the liver (D'Ercole, *et al.*, 1984) but it is not yet known whether the effects of GH on follicular development are exerted directly on the ovary or through induction of hepatic synthesis of IGF-I.

The presence of GH receptors on the ovary of the cow (Tanner and Hauser, 1989) suggests possible direct effects of bST on follicular growth and development. In addition, GH/bST may affect the local production of IGF-I, which acts as an autocrine or paracrine regulator of granulosa cells (Mondschein, Canning, Miller and Hammond, 1989).

### 2.3.2.3- Insulin growth factor-I

Insulin-like growth factors (IGFs) are important mediators of growth, lactation, reproduction and health but their precise mechanisms of action are not known (McGuire, *et al.*, 1992).

Nutritional status affects circulating concentrations of IGF-I in ruminants. IGF-I concentrations change rapidly in response to alterations in intermediary energy metabolism induced by glucose excess or deprivation or by reducing feed intake, with changes in IGF-I being greater than changes in glucose or insulin (Rutter and Manns, 1988b). Rutter and Manns (1989) also showed that serum concentrations of IGF-I were related positively to energy availability in postpartum beef cows. Circulating concentrations of IGF-I were reduced in growing cattle that were severely underfed (Breier, *et al.*, 1986; Ellenberger, Johnson, Carstens, Hossner, Holland, Nett and Nockels, 1989; Elsasser, Rumsey and Hammond, 1989; Houseknecht, Boggs, Campion, Sartin, Kitser and Andrampacek, 1988; Ronge and Blum, 1989) but differences were not apparent when steers were moderately fed (Breier, *et al.*, 1986).

Since IGF-I is critical to the development of the follicle (Adashi, *et al.*, 1985), their low concentration in the plasma may affect postpartum ovarian recrudescence. Although direct effects of low plasma IGF-I on the ovary have not been reported, IGF-I is higher in the blood and follicular fluid of cattle selected for enhanced follicular growth and development (multiple ovulation) (Echternkamp, Spicer, Gregory, Channing and Hammond, 1990). Lucy, *et al.* (1992) also showed that plasma IGF-I was positively correlated to the oestrogen:progesterone ratio in the follicular fluid of dominant follicles of lactating dairy cows and that follicular growth was slower in cows fed a low energy diet; this was associated with reduced plasma IGF-I concentrations but normal LH concentrations.

It is concluded that, since changes in IGF-I concentrations are associated with changes in reproductive activity, it may have an important role in mediating the effects of nutrition on reproductive performance.

### 2.3.3- Endogenous Opioid Peptides

One of the ways through which metabolic hormones and/or metabolites could affect reproductive activity is through an effect on the brain and the production of endogenous opioid peptides (EOP) and so on hypothalamic activity.

There is general agreement that endogenous opioid peptides modulate luteinizing hormone (LH) release by suppressing gonadotrophin releasing hormone (GnRH) pulses, in several species including cattle and sheep (Brooks, Lamming and Haynes, 1986b; Ebling, Scharwtz and Foster, 1989; Gregg, Moss, Hudgens and Malven, 1986; Nanda, Ward and Dobson, 1991; Nett, 1987; Malven, 1986; Whisnant, Thompson, Kiser and Barb, 1986a).

Whilst the opioids are generally thought to act to depress gonadotrophin secretion either at the level of the hypothalamus or higher in the brain, Haynes, Lamming, Yang, Brooks and Finnie (1989) suggested that there may be effects of opioids directly on the pituitary gland. Supporting this idea, recent studies have shown that naloxone (a specific opiate antagonist with distinct affinities to the various types of opiate receptors termed mu, delta and kappa (Pfeiffer and Herz, 1984)) increases the concentration of circulating LH in the postpartum beef cows (Nanda *et al.*, 1991; Whisnant *et al.*, 1986a). It has been postulated by Snyder (1984) that the type of opioid receptor involved in modulation of LH secretion may change during the postpartum period and Malven, Parfet, Gregg, Allrich and Moss (1986) suggested that it is the mu subtype of opioid receptor that is involved in the inhibition of GnRH release.

There is evidence that the opioid inhibition of LH secretion changes with stage of the oestrous cycle (Short, Brooks, Peters and Lamming, 1987). It decreases during

the postpartum interval as indicated by the fact that larger doses of naloxone are required to increase serum concentrations of LH on day 14 than day 28 or 42 postpartum (Whisnant, *et al.*, 1986a). It has been suggested that during the postpartum period the opioidergic suppression of LH is either initiated by suckling or enhanced by it (Haynes, *et al.*, 1989; Malven, *et al.*, 1986; Myers, Myers, Gregg and Moss, 1989) and removal of the suckling stimulus removes the opioid inhibitory tone (Whisnant, Kiser, Thompson and Barb, 1986c).

It has also been suggested that in cows and sheep the inhibition of LH secretion is either parity or age-dependent (Brooks, Lamming, Lees and Haynes, 1986; Mahmoud, Thompson, Peck, Mizinga, Leshin, Rund, Studemann and Kiser, 1989), with inhibition being reduced in older animals.

### 2.3.4- Gonadotrophin-Releasing Hormone and gonadotrophins

EOP exert their effects, at least in part, through control of GnRH secretion by the hypothalamus. Measurement of GnRH secretion *per se* is technically very difficult and most measures of secretion are based on the close link between LH and GnRH profiles (Clarke and Cummins, 1982).

The gonadotrophins, FSH and LH, are glycoproteins with molecular weight of approximately 30kDa (Reichert and Jiang, 1965), and are produced and secreted by the anterior pituitary gland under the control of GnRH, a decapeptide hormone secreted from the hypothalamus (Everett, 1988; Pierce and Parsons, 1981). Gonadotrophins are generally recognized as the primary regulators of ovarian function (Armstrong, Goff and Dorrington, 1979); they regulate the synthesis of ovarian steroids.

The results of Quirk and Fortune (1986) provide evidence *in vivo* that FSH is required for the preovulatory rise in plasma oestradiol. Furthermore, infusion of FSH increases peripheral oestradiol concentrations in ewes (Hudson, McNatty, Ball, M., Heath, Lun, Kieboom and Henderson, 1985). Hypothalamic and pituitary function

are, in turn, partly controlled by the secretion of steroid hormones by the ovary (Nett, Cermak, Braden, Manns and Niswender, 1988).

During late pregnancy, the high concentrations of progesterone and oestradiol present in the circulation exert a negative feedback action on the hypothalamic-hypophiseal axis (Nett, 1987). In particular, they exert an inhibitory effect on the response of the pituitary gland to hypothalamic GnRH (Schallenberger, Schams and Zottmeier, 1978). This causes a depletion of LH in the anterior pituitary gland after parturition and before normal oestrous cycle begins these levels must be restored (Hanzen, 1986).

#### 2.3.4.1- Luteinizing Hormone (LH)

In beef cows, the LH content of the anterior pituitary gland increases following parturition reaching concentrations similar to those present in cyclic animals by day 30 postpartum (Convey, Tucker and Short, 1983; Humphrey, *et al.*, 1983; Moss, Parfet, Marvin, Allrich and Diekman, 1985). The GnRH "pulse generator" is inhibited during the early postpartum period (Short, *et al.*, 1990). Pulses of LH became more frequent as females approach the first postpartum ovulation (Carruthers and Hafs, 1980; Humphrey, *et al.*, 1983; Peters, 1984). Thus, establishment of frequent pulses of LH in plasma of postpartum females appears to be directly related to the initiation of cyclic ovarian function (Rahe, Brown, Marple, Sartin, Bartol, Mulvaney and Reeves, 1988). However, episodic patterns of LH tend to occur less frequently in suckled cows than in milked or non-suckled postpartum cows (see effect of suckling in 2.3.4.3).

During postpartum anoestrus the pulse pattern of LH is typically of a very low frequency (< 1 pulse/4h) (Convey, *et al.*, 1983; Walters, Short, Convey, Staigmiller, Dunn and Kaltenbach, 1982b). Inadequate LH pulse frequency results in low production of androgens in the follicle, and thus the pre-oestrus rise in oestradiol, which is a prerequisite for ovulation, does not occur (Roche, *et al.*, 1992). Ovulation only occurs when an LH pulse occurs every 40-60 min to stimulate maximum

oestradiol production, positive feedback and an ovulatory surge of LH and FSH (Roche, *et al.*, 1992).

Factors that suppress LH pulse frequency in the postpartum cow, such as suckling (see 2.3.4.3), nutrition (see 2.3.4.5) and probably season (see 2.3.4.4), will delay first ovulation.

#### 2.3.4.2- Follicle Stimulating Hormone (FSH)

FSH secretion does not appear to be a limiting factor with respect to the onset of ovarian cycles, since plasma concentrations tend to increase during the first few days after calving (Peters, Lamming and Fisher, 1981; Webb, *et al.*, 1980), and then do not change between 14 to 50 days postpartum in either dairy or beef cattle (Convey, *et al.*, 1983; Schallenberger, *et al.*, 1978; Willians, Kotwica, Slanger, Olson, Tilton and Johnson, 1982). FSH secretion is not secreted in a pulsatile manner throughout the postpartum period unlike LH (Garcia-Winder, Lewis, Deaver, Smith, Lewis and Inskeep, 1986) and, even when pulsed injections of GnRH were given, it has not been possible to detect pulses of FSH (Bolt, Scott and Kiracofe, 1990; McLeod, Peters, Haresign and Lamming, 1985; Riley, Peters and Lamming, 1981). The rapid rise in plasma FSH concentrations after parturition stimulates follicular development (Driancourt, 1991) and Spicer, *et al.* (1986a) reported that large follicles (> 8 mm diameter) were already present by day 7 postpartum.

In summary, and according to Roche, *et al.* (1992), FSH seems to be mainly responsible for recruitment and selection of the dominant follicle (DF) and exposure of an oestrogen-active DF to frequent LH pulses is the key to final maturation and ovulation of the DF.

The synthesis and release of GnRH and gonadotrophins during the postpartum period is affected by several factors, with the most important being suckling, season and nutrition. The effects of these factors are reviewed here.

### 2.3.4.3- Effect of suckling

Suckling suppresses the episodic secretion of LH during the early post-partum period (Garcia-Winder, Imakawa, Day, Zalesky, Kittok and Kinder, 1986a; Walters, Smith, Harms and Wiltbank, 1982d; Willians, *et al.*, 1982). LH and FSH concentrations are greater in non-suckled than in suckled cows (Short, *et al.*, 1972) and serum LH concentrations increase in anoestrus beef cows following weaning (Walters, *et al.*, 1982d). Levels of LH have been reported to be depressed within one hour of the onset of suckling in postpartum beef cows, suggesting that suckling has an acute effect on gonadotrophin secretion (Dunlap, Kiser, Cox, Thompson, Rampacek, Benyshek and Kraeling, 1981; Forrest, Fleeger, Long, Sorensen and Harms, 1980) but Convey, *et al.* (1983) found that suckling did not act in an acute fashion to suppress LH secretion in postpartum beef cows.

Secretion of LH in the postpartum cow appears to be dictated in part by frequency of suckling, since it has been demonstrated to be a major determinant of the length of the postpartum anoestrous period. Once daily-suckling as opposed to suckling *ad libitum* resulted in a shorter postpartum anoestrous interval (Randel, 1981). More severe reductions in suckling frequency such as 48-h and 72-h calf removal, also elevated serum levels of LH and shortened the post partum interval (Dunn, *et al.*, 1985; Walters, *et al.*, 1982d).

One of the mechanisms through which suckling may inhibit the release of GnRH is via endogenous opioids (Whisnant, Kiser, Thompson and Barb, 1986b). However, in addition to this mechanism, suckling is also thought to evoke a neural stimulus that inhibits GnRH release from the hypothalamus and results in depressed plasma LH concentrations (Zalesky, Forrest, McArthur, Wilson, Morris and Harms, 1990).

### 2.3.4.4- Effect of season

Photoperiod affects circulating profiles of LH, prolactin and melatonin in the prepuberal heifer (Critser, Block, Folkman and Hauser, 1987), and cow (Hansen, 1985; McNatty, Hudson, Gibb, Henderson, Lun, Heath and Montgomery, 1984). A seasonal pattern of luteinizing hormone (LH) secretion with the highest concentrations in the winter and the lowest in the summer was recorded in ovariectomized animals (Critser, Miller, Gunsett and Ginther, 1983; Day, Imakawa, Garcia-Winder, Zalesky, Clutter, Kittok and Kinder, 1986; Stumpf, Day, Wolfe, Wolfe, Clutter, Kittok and Kinder, 1988). However, oestradiol does not have a seasonally dependent, differential, positive feedback effect on secretion of LH in the cow (Stumpf, *et al.*, 1988).

The pineal gland plays a central role in mediating effects of photoperiod on reproduction, through the secretion of the indole compound, melatonin (Thatcher and Hansen, 1993). Treatment with melatonin has increased the interval to first postpartum oestrus and ovulation, suggesting an effect of short-day photoperiod on the onset of postpartum activity in beef cattle acting through melatonin secretion (Sharpe, Gifford, Flavel, Nottle and Armstrong, 1986).

The actions of melatonin is thought to mediate the multiple effects of photoperiod by affecting the secretion of hormones from the pituitary gland, with Lincoln (1992) proposing that melatonin acts on the dopaminergic and/or opioidergic neuronal pathways within the mediobasal hypothalamus to influence the release of the peptides and neurotransmiters which normally regulate the pituitary gland.

### 2.3.4.5- Effect of nutrition

An increased pulsatile release of LH is thought to be a prerequisite for resumption of oestrous cycles in postpartum beef cows (Lamming, *et al.*, 1981; Peters and Lamming, 1986), and it has been demonstrated that changes in nutritional state can influence LH secretion (Echternkamp, *et al.*, 1982; Johnson, *et al.*, 1987; Wright, *et*

*al.*, 1987; Wright, *et al.*, 1990; Prado, *et al.*, 1990; Roberson, Stumpf, Wolfe, Kittok, Kinder, 1991; Wright, *et al.*, 1992a).

Perry, *et al.* (1991) showed that, after parturition, cows receiving high levels of energy prepartum had higher mean LH concentrations and a higher pulse frequency than cows fed low levels of energy and had shorter intervals from parturition to ovulation. Feeding levels influence LH pulsatility in both intact (Whisnant, *et al.*, 1985) and ovariectomized cows (Echternkamp, *et al.*, 1982) which suggests that the feeding level can also influence LH pulsatility independently of ovarian steroid feedback mechanisms.

The occurence of higher LH pulse frequencies in cows in high than low BC together with shorter postpartum intervals suggest that the effect of BC on the length of the postpartum anoestrus is mediated through differences in LH pulse frequency (Wright, *et al.*, 1990; Richards, Wettemann, Morgan, 1988). Cows receiving adequate nutrients during the pre- and postpartum periods were shown to have an increasing LH pulse frequency with increasing time postpartum (Nolan, *et al.*, 1989).

Some reports have suggested that the effect of postpartum level of energy on the duration of postpartum anoestrus was limited to cows calving in low body condition (Richards, *et al.*, 1986; Short and Adams, 1988; Wright, *et al.*, 1987). In contrast, no significant effect of level of feeding postpartum on the duration of the postpartum anoestrous was found by Wright, *et al.*, (1992a). Taken together, the results suggest that level of BC at calving is more important than level of postpartum feeding.

Dietary energy restriction has been reported to either increase (Whisnant, *et al.*, 1985), or decrease (Imakawa, Day, Zalesky, Clutter, Kittok and Kinder, 1987; Lishman, Allison, Fogwell, Butcher and Inskeep, 1979; Rutter and Randel, 1984) the release of LH in postpartum cows. Imakawa, *et al.* (1987) proposed that nutritional deprivation lowered pulse frequency by reducing hypothalamic activity and GnRH pulse frequency rather than by altering pituitary response to hypothalamic secretions

and Wright, *et al.* (1990) have demonstrated that body condition did not affect pituitary responsiveness to a physiological dose of GnRH.

The mechanism by which BC influences the hypothalamus remains unclear, but it is unlikely that body fat *per se* determines LH profiles (Schillo, 1992). Metabolic changes associated with changes in body condition probably regulate pulsatile LH release (Schillo, 1992). These metabolic changes may influence LH release via specific blood-borne signals such as metabolites and/or metabolic hormones (Butler and Smith, 1989) which in turn may act through endogenous opioids to provide neural or pituitary inhibition of the pulsatile LH production (Malven, 1986).

#### 2.3.4.6- GnRH treatments to induce ovarian activity

The postpartum period is characterized by a low frequency of pulsatile LH release (Peters and Lamming, 1986), with suckling and low level of nutrition implicated in the prolonged suppression of LH pulses (Myers, *et al.*, 1989). During the preovulatory period of the oestrous cycle, plasma LH concentrations rise and a marked increase in LH pulse frequency is observed (Peters, Pimentel and Lamming, 1985). In order to induce this pattern of LH release postpartum and to advance the first ovulation, repeated injections of low doses of GnRH have been used. Doses of 0.5 to 5 μg administered at intervals of 1 to 2 h, for 48 to 96 h, have been used most commonly (Edwards, Roche and Niswender, 1983; Riley, *et al.*, 1981), and have been shown to induce ovulation in both prepubertal heifers and acyclic cows (McLeod, *et al.*, 1985; Riley, *et al.*, 1981; Walters, Short, Convey, Staigmiller, Dunn and Kaltenbach, 1982c).

McLeod, Haresign and Lamming (1983) suggested that the increase in mean LH concentrations that resulted from an increase in LH pulse frequency was all that was required to promote preovulatory follicle development and, indeed, it has been demonstrated that continuous infusion of GnRH results in a sustained elevation of basal LH secretion rather than an increase in pulse frequency, and that this can induce

ovulation (McLeod, Haresign, Peters, Humke and Lamming, 1988). Continuous infusion of GnRH, consistently induced ovulation in seasonally anoestrous (McLeod, *et al.*, 1983; McLeod, *et al.*, 1988) and postpartum anoestrous sheep (Wright, Geytenbeck, Clarke and Findlay, 1983), but in cattle did not consistently induce ovulation (Jagger, Peters and Lamming, 1987; Lamming and Mcleod, 1988; McLeod, *et al.*, 1988).

McLeod, Dodson, Peters and Lamming (1991) suggested that when sheep or cattle are subjected to a continuous infusion of GnRH, mean LH concentrations are elevated for only 48-72h and, while it is sufficient to promote the final stages of preovulatory follicle development in sheep, a longer period of gonadotrophic stimulation is apparently required in cattle.

The cause of the variability in ovulatory response to GnRH is unknown but Jagger, *et al.* (1987) suggest that the LH response to GnRH treatment is dependent on follicular status in the immediate pretreatment period and this could also determine the capacity of follicles to ovulate.

### 2.3.5- Ovarian Steroids and peptides

Gonadotrophin profiles determine the pattern of ovarian growth and steroidogenesis.

The ovarian sex steroids, progesterone, androstenedione, testosterone and oestradiol, are derived from cholesterol (Short, 1962). The process of ovarian steroidogenesis and its control have been well reviewed by Gore-Langton and Armstrong (1988). Briefly, FSH and LH enhance steroidogenic enzyme activity in granulosa and theca cells after interaction with their receptors sites in the thecal and granulosa cells respectively. They activate the adenylate cyclase-cyclic adenosine monophosphate (cAMP) (Ireland, 1987), which initiates the activation of enzymes responsible for steroidogenesis.

Progesterone is produced by both granulosa and thecal cells of bovine follicles (McNatty, Heath, Henderson, Lun, Hurst, Ellis, Montgomery, Morrison and Thurley 1984) and serves as a substrate for androgen biosynthesis. Only thecal cells possess the 17α-hydroxylase and 17-20 desmolase enzyme system necessary for the production of androgens, thus progesterone is converted into androgens in the thecal cells (Erickson, Magoffin, Dyer and Hofeditz, 1985; Ireland, 1987). The androgens synthesized in the theca interna under the primary control of LH diffuse across the basal lamina to supply the substrate for oestrogen biosynthesis by the aromatase enzyme complex in granulosa cells (Gore-Langton and Armstrong, 1988).

The observations of a temporal relationship between circulating ovarian steroid and pituitary gonadotrophin concentrations during the oestrous cycle (Goodman and Karsch, 1980; Hobson and Hansel, 1972), indicate a probable involvement of ovarian steroids in the control of gonadotrophin secretion (Girmus and Wise, 1991; Price and Webb, 1988). Progesterone and oestradiol may inhibit LH synthesis and secretion through direct action on the pituitary or indirectly by altering GnRH release from the hypothalamus (Fink, 1988).

Supporting the hypothesis of a role for progesterone in the control of the oestrous cycle are observations that progesterone is capable of blocking the oestradiol-induced gonadotrophin surge in cattle (Hobson and Hansel, 1972; Roche and Ireland, 1981) and that high concentrations of endogenous progesterone suppress the gonadotrophin surge in cattle (Short, Randel, Staigmiller and Bellows, 1979). Progesterone seems to inhibit secretion of LH by reducing the frequency of episodes of LH release without affecting the pituitary content of LH or pituitary responsiveness to GnRH, which suggests that progesterone supresses secretion of GnRH from the hypothalamus (Goodman and Karsch, 1980).

Oestradiol-17β can exert either a positive or negative feedback on gonadotrophin secretion. During pregnancy the high circulating concentrations of

progesterone and oestradiol result in a prolonged negative feedback on the hypothalamic-hypophysial axis (Nett, 1987). It has been established that concentrations of plasma LH are low immediately following parturition due to the negative feedback effects of ovarian steroids (Acosta, Tarnavsky, Platt, Hamernik, Brown, Schoenemann and Reeves, 1983; Short, *et al.*, 1972). Only after pituitary stores of LH have returned to their normal levels, by day 30 postpartum in beef cows, are pulses of LH released with sufficient frequency to stimulate follicular growth and secretion of oestradiol (Nett, *et al.*, 1988). Small transient increases in circulating oestradiol concentrations which follow each LH pulse induce more frequent pulses of LH in a positive feedback loop that lead to the preovulatory LH surge and the final stages of follicular development and ovulation (Nett, 1987).

In addition to its role as the major steroid messenger in the circulation, oestradiol also acts within the follicle to regulate its development and function (Fortune and Quirk, 1988). These authors proposed that, as the preovulatory follicle matures, the initial action of oestradiol is that of positive feedback on its own production, via increased androgen synthesis.

In postpartum cows, concentrations of oestradiol in peripheral blood decrease sharply at parturition to basal levels (1 to 8 pg/ml) within 2 to 6 days (Arije, Wiltbank and Hopwood, 1974; Henricks, Dickey, Hill and Johnston, 1972; Humphrey, *et al.*, 1983). During postpartum oestradiol concentrations vary widely, reflecting periods of follicular growth, and Peters and Ball (1987) suggest that ovarian follicles are potentially fully active during early postpartum and although oestrogen concentrations may reach pre-ovulatory levels, these are rarely followed by preovulatory LH surges and ovulation. It is possible that the relatively low concentration of receptors for oestradiol in the hypothalamus and the anterior pituitary render the hypothalamic-pituitary axis less sensitive to the positive feedback effects of oestradiol during the early postpartum period (Nett, 1987; Nett, *et al.*, 1988).

During pregnancy progesterone concentrations are high and a rapid decrease in progesterone levels (due to the preparturient regression of the corpus luteum) begins about three days before calving (Arije, *et al.*, 1974; Humphrey, *et al.*, 1983). Concentrations of progesterone remain low (< 1.0 ng/ml) in cows until the resumption of ovarian activity (Humphrey, *et al.*, 1983; Rawlings, Weir, Todd, Manns and Hyland, 1980). In the postpartum period approximately 50% (40 to 70%) of cows exhibit a transient elevation of plasma progesterone concentrations (not > 2 ng/ml and of 1 to 6 days duration) before the first postpartum oestrus (Humphrey, *et al.*, 1983; Lamming, *et al.*, 1981; Rawlings, *et al.*, 1980) while in others the lifespan of the corpus luteum formed after the first postpartum ovulation is normal.

Both oestradiol and progesterone seems to play an important role in the feedback control of gonadotrophin secretion and, in addition, these two ovarian steroids seem to act in concert, as neither steroid alone can totally account for the cyclic changes in LH concentrations. When at physiological concentrations oestradiol and progesterone were combined, they suppressed LH concentrations to those observed in normal physiological states in ovariectomized heifers (Price and Webb, 1988) and similar results were observed in sheep (Goodman and Karsch, 1980). However, contradictory results were reported for FSH levels and, although Price and Webb (1988) showed that the combination of oestradiol and progesterone in physiological concentrations is able to maintain normal levels of FSH in short-term ovariectomised heifers, other authors showed that steroid treatment of ovariectomised cattle (Roche and Ireland, 1981) and sheep (Goodman, Pickover and Karsch, 1981) fails to suppress FSH concentrations to levels observed in intact controls, which suggests that a further, non-steroidal component of negative feedback may exist.

#### 2.3.5.1- Inhibin and related proteins

While oestradiol concentrations in the follicular fluid are an important index of physiological state and affect the development process, many other factors are

involved. Some of the most commonly studied non-steroid ovarian factors, which may regulate gonadotrophin secretion, are inhibin and its related proteins.

Inhibin is a glycoprotein of gonadal origin recently isolated from ovarian follicular fluid (Franchimont, Hazee-Hagelstein, Charlet-Renard and Jaspar, 1986; Leversha, Robertson, de Vos, Morgan, Hearn, Wettenhall, Findlay and Burger, 1987). It consists of two dissimilar disulphide-linked subunits ($\alpha$ and $\beta$) (Burger, 1988; De Jong, 1987; Hsueh, Dahl, Kaugham, Tucher, Rivier, Bardin and Vale, 1987; Knight, 1991; Tonetta and diZerega, 1989; Ying, 1988).

This hormone was initially considered to be involved in the regulation of gonadotrophin secretion, selectively inhibiting the production of follicle stimulating hormone (FSH) by the anterior pituitary (Burger, 1989; Ling, Ying, Ueno, Esch, Hotta and Guillemin, 1986; McLachlan, Robertson, Healy, de Kretser and Burger, 1986; Quirk and Fortune, 1986; Rivier, Rivier and Vale, 1986; Ying, 1988; Ying, Czvik, Becker, Ling, Ueno and Guillemin, 1987). However, recently, its involvement in the regulation of ovarian function has gained increasing acceptance (Findlay, Sai and Shukovsky, 1990; McNeilly and Baird, 1989).

Inhibin is produced by the granulosa cells in the ovarian follicles (Channing, Gordon, Liu and Ward, 1985; Henderson and Franchimont, 1981; Knight, Castillo, Glencross, Beard and Wrathall, 1990). Production depends on the size and physiological state of follicles. Henderson, Franchimont, Charlet-Renard and McNatty (1984) with cows and Tsonis, Quigg, Lee, Leversha, Trounson and Findlay (1983) and Mann, McNeilly and Baird (1989) with sheep observed that granulosa cells from large healthy follicles produced significantly more inhibin than cells from small follicles and Henderson, *et al.* (1984) have also observed that inhibin production by granulosa cells from large atretic follicles is similar to that of cells from small follicles.

FSH secretion is suppressed by follicular fluid in ovariectomized ewes (Cummins, O'Shea, Bindon, Lee and Findlay, 1983; Findlay, Gill and Doughton, 1985) but in cattle the role of inhibin in the control of FSH secretion is still uncertain.

Results of studies involving treatment of ovariectomized and intact heifers with steroid-free preparations of bFF has been equivocal; some reports indicated that FSH was suppressed (Beard, Castillo, McLeod, Glencross and Knight, 1990; Ireland, Curato and Wilson, 1983; Quirk and Fortune, 1986), while others reported no change (Johnson and Smith, 1985).

Price and Webb (1988) showed that FSH can be maintained in the normal range in short-term ovariectomized heifers with only oestradiol and progesterone, suggesting that bovine follicular fluid and presumably inhibin had no effect. This suggestion is supported by the recent findings of Law (1991) that bovine pituitary cells are unresponsive to inhibin-containing preparations *in vitro*. In addition, in intact heifers, treatment with bFF in which the bioactive inhibin content had been reduced by > 95% by immunoaffinity chromatography failed to suppress peripheral FSH concentrations (Law, Baxter, Logue, O'Shea and Webb, 1992), and they suggest that inhibin-stripped follicular fluid was as potent in delaying oestrus as follicular fluid containing inhibin.

These results support the idea that in cattle, inhibin may not have a major role in controlling FSH secretion, unlike sheep (Martin, Price, Thiéry and Webb, 1988), which require a protein component of follicular fluid, presumably inhibin, to maintain peripheral FSH concentrations within the range of those in intact ewes (Webb, Gong, Law and Rusbridge, 1992).

Several other inhibin-related proteins have also been identified in porcine and bovine follicular fluid.

These include activin, a β-subunit dimer of inhibin (Ling, *et al.*, 1986; McLachlan, Robertson, Burger and de Kretser, 1986), FSP (FSH suppressing protein or Follistatin) (Robertson, Klein, de Vos, McLachlan, Wettenhall, Hearn, Burger and de Kretser, 1987; Ueno, Ling, Ying, Esch, Shimasaki and Guillemin, 1987), a class of proteins with 5-30% of the biological activity of inhibin but with no structural homology to inhibin (Robertson, Foulds, de Vos, Leversha and de Kretser, 1990),

and α-N Peptide of inhibin (αN) (Findlay, *et al.*, 1990; Robertson, Giacometti, Foulds, Lahnstein, Goss, Hearn and de Kretser, 1989). These may also have a role in the regulation of FSH concentrations (Findlay, *et al.*, 1990).

Activin stimulates the basal secretion of FSH by pituitary cells *in vitro*, while not altering the secretion of LH (Ling, *et al.*, 1986; Ying, 1988). FSP suppresses pituitary FSH secretion (O, Robertson and de Kretser, 1989; Robertson, *et al.*, 1987; Ueno, *et al.*, 1987; Ying, *et al.*, 1987).

Findlay, *et al.* (1990), propose that activin can also act as a local regulator within the ovarian follicle. It promotes follicular differentiation and prevents the onset of premature luteinisation, (Hutchinson, Findlay, de Vos and Robertson, 1987), whereas FSP promotes atresia of non-dominant follicles and luteinisation of dominant follicles. Alpha N peptide of inhibin (αN) acts locally in the follicle to facilitate ovulation (Findlay, Robertson, Clarke, Klein, Doughton, Xiao, Russel and Shukovski, 1992). Since most of these studies have been conducted *in vitro*, more research is required *in vivo* to properly define the role of these local regulators.

## 2.4- Intrafollicular growth factors in the ovary

The classical concept of endocrine control of ovarian function has now been extended to a more complex regulatory system which includes autocrine and paracrine regulatory agents (i.e. agents that act in the same cells that produce them or neighbouring cells respectively), many of which act on thecal and granulosa cells in the ovarian follicle (Holly and Wass, 1989; Mondschein, *et al.*, 1989a).

Cellular interactions between thecal cells and granulosa cells have an integral role in the control of oocyte development as well as that of the endocrine status of the animal (Skinner, Keski-Oja, Osteen and Moses, 1987a). There is intercellular communication between theca and granulosa cells (Kotsuji, Kamitani, Goto and Tominaga, 1990). The proliferation of granulosa cells is responsible for the majority of follicle expansion during the maturation process (McNatty, *et al.*, 1984a). The

control of this system is exerted by the production of local agents, growth factors, that stimulate and/or inhibit the actions of gonadotrophins in the responsive cells (Dorrington, Bendell, Chuma and Lobb, 1987).

### 2.4.1- Insulin growth factor-I (IGF-I)

Insulin-like growth factor I is one of several intra-ovarian regulators of ovarian follicular growth and differentiation (Adashi, *et al.*, 1985).

The insulin-like growth factors (IGF-I and IGF-II) are small polypeptides with a molecular weight of approximately 7,500 Da (Clemmons and Underwood, 1991). The IGF-I molecule contains 70 amino acid residues and IGF-II has 67 amino acid residues (Baxter, 1988).

Concentrations of IGF-I in follicular fluid increase with follicular size, indicating local control of IGF-I concentrations, and also suggesting local biosynthesis, alteration in diffusion barriers between blood and follicular fluid, and changes in IGF-I binding proteins (Echternkamp, *et al.*, 1990; Spicer, Echternkamp, Canning and Hammond, 1988). In small follicles IGF-I concentrations correlate with those in circulation, unlike in the dominant follicle, in which IGF-I concentrations are higher than in the circulation and increase further after the LH surge (Eden, Jones, Carter and Alaghband-Zadeh, 1988; Holly and Wass, 1989).

Production by granulosa cells is stimulated by FSH, LH and oestradiol (Hsu and Hammond, 1987; Ui, Shimonaka, Shimasaki and Ling, 1989), inhibin (Carson, Zhang, Hutchinson, Hetherington and Findlay, 1989), and epidermal growth factor/transforming growth factor $\alpha$ (Mondschein and Hammond, 1988). Furthermore Carson, *et al.* (1989) and Dorrington, *et al.* (1987) showed that the granulosa cells produce IGF-I under the influence of GH and the IGF-I in turn maximizes FSH dependent differentiation by augmenting the stimulatory effects of FSH on aromatase activity.

The principal role of the IGFs is to amplify the actions of other hormones and in the presence of gonadotrophins or in the milieu of the pre-ovulatory follicle the cytodifferentiative effects of the IGFs are emphasized (Hammond, Modschein and Canning, 1989; Mondschein, *et al.*, 1989b). This accords with the hypothesis of Dorrington, *et al.* (1987) that the dominant follicle acquires autonomy by generating mechanisms to amplify the LH and FSH signals. At nanomolar concentrations, IGF-I enhances granulosa cells differentiation and proliferation (Adashi, 1992), stimulates biosynthesis of progesterone and oxytocin (Holtorf, Furuya, Ivell and McArdle, 1989; McArdle and Holtorf, 1989; Schams, 1987) and induces LH receptors (Adashi, *et al.*, 1985; Adashi, Resnick, Hernandez, May, Knecht, Svoboda and Judson, 1988).

The mechanism of actions of IGF-I is thought to be mediated at least, in part, by specific high affinity IGF-I receptors in granulosa cells (Carson, *et al.*, 1989; Davoren, Kasson, Li and Hseuh, 1986), such as the type I receptors present in the granulosa cells of the ewe ovary (Monget, Monniaux and Durand, 1989).

Attempts to understand the mechanism of IGF-action in ovarian folliculogenesis are further complicated by the presence in the ovary of IGF-binding proteins (IGF-BP) (Monget, *et al.*, 1989). Until recently the IGF-BP was looked upon only as a carrier for the IGFs in plasma which prolonged the half life of the growth factors, maintained the high IGF levels in the circulation and delivered them to the target tissue (Baxter, 1988). This implied that the binding proteins determined the circulating levels of free IGFs and probably their activity (Holly and Wass, 1989). It is now clear that the BPs have a physiological role; they do not simply act as carrier protein. The most common binding proteins were named IGFBP-I (25-kDa protein), IGFBP-2 (31-kDa protein) and IGFBP-3 (53-kDa glycoprotein) (Clemmons and Underwood, 1991; McGuire, *et al.*, 1992).

IGF-BPs have been found in ovine and bovine follicular fluid (Hammond, Mondschein, Samaras, Smith and Hagen, 1991; Monget, *et al.*, 1989). It has been shown by Mondschein and Hammond (1990) and Mondschein, Hammond and

Etheston (1989b) that granulosa cells from large follicles secrete 10-20 times more IGF-BP than cells from small follicles. They also showed that production of IGF-BP-2 and/or IGF-BP-3 is stimulated by GH and EGF and inhibited by FSH and transforming growth factor β (TGF-β).

### 2.4.2- Epidermal growth factor (EGF)

Epidermal growth factor (EGF) is a single polypeptide chain with 53 amino acids and a molecular weight approximately 6100 (Taylor, Mitchell and Cohen, 1972), which binds to specific high-affinity receptor sites in the surface membrane of granulosa and luteal cells (Chablot, St. Arnaud, Walker and Pelletier, 1986). Furthermore Skinner, Lobb and Donnington (1987b) showed that thecal/interstitial cells are capable of producing an EGF-like substance that would act as a paracrine factor in granulosa cell growth and differentiation.

EGF has a mitogenic action in a variety of cells *in vivo* and *in vitro* (Carpenter and Cohen, 1979; Gospodarowicz, Ill and Birdwell, 1977a). Recently, studies have demonstrated that EGF exerts inhibitory and stimulatory actions on granulosa and thecal cells. EGF has been shown to have potent mitogenic actions on granulosa cells (Hsu, Holmes and Hammond, 1987). The studies of Franchimont, *et al.* (1986) demonstrate the ability of EGF to increase DNA synthesis in the bovine granulosa cells.

EGF affects differentiation of granulosa cell and follicular development through inhibition of the induction of receptors for luteinizing hormone. It suppresses the induction, by FSH, of LH/hCG receptors in cultured rat granulosa cells (Mondschein and Schomberg, 1981) by inhibition of FSH-induced adenylate cyclase (Dodson and Schomberg, 1987).

This EGF-like substance may also act as an autocrine factor within the ovary to regulate steroidogenesis in thecal or interstitial cells (Chablot, *et al.*, 1986; Skinner, *et al.*, 1987b). The initial formation of an antral follicle is accompanied by the activation

of undifferentiated theca-interstitial cells to that of fully differentiated endocrine cells which secrete androgens and androgen secretion is specifically regulated by LH (Erickson and Case, 1983). The same authors also indicated that physiological amounts of EGF will antagonize the LH/hCG-dependent cytodifferentiation of theca cells into active androgen-producing cells, suggesting that the EGF inhibition occurs at an early step in the pathway of the LH-induced cytodifferentiation.

### 2.4.3-Transforming growth factor-β (TGF-β)

Transforming growth factor-β (TGF-β) is a homodimeric polypeptide with subunits containing 112 amino acid residues with a 25000 mol wt (Mr) (Magoffin, Gancedo and Erickson, 1989; Sporn, Robert, Wakefield and Assoian, 1986), ie. it belongs to a family of related peptides which includes inhibin, activin and Mullerian inhibitory substance (Magoffin, *et al.*, 1989; Mason, Hayflick, Ling, Esch, Ueno, Ying, Guillemin, Niall and Seeberg, 1985).

TGF-β is secreted by the thecal-interstitial cells (TIC) of bovine ovaries (Skinner, *et al.*, 1987a), but Adashi, Resnick, Hernandez, May, Purchio and Twardzik (1989) suggest that non-steroidogenic components of the ovary may also be the source of TGF-β mRNA.

TGF-β is a peptide that both stimulates and inhibits cell proliferation as well as enhances cellular differentiation (Carson, *et al.*, 1989; Dodson and Schomberg, 1987; Feng, Catt and Knecht, 1986; Skinner and Coffey, 1988; Skinner, *et al.*, 1987a; Sporn, *et al.*, 1986). TGF-β does not have any effect by itself but exerts a positive effect on follicular development by enhancing follicular responses to the trophic action of FSH (Carson, *et al.*, 1989; Ying, *et al.*, 1987).

TGF-β of intraovarian origin may exert its effects at sites adjacent to its site of synthesis, interacting with specific cell surface receptors to either enhance or inhibit gonadotrophin hormonal action (Feng, *et al.*, 1986). Granulosa cells rather than the

theca-interstitial cells appear to be the site of TGF-β receptors and of hormone action (Adashi, *et al.*, 1989).

In addition to its paracrine role in the ovary, TGF-β produced by thecal cells may act as an autocrine factor to control thecal cell growth and differentiation (Tonetta and diZerega, 1989) and can also regulate EGF receptor formation on granulosa cells, inhibiting EGF-induced mitosis (Feng, *et al.*, 1986).

Magoffin *et al.*, (1989) summarized the action of TGF-β as follows:

1) it modulates the differentiation of a pure population of TIC in serum-free cell culture in a time and dose-dependent manner,

2) the effects of TGF-β on the pattern of TIC steroidogenesis are divergent; the ability of LH to stimulate androgen biosynthesis in the presence of TGF-β is strongly inhibited, while progesterone production is markedly enhanced, (Magoffin and Erickson (1988) showed that LH induction of TIC differentiation is mediated through activation of cAMP, and they have shown that in purified TIC, LH binds to a specific cell surface receptor and stimulates cAMP production) and

3) IGF-I sensitizes the TIC to the inhibitory effects of TGF-β on androgen biosynthesis, indicating that TGF-β action can be modulated by other hormones.

### 2.4.4- Transforming growth factor-α (TGF-α)

Transforming growth factor-α (TGF-α) is a structural analog of EGF. It is a single chain 50-amino acid polypeptide (Massagué and Like, 1985) capable of binding to an apparently common EGF/TGF-α receptor (Marquardt, Hunkapiller, Hood and Todaro, 1984).

Theca cells synthesize and secrete TGF-α as the primary EGF-like activity in the follicle but granulosa cells do not contain TGF-α mRNA or EGF-like material (Skinner and Coffey, 1988).

TGF-α promotes granulosa cell proliferation and theca cell proliferation (Skinner and Coffey, 1988). Skinner, *et al.* (1987a) demonstrated that ovarian theca

cells produce TGF-β and this growth factor can inhibit the ability of EGF to promote the growth of granulosa cells. Thus TGF-β and TGF-α are both produced by theca cells and they can act as growth promoters and inhibitors in the ovarian follicle.

Skinner and Coffey (1988) speculated that the production of TGF-α by theca cells is active predominantly during the growth phase of the follicle, participating in the regulation of cell proliferation, and TGF-β, which can inhibit the ability of EGF to promote granulosa cell growth, would be the predominant influence when growth is inhibited (Skinner, *et al.*, 1987b). These inverse actions of TGF-α and TGF-β may provide an efficient mechanism to control the growth of a tissue that requires both rapid growth stimulation and inhibition as is required by the follicles.

### 2.4.5- Fibroblast growth factor (FGF)

This growth factor was originally identified in the bovine pituitary gland as a 16000 molecular weight protein and it was found to stimulate not only the proliferation of fibroblasts but also a variety of mesoderm-derived cells (Böhlen, Baird, Esch, Ling and Gospodarowicz, 1984; Mormede, Baird and Pigeon, 1985). Recent studies have indicated that it is also produced by ovaries (Neufeld, Ferrara, Schweigerer, Mitchell and Gospodarowicz, 1987)

bFGF stimulates the proliferation of capillary endothelium in bovine granulosa cells (Gospodarowicz, Ill and Birdwell, 1977b; Neufeld, *et al.*, 1987). Treatment of cultured granulosa cells with bFGF inhibits FSH-stimulated LH receptor induction and FSH-induced aromatase activity (Mondschein and Schomberg, 1981).bFGF has also stimulated the proliferation of cultured thecal cells *in vitro* (Gospodarowicz, *et al.*, 1977b).

bFGF may be important in the control of ovulation. It is one of the growth factors known to stimulate protease and collagenase activity in responsive cells (Gospodarowicz and Ferrara, 1989). It is postulated that the effect of FGF on protease/collagenase may be mediated through plasminogen activator (PA) which is

responsible for the disruption of the follicle (Kokolis, Alexaki-Tzivanidou and Smokovitis, 1987). This proposal is based on the fact that ovarian granulosa cells produce PA in a manner which is closely correlated with ovulation (Gospodarowicz and Ferrara, 1989).

Basic FGF is also found in bovine corpum luteum (CL) and has been shown to have mitogenic and angiogenic activity (Gospodarowicz, Cheng, Lui, Baird, Esch and Bohlen, 1985).

### 2.4.6- Platelet-derived growth factor (PDGF)

Platelet-derived growth factor (PDGF) was originally derived from human platelets and later it was found to be synthesized and secreted by many other cells (Ross, 1989).

There is evidence that PDGF is a potent mitogen for cells of mesenchymal origin (Deuel and Huang, 1983). In order to establish possible effects of PDGF at ovarian level experiments have been undertaken using endogenous ornithine decarboxylase activity as a measure of proliferative activity and showing that the decarboxylase activity in pig granulosa cells could only be stimulated in the presence of a relatively crude platelet extract (Baranao and Hammond, 1984). The addition of purified PDGF to these cells had no effect which led to the conclusion that either PDGF does not stimulate granulosa cell ornithine decarboxylase activity or that PDGF action requires the presence of other unidentified components of platelets extracts (Carson, *et al.*, 1989).

Purified PDGF has been shown to enhance FSH-induced progesterone secretion by granulosa cells, adenylate cyclase activity and LH receptor induction (Knecht and Catt, 1983; Mondschein and Schomberg, 1984). This may have implications for LH receptor induction during luteal cell development if PDGF or similar factors are released during follicle rupture or formation of the corpus haemorrhagicum (Mondschein and Schomberg, 1981).

In summary, there is now evidence that a variety of growth factors including IGFs, EGF/TGFα, TGFβ, FGF and PDGF may play an important part in the regulation of ovarian folliculogenesis, exerting stimulatory, differentiatory and inhibitory effects on different types of ovarian cells (see Fig. 1).

It should be emphasized that most of the data describing autocrine and paracrine actions of growth factors and/or ovarian proteins have been obtained in swine or rats, two species in which the ovarian function is clearly different from that of sheep and cattle and so extrapolation to the latter requires caution.

**Fig 1 Summary of the effects of insulin-like growth factor (IGF-I), transforming growth factor-type ß (TGF-ß), platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) on granulosa cell function *in vitro*. Stimulation: ↑; inhibition: ↓; uncertain: ?. (From Carson *et al*, 1989)**

| Growth factor | Mitosis | Differentiation |
|---|---|---|
| IGF-I | ↓ | ↑ |
| TGF-ß | ↓ | ↑ |
| PDGF | ? | ↑ |
| EGF | ↑ | ↓ |

## 2.5- Others factors which may control the resumption of postpartum cyclicity

### 2.5.1- Prostaglandins

After parturition, a massive prostaglandin F2α (PGF2α) release occurs (Fredriksson, Kindahl, Alenius, Carlsson, Cort, Edqvist and Uggla, 1988; Meyer, Enzenhofer and Feck, 1989; Peters and Lamming, 1986). This release of PGF2α is negatively correlated with uterine involution in normal cows and positively correlated in cows with uterine infections (Fredriksson, *et al.*, 1988).

Since ovulation seldom take place before prostaglandin levels return to basal concentrations (Fredriksson, *et al.*, 1988; Peters and Lamming, 1986), it has been suggested that PGF2α release may have an inhibitory effect on ovarian activity (Kastelic, Knopf and Ginther, 1989; Kindahl, *et al.*, 1992). On the other hand, more recently studies have shown that the resumption of ovarian activity is positively correlated with uterine involution. This finding suggests that treatment of cows with PGF2α in the postpartum period can hasten uterine involution and resumption of ovarian activity (Kindahl, *et al.*, 1992).

Contradictory results have been obtained by several authors investigating the effects of inhibitors of prostaglandin synthesis on the onset of ovarian cyclicity. It is concluded that more studies are required before firm conclusions can be drawn (Kindahl, *et al.*, 1992).

### 2.5.2- Cortisol

Suckling induces the release of cortisol (Dunlap, *et al.*, 1981) and it has been shown that, at least in bulls, glucocorticoids suppress LH release (Thibier and Rolland, 1976). Wagner and Li (1982) have also shown that glucocorticoids can inhibit LH secretion in cattle and block GnRH-induced LH release from bovine pituitary cells in primary culture. It is possible that higher concentrations of cortisol associated with suckling could be an important component of the prolonged

postpartum period in beef cows which could act by suppressing secretion of LH (Smith, Convey and Edgerton, 1972). In support of this possibility, concentrations of glucocorticoids have been found to be greater in suckled than in non-lactating beef cows (Schallenberger, Oerterer and Huttser, 1982), and that administration of ACTH, which was followed by cortisol secretion, decreased the number of beef cows which had episodic releases of LH (Dunlap, *et al.*, 1981).

On the other hand, other reports suggest that glucocorticoids are not released by suckling in intact cows but are released in ovariectomized cows, suggesting that suckling-induced glucocorticoid release depends upon the presence of the ovary (Convey, *et al.*, 1983). In view of the contradictory results, further studies are required in order to establish the possible effects of Cortisol on the postpartum period.

### 2.5.3- Prolactin

The role of prolactin in bovine reproduction is still unresolved. Serum concentrations usually increase with suckling (Convey, *et al.*, 1983), and suckling inhibits reproductive activity. However, infusion of prolactin does not alter LH concentrations in ovariectomized heifers (Forrest, *et al.*, 1980), and treatment of postpartum cows with dopamine agonists, while lowering prolactin secretion, do not enhance ovarian activity, reduce the length of the postpartum interval (Willians and Ray, 1980), or enhance postpartum gonadotrophin secretion (Bevers, Dieleman and Kruip, 1988; Schallenberger, *et al.*, 1978). Bevers, *et al.* (1988) have also shown that prolactin is not involved in the growth and development of antral follicles in the cow.

All these results support the conclusions that hyperprolactinaemia is not the cause of the longer postpartum interval (Lamming, *et al.*, 1981) in suckling cows, and that prolactin does not have central effects on gonadotrophin secretion (Curlewis, 1992). However, studies done in sheep (Rhind, Robinson, Chesworth and Phillipo, 1980) and women (Seppala, Hirvonen and Ranta, 1976) indicate that high prolactin levels are often associated with reductions in progesterone production and luteal

function. Further studies are required in order to establish the role of prolactin in ovarian function.

## 2.6- Conclusions

From the review of the literature it is clear that ovarian function in the postpartum period is controlled by complex multiple factors including metabolic hormones and gonadotrophins. There is a need to further elucidate the effects of these factors on ovarian follicular growth and function. In particular, there is a need to determine their roles in mediating the effects of nutrition on follicular size, structure, steroidogenic capacity, receptor concentrations and growth factors.

# CHAPTER 3

## Investigation of the effects of infusion of GnRH pulses and of body condition on the ovarian function of postpartum beef cows.

### 3- Introduction

Some studies (Wright *et al.*, 1987, 1990, 1992a) have shown that cows in low BC have a lower LH pulse frequency than cows in high BC and there is also evidence that the development of highly oestrogenic follicles during the postpartum period is delayed in cows in low BC (Prado *et al.*, 1990). However, the role of LH pulses frequency is not altogether clear since some reports have shown no effect of BC on LH pulse frequency (Rhind *et al.*, 1992) and the significance of differences in LH pulse frequency with respect to ovarian follicle development (size and structure) and function (steroidogenic capacity, hormone receptors and IGF-I production) is unclear.

The aim of this study was to test the hypothesis that infusion of GnRH pulses into postpartum cows in low BC would enhance the rate of follicle growth and increase the granulosa cell population and the capacity of the theca and granulosa tissues to synthesize steroids, compared with animals infused with saline. It was further postulated that the putative changes in follicle function are mediated through changes in steroid receptor concentrations in these tissues.

In addition to effects on gonadotrophin secretion, differences in BC are also associated with differences in energy metabolism and so in circulating GH, insulin and IGF-I. It was postulated that the follicle response to GnRH pulse infusion would be enhanced by exogenous GnRH pulses but that the degree of follicle development would be less than in a high condition cow because the circulating levels of metabolic

hormones were not modified at the same time; thus the hormonal influences on the ovaries were not the same as in high condition cows; i.e. it was postulated that changes in both gonadotrophin and metabolic hormones were required for maximal follicle development.

The infusion was conducted for a minimum of 14 days because the normal duration of the period of follicle growth to maturity (pre-ovulatory status) is about 7 to 10 days (Knopf *et al.*, 1989); thus the treatment was postulated to be sufficient to enhance the development of follicle steroidogenic capacity during at least one wave of growth and probably during a second wave.

## 3.1- Materials and methods

### 3.1.1- Animals and management

Thirty-six multiparous, Blue-Grey (White Shorthorn × Galloway) cows mated at a synchronized oestrus and due to calve in late March were used. At 110 days before the expected date of calving, they had a mean live weight (±sd) of 506 ± 61 kg and a mean body condition score (±sd) of 2.8 ± 0.3. (Body condition score was assessed using the six point scale (0 = emaciated, 5 = obese) of (Lowman, Scott and Sommerville, 1976)).

At that time the cows were allocated randomly within each liveweight and condition score class to three treatments groups except that no cow was required to lose more than 0.5 or gain more than 0.75 condition score units. Two groups of cows were fed to achieve a low target body condition at calving (CS = 2.0; L; n=12 per group). The third group was fed to achieve a high target score (CS =3.0; H; n=12). All cows were fed individually. H cows were fed a mean (±sd) of 22 ± 4.2 kg of silage and L cows were fed 12.8 ± 2.1 kg.

After calving the cows were fed, individually, a variable quantity of concentrate and silage. Fresh water was provided *ad libitum*. These rations supplied an estimated 0.23 MJ of metabolizable energy (ME) per kg live weight per day and were designed

to maintain live weight and body condition (Wright, *et al.*, 1990). The amount of concentrate given to the cows was adjusted weekly according to live weight.

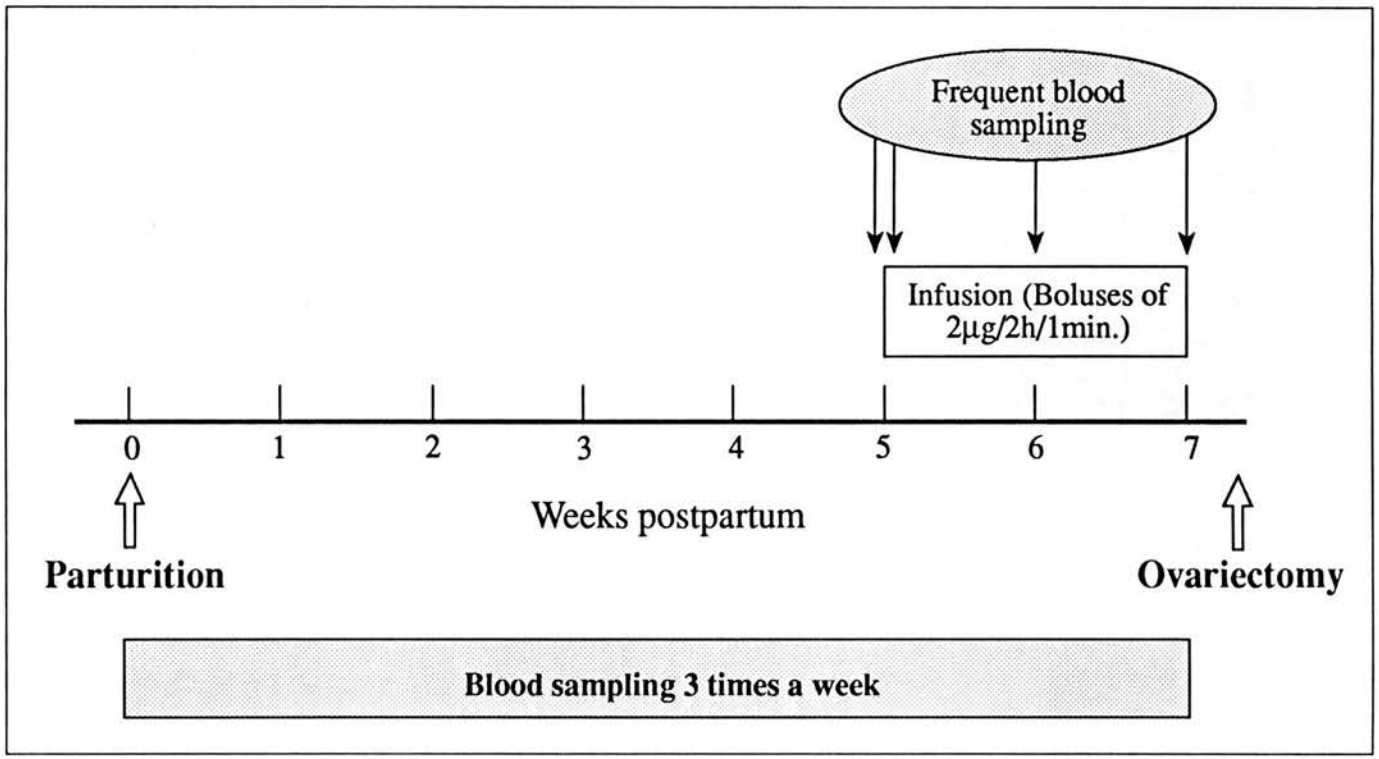
The cows were housed in individual cubicles with their calves in adjacent cubicles, having access to their dam for suckling twice a day (08.00 and 16.00 h.) for 30 min. throughout the experiment.

### 3.1.2- Experimental treatments and procedures

L cows were weighed and body condition recorded within 24 h. of calving and divided into pairs of animals with similar body condition and allocated randomly within pairs to 2 treatment groups, which were to be infused with either saline (LS) or GnRH (LG). The H cows were infused only with saline (HS). The experimental protocol is shown in Fig. 2

Briefly, boluses of 2μg gonadotrophin-releasing hormone (GnRH) in 2ml. of saline were infused into each LG cow using a peristaltic pump (ISMATEC,SA; laboratoriumstechnik, Glatbrugg-Zurich, Germany), connected via transmission tube and spring coil (Stat Large animal IV SET (IV-1000), T.M.S. Consultants, Yeovil, Somerset, UK), to an indwelling jugular catheter. The pump was activated for 1 min. every 2 h. for 14 to 18 days, beginning at approximately 5 weeks postpartum. LS and HS animals were infused with saline only. Blood samples were collected by jugular venepuncture three times a week on Mondays, Wednesday and Fridays. Blood samples were also collected from all cows via an indwelling jugular vein catheter every 20 min. for 10 hours (09.00 to 19.00h), at weeks 5 (on each of two consecutive days; pre-infusion), 6 (day 7 of infusion), and 7 postpartum (day 14 of infusion).

Fig 2 Experimental protocol 1



Jugular cannulation was performed the day before the frequent sample collection.

All blood samples were collected into tubes containing heparin (10 IU/ml blood) and centrifuged within 30 minutes at 1000g for 30 minutes. Plasma was harvested and stored at -20°C until required for hormone assay.

At week seven postpartum ovariectomies were performed in all cows by ecraseur (a surgical device designed to provide haemostasis and section of the ovarian stalk) via high lumbar laparotomy under paravertebral regional anaesthesia (Locovetic, Bimeda, Dublin, Eire).

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

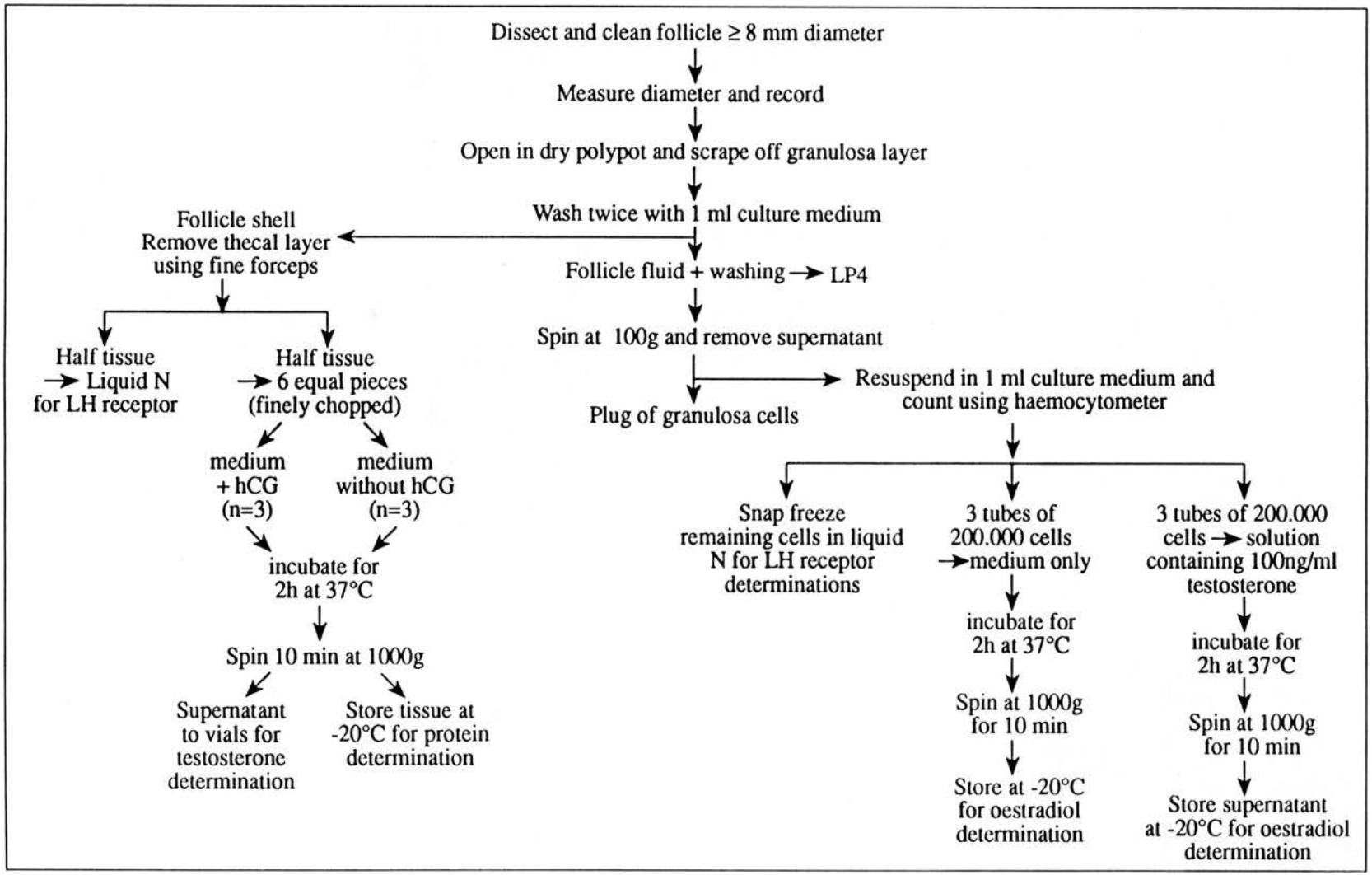
### 3.1.3- Ovarian follicle dissection and associated measurements

The protocol used in follicle dissection and further processing is summarised in the Fig. 3.

After ovariectomy, all follicles measuring ≥ 3 mm. and < 8 mm. in diameter were counted. Follicles ≥ 8 mm. diameter were dissected from the ovarian stroma in a dry petri dish. Follicle diameters were determined to the nearest 0.5 mm., using a stereomicroscope fitted with an ocular graticule; two perpendicular diameters were measured. Follicles ≥ 8 mm. were cut, opened using scissors and granulosa cells were scraped off the adjoining thecal layer, using rounded plastic spatula.

Follicular fluid and granulosa cells were removed to polystyrene tubes (LP4 , Luckham Ltd, Burgess Hill, Sussex, UK). The follicle was then washed twice with 1ml. Medium 199 with HEPES (Flow Laboratories, Rickmansworth, UK) and the remaining granulosa cells and medium were transferred to the LP4 tube.

Fig 3 Protocol for follicle dissection



Granulosa cells were separated by centrifugation for 10 min. at 100g and the supernatant was transferred to cups and stored at -20 °C for subsequent determination of steroid concentrations.

Follicular volume was estimated on the basis of overall mean follicle diameter, assuming follicles to be spherical. Intrafollicular concentrations of steroid hormones were subsequently calculated using the hormone concentrations in the mixture of follicular fluid and culture medium corrected according to the degree of dilution with culture medium.

#### 3.1.3.1- Granulosa cells

Granulosa cells were resuspended in 1 ml culture Medium 199 (containing 0.1% bovine serum albumine (BSA); Sigma Chemical Co Ltd., Poole, Dorset, UK). The number of cells were determined using a haemotocytometer. Up to 6 aliquots, each calculated to contain 200,000 cells, were made up to 1 ml culture medium 199 (n = 3) or medium containing testosterone at a final concentration of 100 ng/ml (n = 3). Remaining culture medium and cells were stored in liquid N for subsequent determination of LH and FSH receptors numbers. Cells were cultured for 2 h. at 37 °C in a water bath, shaking every 15 min. and then spun at 1000g for 10 min. The supernatant was then removed and stored at - 20 °C for oestradiol determination.

#### 3.1.3.2- Thecal tissue

The thecal tissue layer was peeled from the follicle wall using fine forceps. Half of the tissue was snap-frozen in liquid nitrogen for determination of LH and FSH receptors concentrations and the remaining tissue was finely chopped using a scalpel blade and divided into 6 parts (2 or 4 parts if insufficient tissue available) and incubated in Medium 199 with (n=3) or without (n=3) 50 i.u. human chorionic gonadotrophin (hCG (Chorulon, Intervet, UK)). Tubes were incubated in a water bath at 37°C for 2h. shaking every 15 min. All tubes were then spun at 1000g for 10 min.

and the supernatant removed and stored at -20 ºC for testosterone determination. The remaining tissue was stored at -20 ºC for protein determination, to provide an index of the amount of tissue present.

### 3.1.4- Hormone assay procedures and hormone determinations

All plasma samples collected during the 10h. windows were analyzed for LH, and every third sample was analyzed for FSH. GH determinations were performed only on samples collected during the 10 hours window at week 6.

Plasma samples collected 3 times/week were analyzed for progesterone, insulin- like growth factor-I (IGF-I), and insulin.

Follicular fluid from follicles ≥ 8mm diameter were analyzed for testosterone , oestradiol and IGF-I in duplicate.

Granulosa cell and thecal tissue incubates were analyzed in duplicate for oestradiol and testosterone respectively.

Granulosa cell and thecal tissue were analyzed for LH and FSH receptor concentrations.

All assays were analysed on a PC computer using the RIASMART software (Hewlett-Packard, Canberra, UK).

**Hormone determinations**

#### 3.1.4.1- Luteinising hormone

Plasma samples were assayed for LH using the radioimmunoassay techniques described by McNeilly, Jonassen and Fraser (1986). The preparation used for iodination and standards was USDA-bLH-1. The antiserum used was R29 supplied by Prof. A. McNeilly (MRC, Edinburgh). Bound hormone was separated using a precipitation technique with normal rabbit serum (NRS) and donkey anti-rabbit serum (DARS) (Scottish Antibody Production Unit (SAPU), Law Hospital, Carluke,

Lanarkshire, Scotland). The diluent buffer was a 0.075 M sodium phosphate buffer, containing 0.1% BSA.

The sensitivity of the assay was 0.2 ng/ml. Intra-and inter-assay coefficients of variation were 6.7% and 5.8% respectively.

### 3.1.4.2- Follicle-stimulating hormone

FSH determinations were performed using the radioimmunoassay techniques described by McNeilly, McNeilly, Walton and Cunningham (1976). The preparation used for iodination was NIADDIK-oFSH-I-1 and the hormone preparation used as a standard was USDA-b-FSH-B-I. The antiserum used was M94 Rabbit anti-hFSH (supplied by Prof. A. McNeilly, MRC, Edinburgh). Bound hormone was separated using a precipitation technique with NRS and DARS (SAPU). The diluent buffer was a 0.075M sodium phosphate buffer, pH 7.5, containing 0.1% BSA.

The sensitivity of the assay was 2.5 ng/ml and intra- and inter-assay coefficients of variation were 6.9% and 6.2% respectively.

### 3.1.4.3- Growth hormone

Concentrations of growth hormone (GH) were determined by a double antibody precipitation technique. GH was iodinated using $Na^{125}I$ by the iodogen method of Salacinski, McLean, Sykes, Clement-Jones and Lowry (1981). The antiserum to bovine GH was raised against recombinant bGH. Incubation of standards of bGH (AFP11182B, from Dr. Parlow, Harbour-UCLA-Medical Center, California) or sample with first antibody was performed at room temperature before addition of 20,000 cpm of $^{125}I$ bGH. Incubation was continued for a further 24 h. before bound and free hormone were separated using a PEG-assisted second antibody mixture containing appropriate amounts of normal rabbit serum and donkey anti rabbit serum (from SAPU). The diluent buffer was a phosphate buffer, pH 7.4, with 0.5% BSA.

The assay sensitivity was 0.625ng/ml. Intra- and inter-assay coefficients of variation were 11.8% and 12.3% respectively.

#### 3.1.4.4- Insulin-like growth factor I

Concentrations of IGF-I were determined by a radioimmunoassay using the technique of Bruce, Atkinson, Hutchinson, Shakespear and MacRae (1991). Serum samples were extracted using an acid-ethanol mixture. Iodination was performed using the N-Bromosuccinamide technique described by Reay (1982), with recombinant insulin-like growth factor I (N° DGR010, Bachem Ltd., Saffron Walden, Essex, UK). IGF-I (Peninsula Laboratories Ltd., St. Helens, Merseyside, UK) was used for standard preparation. The antiserum used was Anti-IGF/Somatomedin c Rabbit antiserum (UB3-189) supplied by Drs. L. Underwood and Van Wyck, University of North Carolina, Chappel Hill, USA. The bound hormone was separated using 1% gamma globulin solution (Sigma Chemical Co Ltd, Poole, Dorset, UK) and 16.25% Polyethylene glycol 8000 (Sigma Chemical Co Ltd., Poole, Dorset, UK).

The sensitivity of the assay was 0.24 ng/ml. Intra- and inter-assays CVs were 7.4% and 9.8% respectively.

#### 3.1.4.5- Insulin

Insulin concentrations were determined using a radioimmunoassay technique of MacRae, Bruce, Hovell, Hart, Inskter and Atkinson (1991). Antiserum to porcine insulin (ICN Flow, High Wycombe, Bucks, UK) and [$^{125}$I] labelled bovine insulin (Amersham International, Aylesbury, Bucks, UK) were used with porcine insulin (Sigma Chemical Co. Ltd., Pole, Dorset, UK) as standard. The bound and free fractions were separated by the addition of normal-guinea-pig serum and sheep-guinea-pig-serum (SAPU). The assay diluent was 0.05M phosphate buffer, pH 7.4 with 0.5% BSA.

The sensitivity of the assay was 2.6 ng/ml. The inter- and intra assay coefficients of variation were 7.6% and 5.7% respectively.

### 3.1.4.6- Progesterone

Progesterone concentrations were determined using the techniques described by Djahanbakhch, Swanston, Corrie and McNeilly (1981) with modifications described by McNeilly and Fraser (1987). Plasma progesterone concentrations were measured using progesterone antibody R31/12 (supplied by Prof. A. McNeilly, MRC, Edinburgh) and [$^{125}$I] progesterone glucuronide-tyramine (supplied by Prof. A. McNeilly, MRC, Edinburgh). Progesterone (4-Pregnene-3,20-dione) (N° Po103, Sigma Chemical Co Ltd., Poole, Dorset, UK) was used as a standard. The assay buffer was a 0.1M phosphate-citrate buffer, pH 6.0 with 0.1% Gelatin. The bound and free fractions were separated using a solution of 4% Polyethylene glicol and 0.9% saline.

The sensitivity of the assay was 0.08 ng/ml and intra- and inter assay coefficients of variation were 10.5% and 12.5% respectively.

### 3.1.4.7- Oestradiol

Follicle fluid samples and granulosa cell incubates were assayed for oestradiol, using the techniques described by Webb, Baxter, McBride, Nordblum and Shaw (1985). Oestradiol concentrations were measured using an antiserum Rabbit anti-oestradiol #35 (Prof. A.S. McNeilly, MRC, Edinburgh) and [$^{125}$I] -labelled oestradiol, using the Chloramine T method for iodination (Hunter and Greenwood, 1962), and b Estradiol (N° E8875, Sigma Chemical Co Ltd., Poole, Dorset, UK) as a standard. The bound hormone was separated using a double antibody precipitation technique with NRS and DARS (SAPU). The diluent buffer used was 0.1M Phosphate gelatin buffer, pH 7.4.

The assay sensitivity was 0.05 ng/ml. Intra- and inter-assay coefficients of variation were 10.2% and 11.1% respectively.

#### 3.1.4.8- Testosterone

Follicle fluid samples and thecal tissue incubates were assayed for testosterone, using the techniques described by Webb, *et al.* (1985). Testosterone concentrations were measured using an antiserum (Sheep anti-testosterone #505; MRC, Edinburgh) and a tracer (Testosterone-3CMO-Histamine, MRC, Edinburgh). 4-Androsten-17B-ol-3-one (N° T-1500, Sigma Chemical Co Ltd., Poole, Dorset, Uk) was used as the standard. The bound hormone was separated using a double-antibody precipitation technique with Normal Sheep Serum (NSS), and Donkey Anti-Sheep Serum (DASS) from SAPU. The diluent buffer was a Phosphate gelatin buffered saline 0.1M with a pH of 7.4.

The assay sensitivity was 0.025 ng/ml. Intra- and inter-assay coefficients of variation were 6.1% and 7.4% respectively.

### 3.1.5- Protein

Protein contents of the incubated thecal tissue samples were determined using the colorimetric technique described by Lowry, Rosebrough, Farr and Randall (1951) with bovine serum albumin as the standard.

### 3.1.6- Receptors

The binding of [$^{125}$I]- labelled hCG and hFSH to human luteal tissue was measured according the techniques of Bramley, Stirling, Swanston, Menzies and Baird (1987). Briefly the binding of [$^{125}$I]- labelled hCG was measured by incubation of triplicate aliquots of homogenate (0.2-3 µg DNA/tube) in a 1 ml system containing Tris-acetate buffer (40mmol/l; pH 6.5), 0.5% (w/v) BSA and 100,000 c.p.m. [$^{125}$I]-labelled hCG at 20 °C for 16-20h. Non-specific binding was assessed in duplicate by

the inclusion of 50 i.u. hCG (Chorulon) per tube. The binding of [$^{125}$I]- labelled hFSH was measured by incubation of homogenate (0.5-6.5 µg DNA/tube) in a 1 ml system containing Tris-HCl (40 mmol/l; pH 7.4), 0.5% BSA. $MgCl_2$ (25 mmol/l) and 100,000 c.p.m. After incubation, tubes were chilled on ice and bound and free hormone were separated by polyethylene glycol (PEG) precipitation. The results were expressed as picograms of [$^{125}$I] labelled hormone bound specifically per milligram of protein.

### 3.1.7- Statistical analysis

LH pulses were defined according to Baird, Swanston and McNeilly (1981), i.e. a pulse occurred when two consecutive values were higher than the two preceding values, and when the value of the highest (the pulse amplitude) exceeded the mean basal value by at least four times the coefficient of the variation of the assay.

The main effects of body condition and GnRH infusion on metabolic hormones, gonadotrophin profiles, and number of small and large follicles present at ovariectomy were compared by analysis of variance using Genstat 5, version 3.1 (Lawes Agricultural Trust, 1992).

The follicles were arbitrarily divided into 2 classes according to whether they had less than 10,000 pg/ml oestradiol ($E_2$ Low) or more than 10,000 pg/ml oestradiol ($E_2$ High) in the follicular fluid. All statistical comparisons of follicle fluid hormone concentrations, steroid production by granulosa and thecal tissue, receptor concentrations and granulosa cell numbers were performed using restricted maximum likelihood (REML) analysis using the directives of Genstat 5, version 3.1 (Lawes Agricultural Trust, 1992). The model comprised fixed effects for treatment, oestradiol production class and interactions between treatment and oestradiol production class with the random effect of individual cows. All the follicle data were analysed using this model, and when a significant statistical difference was found between treatments by follicle class interaction, a t-test was performed.

Where data had a skew distribution, all values were transformed to a log scale prior to statistical analysis; this resulted in a more symmetrical distribution of the data which meant that they more closely conformed to the assumptions underlying the statistical tests. This transformation was applied to metabolic hormones, gonadotrophins, steroids and IGF-I intrafollicular concentrations, steroid production by granulosa and thecal tissue, receptor concentrations and granulosa cell numbers. For the purposes of presentation and interpretation, mean values were back transformed before presentation. However, standard errors of differences between means are expressed in transformed units for the purpose of statistical comparison.

The Fisher's Exact Probability Test was used to compare the incidence of ovulations.

## 3.2- Results

### 3.2.1- Live weight (LW) and body condition changes

Body condition (BC) and liveweight (LW) at calving were successfully manipulated. At 110 days before the expected mean calving date, cows had a mean (±se) live weight of 506 ± 13.2 kg and mean (±se) body condition score (BC) of 2.8 ± 0.07. While BC and LW were largely maintained until calving in HS cows, LS and LG cows each lost approximately 100 kg.

Following calving BC was largely maintained until ovariectomy at seven weeks postpartum, although there was a small decrease in LW in all groups (Table 1).

Table 1 Mean live weights (kg) and condition scores of cows in high (H) or low (L) body condition and infused with saline (S) or GnRH (G) at parturition and at ovariectomy, 7 weeks postpartum.

| | Live weight | | | | | | Condition Scores | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HS | s.e. | LS | s.e. | LG | s.e. | HS | s.e. | LS | s.e. | LG | s.e. |
| Parturition | 497.9 | 21.60 | 406.0 | 12.47 | 405.5 | 12.48 | 2.81 | 0.08 | 2.18 | 0.05 | 1.95 | 0.06 |
| Ovariectomy | 467.5 | 21.72 | 388.1 | 11.86 | 381.6 | 10.39 | 2.83 | 0.11 | 2.18 | 0.05 | 2.04 | 0.08 |

### 3.2.2- Metabolic hormones profiles during the postpartum period

#### 3.2.2.1- Growth Hormone

At week 6 postpartum, cows in high body condition (HS) had significantly lower mean GH concentrations than cows in low body condition (LS + LG) (0.89 v. 1.92 (ng/ml); s.e.d.=0.127; p< 0.001). Plasma GH concentrations were not significantly affected by GnRH treatment (LS: 2.30 v. LG: 1.54 (ng/ml); s.e.d.=0.127).

#### 3.2.2.2- Insulin-like growth factor-I

Plasma IGF-I concentrations remained stable throughout the experiment (Fig. 4), but from four days postpartum, concentrations were significantly higher ($p<0.05$) in HS than LS+LG cows. Concentrations (ng/ml) were not significantly affected by GnRH treatment (LS: 80.2 v. LG: 90.6; s.e.d.= 1.32).

#### 3.2.2.3- Insulin

Throughout the first 7 weeks postpartum, mean plasma insulin concentrations (μU/ml) in samples collected three times a week were not significantly affected by BC or treatment with GnRH (Fig. 5). However, on Monday of each week throughout the experiment, plasma insulin concentrations before feeding were consistently lower ($p<0.001$) in all the three groups of cows than at the same time on other days. Mean concentrations increased with time postpartum ($p<0.01$). There was a significant treatment × time interaction ($p<0.001$), with large differences between treatments in the pattern of changes in mean concentration over time.

Fig 4 Mean plasma concentrations of IGF-I (back transformed values on a log scale) during first 7 weeks postpartum in cows in high (H) or low (L) body condition and infused with saline (S) or GnRH (G). s.e.d.s (expressed in log (values) units) between treatments: 0.121; between sample collection times: 0.084.

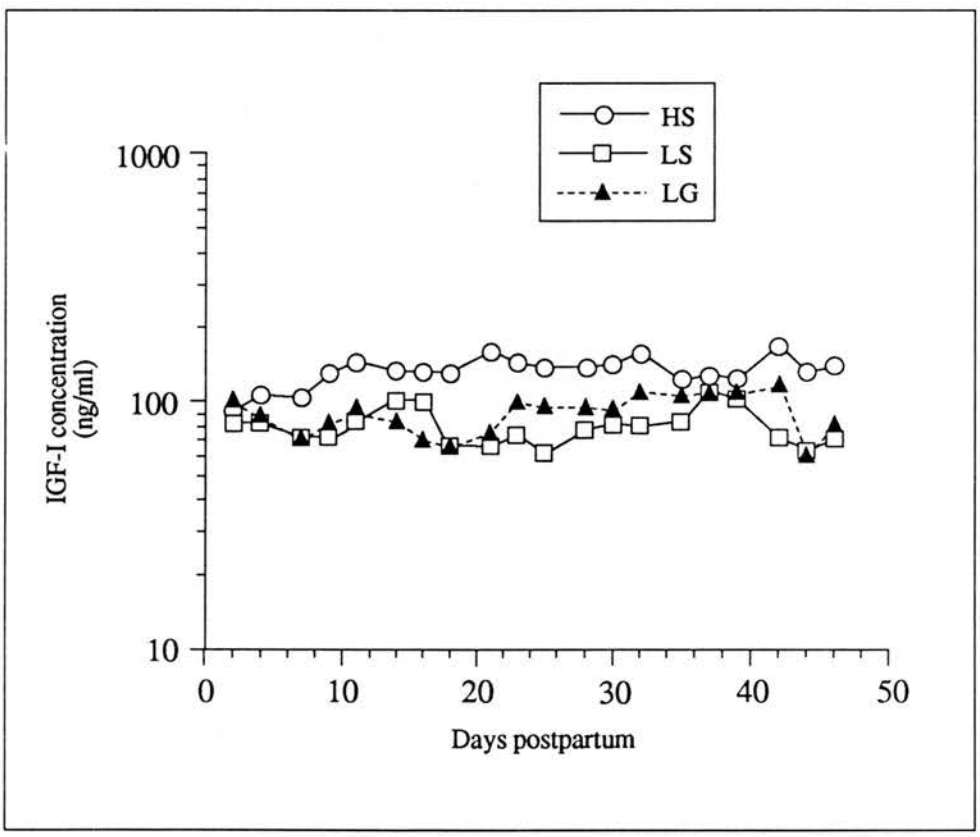
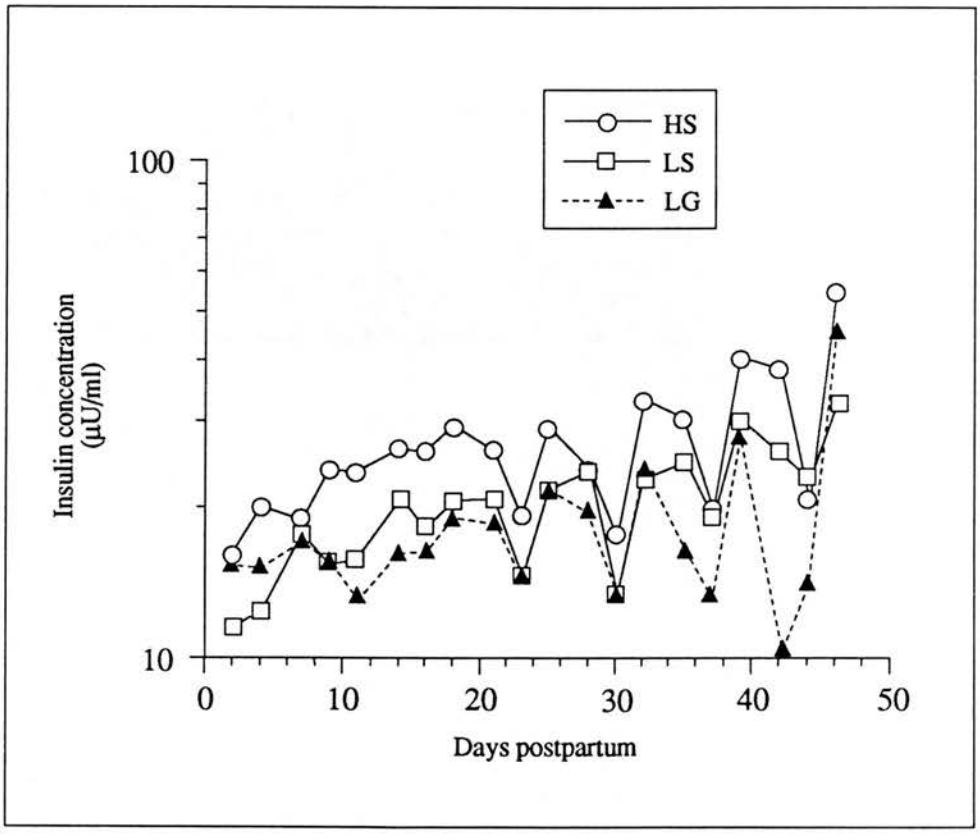


Fig 5 Mean plasma concentrations of insulin (back transformed values on a log scale) during first 7 weeks postpartum in cows in high (H) or low (L) body condition and infused with saline (S) or GnRH (G). s.e.d.s (expressed in log (values) units): between treatments: 0.096; between sample collection times: 0.071.



### 3.2.3- Gonadotrophin profiles at weeks 5, 6 and 7 postpartum

#### 3.2.3.1- Plasma luteinizing hormone (LH), LH pulse frequencies and amplitudes

Overall mean plasma LH concentrations (ng/ml) during the 10-hour sampling periods at weeks 5 (pre-infusion period), 6 (day 7 of infusion) and 7 (day 14 of infusion) postpartum were not affected by either body condition or gonadotrophin releasing hormone (GnRH) infusion (Table 2). However, mean LH concentration were significantly higher at week 6 postpartum compared to weeks 5 and 7 postpartum (1.23 v. 0.98 ng/ml, $p<0.01$).

Mean LH pulse frequency (pulses/hour) and pulse amplitude (ng/ml) were not affected by body condition, GnRH infusion or time postpartum (Table 2). However, at week seven postpartum there was a trend towards a greater LH pulse frequency in the LG animals compared with the HS and LS animals (HS+LS: 0.23 v. LG: 0.32, $p=0.060$).

#### 3.2.3.2- Plasma follicle-stimulating hormone (FSH)

Mean plasma follicle-stimulating hormone (FSH) (ng/ml) concentrations were not affected by body condition, GnRH infusion or time postpartum (Table 3).

### 3.2.4- Ovarian follicle populations and physiological status

#### 3.2.4.1- Follicle population and incidence of ovulation

The effects of differences in BC and of GnRH infusion on follicle populations and ovulation rate at seven weeks postpartum are shown in Table 4. The number of small follicles (3-7.9 mm) was not significantly affected by either BC or infusion of

Table 2 Mean log ( concentrations) of LH (ng/ml) and mean LH pulse frequencies (pulses/hour), and pulse amplitudes (ng/ml) at 5, 6 and 7 weeks postpartum in cows in high (H) or low (L) body condition and infused with saline (S) or GnRH (G); (back transformed values are given in parenthesis; s.e.d.s are expressed in log units).

| | | | | s.e.d | | Significance | | |
|---|---|---|---|---|---|---|---|---|
| | HS | LS | LG | Between Treatments | Between Weeks | Treatment | Week | Interaction |
| LH concentration | | | | | | | | |
| Week 5 | 0.049 (1.12) | 0.071 (1.17) | -0.074 (0.84) | | | | | |
| Week 6 | 0.169 (1.48) | 0.021 (1.05) | 0.078 (1.19) | 0.062 | 0.075 | NS | ** | NS |
| Week 7 | -0.022 (0.95) | 0.010 (1.02) | 0.068 (1.17) | | | | | |
| LH pulse frequency | | | | | | | | |
| Week 5 | 0.23 | 0.22 | 0.20 | | | | | |
| Week 6 | 0.21 | 0.23 | 0.18 | 0.045 | 0.049 | NS | NS | NS |
| Week 7 | 0.22 | 0.27 | 0.32 | | | | | |
| LH pulse amplitude | | | | | | | | |
| Week 5 | 2.63 | 1.92 | 1.85 | | | | | |
| Week 6 | 2.29 | 1.99 | 1.51 | 0.401 | 0.462 | NS | NS | NS |
| Week 7 | 2.09 | 2.24 | 2.52 | | | | | |

Table 3 Mean log (concentrations) of FSH (ng/ml) at 5, 6 and 7 weeks postpartum in cows in High (H) or Low (L) body condition and infused with saline (S) or GnRH (G); (Back transformed values are given in parenthesis; s.e.d.s are expressed in log units).

| | | | | s.e.d | | Significance | | |
|---|---|---|---|---|---|---|---|---|
| | HS | LS | LG | Between Treatments | Between Weeks | Treatment | Week | Interaction |
| Week 5 | 1.131 (13.53) | 1.009 (10.21) | 1.068 (11.70) | | | | | |
| Week 6 | 1.243 (17.51) | 1.042 (11.01) | 0.993 (9.84) | 0.091 | 0.155 | NS | NS | NS |
| Week 7 | 1.180 (15.13) | 0.975 (9.43) | 1.094 (12.43) | | | | | |

Table 4 Mean numbers of small (3 - 7.9 mm diameter) and large (≥ 8 mm diameter) ovarian follicles per cow at week 7 postpartum, numbers of animals which had ovulated and numbers of corpora lutea which were functional (associated with plasma progesterone concentrations ≥ 1.5 ng/ml).

| | | | | | Significance | | |
|---|---|---|---|---|---|---|---|
| | HS | LS | LG | s.e.d. | BC | Treatment | Interaction |
| No. Small follicles | 27.2 | 27.5 | 24.9 | 5.19 | NS | NS | NS |
| No. Large follicles | 1.83 | 2.27 | 1.50 | 0.388 | NS | NS | NS |
| Proportions of cows ovulating | 1/12 | 1/11 | 10/12 | - | NS | *** | * |
| Proportion of cows with functional CL | 1/12 | 1/11 | 8/12 | - | NS | *** | * |

GnRH. Similarly, the number of large follicles (≥ 8 mm) was not significantly affected by BC or treatment with GnRH.

There was no effect of BC on the incidence of ovulation with only one cow of 11 or 12 ovulating in each of the HS + LS groups. However, significantly more cows ovulated in the LG group (10/12; $p< 0.001$); (these animals are physiologically different and so are the follicles). Concentrations of progesterone > 1.5 ng/ml, were recorded in 10 to 12 of the plasma samples collected thrice weekly from parturition to ovariectomy from each of the animals that ovulated, except in 2 of the 10 cows which ovulated in the LG group. Data was not obtained from one animal in the LS group, because ovaries could not be recovered due to ovary adhesions.

Ovulation was deemed to have occured at three days before progesterone values exceeded 1,5 ng/ml. At the time of the window bleed at week 6, no animals had elevated progesterone concentrations while at the time of the window bleed at week 7, levels were elevated in only one animal in the LG group and none of the HS and LS animals. Progesterone measurements in subsequent samples indicated that a further 7 animals in the LG group ovulated during week 7 and one from each of HS and LS groups. The timing of ovulation of 2 animals in the LG group with non functional CL could not be determined because progesterone concentrations were not elevated.

### 3.2.4.2- Number of Granulosa cells per follicle

Neither BC or GnRH infusion affected the number of granulosa cells present in large follicles at week 7 postpartum (Table 5).

Table 5- Mean log (numbers) of granulosa cells present in follicles ≥ 8mm diameter at 7 weeks postpartum in cows in high (H) or low (L) body condition and infused with saline (S) or GnRH (G); (back transformed values are given in parenthesis; s.e.d.s are expressed in log units).

| HS | LS | LG | s.e.d | Significance |
|---|---|---|---|---|
| 6.53 (3,388,442) | 6.43 (2,735,269) | 6.67 (4,764,309) | 0.190 | NS |

### 3.2.4.3- Intrafollicular concentrations of testosterone, oestradiol and IGF-I

The effects of body condition and of infusion of GnRH pulses on steroid and IGF-I concentrations in follicular fluid of large follicles (≥ 8mm diameter) obtained at week 7 postpartum are summarised in Table 6.

Follicular fluid concentrations of testosterone were not significantly affected by either BC, treatment or follicle class (non-oestrogenic (secreting <10,000 pg/ml oestradiol) and oestrogenic (secreting >10,000 pg/ml oestradiol) follicles) (Table 6).

Follicles from cows in high BC had approximately 2 times higher oestradiol concentrations than cows in low BC, but the difference just failed to achieve statistical significance ($p=0.058$). The effect of infusion of GnRH pulses on oestradiol concentration was not statistically significant; however, oestradiol concentrations in the oestrogen-active follicles were almost 50% higher in LG than LS.

There was a significant effect ($p< 0.001$) of follicle class on oestradiol concentration, and a significant class × treatment interaction ($p< 0.05$); this was attributable to the particularly low level of oestradiol production in the non-oestrogenic follicles of HS cows.

IGF-I concentrations in follicular fluid were not affected by BC, GnRH treatment or follicle class (Table 6).

### 3.2.4.4- Steroidogenic capacity of thecal tissue and granulosa cells

Testosterone production by thecal tissue incubated with culture medium alone or with hCG was unaffected by BC or GnRH infusion (Table 7).

Neither BC nor GnRH infusion significantly affected oestradiol production by granulosa cells incubated in culture medium alone or in the presence of testosterone.

Granulosa cells from oestrogen-active follicles produced more oestradiol ($p<0.01$) than cells from non-oestrogenic follicles, both with and without additional

Table 6 Mean log (concentrations) of testosterone (pg/ml) oestradiol (pg/ml) and IGF - I (ng/ml) in follicular fluid of follicles ≥ 8 mm at week 7 postpartum. Follicles were classed as non-oestrogenic ($E_2$ low; secreting <10 000 pg per ml of follicular fluid) and oestrogenic ($E_2$ high; secreting >10 000 pg per ml of follicular fluid). (Back transformed values are given in parenthesis; s.e.d.s are expressed in log units).

| | HS | | LS | | LG | | | Significance | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $E_2$ low | $E_2$ high | $E_2$ low | $E_2$ high | $E_2$ low | $E_2$ high | s.e.d. | Treatment | $E_2$class | Interactions |
| n | 10 | 10 | 13 | 12 | 6 | 10 | | | | |
| Testosterone | 4.172 (14859.4) | 4.193 (15595.5) | 4.145 (13963.7) | 4.267 (18492.7) | 4.247 (17660.4) | 4.431 (26977.4) | 0.175 | NS | NS | NS |
| Oestradiol | 3.102 (1264.7) | 4.821 (66221.6) | 3.346 (2218.2) | 4.487 (30690.2) | 3.509 (3228.5) | 4.650 (44668.4) | 0.186 | p= 0.058 | *** | * |
| IGF-I | 2.288 (194.1) | 2.178 (150.6) | 2.221 (166.3) | 2.362 (230.1) | 2.449 (281.2) | 2.297 (198.2) | 0.139 | NS | NS | NS |

Table 7 Mean log (secretion rates) of testosterone (pg/h/mg protein) by thecal tissue incubated in the presence or absence of hCG and of oestradiol (pg/h/200 000 cells) by granulosa cells incubated in the presence or absence of testosterone. Granulosa cells and thecal tissue were derived from follicles classed as non oestrogenic ($E_2$ low; secreting <10 000 pg per ml of follicular fluid) and oestrogenic ($E_2$ high; secreting >10 000 pg per ml of follicular fluid). (Back transformed values are given in parenthesis; s.e.d.s are expressed in log units).

| | HS | | LS | | LG | | Significance | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $E_2$ low | $E_2$ high | $E_2$ low | $E_2$ high | $E_2$ low | $E_2$ high | s.e.d. | Treatment | $E_2$class | Interactions |
| No of follicles† | 10 | 9 | 11 | 11 | 5 | 10 | | | | |
| Testosterone (thecal tissue) | | | | | | | | | | |
| medium only | 2.959 (909.0) | 3.165 (1462.2) | 3.095 (1244.5) | 3.307 (2027.7) | 3.272 (1870.7) | 3.499 (3155.0) | 0.256 | NS | NS | NS |
| +hCG | 3.146 (1399.6) | 3.263 (1832.3) | 3.041 (1099.0) | 3.183 (1524.0) | 3.341 (2192.8) | 3.498 (3147.7) | 0.252 | NS | NS | NS |
| No of follicles† | 7 | 5 | 11 | 6 | 5 | 4 | | | | |
| Oestradiol (granulosa cells) | | | | | | | | | | |
| medium only | 1.500 (31.62) | 2.844 (698.2) | 1.779 (60.11) | 1.933 (85.7) | 1.535 (34.27) | 3.111 (1291.1) | 0.298 | NS | ** | * |
| + testosterone | 2.398 (250.0) | 3.473 (2971.6) | 2.574 (374.9) | 2.962 (916.2) | 2.211 (162.5) | 3.757 (5714.8) | 0.323 | NS | ** | * |

† - Insufficient tissue available for culture in some follicles

testosterone in the culture medium. There was a significant treatment × follicle class interaction ($p< 0.05$) with respect to oestradiol production in granulosa cells. This was attributable to the low oestradiol output of cells from LS cows. Granulosa cells (incubated with medium alone) from oestrogen active follicles in the HS and LG groups produced significantly more oestradiol than the LS group of cows (HS: 698.2 v. LS: 85.7; s.e.d.=0.299;$p<0.01$) and (LG: 1291.1 v. LS: 85.7; s.e.d.=0.331; $p<0.001$) but no difference was found when comparing HS and LG groups (HS: 698.2 v. LG: 1291.1; s.e.d.=0.318); back transformed values are shown in parenthesis, except for the s.e.d.s.

#### 3.2.4.5- LH and FSH receptor concentration in thecal and granulosa cells

Concentrations of LH receptors in thecal and granulosa tissue were not significantly affected by BC or infusion of GnRH (Table 8).

There were more LH receptors in thecal tissue of the oestrogenic follicles, although the effect was mainly in the HS cows. There was no such difference in granulosa cells. There were a significant ($p< 0.05$) treatment × class interaction in LH receptors in thecal tissue, attributable to the low number of LH receptors in the non-oestrogenic follicles in the HS cows.

FSH receptor numbers in thecal tissue were not measured because limited amounts of tissue were available and large numbers of FSH receptors were not expected in thecal tissue. FSH receptor concentrations in granulosa tissue were not significantly affected by BC or infusion of GnRH (Table 8).There was a significant ($p< 0.05$) effect of follicle class on FSH receptor concentrations in granulosa tissue, with oestrogenic follicles having FSH receptor concentrations at least 4 fold higher than non-oestrogenic follicles.

Table 8 Mean log (concentrations) (pg hormone bound per mg tissue) of LH receptors in thecal and granulosa tissue and FSH receptors in granulosa tissue. Granulosa cells and thecal tissue were derived from follicles classed as non oestrogenic ($E_2$ low; secreting <10 000 pg per ml of follicular fluid) and oestrogenic ($E_2$ high; secreting .>10 000 pg per ml of follicular fluid). (Back transformed values are given in parenthesis; s.e.d.s are expressed in log units).

| | HS | | LS | | LG | | | Significance | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $E_2$ low | $E_2$ high | $E_2$ low | $E_2$ high | $E_2$ low | $E_2$ high | s.e.d. | Treatment | $E_2$class | Interactions |
| No of follicles† | 10 | 10 | 10 | 11 | 4 | 9 | | | | |
| Thecal tissue | | | | | | | | | | |
| LH receptor | 1.191 (15.52) | 1.684 (48.30) | 1.579 (37.93) | 1.605 (40.27) | 1.484 (30.47) | 1.482 (30.33) | 0.160 | NS | * | * |
| No of follicles† | 10 | 10 | 10 | 11 | 6 | 10 | | | | |
| Granulosa tissue | | | | | | | | | | |
| LH receptor | 1.563 (36.55) | 1.644 (44.05) | 0.962 (9.16) | 1.542 (37.83) | 0.686 (4.85) | 1.318 (20.79) | 0.432 | NS | NS | NS |
| FSH receptor | 0.740 (5.49) | 1.657 (45.39) | 0.719 (5.23) | 1.608 (40.55) | 1.037 (10.88) | 1.657 (45.39) | 0.523 | NS | * | NS |

† - Insufficient tissue available for receptors in some follicles

## 3.3- Discussion

In this study, the imposed prepartum levels of feed intake were effective in achieving the prescribed differences in body condition (BC) at calving and this difference was maintained thereafter. This implies that the observed differences in circulating hormone profiles and ovarian function were a consequence of BC *per se*; since, the animals were all in a similar energy balance.

### Metabolic Hormones

The roles of growth hormone (GH), insulin growth factor-I (IGF-I) and insulin in the control of intermediary energy metabolism in ruminants are well known (Breier, Gluckman, McCutcheon and Davis, 1991; Brockman and Laarved, 1986) but recent work has shown that GH treatment increases the population of ovarian antral follicles (2-5 mm diameter) in mature heifers (Gong, *et al.*, 1991) and that natural twinning in cattle is associated with increased concentrations of IGF-I in blood and follicular fluid (Echternkamp, *et al.*, 1990). Downing and Scaramuzzi (1991) showed that increased insulin concentrations induced by infusion of glucose into sheep were associated with an increase in ovulation rate. Collectively, these observations suggest that the metabolic hormones may have a role in mediating the effects of nutrition on reproductive activity in addition to their effects on energy metabolism.

GH concentrations in the present study were similar to those found by Hart, *et al.* (1975) in beef cattle and Hart, *et al.* (1978) in Hereford × Friesian cows with low milk yield. Concentrations of GH recorded during the sixth week postpartum were significantly lower in cows in high BC than in cows in low BC, even with similar energy balance; this finding extends previous studies in heifers and lambs (Breier, *et al.*, 1986; Foster, *et al.*, 1989) which showed that restriction of feed intake resulted in higher circulating concentrations of GH. The present results show that GH concentrations also differ with BC when levels of intake are relatively similar.

IGF-I is an important mediator of growth, lactation, reproduction and health (McGuire, *et al.*, 1992) and GH plays an important role in the regulation of its secretion (Bass, *et al.*, 1992). The IGF-I concentrations in this study were similar to those reported by (Rutter, *et al.*, 1989) for postpartum beef cows. These and other authors also showed that concentrations of IGF-I change with physiological state and nutrient availability (Rutter, *et al.*, 1989), with circulating concentrations of IGF-I being positively associated with intake (Breier, *et al.*, 1986), and BC (Rutter, *et al.*, 1989). The results of the present study are in agreement with Rutter, *et al.* (1989), with higher IGF-I concentrations being present in cows in high BC. IGF-I concentrations are regulated by the action of GH, and the action of GH is mediated by the effect of nutrition on the GH receptors in the liver which explains the paradox that plasma GH is low in well-fed animals when plasma IGF-I is high (Bass, *et al.*, 1992; Hua, Ord, Kirk, Li, Hodgkinson, Spencer, Molan and Bass, 1993) and suggests that secretion of IGF-I becomes "uncoupled" from regulation by GH when nutrient availability is limited (Ellenberger, *et al.*, 1989).

It is possible that circulating concentrations of IGF-I may play a role in the regulation of folliculogenesis, since Echternkamp, *et al.* (1990) found that twinning is associated with increased concentrations of IGF-I in blood and follicular fluid. Since the duration of the postpartum anestrous period in beef cows is influenced by BC at calving, with high levels of BC being associated with shorter anoestrous periods (Wright, *et al.*, 1987; Wright, *et al.*, 1992a; Wright, Rhind, Whyte and Smith, 1992b), it is possible that IGF-I concentrations might be inversely related to the duration of the postpartum anoestrus. GH, however, does not operate in isolation but in concert with insulin and other metabolic hormones.

While insulin concentrations in the present study were not affected by BC or GnRH infusion throughout the postpartum period, every Monday the concentrations of insulin was lower. Due to small changes in the cows management at weekends, the interval between meals was slightly longer between Sunday and Monday. Since

ruminants have depressed levels of insulin after a period of fasting (Basset, 1974; Easdon, *et al.*, 1985) it is likely that the greater interval between feeding times on Sunday and Monday resulted in lower insulin concentrations on Mondays than on other sampling days.

Insulin levels increased consistently and markedly with time postpartum. Since the nutrient requirement for milk production decreases with time postpartum, with more nutrients being directed towards the tissues, the trend towards higher insulin concentrations with increasing time postpartum in this study is consistent with increased anabolism.

In summary, the differences in BC that were achieved in this study resulted in differences in metabolic hormones. This indicates that the known effects of BC on reproductive activity could be mediated through differences in metabolic hormone profiles. However, it should be noted that the relationship between the catabolic hormone, GH, and the anabolic hormone, insulin, and their effects on nutrient partitioning could be as important as the direct action of the individual hormones.

**Gonadotrophin profiles**

One mechanism through which body condition and metabolic hormones may affect reproductive activity is changes in gonadotrophin profiles.

Gonadotrophin profiles control ovarian follicle development (Ireland, 1987). In particular, associations between LH pulse frequency and both level of energy intake (Echternkamp, *et al.*, 1982; Terqui, *et al.*, 1982) and body condition (Wright, *et al.*, 1990) have been identified. The mean LH and FSH concentrations recorded in this study were similar to those reported previously (Wright, Rhind, Smith and Whyte, 1992c; Wright, *et al.*, 1990; Wright, *et al.*, 1992b). However, the absence of differences in LH pulse frequency with BC, in this study, contrasts with the results of Wright, *et al.* (1990), but supports the findings of Rhind, *et al.* (1992). Collectively,

these results suggest that the effects of BC on reproductive activity are not mediated through differences in this factor alone.

The postpartum anoestrous period in beef cows is characterized by a low frequency pulsatile secretion pattern of LH and presumably GnRH (Short, *et al.*, 1990) and it has been suggested that an increasing LH pulse frequency is a prerequisite for the onset of ovulation (Peters, *et al.*, 1981). In contrast to the view that a pulsatile pattern of LH can be induced by giving low-dose injections of GnRH (McLeod, *et al.*, 1985; Riley, *et al.*, 1981), in this study, the pulsatile infusion of GnRH did not induce a significant increase in the LH pulse frequency or mean LH concentrations recorded; similar results have been obtained by Jagger, *et al.* (1987).

Although the recorded LH pulse frequency in the LG group was not statistically different to that of HS and LS cows, 10 out of 12 cows in the LG group ovulated compared with only one in each of the other groups. This latter result confirms previous work in which ovulation was induced in both prepubertal heifers and acyclic cows by administering repeated injections of GnRH (McLeod, *et al.*, 1985; Riley, *et al.*, 1981; Walters, *et al.*, 1982c), and indicates that the infusion had affected ovarian function. Furthermore, it is known that each pulse of GnRH released from the hypothalamus is associated with an LH pulse (Clarke and Cummins, 1982) and so the failure to detect a significant change in LH pulse frequency is almost certainly attributable to the sampling regime and not to a failure to induce a response.

Elevated progesterone concentrations were not recorded until after the times of window bleeds to determine LH pulse frequency and so the absence of an increase in LH pulse frequency in the LG group at weeks 6 and 7 cannot be attributed to a negative feedback effect of progesterone resulting in reduction in LH pulse frequency. However, the timing of ovulations (as indicated by progesterone profiles) was such that the ovarian production of oestradiol was likely to be elevated at the time of the window bleeds, particularly in week 7. Consequently, the steroidal influence could

have been greater in LG animals and so the inhibitory effect on LH pulse frequency may also have been greater in LG than HS or LS cows.

Other possible reasons for the lack of evidence of additional LH pulses include: a) an excessive interval (up to 20 min.) between the infusion of a pulse of GnRH and sample collection; b) the small numbers of pulses occuring during the 10 h sampling period; c) undetected failure of pumping? and d) the definition of an LH pulse used in this work may have been conservative.

Recent studies suggest that short-term changes in FSH secretion can not be explained on the basis of changes in GnRH pulse frequency (Chappel, 1985). GnRH appears to provide a trophic stimulus for FSH secretion but the direct relationship that exists between GnRH and LH secretion (Clarke and Cummins, 1982) is absent for FSH.

The results of the present experiment support the earlier observations; on ovariectomized sheep (Clarke, Burman, Doughton and Cummins, 1986; Clarke, Cummins, Findlay, Burman and Doughton, 1984) in which pulsatile infusion with GnRH did not affect mean concentrations of FSH.

**Follicles**

Effects of nutritional treatment and gonadotrophin profiles on the incidence of ovulation must be mediated through changes in follicle development and steroidogenic capacity. It has been suggested that increased numbers of medium sized follicles (4-7.9 mm. diameter) may provide a pool from which ovulatory follicles are selected; this selection process is one of the necessary steps towards the first postpartum ovulation (Spicer, *et al.*, 1986a; Spicer, Matton, Echternkamp, Convey and Tucker, 1987). The number of small (3-7.9 mm diameter) and large (≥ 8mm diameter) follicles observed in this study at week 7 postpartum, were comparable with those reported previously (Prado, *et al.*, 1990) and were not affected by BC, as reported by Prado, *et al.* (1990) in a study of beef cows at 9 weeks postpartum. In the same study, at 5 weeks

postpartum, cows in high BC, had an greater number of small follicles than cows in low BC, perhaps resulting in differences in intrafollicular and endocrine signals. Therefore, Prado (1989) concluded that the physiological mechanisms involved in the recruitment, growth and development of ovarian follicles may become fully functional at an earlier stage in cows that calve in high BC. However, in both the present work and that of Prado (1989) differences in the physiological state of the follicles at weeks 7 or 9 postpartum, which were subsequently reflected in ovulatory activity, were not reflected in follicle size. This highlights a limitation of ultrasonographic observations (Gong, *et al.*, 1993; Knopf, *et al.*, 1989; Pierson and Ginther, 1984; Savio, *et al.*, 1988; Sirois and Fortune, 1988); while they have the advantage that they can be used to measure change over time without damaging the ovary, they cannot provide information on the intrafollicular hormone levels and follicles "health".

Only follicles > 8mm have the capacity to became dominant and ovulate (Spicer, *et al.*, 1986a) and, in this study, the ability of the follicles to grow and achieve a potentially ovulatory size was not limited by BC at calving, at least within the range applied in this study, since cows of both groups had similar numbers of follicles ≥ 8 mm. Similarly, the presence of large follicles at week 7 postpartum in both the LS and HS groups of cows suggests that follicular growth *per se* was not inhibited during the postpartum anoestrous period, and was not a limiting factor in the reestablishment of postpartum cyclicity. This is in agreement with previous work in cattle in which follicular growth was shown to increase markedly after the first week postpartum with large follicles appearing several weeks before the first postpartum ovulation (Moss, *et al.*, 1985; Spicer, *et al.*, 1986a).

In summary the results of this study indicate that the capacity of follicles to grow and ovulate is not a limiting factor in postpartum anoestrus. Ovulation only occurs when a large oestrogenic follicle is exposed to the correct LH pulse profile

(Roche, *et al.*, 1992). However, the ability of these large follicles to produce enough oestradiol to induce ovulation may be the limiting factor.

**Follicle steroidogenic capacity *in vivo***

Determinations of steroid concentrations in follicular fluid from follicles of known physiological status provide information on the synthetic capabilities of granulosa and thecal cells at particular stages of differentiation, and knowledge of steroid concentrations in follicular fluid can help in understanding the control of follicular function (Fortune and Hansel, 1985).

The conversion of androgens to oestrogens and the response of granulosa cells to these steroids are key factors in determining whether a follicle ovulates or becames atretic. However, the control of this system is more complicated and also involves the production of local agents, growth factors, such as IGF-I that stimulate and/or inhibit the actions of the gonadotrophins in responsive cells (Dorrington, *et al.*, 1987).

In this study, testosterone concentration (an oestrogen precursor) in the follicular fluid of large follicles (≥ 8 mm. diameter) was not affected by BC, GnRH infusion and follicle class at week 7 postpartum. This is consistent with the results of Rhind, *et al.* (1992) who found that BC did not affect follicle fluid concentrations of testosterone. However, studies *in vitro* conducted by Prado, *et al.* (1990) showed that cows in high BC had higher concentrations in testosterone than cows in low BC.

The oestrogen content of follicles is an important determinant of their ability to respond to gonadotrophins and so to undergo the final stages of development and maturation (Gore-Langton and Armstrong, 1988). In this study, oestradiol concentration in the follicular fluid of follicles ≥ 8 mm. diameter at 7 weeks postpartum was not affected by BC or GnRH infusion, as reported by Rhind, *et al.* (1992). However, the observation that oestradiol concentrations in cows in high BC were twice as high as in cows in low BC is consistent with the findings of Prado, *et*

*al.* (1990) in which the incidence of highly oestrogenic follicles was found to be greater in cows in high BC compared with those in low BC.

Thus, it is concluded that body condition does not affect ovarian capacity to produce large follicles, but it seems that some of these large follicles do not have the ability to produce large amounts of oestradiol necessary for ovulation. The reduced oestradiol production in the non-oestrogenic follicles, particularly in cows in high BC, suggests that such follicles have not yet developed the capacity to convert androgens to oestrogen, and it seems that this was not due to a lack of aromatizable androgens which were similar in both oestrogenic and non-oestrogenic active follicles.

The effect of GnRH infusion on oestradiol concentration in large follicles was not significant, although it was almost 50% greater than in LG group than cows in the LS group. Since 10 out of 12 cows in the group of cows infused with GnRH ovulated, it is clear that ovarian function was altered and that these cows were in a different physiological state from those on the HS and LS treatments as indicated by the fact that some of them had already matured to the point that they could ovulate. The results suggest that oestradiol synthesis may have been enhanced by GnRH infusion.

IGF-I is present and secreted in the ovary (Hammond, *et al.*, 1991; Spicer, Alpizar and Echternkamp, 1993) and can stimulate follicular and luteal steroidogenesis in cattle (McArdle and Holtorf, 1989; Savion, *et al.*, 1981). In this study, IGF-I concentrations in the follicular fluid were similar to those found by Echternkamp, *et al.* (1990) in beef cows, and were not affected by BC or GnRH infusion. Echternkamp, *et al.* (1990) suggests that levels of IGF-I in blood may regulate follicular growth and differentiation, since they have found that natural twinning in cattle is associated with increased concentrations of IGF-I in both blood and follicular fluid. Furthermore, it is also known that plasma IGF-I concentrations change in response to alterations in intermediary energy metabolism induced by glucose excess or deprivation or by reducing feed intake (Rutter and Manns, 1988b) as well by changes in BC (Rutter and Manns, 1989). In this study, BC affected plasma concentrations of IGF-I, with cows

in high BC having higher levels of IGF-I. However, the higher circulating concentrations were not reflected in higher concentration of IGF-I in follicular fluid. Rhind, Schemm and Schanbacher (1993) found an absence of measurable amounts of IGF-I in a culture medium with follicles, and suggested that circulating concentrations are unlikely to be affected by secretion from this source. If the transfer of IGF-I from the circulation to the follicular fluid is also limited, it is unlikely that changes in circulating concentrations may affect follicle physiology in the short term.

**Follicle steroidogenic capacity *in vitro***

Steroid production from follicles cultured *in vitro* reflects steroid production *in vivo* (Hillier, *et al.*, 1981; Webb and Gauld, 1985). In addition, a high positive correlation exists between follicular cell steroid production *in vitro* and concentration of steroids in follicular fluid (Spicer and Echternkamp, 1986). Although follicle steroid production *in vitro* reflects steroid production *in vivo*, differences in steroid content of the follicle fluid are not always reflected in plasma steroid concentrations (Rone, Henricks and Echterkamp, 1983; Walters, Kaltenbach, Dunn and Short, 1982). Nevertheless measurement of steroid production *in vitro* can be a valuable aid to understanding how BC or GnRH pulse infusion affects ovarian steroidogenesis.

Testosterone production by thecal tissue incubated in medium alone or with hCG was not affected by BC, GnRH infusion or follicle class. In bovine antral follicles, specific receptors for human chorionic gonadotrophin (hCG/LH) are present in the theca interna at all stages of the development of the follicle (McNatty, Lun, Kieboom and Henderson, 1985). Thus the absence of any change in testosterone production after adding hCG to thecal tissue in cows in different BC and GnRH or saline suggests that gonadotrophin support was not deficient in cows of any treatment.

Different results were obtained by Prado, *et al.* (1990) who reported that beef cows in high BC had an higher overall follicular testosterone production and by Rone, *et al.* (1983) who reported a reduced testosterone concentration in follicular fluid of

beef cows with a low feed intake, even although feed intake was totally independent of BC.

Oestradiol production by granulosa cells when incubated in medium alone or with testosterone was not affected by either BC or GnRH infusion. However, cells from oestrogen active follicles (incubated in medium alone) of cows in high BC had a significantly higher rate of oestrogen secretion than those from cows in low BC. These results support the earlier observations of Prado, *et al.* (1990) which also demonstrated a greater oestrogen production in follicles of beef cows in high BC than animals in low BC.

Wright, *et al.* (1987) showed that cows calving with low BC have a longer postpartum anoestrus period than cows in high BC, and Prado, *et al.* (1990) suggested that this may be a result of poorly developed aromatase systems in the large follicles of cows in low BC, as indicated by the reduced ability to convert testosterone (which was present in large amounts) to oestradiol. The results of the present study broadly confirm and extend these results, indicating that testosterone concentrations in the follicular fluid were adequate and not responsible for the treatment differences in oestradiol production in the oestrogenic active follicles of cows in different BC, since differences in the ability of the granulosa cells to convert supplementary testosterone into oestradiol were proportionately similar to the differences obtained with granulosa cells incubated with medium alone.

A significantly higher rate of oestradiol production by granulosa cells from oestrogen active follicles incubated with medium was also recorded in cows infused with GnRH.

Elevated oestradiol concentrations are necessary for the initiation of the preovulatory LH surge (Hanzen, 1986) and Prado (1989) suggested that the higher oestradiol production of cows in high BC indicates that these animals have more mature follicles and their oestradiol concentrations are approaching levels necessary to induce the LH surge and ovulation and that ovulation takes place earlier in these cows

than cows in low BC. However, this study does not agree with the suggestion of Prado (1989), since at 7 weeks postpartum, only one cow had ovulated in each of the high and low BC groups, even with large differences in oestradiol production in the follicular fluid of large follicles and with significant differences in the secretion rates of oestradiol from granulosa cells from oestrogenic active follicles. Furthermore, 10 out of 12 cows did ovulate in cows in low BC submited to GnRH infusion, and with the oestradiol follicular fluid production and secretion being similar with cows in high BC. This suggests that higher oestradiol concentration in the follicular fluid *per se* is not the only factor, responsible for the ovulation. However, it should always kept in mind that when granulosa cells are in culture, they are not under regulatory pressure as within the follicle (Henderson, McNatty, Smith, Gibb, O'Keefe, Lun, Heath and Prisk, 1987) and, therefore, results need to be interpreted with caution.

**FSH and LH receptors**

The conversion of androgens (androstenedione and testosterone) to oestradiol by granulosa cells is FSH dependent (Hansel and Convey, 1983) and, thus, oestradiol synthesis is dependent on the presence of adequate concentrations of FSH receptors in the granulosa cells. Similarly, since thecal cells secrete androgens under the influence of LH (Gore-Langton and Armstrong, 1988) and oestradiol synthesis depends of an adequate supply of androgen substrate (Hansel and Convey, 1983), the presence of adequate concentrations of LH receptors in thecal tissue is another prerequisite for the normal follicle development.

In this study there was no effect of either BC or GnRH infusion on LH receptor concentrations in either thecal or granulosa tissues. However, there was a significant difference with follicle class with oestrogen-active follicles in cows in high BC having more LH receptors than non-oestrogenic follicles. FSH receptor concentrations in granulosa cells were not affected by BC or GnRH infusion. The present results extend the observations of Rhind, *et al.* (1992); they reported a higher

incidence of oestrogenic follicles in cows in high BC than low BC at 9 weeks postpartum but could not determine whether or not this was a function of a higher incidence of follicles with large numbers of FSH receptors at an earlier time postpartum. The present experiment, performed at week 7 postpartum shows that the number of FSH receptors were not different with BC and probably were not responsible for the increase in oestradiol production in cows in high BC. Rhind, *et al.* (1992) also showed no differences in FSH receptor concentrations with BC. The observation of lower FSH receptors concentrations in the non-oestrogenic follicles than in oestrogenic follicles agrees with the findings of Ireland and Roche (1982) who reported similar differences in gonadotrophin binding during the periovulatory period in cyclic heifers and that of Rhind, *et al.* (1992) in postpartum beef cows. FSH receptors in thecal tissue were not measured due to the limited amount of tissue available and large numbers of FSH receptors were not expected in thecal tissue (Staigmillar, *et al.*, 1982).

In summary, the differences in oestradiol production and secretion rates from follicular fluid and granulosa and thecal tissue incubates with BC, are unlikely to be mediated through differences in gonadotrophin receptor concentrations.

**Number of granulosa cells**

The capacity of the follicle to produce oestradiol depends not only on oestradiol output of cells but also on the number of granulosa cells present in large follicles (Hillier, 1990).

At week 7 postpartum the number of granulosa cells in this study was not affected by BC or GnRH infusion. The numbers of granulosa cells present in follicles $\geq$ 8 mm. diameter at 7 weeks postpartum in this study were smaller than those reported in cyclic heifers (Ireland and Roche, 1983a) and cows (McNatty, *et al.*, 1984a), and were similar to the numbers present in oestrogen-inactive follicles and atretic follicles in heifers (Ireland and Roche, 1983b). Ireland and Roche (1983a) also suggests that

the loss of capacity of an oestrogen-active follicle to produce oestradiol was associated with a loss of granulosa cells and a reduction in the number of FSH and LH receptors per granulosa or thecal tissue. The number of granulosa cells present at week 7 postpartum, regardless of BC, suggests that at this time in the postpartum period the follicles had not fully developed, at least in HS and LS animals. Treatment with GnRH may have resulted in development of additional granulosa cells in follicles that subsequently ovulated. However, follicles present at ovariectomy had apparently not been sufficiently stimulated for this to happen.

The failure in two of the cows in the LG group that had ovulated, to obtain a functional CL (as indicated by the low levels of progesterone) could be due to a failure to develop enough granulosa cells and to a lack of adequate oestradiol. This finding is similar with work in prepubertal heifers (McLeod, *et al.*, 1985) and seasonally anoestrous ewes in which absence of normal luteal function was also found as a proportion of GnRH-induced ovulations, unless the animals are pretreated with progesterone (McLeod, Haresign and Lamming, 1982). It has been suggested that a short-lived corpus luteum is at least partly due to altered follicular development (Braden, King, Odde and Niswender, 1989).

## Conclusions

The results of the experiment support the hypothesis that infusion of GnRH pulses into postpartum cows in low BC would enhance follicle development, as indicated by the fact that 10/12 treated animals had ovulated. However, while GnRH treatment may have enhanced follicular oestradiol production, it did not increase production to the level recorded in HS animals; these results may not show the true nature of the ovarian response to GnRH since cows in the LG group had already ovulated and the follicles present at the time of ovariectomy were therefore in a different physiological state from those of HS and LS cows. If follicles had been examined before ovulation a higher level of oestradiol production might have been

recorded. The occurence of ovulation in most treated animals combined with the trend towards elevated oestradiol concentrations in the follicles of LG cows indicate that GnRH treatment enhanced follicular steroidogenesis without modification of metabolic hormones profiles to that of high condition animals. However, these differences were not apparently attributable to increase in number of granulosa cells, the capacity of the theca to synthesize testosterone or differences in gonadotrophin receptor populations.

Body condition was associated with differences in GH and IGF-I and also associated with differences in oestradiol in follicular fluid but it was not possible demonstrate a cause and effect. Therefore the need of further investigations of a role of metabolic hormones on ovarian function are required.

# CHAPTER 4

## Investigation of the role and mode of action of bST in the control of ovarian function in postpartum beef cows

### 4- Introduction

Since the relationship between LH pulse frequency and BC is inconsistent, it appears that other factors are involved in the resumption of ovarian activity postpartum. Evidence of a stimulatory role of GH in ovarian function has been provided by studies on a range of species including cows (Gong *et al.*, 1991), rabbits (Yoshimura *et al.*, 1993) and humans (Barreca *et al.*, 1993). bST has been reported to increase numbers of small ovarian follicles (Gong *et al.*, 1991); this may affect the intraovarian signals with possible consequences for the growth and function (steroidogenic capacity, hormone receptors and IGF-I production) of a dominant follicle i.e. the bST treatment may enhance the hormonal signals required for maturation and ovulation of the dominant follicle. Specifically, GH is likely to affect the oestrogenicity of the dominant follicle (Yoshimura *et al.*, 1993; Barreca *et al.*, 1993); this effect may be mediated through changes in metabolic hormones, gonadotrophins, hormone receptors and/or numbers of granulosa cells.

The aim of this experiment was to test the hypothesis that GH has a stimulatory effect on steroidogenesis and the capacity to generate a pre-ovulatory follicle and that this effect is mediated in part by altered intraovarian signals associated with changes in the population of small follicles.

## 4.1- Materials and methods

The analytical procedures carried out were similar to those described in Chapter 3 where they are described in detail; differences in procedures for this experiment are indicated below.

### 4.1.1- Animals and management

Thirty-four Blue-Grey (White Shorthorn × Galloway) cows which had been mated at a synchronized oestrus to calve in late March were used. One hundred and ten days before the expected date of calving, the cows had a mean (±sd) live weight of 485±62 kg. and a mean body condition score of 2.9±0.3 respectively.

During the last 4 months of pregnancy cows were weighed and condition scored (Lowman, *et al.*, 1976) weekly and fed different amounts of silage (Table 9) according to body condition (BC) so that they achieved a moderately low body condition at calving.

Table 9- Silage and straw rations according to BCS during last 4 months of pregnancy.

| Condition Score | Silage (kg/day) | Straw (kg/day) |
|---|---|---|
| 2 | 30 | - |
| 2.25 | 25 | - |
| 2.5 | 12 | 1 |
| >2.5 | 10 | 1 |

At calving the cows had a mean (±sed) BCS of 2.2±0.31. Thereafter they were individually fed rations designed to maintain weight and body condition (Wright, *et al.*, 1990). All cows were fed 25 kg silage together with a variable quantity of

concentrate (1-3 kg); these rations supplied an estimated 0.23 MJ of ME per kg live weight per day. The amount of concentrate fed was adjusted weekly according live weight changes. Fresh water was provided *ad libitum*. Animals in the two treatments were fed similarly irrespective of changes in milk yield associated with bST treatment.

The cows were housed in individual cubicles with their calves in adjacent cubicles, having acess to their dam for suckling twice a day (08.00 and 16.00 h.; for 30 min at each time) thoughout the experiment.

During the experiment two cows from the control group died. Data from these animals were not included in any of the analyses.

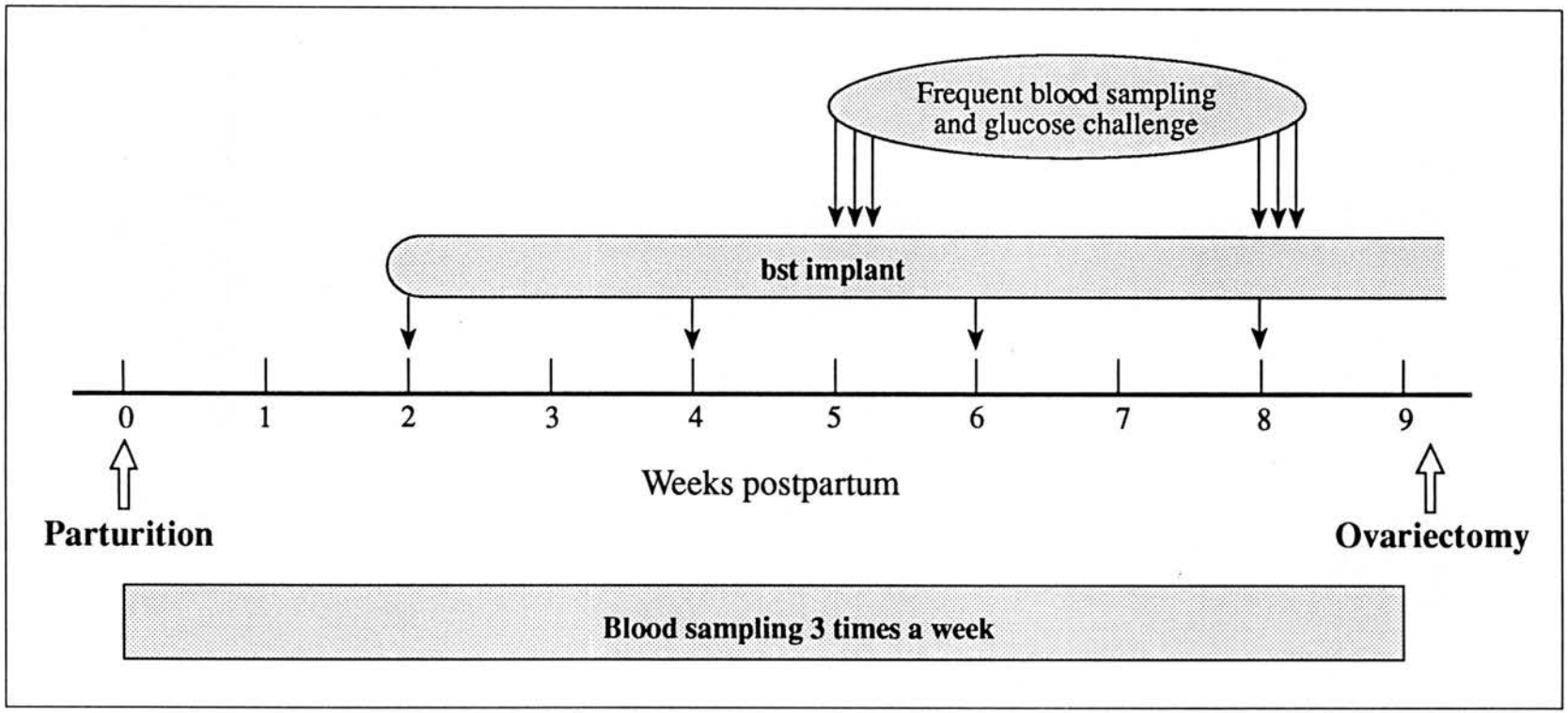
### 4.1.2- Experimental treatments and procedures

As cows calved, over 7 day period, they were allocated randomly within each condition score class to one of two treatment groups of 17 animals: a Control (C) group and one treated with the synthetic growth hormone, bovine somatotropin (T). The experimental protocol is summarised in Fig. 6.

At weeks 2, 4, 6 and 8 postpartum (± 4 days), T animals received a depot injection containing 320 mg bST in sesame oil (Optiflex; Elanco Animal Health Research, Basingstoke), a dose designed to release 23 mg/d. for 14 days. Animals in the C group were injected only with the carrier (sesame oil; Anglia Oils Ltd., Kingston-Upon-Hull, North Humberside, UK) at the same times. Blood samples were collected by jugular venepuncture three times a week (on Mondays, Wednesdays and Fridays) throughout the study.

At weeks 5 and 8 postpartum, blood samples were collected from an indwelling jugular venous catheter every 20 min. for 10 h. on each of two consecutives days. On the following day on each occasion, a bolus injection comprising 300 ml. of a 40% solution of glucose (Aldrich Chemical Ltd., Gilligham, Dorset, UK) solution was administered (i.v.). Blood samples were collected via

Fig 6 Experimental protocol 2



jugular catheters at 15 min. intervals for 30 min. before injection and for 3 h. after injection, in order to assess the rate of disappearance of glucose.

At week 9 postpartum all animals were surgically ovariectomised using techniques described in Chapter 3.

### 4.1.3- Hormone assays

Thrice weekly plasma samples were analyzed for progesterone, using an ELISA kit (Ridgeway Science Ltd., Alrington, Gloucertshire, UK). Growth hormone, IGF-I and insulin concentrations in these samples were also determined using the same techniques as in Chapter 3.

Samples collected at frequent intervals during the 10h. 'windows', were assayed for LH and every third sample was assayed for FSH.

Samples collected at frequent intervals following the glucose injection were assayed for glucose and insulin.

Intra- and inter-assay coefficients of variation for the respective hormones are summarised in Table 10.

Table 10- Intra- and Inter-assay Coefficient of Variation for LH, FSH, GH, IGF-I, Insulin, Progesterone, and Glucose

| | Intra-assay (%) | Inter-assay (%) |
|---|---|---|
| LH | 5.6 | 8.4 |
| FSH | 9.7 | 11.0 |
| GH | 10.2 | 11.2 |
| IGF-I | 7.8 | 10.2 |
| Insulin | 6.8 | 8.8 |
| Progesterone | 7.4 | 8.7 |
| Glucose | 1.4 | 3.2 |

#### 4.1.3.1- Steroid hormones

All procedures were as described in Chapter 3. Intra-and inter-assay coefficients of variation were respectively 6.2% and 8.4% for oestradiol and 6.0% and 7.7% for the testosterone.

#### 4.1.3.2- Glucose

Glucose was measured by a glucose oxidase method, using a modification of Trinder's method for an Auto Analyzer (Richardson, 1977). This method was adapted for use on an Alpken Rapid Flow Analyser.

### 4.1.4- Follicle procedures

All procedures were as described in Chapter 3, except that pooled follicular fluid from all the follicles measuring $\geq 3$ mm and $< 8$ mm diameter was collected for steroid determinations.

### 4.1.5- Statistical analysis

The main effects of bST treatment on metabolic hormones, gonadotrophin profiles, number of small and large follicles, steroid concentrations in small follicles and calves weights were compared using analysis of variance. For each of the metabolic hormones, concentrations before and after the start of bST treatment were also compared by analysis of variance.

Fisher's Exact Probability test was used to compare the incidence of ovulations.

All follicle data were analysed using the same statistical procedures as described in Chapter 3.

## 4.2- Results

### 4.2.1- Live weight (LW) and body condition changes

At 110 days before the expected mean calving date, cows had a mean (±se) live weight of 487 ± 11.7 kg and mean (± se) body condition (BC) of 2.9 ± 0.06.

The feeding regimes applied during late pregnancy resulted in moderately low body condition at calving (2.21; s.e.= 0.075). Following calving BC and LW were largely maintained in cows of both the bST treated and control groups, until ovariectomy at nine weeks postpartum (Table 11).

There were no significant differences between treatments in calf weights throughout the period of bST treatment (week 2: C: 54.0 kg v. T: 52.5 kg, s.e.d.= 2.68; week 9: C: 88.8 kg v. T: 92.9 kg, s.e.d.= 4.26). However, the average daily weight gain over this period, which represents an index of milk yield was significantly higher in bST treated cows than control cows (T: 0.904g v. C: 0.785g; s.e.d.= 0.052; $p< 0.05$).

### 4.2.2- Metabolic hormone profiles during the postpartum period

#### 4.2.2.1- Growth Hormone

GH concentrations were similar in cows of the two treatment groups before the start of the bST treatment. Following bST injection, T cows had higher peripheral GH concentrations throughout the treatment period compared with C cows ($p< 0.001$).

Table 11 Mean live weights (kg) and condition scores at parturition and at ovariectomy (nine weeks postpartum) of cows injected with bovine somatotrophin (Group T) or vehicle only (Group C)

| | Live weight | | | | Condition score | | | |
|---|---|---|---|---|---|---|---|---|
| | C | s.e | T | s.e | C | s.e | T | s.e |
| Parturition | 399.4 | 17.25 | 411.8 | 11.16 | 2.20 | 0.08 | 2.22 | 0.07 |
| 9 weeks postpartum | 392.8 | 15.88 | 408.5 | 10.44 | 2.30 | 0.08 | 2.30 | 0.06 |

There was a significant time × treatment interaction ($p < 0.001$) following bST treatment attributable mainly to the increases in GH concentrations after each bST injection in the T animals. Peripheral GH concentrations increased within 24 hours after each injection (Fig. 7), and remained elevated ($p<0.001$) throughout the postpartum period compared with levels in C cows. The difference was most marked during the first 7-8 days after each injection.

#### 4.2.2.2- Insulin-like growth factor I

Since IGF-I is GH dependent and GH affects intermediary metabolism, concentrations of IGF-I in plasma may be an index of nutritional status and may affect reproductive activity.

Mean plasma IGF-I concentrations remained fairly constant throughout the postpartum period in C cows (Fig. 8). T cows had similar IGF-I concentrations before implantation of bST, but following bST implantation IGF-I concentrations were elevated within 48 hours, and remained elevated during the postpartum treatment period, compared with C cows ($p < 0.001$). Following bST treatment there was a significant time × treatment interaction ($p < 0.001$) attributable to an increase in IGF-I after each bST injection. IGF-I concentrations in the T cows followed a profile broadly similar to that of GH following bST implantation.

#### 4.2.2.3- Insulin

Peripheral insulin concentrations of C and T cows were not significantly different throughout the experiment (Fig. 9). At the beginning of each week during the period in which bST was administered, mean insulin concentrations were depressed ($p < 0.01$) in cows of both treatment groups. The treatment × time postpartum interaction was significant ($p < 0.001$) following bST injection, with mean values increasing slightly but significantly through the postpartum period.

Fig 7 Mean plasma concentrations of GH (back transformed values on a log scale) from parturition (day 0) to ovariectomy at day 65. Injection of bST or vehicle is indicated by the arrows. s.e.d.s (expressed in log (values) units): between treatments: 0.151; between sample collection times: 0.139.

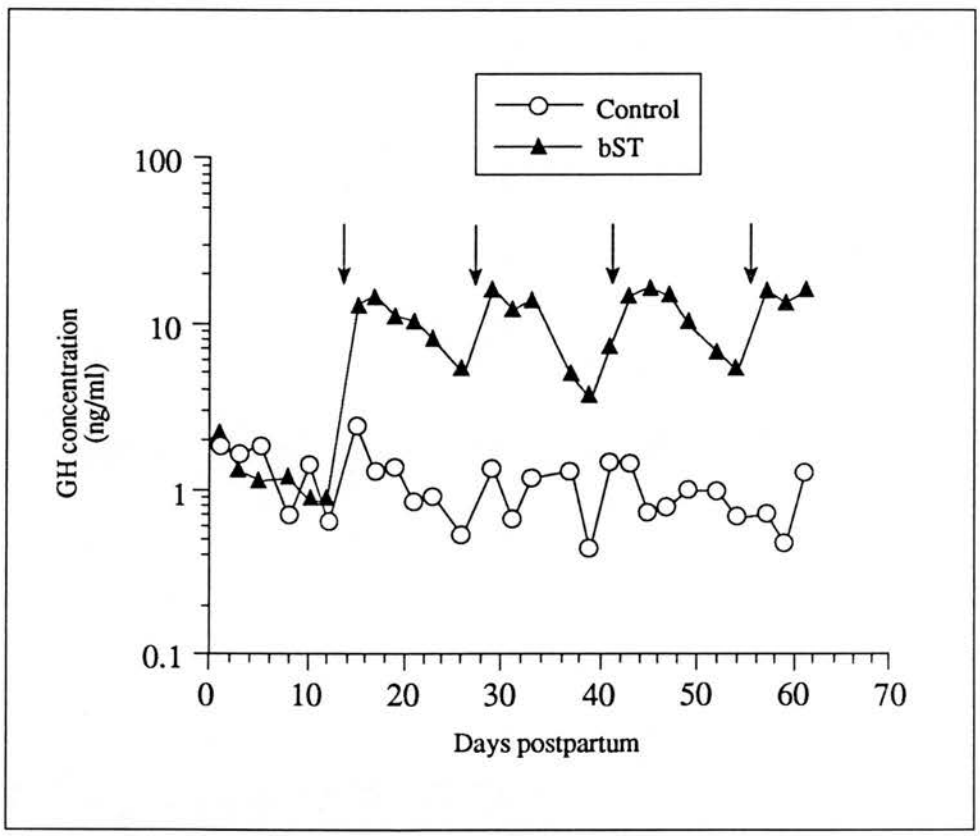


Fig 8 Mean plasma concentrations of IGF-I (back transformed values on a log scale) from parturition (day 0) to ovariectomy at day 65. Injection of bST or vehicle is indicated by the arrows. s.e.d.s (expressed in log (values) units): between treatments: 0.099; between sample collection times: 0.071.

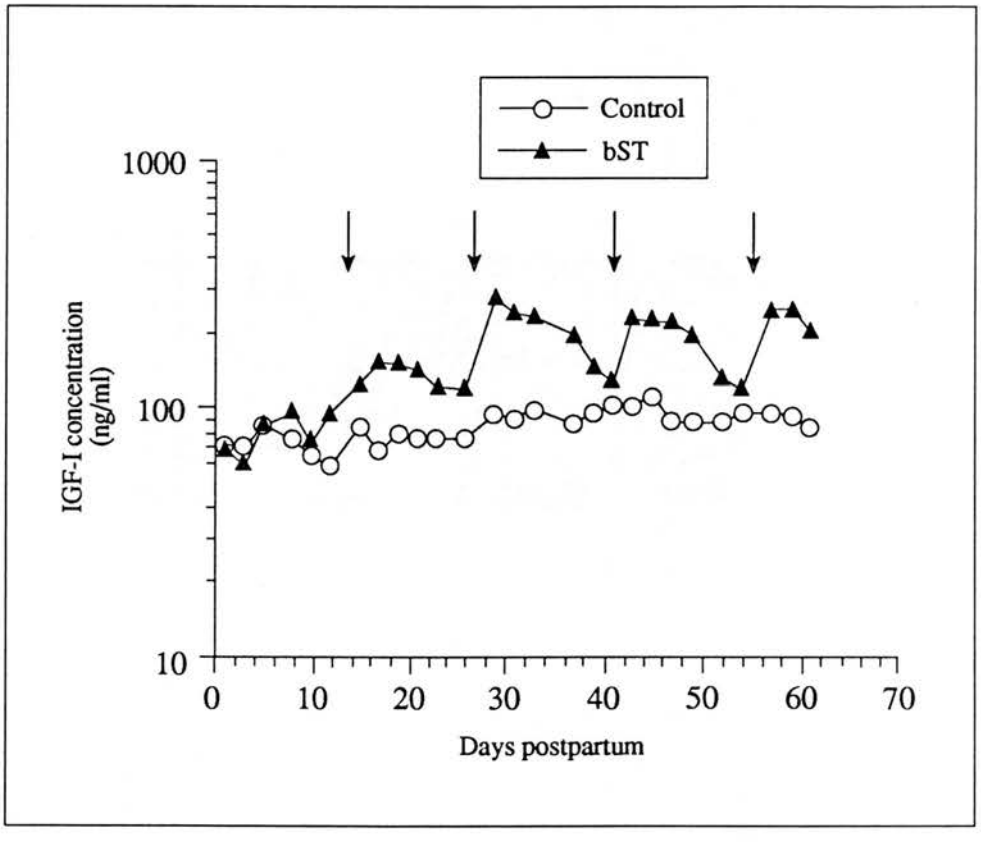
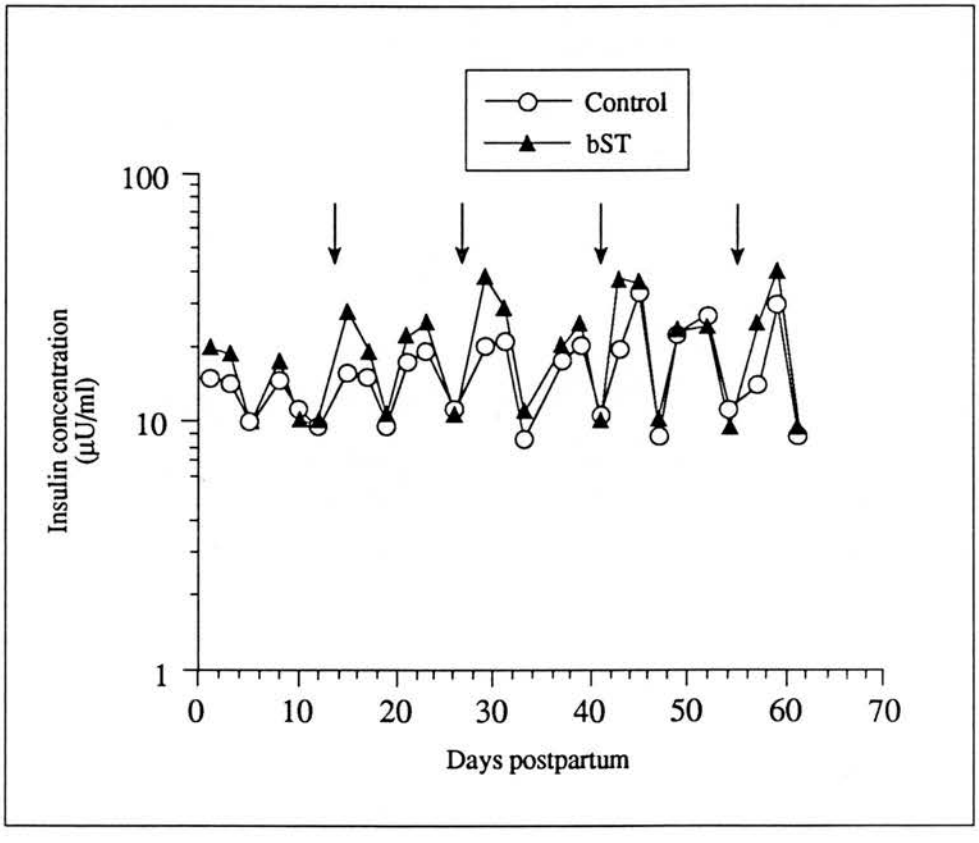


Fig 9 Mean plasma concentrations of insulin (back transformed values on a log scale) from parturition (day 0) to ovariectomy at day 65. Injections of bST or vehicle is indicated by the arrows. s.e.d.s (expressed in log (values) units): between treatments: 0.079; between sample collection times: 0.059.



#### 4.2.2.4- Insulin/GH ratio

Before bST injection, T cows had similar insulin/GH ratios. After bST injection, T cows had a lower Insulin/GH ratio ($p< 0.001$) than C cows (Fig. 10). Differences with time postpartum in the insulin/GH ratio were significant ($p< 0.001$) but did not show a consistent pattern in either treatment group. There was a significant ($p< 0.001$) treatment $\times$ time interaction, due mainly to the effects of bST injections in the T cows.

### 4.2.3- Glucose challenge test

#### 4.2.3.1- Glucose profiles

The treatment differences in metabolic hormone profiles were not associated with differences in the rate of clearance from the circulation of the energy-providing substrate, glucose, as assessed by repeated measurements of concentrations following injection of a bolus comprising 300 ml. of a 40% glucose solution.

Plasma glucose concentrations before the bolus injection of glucose at both weeks 5 and 8 were similar in the T and C cows (Fig. 11). Profiles were also similar in C and T cows after glucose injection at both times. Maximal glucose values were recorded at fifteen minutes after injection. The mean peak value was higher but not significantly at week 8 than week 5 (13.0 v. 10.6 mmol/l; s.e.d= 2.03). Glucose concentrations returned to pre-infusion concentrations by approximately 90 minutes after injection in all animals.

Fig 10 Mean ratio of plasma insulin concentration/GH concentration (back transformed values on a log scale) from parturition (day 0) to ovariectomy at day 65. Injection of bST or vehicle is indicated by arrows. s.e.d.s (expressed in log (values) units): between treatments: 0.172; between sample collection times: 0.151.

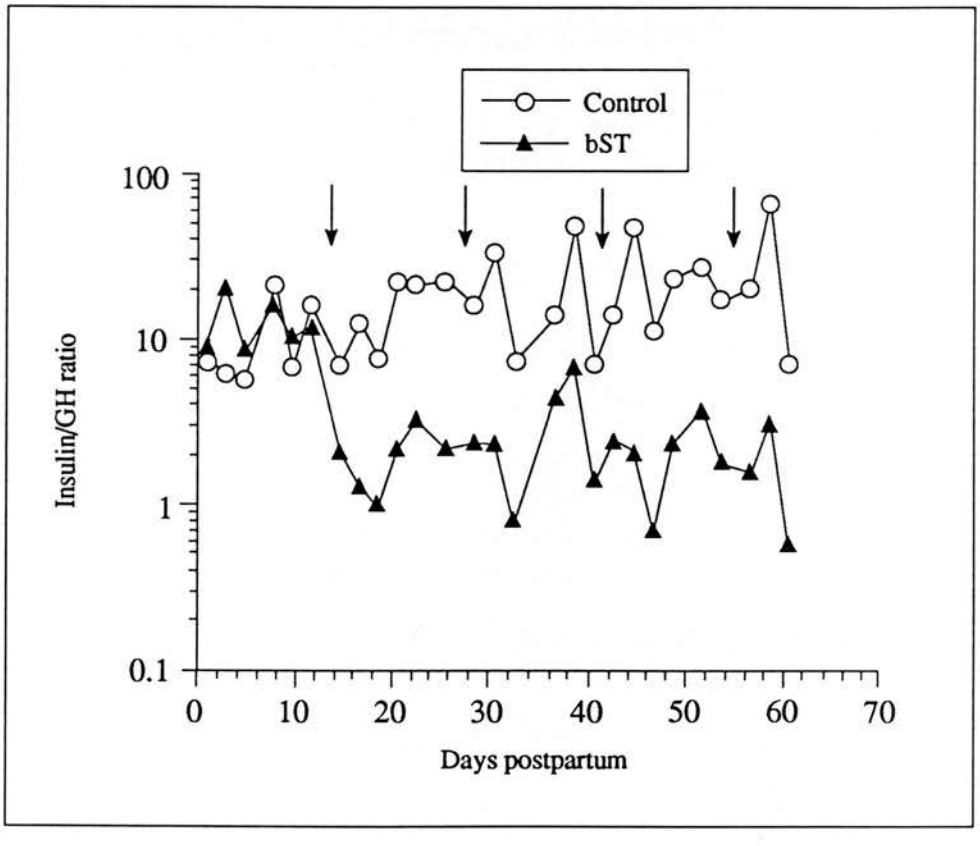
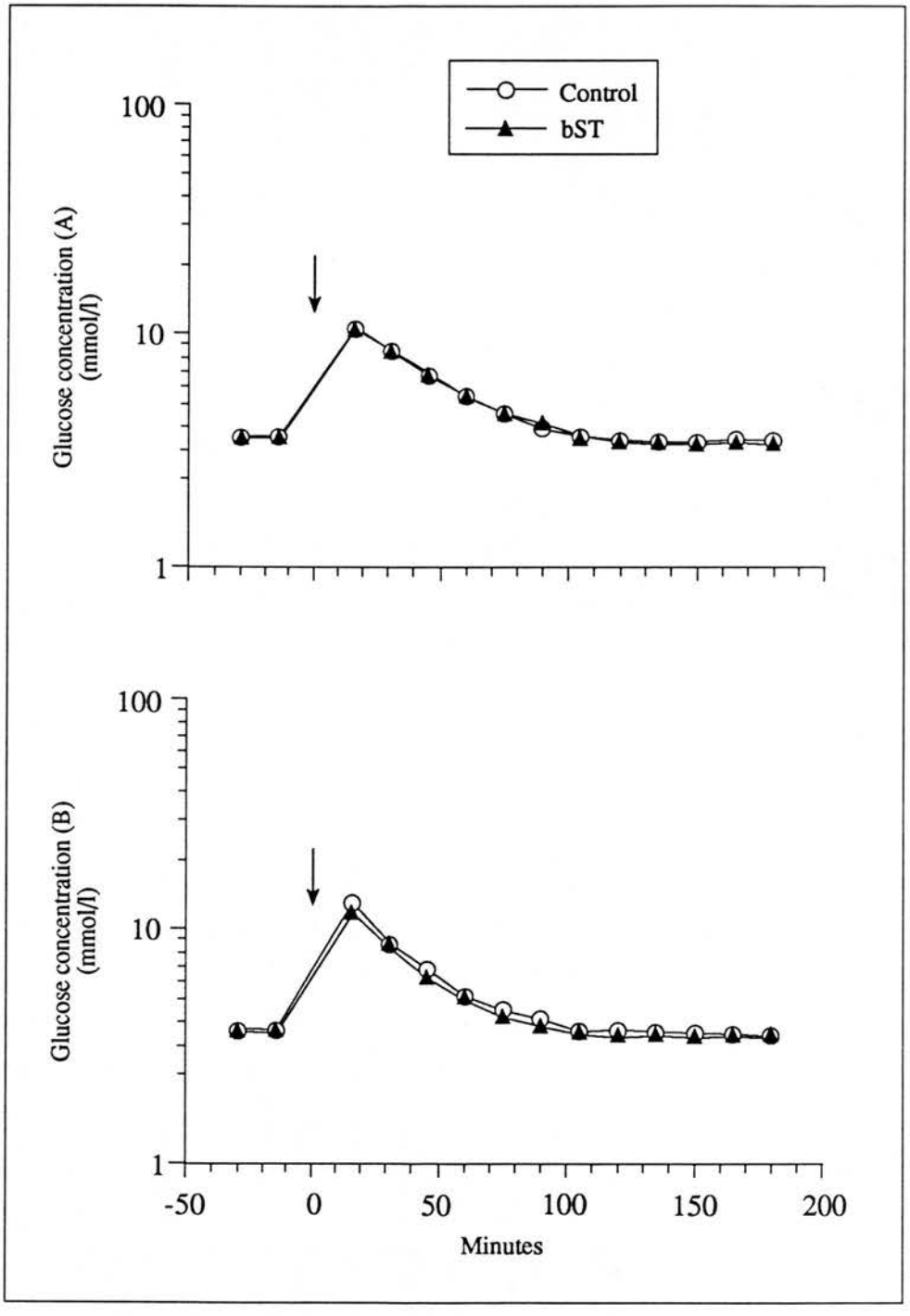


Fig 11 Mean plasma concentrations of glucose (back transformed values on a log scale) from 30 min before to 3 h. after injections of glucose (↓), at weeks 5 (A) and 8 (B) postpartum. s.e.d.s (expressed in log (values) units) were:
A: between treatments: 0.025; between sample collection times: 0.015
B: between treatments: 0.030; between sample collection times: 0.028.



#### 4.2.3.2- Insulin profiles

Plasma insulin profiles before and after glucose challenge at weeks 5 and 8 postpartum are summarised in Fig. 12 . At week 5 postpartum before glucose injection there was no significant difference between T and C cows in peripheral insulin concentrations. Following the injection of glucose, insulin profiles were similar; there was a significant increase ($p< 0.001$) in concentrations with maximal values being reached at 15 minutes after injection and returning to pre-infusion levels by 2 hours after injection.

At week 8 postpartum, plasma insulin concentrations (μU/ml) were higher in T than C cows both before (C: 11.80;T: 13.45; s.e.d. = 0.080; $p< 0.05$) and after glucose infusion (C: 97.05; T: 116.94; s.e.d.= 0.081; $p< 0.05$); (back transformed log (units) concentrations in parenthesis, except for the s.e.d.s which are in log (units)). Maximal insulin values (μU/ml) were recorded at 15 min. after glucose injection (C: 114.6 v. T: 187.4) and concentrations returned to pre-infusion levels within 2 hours in both treatments.

### 4.2.4- Gonadotrophin profiles at weeks 5 and 8 postpartum

#### 4.2.4.1- Plasma luteinizing hormone (LH) concentrations, LH pulse frequencies and amplitudes

Overall mean plasma LH (ng/ml) concentration and LH pulse frequency (pulses/hour) during the periods of frequent sample collection were not affected by treatment with bST (Table 12). However, there is a trend towards higher LH concentrations with increasing time postpartum (week 5: 0.37 v. week 8: 0.42 ng/ml; $p= 0.064$) and the LH pulse frequency was higher at week 8 than at week 5 postpartum (week 5: 0.133 v. week 8: 0.170; $p< 0.05$).

Mean LH pulse amplitude (ng/ml) was not affected by the bST treatment or

Fig 12 Mean plasma concentrations of insulin (back transformed values on a log scale) from 30 min before to 3 h. after injections of glucose (↓), at weeks 5 (A) and 8 (B) postpartum. s.e.d.s (expressed in log (values) units) were:
A: between treatments: 0.087; between sample collection times: 0.048
B: between treatments: 0.079; between sample collection times: 0.053.

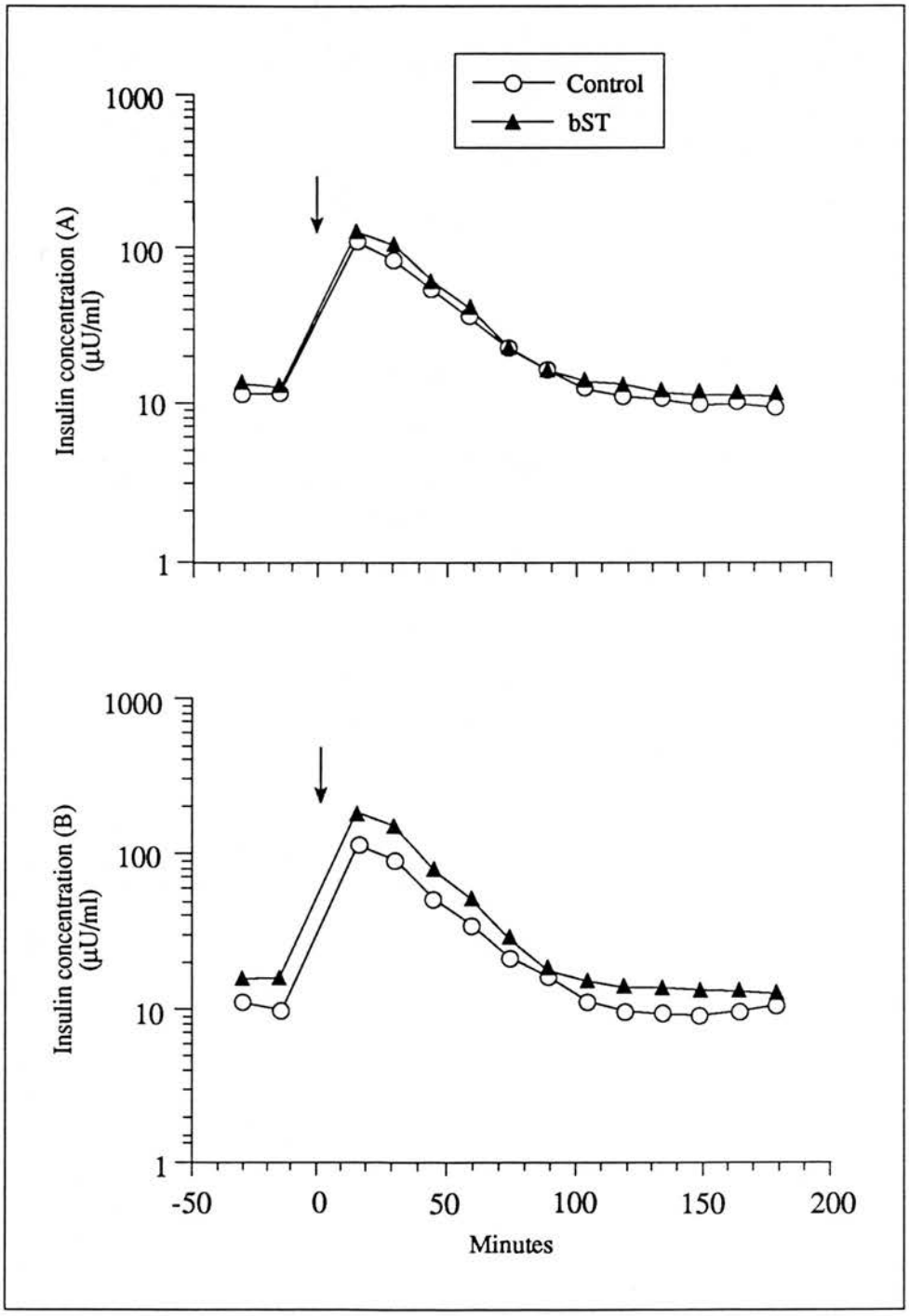


Table 12 Mean log (concentrations), of LH (ng/ml) and mean LH pulse frequencies (pulses/hour), and pulse amplitudes (ng/ml) at 5 and 8 weeks postpartum in control (C) and bST treated (T) cows. (Back transformed values are given in parenthesis; s.e.d.s are expressed in log units).

| | | | s.e.d. | | Significance | | |
|---|---|---|---|---|---|---|---|
| | C | T | Between treatments | Between weeks | Treatment | Week | Interaction |
| LH concentration | | | | | | | |
| week 5 | -0.428 (0.37) | -0.418 (0.38) | 0.033 | 0.055 | NS | p=0.064 | NS |
| week 8 | -0.411 (0.39) | -0.351 (0.45) | | | | | |
| LH pulse frequency | | | | | | | |
| week 5 | 0.13 | 0.14 | 0.020 | 0.029 | NS | * | NS |
| week 8 | 0.17 | 0.17 | | | | | |
| LH pulse amplitude | | | | | | | |
| week 5 | 1.28 | 1.24 | 0.163 | 0.189 | NS | NS | NS |
| week 8 | 1.38 | 0.92 | | | | | |

time postpartum (Table12).

#### 4.2.4.2- Plasma follicle stimulating hormone (FSH)

Mean plasma FSH (ng/ml) concentration during the periods of frequent sample collection at week 5 and week 8 postpartum were not affected either by treatment with bST or with time postpartum (Table 13).

### 4.2.5- Ovarian follicle populations and their physiological status

#### 4.2.5.1- Follicle populations and incidence of ovulation

The presence of corpora lutea on the ovaries of four cows, all in the bST treated group, indicated that they had ovulated before ovariectomy; the ovaries of these cows were considered to be in a different physiological state and so they were excluded from all subsequent analyses.

There was no significant difference between T and C cows in the numbers of small (3-7.9 mm diameter) or large (≥ 8 mm diameter) follicles present at nine weeks postpartum (Table 14). The incidence of ovulations in the C and T cows was not significantly different but ovulations were recorded only in T cows (0/15 v. 4/17; p=0.10).

Progesterone concentrations > 1.5 ng/ml were recorded in samples collected thrice weekly from parturition to ovariectomy in only 3 of the 4 animals that had ovulated in the bST treated group.

Table 13 Mean log (concentrations) of FSH (ng/ml) at 5 and 8 weeks postpartum in control (C) and bST treated (T) cows (Back transformed values are given in parenthesis; s.e.d.s are expressed in log units).

| | C | T | s.e.d. | | Significance | | |
|---|---|---|---|---|---|---|---|
| | | | Between treatments | Between weeks | Treatment | Week | Interaction |
| week 5 | 1.233 (17.10) | 1.226 (16.85) | 0.038 | 0.096 | NS | p=0.056 | NS |
| week 8 | 1.148 (14.07) | 1.200 (15.88) | | | | | |

Table 14 Mean number of small (3 - 7.9mm diameter) and large (≥ 8 mm diameter) ovarian follicles per cow at week 9 postpartum, number of animals which had ovulated and numbers of corpora lutea which were functional (associated with plasma progesterone concentrations ≥ 1.5 ng/ml).

| | C | T | s.e.d | Significance |
|---|---|---|---|---|
| No. Small follicles | 28.3 | 37.4 | 5.62 | NS |
| No. Large follicles | 1.7 | 1.8 | 0.29 | NS |
| Proportions of cows ovulating | 0/15 | 4/17 | - | p=0.10 |
| Proportion of cows with functional CL | 0/15 | 3/17 | - | NS |

### 4.2.5.2- Number of granulosa cells per follicle

At nine weeks postpartum the number of granulosa cells present in the large follicles were not significantly affected by bST treatment (Table 15).

Table 15- Mean log (numbers) of granulosa cells present in follicles ≥ 8 mm diameter at 9 weeks postpartum in control and bST treated cows. (Back transformed values are given in parenthesis; s.e.d.s are expressed in log units).

| Control | bST | s.e.d. | Significance |
|---|---|---|---|
| 7.095 (12,473,835) | 6.847 (7,046,930) | 0.262 | NS |

### 4.2.5.3- Intrafollicular concentrations of testosterone, oestradiol and IGF-I

The physiological state of the follicles was assessed by measurement of the steroid content of the follicular fluid. Measurements were made in the pooled follicular fluid from small follicles (3 - 7.9 mm diameter) and of individual large follicles (≥ 8mm diameter).

Testosterone and oestradiol concentration (pg/ml of fluid) in the pooled follicular fluid from small follicles were not affected by bST treatment (Testosterone: C: 13.53, T: 10.09; s.e.d.= 5.00; $p > 0.05$; Oestradiol: C: 57.07, T: 56.84; s.e.d.= 11.23; $p > 0.05$).

Testosterone concentrations in the follicular fluid of large follicles were not significantly affected by either bST treatment or follicle class (Table 16). Oestradiol concentrations were significantly higher ($p < 0.05$) in T than C cows in both non-oestrogenic (secreting < 10,000 pg/ml oestradiol) and oestrogenic (secreting > 10,000 pg/ml oestradiol) follicles (Table 16).

Table 16 Mean log (concentrations) of testosterone (pg/ml), oestradiol (pg/ml) and IGF-I (ng/ml) in follicular fluid of follicles ≥ 8mm at week 9 postpartum. Follicles were classed as non-oestrogenic ($E_2$ low; secreting < 10 000 pg per ml of follicular fluid) and oestrogenic ($E_2$ high secreting > 10 000 pg per ml of follicular fluid). (Back transformed values are given in parenthesis; s.e.d.s are expressed in log units).

| | C | | T | | | Significance | | |
|---|---|---|---|---|---|---|---|---|
| | $E_2$ low | $E_2$ high | $E_2$ low | $E_2$ high | s.e.d. | Treatment | $E_2$class | Interactions |
| n | 7 | 18 | 8 | 16 | | | | |
| Testosterone | 4.071 (11776.1) | 4.060 (11481.5) | 4.166 (14655.5) | 4.163 (14554.6) | 0.152 | NS | NS | NS |
| Oestradiol | 3.448 (2805.4) | 4.588 (38725.8) | 3.665 (4623.8) | 4.875 (74989.4) | 0.167 | * | * | NS |
| IGF-I | 1.972 (93.75) | 2.053 (112.97) | 2.417 (261.2) | 2.268 (185.4) | 0.117 | ** | NS | NS |

IGF-I concentrations in follicular fluid were higher in T cows ($p< 0.01$). There was no significant effect of follicle class (Table 16).

#### 4.2.5.4- Steroidogenic capacity of thecal tissue and granulosa cells

The underlying causes of the treatment differences in steroidogenic capacity were investigated by measuring the capacities of thecal and granulosa tissues to secrete testosterone and oestradiol respectively.

bST treatment did not affect testosterone production by thecal tissue incubated with or without supplementary hCG (Table 17). Tissue from oestrogenic follicles incubated with hCG had a higher rate of testosterone production than that of non-oestrogenic follicles ($p< 0.05$) (Table 17). A similar trend was recorded in tissues incubated in medium alone but it was not statistically significant.

Oestradiol production in granulosa cells incubated either in culture medium alone or with supplementary testosterone was not significantly affected by treatment or follicle class.

#### 4.2.5.5- LH and FSH receptor concentration in thecal and granulosa cells

Concentrations of LH and FSH receptors in thecal tissue were not significantly affected by bST treatment (Table 18). The concentration of LH receptors was higher ($p< 0.05$) in the thecal tissue of oestrogenic follicles than non-oestrogenic follicles, but there was no differences with follicle class in FSH receptors concentrations in thecal tissue.

LH receptor concentrations on granulosa tissue (Table 18) were lower ($p< 0.01$) in bST treated animals than control animals. LH receptor concentrations were higher ($p< 0.01$) in oestrogenic than non-oestrogenic follicles for both T and C cows.

Table 17 Mean log (secretion rates) of testosterone (pg/h/mg protein) by thecal tissue incubated in the presence or absence of hCG and of oestradiol (pg/h/200 000 cells) by granulosa cells incubated in the presence or absence of testosterone. Granulosa cells and thecal tissue were derived from follicles classed as non-oestrogenic ($E_2$ low; secreting < 10 000 pg per ml of follicular fluid) and oestrogenic ($E_2$ high; secreting >10 000 pg per ml of follicular fluid). (Back transformed values are given in parenthesis; s.e.d.s are expressed in log units).

| | C | | T | | | Significance | | |
|---|---|---|---|---|---|---|---|---|
| | $E_2$ low | $E_2$ high | $E_2$ low | $E_2$ high | s.e.d. | Treatment | $E_2$ Class | Interactions |
| No of follicles † | 5 | 18 | 6 | 16 | | | | |
| Testosterone (thecal tissue) | | | | | | | | |
| medium only | 2.112 (129.4) | 2.552 (356.5) | 2.207 (161.1) | 2.425 (266.0) | 0.292 | NS | NS | NS |
| + hCG | 2.326 (211.8) | 2.871 (743.0) | 2.321 (209.4) | 3.168 (1472.3) | 0.365 | NS | * | NS |
| No of follicles † | 4 | 14 | 4 | 12 | | | | |
| Oestradiol (granulosa cells) | | | | | | | | |
| medium only | 1.801 (63.24) | 1.959 (90.99) | 1.806 (63.97) | 2.217 (164.81) | 0.381 | NS | NS | NS |
| + Testosterone | 2.691 (490.9) | 2.751 (563.6) | 2.633 (429.5) | 2.792 (619.4) | 0.161 | NS | NS | NS |

† - insufficient tissue available for culture in some follicles

Table 18 Mean log (concentrations) (pg hormone bound per mg tissue) of LH and FSH receptors in thecal and granulosa tissue. Granulosa and thecal tissue were derived from follicles classed as non-oestrogenic ($E_2$ low; secreting <10 000 pg per ml of follicular fluid) and oestrogenic ($E_2$ high; secreting >10 000 pg per ml of follicular fluid). (Back transformed values are given in parenthesis; s.e.d.s are expressed in log units).

| | C | | T | | | Significance | | |
|---|---|---|---|---|---|---|---|---|
| | $E_2$ low | $E_2$ high | $E_2$ low | $E_2$ high | s.e.d. | Treatment | $E_2$ Class | Interactions |
| No of follicles † | 6 | 18 | 5 | 12 | | | | |
| Thecal tissue | | | | | | | | |
| LH receptor | 1.758 (57.3) | 1.937 (86.5) | 1.602 (39.9) | 1.900 (79.4) | 0.118 | NS | * | NS |
| FSH receptor | 0.802 (6.34) | 0.521 (3.32) | 0.291 (1.95) | 0.569 (3.70) | 0.464 | NS | NS | NS |
| No of follicles † | 7 | 18 | 5 | 13 | | | | |
| Granulosa tissue | | | | | | | | |
| LH receptor | 1.713 (51.64) | 1.975 (94.40) | 0.087 (1.22) | 1.806 (63.97) | 0.279 | ** | ** | ** |
| FSH receptor | 1.612 (40.92) | 1.757 (57.14) | 1.040 (10.96) | 1.834 (85.90) | 0.425 | NS | NS | NS |

† Insufficient tissue available for receptors in some follicles

There was a significant ( $p< 0.01$) treatment × class interaction with respect to LH receptor concentrations in granulosa cells; this was attributable to the very low value in the non-oestrogenic follicles of T cows.

Concentrations of FSH receptor in granulosa tissue were unaffected by bST treatment (Table 18). There was no significant effect of follicle class and no significant treatment × follicle class interaction.

## 4.3- Discussion

In this study, the imposed prepartum levels of feed intake were effective in achieving a moderately low level of body condition (BC) at calving, and this was maintained thereafter. Reproductive responses to contemporary nutritional treatments are maximal in low condition cows (Wright, *et al.*, 1992b) and so it is postulated that the effects of GH may also be maximal in animals in low body condition.

bST treatment induced a higher rate of milk production that was reflected in an increase in the average daily weight gain of calves of bST treated cows. The difference (13.2%) in daily weight gain of C and T calves from week 2 until week 9 postpartum is similar to the reported increase in milk production (15%) after bST treatment in dairy cows (McDowell, 1991). Clearly, since T cows had produced more milk but have the same feed intake, they must have been subjected to a greater degree of negative energy balance. The degree of change in GH profiles attributable to such differences in energy status would probably have been small compared with that due to bST treatment *per se*. It is therefore likely that the observed differences in ovarian function would be primarily attributable to bST treatment and its associated effects on hormone profiles.

### Effects of bST treatment on metabolic hormone profiles and IGF-I profiles and their significance

The GH concentrations for both bST and vehicle-treated cows in this study were lower than those reported for heifers (Gong, *et al.*, 1993), but similar to those

reported for cows (McShane, Schillo, Estienne, Boling, Bradley and Hall, 1989; Plouzek and Trenkle, 1991; Schams, Winkler, Theyerl-Abele and Prokopp, 1989) indicating that GH levels may be lower in adult cows than in young or growing cattle.

The observations that bovine somatotrophin (bST) increases twinning rates in lactating dairy cows (Butterwick, *et al.*, 1988), and that in heifers treated with bST there is an increase in the population of antral follicles, without changes in circulating gonadotrophin concentrations (Gong, *et al.*, 1991), indicate that GH may be involved in the control of ovarian function in cattle.

In the present study bST treatment was effective in raising peripheral GH concentrations within 24 h. of insertion of each new, slow release, depot injection, as reported by Gong, *et al.* (1993) and elevated concentrations were maintained through the postpartum period. In the control animals, GH concentrations were relatively constant throughout the experiment, and the small changes observed, reflect the pulsatile nature of the GH (Bass, *et al.*, 1992; Ross and Buchanan, 1990).

GH has an important role in the regulation of IGF-I in the liver, the most important site of IGF-I synthesis (D'Ercole, *et al.*, 1984; Hua, *et al.*, 1993). In this study, bST treatment increased peripheral concentrations of IGF-I within two days of each injection and concentrations of IGF-I in plasma were increased approximately 2 to 3-fold by bST treatment. Higher values were recorded throughout the experiment in treated cows compared with control cows. Both basal and bST-elevated IGF-I concentrations in this study were similar to those reported in previous studies in cattle (Cohick, Plaut, Sechen and Bauman, 1989; De La Sota, Lucy, Staples and Thatcher, 1993; Gong, *et al.*, 1991; Gong, *et al.*, 1993).

bST is known to act as a counter-regulatory hormone to insulin (Prosser and Mephan, 1989) but bST treatment usually has no effect on serum insulin concentrations in lactating animals (McDowell, 1991) and the results of the present experiment broadly confirm this. However, the recent studies of Gong, *et al.* (1991)

and Gong, *et al.* (1993) in heifers, showed that treatment with bST significantly increased peripheral insulin concentrations and in the present study the data showed a similar trend. The reasons for the discrepancies in insulin levels after bST treatment remain unclear, but differences in the energy status of the animals may affect the response.

In this study, the pattern of insulin concentrations during the postpartum period was similar to that of the previous experiment; the greater interval between feeding times on Sunday and Monday resulted in lower insulin concentrations every Monday than on other sampling days, and the trend towards higher insulin concentrations with increasing time postpartum is consistent with increased anabolism.

Several lines of evidence suggest that GH and possibly IGF-I may play a significant role in regulating the development and maturation of ovarian follicles (Adashi, *et al.*, 1985; Butterwick, *et al.*, 1988; Gong, *et al.*, 1991). Indeed, in this study, oestradiol concentration in the follicular fluid of large follicles was significantly increased by bST treatment. This finding is consistent with that of Yoshimura, *et al.* (1993) which showed that GH acts on the rabbit ovary to stimulate ovarian oestradiol production *in vitro* and with those of Barreca, *et al.* (1993) which showed that GH treatment increases oestradiol production *in vivo* and *in vitro* in human follicles.

In this study, bST treatment was shown to elevate circulating IGF-I levels probably by stimulating the production of IGF-I in the liver (D'Ercole, *et al.*, 1984) and at the same time enhanced IGF-I concentrations in follicular fluid of large follicles. Studies *in vitro* have shown that ovarian granulosa cells can secrete IGF-I (Adashi, *et al.*, 1985; Hammond, Hsu, Monsdchein and Canning, 1988; Schams, Koll and Li, 1988; Spicer, *et al.*, 1988) and that IGF-I stimulates granulosa cell mitosis and steroidogenesis. In the present study, bST not only increased the concentrations of oestradiol in follicular fluid but also induced ovulation in 4 of 17 animals, a further indicator that it enhanced ovarian function. This effect could be due to a direct action of bST or to an indirect effect on metabolism and nutrient availability.

The relationship between insulin and GH concentrations is a key factor in the control of nutrient utilization; insulin decreases the availability and/or tissue utilization of energy providing substrates while GH increases it (Trenkle, 1981). Changes in the insulin:GH ratio may affect reproductive function. Downing and Scaramuzzi (1991) showed that availability of energy-providing substrates can affect reproduction, since they demonstrated that increases in ovulation rate in sheep, rather than being related to changes in gonadotrophin profiles, correlated with the magnitude of increases in plasma insulin concentrations after infusion of glucose or branched chain amino acids. In this study, the insulin:GH ratio before bST treatment was similar for the two groups of cows. After bST implants were inserted, the higher GH level in bST treated cows, combined with low and fairly stable insulin levels throughout the experiment, resulted in a lower insulin:GH ratio in T than C cows. Thus availability to tissue of energy-providing substrates may also have been higher in T cows and their pattern of utilization may have been changed.

Glucose is the main source of energy utilized by the neural system and this is intimately involved in the control of reproductive activity (Schillo, 1992). Availability of glucose is, arguably, a crude index of the availability to tissues of oxidizable metabolic fuels. Plasma glucose concentrations have been found to be higher in high condition/well fed sheep injected with ovine somatotropin than in control animals (Bassett and Wallace, 1966). By contrast in the present study neither basal levels of glucose nor the rate of disappearance of glucose after infusion differed between T and C cows at either week 5 and 8 postpartum.

McDowell (1983) has suggested that bST may affect plasma concentration of glucose indirectly via its insulin-antagonizing activity or directly by promoting gluconeogenesis. The similar rate of glucose clearance combined with similar basal concentrations suggest that glucose production was not altered by bST treatment. This may be because the cows were in low body condition, the demands for lactation were

also high and the rations supplied only liveweight maintenance and milk production requirements, and enhanced glucose production may have been an inappropriate metabolic response. However, irrespective of the reasons for this response to bST, it may be concluded that bST did not affect ovarian function through changes in availability of this source of energy providing substrates.

bST could also affect ovarian function through changes in insulin profiles *per se*. Supporting the idea that insulin may affect ovarian function, Langhout, *et al.* (1991) in a recent study have shown that GH stimulated bovine granulosa cells proliferation and steroidogenesis *in vitro* only when given together with insulin. Since insulin can bind to type-I IGF-I receptors with a lower affinity (Adashi, *et al.*, 1985), it was proposed that the effects of insulin on granulosa cells were mediated via cross reaction with type-I IGF-I receptors (Davoren, *et al.*, 1986). However Gong (1992) suggested that the effect of insulin on ovarian function could be mediated through its own receptor, rather than by cross-reacting with type-I IGF-I receptors. Several other studies also suggest that insulin plays a role in the control of ovarian function (Adashi, *et al.*, 1985; Gong, *et al.*, 1991; Poretsky and Kalim, 1987; Webb and McBride, 1991).

The effect on insulin concentrations in thrice weekly samples in the present study was equivocal with a trend towards higher concentrations in bST treated cows. Neither basal levels of insulin nor the rate of disappearance of insulin after glucose infusion differed with treatment group at week 5 postpartum. At week 8 postpartum the basal insulin concentrations were higher in T cows both before and after glucose infusion, although the rate of disappearance was similar. The results of the present study are not clear cut, but it is possible that insulin is directly involved in mediating the effects of bST on ovarian function since it may have an effect which is independent of the insulin:GH ratio and effects on nutrient utilization. This requires further investigation.

In summary, bST treatment affected ovarian function (enhancing oestradiol production) and this could be due to a direct action of GH. The higher levels of IGF-I in circulation and in follicular fluid in bST treated cows also suggest that the effect of GH was mediated through changes in IGF-I profiles. bST did not affect ovarian function through changes in availability of energy providing substrates such as glucose.

**Effects of bST treatment on gonadotrophin profiles and their significance**

In addition to putative direct effects, bST treatment could affect reproductive activity through changes in gonadotrophin profiles which control ovarian follicle development (Ireland, 1987). The postpartum anoestrous period in beef cows is characterized by a low frequency pulsatile secretion of LH (Short, *et al.*, 1990). In this study, bST treatment did not change mean LH and FSH concentrations, LH pulse frequency or LH pulse amplitude compared with control animals, a finding which extends the observations of Gong, *et al.* (1991) on heifers. On the other hand, Schemm, Deaver, Griel and Muller (1990), working on lactating dairy cows, indicated that LH pulse frequency during the early follicular phase of the first treatment oestrous cycle was slightly increased ($p=0.06$) by bST treatment. However, by the third oestrous cycle these treatment differences were no longer present. Failure to detect any difference in LH pulse frequency in the present study could be due to the fact that changes following bST treatment were transient or to an inadequate sampling regime. However, studies of Wright, *et al.* (1987) and Wright, *et al.* (1992c), using the same sampling regime were effective in detecting differences in LH pulse frequencies, indicating that the regime was adequate.

In summary, although bST treatment enhanced ovarian function in the present experiment, gonadotrophin profiles were not affected. It is therefore concluded that the

effects of bST on ovarian function are unlikely to be mediated primarily through changes in circulating gonadotrophin concentrations.

**Effects of bST on gonadotrophin receptors**

Gonadotrophin action depends not only on circulating concentrations but also on the capacity of the target tissue to 'read' the signal. This depends, in part, on the concentration of receptors in the target tissue.

LH receptor concentrations in thecal tissue were not affected by bST treatment as reported by Gong, *et al.* (1991) who also showed no effect of bST treatment on LH receptors in granulosa cells from heifer ovaries. IGF-I acts synergistically with FSH in granulosa cells to induce receptors for LH (Adashi, *et al.*, 1985). In this study, despite the presence of elevated IGF-I concentrations in both plasma and follicular fluid of bST treated cows, there were fewer LH receptors in granulosa cells of follicles of T cows compared with those from follicles of control animals, suggesting that bST treatment depressed LH receptor concentrations in granulosa cells.

FSH receptors in both thecal and granulosa tissue were also unaffected by bST treatment as reported for heifers by Gong, *et al.* (1991). The low concentration of FSH receptors in thecal cells compared to granulosa cells may be considered normal (Carson, Findlay, Burger and Trounson, 1979; Gong, *et al.*, 1991; Staigmillar, *et al.*, 1982).

Since numbers of LH and FSH receptors in both theca and granulosa tissue were not influenced by bST treatment, it may be concluded that the effects of bST on ovarian function were not mediated through changes in these or indeed through changes in the influence of gonadotrophins.

**Effects of bST treatment on follicles**

The mean number of small follicles (3-7.9 mm. diameter) present in this study at nine weeks postpartum was not affected by bST treatment, although there was a

trend towards a greater number of follicles in bST treated cows. This observation contrasts with the findings of Lucy, *et al.* (1993) in lactating dairy cows and with the studies of Gong, *et al.* (1991) and Gong, *et al.* (1993) in which bST treatment increased the population of small antral follicles (2-5 mm. diameter) in mature heifers. The numbers of small follicles were positively correlated with the peripheral concentrations of IGF-I and insulin (Gong, *et al.*, 1991).

One possible reason for the differences in response between the present study and those of Gong, *et al.* (1991) and Lucy, *et al.* (1993) is the difference in GH profiles. In the present study there was a decline in circulating GH concentrations following bST injection, while in the earlier studies, in which daily injections of 25 mg of bST were administered, concentrations were more consistently elevated. A second possibility is the fact that there were differences in the classifications of small follicles used in the two studies. In the present study follicles less than 8 mm. diameter (3-7.9 mm. diameter) were included in the "small class", because follicles below this size cannot ovulate (Spicer, *et al.*, 1986a). Different classifications of small follicles were used by Gong, *et al.* (1991) (2-5 mm. diameter) and Lucy, *et al.* (1993) (3-5 mm. diameter). This may also have contributed to the difference in response.

In this study, bST treatment did not change the number of large follicles ($\geq$ 8 mm. diameter) present at ovariectomy compared with control cows. This suggests that follicular growth *per se* was not enhanced by bST treatment. This observation is consistent with the findings of Murphy *et al.* (1990) who reported that the prolonged anoestrus of postpartum beef cows was not due to a lack of large morphologically dominant follicles but was presumably due to a physiological inadequacy. However, measurements of this sort do not reveal effects of treatment on the underlying physiology of the follicles.

The mean numbers of granulosa cells per follicle at week 9 postpartum present were similar to those reported by Ireland and Roche (1983a) in cyclic heifers and

McNatty, *et al.* (1984a) in cyclic cows. The observed trend towards smaller numbers of granulosa cells in large follicles of bST cows compared with control cows is consistent with the previous work of Gong (1992), which showed that bST treatment inhibited the proliferation of granulosa cells from large follicles *in vitro*. Carson, *et al.* (1989) has shown that mitosis of granulosa cells is inversely related to their differentiation. The results from the present study suggests that granulosa cells were more differentiated, since they were producing more oestradiol, and this in turn becames inhibitory to mitosis reducing the number of granulosa cells. Since the numbers of granulosa cells present in the large follicles at week 9 were similar to the numbers of granulosa cells present in cyclic animals (Ireland and Roche, 1983a; McNatty, *et al.*, 1984a), it may be concluded that this was not a limiting factor with respect to follicle function and capacity to ovulate.

Like follicle size, granulosa cell numbers do not provide a full description of follicle function. One important measure of follicle physiology is the capacity to synthesize steroids and, in particular, oestradiol (Hanzen, 1986). Steroid production depends on the influence of gonadotrophins which act via the local production of steroid hormones and a wide variety of growth factors within the follicle (Tonetta and diZerega, 1989).

In the present study, the rates of production of testosterone and oestradiol by small follicles (3-7.9 mm. diameter) were not affected by bST treatment as indicated by intrafollicular concentrations. Thus it is unlikely that it is through changes in steroid concentrations in the follicular fluid of small follicles that bST affects the development of large follicles.

In this study, oestradiol concentrations in the follicular fluid of large follicles ($\geq$ 8 mm. diameter) at week 9 postpartum were significantly increased by bST treatment in both oestrogenic and non-oestrogenic follicles. These results suggests that bST enhanced ovarian function.

Since oestradiol production was enhanced in both non-oestrogenic and oestrogenic/potentially ovulatory follicles, it is likely that bST caused a general increase in oestradiol synthesis and did not merely accelarate the development of pre-ovulatory follicles.

Testosterone concentrations in the follicular fluid of large follicles were not affected by bST treatment and, since testosterone is the primary precursor for oestrogen synthesis, it is concluded that the observed enhancement of oestradiol levels in follicles of bST treated cows was not due to the presence of greater concentrations of testosterone precursor. It remains to be determined whether rate of testosterone synthesis was enhanced (since this does not necessarily equate with intrafollicular concentration), although the absence of any difference with treatment in follicular testosterone concentrations may indicate that this is unlikely.

The intrafollicular concentration of IGF-I in both oestrogenic and non-oestrogenic follicles, like oestradiol concentrations, were significantly increased by bST treatment. Oestrogenic follicles produced more oestradiol and less IGF-I than non-oestrogenic follicles as reported by Wiedemann, Schwartz and Frantz (1976) who found that IGF-I production can enhance oestradiol production, but when higher levels of oestradiol are reached they can inhibit IGF-I production. The results of Spicer, *et al.* (1988) do not support the hypothesis that IGF-I levels in follicular fluid enhance oestrogen biosynthesis *in vivo*, since they found that large increases in concentrations of oestradiol in follicular fluid were not associated with increases in levels of IGF-I during spontaneous and GnRH-induced follicular development in cattle. However, it is possible that changes can occur in the interrelationship between IGF-I and oestradiol with changing of stage of maturation. Is not clear whether or not the higher concentrations of IGF-I in follicular fluid, stimulated by bST treatment, affected ovarian function. However, the enhanced oestradiol concentrations in these follicles suggests that a role of IGF-I in the ovary is likely to exist.

The observations from the present study have not shown whether the effects of GH on oestradiol production are exerted directly on the ovary or mediated through increases in the hepatic and/or intrafollicular production of IGF-I. Yoshimura, *et al.* (1993) working on rabbits suggested that there was an effect of GH on oestradiol production on the ovary, independent of the effect of gonadotrophins. However, they also observed that addition of GH to the ovary perfusate also stimulated ovarian tissue concentration of IGF-I. The observation of higher IGF-I concentrations in bST-treated cows in the present study supports this finding. Barreca, *et al.* (1993) suggested that GH exerts its effect on the ovary, not only by inducing IGF-I synthesis but also by inducing the initial differentiation that makes the cells more sensitive to the actions of IGFs. It is concluded that in the present study the effect of GH on the ovary was probably mediated partly through IGF-I.

It is not clear whether or not the higher levels of IGF-I in circulation in bST treated cows can affect intrafollicular IGF-I concentrations and follicular function. However, support for the suggestion that IGF-I production by liver and granulosa cells may be differentially regulated is provided by the observations of Spicer, Crowe, Prendiville, Goulding and Enright (1992) that during short periods of feed withdrawal in cattle, IGF-I concentrations in blood decreases, whereas IGF-I concentrations in follicular fluid remain unchanged. Further work is required in order to establish the nature of relationship between IGF-I in circulation and intrafollicular IGF-I.

### Steroidogenic production *in vitro*

The effects of bST/GH and/or IGF-I on intrafollicular oestradiol concentrations depend on rate of synthesis and rate of loss from the follicle fluid. The capacity of the granulosa cells to synthesize testosterone and oestradiol was investigated *in vitro*, since this is an environment largely devoid of GH and IGF-I influences.

Testosterone production by thecal tissue incubated in medium alone or with hCG was not affected by bST treatment in this study since the pattern of testosterone

synthesis reflected intrafollicular concentrations. Addition of hCG to the thecal tissue did not affect the response to treatment but enhanced testosterone production. This suggests that gonadotrophic support to thecal tissue was not differentially deficient in the two treatments.

Oestradiol production by granulosa cells when incubated in medium alone or with testosterone, was not affected by bST treatment or follicle class in apparent contradiction to the observed differences in follicle fluid oestradiol concentrations. However, it is known that when granulosa cells are in culture, they are not under normal regulatory pressures (Henderson, *et al.*, 1987). Since bST treatment raised GH concentrations and resulted in increased oestradiol production within the follicle but was not able to increase oestradiol secretion *in vitro*, these observations are indicative of an effect of GH within the follicle which was absent from the culture *in vitro*. It is postulated that the absence of the bST-enhanced IGF-I stimulus in the culture may be the reason for the lack of treatment effect on oestradiol synthesis in culture, while in the intact follicle, in which IGF-I concentrations were elevated by bST treatment, oestradiol concentrations were elevated.

Speculation concerning IGF action requires care, since their effects depend on the insulin-like growth factor binding proteins (IGFBPs), high affinity carrier proteins that circulate in blood and extracellular fluids and control IGF transport and the availability of IGFs to tissues (Clemmons and Underwood, 1991); some studies have demonstrated inhibitory effects of IGFBP on IGF actions (Baxter, 1988). Further work is required to elucidate these mechanisms.

**CL function**

Preovulatory follicles which are not fully functional may result in abnormal CL. One of the bST treated cows that had ovulated failed to develop a functional CL. Since oestradiol concentrations are indicative of the "health" of the follicles and the "health" of the follicles indicates the "health" of the CL, it is suggested that lack of

adequate oestradiol concentrations could lead to an altered follicular development and resulted in non functional CL. Failure to develop a functional CL could also be due to a deficiency in granulosa cells populations since McNatty, *et al.* (1984a) suggested that larger CL were derived from preovulatory follicles with greater numbers of granulosa cells than small CL; there is no evidences in the literature but it is possible that larger CL may also produce more progesterone.

## Conclusions

In conclusion the results from the experiment indicate that bST treatment enhanced follicular steroidogenesis and follicle maturation as postulated. This is indicated by the fact that 4 of the bST treated cows had ovulated and that follicular oestradiol concentrations were significantly enhanced. This effect does not seem to be mediated through changes in gonadotrophin profiles, gonadotrophin receptor levels, numbers of granulosa cells present in large follicles or through changes in the numbers of small follicles or their function. bST may act via increased IGF-I concentrations in plasma and/or follicular fluid resulting in enhanced oestradiol synthesis or through direct effects of bST at the ovarian level. However the exact mechanism through which bST or GH affects ovarian function requires further study.

# CHAPTER 5

## General Discussion

While the application of ultrasound observations makes possible the determination of growth patterns of ovarian follicles without damaging the ovary (Knopf, *et al.*, 1989; Pierson and Ginther, 1984; Savio, *et al.*, 1988; Sirois and Fortune, 1988), they do not provide information on the intrafollicular hormone concentrations and physiological state of follicles that are provided by studies involving ovariectomy and follicle dissection. However, ovariectomy has the disadvantage that information is only obtained at a single time point of follicle growth. Further progress in understanding the mechanisms that affect ovarian function requires both approaches.

Neither bST treatment nor BC affected number of follicles, one of the few measurements that can be made in the intact animal; this highlights the limitations of the "non-destructive" approach. More detailed study showed that, while the numbers of granulosa cells, were not affected by treatments, across the two experiments, there was a trend towards a greater number of granulosa cells in large follicles at week 9 postpartum than at week 7 irrespective of treatment and BC. This suggests that number of granulosa cells increase with time postpartum. This may be a necessary part of follicle maturation and thus ovulation.

The experiment in Chapter 3 demonstrated that infusion of GnRH pulses enhanced ovarian function as indicated by the occurrence of ovulation in 10 out of 12 treated cows, compared with only 1 out of 11 or 12 controls. These results appear consistent with the suggestion of Wright, *et al.* (1987) and Wright, *et al.* (1990) that

the effect of body condition on the length of the postpartum anoestrous period might be mediated through LH pulse frequency. On the other hand, in apparent contradiction to the involvement of LH pulse frequency in mediating the effects of body condition on reproductive activity, the results from the second experiment and those obtained by Rhind, *et al.* (1992) showed no differences in LH pulse frequency with bST treatment or BC, which suggests that the effect of body condition on follicle development is not mediated through differences in gonadotrophin profiles alone. Furthermore, the effects on ovarian function of BC or bST treatment acting in conjunction with the gonadotrophins cannot be explained by changes in FSH or LH receptor concentration in either granulosa or thecal tissue. The results are not irreconcilable; ovarian function is dependent on gonadotrophin support (Roche, *et al.*, 1992) and the results of the GnRH infusion study indicate that in the postpartum cow that support is normally insufficient to achieve maturation and ovulation, particularly in low body condition cows. Furthermore supplementation of the support through GnRH infusion enhanced the relatively slow process of follicular development in cows in low BC. However, ovarian responses to gonadotrophins are clearly modified by other components of the hormonal milieu, as indicated by the differential ovarian response of bST treated and control cows which had similar gonadotrophin profiles.

While availability of energy-providing substrates can affect reproduction (Downing and Scaramuzzi, 1991), concentrations of glucose, the main source of energy utilized by the neural system, were not affected by bST treatment and it may be concluded that bST did not affect ovarian function through changes in circulating levels of this substrate. Although, bST treatment lowered the insulin:GH ratio and might be expected to have modified the availability to tissue of energy providing substrates, the rate of clearance from the circulation of glucose was not affected and so it is concluded that changes in this factor were not responsible for the observed differences in ovarian function. The effects of the metabolic hormones on follicle

physiology are likely to be due to the action of the hormones themselves and not to any associated changes in nutrient utilization.

In the first experiment, the differences in BC that were achieved resulted in differences in metabolic hormones but these differences were not reflected in significantly altered ovarian function, although there was a trend towards higher oestradiol concentrations in follicular fluid in cows in high BC. These findings are consistent with the results of Prado, *et al.* (1990), which were based on small numbers of follicles but which also showed a greater incidence of highly oestrogenic follicles in cows in high BC compared with those in low BC. The results from the first experiment show a clear link between BC and metabolic hormone profiles but the relationship between BC and oestradiol remains unclear. Similarly, in the same experiment it was not possible to identify a clear relationship between metabolic hormones and oestradiol. In the second experiment, on the other hand, oestradiol concentration in the follicular fluid of large follicles were significantly enhanced by bST treatment, suggesting that GH affected ovarian function through effects on oestradiol synthesis, an index of follicle physiological state. Further research is required in order to learn more about the precise nature of the relationships between BC, metabolic hormones and steroid production.

The effect of bST on ovarian function did not appear to be mediated through changes in circulating gonadotrophin concentrations, ovarian gonadotrophin receptors, numbers of follicles, number of granulosa cells or a deficiency of testosterone substrate but bST treatment did significantly increase peripheral concentrations of GH and IGF-I and perhaps enhanced insulin, suggesting that these metabolic hormones may be involved in the control of ovarian function and oestradiol synthesis as indicated by previous studies (Adashi, *et al.*, 1985; Barreca, *et al.*, 1993; Poretsky and Kalim, 1987; Yoshimura, *et al.*, 1993).

It remains uncertain whether the effects of GH on oestradiol production are exerted directly on the ovary or mediated through increases in the hepatic and/or intrafollicular production of IGF-I. The observations from the present study support the idea that the effect of GH on the ovary was mediated through IGF-I. but it remains unclear whether the circulating levels of IGF-I can affect intrafollicular concentrations of IGF-I. Furthermore, the exact role of this peptide (IGF-I) in ovarian folliculogenesis is not fully understood. It may act as a local modulator of granulosa cell growth and function (Carson, *et al.*, 1989) but its role in the mediation of effects of BC or bST on follicle function require further investigation.

In summary, both gonadotrophins and metabolic hormones enhance ovarian function, increasing oestradiol production. Increased follicular IGF-I concentrations are thought likely to be responsible for the enhanced oestradiol production of large follicles in cows subject to metabolic hormone stimuli such as exogenous GH or changes in circulating concentrations induced by altered nutritional state.

**Futures approaches**

The limitations of ultrasound studies and of the more "in depth" approach of the present study have been highlighted. Ideally, it would be possible to repeatedly sample follicle fluid without destroying the follicle. Such a technique would make possible the description of sequential changes in steroid and growth hormone content of the intrafollicular fluid at different stages of postpartum period. Furthermore, it would be possible to introduce hormones and growth factors into the follicle and measure changes in physiological state of the follicle.

At present, such techniques have not been developed. However, some progress may be possible using less sophisticated techniques such as attaching microimplants containing hormones such as oestradiol or growth factors such as IGF-I, EGF, TGFs to the selected target organ and measuring responses (Hutz, 1989)

including number of follicles, number of granulosa cells, gonadotrophin receptors, steroid and/or proteins concentrations and growth factors.

Understanding of the effects of circulating hormones on the ovary may also be improved by further studies of ovarian tissues *in vitro*. The experimental protocols required to investigate the relationships between the several components of follicular fluid are much more complex than simple cultures, because when granulosa cells are in culture they are not usually under normal regulatory pressure (Henderson, *et al.*, 1987). This is supported by the results of the second experiment which showed significantly enhanced intrafollicular concentrations of oestradiol in bST treated cows but no significant effects on oestradiol production by granulosa cells cultured *in vitro*.

There is a need to assess the roles of a variety of growth factors such as IGF-I, EGF, TGFs and PDGF in processes of stimulation and inhibition of cell multiplication, differentiation and steroidogenesis. The effects of individual growth factors and combination of growth factors each in conjunction with various hormone concentrations (reflecting different stages of follicle development and metabolic states) need to be investigated using increasingly complex culture media 'recipes'.

Another aspect which requires investigation is the relationship between circulating IGF-I and intrafollicular IGF-I. This may be possible through the use of IGF-I radioisotopes to determine whether or not the effects of GH on the ovary are mediated through increases in hepatic IGF-I production, resulting in higher circulating IGF-I concentrations and in turn inducing an increased rate of transfer to the follicle. Alternatively the intrafollicular concentrations of IGF-I may be simply a function of intrafollicular IGF-I synthesis and unrelated to hepatic IGF-I production. Understanding of the mechanisms through which nutrition may affect ovarian function requires an understanding of the relationship between hepatic and ovarian IGF-I concentrations.

The prolonged postpartum period in beef cows is associated with infrequent LH pulses in early postpartum due to an infrequent GnRH pulse frequency. In order to overcome this problem and try to minimize the economic losses due to long postpartum periods in beef cows, GnRH treatments have been used. However, infusions of GnRH, as used in Experiment 1 is clearly not a pratical option for use in large beef herds. Until now, responses to injections or implants of GnRH have been variable. Treatments were applied when the follicular status of the animal was not known and Jagger, *et al.* (1987) suggests that the LH response to GnRH treatment is dependent on follicular status in the immediate pretreatment period. Furthermore, the ovarian response to LH will in turn depend on follicle status at the time.

While the application of slow release implants of GnRH for longer periods during postpartum may be sufficient to promote follicle maturation, it should be noted that delayed ovulation in cows in low BC could be regarded as a strategy for survival on the part of a cow during periods of environmental or physiological stress and manipulation of reproductive activity could affect other physiological mechanism in the cow such as subsequent milk production and reproductive activity. A better understanding of the endocrine control of ovarian activity will not only facilitate identification of suitable GnRH treatment regimes (times, doses) but may also provide insight into likely secondary effects.

It is now generally accepted that growth hormone either directly or indirectly stimulates anabolic processes, such as cell division, skeletal growth, and protein synthesis (growth promoting and galactopoietic activity) (Lean, Trout, Bruss and Baldwin, 1992). In dairy cows increases in milk yield due to bST treatment are associated with a decrease in pregnancy rate and an increase of days from parturition to conception (Butler and Smith, 1989) due to the reduction in the energy balance that occurs at this early stage of lactation (Bauman, 1992).

Recent findings suggest that in addition to these effects on tissue growth and metabolism, GH, alone or in combination with other factors, may have an important role in follicular growth. Indeed, in the second experiment bST was effective in enhancing follicular oestradiol production and to some extent, ovulation. bST treatment has also been reported to increase the number of small antral follicles (Gong, *et al.*, 1991; Gong, *et al.*, 1993) and enhance the superovulatory response of heifers to PMSG (Gong, 1992). Similarly, treatment of lactating dairy cows in early postpartum with bST increases the incidence of twin-calving (Butterwick, *et al.*, 1988).

While the desirability of multiple births, particularly in dairy cattle, is open to question (Johnson, Turman and Stephens, 1975; Reid, Wilton and Walton, 1986) the economics of this approach has been investigated (Davis, Harvey, Bishop and Gearheart, 1989) and twinning has been shown to be a viable economic proposition. Further studies are required in order to establish the potential of bST treatment for the reduction of the postpartum interval, induction of twinning or as a basis for improved and more predictable superovulation regimes.

A better understanding of the mechanisms controlling ovarian function will help maximize economic returns; the results presented in this thesis provide some new insight and provide a basis for further work.

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