POSTPARTUM ANOESTRUM

IN THE PASTURE GRAZED

NEW ZEALAND DAIRY COW

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Postpartum Anoestrum in the Pasture Grazed New Zealand Dairy Cow

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Abstract

Postpartum anoestrum (PPA) is that period following parturition before ovulatory ovarian cycles have been re-established. This period lasts 20 to 30 days in normal, well-fed dairy cattle. To achieve an average interval between calvings of 365 days so that calving continues to occur at an appropriate time of the year, cows must resume cyclic activity, display behavioural oestrus, be mated and conceive by 83 days postpartum. An extended period of PPA compromises achievement of this target. Extended periods of PPA may result from either a failure to resume ovulations (anovulatory anoestrus) or a failure of expression or detection of behavioural oestrus (non-detection of oestrus).

The study population for this thesis was drawn from the research herds of the Dairying Research Corporation, Hamilton and from commercial herds from the Waikato region around Hamilton.

All herds calved seasonally between July and September and were milked twice daily. First calving occurred at approximately 2 years of age (i.e. heifers) and thereafter at 365 d intervals. Nutrition was predominantly from ryegrass/white clover pasture. The 10 year average rainfall of the distribution 1230 mm with higher rainfall in spring than summer. Average daily temperature ranges from a low of 8-9 °C in July to a maximum of 18.3 °C in January.

It was demonstrated that primiparous cattle had a longer PPA (40.2 vs. 27.2 ± 6.2 days for 2 year old and older cows, mean ± SED, respectively) and that Friesians had longer PPA intervals than Jerseys (39.3 ± 3.1 vs. 27.9 ± 2.7 days, respectively). Increasing the stocking rate resulted in an increased PPA interval (30.2 ± 2.8 vs. 27.1 ± 2.9 days, for high and low stocking rates, respectively). Body condition score (1 = thin, 10 = fat) at calving was inversely related to the PPA interval (regression slope = -7.9 days, $P<0.05$). Cows that had not commenced cycling 1 week before the planned start of mating (PSM) had lower condition scores (-0.3 ± 0.1), smaller total ovarian mass (-1.3 ± 0.2, arbitrary units), higher serum urea concentrations (0.31 ± 0.16 mmol/L) and lower blood glucose concentrations (-0.14 ± 0.06 mmol/L) than cows which had ovulated by this time. Significant differences in the proportion of cows not detected in oestrus and anovulatory anoestrus were demonstrated among the 8 farms studied. These data indicate that age, breed, stocking rate, body condition score and between farm factors influenced the PPA interval.

Large ovarian follicles (>9 mm) were present by 10.3 (± 0.7) days postpartum and regular turnover of follicles occurred in the ovaries of all cows examined by daily transrectal ultrasound. An average of 4.2 (± 0.6) large follicles appeared before the first ovulation which occurred at 43.4 (± 5.3) days postpartum. The largest follicle in anovulatory cows had lower intrafollicular concentrations of oestradiol (E_2), testosterone (T) and progesterone (P_4) than in cycling cows when ovariectomy occurred at approximately 60 days postpartum (47 vs. 372 ± 2.1 ng/ml; 1.4 vs. 10.0 ± 2.3 ng/ml, and 7.8 vs. 16.0 ± 1.8 ng/ml for E_2, T and P_4, respectively). However, there were no differences in the diameters, the number of granulosa cells or the rates of growth of the largest follicles between the anovulatory and cycling cows.

A luteinising hormone (LH) surge and ovulation was induced in 10 of 10 and 9 of 10 anovulatory heifers, respectively, following treatment with 250 µg of gonadotrophin releasing hormone (GnRH) when the largest follicle was >10 mm in diameter and growing, at 3 to 4 weeks postpartum. Sufficient GnRH receptors and releasable LH must have been present in the pituitary and the largest follicle must have been sufficiently mature to ovulate in response to the LH surge. However, only 3 of 9 ovulating heifers continued to ovulate beyond the first, short (<10 day) cycle.

Oestradiol treatment (0.5 mg i.m.) of PPA cows when either a small, growing follicle (5 to 9 mm) or a large plateau follicle (>10 and ± 1 mm for 72 h) was present on the ovary resulted in 8 of 15 and 5 of 15 cows having an LH surge and ovulating, respectively. This illustrates that the E_2 positive feedback mechanism, a prerequisite for ovulation in a normal oestrous cycle, failed in nearly half of these PPA cows.

PPA cows had a lower LH pulse frequency and a higher LH pulse amplitude but similar mean LH concentration before and 3 and 10 days after ovariectomy compared to cyclic cows when ovariectomy occurred approximately 60 days postpartum. The LH parameters increased by a similar amount in the PPA and the cycling cows following ovariectomy. Exogenous E_2 treatment at 10 days post-ovariectomy resulted in a significant decrease in LH pulse frequency and an increase in LH pulse amplitude in the PPA but not the cycling cows. The GnRH pulse generator in the PPA cows appears to be suppressed by both ovarian and extra-ovarian factors. Additionally, hypersensitivity to negative E_2 feedback on LH pulse frequency was observed. Undernutrition and low body condition score have been hypothesised as contributing to increased negative E_2 feedback in cattle.

Treatment of anovulatory cows with P_4 for 5 days at 2 to 3 weeks postpartum resulted in a significant shortening of the intervals from calving to first ovulation, calving to first oestrus and calving to conception (30.7 ± 0.4 vs. 34.2 ± 1.0, 35.8 ± 2.6 vs. 40.0 ± 1.8 and 85.0 ± 3.0 vs. 93.4 ± 2.3 days, respectively, $P<0.05$) when compared with herdmates. Progesterone treatment produced a 'priming' effect, as the duration of the first postpartum luteal phase (9.5 ± 0.4 vs. 5.6 ± 0.9 days) and the proportion of cows detected in oestrus at the first postpartum ovulation (83.3 vs. 37.0, $P<0.05$) were both increased.

Treatment of anovulatory cows for 7 days with P_4 and 400 i.u. of equine chorionic gonadotrophin (eCG) increased the probability of first service and conception occurring, compared to untreated cows. Low body condition score at the time of treatment reduced the probability of first service and conception, but the increase in probability of first service or conception following P_4 and eCG treatment was the same among cows with either low or medium body condition score.

Supplementation of a white clover/ryegrass pasture diet with pasture silage did not alter the intervals from calving to first ovulation, calving to first oestrus or calving to conception when compared with control cows fed pasture only. Silage supplementation did reduce first service conception rate (37.5% vs. 53.3%, $P = 0.09$).

The proportion of cows not in oestrus by the date of the planned start of mating varied among herds possibly due to differences in the age structure, breed and nutritional management. Further research is required to identify management and animal factors associated with an unacceptably high proportion of the herd not detected in oestrus by this date. Failure of the E_2 positive feedback mechanism, low LH pulse frequency and low intrafollicular steroid concentrations were identified in PPA cows. Increased sensitivity of the E_2 negative feedback mechanisms due to depleted body fat reserves and/or poor postpartum nutrition associated with prolonged periods of negative energy balance postpartum may be the major mechanism for extended PPA. An understanding of the control of GnRH and LH release from the hypothalamus and pituitary respectively, will be required before the patho-physiology of PPA can be fully understood. Treatment of anovulatory cows either early (2 to 3 weeks) postpartum or immediately before the planned start of mating shortened PPA intervals. The mechanisms appear to involve a 'priming' effect on expression of behavioural oestrus and on the length of the first luteal phase.

This thesis increases the understanding of the factors that influence the PPA interval, the endocrinology of PPA and the treatment of PPA cows.

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McDougall S, Clark DA, Macmillan KL and Williamson NB (1994) Some effects of feeding pasture silage as a supplement to pasture on reproductive performance in lactating dairy cows *New Zealand Veterinary Journal*

McDougall S, Burke CR, Macmillan KL and Williamson NB (1994) Follicle patterns during extended periods of postpartum anovulation in pasture-fed dairy cows *Research in Veterinary Science*

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Abbreviations

AA	Anovulatory anoestrus
BOH	β-hydroxy-butyrate
BSA	Bovine serum albumin
C_con	Calving to conception interval (days)
C_h1	Calving to first oestrus interval (days)
CI	Confidence interval(s)
CL	Corpus luteum
C_ovn1	Calving to first ovulation interval (days)
C_s1	Calving to first service interval (days)
CS	Body condition score
DF	Dominant follicle
DMD	Dry matter disappearance
DMI	Dry matter intake (kg/cow/day)
E_2	Oestradiol 17-β
eCG	Equine chorionic gonadotrophin
EDTA	Ethylenediamine tetraacetate
EV	Oestradiol valerate
FSH	Follicle Stimulating Hormone
GH	Growth Hormone
GnRH	Gonadotrophin Releasing Hormone
h	hours
H-P-O	Hypothalamic-pituitary-ovarian
i.m.	intramuscular
i.v.	intravenous
IGF	Insulin-like Growth Factor
IGFBP	Insulin-like Growth Factor Binding Proteins
LH	Luteinising Hormone
MPA	Medroxyprogesterone acetate
NDO	Not detected in oestrus
NEB	Negative energy balance
NEFA	Non Esterified Fatty acids
ODB	Oestradiol benzoate
P_4	Progesterone

PBS	Phosphate buffered saline
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PP	Postpartum
PPA	Postpartum anoestrum
PSC	Planned start of calving
PSM	Planned start of mating
PSM-7	7 days before the planned start of mating
PSM_con	Planned start of mating to conception
PSM_s1	Planned start of mating to first service
RIA	Radioimmunoassay
s.c.	Subcutaneous
s/c	Services/successful conception
sem	Standard error of the mean
SED	Standard error of the difference
SR	Stocking rate
T	Testosterone
TT4	Total thyroxine

CHAPTER 1:

Introduction

What is postpartum anoestrum (PPA)?

Cows undergo ovulation and oestrus at regular intervals of around 21 days following puberty. These cycles are interrupted by pregnancy and for a variable period of time after calving. This period following calving before the ovulatory cycles are re-established is termed postpartum anoestrum (PPA). During PPA, the normal relationships among the hypothalamic-pituitary-ovarian (H-P-O) axis and uterus must be re-established before ovulatory cycles can re-occur (reviewed by Lamming *et al.*, 1981; Butler and Smith, 1989; Short *et al.*, 1990; Peters and Lamming, 1991). Uterine involution occurs simultaneously (Morrow *et al.*, 1966), although there is a poor correlation between the duration of the involution process and the PPA interval (Short *et al.*, 1990).

Postpartum anoestrum may be defined as the interval between calving and the first postpartum ovulation. However, as this ovulation may not be accompanied by behavioural oestrus, PPA is often defined as the interval from calving to first detected postpartum oestrus. In the seasonal calving and mating systems common in the New Zealand dairy industry (Holmes and Wilson, 1987), PPA may also be defined as occurring where a cow is not detected in oestrus by the date when the mating period commences. Cows that calve 'late' in the calving season may have been calved only a few weeks when mating commences. Thus, "anoestrous" cows in this wider context includes animals still undergoing the normal postpartum re-establishment of the relationships among the H-P-O axis and uterine involution as well as those cows with abnormally long periods of PPA.

Length of PPA

The interval from calving to first postpartum ovulation has been variously reported as 19.1 (Carruthers and Hafs, 1980), 23.6 (Britt *et al.*, 1974), 24.3

(Lamming and Bulman, 1976) and 30.6 days (Fagan and Roche, 1986) and with between 7% and 14% of cows not having ovulated by 50 days postpartum (Lamming and Bulman, 1976; Boyd and Munro, 1979; Fagan and Roche, 1986). The interval from calving to first oestrus is generally reported as 2 to 3 weeks longer (e.g. 35.7 and 39.0 days, Britt *et al.*, 1974; Carruthers and Hafs, 1980, respectively). Longer intervals from calving to first oestrus than from calving to first ovulation are at least partially due to a failure of detection of behavioural oestrus at first postpartum ovulation. Only 29.3% of first postpartum ovulations were accompanied by detected behavioural oestrus compared to 62.9% and 84.2% at the second and third postpartum ovulations respectively (Lamming and Bulman, 1976). However, these intervals are also affected by genetic, physiological and managerial factors.

Factors affecting the duration of PPA

Suckling of cows prolongs their period of PPA in comparison to machine milked cows (Smith *et al.*, 1981). Season of calving may also affect the PPA interval, both due to indirect effects of seasonal differences in nutrient quality and quantity and by direct effects of photoperiod (Bulman and Lamming, 1978; Savio *et al.*, 1990; Short *et al.*, 1990). The PPA interval for Jersey cows is shorter than for Friesians (Fonseca *et al.*, 1983), or Friesian cross Jersey cows (Macmillan and Clayton, 1980). Young cows have longer PPA intervals than older cows (Fonseca *et al.*, 1983; Macmillan and Clayton, 1980). Extended periods of PPA have been associated with dystocia and cystic ovaries in heifers and retained foetal membranes, ovarian cysts and metritis in cows (Erb *et al.*, 1985; Etherington *et al.*, 1985). Additionally, cows diagnosed as anoestrus (not detected in oestrus by 50 or 70 days postpartum) had longer intervals to first service and conception (Etherington *et al.*, 1985), lower first and total service conception rates (Francos and Mayer, 1988) and a higher probability of being culled (Erb *et al.*, 1985; Etherington *et al.*, 1991). Large, unexplained variation among herds in the proportion of a herd not detected in oestrus by 60 days postpartum (5% to 40%) has been reported (Francos and Mayer, 1988).

Body condition score (CS) is an estimate of the percentage of the body that is adipose tissue based on the external appearance of the animal (Macdonald and Macmillan, 1993). Cows in lower CS at calving have a longer period of PPA than cows in higher CS (Grainger *et al.*, 1982).

Following calving, cows undergo a series of physiological changes associated with lactation. The transition from one state to another (e.g. gestation to lactation) is controlled by the homeorhetic mechanisms (Bauman and Currie, 1980) which orchestrate the required changes in metabolic processes. The daily nutrient intake of many cows is less than that required to support milk production during the first 2 to 4 months of lactation. During this period of negative energy (and protein) balance (NEB; Butler *et al.*, 1981), body tissue is mobilised to meet the energy deficit. In opposition to the homeorhetic processes, the homeostatic processes attempt to maintain the body in its existing state. If excess body tissue is being mobilised in the early postpartum period, the homeostatic mechanisms may be activated so that milk production, the major energy requiring process, is reduced. The duration and depth of NEB have been correlated with the duration of PPA (Butler *et al.*, 1981; Butler and Smith, 1989; Canfield and Butler, 1990; Staples *et al.*, 1990; Lucy *et al.*, 1992). The duration and depth of NEB is a result of the complex interactions between the level of milk production, nutrient intake and the amount of body tissue available for mobilisation. Apparently contradictory relationships among milk production, postpartum liveweight and body CS change with the PPA interval reported in reviews (Esslemont, 1979; Butler and Smith, 1989) may be explained by differences among cows in how they adjust to the increased nutrient demands of lactation, i.e. by differences among cows in the balance of their homeorhetic and homeostatic mechanisms. Variation among cows in the rate of increase of postpartum production, nutrient intake and body tissue mobilisation has been reported (Staples *et al.* 1990; Lucy *et al.*, 1992). The interaction between the homeostatic and homeorhetic mechanisms is not well understood as there is a lack of experimental data with which to construct models to explain the complex interactions (Sauvant, 1994). For example, the control of homeostatic and homeorhetic mechanisms differs between cows selected on the basis of production. Cows selected for high production have

higher nutrient intake and mobilise more tissue than cows unselected for production despite being offered equivalent amounts of feed (Bryant and Trigg, 1981; Grainger *et al.*, 1985).

In the group-grazing, pasture-fed dairy systems of New Zealand, estimation of individual pasture intake and hence energy balance is technically difficult. Condition score, liveweight and blood concentrations of carbohydrate, lipid and protein metabolites (Payne and Payne, 1987) may be useful indirect measures of energy balance in grazing cows.

Ovarian follicular development during PPA

Ovarian follicular development is an essential precursor to ovulation and behavioural oestrus and hence to mating and conception. Transrectal B-mode ultrasound has been used to assess ovarian structures and demonstrate the presence of waves of follicles in cattle (Pierson and Ginther, 1984). These waves occur in prepubertal (Hopper *et al.*, 1993) and postpubertal (Sirois and Fortune, 1988) heifers as well as during early pregnancy (Ginther *et al.*, 1989a). Each wave consists of a group of follicles recruited from a gonadotrophin-dependent pool of antral follicles (Scaramuzzi *et al.*, 1993). One follicle is selected to become the largest or dominant follicle (DF). If this follicle does not ovulate, it becomes atretic, allowing emergence of a new follicle wave. This sequence occurs approximately every 10 days (Sirois and Fortune, 1988; Ginther *et al.*, 1989b). During PPA, large follicles were present within 11 days of calving in well-fed dairy cows. In three-quarters of these cows this first postpartum DF ovulated, with the second or third postpartum DF ovulating in the remaining cows (Rajamahendran and Taylor, 1990; Savio *et al.*, 1990). However, in suckled beef cows only 2 of 18 first postpartum DF ovulated and an average of 3.2 (range 1 to 6) DF's occurred before ovulation at 35.9 days postpartum (Murphy *et al.*, 1990). This indicates that a large DF can develop in cattle in the postpartum period without ovulation necessarily ensuing.

Endocrinology of PPA

The normal ovulatory ovarian cycles and the relationships within the H-P-O axis are disrupted by pregnancy and take time to re-establish postpartum.

In the normally cycling cow, there are a series of inhibitory and stimulatory feedback loops operating among the components of the H-P-O axis. During the luteal phase of the cycle, progesterone (P_4; produced by the corpus luteum (CL)) and oestradiol-17β (E_2; produced predominantly by large ovarian follicles; McNatty *et al.*, 1984a) combine to inhibit luteinising hormone (LH) release (Price and Webb, 1988; Stumpf *et al.*, 1993). Follicle stimulating hormone (FSH) concentration in the ewe appears to be modulated by both E_2 and the ovarian protein, inhibin (Findlay *et al.*, 1992), but the control of FSH is less clearly understood in cows (Webb *et al.*, 1992). Uterine release of the luteolytic hormone prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is followed by a decline in P_4 concentrations (Peterson *et al.*, 1975). As the P_4 concentration declines, increasing LH pulse frequency and amplitude stimulate final follicular maturation and increase E_2 production (Peterson *et al.*, 1975). In the absence of P_4, the increasing E_2 concentration stimulates release of gonadotrophin releasing hormone (GnRH) from the hypothalamus and finally the ovulatory gonadotrophin (LH and FSH) surge (Karsch *et al.*, 1992). Oestradiol is both inhibitory (in the presence of P_4 concentrations of >0.5 ng/ml; Nanda *et al.*, 1988) and stimulatory (in the absence of P_4). Follicular E_2 production is dependent on both LH, to stimulate androgen production by the theca interna cells, and on FSH to stimulate granulosa cell production of the aromatase enzyme that converts the androgens to E_2 (Fortune, 1994).

The pulse frequency of LH increases with time postpartum (Lamming *et al.*, 1981; Schallenberger *et al.*, 1982). Cows that have an extended postpartum anovulatory interval have a lower LH pulse frequency and amplitude than cows commencing cycling earlier postpartum (Fisher *et al.*, 1986; Wright *et al.*, 1990). Low LH pulse frequency has been suggested as a factor limiting resumption of postpartum ovulatory activity (Lamming *et al.*, 1981; Roche *et al.*, 1981; Schallenberger *et al.*, 1982). Virtually every LH pulse in sheep is preceded by a pulse of GnRH from the hypothalamus (Clarke and

Cummins, 1985; Karsch *et al.*, 1992). This suggests that low LH pulse frequency may be due to a lack of, or inhibition of, pulsatile GnRH release. The hypothalamic concentration of GnRH in postpartum cows is similar to that found in cycling cows (Moss *et al.*, 1985; Nett *et al.*, 1988). Peripheral and pituitary concentrations of FSH return to normal within days of parturition and are not considered to limit resumption of cyclic activity postpartum (Schallenberger *et al.*, 1982; Moss *et al.*, 1985). However, pituitary LH concentration may take up to 30 days to return to levels similar to those found in cycling cows (Moss *et al.*, 1985; Nett *et al.*, 1988). LH release following exogenous GnRH treatment increases with time postpartum (Kesler *et al.*, 1977; Fernandes *et al.*, 1978; Alam and Dobson, 1987), indicating either greater LH production or increased sensitivity to GnRH. Exogenous E_2 fails to induce a surge-like LH release early in the postpartum period (Schallenberger and Prokopp, 1985; Alam and Dobson, 1987), suggesting that the E_2 stimulatory feedback loop fails in the early postpartum period.

GnRH and LH release is modulated by ovarian steroids (P_4 and E_2) and by extra-ovarian factors (reviewed by Short *et al.*, 1990). Extra-ovarian factors such as photoperiod (Montgomery *et al.*, 1985; Goodman, 1988), level of nutrition and body condition (Imakawa *et al.*, 1986), suckling (Garcia-Winder *et al.*, 1984, 1986a) and elevated temperatures (Madan and Johnson, 1973) can depress mean LH concentration and/or LH pulse frequency independently of the ovary. There is also evidence for interactions between the sensitivity of E_2 inhibition and season in sheep (Legan *et al.*, 1977).

Endogenous opiates may extend PPA, as infusion of the opiate antagonist, naloxone, increased the LH pulse frequency in ovariectomised, suckled beef cattle (Rund *et al.*, 1989). However, treatment of machine milked dairy cows with naloxone did not effect LH parameters following pulsatile infusion (Canfield and Butler, 1991) or bolus injection (Nanda *et al.*, 1991).

In women, hyperprolactineamia, associated with breast feeding or functional pituitary tumours inhibits ovulation (Greenspan, 1991). In cattle no correlation between prolactin concentrations and PPA interval could be demonstrated (Lamming *et al.*, 1981) and treatment with bromocriptine, which

inhibits prolactin concentrations, did not shorten the PPA interval (Williams and Ray, 1980).

Nutritional effects on the hypothalamo-pituitary-ovarian axis

How nutrition modulates the H-P-O axis is unknown. Reduction of LH concentration and pulse frequency have been observed in animals that are underfed and in NEB (Canfield and Butler, 1990; 1991). The underfeeding was associated with reduced blood glucose and insulin concentrations and elevated non-esterified fatty acid concentrations (Canfield and Butler, 1990). Low blood glucose has been associated with poor expression of behavioural oestrus and lowered conception rates (McClure *et al.*, 1978) and LH concentration was reduced by pharmacologically-induced hypoglycaemia (Rutter and Manns, 1987). Undernutrition can exert inhibitory effects on the H-P-O axis independent of the ovary, as ovariectomised, underfed sheep (Rhind *et al.*, 1989) and cattle (Imakawa *et al.*, 1986) have lower LH concentrations than fully-fed controls. Whether these effects are due to direct metabolic substrate deficiencies at the hypothalamic/pituitary level, the effects of hormones involved in metabolic regulation (e.g. insulin, growth hormone, insulin-like growth factor, thyroxine etc.) or to production or release of specific neurotransmitters is not known (Schillo, 1992). Specific dietary amino acids may modulate neurotransmitter concentrations and hence influence hypothalamic function (Schillo, 1992; Kalra, 1993). There is also evidence that undernutrition may enhance the effects of E_2 inhibition of GnRH release in cattle (Imakawa *et al.*, 1986).

Treatment of PPA

A variety of hormonal and managerial approaches to the treatment of anoestrum have been attempted. There is wide variation among treatment trials in the diagnostic techniques used and in the definitions of anoestrum as well as of 'success'. Many trials have defined PPA as failure of a cow to be

detected in behavioural oestrus by some specific number of days postpartum. Thus, cows presented for treatment of PPA may have ovulated and expressed behavioural oestrus, but not been detected in oestrus by the herd manager; they may have ovulated without expressing behavioural oestrus concurrently with ovulation ('silent' ovulations); or, they may not have ovulated or expressed behavioural oestrus (anovulatory anoestrus, AA; Radostits and Blood, 1985). Rectal examination of the ovaries for the presence of a CL, indicating that ovulation has occurred, has been used to classify PPA cows. In a New Zealand study of 335 pasture-fed PPA cows, 88.9%, 82.5% and 47.0% of 2 year old, 3 year old and older cows had no palpable CL (Fielden *et al.*, 1973). In contrast, 87.8% and 72.4% of PPA cows had P_4 profiles indicating that cyclic activity had commenced in North American and British studies, respectively (Etherington *et al.,* 1991; McCleod and Williams, 1991). These differences may be due to diagnostic methodology (palpation vs. P_4 analysis) or due to true differences in the populations in the frequency of AA. Sensitivity and specificity of rectal palpation for detection of a CL varies among veterinarians and is reported to range between 70% to 89% and 50% to 97%, respectively (Kelton *et al.*, 1988; Kelton, 1989). Incorrect classification of PPA cows may result in inappropriate treatments being applied and hence incorrect conclusions about treatment efficacy being drawn. For example, 18% and 26.5% of PPA cows were incorrectly diagnosed as having a CL and treated with PGF_{2_α} to induce luteolysis (Ott *et al.*, 1986; Etherington *et al.*, 1991). Incorrect diagnosis of 58.1% of a group of cows not detected in oestrus as AA has been reported (McCleod and Williams, 1991). Veterinary access to serial milk P_4 data resulted in an increased proportion of PPA cows being diagnosed as "cycling normally", and a reduced proportion of cows being examined in the postpartum period (Williams and McCleod, 1992). Results of treatment trials of PPA cows must be interpreted carefully in the light of the range of definitions of PPA and of 'success' used.

Endocrine treatments of PPA

GnRH treatment has been widely used in PPA cattle. A single injection designed to induce an LH surge and ovulation has been used (Britt *et al.*, 1974; Britt *et al.*, 1977; Webb *et al.*, 1977; Benmrad and Stevenson, 1986). To be successful this single treatment must evoke an LH surge. Also a follicle at a stage of development at which it can respond to the LH surge must be present. In a population of PPA cows, follicles will be at a range of stages of development at the time of treatment resulting in a highly variable response. Field trials with single GnRH treatments have shortened the intervals from calving to first ovulation (Britt *et al.*, 1974), to first service and/or to conception (Webb *et al.,* 1977; Benmrad and Stevenson, 1986; Nash *et al.,* 1980) in some trials; but had no effects in others (Kesler *et al.*, 1977; Kesler *et al.*, 1978; Roche *et al.*, 1981; Ball and Lamming, 1983). Injection of physiological (1 to 5 μg) doses of GnRH hourly or 2 hourly have been tested (Edwards *et al.*, 1983; Jagger *et al.*, 1987; Jagger *et al.*, 1989). These treatments result in increases in LH pulse frequency which stimulate increased E_2 production which eventually evokes the pre-ovulatory LH surge (Jagger *et al.*, 1987). Such pulsatile treatment systems are impractical for field use. Subcutaneous continuous infusion systems have been tested, but with inconsistent results (Jagger *et al.*, 1989).

Single, bolus injections of various oestradiol esters have also been used to induce an LH surge and ovulation in PPA cows. Small doses (0.4, 0.5 or 1.0 mg) failed to induce an LH surge, oestrus or ovulation in some PPA cows (Radford *et al.*, 1978; Roche *et al.*, 1981; Schallenberger and Prokopp, 1985; Alam and Dobson, 1987); and larger doses (3 or 4 mg) invariably resulted in oestrus, but it is not clear that ovulation was always induced as conception rates were low (Fielden *et al.*, 1973). Failure of the low dose treatment may have been due to an inability of E_2 to induce the $\bar{LH}$ surge indicating refractoriness of the hypothalamus to E_2 positive feedback (Radford *et al.*, 1978; Roche *et al.*, 1981). Alternately, there may have been no follicle at a suitable stage of development to respond to the induced LH surge present in the ovary.

Progesterone alone or in combination with oestrogens, GnRH or equine chorionic gonadotrophin (eCG) has been widely used to treat PPA. Trials using small numbers of animals demonstrated that intravaginal P_4 treatment could stimulate some PPA cows to ovulate and express behavioural oestrus (Lamming and Bulman, 1976; Bulman and Lamming, 1978; Bulman *et al.*, 1978; Bulman and Wood, 1980; Roche *et al.*, 1981). Use of a subcutaneous implant containing a synthetic progestagen (norgestemet) and oestradiol valerate (EV; Synchromate, Intervet) increased conception rate within 14 days of treatment in PPA dairy cows that were >60 days postpartum (Galloway *et al.*, 1987). Intravaginal devices containing P_4 have been shown to increase the proportion of cows inseminated within 14 days of device removal (Macmillan and Day, 1987), and to shorten the calving to conception interval (Ball and Lamming, 1983). Treatment of at least 7 days using a controlled internal drug releasing device containing 1.9 g of P_4 (CIDR-B, InterAg, Hamilton, NZ) and 400 i.u. of eCG at device removal were found to optimise the proportion of cows inseminated within 14 days of device removal (Macmillan and Day, 1987). However, a multi-farm, clinical trial run in Australia using the same regime resulted in no increase in the percentage of animals in oestrus within 14 days of treatment and no shortening of the treatment to conception interval compared with control cows (Jubb *et al.*, 1989). Significant between farm variation in response to P_4 treatment has been demonstrated (Macmillan and Peterson, 1993) perhaps due to differences in nutritional management or sensitivity and specificity of oestrus detection. Other factors such as the definitions of PPA used, the definitions of successful treatment outcome, the rate of spontaneous recovery of the control cows and the timing of treatment relative to the commencement of the mating period may explain differences among trial results.

Conception rate has been positively related to the number of premating oestrus events (Thatcher and Wilcox, 1973). Several recent studies have investigated the effect of stimulating early (1 to 3 weeks) postpartum resumption of ovulatory activity with P_4 (Kyle *et al.*, 1992; Stevenson and Pursley, 1994) with no effect on the intervals from calving to first ovulation or first oestrous or on conception rates.

The mechanism for the effects of P_4 on PPA cows is unclear (Lamming *et al.*, 1979). Progesterone may inhibit LH pulse frequency as occurs in cycling cows (Price and Webb, 1988). Removal of the P_4 may allow an increase in LH pulse frequency resulting in stimulation of follicle development, increased E_2 production and hence a pre-ovulatory LH surge.

Nutritional and biostimulatory treatments of PPA

The recognition that low CS at calving (Grainger *et al.*, 1982) and long periods of NEB extend the PPA period have led to attempts to manipulate pre- and postpartum nutrition to shorten PPA. Each reduction of one CS at calving extends the PPA interval by 5.7 days (Grainger *et al.*, 1982). Feeding extra pasture over the first 5 or 10 weeks of lactation may shorten the PPA interval, but the effect of CS at calving appears to be independent of postpartum nutrition (Grainger and Wilhelms, 1979; Grainger *et al.*, 1982). In an attempt to minimise duration of NEB postpartum, diets with high energy densities have been fed. For example, the feeding of protected lipids has been shown to increase the total energy intake in early lactation; however, this increase in energy intake is accompanied by increasing production, no reduction in the NEB and no reduction in PPA interval (Lucy *et al.*, 1992; Sklan *et al.*, 1994).

Presence of teaser bulls has been shown to shorten the PPA interval in some trials. In only 1 of 5 herds, in which half the animals were exposed to a teaser bull for 3 weeks before the start of the mating period, did the presence of the bull increase the number of cows mated (Macmillan *et al.*, 1979). Cows with low, but not high CS at calving, had shorter PPA intervals following exposure to bulls (Stumpf *et al.*, 1992). Exposure to bull urine, rather than the bulls themselves, did not shorten the PPA of beef heifers (Taylor *et al.*, 1992).

PPA in New Zealand

The New Zealand dairy industry is a 'low cost, low return' industry based on seasonal calving (90% of cows calve in spring) and a diet which is predominantly white clover/ryegrass pasture (Holmes and Wilson, 1987). The

pasture may vary markedly in quantity and quality from season to season and year to year. This can result in seasonal variation in milk production, cow body condition, energy balance and hence reproductive performance. In this respect, the New Zealand dairy production system differs from that of North America and most of Europe where nutrient intake is controlled by feeding supplements or total mixed rations.

Reproductive failure, i.e. failure to conceive or conception that occurs unacceptably late in the breeding period, is the second largest cause of removal of cows from New Zealand dairy herds (1.4% to 3.2% of cows in the herd/annum depending on cow age; Harris, 1989). Cows calving late in the calving season have a higher risk of being removed from the herd (Harris, 1989). Late calving results in less time for uterine involution and re-establishment of the relationships within the H-P-O axis and hence a reduced probability that insemination and conception will occur within the planned mating period. However, cows that calve early in the calving period and that have extended periods of PPA, may also not be inseminated or conceive within the mating period, or may conceive late in the mating period resulting in late calving in the subsequent year. Moller (1970) demonstrated, by repeated transrectal ovarian palpation, that the calving to first ovulation interval was 35 days for cows >4 years old, but >50 days for cows that were 2 or 3 years old. The calving to first oestrus interval has been reported as 47 days for one New Zealand dairy herd (Macmillan and Clayton, 1980). By 4 weeks into the seasonal mating period, 14% of all cows had not been inseminated and of these animals, 73.7% had calved more than 50 days earlier (Fielden *et al.*, 1973). Rectal palpation of the ovaries of these cows indicated that >85% of 2 and 3 year old cows had no palpable CL, suggesting that failure of resumption of postpartum ovarian activity, rather than failure of detection or expression of behavioural oestrus was the underlying problem (Fielden *et al.*, 1973). These data indicate that the PPA interval of pasture-fed New Zealand dairy cows may be considerably longer than that of cows under different management systems. A significant proportion of the national herd may fail to commence ovulatory activity by the designated start of the mating period. Factors associated with extended periods of PPA have not been examined systematically in New

Zealand dairy cows. No data on ovarian follicular turnover or endocrine status are available for New Zealand cows. Although treatment responses of cows with extended periods of PPA have been reported (Macmillan and Day, 1987; Macmillan and Peterson, 1993), the factors contributing to the failure of up to 25% of cows to be detected in oestrus and the large variation in treatment responses between farms have not been identified.

Aim and scope of this thesis

This thesis has the objective of examining managerial, endocrinological and treatment aspects of PPA in New Zealand dairy cattle. The extended periods of PPA that occur in the pasture-fed New Zealand dairy cow are in contrast with those of the well-fed North American and European dairy cows.

The thesis is structured in 3 sections:

(a) The characteristics of PPA

It was hypothesised that:

- Younger cows, Friesian cows and cows managed at a high stocking rate would have extended PPA intervals (Chapter 2); and that
- Extended periods of PPA would be associated with poor body condition, low blood glucose and high concentrations of metabolites associated with mobilisation of protein and adipose tissue (Chapter 3).

(b) The follicular and endocrine status of the postpartum cow

It was hypothesised that:

- Anovulatory cows would have large ovarian follicles and that follicle turnover would occur, but that despite the presence of these follicles ovulation would not occur (Chapter 4); that
- Anovulatory cows would have lower intrafollicular concentrations of E_2, P_4, testosterone, and insulin-like growth factors than cycling cows (Chapter 5); that

- Anovulatory cows would release LH from the pituitary in response to treatment with GnRH and the DF would ovulate in response to this LH release (Chapter 6); that
- Anovulatory cows would fail to release LH and not ovulate following treatment with E_2 (Chapter 7); and that
- Anovulatory cows would have lower mean LH concentration and LH pulse frequency and amplitude than ovulating cows, both in the presence and absence of the ovaries (Chapter 8).

(c) The treatment of PPA

It was hypothesised that:

- Treatment of cows early in the postpartum period (2 to 3 weeks) with E_2, P_4, or a combination of both would stimulate oestrus and ovulation (Chapter 9); that
- Treatment of cows not detected in oestrus by 1 week before the start of the mating period with P_4 and eCG would result in more cows being mated and conceiving than untreated controls (Chapter 10); and that
- Cows fed pasture silage in addition to white clover/ryegrass pasture for the first month of lactation would have shorter calving to first oestrus and ovulation intervals than cows fed pasture alone (Chapter 11).

Testing these hypotheses aimed to increase the understanding of the endocrine and managerial factors contributing to PPA so that more effective treatment approaches would be available to New Zealand herd owners.

CHAPTER 2:

The Effect Of Stocking Rate And Breed On The Period Of Postpartum Anoestrum In Grazing Dairy Cattle

Abstract

The effects of stocking rate, breed and age on the intervals from calving to first ovulation and to first oestrus were assessed in pasture grazed dairy cattle.

Four herds, in a 2 x 2 factorial arrangement of 2 stocking rates (low, L; and high, H) and 2 breeds (Jersey, J; and Friesian, F) were formed on June 1 1990. Cows were stratified on age, expected calving date, liveweight, CS, previous production and breeding index.

Milk samples for P_4 analysis were collected twice weekly from 20 cows in each herd following commencement of calving on July 2, 1991. Sampling ceased following the third postpartum ovulation or the planned start of mating (PSM; 3 October, 1991). All observed oestrous events were recorded from calving onwards, and any cow seen standing to be mounted by another cow or having >50% of her tail paint removed was defined as having been in oestrus. Body CS and liveweight were recorded on a fortnightly basis and milk production was recorded on a weekly basis. Blood samples were drawn from the coccygeal vessels of 8 animals in each herd on a weekly basis from 4 to 14 weeks postpartum. These were analysed for albumin, β-hydroxy-butyrate (BOH), glucose, non-esterified fatty acid (NEFA) and urea concentrations.

Cows in the FH herd had longer intervals from calving to first postpartum ovulation (C_ovn1; 49.2 vs. 24.7, 31.1 and 29.4 days) and calving to 1st oestrus (C_h1; 52.2 vs. 30.9, 38.9 and 35.3 days) than the JL, JH and FL herds respectively. Two year old cows had longer intervals from calving to first ovulation (40.2 vs. 27.2 days), and from calving to 1st oestrus (47.1 vs. 32.5 days) than older cows. By 50 days postpartum, 100%, 84.9%, 90.9% and 37.9% of cows in the JL, JH, FL and FH herds respectively, had been detected in oestrus.

The high stocking rate herds had reduced CS, liveweight and milk production compared to the low stocking rate herds. Condition score, glucose concentration at week 11 postpartum and milksolids production at week 4 postpartum were inversely related to the intervals from calving to first postpartum ovulation and/or to first oestrus.

Increased stocking rates were associated with longer PPA. This may have been due to reduced individual cow pasture intake both pre- and postpartum, resulting in a greater duration of NEB and lower reserves of body fat. There was a significant breed by stocking rate interaction, with the FH having a longer period of PPA than the JH herd, indicating that Friesians may have been less able to adjust to the nutrient restrictions imposed in this trial.

Introduction

Cows not detected in oestrus by 60 days postpartum have a lower conception rate and a higher risk of being removed from the herd for failing to conceive than cows detected in oestrus before 60 days postpartum (Francos and Mayer, 1988). Postpartum anoestrum has been identified as a problem in pasture-grazed New Zealand dairy herds since 14.4% of cows were not detected in oestrus and inseminated by 4 weeks into the seasonal mating period (Fielden *et al.*, 1973), and the PPA interval was reported as >50 days for 2 and 3 year old cows (Moller, 1970).

Cows will not normally show oestrus if they have not commenced ovulating. Others may ovulate but not display the physical signs of oestrus, but commonly there is a human failure to detect these signs (Radostits and Blood, 1985). Failure to express oestrus occurred in over 70% of cows at the first postpartum ovulation, but this reduced to 37.1% and 15.8% at the second and third postpartum ovulation in a survey of British dairy herds (Lamming and Bulman, 1976). Whether a similar percentage of New Zealand dairy cows fail to express oestrus at the first and second postpartum ovulation is not known.

Postpartum anoestrum is a multifactorial condition which has managemental (for example heat detection), physiological (season, age, breed, CS and postpartum weight loss), pathological (retained foetal membranes,

cystic ovaries, endometritis/pyometron) and nutritional (NEB, feed intake, feed quality) components (Callahan *et al.*, 1971; Macmillan and Clayton, 1980; Fonseca *et al.*, 1983; Erb *et al.*, 1985; Francos and Mayer, 1988; Etherington *et al.*, 1991). Following parturition, energy provided by feed intake is insufficient to meet the demands for milk production, so that cows are in NEB (Butler *et al.*, 1981). Both the extent and duration of NEB have been associated with the C_ovn1 interval (Canfield and Butler, 1990; Staples *et al.*, 1990; Lucy *et al.*, 1992). Estimates of individual cow pasture intake and hence of energy balance under grazing conditions are difficult to make. Measurement of the blood concentration of various essential metabolites, a 'metabolic profile test' (Payne and Payne, 1987) offers a way of indirectly estimating energy balance. NEFA, glucose and insulin concentrations have been correlated with energy balance (Canfield and Butler, 1991). Liveweight, CS and milk production also provide information on the energy balance of a cow. Cows with extended periods of PPA have been shown to be in NEB longer, to lose more CS, to eat less and produce less milk than cows ovulating earlier postpartum (Staples *et al.*, 1990).

A farms' stocking rate is positively correlated with the total economic return to the farm-owner (R^2 = 0.93; Deane, 1993) which has led to increases in stocking rate on many New Zealand farms. Increased stocking rates may be associated with reduced feed intake for individual cows. This may prolong the period of postpartum NEB and PPA.

The aims of the present trial were to quantify factors affecting variation in the C_ovn1 and C_h1 intervals and the proportion of ovulations not accompanied by behavioural oestrus in cows of different breeds and ages grazed at different stocking rates.

Materials and Methods

One hundred and ten mixed age dairy cows were formed into 4 herds on 1 June, 1990 in a 2 by 2 factorial arrangement on the basis of breed (Jersey, J; Friesian, F) and at 2 stocking rates (Low, L; High H; Table 2.1) following stratification on the basis of age, CS, liveweight, expected calving date, previous production and breeding index (Albhorn and Bryant, 1992).

Table 2.1. The breed, numbers, stocking rate and metabolic weight of the four experimental herds.

Herd	Abbreviated herd name	n	SR† (cows/ha)	Metabolic weight ($kg^{0.75}$) *
Low Stocked Jersey	JL	26	3.5	1858[a]
High Stocked Jersey	JH	33	4.5	2305[b]
Low Stocked Friesian	FL	22	3.0	1879[a]
High Stocked Friesian	FH	29	4.0	2308[b]

* Total mass of the herd (mean of 6 weighings from 17/8/91 to 26/10/91)

† Stocking rate

Each herd was designated to a farmlet (7.5 ha) which consisted of 18 equal-sized paddocks, of predominantly ryegrass/white clover pasture. Each herd grazed its allocated paddocks in rotation. Pasture mass was estimated before and after grazing three times weekly and the dry matter disappearance /cow (DMD) rate estimated by:

$$DMD = \frac{(pregrazing\ (kg\ DM\ /\ ha) - postgrazing\ (kg\ DM\ /\ ha))\ x\ area\ grazed\ (ha)}{n\ cows}$$

Stock were culled on the basis of failure to conceive or poor production and replaced with 2 year old stock at a rate of approximately 20% per annum.

Calving occurred between 2 July and 27 August 1991 (mean = 24 July ± 1.9 days). Three cows (2 x FL and 1 x FH) were induced to calve by injection of long-acting corticosteroids. Estimates of volume of milk production occurred on a weekly basis and a composite afternoon and morning milk sub-sample

was analysed for milkfat and protein concentration by infrared spectrophotometry (Milk-o-Scan, N. Foss Electrical, Hillerod, Denmark). Twice weekly milk samples (20 ml) were taken for subsequent P_4 analysis from calving to 3 weeks after the commencement of the mating. Cows were selected after age stratification (4 x two year olds, 4 x three year olds and 12 x > three year olds) from each of the 4 herds.

All cows were weighed and CS (1 = thin, 10 = fat; Macdonald and Macmillan, 1993) was estimated at fortnightly intervals from June onwards at the same time of day and by the same operators.

Eight animals (4 x two year olds, 2 x three year olds and 2 x > three year olds) in each herd were blood sampled by venipuncture of the ventral coccygeal vessels, at weekly intervals for 10 weeks from late July. The serum was analysed for albumin, BOH, glucose, NEFA and urea concentrations.

Oestrus detection occurred twice daily as cows moved to and from the milking shed. Oestrus was defined as occurring if a cow stood to be mounted (Williamson *et al.*, 1972) and/or if >50% of the tail paint, applied soon after calving, had been removed (Macmillan *et al.*, 1988). Any cow detected in oestrus between 2 October, the PSM, and 19 November was submitted for artificial insemination by an experienced technician using commercially available semen. Between 20 November and 23 December (for the Friesian herds) or 4 January 1992 (for the Jersey herds), a bull was run with each herd and any matings were recorded. Pregnancy status of each animal was established by manual palpation of the reproductive tract per-rectum between 35 and 50 days after the final recorded mating and again at >100 days after mating.

Laboratory analyses

Metabolite concentrations (albumin, BOH, glucose, NEFA and urea) were determined at the Ruakura Animal Health Laboratory (Hamilton, New Zealand) using a Hitachi 717 auto-analyser run at 30 ^{o}C. The within- and between-assay co-efficients of variation were <5% for all tests.

Milk P_4 concentration was determined in the fresh milk samples using a commercial ELISA kit (Ovucheck, Cambridge Veterinary Sciences, Ely, Cambridgeshire). The within- and between-assay coefficients of variation for a sample containing approximately 10 ng/ml of P_4 were 5.8% and 12.7%, respectively. The sensitivity (upper 95% confidence interval around the mean of 10 samples from ovariectomised cows analysed in sextuplet) was 0.39 ng/ml. A concentration of >2.5 ng/ml was defined as indicative of luteal activity.

Ovulation without behavioural oestrus was defined as having occurred when oestrus was not recorded 3 to 8 days before the first milk sample with a P_4 concentration of >2.5 ng/ml following calving, or following the decline of P_4 concentration to <2.5 ng/ml for at least one sampling following a previous luteal phase.

Statistical analyses

The effect of age (2, 3, >3 years), breed (Jersey or Friesian) and stocking rate (Low and High) on the C_ovn1 and C_h1 intervals were examined by general linear models (GLM, SAS Institute Ltd., SAS Campus Drive, Cary, NC). The calving date was included as a covariate in all models. All interactions were initially fitted and then removed if not significant (P> 0.15). In both models the interactions of stocking rate and breed approached significance and was included in the final model.

The intervals from the PSM to first service (PSM_s1) and to conception (PSM_con) were analysed by calculating survival functions using the product-limit method (Proc lifetest, SAS Institute Ltd., SAS Campus Drive, Cary, NC). This was necessary as the data were not normally distributed and were right censored by the end of the mating period due to 4 cows failing to conceive. The effects of stocking rate, breed and their interaction were evaluated by comparing survival functions with log-rank tests.

Independent variables (e.g. CS, change in CS from calving to 4 weeks after calving, weight, milksolids (i.e. milkfat and protein) production and the metabolite concentrations) were aligned by calving day, then analysed by a GLM with the weekly (or fortnightly) sampling period as a repeated measure.

Age, breed, stocking rate and the stocking rate by breed interaction were included in the model.

The relationships among the independent variables and the C_ovn1 and C_h1 intervals were initially examined by stepwise regression with breed and calving date included in the models. Any variable that was associated ($P<0.15$) was then included in a GLM with the main effects: calving date, age, breed and stocking rate. Each non-significant independent variable was then removed to produce the final model.

The rate of dry matter disappearance was analysed by GLM with month of sampling and herd as the main effect.

Categorical data were analysed by χ^2 analysis. Group means are quoted as least square means ± standard error of the mean (sem) or least square means ± standard error of the difference (SED). The SED presented is a conservative estimate calculated using the n of the smallest group.

Results

Intervals from calving to first ovulation and first oestrus and from planned start of mating to first service and to conception

Jerseys had shorter C_ovn1 and C_h1 intervals than Friesians as did the low stocking herds compared to the high stocking rate herds (Table 2.2). The interaction among stocking rate and breed approached significance for both intervals as the effect of stocking rate was more pronounced in Friesians than in Jerseys (Table 2.2; Figure 2.1). The 2 year old cows had longer C_ovn1 and C_h1 intervals than older cows (>3 years; Table 2.3). Because the calving date by breed interaction was significant for the C_h1 interval, separate models were fitted for each breed. The C_h1 interval was significantly related to calving date in the Jersey (-0.41 ± 0.13, $P<0.01$) but not the Friesian cows (-0.03 ± 0.18). The proportion of cows that had not ovulated (No ovn) or had not been detected in oestrus (No oestrus) by 50 days postpartum differed among herds ($P<0.01$; Table 2.2).

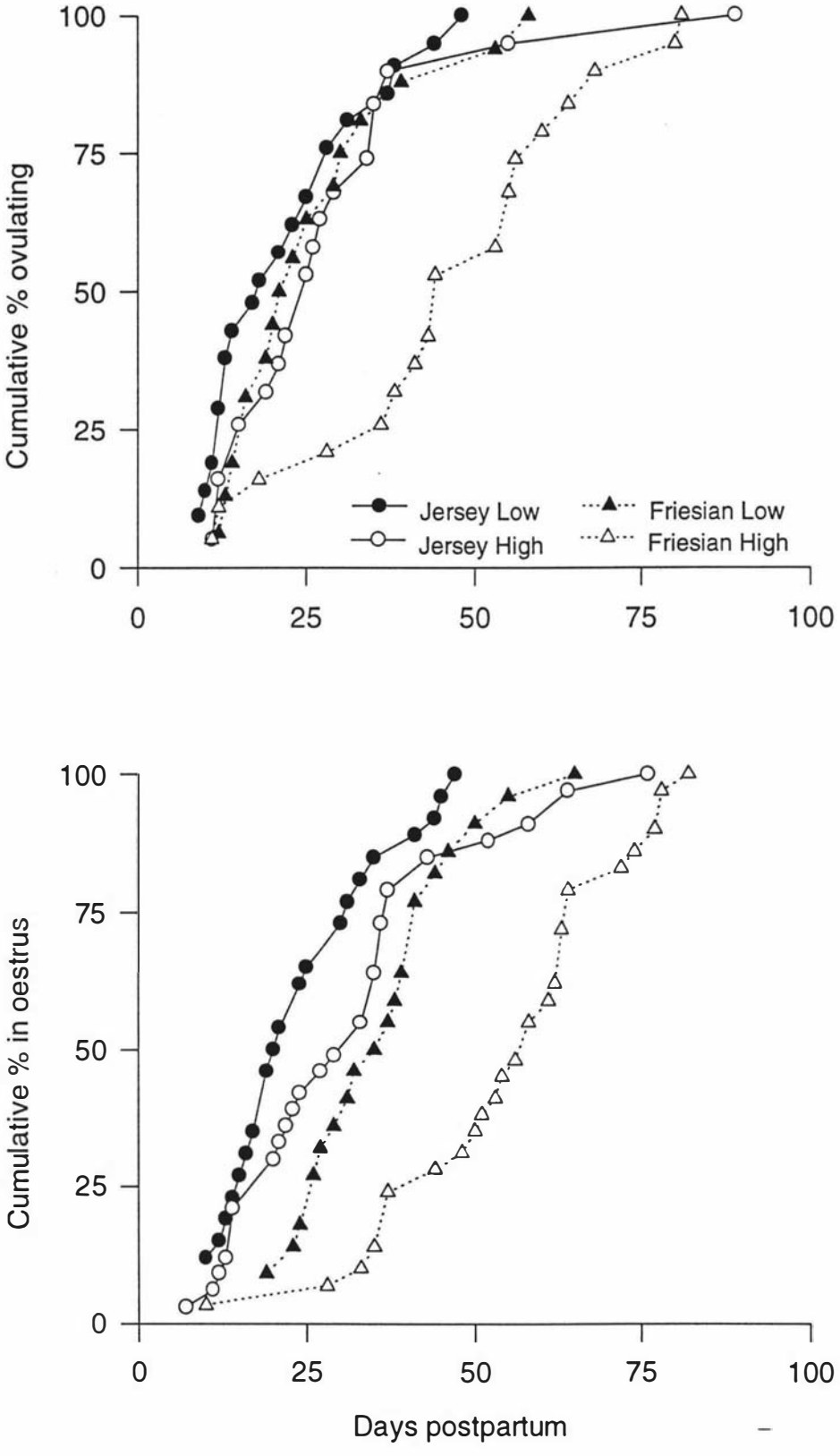

Figure 2.1. The cumulative percentage of cows ovulating (top panel) and detected in oestrus (bottom panel) for the first time after calving in the 4 herds.

The PSM_s1 interval was longer in high stocking rate than low stocking rate herds (12 (7-13) vs. 7 (4-11) days, median (95% CI), P<0.001; Figure 2.2).

Table 2.2. The intervals from calving to first ovulation and from calving to first oestrus and the percentage of cows not ovulating or not detected in oestrus by 50 days postpartum for the 4 herds.

	JL	JH	FL	FH	SED	Age	Breed	Cd†	SR¯	SR‡Breed
C_ovn1 (days)	24.7 [b]	31.1 [b]	29.4 [b]	49.2 [a]	5.0	*	*	*	**	0.11
C_h1 (days)	30.9 [c]	38.9 [b]	35.3 [bc]	52.2 [a]	3.6	0.08	**	**	**	0.12
No ovn (%)	0	10.6	12.5	50.0						
No oestrus (%)	0	15.1	9.1	62.1						

[abc] Means with different superscripts within a row differ significantly (P<0.05)
† Calving date as a covariate (slope = -0.05 and = -0.25 for C_ovn1 and C_h1, respectively)
¯ Stocking rate
‡ Stocking rate by breed interaction
*,** P<0.05, P<0.01, respectively

Table 2.3. The effect of age on the intervals from calving to first ovulation and to first oestrus.

Age (years)	2		3		>3		SED
C_ovn1 (days)	40.2 [a]	(16)†	33.5 [ab]	(13)	27.2 [b]	(48)	6.2
C_h1 (days)	47.1 [a]	(22)	38.4 [ab]	(17)	32.5 [b]	(71)	4.6

[ab] Indicate means within a row with different letters are significant different P<0.05
† Numbers in parenthesis indicate the number of observations

The PSM_con interval was not affected by either stocking rate (16 (12-30) vs. 17.5 (16-29) days, median (95% CI) for L and H stocking rate, respectively) or breed (17 (12-29) vs. 17 (15-31) days, median (95% CI) for J and F, respectively; Figure 2.2).

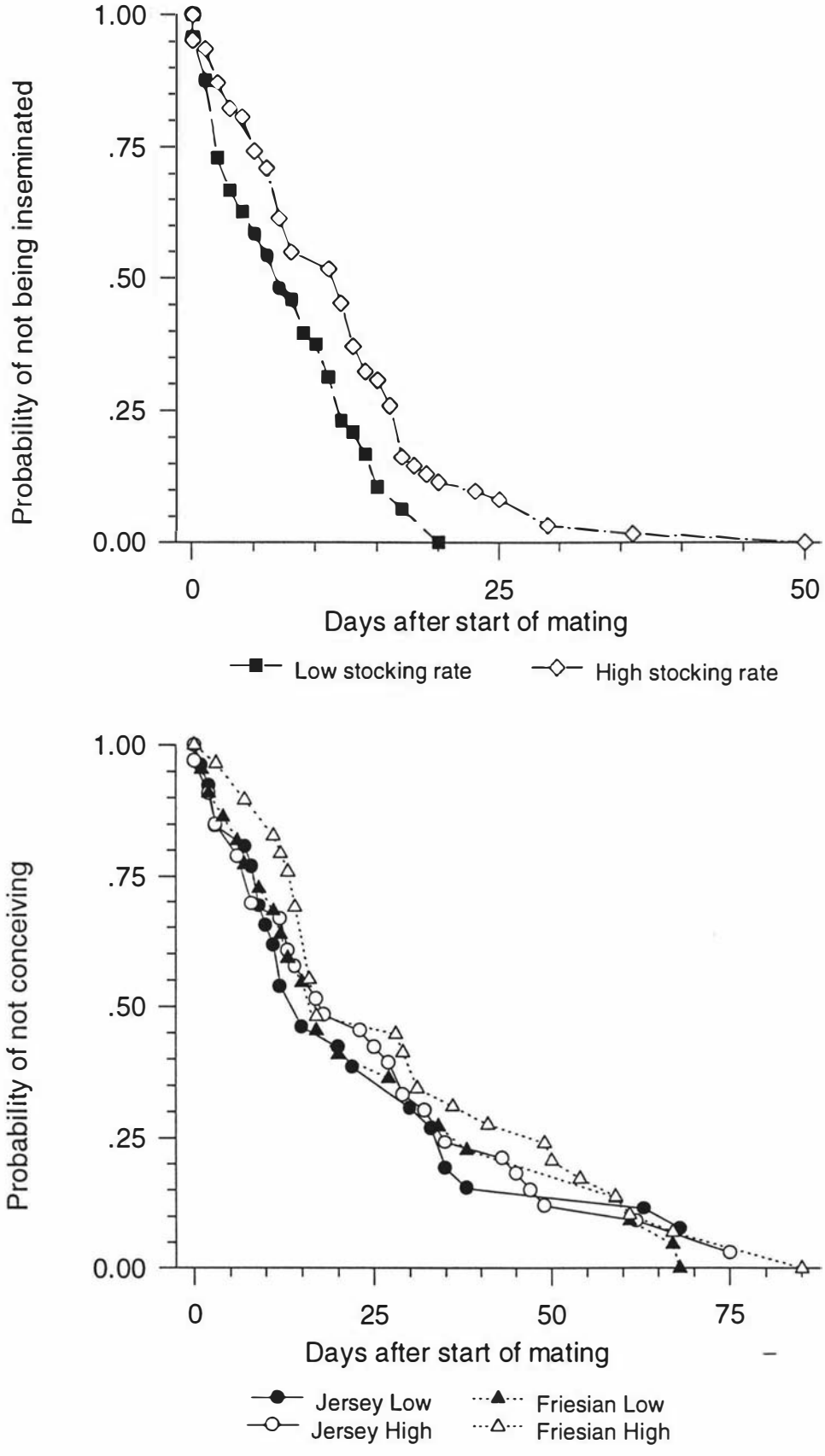

Figure 2.2. The probability of not being inseminated analysed by stocking rate (top panel) and the probability of not conceiving (bottom panel) for the 4 herds.

Condition score, liveweight, milk solids production and blood metabolite concentrations

The average CS was higher for Jerseys than Friesians (4.7 ± 0.1 vs. 4.4 ± 0.1) and for cows at the low stocking rates compared to animals at the higher stocking rates (4.8 ± 0.1 vs. 4.4 ± 0.1). However, the stocking rate by breed interaction approached significance since the Jersey herds did not differ in CS as much (4.9 ± 0.1 vs. 4.6 ± 0.1) as the Friesian herds (4.7 ± 0.1 vs. 4.1 ± 0.1; Table 2.4; Figure 2.3). The high stocked herds lost more CS over the first

Table 2.4. The average CS, liveweight and milksolids production, and blood metabolite concentrations in the peri-partum period for the 4 herds.

	CS‡	WT‡ (kg)	Solids§ (kg/cow/day)	Albumin~ (g/L)	BOH~ (mmol/L)	Glucose~ (mmol/L)	NEFA~ (mmol/L)	Urea~ (mmol/L)
JL	4.9 [a]	342 [c]	1.6 [c]	34.1 [a]	0.9 [a]	3.0 [ab]	0.20 [a]	9.4 [a]
JH	4.6 [a]	333 [c]	1.4[c]	34.7[a]	0.9 [a]	3.2 [a]	0.15 [b]	8.8 [a]
FL	4.7 [a]	428 [a]	2.7[a]	33.5[b]	0.7 [b]	3.1 [a]	0.08 [c]	7.1 [b]
FH	4.1 [b]	393 [b]	1.8[b]	32.8[b]	0.9 [a]	2.9 [b]	0.08 [c]	6.8 [b]
SED	0.1	11	0.3	0.5	0.1	0.1	0.01	0.5
Age[ø]	ns	***	***	**	***	***	***	ns
Breed	**	***	***	***	†	ns	***	***
SR	***	**	***	ns	†	ns	*	ns
SRIB[z]	†	†	***	ns	ns	*	*	ns
P[y]	***	***	***	***	***	***	***	***
R^{2y}	26.0	70.6	70.4	60.8	57.2	52.2	63.6	85.6

[abc] Means within a column with different letters differ significantly (P<0.05)
‡ Average of fortnightly assessments of condition score (CS) and liveweight (WT) from 4 weeks before to 8 weeks postpartum
§ Average of estimates of milkfat and protein production from 1 to 10 weeks postpartum
~ Average of weekly samples from 4 to 14 weeks postpartum, n = 8 cows/herd
† ns,*,**,***, not significant, P<0.1, P<0.05, P<0.01, P<0.001, respectively
ø Age classified as 2, 3 or >3 years
z Stocking rate (SR) by breed (B) interaction
y P value and fit (%) of the final model

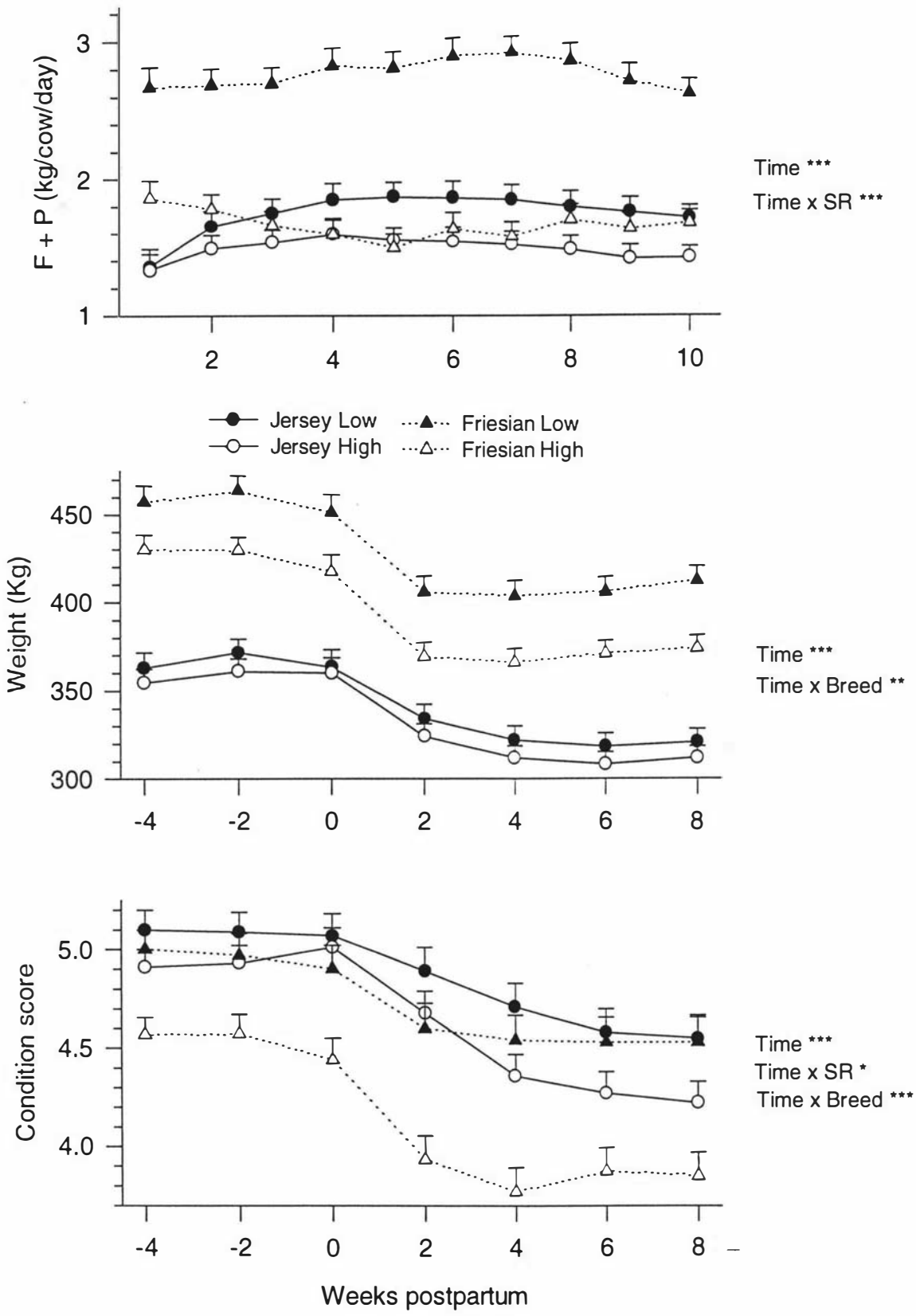

Figure 2.3. The average weekly milksolids production (top panel) and fortnightly liveweight (middle panel) and CS (bottom panel) for the 4 herds.

month of lactation than the low stocked herds (0.7 vs. 0.4 ± 0.1, P<0.01). Average liveweight increased with age (Table 2.5). Jerseys were lighter than Friesians (337 ± 5 vs. 411 ± 6 kg), and cows at the higher stocking rates were lighter than animals at the lower stocking rates (363 ± 5 vs. 385 ± 6 kg). However, the stocking rate by breed interaction approached significance since the Jersey herds differed less (342 ± 8 vs. 333 ± 7) than the Friesian herds (393 ± 7 vs. 428 ± 8; Table 2.4; Figure 2.3).

Table 2.5. The average CS, liveweight and milksolids production, and metabolite concentration for three age-groups.

Age	CS‡	WT‡ (kg)	Solids§ (kg/cow/day)	Albumin~ (g/L)	BOH~ (mmol/L)	Glucose~ (mmol/L)	NEFA~ (mmol/L)	Urea~ (mmol/L)
2	4.6	331 ^{a}	1.2 ^{a}	33.4 ^{a}	0.7 ^{a}	3.4 ^{a}	0.17 ^{a}	8.1 ^{ab}
3	4.5	369 ^{b}	2.0 ^{b}	33.5 ^{a}	1.0 ^{b}	3.0 ^{b}	0.12 ^{b}	8.6 ^{a}
>3	4.6	422 ^{c}	2.6 ^{c}	34.9 ^{b}	1.0 ^{b}	2.8 ^{b}	0.10 ^{b}	7.5 ^{b}
SED	0.2	12	0.3	0.5	0.1	0.1	0.01	0.5

^{abc} Means within a column with different letters differ significantly (P<0.05)

‡ Average of fortnightly assessments of condition score (CS) and liveweight (WT) from 4 weeks before to 8 weeks postpartum

§ Average of solids in the weekly sum estimates of milkfat and protein production from 1 to 10 weeks postpartum

~ Average of the weekly samples from 4 to 14 weeks postpartum, n = 8 cows/herd

Milksolids production increased with age (Table 2.5). Jerseys had lower production than Friesians (1.6 ± 0.1 vs. 2.2 ± 0.1, kg milksolids/cow/day) and cows at the higher stocking rates produced less than animals at the lower stocking rates (1.6 ± 0.1 vs. 2.3 ± 0.1 kg; Table 2.4; Figure 2.3).

Two year old cows had lower albumin and BOH concentrations and higher glucose and NEFA concentrations than older cows (Table 2.5). Jerseys had higher albumin and NEFA concentrations and lower urea concentrations than Friesians (Table 2.4; Figure 2.4). Lower NEFA concentrations were associated with high stocking rate.

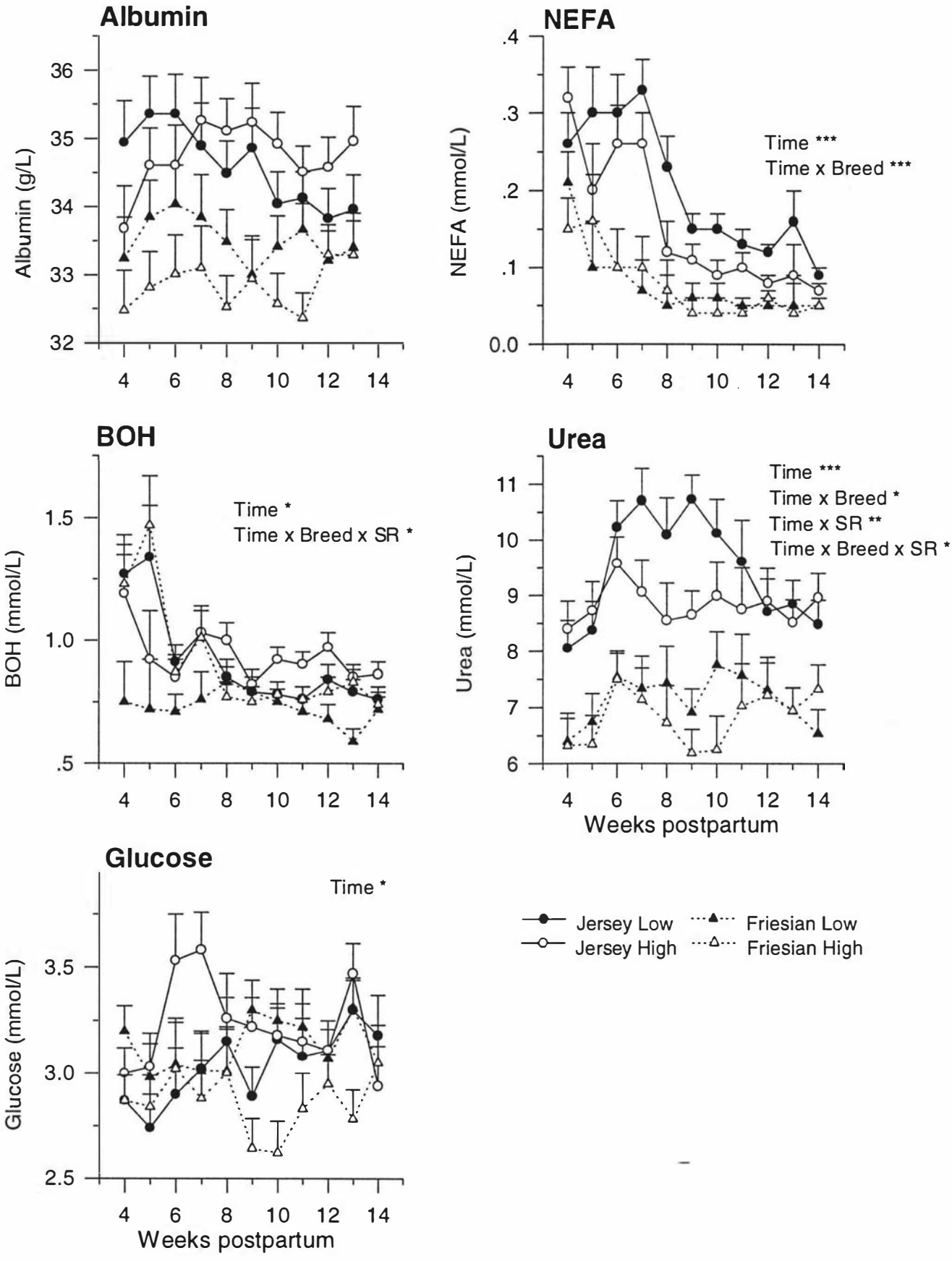

Figure 2.4. The average weekly blood metabolites from 8 cows from each of the 4 herds.

Dry matter disappearance (DMD) was lower in the high stocked herds (9.3 vs. 10.8 and 9.7 vs. 12.1 ± 0.2, kg DM/cow/day, for JH vs. JL and FH vs. FL, P<0.05, respectively) than the low stocked herds (Figure 2.5). The high stocked Friesians had lower DMD/cow than the low stocked Friesians in each month except July and the high stocked Jerseys had lower DMD/cow than the low stocked Jerseys in July, September and October.

Relationships among CS, liveweight, milksolids production, blood metabolite concentrations and the intervals from calving to first ovulation and to first oestrus

The CS at week 8 was related to the C_ovn1 interval (slope = -4.95, P = 0.15). The BOH concentrations at weeks 6, 8, 9 and 12 were significantly related to C_ovn1 interval with weeks 6 and 12 being positively associated (regression co-efficient = 62.7, P<0.01 and regression co-efficient = 62.2, P<0.01, respectively) while weeks 8 and 9 were negatively associated (regression co-efficient = -52.6, P<0.05; regression co-efficient = -62.5, P<0.05, respectively). The mean CS was not significantly related to the C_ovn1 interval in the full model, but when this relationship was examined within each breed, the mean CS in Friesians was negatively related to the C_ovn1 interval (-15.9, P<0.1), but not related in Jerseys (1.7).

Mean CS (regression co-efficient = -7.9, P<0.05), glucose concentration at week 11 postpartum (regression co-efficient = -11.6, P<0.05) and the milksolids production at week 4 (regression co-efficient = -14.2 P<0.001) postpartum were significantly related to the C_h1 interval. A breed by mean CS interaction occurred with the mean CS being negatively related to the C_h1 interval in Friesians (-14.7; P<0.01), but not in Jerseys (-3.9).

Oestrus detection at the first, second and third postpartum ovulation

Oestrus was detected at fewer first postpartum ovulations (22 of 75; 29.3%) than second (63 of 74; 88.7%) or third postpartum (49 of 59, 83.0%) ovulations (χ^2 = 63.0, P<0.001). More Jersey than Friesian cows were

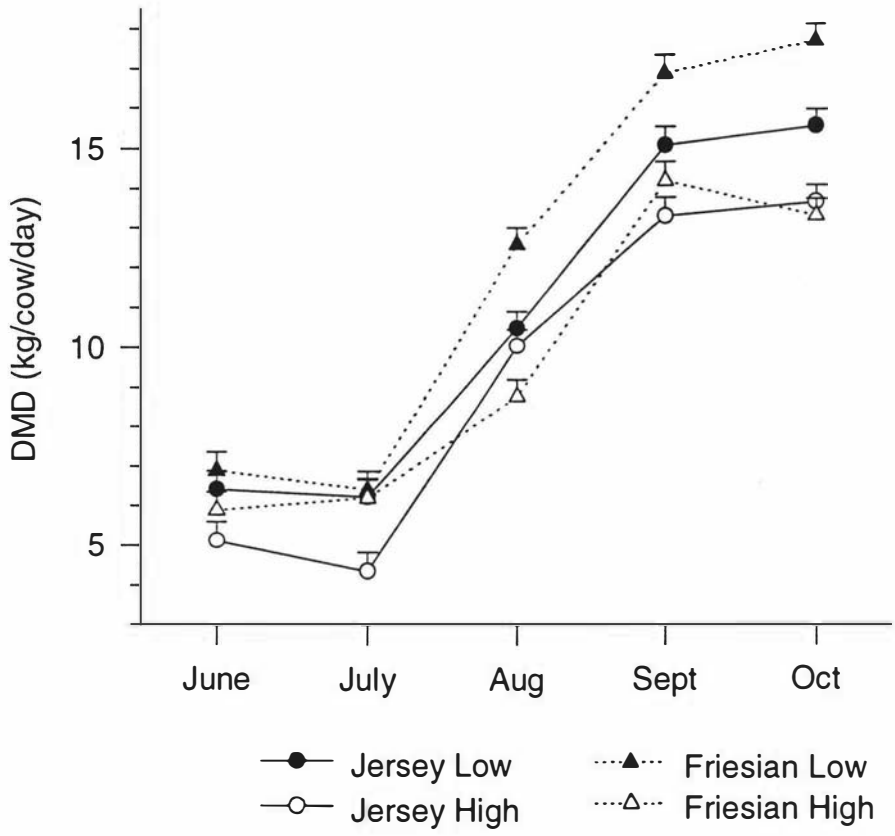

Figure 2.5. Dry matter disappearance/cow (DMD) for the 4 herds.

detected in oestrus at their first postpartum ovulation (18 of 40 vs. 4 of 35, P<0.01). Low stocking rate cows were detected in oestrus at the third postpartum ovulation more often than high stocking rate cows (31 of 33 vs. 18 of 26, P<0.01).

Discussion

The PPA interval was longer in Friesian than Jersey cows especially at higher stocking rates. Younger cows (2 years old) had a longer period of PPA than older (>3 year old) cows with 3 year old animals being intermediate.

The PPA intervals found in the present trial involving pasture fed dairy cows were longer than those reported for cows from dairy systems where supplementary feed is more widely used. Over 93% of cows had ovulated by 40 days postpartum in a British study (Lamming and Bulman, 1976) and 98.6% of Jerseys and Friesians had ovulated by 45 days postpartum in a North American study (Fonseca *et al.*, 1983). This can be compared to only 50% of the cows in the FH herd having ovulated by 50 days postpartum in the present trial.

Calving date influenced the duration of PPA, with cows calving later in the calendar year having shorter C_ovn1 (0.05 days less for each day later that calving occurred) and C_h1 (0.25 days less for each day later that calving occurred) intervals. A breed difference was detected in the effect of calving date on the C_h1 interval, as for each day later in the year that calving occurred in Jerseys the C_h1 interval was 0.4 day shorter but there was no effect of calving date on C_h1 in Friesians. This contrasts with an earlier study where Jersey-Friesian cross cows had a steeper regression co-efficient than Jersey cows for the C_h1 interval (-0.28 and -0.48 for Jersey and Jersey-Friesian cross, cows respectively; Macmillan and Clayton, 1980). Beef cows have also been reported to have negative relationship between calving date and C_h1 relationship but it appears to be more variable but generally steeper than for dairy cows (-0.3 to -0.8, Knight and Nicoll, 1978). This negative relationship may be due to an increased mass or quality of feed being available

as spring progresses, or due to increased vigilance in oestrus detection as the start of mating is approached, in the case of the C_h1 interval.

Jersey cows had a shorter PPA interval than Friesians as has been previously reported (Fonseca *et al.*, 1983). The significant stocking rate by breed interaction for the PPA intervals suggests that Friesians may be more sensitive to nutritional restriction effects on the resumption of cyclic activity than Jerseys. The breeds also differed in the relationship between calving date and C_h1 interval and in the relationship between mean CS and the C_ovn1 and C_h1 intervals. Jerseys appear to be less affected by low CS than Friesians, as the C_ovn1 interval in Jerseys was not related to mean CS and the regression co-efficient of the relationship between mean CS and C_h1 interval was less negative than for Friesians. Differences in feed intake, milk production and some blood metabolites suggest that there may be differences between the breeds in the magnitude of NEB, in the way that partitioning of nutrient intake occurs and in mobilisation of body tissue during NEB.

Increased stocking rate resulted in a prolonged PPA period. Increased stocking rate was also associated with reduced individual cow feed intake, reduced CS, liveweight and milk production. The PPA interval is positively related to the magnitude of NEB after calving (Canfield and Butler, 1990; Staples *et al.*, 1990). In trials where individual feed-intakes and production of individual cows have been monitored, extended periods of PPA are associated with reduced feed intakes, reduced production and increased loss of liveweight (Staples *et al.*, 1990; Lucy *et al.*, 1992). The prolonged period of PPA observed in the high stocked herds in the present trial may be due to reduced feed intake and hence prolongation of the period of NEB in these herds. The homeorhetic mechanisms induced by calving and commencement of lactation (Bauman and Currie, 1980) tend to encourage milk production at the expense of body reserves. Milksolids production in the high stocked herds continued despite the lower availability of nutrient and potentially greater extent and duration of NEB. The homeostatic mechanisms that preserve physiological status (Bauman and Currie, 1980) may eventually override the homeorhetic drives. Increased nutrient intake has been reported in cows calving in low CS (Garnsworthy and Topps, 1982) and cows that have reduced voluntary nutrient

intake in the early postpartum period have lower milk production relative to cows with higher nutrient intake (Staples *et al.*, 1990). The high stocked herds had lower milk production than the low stocked herds indicating that homeostatic mechanisms may have been invoked, resulting in reduced milk production as the body reserves became depleted in early lactation.

Mean CS before and at calving were inversely related to the C_h1 interval. Extension of the PPA interval has been demonstrated following restrictions of feed intake before and after calving. Grainger *et al.*, (1982) demonstrated that manipulation of feed intake to produce a range of CS between 3 and 7 at calving, resulted in an extension of the C_h1 interval by about 5.7 days for each unit decline in CS. Additionally, feeding at two different levels for the first 5 weeks after calving (7 vs. 14 kg of pasture) resulted in a reduction of the C_h1 interval by 1.2 days for each additional kg of pasture fed/cow/day. Both pre- and postpartum restriction of feed intake resulting from the higher stocking rates are likely to have influenced the C_h1 intervals in the present trial. The C_h1 interval was associated with an increase of 7.5 days for each unit decline in CS at calving. This is likely to have involved the combined effects of restricted nutrient intake and lowered CS. The relationship between body CS and NEB has not been clearly established. Whether cows in lower CS at calving are more likely to invoke homeostatic mechanisms such as reduced milk production and/or increased nutrient intake and hence minimise NEB is not known. The FH herd calved at a lower CS than the other herds and had reduced milk production but still had a prolonged PPA period. This may indicate reduced effectiveness of the homeostatic mechanisms, resulting from a reduction of body fat mass to a level below some critical point. In humans, excess loss of body fat, either due to physical training or due to eating disorders results in amenorrhoea. It has been hypothesised that both an absolute mass of adipose tissue and a minimum percentage of body fat related to body mass and height are required before ovulatory activity can commence or be maintained (Frisch, 1994).

Milksolids production at 4 weeks postpartum was negatively related to the C_h1 interval. For each increase of 1 kg in milksolids production, the C_h1 interval was reduced by 14.2 days. Previous studies of both pasture based

systems (Wilson *et al.*, 1985) and those with individual feeding of a total mixed rations have produced a similar relationship (Fonseca *et al.*, 1983; Staples *et al.*, 1990; Lucy *et al.*, 1992). In the group pasture grazing system used in the present trial, competition for limited pasture resources may have occurred, with the most successful cows having higher milk production as well as being in less severe NEB.

Glucose concentration at 11 weeks postpartum was negatively related to the C_h1 interval. This may indicate a greater degree of NEB in the cows with longer periods of PPA as glucose concentration has been correlated with energy balance (Canfield and Butler, 1990). NEFA concentrations have been negatively related to the depth of NEB (Canfield and Butler, 1991). In this trial, NEFA concentrations were not related to the C_ovn1 or the C_h1 intervals. As NEFA concentrations are associated with adipose tissue mobilisation, cows in the high stocked herds which were in lower CS, may not have had as much adipose tissue to mobilise as cows in the lower stocked herds. BOH concentrations, another marker of adipose tissue mobilisation (Lean *et al.*, 1992) were greater in the high than in the low stocked herds. BOH concentrations were both positively (weeks 6, 12 and 13 postpartum) and negatively (weeks 8 and 9 postpartum) associated with the C_ovn1 and C_h1 intervals. There were also large changes in BOH concentration within- and between herds on a weekly basis (e.g. weeks 4 to 6 postpartum, Figure 2.4). This illustrates that these relationships may be spurious or influenced by other unmeasured factor(s). Some previous studies have failed to find consistent relationships among nutrient intake, NEB and measures of reproductive performance (Parker and Blowey, 1976; Staples *et al.*, 1990). The tight homeostatic control of metabolites is likely to mean that very large numbers of animals may be required to examine the relationships among metabolites and reproductive performance. Measuring nutrient flux may be a more sensitive indicator of nutrient status than a one-off measurment of the concentration of a particular nutrient and may be more closely related to reproductive performance.

Younger cows took longer to commence cycling after calving than did older cows which supports the earlier New Zealand work of Macmillan and

Clayton (1980). The younger cows in the present trial had lower liveweight, milksolids production, BOH and albumin but higher glucose and NEFA concentrations than did the older animals. As these animals were still growing, they required nutrients not only for maintenance and production but also for growth. These animals may have been in greater NEB than the older cows. It may also be hypothesised that younger animals are in a lower position in the social dominance hierarchy within the herd resulting in less access to pasture and longer time spent in the milking yards which may also contribute to the poorer reproductive performance of these younger animals.

Only 29.3% of cows were detected in oestrus at first postpartum ovulation, compared to >80% at the second and third postpartum ovulations. These results are similar to those of Lamming and Bulman (1976) who found 29.3%, 62.9% and 84.2% of first, second and third postpartum ovulations, respectively, were accompanied by behavioural oestrus. An absence of P_4 'priming' before the first postpartum ovulation may account for the low percentage of cows being detected in behavioural oestrus (McDougall *et al.*, 1992). Although Jerseys were more likely to be detected in oestrus at the first postpartum ovulation than Friesians (45.0% vs. 11.4%) there was no difference among breeds in expression of oestrus at subsequent ovulations. A higher proportion of Jerseys were also detected in oestrus in a North American study (Fonseca *et al.*, 1983).

Cows in the high stocked herds were less likely to be detected in oestrus at the third postpartum ovulation (69.2% vs. 92.9%) than those in the low stocked herds. The level of NEB at the time of ovulation may influence expression of behavioural oestrus but no relationships were found among CS, liveweight, milk production or blood metabolite concentrations and expression of behavioural oestrus which agrees with the findings of Villa-Godoy *et al.*, (1990) and Fonseca *et al.*, (1983).

The PPA interval was prolonged at higher stocking rates, in younger cows and in Friesians. Friesians were less likely to be detected in oestrus at their first postpartum ovulation. Reduced CS at calving, coupled with reduced pasture availability and hence a greater duration/extent of NEB may account for the prolongation of the postpartum period of anoestrum in the high stocked

herds. The longer C_ovn1 and C_h1 intervals of cows in the FH herd indicate that Friesians may be more sensitive to the effects of higher stocking rates than Jerseys. Mean CS from 1 month before calving to 2 months after calving was negatively related to the C_h1 interval, but this relationship was stronger in Friesian than in Jersey cows. The use of 'metabolic profiles' to predict reproductive performance was of limited value as few, and at times contradictory relationships among metabolite concentrations and C_ovn1 and C_h1 intervals were observed. Both a failure to commence ovulatory activity and a failure to express behavioural oestrus occurred in some cows that were not detected in oestrus for extended periods postpartum.

CHAPTER 3:

A Case Control Study Of Postpartum Anovulation In New Zealand Dairy Cows

Abstract

The proportion of postpartum dairy cows not undergoing regular oestrous cycles by the PSM and the factors influencing this have not been accurately determined for seasonally calving New Zealand dairy herds. Overseas studies have shown that low body CS at calving and extended periods of NEB are associated with prolonged PPA. Cows with extended periods of PPA may mobilise more body reserves and have lower glucose concentrations and higher concentrations of metabolites associated with body tissue mobilisation than cows cycling earlier in the postpartum period. The following study used a case-control design to examine the differences in body CS and blood metabolites among cycling and anovulatory dairy cattle.

Eight herd owners presented all cows not detected in oestrus (NDO) for blood sampling for P_4 analysis at weekly intervals for 3 weeks until 1 week before the herd PSM. One week before PSM an additional blood sample was drawn for analysis of a range of blood metabolites and the CS was assessed. At the same time, the reproductive tract was palpated per-rectum to estimate the ovarian size, to detect the presence of a CL and to detect any ovarian or uterine pathology. From a total of 1596 cows, 275 (17.2%) had been calved >45 days and were still NDO by 1 week before the PSM. Between 8.2% and 38.0% of animals within individual herds were NDO. Only 90 (32.7% of all NDO cows) were AA. Two year old cows were more likely to be AA than older cows (9.4% vs. 6.4% vs. 3.9% of 2, 3 and >3 year olds, respectively; $\chi^2 = 15.3$, $P<0.05$) and more Friesians and crossbreds were AA than Jerseys (6.4% vs. 6.2% vs. 2.1%, respectively; $\chi^2 = 6.9$, $P<0.05$).

The 90 AA cows were paired with ovulating cows of the same age (2, 3, >3 years) and breed (Friesian, Jersey and crossbred). AA cows had lower CS (-0.3 ± 0.1, difference ± standard error the difference; $P<0.001$), had smaller

ovarian size scores (-1.3 ± 0.2, P<0.001), were more likely to have an ovarian follicle (61% vs. 38%, P<0.01), had a higher urea concentration (+0.31 ± 0.16 mmol/L, P = 0.06) and a lower glucose (-0.14 ± 0.06 mmol/L, P<0.05) and total thyroxine (-3.35 ± 1.82 nmol/L, P = 0.07) concentration in their plasma than their cycling pairs.

A large variation in the proportion of cows that were NDO and AA occurred among herds. The factors contributing to this included age, breed and the interval before the PSM that oestrus detection commenced. The CS and metabolite data suggest that anovulatory cows may be in negative energy balance for longer in the postpartum period than cycling herdmates resulting in greater mobilisation of body tissue.

Introduction

PPA can be defined as a failure of a cow to exhibit oestrus by the PSM. Anoestrum may be due to a failure to detect oestrus by the herd manager, to the cow not expressing behavioural oestrus concurrently with ovulation ('silent' ovulations) or to a failure to undergo ovulatory activity (i.e. anovulatory anoestrus, AA; Radostits and Blood, 1985). NDO cows presented for veterinary examination may be categorised by rectal palpation. Detection of a CL indicates that ovulation has occurred while failure to detect a CL indicates an anovulatory state. Between 47.0% and 88.9% (for >3 and 2 year olds, respectively) of NDO cows did not have a palpable CL in a study involving 14 New Zealand dairy herds (Fielden *et al.*, 1973). In contrast, more than 80% and 70% of NDO cows in North American and British studies, respectively, had elevated P_4, indicating ovulation had occurred (Etherington *et al.*, 1991; McCleod and Williams, 1991). This suggests that AA is the most important category of NDO in New Zealand. Lower sensitivity and specificity of rectal palpation relative to serial milk P_4 analysis in detecting ovulation, may also account for these differences (Kelton, 1989)

Early in lactation, energy requirements for production and maintenance exceed energy intakes resulting in cows being in NEB with resultant mobilisation of body tissue to meet this deficit. Cows resume cyclic activity

while still in NEB, about 10 days after the nadir of NEB (Butler *et al.*, 1981). It is difficult to measure energy balance in pasture fed cows because of problems in measuring the individual feed intake. Concentration of various blood metabolites and minerals and CS have been used as indirect indicators of NEB (Payne *et al.*, 1970; Payne and Payne, 1987; Canfield and Butler, 1990).

Cows with extended periods of AA may be in NEB for a longer period and/or to a greater extent than cows that commence cycling earlier in the postpartum period. Greater mobilisation of body tissue may be expected in AA animals. The blood concentrations of metabolites associated with tissue mobilisation (e.g. BOH, urea and NEFA) may be higher and glucose lower in AA cows than in cycling herdmates. Additionally, body CS, as a crude marker for tissue mobilisation, may be expected to be lower in cows with an extended period of NEB. Elemental copper, elemental selenium and total thyroxine (TT4) concentrations in blood have been associated with poor conception rates (Kappel *et al.*, 1984; McClure *et al.*, 1986) and may influence the duration of anoestrum.

This experiment aimed to accurately quantify the proportion of cows that were NDO and AA in 8 selected dairy herds. Additionally, differences in blood metabolite and mineral concentrations and CS between AA and ovulating pairs of animals were quantified.

Materials and Methods

Animals and Design

Cows (n = 1596) from 8 herds of varying size, age composition and breed structure (Table 3.1), located in the Central Waikato region, were selected for this experiment on the basis that the herd managers were willing to cooperate and collect the required reproductive data. Cows were observed for signs of behavioural oestrus, starting at least 1 month before the PSM (44.0 ± 5.5, mean ± sem, range = 28 to 63 days before PSM) by the herd managers using their normal practices. Any cow that had not been detected in oestrus by 3 weeks before the PSM had blood samples (10 ml) removed at weekly

intervals for 3 weeks from the ventral coccygeal vessel into a plain evacuated glass tube (Vacutainer, Salmond Smith Biolab, Auckland, New Zealand) for determination of P_4 concentration. The breed, age, calving dates, and any oestrous data were collected for every cow in each herd 10 days before the PSM. At this time each NDO calved >45 days was paired with a cycling cow on the basis of breed (Friesian, Jersey or crossbred), age (2, 3, >3) and calving date (± 7 days). The reproductive tracts of these pairs of cows were palpated per-rectum one week before the PSM (PSM-7). Ovarian size was assessed on a subjective scale (1 = small, 7 = large), presence of a follicle and/or of a CL was determined and any palpable reproductive tract pathology noted. Body CS was assessed on a 1 (= thin) to 10 (= fat) scale (Macdonald and Macmillan, 1993). Any cow with reproductive tract pathology and any NDO cow with a CL, and their pairmates, were removed from the trial at this point. Blood (20 ml) was drawn at this time from the coccygeal vessels into one plain and one EDTA containing evacuated glass tube for analysis of a range of blood metabolites and minerals.

Table 3.1. Details of the 8 herds used in this study.

Herd	Cows	Late calv†	Age~			Breed‡			Date of First:	
	n	(%)	2	3	>3	F	J	XB	Calving	Mating
1	355	14	24	15	61	4	37	60	2-Jul	19-Oct
2	170	18	20	15	65	65	35	0	12-Jul	5-Oct
3	103	23	25	22	53	92	8	0	5-Jul	2-Oct
4	128	26	26	18	56	20	3	77	21-Jul	17-Oct
5	95	33	19	18	63	100	0	0	28-Jun	10-Oct
6	226	7	31	23	46	43	1	56	20-Jul	1-Nov
7	146	23	19	21	60	24	22	54	20-Jul	21-Oct
8	283	24	19	20	61	66	2	32	9-Jul	19-Oct
mean	185.1	20.9	23.0	19.1	57.9	51.8	13.3	34.9	10-Jul	15-Oct
sem	32.6	2.8	1.6	1.1	2.3	12.3	5.5	11.1	3.1	3.4

† i.e. % of cows calved <45 days 1 week before the PSM
~ % of each herd which were 2, 3 >3 years old
‡ F = Friesian, J = Jersey; XB = crossbred

Laboratory analyses

Metabolite and mineral concentrations were determined at the Ruakura Animal Health Laboratory (Hamilton, New Zealand) using an Hitachi 717 auto-analyser (BOH, glucose, NEFA, urea) at 30 °C. Copper and Selenium concentrations were determined by nitric/perchloric acid digestion followed by atomic absorbence spectrophotometry and TT4 concentration by solid phase RIA (Quanticoat, Kallestad diagnostics, Chaska, Mn, USA). The within- and between-assay coefficients of variation were <5% for all tests. The metabolite and mineral concentrations were measured in samples from each pair of animals, except for selenium for which a random sample of about half of the pairs in each herd was selected.

Progesterone concentration was determined using a solid phase I^{125} RIA (Coat-a-Count, DPC, Los Angeles, Calif, USA). The sensitivity of the assay was <0.2 ng/ml and the within- and between-assay coefficients of variation were 13.0% and 10.9% and 14.7% and 16.6% for pooled samples with mean concentrations of 3.9 and 1.7 ng/ml of P_4, respectively, analysed in sextuplet in 4 assays. A concentration of >1 ng/ml was arbitrarily defined as indicative of luteal activity.

Milk volume (litres; l), milkfat (kg), protein (kg) and length of lactation (days in milk; DIM) data for each cow in 6 of the 8 herds were obtained from the Livestock Improvement Corporation's (Hamilton, New Zealand) data base.

Statistical analyses

Cows presented as NDO at PSM-7 and which had been calved for over 45 days at this time were classified on the basis of rectal palpation and retrospectively on the basis of serum P_4 concentration at 1, 2 and 3 weeks before PSM into one of three categories:

(a) Cycling (C): i.e. a CL was detected upon palpation and/or the serum P_4 concentration was >1 ng/ml in at least one of the weekly blood samples;

(b) Pathological: i.e. abnormalities of the ovary (adhesions, cysts) or uterus (endometritis, pyometron) were detected upon palpation; and

(c) Anoestrous anovulatory (AA): i.e. cows not having been detected in behavioural oestrus before PSM-7, not having a CL or palpable uterine or ovarian pathology and all 3 samples having P_4 concentrations of less than 1 ng/ml.

The proportions of NDO cows in each of the categories were analysed by χ^2 analysis. The proportion of AA cows in each age group and breed were compared by χ^2 analysis. Relationships involving the size of the herd, the proportion of the herd that was Friesian or 2 year old, the number of days before the PSM that oestrus detection started, and the proportion of the herd that was NDO and AA were analysed by linear regression.

The body CS, ovarian size and mineral and metabolite concentrations for each AA cow was subtracted from the observed value from its paired C cow. This difference was then analysed by paired t-Test.

Analyses were performed in Minitab version 8.2 (Minitab Inc., State College, Pa, USA). Data are presented as a mean ± standard error of the mean (sem), unless otherwise indicated.

Results

A total of 460 of 1596 (28.8%) cows had not been observed in oestrus by PSM-7. An average of 19.5% of all cows calved >45 days were NDO with a range of 8.6% to 29.3% among herds (Table 3.2). Of these, 32.7% were AA, 2.5% had pathology and 64.7% were cycling (Table 3.2). The proportion of NDO and AA cows varied among herds (χ^2 = 35.1 and 10.6, $P<0.001$, $0.05<P<0.1$; respectively). The proportion of NDO classified as AA was positively related to the number of days before the PSM that oestrus detection had commenced (%AA/NDO = 1.4 x days before PSM -7.8, R^2 = 58.4%, $P<0.05$). There was no relationship between the size of the herd, the proportion of the herd that was Friesian or the proportion of the herd that was 2 years old, and the proportion of NDO or AA cows. More 2 year old cows were AA than older cows (9.4% vs. 6.4% vs. 3.9% of all 2, 3 and >3 year olds,

Table 3.2. Findings from examination of NDO cows from 8 herds.

Herd	NDO		Cycling		Pathology		AA	
	n	%	n	(% of NDO)	n	(% of NDO)	n	(% of NDO)
1	90	29.3	68	(75.6)	1	(1.1)	21	(23.3)
2	12	8.6	3	(25.0)	0	(0.0)	9	(75.0)
3	17	21.3	9	(52.9)	1	(5.9)	7	(41.2)
4	43	23.2	20	(46.5)	3	(7.0)	20	(46.5)
5	12	17.9	6	(50.0)	0	(0.0)	6	(50.0)
6	31	14.7	19	(61.3)	1	(3.2)	11	(35.5)
7	20	17.7	16	(80.0)	1	(5.0)	3	(15.0)
8	50	23.4	37	(74.0)	0	(0.0)	13	(26.0)
Sum	275	20.9*	178	64.7†	7	2.5†	90	32.7†
mean‡	34.4	19.5	22.3	58.2	0.9	2.8	11.3	39.1
sem‡	9.4	2.2	7.5	6.5	0.4	1.0	2.3	6.7

* % of all 1317 cows that had been calved 45 d by PSM-7,
† As a percentage of the total number of NDO cows that had been calved more than 45 days at PSM-7
‡ Mean and sem among herds

respectively; χ^2 = 15.3, P<0.01) and more Friesians and crossbreds were AA than Jerseys (6.4% vs. 6.2% vs. 2.1%, respectively; χ^2 = 6.9, P<0.05).

The 90 AA cows had a lower body CS (-0.3 ± 0.1; mean difference ± standard error of the difference), higher urea concentration (+0.31 ± 0.16 mmol/L) and a lower glucose (-0.14 ± 0.06 mmol/L) and TT_4 (-3.35 ± 1.82 nmol/L; Table 3.3) concentration than C cows. AA cows had smaller ovaries than C cows (-1.3 ± 0.2; Table 3.3). An ovarian follicle was more likely to be detected on the ovary of an AA cow than a C cow (82.2% vs. 52.2%, respectively, χ^2 = 18.4, P<0.005). There were no differences among AA and C cows in the length of lactation, the milksolids, milkfat, protein or volume of production across the lactation (Table 3.3).

Table 3.3. Differences between anovulatory anoestrous and cycling cows for a range of physical, metabolic, mineral and production measures.

Variable	Units	n	Mean	Diff[§]	SED[~]	P	[‡]
Days pp[ϕ]		90	64.0	-0.4	0.3	0.410	
Ovarian size[Δ]		90	3.7	-1.3	0.2	0.000	***
Condition score		88	4.2	-0.3	0.1	0.000	***
BOH	mmol/L	84	0.6	-0.02	0.05	0.606	
Copper	µmol/L	84	11.8	0.65	0.43	0.132	
Glucose	mmol/L	84	3.3	-0.14	0.06	0.022	*
NEFA	mmol/L	84	0.3	-0.06	0.04	0.103	
Selenium	nmol/L	45	1416.1	77.6	86.1	0.373	
TT_4	nmol/L	84	57.5	-3.35	1.82	0.069	†
Urea	mmol/L	84	7.1	0.31	0.16	0.055	†
Lactation length	days	54	239.2	-7.8	5.1	0.132	
Milksolids	Kg	54	326.4	-1.8	9.4	0.849	
Milkfat	Kg	54	188.0	0.1	5.6	0.992	
Protein	Kg	54	138.4	-1.9	4.1	0.654	
Volume	L	54	3792.2	20.0	125.7	0.874	

[§] Cycling - Anovulatory anoestrus value
[~] Standard error of the difference
[‡] †, *, **, *** P<0.1, P<0.05, P<0.01 and P<0.001 respectively
[ϕ] Days postpartum at the planned start of mating
[Δ] Ovarian size on a subjective 1 to 7 scale (Morris and Day, 1994)

Discussion

Approximately a fifth (20.9%) of the cows calved for more than 45 days had not been detected in oestrus. Of these cows 65% had commenced ovulating while 32% had yet to commence ovulatory activity. That is

approximately 7% of the total number of cows in the herds that were >45 days postpartum at PSM-7 had not commenced ovulatory activity. By comparison, 7% to 15% of Irish and British dairy cows had not commenced ovulating by 50 days postpartum (Lamming and Bulman, 1976; Boyd and Munro, 1979; Fagan and Roche, 1979).

Earlier estimates of the proportion of NDO cows that had commenced cycling in New Zealand dairy herds were lower than the present study (11.1% to 53.0%, Fielden *et al.,* 1973). That study included all cows, irrespective of the time postpartum and rectal palpation was used to determine ovulatory status. However, rectal palpation detects only 70% to 89% of CL's (Kelton, 1989), thus the proportion of AA cows may have been overestimated by Fielden *et al.,* (1973).

In the present study farms varied significantly in the proportion of NDO, AA and the ratio of AA to NDO cows, suggesting differences among farms in either the sensitivity of oestrus detection or in the proportion of cows exhibiting oestrus at ovulation. The proportion of ovulating cows detected in oestrus may be influenced by the herd manager's ability to detect oestrus, the frequency and duration of observation (Esslemont *et al.,* 1985), the number of cows in oestrus simultaneously (Williamson *et al.,* 1972; Kilgour *et al.,* 1977; Pennington *et al.,* 1985), the breed of the cattle (Fonseca *et al.,* 1983), the use of oestrus detection aids (e.g. tail paint, Macmillan *et al.,* 1988) and climatic conditions and physical surroundings (Wolff and Monty, 1974; Pennington *et al.,* 1985). Time postpartum may also effect oestrus detection as only 10 to 30% of dairy cows express behavioural oestrus at the first postpartum ovulation, increasing to between 50% and 70% at subsequent ovulations (Williamson *et al.,* 1972; King *et al.,* 1976; Stevenson and Britt, 1977; Bulman and Lamming, 1978; Peter and Bosu, 1986). Lack of P_4 'priming' before ovulation may account for this low percentage of cows expressing behavioural oestrus at the first postpartum ovulation (McDougall *et al.,* 1992). Farms on which premating oestrus detection commenced early, had a higher proportion of NDO cows which were AA. This may indicate that oestrus was missed in fewer cows on these farms. Other farm management decisions such as cow stocking rate, age structure of the herd, breed of cow, nutritional management

and oestrus detection policy may influence the proportion of the herd that is NDO and/or AA but these have yet to be examined on New Zealand farms.

The lower body CS, higher urea and lower glucose concentrations found in this study support the hypothesis that AA cows may be in lower energy balance than their C herd mates. The lower body CS may be a result of either a longer or more severe period of NEB and hence a greater requirement to mobilise body tissue in the postpartum period, or a reflection of lower CS at calving. Low body CS at calving is associated with an extended interval to resumption of ovulatory activity, but cows fed restricted amounts of pasture postpartum lost more body CS and had a longer period of PPA than better fed animals (Grainger *et al.,* 1982). Whether the AA cows calved in low CS and remained so until the PSM, or whether they underwent postpartum loss of CS could not be determined from this study.

The lower glucose concentrations in AA than C cows supports the hypothesis of lower NEB as it indicates lower feed intake by AA cows. Reduced dry matter intake (DMI) reduces blood glucose concentration in beef cattle (Richards *et al.*, 1989) and supplementing pasture fed dairy cattle with hay increased blood glucose and conception rate (McClure, 1965).

There is a positive relationship between dietary nitrogen intake and blood urea concentrations (Gordon and McMurray, 1979). Urea concentration has been correlated with the length of PPA (Eldon *et al.,* 1988); however, the relationship was positive for the first 20 days postpartum and negative for the rest of the postpartum period. Another study failed to find this association (Armstrong *et al.,* 1990). Cows in poor CS at calving have a higher DMI postpartum than cows calving at higher CS (Garnsworthy and Topps, 1982). A higher DMI may lead to increased protein intake and hence higher urea concentrations. However, Lucy *et al.*, (1992) demonstrated that cows with longer periods of PPA have lower DMI than cows ovulating earlier in the postpartum period. Reduced DMI by anoestrous cows may lead to a negative protein balance and hence protein catabolism resulting in elevated urea concentrations. Studies examining the interactions between protein intake, protein catabolism and urea concentrations in cows with extended periods of PPA are required.

Thyroxine is an important modulator of intermediate metabolism and is involved indirectly in the clearance of steroid hormones (Greenspan, 1991). Energy restriction has been reported to reduce TT_4 concentrations in growing pigs (Giesmann *et al.,* 1989). The lower TT_4 concentrations in AA than C cows in the present experiment may reflect lower DMI by AA cows than C cows. The thyroid gland does not appear to influence expression of behavioural oestrus as the duration and intensity of oestrous activity was not different between thyroidectomised-ovariectomised cows and cows that were only ovariectomised (Stewart *et al.*, 1993). If TT_4 does play any role in the resumption of behavioural oestrus, it may be due to its role in intermediate metabolism, rather than any direct effect on oestrous behaviour.

Cows in NEB in the early postpartum period have elevated NEFA concentrations (Canfield and Butler, 1990). No difference in NEFA concentrations between AA and C cows was demonstrated in the present study. These cows had been calved 63 days on average at the time of sampling and may have been in increasing energy balance by this time. Alternatively, all of the mobilisable adipose tissue may have been utilised by these cows by the time of sampling. Sampling earlier in the postpartum period, when animals may be expected to be in a greater NEB, may have revealed larger differences between C and AA cows. However, the strong homeostatic controls on many of the blood metabolites and minerals (Parker and Blowey, 1976; Adams *et al.,* 1978) may preclude larger differences in C and AA cows being demonstrated, whatever the sampling regime employed.

AA cows had less ovarian tissue than C cows. This may have been due to the presence of a CL in the C cows. However, the AA cows were more likely to have had an ovarian follicle detected than C cows. Large follicles (greater than 10 mm in diameter) and an active process of turnover of follicles occurs in early postpartum, non-ovulating dairy cows (Savio *et al.,* 1990; Chapter 4) similar to that which occurs in cycling cows (Ginther *et al.,* 1989b). In a random selection of cows it may be expected that an equal number of C and AA cows would be at similar stages of follicle development. Consequently, equal numbers of cows with and without palpable follicles would have been expected within the 2 categories. The finding that AA cows were more likely to have a

palpable follicle than C animals is surprising. This may have occurred if CL and ovarian follicles were not distinguished. Alternately, the presence of a CL may make the presence of ovarian follicles more difficult to identify upon palpation.

In conclusion over 20% of cows that had been calved >45 days had not been detected in oestrus by 7 days before the PSM and there were large unexplained differences in the proportion of NDO and AA cows among herds. Managerial factors such as specificity and sensitivity of oestrus detection and the duration of oestrus detection before the PSM may have contributed to these differences.

AA cows had a lower CS, higher urea concentration and a lower glucose and TT_4 concentration than C cows 1 week before the PSM. These data support the hypothesis that AA cows are in NEB for a longer period and/or to a greater degree than their C pairmates. This may have been due to reduced DMI intake by the AA cows before or at the time of sampling.

CHAPTER 4:

Follicle Patterns During Extended Periods Of Postpartum Anovulation In Pasture-Fed Dairy Cows

Abstract

Pasture-fed dairy cows can experience extended periods of PPA which delay mating and make maintenance of a 365 days intercalving interval difficult to attain. This study describes ovarian activity occurring during PPA.

The ovaries of 17, mixed age (2 to 7 years) Friesian cows were examined daily by transrectal ultrasound from 1 week postpartum. The positions and sizes of follicles greater than 2 mm in diameter were recorded. A large (>9 mm in diameter) follicle was present on at least one ovary by 10.3 ± 0.7 (range 6 to 17) days postpartum. This first large follicle ovulated in 2 (12%) cows, with the remaining cows having from two to 9 large follicles before ovulation. The interval from calving to first postpartum ovulation was 43.4 ± 5.3 (range 13 to 93) days following 4.2 ± 0.6 waves of follicles.

The absence of large follicles is not the limiting factor in resumption of postpartum ovulatory activity.

Introduction

Follicular development is an essential precursor to behavioural oestrus and ovulation. Follicles develop in a series of waves which occur in pre-pubertal, postpartum and cycling cattle (Sirois and Fortune, 1988; Ginther *et al.,* 1989a; Murphy *et al.,* 1990; Savio *et al.,* 1990; Hopper *et al.,* 1993). Each wave consists of a group of follicles recruited from a gonadotrophin-dependent pool of antral follicles (Scaramuzzi *et al.,* 1993). One follicle is selected to become the largest or dominant follicle (DF). If this follicle does not ovulate, it undergoes atresia, which allows the emergence of a new follicle wave. This sequence occurs approximately every 10 days (Sirois and Fortune, 1988; Ginther *et al.,* 1989b).

Large follicles are present on the ovaries within 11 days of calving, three-quarters of which may ovulate in well-fed dairy cows (Savio *et al.,* 1990). Initial studies reported that the remaining cows ovulate by the third postpartum DF, at an average of 27 days postpartum (Savio *et al.,* 1990). However, a proportion of pasture-fed cows in New Zealand have extended periods (>60 days) of PPA (Macmillan and Clayton, 1980). Rectal examination of these cows revealed that over half had no palpable ovarian structures, suggesting that follicular development may not have been occurring (Fielden *et al.,* 1973).

Transrectal B-mode ultrasound has been used to assess ovarian structures and demonstrate the presence of follicular waves in cattle (Pierson and Ginther, 1984). The technique has been validated by examining by ultrasound, ovaries recovered following slaughter and then dissecting follicles from the ovary and measuring the external diameter. Quirk *et al.,* (1986) reported a high degree of agreement ($r = 0.98$, $P<0.001$) between the measurements.

The objectives of the present trial were:

(a) to validate the use of B-mode ultrasound for measurement of follicle diameter and detection of the corpus luteum (CL);

(b) to determine when large (>9 mm) DF's were first present on the ovaries of postpartum cows;

(c) to determine when the sequential emergence of DF's, indicative of wave turnover commenced; and,

(d) to determine the number of DF occurring before ovulation in pasture-fed dairy cows.

Materials and Methods

Experiment 1. Validation of ultrasound measurement of ovarian structures

The ovaries of 7 adult milking Friesian cows and 19 adult beef cows were scanned less than 30 min. before slaughter with B-mode ultrasound (ALOKA SSD-210DX, Medtel, Auckland) with a 7.5 MHz linear-array probe. The diameter of each follicle greater than 2 mm in diameter was estimated by taking the average of the horizontal and vertical diameters estimated with reference to the grid on the ultrasound screen. The presence or absence of a CL was noted. The position of each follicle greater than 4 mm in diameter and every CL was noted to facilitate identification of individual structures following slaughter. Each follicle greater than 4 mm in diameter was dissected following slaughter and the external follicle diameter measured in two dimensions with callipers.

The relationship between the diameter estimated by transrectal ultrasound and the diameter measured post-slaughter was investigated by regression analysis. Follicles were also categorised as small (3-5 mm), medium (6-9 mm) or large (>9 mm), and the number of follicles in the size classes detected by ultrasound and dissection were compared using a paired Student's t-Test.

Experiment 2. Daily transrectal examination of the ovaries of postpartum dairy cows.

Seventeen, mixed age cows (11, 2 year olds; three, 3 year olds; and three, older cows) which calved between 10 July and 9 September, 1992, were managed as a single group in a rotationally grazed pasture system and were milked twice daily. From 6.7 ± 0.7 (range 4 to 12) days postpartum, the ovaries of each animal were examined daily by transrectal ultrasound. The position of each follicle greater than 2 mm in diameter was recorded and graphs prepared for each ovary of each animal using the follicle 'identity' method (Ginther,

1993). Ultrasound examination ceased following the third postpartum ovulation except where the first inter-ovulatory interval was greater than 16 days and included at least two follicle waves (i.e. a 'normal' cycle length), or where artificial insemination occurred at the second ovulation in which case it ceased after the second postpartum ovulation.

All cows were weighed and scored for body condition (0 to 10 scale; Macdonald and Macmillan, 1993) each fortnight by one operator. Milk samples were taken at a morning and evening milking weekly for estimation of milk production. A sub-sample was taken for determination of milkfat and protein composition. Composite milk samples (20 ml) were taken twice weekly to measure the P_4 concentration using a commercial ELISA assay (Ovucheck, Cambridge Veterinary Sciences, Ely, Cambridge). The sensitivity of the assay (95% confidence interval around the 0 standard tube) was 0.4 ng/ml. The within- and between-assay coefficients of variations were 5.8% and 12.7%, respectively, for a pool containing approximately 5 ng/ml of P_4. Concentrations of milk P_4 >2 ng/ml were taken as indicative of luteal activity.

The DF was defined as the follicle achieving the largest diameter in any one wave. The time of emergence of the wave was defined, retrospectively, as the day that the DF was first >4 mm in diameter. The maximum diameter, the day of emergence, the number of days that the DF was greater than 9 mm and the growth rate ((maximum diameter - diameter on first day of emergence)/(day of maximum - day of emergence); mm/day) were calculated for each DF. These parameters were analysed for both the second postpartum DF and the ovulatory DF by one-way analysis of variance with the wave number (1 + 2, 3, 4, 5 + 6 and >6) as the main effect. The diameter (mm) of the second postpartum DF on each day following emergence, the mean difference in the maximum diameter of the first DF and the ovulatory follicle divided by the number of DF to ovulation (mm/DF) and the interval between the emergence of the second and third DF (days) were analysed by one-way analysis of variance with the wave number (1 + 2, 3, 4, 5 + 6 and >6) as the main effect. Differences in means were compared using the least significant difference technique. The relationship among the maximum diameter, the number of days that the DF was greater than nine mm, the growth rate of the second

postpartum DF and the number of DF's preceding ovulation were examined by regression analyses.

The weekly estimates of average daily milk volume production (kg), milksolids production (i.e. milkfat (kg) plus protein (kg)) and fortnightly CS and liveweight (kg) for each cow were aligned by calving date (week 0). The data were then analysed by one-way analysis of variance with the number of follicle waves (1 + 2, 3 + 4 or >4) as the main effect.

All analysis was performed using SAS (SAS Institute Limited, SAS Campus Drive, Cary, NC). Data are presented as least square means and standard errors of the mean (sem) unless otherwise indicated.

This experiment was approved by the Animal Ethics Committee, AgResearch, Ruakura, as experiment number DRC 003/02.

Results

Experiment 1

The estimate of follicle diameter by ultrasound was positively correlated with the diameter of the dissected follicles ($P<0.001$; Figure 4.1). Ultrasound underestimated the number of small (3 to 5 mm) follicles (Table 4.1). A CL was identified on one ovary of every cow by ultrasound and this was confirmed upon dissection.

Table 4.1. The number of small, medium and large follicles detected upon transrectal ultrasound and ovarian dissection from 26 mixed age cows.

Method	small†	medium†	large†
Ultrasound	19.3 [a]	1.4	1.3
Dissection	26.7 [b]	1.6	1.1
SED¶	2.2	0.3	0.1

[ab] Indicates significant differences ($P<0.05$) within a column
† Small = 3 to 5 mm; medium = 6 to 9 mm, large = >9 mm
¶ Standard error of the difference

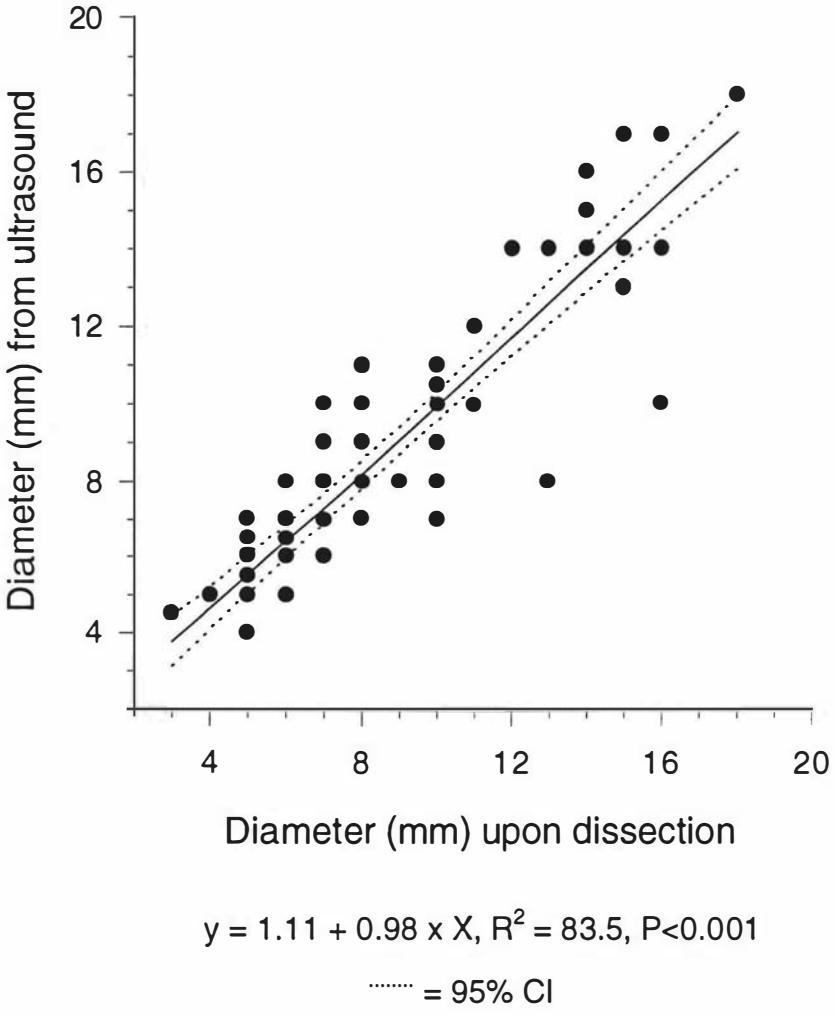

Figure 4.1. The relationship between the diameter (mm) of follicles (n = 70) estimated by transrectal ultrasound and by follicle dissection.

Experiment 2

The first follicle with a diameter of greater than 9 mm was detected 10.3 (± 0.7; range 6 to 17) days postpartum. This follicle ovulated in 12% of cows. All other cows had a sequence of DF's from calving onwards (Figure 4.2). First ovulation occurred 43.4 ± 5.3 (range 13 to 93) days postpartum following 4.2 ± 0.6 (range 1 to 9) waves of follicles. The average interval between the first and second postpartum ovulations was 12.7 ± 1.7 (range 6 to 32) days, with 75% of first inter-ovulatory intervals being less than 18 days, 18.8% within 18 to 24 days and 6.3% greater than 24 days. Between the second and third postpartum ovulations, 5 of 7 cows had two DF's and the remaining two cows, three DF's. The interval between the second and third postpartum ovulations was 19.2 ± 0.4 and 25.0 ± 4.0 days for cows having two and three DF's between ovulations, respectively.

Every first postpartum ovulation detected by ultrasound was followed by a rise in P_4 concentration exceeding 2 ng/ml within 3.7 ± 5.3 days. Two cows each had an individual P_4 concentration of greater than 2 ng/ml (5.8 and 3.3 ng/ml) in one sample either 4 or 6 days before ovulation (as determined by disappearance of the DF) was detected by ultrasound.

The maximum diameter of a DF increased by 1.8 ± 0.7 mm between successive DF's, and there was no difference in this rate of increase among cows ovulating following different numbers of DF's postpartum. The maximum diameter of the DF tended to increase with the number of waves postpartum (Table 4.2, Figure 4.3). The maximum diameter of the second DF, the number of days the second DF was greater than 9 mm in diameter and the interval from second to third postpartum DF emergence were larger in cows having fewer DF's to ovulation (Table 4.2; Figure 4.4), and were inversely associated with the number of DF's occurring before ovulation (Figure 4.5; Number of DF = -0.39 x days DF>9 mm + 9.05; R^2 = 64.2%, P<0.005). There was no difference in the growth rate of the second DF among cows ovulating after two to nine DF's postpartum.

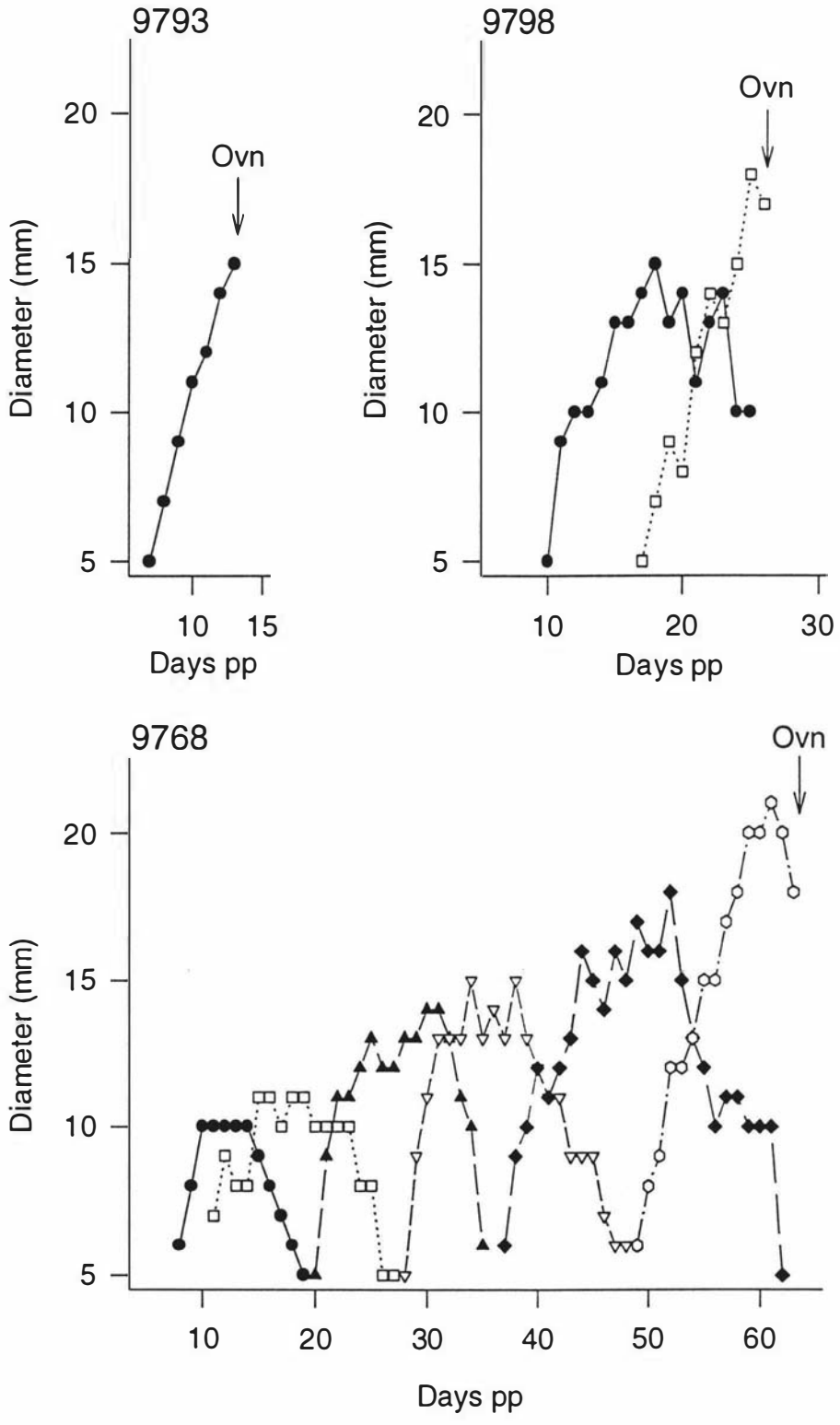

Figure 4.2. The daily diameter (mm) of the DF's from three cows which ovulated the first (9793), second (9798) or sixth (9768) postpartum DF.

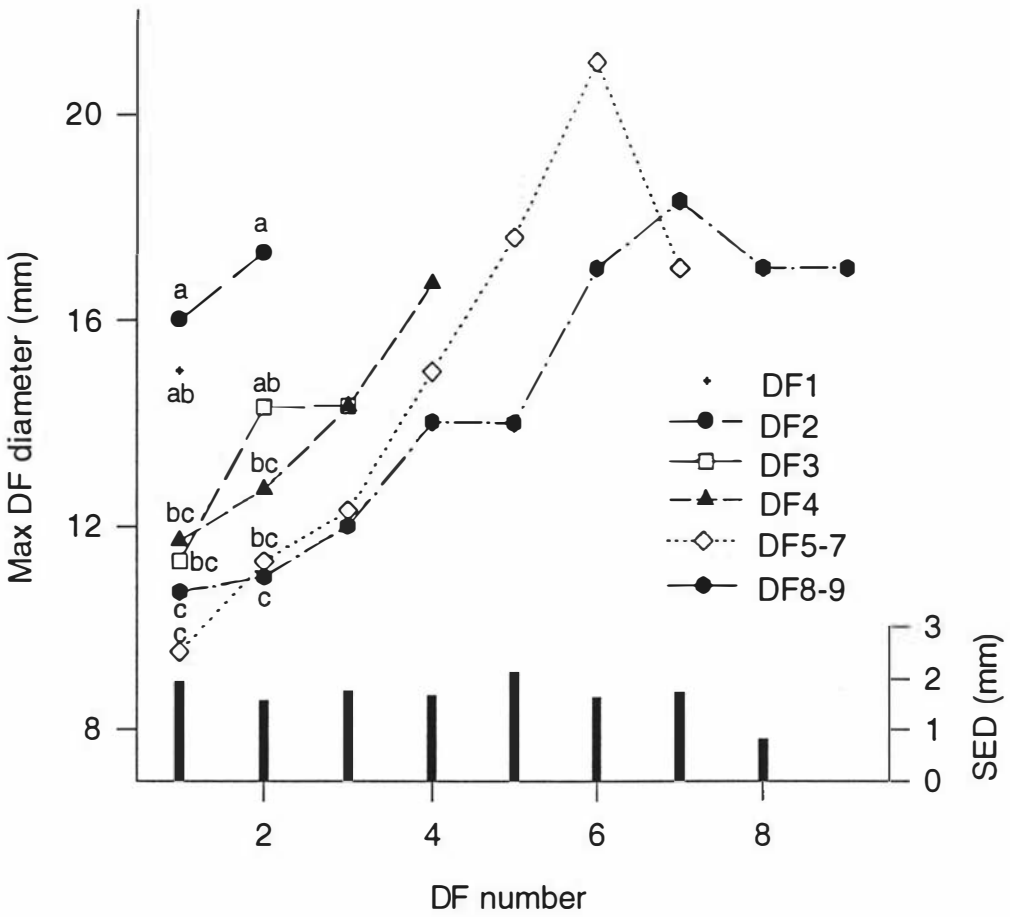

Figure 4.3. The maximum diameter (mm; standard error of the difference as bars along the x axis) of the DF for cows ovulating the first, second, third, fourth, fifth to seventh or eighth and ninth DF postpartum. [abc] Different letters among diameters at the same DF number indicates significant difference ($P<0.05$).

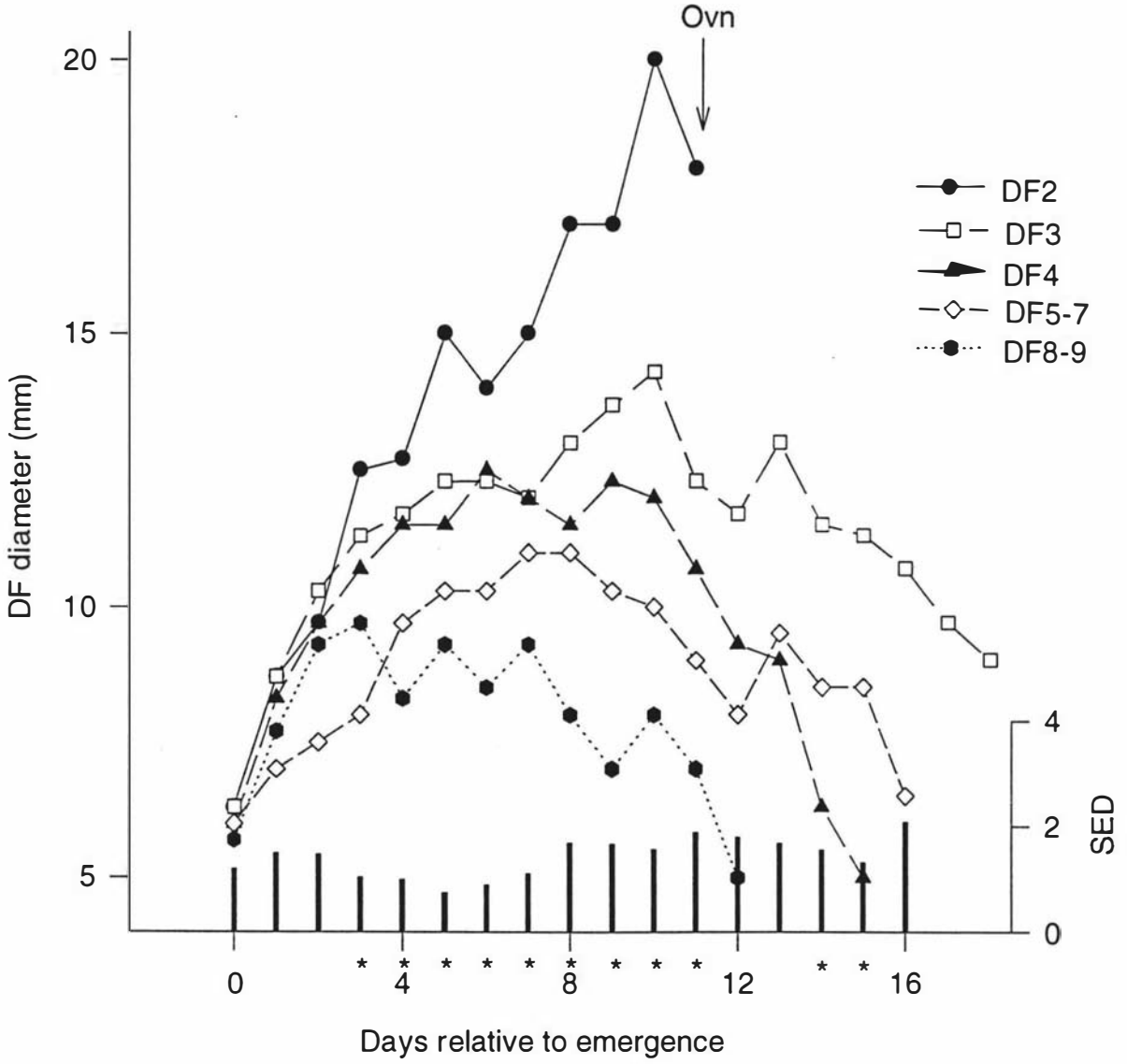

Figure 4.4. The diameter and SED (bars along the x axis) of the second postpartum DF for each day following emergence (d = 0) from cows ovulating the second, third, fourth, fifth to seventh or eighth and ninth DF postpartum. * Indicates significant difference ($P<0.05$) among means on that day following emergence.

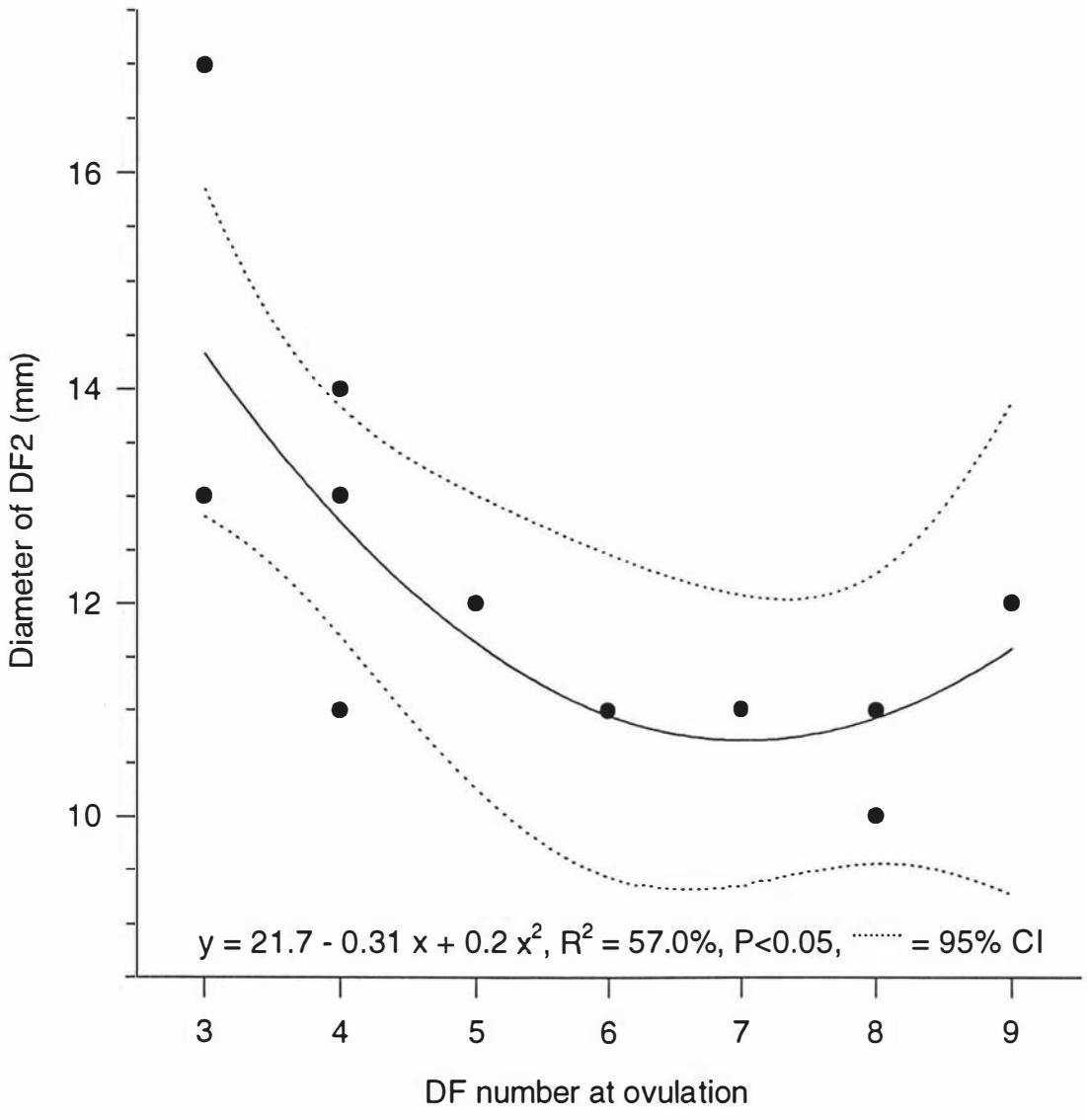

Figure 4.5. The relationship between the maximum diameter (mm) of the second postpartum DF (DF2) and the number of the DF ovulating.

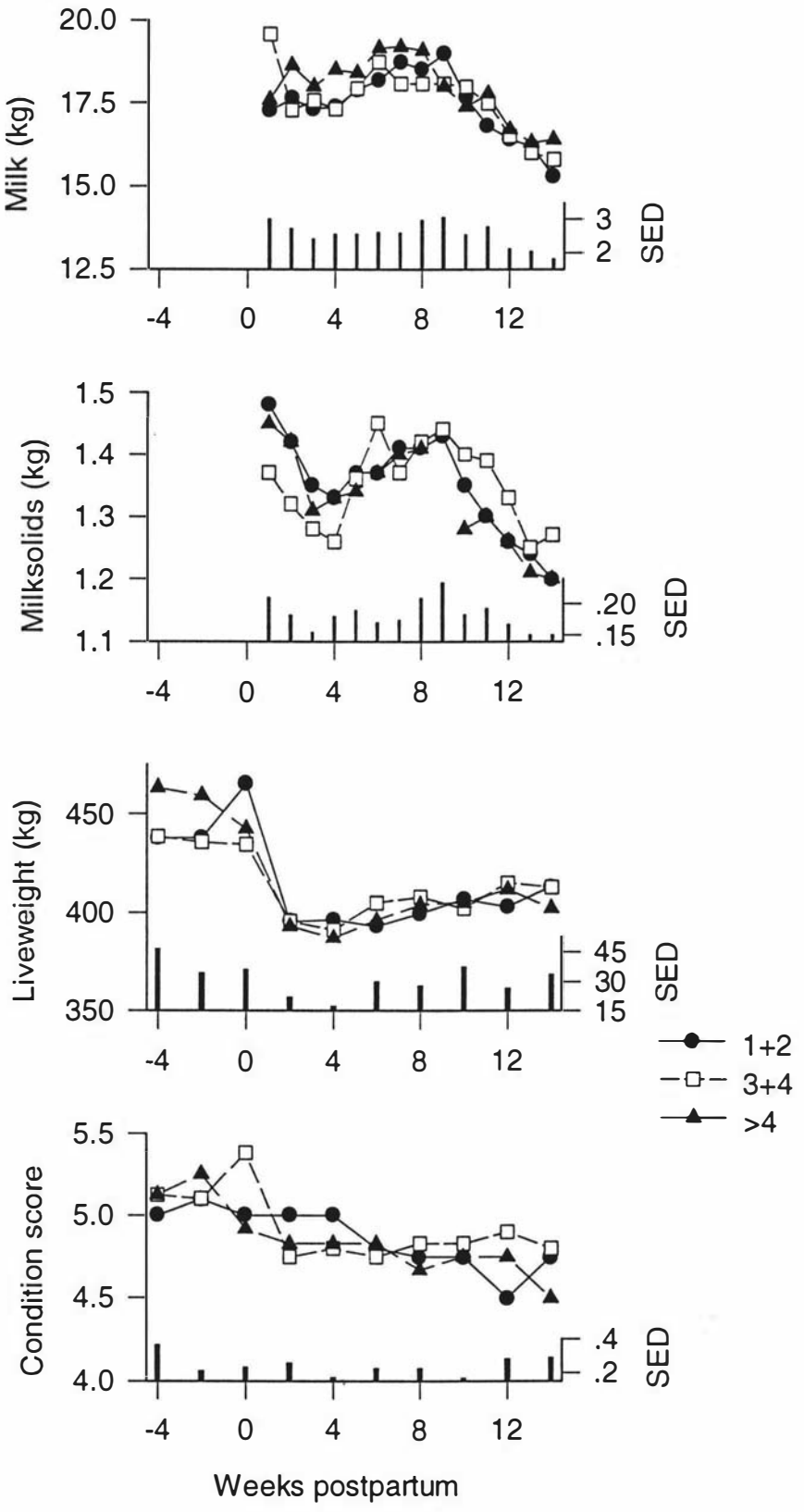

Figure 4.6. The mean and SED (as bars on x axis) daily milk production and milksolids production, and liveweight and condition score at weekly or fortnightly intervals from 4 weeks prepartum to 14 weeks postpartum for cows having 1+2, 3+4 and >4 DF's before ovulation.

Table 4.2. Growth characteristics of the second and ovulatory dominant follicle from 17 mixed age cows examined by ultrasound.

DF at ovn	n	E 2† (day pp)	Gr 2† (mm/day)	Max 2† (mm)	DF>9† (days)	E2-3~ (days)	DF changeØ (mm/DF)	E ovn‡ (day pp)	Max ovn‡ (mm)
1	2	-	-	-	-	-	-	6.0	15.6 ^{b}
2	3	19.3	1.5	17.3 ^{a}	-	-	1.3	19.3	17.3 ^{ab}
3	3	11.7	1.0	14.3 ^{ab}	16.0 ^{a}	9.0 ^{ab}	2.0	24.0	15.3 ^{b}
4	3	11.5	0.9	12.7 ^{b}	10.0 ^{ab}	8.0 ^{ab}	2.7	28.7	16.6 ^{ab}
5,6 + 7	3	16.0	1.0	11.3 ^{b}	7.3 ^{bc}	9.7 ^{a}	2.1	49.3	19.7 ^{a}
8 + 9	3	16.0	1.7	11.0 ^{b}	5.0 ^{c}	5.5 ^{b}	0.9	71.3	17.3 ^{ab}
SED	-	3.3	0.4	1.6	1.7	1.5	2.0	6.5	1.9

† Day of emergence (E 2), growth rate (Gr 2), maximum diameter (Max 2) and the number of days >9 mm in diameter of the second postpartum dominant follicle

~ The interval between emergence of the second and third postpartum dominant follicle

Ø Change in maximum diameter of the dominant follicle from the first to the ovulatory DF divided by the number of dominant follicles before ovulation

‡ Day postpartum of emergence and the maximum diameter of the ovulating dominant follicle

abc Indicates significant differences among means with different superscripts ($P<0.05$) within a column

There was no difference in the milk production (volume or milksolids), liveweight or CS among cows having 1 and 2, 3 and 4 or >4 DF's before ovulation despite there being significant differences in the intervals to first postpartum ovulation (23.0, 34.3 and 69.1 ± 5.6 days for cows having 1 + 2, 3 + 4 and >4 DF's before ovulation, respectively, $P<0.001$; Figure 4.6).

Discussion

Use of the ultrasound allowed accurate estimation of the diameter of ovarian follicles and detection of the presence of the CL in agreement with an earlier study (Quirk *et al.*, 1986). However, the ultrasound technique underestimated the number of small (3-5 mm) follicles by approximately 25%. In our hands, ultrasound was best used for *in-vivo* estimation of the number and diameters of follicles greater than 5 mm.

The first DF was detected within 11 days of calving, as had been reported previously (Rajamahendran and Taylor, 1990; Savio *et al.,* 1990). This DF ovulated in only a few cases (12%), with the remaining cows having a series (two to 9) of large DF's before ovulating. Absence of a large DF was not the factor limiting resumption of cycling activity in this group of cows. A similar conclusion was reached for the suckling beef cow (Murphy *et al.,* 1990).

The interval to first ovulation was longer and there were more DF's before first ovulation than has been previously reported in dairy cows (Rajamahendran and Taylor, 1990; Savio *et al.,* 1990). Several environmental and genetic factors may contribute to this difference. Lower dietary intakes of energy with resultant longer periods of NEB could be one explanation. The nadir of NEB has been associated with the interval to resumption of cyclic activity (Butler *et al.,* 1981), and the number of large (>9 mm) follicles positively associated with energy balance in postpartum cows (Lucy *et al.,* 1991). Undernutrition also has been shown to reduce the size of follicles in cyclic cows (Murphy *et al.,* 1991).

The gonadotrophins (FSH and LH) are required for the development of follicles to >2.5 mm in sheep (Scaramuzzi *et al.,* 1993) and to >6 mm in cattle (Webb *et al.,* 1994). Sufficient gonadotrophin concentrations appear to have been present in the cows in the present experiment to allow development of large follicles. However, despite the presence of large follicles and low P_4 concentrations (mainly less than 1.0 ng/ml), ovulation did not occur. The final maturation of the ovulatory follicle involves development of LH receptors in the granulosa cells (an FSH-dependent event) and production of sufficient E_2 from the androgen precursors (androgen production is LH dependent) to induce a pre-ovulatory gonadotrophin surge. Both FSH and LH may be potentially limiting return to cyclic activity in the postpartum period. Whereas FSH concentrations return to luteal-phase concentrations within a few days of calving, LH concentrations are low for variable periods postpartum (Schallenberger, 1985). Additionally, cows with extended periods of NEB and prolonged intervals to ovulation also have a low LH pulse frequency and amplitude (Canfield and Butler, 1990). Low LH concentrations may reduce thecal androgen production (McNatty *et al.,* 1984b), and consequently E_2

synthesis. Insufficient E_2 would prevent the positive feedback release of gonadotrophins required to achieve ovulation.

There was a large range in the number of DF's recorded before ovulation in this trial (Figures 4.2 to 4.5). Cows with larger second DF's which persisted at greater than 9 mm for longer periods and which had longer intervals between second and third postpartum DF emergence had shorter intervals to ovulation. This may indicate greater 'dominance' by the DF's of these cows. Given that follicular growth and steroid production are gonadotrophin dependent, the cows with larger second postpartum DF's may have had higher gonadotrophin concentrations than cows ovulating later postpartum. These higher gonadotrophin levels may then have stimulated the higher production of androgen and E_2 required for ovulation.

In conclusion, post-slaughter dissection of follicles from ovaries which were from previously examined and measured by ultrasound, produced a high correlation in estimated diameters. Ultrasound is thus a valid tool for estimation of follicle size *in-vivo*.

The absence of large follicles is not the limiting factor to resumption of postpartum ovulation in pasture-fed dairy cows, as large follicles were present within 11 days of calving. However, despite the presence of these large follicles and low concentrations of P_4, ovulation did not occur for an extended period in many individuals. This suggests that a failure of the preovulatory gonadotrophin surge, perhaps due to insufficient E_2 production by these follicles, is the mechanism preventing resumption of cyclic activity.

CHAPTER 5:

Concentrations Of Steroids, Insulin-Like Growth Factor And Insulin-Like Growth Factor Binding Proteins In The Ovarian Follicles Of Anovulatory And Cycling Dairy Cows

Abstract

Some dairy cows have extended periods of PPA when large follicles are present, but ovulation does not occur. This may be related to low LH pulse frequencies leading to a failure of E_2 production by these large follicles and hence a failure of the pre-ovulatory gonadotrophin surge. Anovulatory and cycling cows (n = 14/group) were ovariectomised when the largest follicle was growing or when it had ceased growing (n = 14/group). The concentrations of E_2, testosterone (T), P_4, insulin-like growth factor (IGF) and the insulin-like growth factor binding proteins (IGFBP) were measured in the largest growing or plateau follicle present at ovariectomy. The LH concentration was determined in samples collected at 15 min intervals for 8 h preceding ovariectomy.

The largest follicle from anovulatory cows had lower E_2 (47.0 vs. 372.1) T (1.4 vs. 10.0) and P_4 (7.8 vs. 16.0) concentrations than the largest follicle from cycling cows. There was no difference in the ratio of E_2 to T, IGF and IGFBP concentrations among anovulatory and cycling cows.

These data suggest that low LH pulse frequency may result in low T and hence E_2 production. Insufficient E_2 may be produced to induce the pre-ovulatory gonadotrophin surge so that the large follicles which are present following calving fail to ovulate and undergo atresia, allowing new follicles to develop.

Introduction

Some pasture-fed dairy cows in New Zealand have extended periods of PPA (Fielden *et al.*, 1973). During this period, a series of DF develop but they

fail to ovulate until 4 to 5 DF's have occurred around 44 days postpartum (Chapter 4). In the cycling cow, similar large follicles develop (Sirois and Fortune, 1988; Knopf *et al.*, 1989) and in the absence of P_4 the DF produces sufficient E_2 to induce the pre-ovulatory gonadotrophin surge and hence ovulation (Peterson *et al.*, 1975). The DF is selected from a pool of antral follicles and suppresses the growth of both the concurrent and subsequent cohorts of follicles (Ko *et al.*, 1991; Badinga *et al.*, 1992). As a DF increases in diameter the concentration of T declines and E_2 and P_4 increase. Where a follicle fails to ovulate, dominance wanes and atresia occurs which is associated with declining E_2 and rising P_4 concentrations within the follicle (Ireland and Roche, 1982; McNatty *et al.*, 1984a). Androgens (T and androstenedione) are produced by cells of the theca interna under LH stimulus and are then converted to E_2 by the FSH dependant aromatase enzyme present in the granulosa cells (McNatty *et al.*, 1984b; Fortune, 1986).

Undernutrition and low body condition have been associated with extended PPA (Grainger *et al.*, 1982). Low CS at calving due to low prepartum intake of nutrients, reduces the number of small follicles and the E_2 concentration in the DF 5 to 9 weeks after calving in suckled beef cows (Prado *et al.*, 1990). This may result from inhibition of GnRH release from the hypothalamus causing a low peripheral LH pulse frequency (Canfield and Butler, 1990; Wright *et al.*, 1990). Undernutrition is also associated with reduced peripheral concentrations of IGF in cattle (Granger *et al.*, 1986; Houseknecht *et al.*, 1988; Spicer *et al.*, 1990). Treatment with growth hormone increases plasma IGF concentrations and results in increases in the number of small (2 to 5 mm) follicles (Gong *et al.*, 1991). IGF is mitogenic for granulosa cells, stimulates steroid production and interacts synergistically with the gonadotrophins in cell culture (Adashi *et al.*, 1991; Gong *et al.*, 1993; Spicer *et al.*, 1993). However, intrafollicular IGF concentrations are similar in underfed and fully-fed cattle (Rutter and Manns, 1991; Spicer *et al.*, 1991). Additionally, IGF concentration is similar in follicles with a wide range of E_2 concentrations (Spicer *et al.*, 1988). IGF may be bound to one of a family of at least 6 IGFBP's (Baxter, 1991; Clemmons, 1993). These have been characterised in the follicular fluid from various mammalian species and the occurrence of

individual IGFBP's differs among species (Ling *et al.*, 1993). Within a species, the IGFBP concentrations vary with physiological status, with more lower molecular weight IGFBP's present in atretic follicles in the rat (Ling *et al.*, 1993), human (Catlado and Guidice, 1992), pigs (Howard and Ford, 1992) sheep (Monget *et al.*, 1993) and cow (Echkterncamp and Howard, 1992). This observation has suggested that the IGFBP's may be modulating the effect of gonadotrophins on steroidogenesis, by altering the concentration of the free, biologically active IGF concentration (Ling *et al.*, 1993).

The aim of the present study was to determine the diameter of the DF, the total number of follicles, the intrafollicular concentrations of E_2, T, P_4, IGF and the IGFBP's at two different stages of follicle development in anovulatory and cycling dairy cows.

Materials and Methods

The experiment was a 2 by 2 factorial design, comparing follicular function in anovulatory and cycling cows at growing and plateau phases of follicular development. The experiment involved 28 cows over a period of 2 years (n = 16 in 1991 and n = 12 in 1992). Cows were selected from a spring calving, pasture-fed, dairy herd with a planned start of calving of 15 July. Oestrus detection and milking were carried out twice daily from calving onwards. Six weeks after the planned start of calving, cows were selected as cycling or anovulatory based on oestrus detection records. A cycling and an anovulatory cow were paired based on calving date (± 7 days), breed (Jersey or Friesian) and age (2, 3 or >3 years old). The ovaries of each cow were examined daily by transrectal ultrasound using an ALOKA 210Dx ultrasound with a 7.5 MHz linear array transducer (Medtel, Auckland, NZ). The position and size of each follicle >2 mm in diameter were recorded and a longitudinal graph drawn for each ovary of each cow (Ginther, 1993). The cows were weighed and body condition scored (CS; 1 = thin to 10 = fat scale) during the week ultrasound examination commenced.

Milk samples (20 ml) were collected twice weekly for determination of P_4 concentration. Any cow selected for inclusion in the anovulatory group that had

a milk P_4 concentration of >2 ng/ml or was detected as having ovulated by ultrasound (a large follicle was not detectable on consecutive daily examinations) was removed from the trial along with its cycling pair. Half of the pairs of cows were randomly assigned to be ovariectomised when in the growing phase of follicle development and the other half in the plateau phase. The growing phase was defined as when the DF (the largest growing follicle in a cohort of follicles) was between 5 and 9 mm in diameter. The plateau phase was defined as when the DF was >10 mm in diameter and its diameter had not altered by more than 1 mm for 72 h. Ovariectomy in the plateau phase for the cycling cows occurred during the presence of the first DF after ovulation (8.3 days after ovulation) and in the growing phase during the presence of the second DF following ovulation (11.1 days after ovulation). Twenty-four h before ovariectomy all cows were injected with 25 mg of dinoprost tromethamine (Lutalyse, Upjohn, Auckland NZ) i.m., to ensure that the DF would be the presumptive pre-ovulatory follicle. In the 8 hours preceding ovariectomy, blood samples (10 ml) were drawn at 15 min. intervals for subsequent assay for LH concentration. Bilateral ovariectomy was performed using a standing left flank approach following sedation with xylazine (Rompun 2% solution, Bayer New Zealand Limited), infusion of lignocaine hydrochloride (Lopaine 2%, Troy Laboratories, Ethical Agents, Auckland, NZ) for local anaesthesia and surgical preparation of the site.

The recovered ovaries were placed on ice and within one h they were trimmed of adventitia, weighed and the CL removed and weighed. The ovaries were then immersed in water, examined by ultrasound and all follicles >2 mm in diameter were located and their position recorded. Each follicle >4 mm in diameter was dissected free of the stroma, its diameter measured and the follicular contents aspirated with a 25 gauge needle attached to a tuberculin syringe. Where the aspirated volume was less than 500 µl, it was made up to 500 µl with buffer (0.2 g/L KCl, 0.2 g/L KH_2PO_4, 8.0 g/L NaCl, 2.16 g/L $Na_2HPO_4.7H_2O$; pH 7.2). Four, 100 µl samples were stored at -20 °C until assay for P_4, E_2, T, IGF and IGFBP concentrations. Following aspiration the follicles were cut in half, and using a plastic microbiology loop (Looplast, LP Italiana SPA, Milan, Italy) the granulosa cells were gently scraped off into a well

of a 24-well tissue culture plate (Falcon 3047, Becton Dickinson, Lincoln Park, New Jersey, USA), re-suspended in 0.9% NaCl and counted using a haemocytometer (McNatty *et al.*, 1984a).

Hormone assays

The concentrations of E_2, P_4, T and IGF were measured in validated radioimmunoassays (RIA) after the follicular fluid was diluted with buffer to between 1:25 and 1:100, 1:1 to 1:50, 1:10 to 1:500 and 1:1 to 1:25 for E_2, P_4, T and IGF respectively, before assay in duplicate. This ensured that the hormone concentration was determined within the linear part of the standard curve.

Progesterone and Testosterone assays

P_4 and T were assayed without extraction in commercial solid-phase RIA's (Coat-a-count, DPC, Calif, USA) with all samples assayed in a single assay. Parallelism of serially diluted follicular fluids with the standard curves was demonstrated. The recoveries following addition of 10, 5 and 2 ng/ml of P_4 and T to charcoal-stripped follicular fluid were 97.2%, 90.4%, and 94.9% and 94.9%, 98.9% and 104.8%, respectively. The sensitivities were 0.04 and 0.07 ng/ml for the P_4 and T assay, respectively. The within-assay variation (n = 6) was 17.1% for a sample of mean concentration of 2.5 ng/ml in the P_4 assay, and 5.8% for a sample of mean concentration of 0.5 ng/ml in the T assay.

Oestradiol assay

The E_2 concentration was determined without extraction (McNatty *et al.*, 1982). Parallelism was demonstrated by serially diluting a pool of follicular fluid with charcoal-stripped follicular fluid. The recoveries following addition of 12.5 and 50 pg/ml of E_2 to assay buffer were 117.2 ± 4.2% and 99.4 ± 5.9% (mean ± sem for duplicate estimations in 3 assays), respectively. The within- and between-assay coefficients of variation were 13.7% and 6.6%, and 21.4% and

14.2%, respectively, for quality control pools containing 77 and 20 ng/ml of E_2 (n = 4 assays). The sensitivity was 3.4 ± 0.3 pg/ml (6 replicates in 4 assays).

IGF assay

The intrafollicular IGF concentrations were determined by formic acid/methanol extraction (Bruce *et al.*, 1991) followed by double antibody RIA using an I^{125} labelled human recombinant IGF-1. The rabbit anti-human IGF-1 was a gift from Drs Underwood and Van Wyck, distributed through the NIH hormone distribution program. The concentrations were determined in one assay that had a within-assay coefficient of variation of 6.0% and an extraction efficiency of 96 ± 6 %.

IGFBP determinations

Follicular fluids (2 µl) were subjected to SDS-page electrophoresis under non-reducing conditions and the IGFBP's detected by Western-ligand blotting using iodinated IGF-2 (Hossenlopp *et al.*, 1986). They were quantified by densitometry (Molecular Dynamics, USA) and the band density (OD) was standardised against the OD of the same band from a control ovine plasma and cerebral spinal fluid run in parallel on the same Western ligand blot. The identity of three IGFBP's was confirmed by immunoblotting with anti-bIGFBP2 and anti-hIGFBP4 (U.B.I., Lake Placid, New York) and anti-gIGFBP3 (McLaren and Prosser, 1994).

Milk Progesterone assay

The milk P_4 concentrations were determined by direct assay using a validated solid-phase RIA (Coat-a-count, DPC, Calif, USA).

LH assay

Serum LH concentrations were determined in a validated (Chapter 6), heterologous double antibody, I^{125} RIA with a sensitivity of <0.08 ng/ml and with

within- and between-assay coefficients of variation of 4.0% and 5.0% and 3.6% and 3.1% for pooled samples containing 1.2 and 5.7 ng/ml of LH, respectively.

Statistical analyses

Continuous data were analysed using general linear models (GLM; SAS Institute Ltd, SAS Campus Drive, Cary, NC, USA). Least square means for the main effects were compared by the least significant difference technique.

The diameters of the DF and sub-dominant follicle (i.e. the follicle with the second largest maximum diameter within a cohort of follicles) for each day before ovariectomy, were analysed by a GLM following alignment of the daily profiles to the day of ovariectomy. The total number of follicles and the numbers of small (3 to 5 mm diameter), medium (6 to 9 mm) and large (>9 mm) follicles were analysed by GLM following alignment by the day of emergence of the DF (i.e. the first day the DF >3 mm). The diameter of the DF, the growth rate of the DF over the 3 days before ovariectomy and the density of granulosa cells were also analysed by a GLM. In each model the status (cycling or anovulatory), the phase of DF development (growing or plateau) and the status by phase interaction were included as factors.

The density (d) of granulosa cells was calculated as:

$$d = \frac{count}{\pi d^2}$$

where count was the number of granulosa cells (x 10^3) and πd^2 was the surface area of a follicle with a diameter of d (mm).

The steroid and IGF concentrations within the DF were log-transformed before being analysed by year, status, phase and the status by year interaction. Each IGFBP was analysed as a log-concentration and as a percentage of the sum of each of the IGFBP's. The IGF and IGFBP concentrations were also analysed in a model using a classification of all aspirated follicles as either a DF or a non-dominant follicle as the main effect.

Following analysis, log-transformed data were back-transformed to produce geometric means and the least significant ratio (LSR) was used to compare treatments (Steel and Torrie, 1980). Other data are expressed as

mean ± standard error of the mean (sem) or mean ± standard error of the difference (SED).

Results

Ovariectomy occurred 61.0 ± 2.3 days after calving, with no difference in days postpartum between phase or status. The cows weighed 346 ± 10 kg and had a CS of 4.6 ± 0.1 during the week ultrasound examination commenced, with no difference between phase or status.

Follicle numbers and sizes before ovariectomy

The diameter of the DF and the sub-dominant follicle did not differ among cycling and anovulatory cows on any day before ovariectomy (Figure 5.1).

The average number of small and large follicles over the 14 days before ovariectomy was higher in anovulatory than cycling cows (12.2 vs. 10.0 ± 1.2, P = 0.07 and 1.6 vs. 1.1 ± 0.1, P<0.001; respectively) with no difference in the number of medium follicles (1.7 vs. 1.4 ± 0.3). Ovaries of anovulatory cows contained more small follicles on days -3 and +8 and more large follicles on days -7, -6, -5, -2, 1 and 3 relative to emergence than did cycling cows (P<0.05; Figure 5.2).

Ovarian weights and follicle numbers after ovariectomy

Average paired ovarian weight was higher in cycling than anovulatory cows (16.1 ± 4.3 g vs. 12.1 ± 2.5 g, respectively; P<0.01). However, when the weight of the CL was subtracted, there was no difference between cycling and anovulatory cows (10.4 ± 2.5 vs. 12.1 ± 2.5 g, respectively). There were no differences in ovarian weight between the phases at ovariectomy irrespective of presence or absence of the CL.

Eighty seven follicles were dissected from the ovaries (19 from cows in the anovulatory plateau phase, 35 from anovulatory growing phase, 9 from

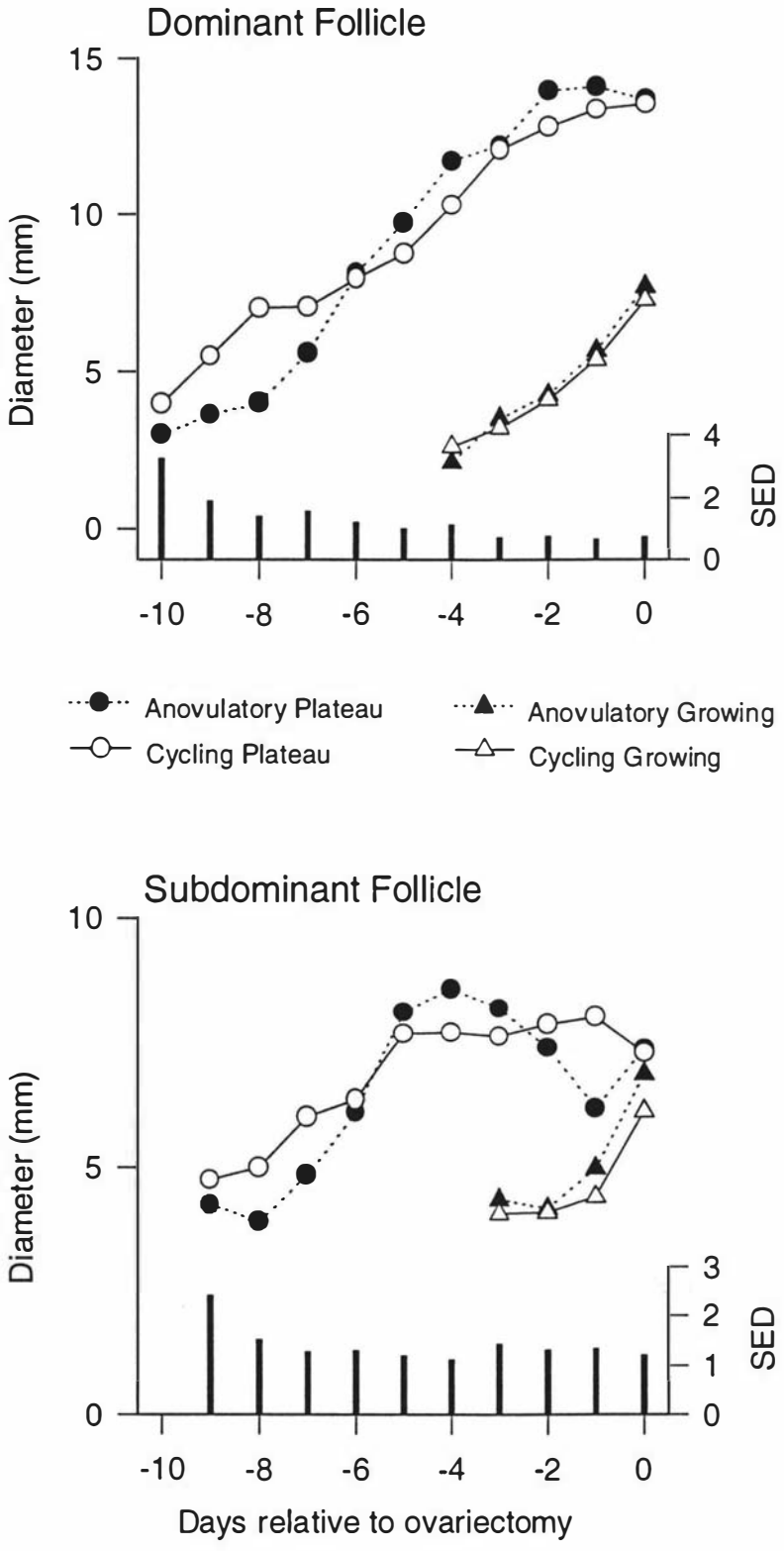

Figure 5.1. The least squares mean (SED as vertical bars on the x axis) of the dominant and sub-dominant follicle diameter aligned by day of ovariectomy from anovulatory or cycling cows, ovariectomised at the growing or plateau phase of follicle development.

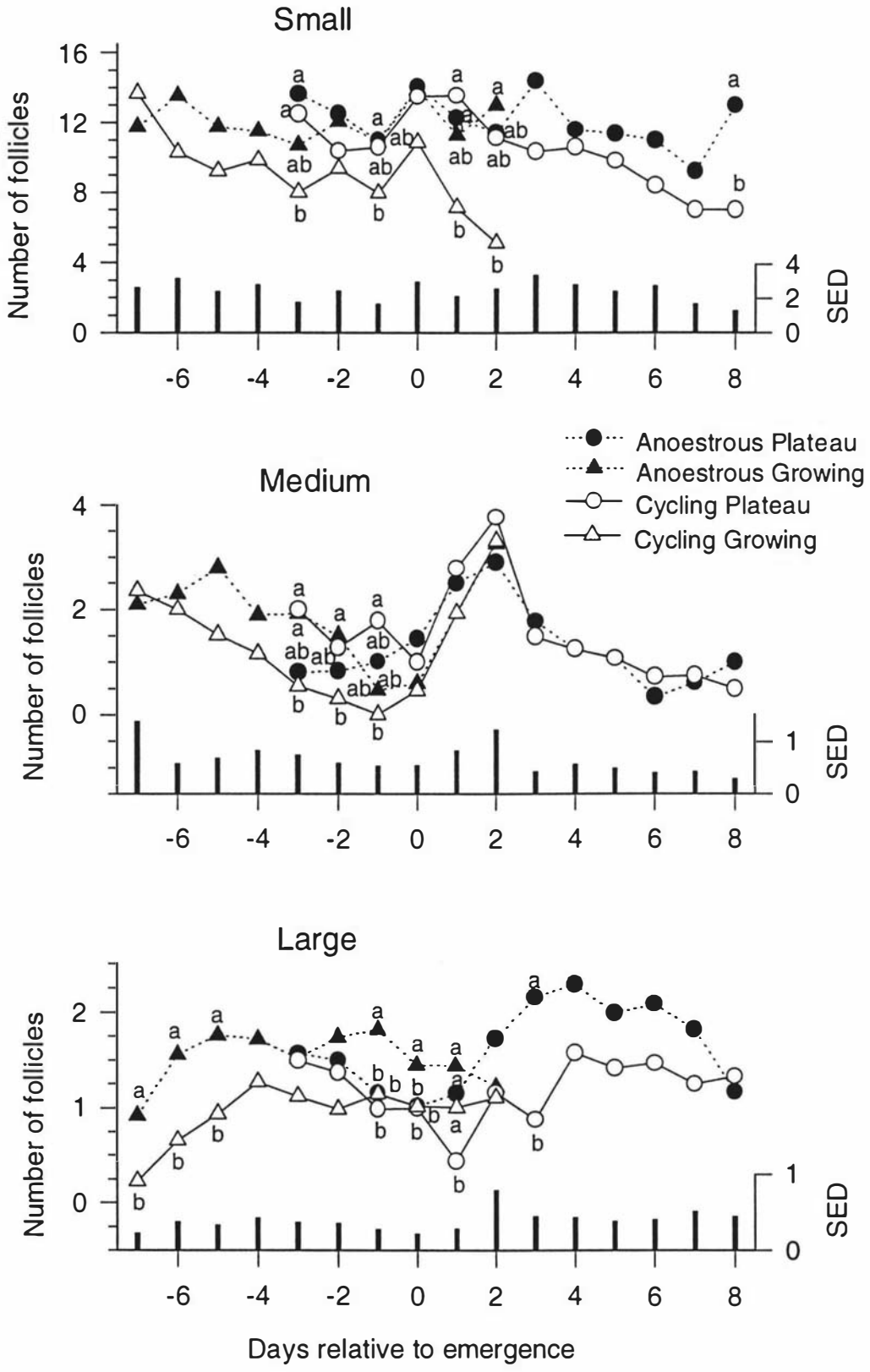

Figure 5.2. The least squares mean (SED as vertical bars on the x axis) number of small (3 to 5 mm), medium (6 to 9 mm) and large (>9 mm) follicles per cow in anovulatory or cycling cows ovariectomised at the growing or plateau phase of follicle development. ^{ab} Means within day with different superscripts differ by $P<0.05$.

cycling plateau phase and 24 from cycling growing phase; respectively). Ten follicles were ruptured during ovariectomy or subsequent dissection including 5 DF's from cycling cows. A further 5 DF's could not be positively identified because there was more than one large follicle of the same diameter on a single ovary.

The average diameters of the DF's from cows ovariectomised in the plateau phase were larger (15.6 vs. 10.7 ± 1.5 mm, respectively) and the growth rate slower (0.8 vs. 2.0 ± 0.4 mm/day, respectively) than DF's from cows ovariectomised in the growing phase. There were no differences in diameters of the DF's or the growth rate of DF's from anovulatory or cycling cows (Figure 5.3). The density of granulosa cells was not affected by phase or status of the cow.

Hormone concentrations in follicular fluid

There was no difference in E_2 concentration but T concentration was lower and P_4 concentration was higher in DF's from cows in the plateau compared to growing phase at ovariectomy (Figure 5.4). Oestradiol, T and P_4 concentrations were higher in DF's from cycling cows than anovulatory cows (Figure 5.4). The ratio of E_2 to T (on a mass basis) in the DF's did not differ among cycling and anovulatory cows but was higher in DF's recovered during the plateau than at the growing phase (Figure 5.4).

Western-ligand blotting revealed a full array of IGFBP's binding to IGF-II (Figure 5.5). Binding at 200 kDA was presumably to the truncated mannose-6-phosphate receptor. Immunoblotting revealed the doublet at 40-43 kDA to be IGFBP3 and binding at 34 kDA to be IGFBP2. Immunoblotting against anti-hIGFBP4 revealed binding at 30 and 24 kDA, the latter BP also cross-reacted with anti-bIGFBP2. Thus, the absolute identity of the IGFBP's at 24 and 30 kDA remains uncertain.

The IGF, IGFBP concentrations and the percentage of each BP present in the DF was not related to the phase or status of the cow at ovariectomy (Table 5.1), although there was a tendency ($P<0.1$) for the IGF concentration to be lower in anovulatory than cycling cows.

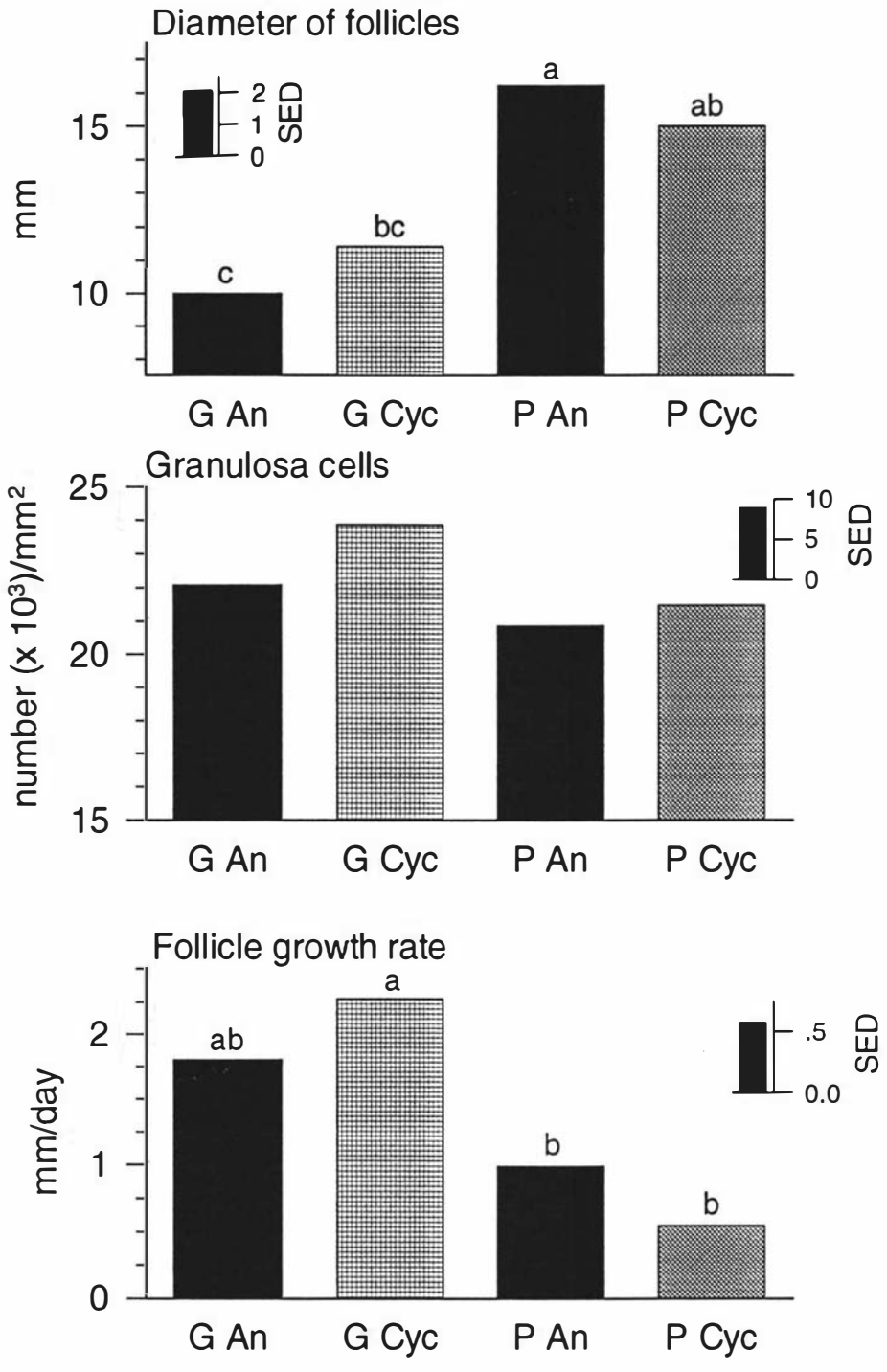

Figure 5.3. The least squares mean (and SED) of DF diameter, the density of granulosa cells and the follicular growth rate over 3 days in anovulatory (An) or cycling (Cyc) cows ovariectomised when the DF was in growing (G) or plateau (P) phase of development. ^{abc} Means within panel with different superscripts differ by $P<0.05$.

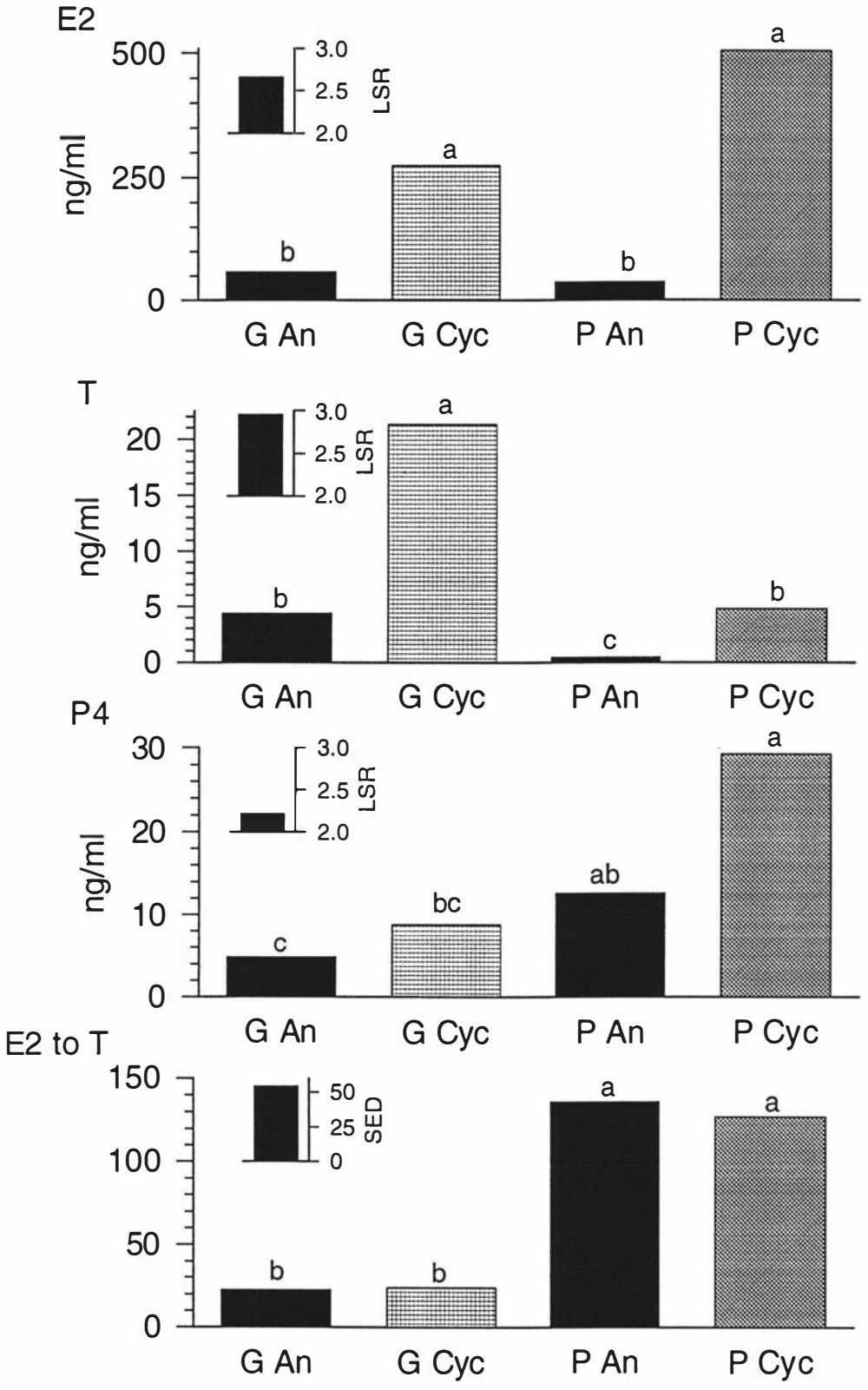

Figure 5.4. The geometric mean and least significant ratio (LSR) of the E_2, T and P_4 concentrations and the E_2 to T ratio (E_2 to T) in the DF from anovulatory (An) or cycling (C) cows ovariectomised when the DF was in growing (G) or plateau (P) phase of development. ^{abc} means within panel with different superscripts differ by $P<0.05$.

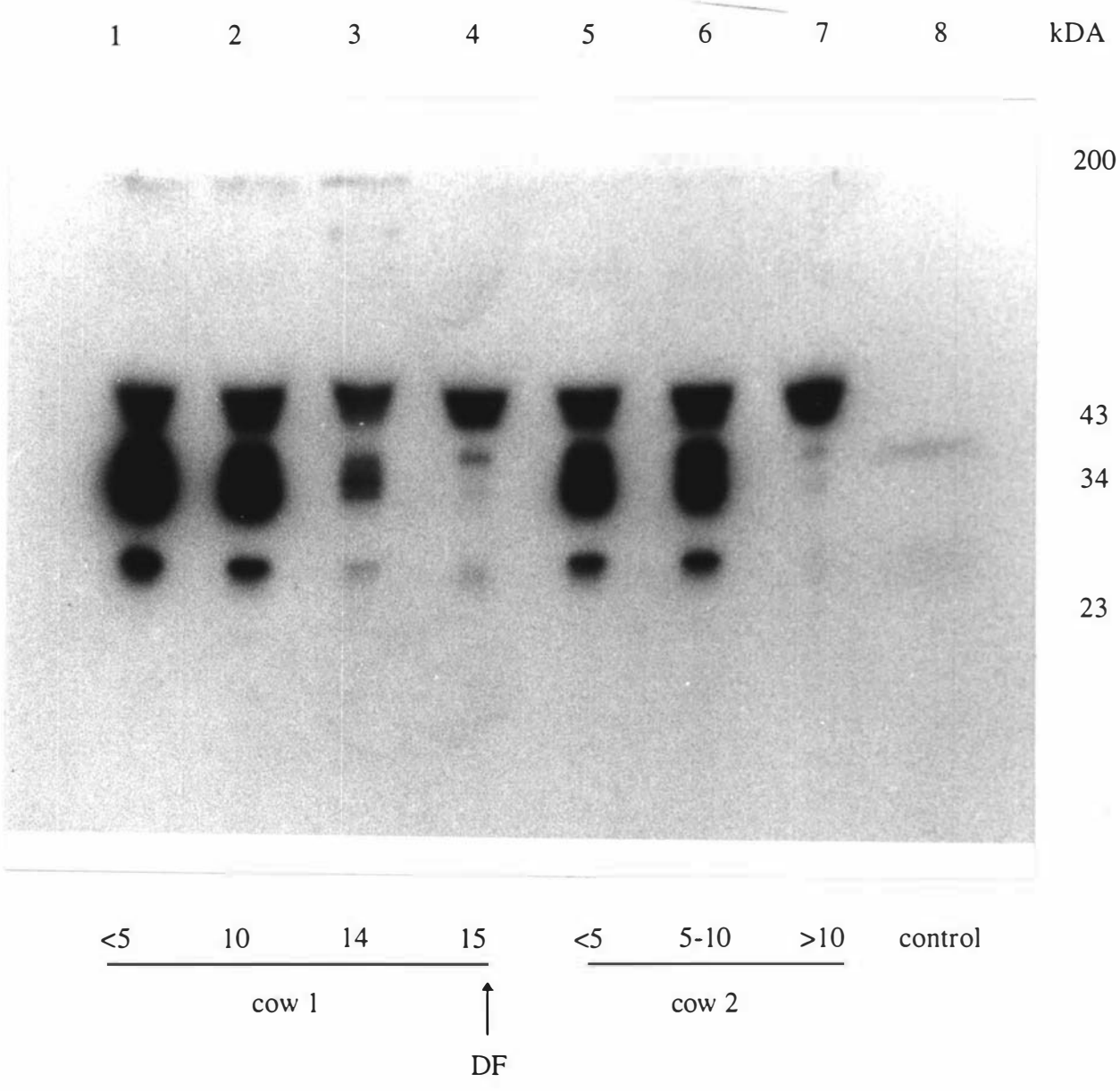

Figure 5.5. The IGFBP patterns from 7 follicular fluids (lanes 1 to 7) and ovine cerebrospinal fluid (Lane 8, control) following SDS-page electrophoresis and western-ligand blotting with IGF-II. Lanes 1 to 4 are from 1 cow with lane 4 being the identified dominant follicle, and lanes 5 to 7 from a second animal. The molecular weight (kDA) are indicated down the right margin.

Table 5.1. Concentration of insulin-like growth factor and insulin-like growth factor binding proteins of anovulatory or cycling cows ovariectomised at the growing or plateau phase of DF development.

			BP (kDa)							
Phase	Status	IGF	24		30		34		43	
		(ng/ml)	OD‡	%~	OD	%	OD	%	OD	%
Growing	Anovulatory	80.1	58.5	6.1	305.5	38.4	88.7	11.5	449.5	43.8
	Cycling	116.5	31.8	3.9	254.3	45.2	41.2	5.8	273.5	45.0
Plateau	Anovulatory	76.9	44.7	3.8	243.6	44.1	62.0	13.3	284.6	35.8
	Cycling	107.5	43.4	5.6	336.7	38.2	63.6	11.8	493.4	44.4
LSR/SED[Ø]		1.7	3.7	2.2	1.9	7.7	4.4	4.7	4.1	8.0
Status		†	ns	ns	ns	ns	ns	ns	ns	ns
Phase		ns	ns	ns	ns	ns	ns	ns	ns	ns
Interaction		ns	ns	ns	ns	ns	ns	ns	ns	ns

ns = Not significant (P>0.1), † P<0.1
‡ Geometric means of arbitrary optical density units
~ % of sum of the 4 classes of IGFBP's
Ø Least significant ratios for IGF and IGFBP's OD's and standard error of the difference for the IGFBP percentages

Dominant follicles had lower concentrations of the IGFBP's at 24, 34 and 43 kDA and a lower percentage of the IGFBP's at 24 and 34 kDA but a higher percentage of the IGFBP at 30 kDA than non-dominant follicles (Figure 5.6). Dominant follicles also were larger (13.0 vs. 8.6 ± 0.5 mm), grew more quickly in the 3 days before ovariectomy (1.5 vs. 0.4 ± 0.2 mm/day) and had a higher E_2 concentration (115 vs. 3 pg/ml, LSR = 1.3) than non-dominant follicles.

There was a positive relationship among the log intrafollicular E_2, T and IGF concentrations, but not the log P_4 concentration, and the number of LH pulses in the 8 h preceding ovariectomy (Figure 5.7).

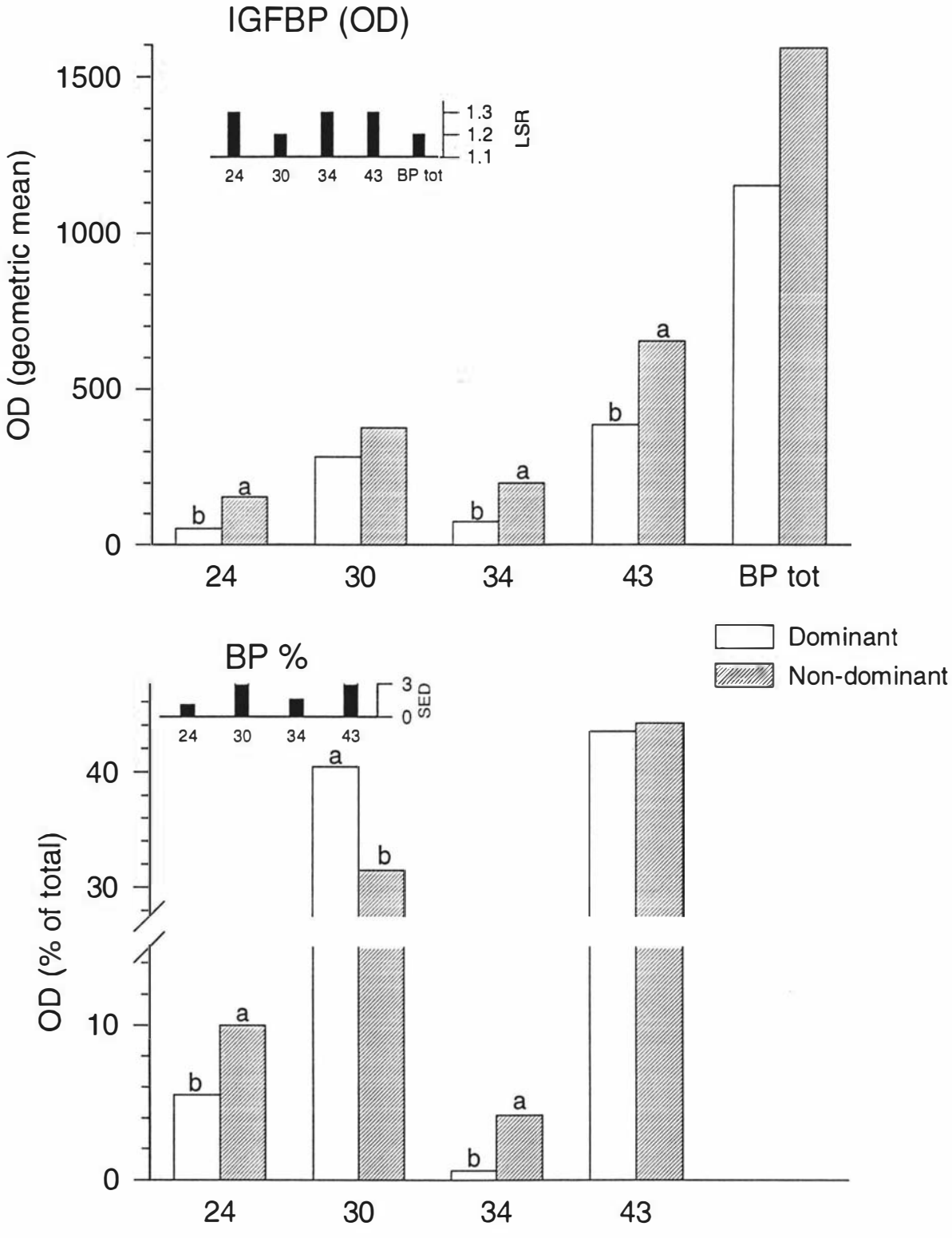

Figure 5.6. The geometric mean optical density (and LSR) for the individual IGFBP's and the sum of all BP's (upper panel) and the relative percentage OD (SED; lower panel) of each IGFBP of molecular weight (MW) 24, 30, 34 and 43 kDA from follicles defined as either dominant or non-dominant. ^{ab} Means within each BP with different superscripts differ by $P<0.05$.

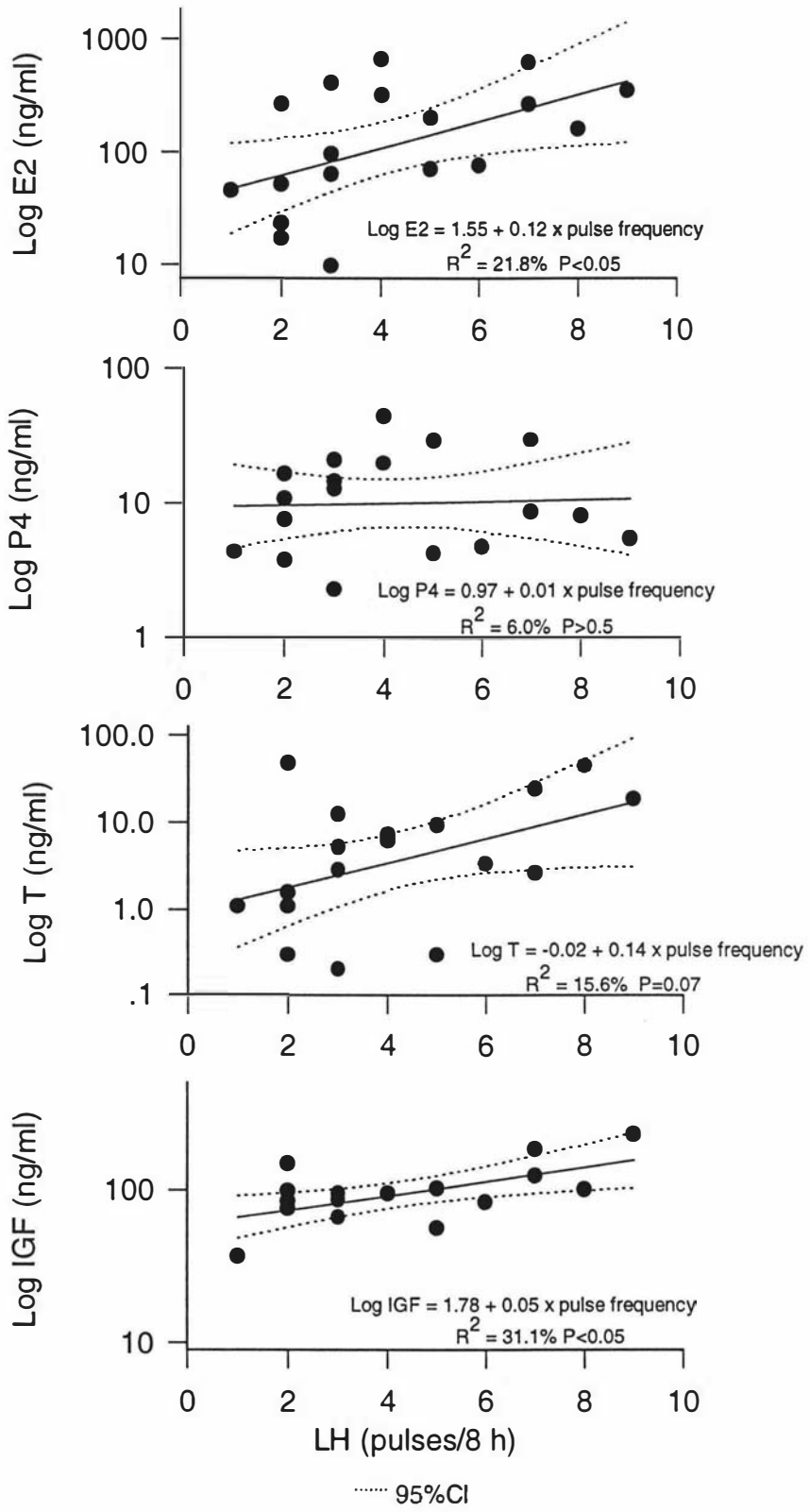

Figure 5.7. The log E_2, P_4, T and IGF concentrations within the DF and the number of LH pulses in the 8 h preceding ovariectomy.

Discussion

Ovaries of anovulatory cows had more small and large follicles than cycling cows. Dominant follicles from anovulatory cows had the same diameters, density of granulosa cells and growth rates as DF from cycling cows. However, DF from anovulatory cows had lower intrafollicular E_2, T and P_4 concentrations than DF from cycling cows. The size, growth rate and steroid concentration of the DF differed with the stage of development. However, these differences were similar between the anovulatory and cycling cows, as indicated by the lack of significant phase by status interactions.

The larger number of follicles observed in the anovulatory cows may reflect differences in the degree of dominance of the largest growing follicle. Removal of the largest growing follicle is followed by emergence of many new, small follicles (Ko *et al.*, 1991; Badinga *et al.*, 1992), which is preceded by an increase in FSH concentration (Adams *et al.*, 1993). The lower E_2 concentration in the DF's of anovulatory cows may cause less inhibition of FSH release and hence allow more follicles to emerge when compared to cycling cows.

In agreement with earlier studies, as follicles increase in diameter and approach ovulation, their steroid concentrations increase (Ireland and Roche, 1982, 1983; McNatty *et al.*, 1984a). The low intrafollicular T concentration and the positive relationship among T, E_2 and the number of LH pulses in the 8 h preceding ovariectomy suggests that anovulatory cows had lower LH pulse frequency than cycling cows resulting in lower theca interna T production (Fortune, 1986). The E_2 to T ratio in the DF did not differ between cycling and anovulatory cows suggesting that they had similar capabilities of aromatisation of T to E_2, an FSH-dependant function (Fortune, 1994). This suggests that LH-dependant T production rather than the FSH-dependant aromatisation of T to E_2 was limiting E_2 production in the anovulatory cows. Insufficient E_2 production may lead to a failure to induce the pre-ovulatory gonadotrophin surge resulting in DF atresia which allows another cohort of follicles to emerge. Similarly, low intrafollicular E_2 and T concentrations are reported to occur in suckled beef cows with prolonged PPA and low peripheral LH pulse

frequencies (Prado *et al.*, 1990; Wright *et al.*, 1990). Anovulation in both suckled beef cows and the dairy cows in the present experiment appears to be associated with the same, low LH pulse frequencies and follicular steroid concentrations.

Anovulatory cows tended ($P<0.1$) to have lower intrafollicular IGF concentrations than cycling cows. Differences in intrafollicular IGF concentration have not previously been demonstrated between anovulatory and cycling cows or among follicles of different diameters under a wide range of nutritional and physiological states (Spicer *et al.*, 1988; Rutter and Manns, 1991; Spicer *et al.*, 1991; Badinga *et al.*, 1992; Rhind *et al.*, 1993). Neither time postpartum (Spicer *et al.*, 1988) nor restriction of feeding postpartum (Rutter and Manns, 1991) has been reported to effect IGF concentration within follicles. Differences in cow breed (beef vs. dairy), amount of body tissue available for mobilisation, or in the quality or quantity of the diet may explain why a difference was observed in the present experiment and not the previous reports. Granulosa cell IGF production in cattle is not affected by growth hormone, insulin, FSH or epidermal growth factor *in-vitro* (Spicer *et al.*, 1993). However, in this experiment there was a positive association between intrafollicular IGF concentration and LH pulse frequency, suggesting LH may play a role in IGF production by granulosa cells *in-vivo* or in influx of IGF from peripheral circulation.

Dominant follicles had lower concentrations of IGFBP's at 20, 34 and 43 kDA and a lower percentage of IGFBP's at 24 and 34 kDA than non-dominant follicles. Similarly, higher concentrations of the lower molecular weight IGFBP's have been observed in non-dominant or immature follicles from sows (Howard and Ford, 1992) and ewes (Monget *et al.*, 1993). IGF acts synergistically with the gonadotrophins to increase steroid production in rat, and large (>8 mm) bovine, follicles (Adashi *et al.*, 1991; Spicer *et al.*, 1993). In the present experiment, the total IGF concentration did not vary among sizes or classes of follicles but the decreased IGFBP concentrations may have increased the concentration of free, biologically active IGF leading to enhanced steroid production by the DF's. Intrafollicular control of IGFBP concentrations is a mechanism by which the DF may have higher E_2 production than non-

dominant follicles despite being exposed to the same concentrations of gonadotrophins. Changes in the relative proportions of the IGFBP's suggests different control mechanisms for the individual IGFBP's, as has been reported in porcine granulosa cells (Grimes *et al.*, 1994). This differential control suggests that they play varying roles within the follicle.

In conclusion, the ovaries from anovulatory cows contained more follicles than those from cycling cows. However, the maximum size of the DF's, their growth rate and the density of granulosa cells within the DF's did not differ from those of cycling cows. The low E_2 and T concentrations in anovulatory DF's suggest that low, LH-dependant, T production may limit E_2 production. Ovulation of these large DF's present in PPA cows may fail to occur as insufficient E_2 is produced to induce the pre-ovulatory gonadotrophin surge.

The concentration of IGF but not the IGFBP's differed among cycling and anovulatory cows. The reduced intrafollicular IGF concentrations may be contributing to the lower E_2 and T concentrations recorded in the anovulatory cows. The lower concentrations of IGFBP's at 24, 34 and 43 kDA in DF's compared to non-dominant follicles suggests a role for IGFBP's in selection and maintenance of DF's which may be mediated by enhancement of steroid production.

CHAPTER 6:

GnRH Induces Ovulation Of A Dominant Follicle In Dairy Heifers Undergoing Turnover of Anovulatory Follicles

Abstract

This trial examined the effect of an injection of GnRH administered approximately 3 weeks postpartum on induction of an LH surge and ovulation of large (>10 mm) follicles in the ovaries of lactating dairy heifers likely to have extended periods of PPA.

At 2 weeks postpartum daily transrectal ultrasound of ovarian follicles commenced in 20 dairy heifers which were randomly assigned to be injected with 250 μg GnRH or saline, intramuscularly (i.m.). Treatment was given the day after the largest growing follicle was >10 mm in diameter. Blood samples were drawn hourly following treatment to measure plasma concentrations of LH. Milk samples were taken thrice weekly from 2 weeks postpartum to 3 to 4 weeks after treatment for analyses of P_4 concentration.

Every heifer treated with GnRH had an LH surge (>10 ng/ml maximum concentration) which was maximal 2 h after treatment. Nine of the 10 heifers treated with GnRH and one saline-treated heifer ovulated within 4 days of treatment. However, only three of the heifers treated with GnRH continued to ovulate following the first, short (<10 day) luteal phase.

These data indicate that sufficient releasable pituitary stores of LH were present at the time of treatment with GnRH and that large follicles were capable of ovulation. Hypothalamic release of GnRH, rather than pituitary or ovarian insufficiency, appears to be the factor limiting resumption of cyclic activity in these heifers. Induction of ovulation may not lead to the resumption of normal cyclicity.

Introduction

Large (>10 mm) follicles are present in the ovaries of postpartum dairy cows from 11 days postpartum (Rajamahendran and Taylor, 1990; Savio *et al.,* 1990). The first postpartum DF ovulates in a majority of well-fed dairy cows (Rajamahendran and Taylor, 1990; Savio *et al.,* 1990). Ovulation does not occur until 42.3 ± 4.2 days postpartum following 4.2 ± 0.6 (range 1 to 9) DF's in pasture-fed New Zealand dairy cows (Chapter 4). The absence of large follicles does not appear to be the factor limiting resumption of ovulation in these cows.

Ovulation is preceded by a surge of GnRH from the hypothalamus which stimulates the release of LH. The release of GnRH is in response to the positive feedback effects of rising E_2 concentrations produced by large, pre-ovulatory follicles. The E_2 production is in turn dependent on increasing LH concentrations (McNatty *et al.,* 1984b). Low mean concentrations, pulse frequencies and amplitudes of LH have been associated with extended periods of PPA (Lamming *et al.,* 1981; Schallenberger *et al.,* 1982; Wright *et al.,* 1990). These low LH concentrations may lead to insufficient production of follicular E_2 to induce a GnRH and hence an LH surge (Prado *et al.,* 1990; Roche *et al.,* 1992).

Exogenous GnRH can induce an LH concentration similar to that of a normal pre-ovulatory surge in suckling beef and dairy cows 2 to 4 weeks postpartum (Kesler *et al.,* 1977; Webb *et al.,* 1977; Fernandes *et al.,* 1978; Carter *et al.,* 1980). Treatments by single injection, multiple injection or continuous infusion have been shown to induce ovulation in 10-100% of postpartum cows (Britt *et al.,* 1974; Kesler *et al.,* 1977; Webb *et al.,* 1977; Foster *et al.,* 1980; Edwards *et al.,* 1983; Peters *et al.,* 1985; Benmrad and Stevenson, 1986; Jagger *et al.,* 1987; Crowe *et al.,* 1993). The variability in the responses may be associated with differences in the phase of the follicle wave at the time of GnRH treatment, as large follicles (>12 mm) measured by laparoscopy or palpation are more likely to ovulate than smaller ones following treatment (Lishman *et al.,* 1979; Garverick *et al.,* 1980).

The aim of the experiment was to determine if a single injection of a selected dose of GnRH induced an LH surge and ovulation of a large (>10 mm), growing follicle in dairy heifers likely to have an extended period of PPA.

Materials and Methods

Animals and treatment

Twenty, 2-year old, lactating, Friesian heifers which had calved between 29 June and 18 July at a CS of 4.1 ± 0.4 (0 to 10 scale) and a postpartum weight of 340 ± 43 kg, were used for the experiment. The heifers were grazed on white clover/ryegrass pasture for the duration of the experiment. Daily transrectal ultrasound examination of the ovaries commenced 2 weeks postpartum using an ALOKA 210Dx ultrasound with a 7.5 MHz linear array transducer (Medtel Ltd, Auckland, NZ). The position of each follicle at least 3 mm in diameter was recorded and sequential daily graphs of individual follicles on each cow's ovaries were prepared for each animal (Ginther, 1993). Animals were randomly assigned to two groups (n = 10/group) and treated i.m. with either 2.5 ml of 0.9% NaCl (control) or 250 μg of a synthetic GnRH analogue, gonadorelin (treated; Fertagyl, Intervet, NZ). There was no difference among treatment groups in calving date, CS or liveweight at calving. No heifer had a milk P_4 concentration of >0.8 ng/ml or a CL before treatment. Treatment was applied the day after the DF, whose emergence had been observed, was at least 10 mm in diameter. Treatment occurred when the DF was 11.2 ± 1.0 mm in diameter at 23.8 ± 2.7 days postpartum. The DF present at treatment was likely to be the second or third postpartum DF as previous studies (Chapter 4) found that the second and third DF emerged at 15.1 ± 4.6 (range 10 to 28) and 22.0 ± 2.3 (range 18 to 26) days postpartum, respectively. Ten ml of blood was removed from pre-placed jugular catheters at 0800 and 0830 and treatment followed the latter sample. Another 10 ml of blood was removed hourly for the next 8 h. Each blood sample was immediately placed into a 10 ml lithium heparin glass tube (Vacutainer, Salmond Smith-Biolab LTD,

Auckland, NZ) and kept on ice. They were centrifuged within 4 h and the plasma samples stored at -20 °C before being analysed for LH concentrations.

Daily ultrasound examinations continued until ovulation or the emergence of the next follicle wave (i.e. the maximum diameter of the largest follicle in the next cohort was at least 10 mm). Composite milk samples (20 ml), were collected thrice weekly from 14 days postpartum until 22 to 25 days after treatment for P_4 assay. The milk samples were stored at 4 °C until assayed within 3 days of collection.

Hormone assays

LH concentration was determined using a double antibody radioimmunoassay. The standards and LH for iodination were of ovine origin (CY1085, INRA, Nouzilly). The primary antibody was raised in a rabbit against the same LH (R#2; AgResearch, Invermay) and used at a final dilution of 1:200,000 having been diluted with a 0.01M PBS, 0.05M EDTA, and 0.1% BSA assay buffer also containing 1:240 normal rabbit sera. The second antibody (sheep anti-rabbit) was also raised at AgResearch, Invermay and was used at a dilution of 1:30. One hundred μl of a plasma from a ram treated with 10 mg of MPA (Promone-E, Upjohn, Auckland, NZ) was added to each standard tube. The LH concentration of this plasma was below the sensitivity of the assay. Serial dilutions of a cow plasma containing 10 ng/ml of LH with bovine plasma containing less than 0.1 ng/ml of LH, produced a curve parallel to the standard curve. All samples were assayed in one assay. The within-assay coefficients of variation were 8.4%, 9.2% and 9.1% for three quality control plasma's with mean concentrations of 0.7, 3.8 and 7.8 ng/ml, respectively, each assayed seven times in duplicate in the assay. The sensitivity (upper 95% CI of the zero standard) was 0.04 ng/ml.

Progesterone concentrations were measured directly using a commercial, solid phase, I^{125} label RIA (Coat-a-Count, DPC, Los Angeles, Calif., USA). The cross-reactivity of the antibody was 2.4% with 11-deoxycortisol, 1.7% with 11-deoxycorticosterone, 2.0% with 20α-

dihydroprogesterone 1.3% with 5β-pregnan-3,20-dione and less than 0.5% with a range of other steroids tested (DPC, Coat-a-count manual, 1993). The recovery of 5, 10 and 20 ng/ml of P_4 added to milk from an ovariectomised cow were 85.1 ± 1.8%, 88.7 ± 2.8% and 99.4 ± 4.0%, respectively (mean ± sem). A milk sample containing approximately 12 ng/ml was serially diluted with milk from an ovariectomised cow and each dilution was assayed in duplicate. The resultant curve was parallel to that of the standard curve. Two quality control pools were run in sextuplet in each assay and the within-assay and between-assay coefficients of variation were 5.3% and 15.2%, and 6.0% and 9.0% from samples with mean concentrations of 3.7 and 1.7 ng/ml, respectively, over four assays. The sensitivity was 0.08 ± 0.02 ng/ml. A P_4 concentration of at least 1 ng/ml was defined as indicative of luteal activity.

Statistical analyses

The day of emergence of a cohort of follicles was defined as the first day on which a 4 mm follicle was identified which subsequently grew to be at least 10 mm in diameter.

An LH surge was defined as occurring when at least one post-treatment sample had a concentration of >10 ng/ml. The duration of the LH surge was defined as the number of hours during which the concentration of LH was >2 ng/ml.

Ovulation was defined as having occurred where a previously visible large follicle was not located by ultrasound examination from 1 to 4 days after treatment, where a CL was subsequently identified on the same ovary and where the milk P_4 concentration was greater than 1 ng/ml within 3 to 9 days of the follicle disappearing.

The duration of the induced luteal phase was defined as the number of days that the concentration of milk P_4 was >1 ng/ml. Continuity of cyclic activity after the first ovulation was defined as occurring where the milk P_4 concentration increased to >2.5 ng/ml within 7 days of the decline in milk P_4 to <1 ng/ml at the end of the first, induced, luteal phase.

Proportional data were analysed by χ^2 test and continuous data by one-way analysis of variance with treatment as the main factor. All analyses were performed using Minitab version 8.2 (Minitab inc., State College, Pa, USA). Data are presented as mean ± standard deviation unless otherwise stated.

Results

A regular pattern of DF growth, plateau and atresia was observed in all heifers. An LH surge occurred in every heifer treated with GnRH with the mean maximum LH concentration being 20.6 ± 2.8 (range 10.6 to 35.0) ng/ml which occurred 2 h after treatment. The duration of the LH surge was 3.6 ± 0.8 (range 3 to 5; Figure 6.1) h. Ovulation occurred in nine of the ten treated heifers and only in one of the ten control heifers (χ^2 = 9.8; P<0.01). In the treated heifers, ovulation occurred between 24 and 48 h after treatment, while the one control heifer ovulated 4 days after treatment.

The first post-treatment DF emerged sooner in the treated than in the control heifers (0.7 ± 1.1 vs. 3.6 ± 1.0 days, respectively; P<0.001). The post-treatment DF was larger in treated than in control heifers, 2, 3 and 4 days after treatment (Figure 6.2).

The P_4 concentration was >1 ng/ml for 2.6 ± 2.3 (range 1 to 9) days following ovulation. A second ovulation occurred in three of the nine treated heifers (Figure 6.3). The maximum P_4 concentration during the first luteal phase was higher in heifers whixh continued to cycle than those which did not (6.0 ± 1.2 and 3.1 ± 0.4 ng/ml, respectively; P<0.05).

A further four control heifers ovulated before the end of the milk sampling regime at 22 to 25 days post-treatment. Overall, nine of ten treated and five of ten control heifers ovulated before 45 days postpartum.

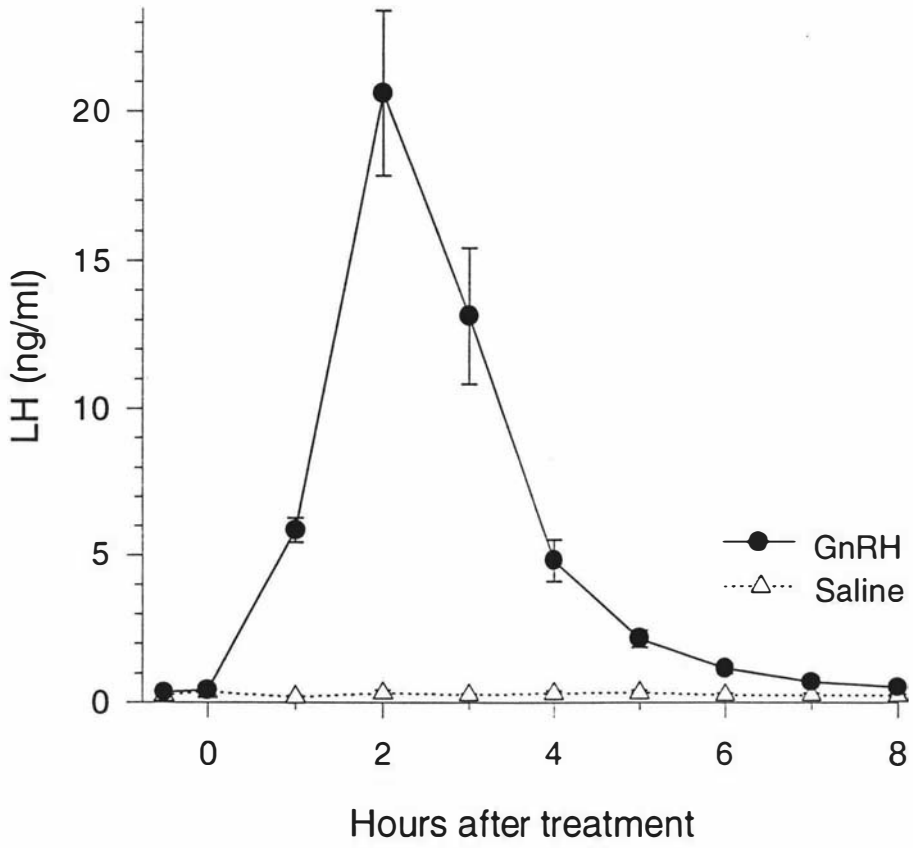

Figure 6.1. Concentrations of LH (mean ± sem) in plasma of lactating heifers treated with either 250 μg of GnRH or 2.5 ml of saline.

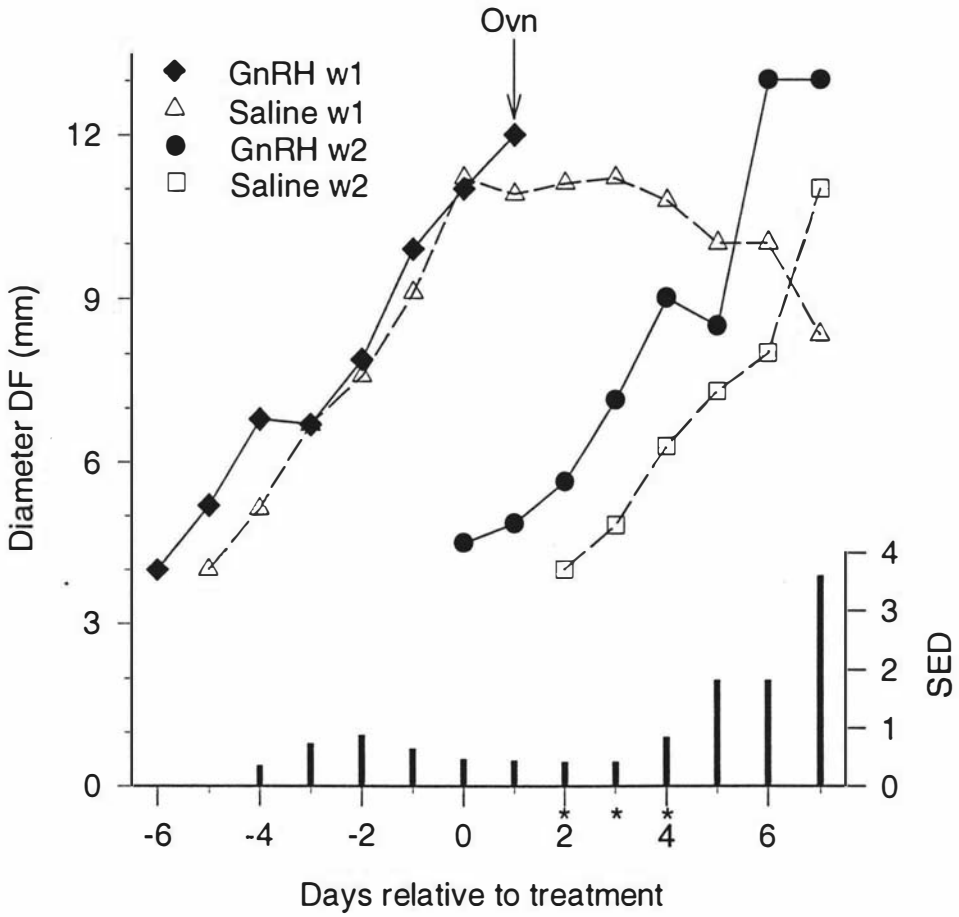

Figure 6.2. The mean (SED as bars along x axis) diameter of 2 dominant follicles (treatment DF = w1 and subsequent DF = w2) following treatment with 250 μg of GnRH or 2.5 ml of saline. * Indicates significant ($P<0.05$) differences within day.

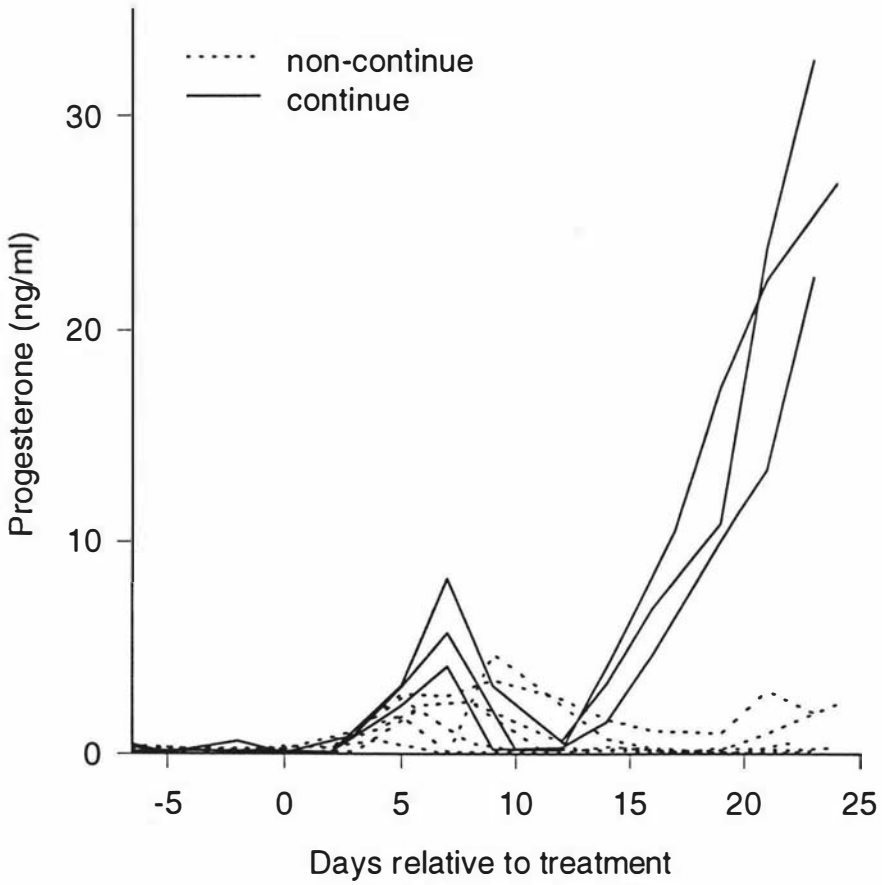

Figure 6.3. The milk progesterone concentration of nine cows which ovulated following injection with 250 μg of GnRH, divided into those that continued to ovulate (continue) and those that did not (non-continue) following the induced, first postpartum ovulation.

Discussion

This study confirmed that an LH surge could be induced by treatment with GnRH and demonstrated that large (>10 mm) follicles ovulated after this surge in dairy heifers which were likely to have extended periods of PPA.

Follicle turnover was evident and a follicle of at least 10 mm in diameter was present in all heifers preceding treatment in agreement with previous studies (Murphy *et al.,* 1990; Savio *et al.,* 1990; Chapter 4). Despite the presence of these large follicles, none of the heifers had ovulated by the time of treatment (19 to 31 days postpartum) and only half of the control heifers had ovulated by 45 days postpartum. As in beef heifers (Murphy *et al.,* 1990), the absence of large follicles does not appear to be the factor limiting the resumption of cyclic activity in this group of non-suckled dairy heifers. This demonstrates that factors other than suckling can prevent ovulation despite the presence of large follicles.

Every heifer treated with GnRH had an LH surge. The maximum LH concentration was equivalent to that of an endogenous pre-ovulatory surge (Chenault *et al.,* 1975; Peterson *et al.,* 1975; Rahe *et al.,* 1980) and to that following treatment with GnRH with the same GnRH dose in cycling cows (Webb *et al.,* 1977). The duration of the LH surge was shorter than the 10 h reported for the endogenous LH surge (Chenault *et al.,* 1975; Peterson *et al.,* 1975; Rahe *et al.,* 1980), but similar to that reported following treatment with the same GnRH dose in cycling cows (Webb *et al.,* 1977). This suggests that the shorter duration of the LH surge was due to the delivery and/or dose of the GnRH rather than the physiological status of the animal. The pituitary concentration of LH (Moss *et al.,* 1985) and the LH release following a GnRH injection (Britt *et al.,* 1974; Kesler *et al.,* 1977; Fernandes *et al.,* 1978) return to levels similar to those of cycling cows by 10 to 30 days postpartum, following the postpartum depression of pituitary LH concentration and release. The ability of the pituitary to release LH appears not to be a limiting factor to resumption of cyclic activity in postpartum cattle. Inhibition of release of GnRH and hence low LH pulse frequencies by factors such as low CS at calving (Grainger *et al.,* 1982) and extended periods of NEB postpartum (Canfield and

Butler, 1990) may be factors delaying the resumption of cyclic activity in these heifers.

Ovulation of a large DF was induced in nine of ten heifers. Similarly, 12 of 12 growing DF's ovulated in suckled beef heifers treated with GnRH (Crowe *et al.*, 1993). This demonstrates that the large anovulatory follicles present in postpartum animals can ovulate following an appropriate LH surge. Despite inducing an LH surge, previous trials using GnRH have reported variable proportions of cows ovulating (Britt *et al.*, 1974; Kesler *et al.*, 1977; Webb *et al.*, 1977; Foster *et al.*, 1980; Edwards *et al.*, 1983; Peters *et al.*, 1985; Benmrad and Stevenson, 1986; Jagger *et al.*, 1987). This may be due to variability in the phase of development of the DF at the time of treatment. Crowe *et al.*, (1993) were unable to demonstrate a difference in ovulation rate among cows treated with GnRH when a growing or plateau phase DF (12 of 12 ovulated) or atretic DF (7 of 12 ovulated) was present. However, they used 20 μg of the synthetic GnRH, buserelin (Receptal, Hoechst) which because of its high biopotency is equivalent to between 500 and 4000 μg of native GnRH (Chenault *et al.*, 1990). This dose may have induced a supra-physiological LH surge, resulting in follicles that were approaching atresia, and perhaps not responsive to more physiological doses of GnRH, ovulating.

All induced ovulations were followed by short (1 to 9 day) luteal phases. Similar short luteal phases occur following 50% of spontaneous first postpartum ovulations in dairy cattle (Lamming *et al.*, 1981) which are then followed by normal (18 to 24 day) interovulatory periods in the majority of cows. Progesterone treatment before induced ovulation produces a normal first interovulatory interval in suckled beef cows (Troxel and Kesler, 1984) suggesting a role for P_4 in 'priming' luteal function. Ovulations continue to occur following spontaneous (Lamming *et al.*, 1981) and induced (Britt *et al.*, 1974; Benmrad and Stevenson, 1986) first postpartum ovulations in around 95% of well-fed dairy cows. In the present experiment, only 33% of the heifers which were induced to ovulate showed continued ovulatory activity. Cows in low CS with extended periods of PPA have low LH pulse frequencies (Wright *et al.*, 1990) and low E_2 concentrations within the DF (Prado *et al.*, 1990). Insufficient E_2 production may lead to failure of the of the E_2-induced positive

feedback release of GnRH and hence of LH, essential for spontaneous ovulation (Roche *et al.,* 1992). Following treatment, heifers returning to an anovulatory state may have had an insufficient LH pulse frequency to induce final follicular maturation and ovulation, despite having been exposed to P_4 and being further postpartum following treatment. The low P_4 concentration during the induced luteal phase may be indicative of a low LH pulse frequency during the pre-treatment development of the DF induced to ovulate and/or during the subsequent luteal phase.

The earlier emergence of the subsequent cohort of follicles in the animals treated with GnRH suggests either that the DF suppresses emergence of subsequent follicles and hence its removal allowed earlier emergence of follicles or that GnRH-released FSH stimulated follicle emergence. Removal of the DF by electrocautery or unilateral ovariectomy also leads to earlier emergence of the next cohort (Ko *et al.,* 1991; Badinga *et al.,* 1992), probably due to increased FSH concentrations following DF removal (Adams *et al.,* 1993). Thus, increased FSH concentration due to both the stimulatory effect of GnRH and the removal of inhibition from ovarian products probably contributed to earlier emergence of the subsequent DF.

In conclusion, exogenous GnRH induced an LH surge in ten of ten and ovulation in nine of ten postpartum dairy heifers drawn from a population of heifers likely to have extended periods of PPA. The dose of GnRH (250 μg) selected produced a maximum LH concentration equivalent to those seen at the pre-ovulatory surge in normally cycling cows. This indicates that sufficient GnRH receptors and LH are present in the pituitary of these heifers. Additionally, the DF ovulated in 90% of these animals. Insufficient GnRH release, due to insufficient GnRH production or suppression of release by nutritional factors may limit the resumption of cyclic activity in these heifers, rather than a failure of pituitary or ovarian function. The failure of 66% of the heifers induced to ovulate to continue to cycle, indicates that the factors inhibiting spontaneous ovulation may still persist, despite one ovulation having been induced.

CHAPTER 7:

The Effects Of Oestradiol On Release Of Luteinising Hormone And The Ovulatory Response At Two Stages Of Follicular Development In The Postpartum Dairy Cow

Abstract

The effects of oestradiol benzoate (ODB; 0.5 mg) or saline (Saline; 2.5 ml, i.m.) treatment of anovulatory postpartum dairy cows (n = 32) on LH concentration, the occurrence of ovulation or alteration in the growth rate of the DF and timing of emergence of the subsequent DF were examined. The ovaries were examined by transrectal ultrasound on a daily basis from 2 weeks postpartum and were treated when the DF was growing or had ceased growing.

ODB treatment induced an LH surge and ovulation in 8 of 15 and in 5 of 15 cows, respectively. The growth rate of the DF was slower (0.2 vs. 1.1 mm/day), the maximum DF size smaller (10.9 vs. 14.0 mm) and the emergence of the subsequent follicle wave occurred sooner (4.3 vs. 6.5 days) in cows treated with ODB when the DF was still growing compared to cows treated with saline at the same stage of follicular development.

Failure of both an LH surge and ovulation following ODB treatment indicated that at least two points of the H-P-O axis were dysfunctional in some of these anovulatory dairy cows. It was also shown that ODB alone can limit growth of the DF and timing of emergence of the subsequent DF.

Introduction

Parturition is followed by a period of anovulation which may extended beyond 50 days in some pasture-fed New Zealand dairy cows (Fielden *et al.,* 1973). During this period, there is a gradual re-establishment of the normal H-

P-O functions and thus ovulatory cycles (Lamming *et al.,* 1981; Roche *et al.,* 1981). The factor(s) limiting resumption of ovulatory activity has not been identified with certainty. An LH surge can be induced by GnRH in a majority of cows by 10 to 20 days postpartum (Fernandes *et al.,* 1978; Alam and Dobson, 1987). The hypothalamic concentration of GnRH is not reduced postpartum (Moss *et al.,* 1985; Nett *et al.,* 1988). However, the pituitary concentrations of GnRH receptors and LH take 10 to 30 days postpartum to return to levels equivalent to those in cyclic cows (Moss *et al.,* 1985; Nett *et al.,* 1988). The ability of GnRH to release LH appears to return earlier postpartum than the ability of ODB to release LH (Alam and Dobson, 1987). Additionally, blood concentrations of E_2 equivalent to those of the follicular phase of an ovulatory cycle, are present in the early postpartum period without ovulation occurring (Gyawu and Pope, 1990). Thus, failure of E_2 to induce a release of GnRH and then LH may be the limiting factor to resumption of cyclic activity in the postpartum period (Schallenberger and Prokopp, 1985; Alam and Dobson, 1987).

Antral follicular growth occurs in a wave-like fashion in cycling cows with follicles undergoing phases of increase (i.e. growth), maintenance (i.e. plateau) and decrease in diameter (i.e. atresia; Sirois and Fortune, 1988). This pattern has also been demonstrated in postpartum dairy (Savio *et al.,* 1990) and beef cows (Murphy *et al.,* 1990). The DF develops from a cohort of follicles and appears to suppress the growth rate of other follicles within the cohort and the emergence of the subsequent DF (Ko *et al.,* 1991; Badinga *et al.,* 1992). Dominance by an individual follicle may be mediated by products of the DF such as E_2, inhibin or other hormones.

Exogenous ODB or EV treatment disrupts the normal pattern of follicular growth in cycling cows (Nadaraja and Hansel, 1976; Engelhardt *et al.,* 1989; Rajamahendran and Walton, 1990). The phase of development of the DF at the time of treatment affects the response. When the DF is growing, treatment suppresses further growth and results in an earlier emergence of a new DF. In contrast, treatment when the DF has ceased growing does not change its growth and delays emergence of the subsequent DF (Bo *et al.,* 1993). However, these effects of treatment with exogenous ODB or EV may be

confounded by coincidental changes in peripheral concentrations of P_4 in the cycling cow.

The aim of this experiment was to investigate whether ODB treatment would induce an LH surge and ovulation in PPA dairy cows. Additionally, the effects of ODB on the growth rate of the DF and the timing of emergence of the subsequent DF after treatment at different phases of follicle development were investigated. The postpartum cow was used as a model because these animals had low P_4 peripheral concentrations thereby allowing the effect of ODB to be examined independently of P_4.

Materials and Methods

Animals and design

Thirty-two mixed age Friesian (n = 22) and Jersey (n = 10) cows were used. They calved in spring (25 August to 21 September, 1992), with a weight of 386 ± 9.8 kg (mean ± sem) and a CS of 4.5 ± 0.1 (1 = thin; 10 = fat) on the day following calving.

Cows were blocked by age (2, 3 or >3 years) and breed then randomly assigned to treatment in a 2 by 2 factorial design. Treatment consisted of either 0.5 mg of oestradiol benzoate in arachis oil (Oestradiol Benzoate SA, Intervet, Sydney, Australia) or 2.5 ml of saline by injection into the gluteal muscles. One half of the cows were treated when the DF was 8.3 ± 0.1 mm at 21.3 ± 0.2 days postpartum (growing) and the remainder when the DF was >10 mm in diameter and had changed by <1 mm over 48 h (diameter = 12.8 ± 0.1 mm, 42.3 ± 0.3 days postpartum; plateau). The ovaries of each cow were examined daily from 2 weeks postpartum by transrectal ultrasound, using an ALOKA 210Dx ultrasound with a 7.5 MHz linear array transducer (Medtel, Auckland, NZ). The position of each follicle greater than 2 mm in diameter was recorded and daily graphs of the individual follicles were prepared for each ovary of each animal (Ginther, 1993). From these graphs the stage of follicular development for treatment was determined. Ultrasound examination of the

ovaries continued daily until ovulation of the DF present at the time of treatment or the emergence of the second DF after treatment (i.e. the maximum diameter of the DF in the second cohort was at least 6 mm in diameter).

A jugular catheter (60 cm, 1.1 x 1.7 mm, 18g catheter; Cavafix, Braun, Salmond Smith-Biolab, Auckland, NZ) was fitted on the morning of treatment. Starting at midday, blood samples (10 ml) were taken at 4 h intervals for 48 h for LH assay. Samples were immediately placed into 10 ml heparinised glass tubes (Vacutainers, Salmond Smith-Biolab LTD, Auckland, NZ) and kept on ice, centrifuged within 4 h of collection and the plasma then stored at -20 ^{o}C before analysis. Treatment (ODB or saline) was given immediately after the first blood sample had been taken.

Milk samples (20 ml) were collected thrice weekly from 2 weeks postpartum until 3 weeks after treatment for P_4 assay. A potassium dichromate preserving tablet (E. Merck, Darmstadt, Germany) was added and the milk samples were held at 4 ^{o}C until assay within 3 days of collection.

Hormone assays

Progesterone concentration was determined by direct assay of whole milk in a commercial, solid phase, I^{125} labelled radioimmunoassay (RIA; Coat-a-Count, DPC, Los Angeles, Calif., USA; Chapter 6). Two quality control pools were run in sextuplet in each assay and the within-assay and between-assay coefficients of variation were 5.3% and 15.2%, and 6.0% and 9.0% from samples of mean concentrations of 3.7 and 1.7 ng/ml, respectively, over four assays. The sensitivity (upper 95% confidence interval around the 0 standard) was 0.08 ± 0.02 ng/ml.

LH concentration was determined using a validated RIA (Chapter 6). All samples were processed in one assay which had within-assay coefficients of variation of 11.8%, 7.4% and 4.8% for three quality control plasma's with concentrations of 1.15, 3.8 and 7.2 ng/ml respectively, each assayed 10 times in duplicate. The sensitivity of the assay was 0.05 ng/ml.

Definitions

The DF was defined as the largest of a group of follicles first detected within 2 days of each other, growing or static in diameter. The day of emergence of the DF was defined as the day on which it was first greater than 3 mm in diameter. The growth rate of the DF was calculated from emergence to the day of treatment, and over the 3 days following treatment or to ovulation where this occurred in less than 3 days. Ovulation was defined as having occurred when a previously present large (>10 mm) follicle was subsequently not detected by ultrasound. It was confirmed by a rise in milk P_4 concentration (to >2.5 ng/ml) within 2 to 5 days. Two cows (one ODB- and one saline-treated cow) ovulated within 24 h of treatment and these ovulations were regarded as spontaneous and unrelated to treatment. Their data were not included in subsequent analysis.

An LH surge was defined as having occurred where the maximum LH concentration was >3 ng/ml in at least one sample after treatment.

Statistical analyses

For proportional data, χ^2 analyses were used except where any cell size was 5 or less, in which case Fishers' exact test was used. Continuous data were analysed by a General linear model (GLM) in SAS (SAS Institute Inc., Cary, NC) with treatment, phase and the treatment by phase interaction as the factors. The residuals were tested for normality of distribution using the Shapiro-Wilks test and visually inspected to check for homogeneity of variance within each treatment group. Where significant ($P<0.05$) differences were detected, least square means of the main effects were compared using the least significant difference technique. Data are presented as the mean ± standard error of the mean (sem) unless otherwise stated.

Results

The concentration of LH was lower in ODB- than saline-treated cows at 12 h after treatment (0.3 ± 0.1 vs. 0.6 ± 0.1 ng/ml; $P<0.05$; Figure 7.1). This was due to a tendency for LH concentration to decline in the ODB-treated cows (-0.14 ± 0.11 ng/ml) and to increase in the saline-treated cows (0.14 ± 0.13 ng/ml) over the 12 h following treatment. An LH surge occurred in 8 of the 15 cows treated with ODB and in none of the saline-treated cows. The average maximum LH concentration, in those cows with an LH surge, was 17.1 ± 2.7 ng/ml which occurred 32.5 ± 2.4 h after treatment with ODB (Figure 7.1). There was no effect of phase of DF development on the maximum LH concentration.

Every cow that ovulated within 3 days of treatment had a detectable LH surge. Three cows with LH surges did not subsequently ovulate within 3 days of treatment. The DF's in two of these three cows (one treated when the DF was growing and the other when the DF had ceased growing) continued to grow and were >10 mm for >15 days. Both cows had P_4 concentrations >1 ng/ml for at least 7 days while the DF was present. Progesterone concentrations in the third cow (growing phase) which did not ovulate remained <1 ng/ml for at least 10 days following treatment.

Treatment with ODB increased the number of cows ovulating within 3 days (5/15 vs. 0/15) and over the entire period of daily ultrasound examinations (10/15 vs. 5/15; respectively; $P<0.05$) compared to saline-treated cows. There was a tendency for more cows treated with ODB to ovulate within 3 days of treatment when growth of the DF had ceased than when the DF was still growing (4/7 vs. 1/8; respectively; $P<0.1$).

The growth rate of the DF's not ovulating after treatment was reduced in cows treated with ODB when the DF was still growing, but not in cows treated when the DF had ceased growing compared with the saline-treated controls at the same phase of follicular development (Table 7.1, Figure 7.2). This reduced the maximum diameter of the DF in the cows treated with ODB when the DF was still growing (Table 7.1). Treatment with ODB when the DF was growing reduced the interval to emergence of the subsequent DF (Table 7.1, Figure 7.2).

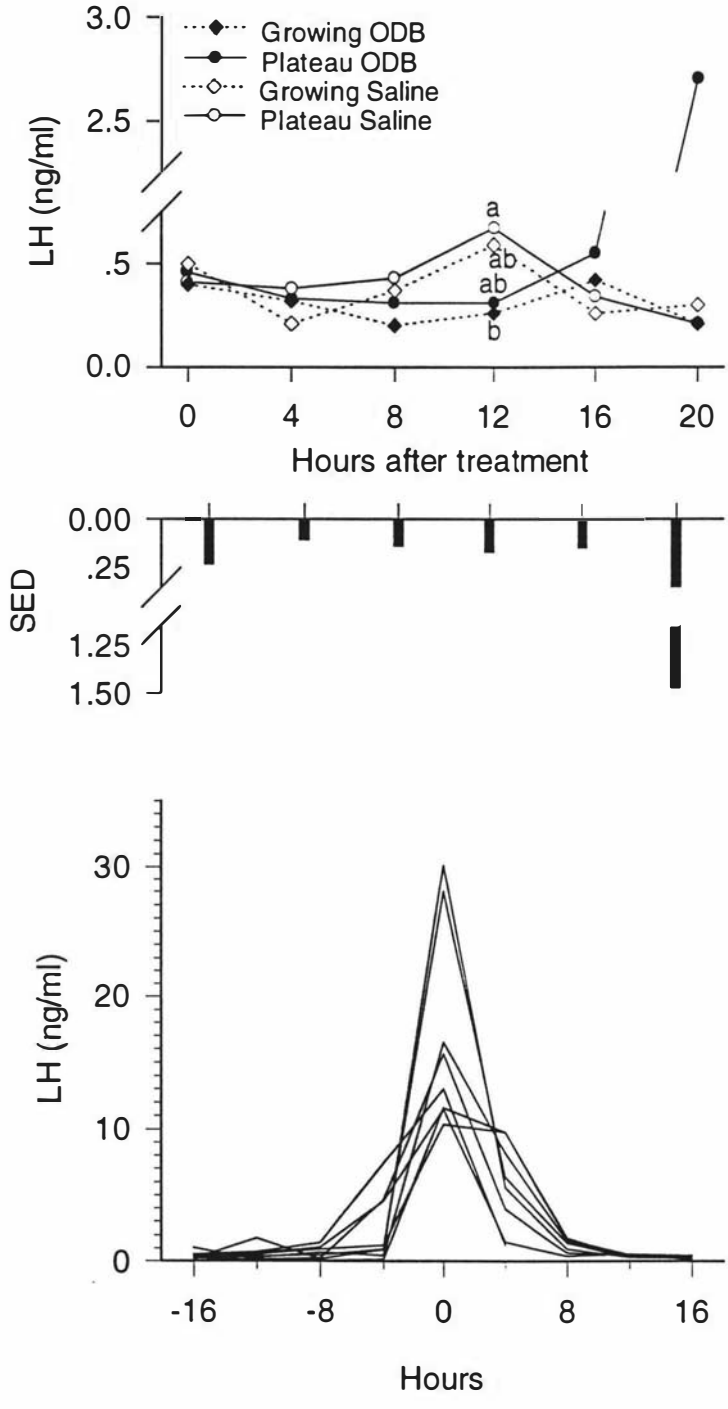

Figure 7.1. Luteinising Hormone concentration (top panel and SED, middle panel) for the first 20 h after treatment and for those having an LH surge (bottom panel, aligned by time of maximum concentration) following treatment with 0.5 mg oestradiol benzoate (ODB), or saline (saline) when the DF was either growing (Growing) or had ceased growing (Plateau). ^{abc} Indicate significant differences ($P<0.05$) among means on that day.

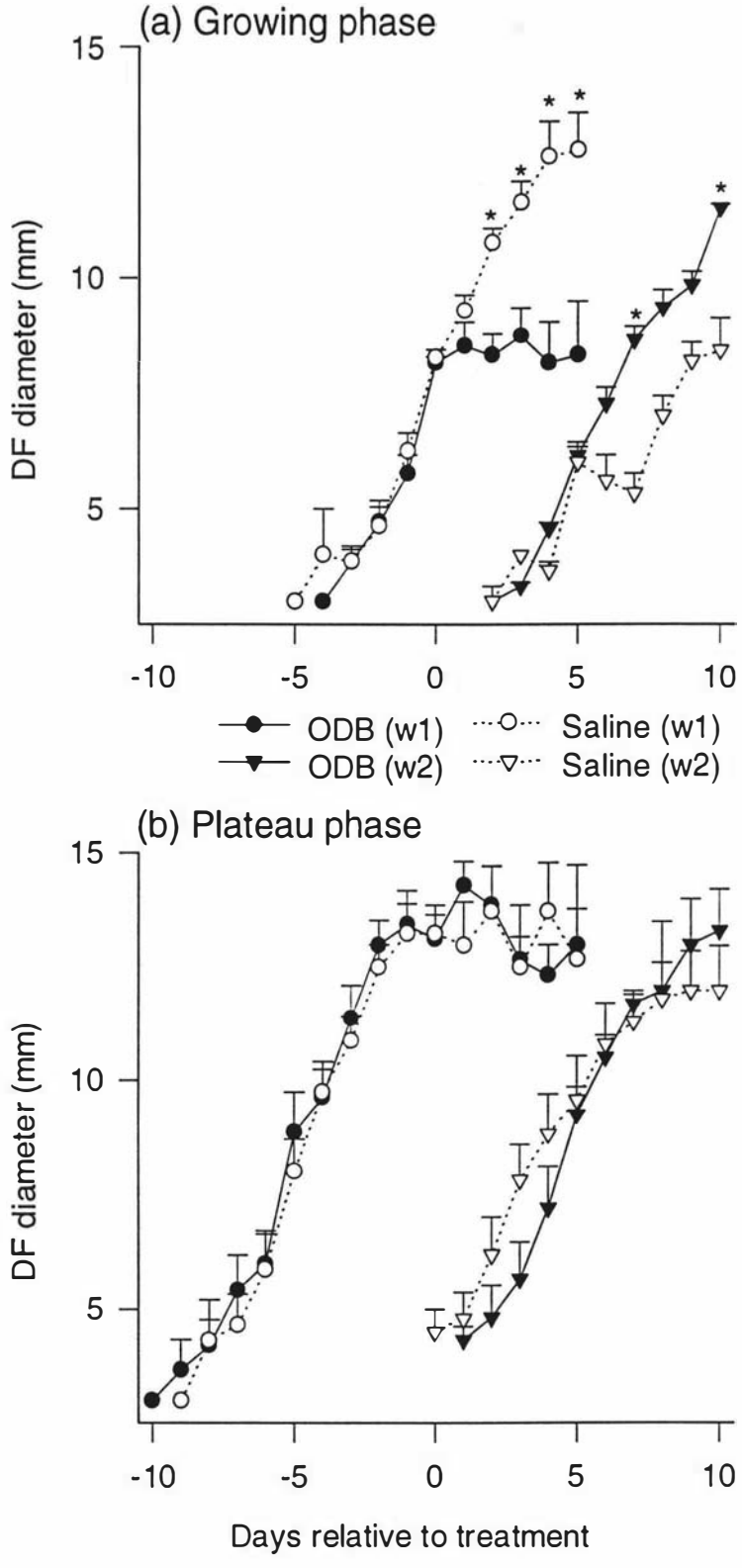

Figure 7.2. The mean diameter (+ sem) of the DF of the wave during which treatment was instituted (w1) and the subsequent wave (w2) following treatment with oestradiol benzoate (ODB) or saline (saline) when the DF was either growing (Growing) or had ceased growing (Plateau). * Indicates differences among treatments ($P<0.05$) on that day.

Table 7.1. The influence of oestradiol benzoate or saline on follicular growth when a dominant follicle was in growing or plateau phase of development.

		GRpost[†]			Maximum diam[‡]			Emergence[¶]		
Treatment	Phase	n	mm/day	sem	n	mm	sem	n	days	sem
ODB	Growing	7	0.2[b]	0.2	8	10.9[b]	1.3	8	4.3[b]	0.5
	Plateau	3	-0.1[b]	0.3	7	16.9[a]	2.1	7	2.7[bc]	0.3
Saline	Growing	8	1.1[1]	0.2	8	14.0[ab]	1.2	8	6.5[b]	1.0
	Plateau	7	0.2[b]	0.2	7	14.7[ab]	1.0	7	1.9[c]	0.7

[abc] Indicates significant difference ($P<0.05$) among means with different superscripts within a column

[†] Growth rate of the non-ovulating DF over the 3 days following treatment

[‡] Maximum diameter of the DF following treatment

[¶] Days to emergence of the subsequent DF (i.e. first day on which the new DF was >3 mm in diameter)

Discussion

Treatment with ODB induced an LH surge and ovulation in only 8 of 15 and 5 of 15 of the pasture-fed dairy cows used in this experiment, respectively. The growth of the DF was slowed and the interval to emergence of the next DF was shortened by treatment with ODB when the treatment occurred while the DF was growing but not when the DF had ceased growing (Table 7.1, Figure 7.2).

The failure of 7 of 15 cows to release LH following treatment with ODB despite having calved more than 3 weeks previously is consistent with previous data showing that ODB may not stimulate LH release in the early postpartum period (Short *et al.,* 1979; Alam and Dobson, 1987; Schallenberger and Prokopp, 1985). A similar failure of E_2 to induce an LH surge can occur in lactating women (Baird *et al.,* 1979). Oestradiol-induced release of GnRH and hence LH, is mediated by a series of excitatory and inhibitory neuropeptides (Kalra, 1993). Alterations in the concentrations of one or more of these by

carryover effects of pregnancy, undernutrition or low CS are possible mechanisms for the failure of ODB to induce LH release.

However, in three of the 8 animals with an induced LH surge, ovulation did not occur. The final maturation of the pre-ovulatory follicle includes acquisition of increasing numbers of LH receptors in the granulosa cell (Ireland and Roche, 1983) and this process is gonadotrophin dependent. Some cows may have had insufficient gonadotrophin support before treatment to develop follicles capable of responding to an LH surge. Cows with extended periods of PPA have been reported to have a low LH pulse frequency (Fisher *et al.,* 1986; Wright *et al.,* 1990). Our data indicate that dysfunction may have occurred at two points of the H-P-O axis in these pasture-fed anovulatory dairy cows.

The phase of follicular development at the time of treatment affected the growth rate of the non-ovulating DF, the timing of emergence of the subsequent DF and the proportion of cows ovulating within 3 days of treatment. Depression of the growth rate of the DF treated while still growing has been reported in cycling cows following treatment with 5 mg of EV (Bo *et al.,* 1993). However, in that study the endogenous P_4 concentrations were also increasing coincidentally with growth of the DF. In the present study, the P_4 concentrations were below 1 ng/ml, demonstrating that ODB alone was able to perturbate follicle development. The use of only 0.5 mg of ODB produced depression of DF growth rate similar to that seen following treatment with 5 mg of EV in cycling cows. Whether this lower dose would produce the same response in cycling cows has not been studied.

Emergence of the next DF occurred 4 days after ODB treatment when the DF was growing, 2 days earlier than in saline-treated cows. A similar interval is reported in cycling cows (Bo *et al.,* 1993). Premature atresia of the DF is the likely explanation for this early cessation of growth and earlier emergence of the next DF. Atresia of the DF following 5 mg EV has been demonstrated following histological examination and assay of follicular steroid concentrations (Engelhardt *et al.,* 1989). Similarly, removal of the growing DF by electrocautery or ovariectomy has resulted in earlier emergence of the subsequent DF (Ko *et al.*, 1991; Badinga *et al.,* 1992). In contrast to studies in cycling cows (Bo *et al.,* 1994), treatment with ODB when the DF had ceased

growing had no effect on the emergence of the subsequent DF. The difference may be due to the dose of ODB used (i.e. 5 mg by Bo *et al.,* 1994 compared with 0.5 mg in this experiment). The larger dose may have produced sustained elevation of peripheral ODB concentrations, thus inhibiting follicular emergence (Bo *et al.,* 1993).

The growth rate of the DF and the time of emergence of the subsequent DF was not affected following ODB treatment when the DF had ceased growing. This may have been because the DF had already, or was about to become, atretic. ODB may not be able to accelerate the process of atresia, in follicles where this process has commenced spontaneously.

Oestradiol may affect follicles directly (Hunter *et al.,* 1992) as it has direct negative effects on steroid production by bovine thecal and granulosa cells *in-vitro* (Fortune and Hansel, 1979; Henderson *et al.,* 1987).

Alternatively, ODB may affect follicles indirectly via modulation of gonadotrophin concentrations (Adams *et al.,* 1993). Follicular growth to the pre-ovulatory stage is dependent on presence of gonadotrophins (McNeilly *et al.,* 1986; McNatty *et al.*, 1990; Webb *et al.,* 1994). Emergence of a new DF is preceded by an increase in FSH concentration (Adams *et al.,* 1992), and depression of FSH concentration by follicular fluid injection may delay DF emergence (Turzillo and Fortune, 1990). ODB treatment induces a bi-phasic decrease and then increase in gonadotrophin concentration (Kesner *et al.,* 1981; Schallenberger and Prokopp, 1985) in the absence of serum P_4 concentrations of more than 0.5 ng/ml (Nanda *et al.,* 1988). This bi-phasic pattern of LH release was demonstrated in 8 of 15 ODB-treated cows in this study. The cessation of DF growth in the present study may have been caused by the decrease, the increase or both in FSH and/or LH concentration following treatment. A decrease in LH pulse frequency induced by increases in exogenous P_4 concentration in cycling cows resulted in a decline in the diameter of the DF and emergence of the next DF (Savio *et al.,* 1993). This depression in LH pulse frequency was associated with a decline in circulating E_2 concentration indicating that atresia of the DF had occurred. However, the LH surge may itself induce atresia as the intrafollicular concentration of E_2 in the DF and in non-ovulating follicles decline following the surge (Staigmiller and

England, 1982; Ireland and Roche, 1983). Additionally, infusion of pulsatile LH in addition to FSH in the follicular phase reduce the number of ovulatory follicles in hypothalamo/pituitary disconnected sheep (McNeilly *et al.,* 1992). The LH surge may initiate atresia in follicles that do not ovulate, reducing growth rate as well as allowing earlier emergence of the subsequent DF. ODB treatment without preceding progestogen treatment results in an LH surge but no effect on the growth rate of the growing DF, whereas ODB treatment preceded by progestogen treatment produced no LH surge but depressed the growth rate of the DF in cycling cows (Bo *et al.,* 1994). This suggests that it may be the depression of gonadotrophin concentrations, rather than the surge that is important in modifying DF growth rate. Why the ODB treatment depressed DF growth rate in the present experiment, but not in the experiment of Bo *et al.*, (1994) may be related to the timing and dose of ODB used. Five mg of ODB depressed gonadotrophin concentrations for only about 6 h and this was followed by the LH surge, which was maximal at 18 h after ODB treatment. In contrast, in the present experiment, the LH concentration was depressed for at least 12 h before the LH surge which was maximal at 32 h. Bo *et al.*, (1994) state that depression of gonadotrophins must last for 24 h before DF growth rate is affected in cycling cows. The present experiment demonstrates that shorter periods may be sufficient to depress DF growth rate, perhaps indicating that the DF's of anovulatory cows are more sensitive to changes in gonadotrophins than those of cycling animals.

Two cows had abnormally large follicles following treatment with ODB. Partial luteinisation of granulosa cells appeared to have occurred as the peripheral P_4 concentration exceeded 1 ng/ml for at least 7 days after treatment. Similar structures have been experimentally induced by EV treatment in late dioestrus (Engelhardt *et al.,* 1989; Rajamahendran and Walton, 1990). Low maximum LH concentrations and a prolonged interval from E_2 treatment to the LH surge have been associated with the formation of these structures (Nadaraja and Hansel, 1976). However, both cows in the present experiment had maximum LH concentrations >10 ng/ml at 32 and 44 h, respectively and the LH concentrations were >2 ng/ml for at least 4 h which were figures similar to those from cows which ovulated. This supports a

previous observation (Engelhardt *et al.*, 1989) that the maximum LH concentration and the timing of the LH surge may not be important in formation of these follicular structures.

Conclusion

Failure to release LH following treatment with ODB and of ovulation where an LH surge was induced occurred in some postpartum anovulatory cows indicating dysfunction occurred at two points of the H-P-O axis in some animals.

Treatment with ODB when the DF was growing reduced DF growth, resulted in earlier emergence of the subsequent DF and resulted in fewer ovulations than in cows treated when the DF had ceased growth. Differences in the maturity of the DF at these different stages of follicular development may account for the observed difference in response. Treatment with ODB appears to have induced atresia in the growing DF in the absence of P_4. Atresia was induced directly by treatment with ODB or indirectly by alteration in LH and/or FSH concentration.

CHAPTER 8:

Anovulatory Postpartum Dairy Cows Have Lower LH Pulse Frequency Than Cycling Cows Before And After Ovariectomy

Abstract

Beef and dairy cows with extended periods of PPA have been shown to have a low mean concentration and a low pulse frequency of LH. This may be due to either increased sensitivity of the hypothalamus to the inhibitory effects of ovarian E_2 or to ovary-independent inhibition of GnRH and/or LH release. Increased sensitivity to E_2 inhibition of LH release occurs in seasonally anoestrous sheep and in undernourished cattle. Increasing E_2 concentration with increasing diameter of the DF may result in reduced mean concentration and pulse frequency of LH. The mean LH concentration and LH pulse frequency and amplitude were examined in anovulatory and contemporary cycling cows at 2 stages of follicle development, before and after ovariectomy. Additionally, the effect of treatment with E_2 on these LH parameters was examined following ovariectomy.

Fourteen, anovulatory cows were paired with 14 cycling cows. Ovariectomy was performed on half of the pairs when the DF was growing and 5 to 9 mm in diameter (growing phase), while the remaining pairs were ovariectomised when the DF had not altered in diameter by more than 1 mm in the preceding 72 h (plateau phase). Blood samples were drawn at 15 min. intervals for 8 h immediately preceding (d0), and 3 (d3) and 10 (d10) days after ovariectomy. Additionally, 6 pairs of cows had two dermal patches containing E_2 applied following sampling on d10 and had blood samples drawn 2 days later at 15 min. intervals for 8 h (d12). The LH concentrations were determined in each of these samples.

Anovulatory cows had lower LH pulse frequencies and higher pulse amplitudes than cycling cows before and after ovariectomy. The mean

concentration and the LH pulse frequency increased from d0 to d3 and from d3 to d10 in both cycling and anovulatory cows. Phase of follicular development at the time of ovariectomy did not affect any LH parameter, before or after ovariectomy. Exogenous E_2 reduced LH pulse frequency and increased LH pulse amplitude in anovulatory, but not cycling, cows.

These data indicate that anovulatory cows have lower LH pulse frequency than cycling cows. Both ovarian and extra-ovarian factors inhibited the release of LH in anovulatory cows and anovulatory cows were more sensitive to the inhibitory effects of E_2 than cycling cows.

Introduction

Some pasture-fed New Zealand dairy cows have an extended periods of PPA (Fielden *et al.*, 1973; Chapter 2). This anovulatory period is characterised by a series of large ovarian follicles which develop but fail to ovulate until an average of 4.2 (± 0.6) waves of follicles has occurred, corresponding to 42.3 (± 4.2) days postpartum (Chapter 4).

In the cycling cow, ovulation is preceded by an increasing concentration and pulse frequency of LH (Peterson *et al.*, 1975) and it has been hypothesised that a similar, increasing, LH pulse frequency and concentration is essential for the resumption of ovulatory activity in the postpartum period (Lamming *et al.*, 1981; Roche *et al.*, 1981; Schallenberger *et al.*, 1982). The LH pulse frequency increases with time postpartum (Schallenberger *et al.*, 1982; Wright *et al.*, 1990). Cows that have an extended period of PPA (>40 days) have a lower LH pulse frequency and amplitude than cows ovulating earlier (<40 days) postpartum (Fisher *et al.*, 1986).

There are cyclical changes in mean LH concentration and LH pulse frequency (Rahe *et al.*, 1980; Schallenberger *et al.*, 1985) which are controlled by ovarian steroid inhibition of the hypothalamus and/or pituitary in cycling cows (Price and Webb, 1988; Stumpf *et al.*, 1993). Progesterone appears to be the major controlling steroid in cycling cows as natural or induced luteolysis and unilateral ovariectomy of the ovary containing the CL results in an increase in mean LH and in LH pulse frequency (Ireland and Roche, 1982;

Schallenberger *et al.*, 1984; Badinga *et al.*, 1992). Treatment of ovariectomised cattle with P_4 results in suppression of mean LH concentration and LH pulse frequency (Price and Webb, 1988; Stumpf *et al.*, 1993).

The role of E_2 in the control of LH is less well defined. The circulating concentration of E_2 increases following luteolysis as a result of increased LH and FSH stimulation of E_2 production by large follicles (McNatty *et al.*, 1984b). This increase in E_2 concentration is associated with increasing LH pulse frequency and mean LH concentrations resulting in the preovulatory LH surge (Schallenberger *et al.*, 1984). Experimentally, the effect of E_2 is mediated by the concentration of P_4 at the time of treatment. Combined treatment of ovariectomised cows with P_4 and E_2 results in greater depression of LH mean concentration and LH pulse frequency than either P_4 or E_2 alone (Price and Webb, 1988; Stumpf *et al.*, 1993). However, an injection or a subcutaneous implant of E_2 results in a bi-phasic, decrease for 2 to 9 h, followed by a surge-like increase, peaking at 15 to 24 h, in LH mean concentration before a return to basal levels in PPA or ovariectomised cattle (Beck and Convey, 1977; Kesner *et al.*, 1981; Schallenberger and Prokopp, 1985). Chronic implantation of subcutaneous E_2 into ovariectomised, previously cycling cattle, results in either increases (Kinder *et al.*, 1991) or decreases in mean LH concentration and pulse amplitude (Price and Webb, 1988) with no change in LH pulse frequency.

In the ewe, although P_4 is the major inhibitor of LH release, E_2 also plays a role (Goodman, 1988). The sensitivity to E_2 inhibition increases markedly during the anoestrous season in comparison to the normal breeding season (Legan *et al.*, 1977). An increase in sensitivity to E_2 feedback has been reported in undernourished, compared to fully fed, beef cattle (Imakawa *et al.*, 1987).

Follicles develop in a series of waves with 1 to 4 large follicles between ovulations (Sirois and Fortune, 1988; Ginther *et al.*, 1989b). The intrafollicular concentration of E_2 increases with diameter until atresia or ovulation occurs (Ireland and Roche, 1983; McNatty *et al.*, 1984a; Badinga *et al.*, 1992). In proestrus, increasing circulating E_2 concentrations produced by the pre-ovulatory follicle, are associated with increasing LH pulse amplitude and mean

LH concentration. In contrast, in anovulatory cows, if there is an increased sensitivity to E_2 feedback, then the LH pulse amplitude and mean LH concentration may be lower in the presence of large, E_2 producing follicles.

Ovariectomy of cycling cows is followed by a rapid increase in LH concentration (Hobson and Hansel, 1972) and pulse frequency (Schallenberger and Peterson, 1982). Ovariectomy of early (4 day) postpartum cows does not lead to comparable increases in LH concentration and pulse frequency (Schallenberger and Peterson, 1982). This suggests that extra-ovarian factors may be inhibiting release of LH in anovulatory cows. Ovariectomy followed by imposition of two levels of nutrition has shown that nutrition has direct effects on the rate of increase of LH concentration and pulse frequency following ovariectomy (Imakawa *et al.*, 1987). Cows which calve at low body CS have lower LH pulse frequencies before and after ovariectomy than cows calving in high CS (Wright *et al.*, 1990) indicating that CS effects LH release, independent of the ovary.

The following experiment was designed to determine the LH concentration, pulse frequency and amplitude in lactating, anovulatory, dairy cows, in the presence and absence of the ovaries and at two stages of follicular development when differences in E_2 concentration may be expected. Additionally, the effect of E_2 treatment, 10 days after ovariectomy on LH parameters was examined.

Materials and Methods

Animals and procedures

The experiment was a 2 by 2 factorial design, involving 28 cows over 2 years (n = 16 in 1991 and n = 12 in 1992). Cows were selected from a spring calving, pasture-fed, dairy herd with a planned start of calving of 15 July. Oestrus detection and machine milking were carried out twice daily from calving onwards. Six weeks after the planned start of calving, cows were selected as anovulatory or cycling based on oestrus detection records. An

anovulatory and cycling cow were then paired by calving date (± 7 days), breed (Jersey or Friesian) and age (2, 3 or >3 years old). The ovaries of each cow were then examined daily by transrectal ultrasound using an ALOKA 210Dx ultrasound with a 7.5 MHz linear array transducer (Medtel, Auckland, NZ).

Milk (20 ml) was collected twice weekly for measurement of P_4 concentration. Anovulatory cows that had a milk P_4 concentration >2 ng/ml or which were detected as having ovulated by ultrasound (a large follicle was not detectable on consecutive daily examinations) were removed from the trial along with their cycling pairs. Half of the pairs of cows were randomly assigned to be ovariectomised when in the growing phase of follicle development and the other half in the plateau phase. The growing phase was defined as when the growing DF was between 5 and 9 mm in diameter. The plateau phase was defined as when the DF was greater than 10 mm in diameter and its diameter had not altered by more than 1 mm for 72 hours. For the cycling cows, ovariectomy in the plateau phase occurred during the presence of the first DF after ovulation (i.e. 8.3 days after ovulation) and in the growing phase during the presence of the second DF following ovulation (i.e. 11.1 days after ovulation). Each cow was injected with 25 mg of dinoprost tromethamine (Lutalyse, Upjohn, Auckland NZ) i.m., 24 h before ovariectomy to ensure that the DF would be the presumptive pre-ovulatory follicle. Bilateral ovariectomy was performed using a standing left flank approach following sedation with xylazine (Rompun 2% solution, Bayer New Zealand Limited), infusion of lignocaine hydrochloride (Lopaine 2%, Troy Laboratories, Ethical Agents, Auckland, NZ) for local anaesthesia and surgical preparation of the site. Ovariectomy occurred 61.0 (± 2.3) days postpartum.

Blood samples (10 ml) were removed at 15 min. intervals from preplaced jugular catheters from 0800 to 1600 h during the 8 h preceding ovariectomy (d0), and 3 (d3) and 10 (d10) days after ovariectomy.

In 1992, all cows (n = 12) had two transdermal patches containing E_2 (Estraderm TTS 50, Ciba-Geigy NZ Ltd, Auckland, NZ) applied following the blood sampling on d10. These patches are designed to deliver 50 μg/day of E_2 for 3 to 4 days and are used for hormone replacement therapy for post-menopausal women. The patches were applied to the dorso-caudal aspect of

the udder following clipping, cleaning with povidone-iodone solution and drying of the area. Blood samples (10 ml) were collected at 15 min. interval over 8 h, two days later (d12).

Blood samples were immediately placed into a 10 ml lithium heparin glass tube (Vacutainer, Salmond Smith-Biolab LTD, Auckland, NZ) and kept on ice for up to 4 h before centrifugation and storage at -20 °C pending analysis. Every sample was assayed for LH concentration and the fourth sample on each day was analysed for P_4 concentration.

Hormone assays

Plasma LH concentrations were determined using a validated RIA (Chapter 6). The within-assay and between-assay coefficients of variation were 4.0% and 5.0% and 3.6% and 3.1% for two quality control plasma's with mean concentrations of 1.2 and 5.7 ng/ml, respectively, each assayed 10 times in duplicate in each of 8 assays.

Progesterone concentrations in milk and plasma were measured directly using a commercial, solid phase, I^{125} label assay (Coat-a-Count, DPC, Los Angeles, Calif., USA, Chapter 6). The within-assay and between-assay coefficients of variation were 5.3% and 15.2%, and 6.0% and 9.0% for 2 quality control pools run in sextuplet in each assay with mean concentrations of 3.7 and 1.7 ng/ml, respectively, over four assays. The sensitivity was 0.08 ± 0.02 ng/ml. A P_4 concentration of at least 2 ng/ml was defined as indicative of luteal activity.

Statistical analyses

The LH data were analysed using the algorithm of Merriam and Wachter (1982) using the default G settings (G1 = 3.8, G2 = 2.6, G3 = 1.9 G4 = 1.5, and G5 = 1.2) and with a linear within-assay error structure ($y = 3.388 + 2.93x$ and $0x^2$).

The serum P_4 concentration, mean LH concentration, the number of pulses of LH/8 h and the mean pulse amplitude were analysed using a General Linear Model (GLM; SAS, SAS Institute Inc., Cary, NC) with status (anovulatory or cycling), phase (growing or plateau) and the status by phase interaction as the main effects. A separate model was run for each day (0, 3, 10) relative to ovariectomy. Least square means were compared using the least significant difference technique. The difference in each of these parameters for d3-d0, d10-d3 and d12-d10 (1992 only) was calculated and the difference analysed in a similar model. The effects of phase and status were compared as above. The mean change across days (d3-d0, d10-d3, d12-d10) for each parameter was compared by Student's t-Test.

Results

Progesterone concentrations

Each anovulatory cow had milk and plasma P_4 concentrations of <1 ng/ml throughout the experiment and each cycling cow had >2.5 ng/ml of milk P_4 before ovariectomy. On the day of ovariectomy, 27 of the 28 cows had plasma P_4 levels of <1 ng/ml, with one cycling cow having a P_4 concentration of 2.8 ng/ml. Cycling cows had higher plasma P_4 concentrations than anovulatory cows on the day of ovariectomy (0.81 ± 0.13 vs. 0.03 ± 0.12; $P<0.01$), but there was no difference following ovariectomy between groups (0.13 ± 0.05 vs. 0.11 ± 0.05; 0.20 ± 0.11 vs. 0.11 ± 0.11 for d3 and d10, respectively). The P_4 concentration declined significantly between d0 and d3 in the cycling cows, but there was no difference in concentration between d3 and d10 in the cycling cows or between the d0, d3 or d10 in the anovulatory cows. Three cows (2 cycling, 1 anovulatory) had milk or plasma P_4 concentrations of >2 ng/ml following ovariectomy. This indicates the likely presence of luteal tissue, so data following ovariectomy for these 3 cows were removed from subsequent analyses.

LH data

At least one pulse of LH in 8 h was detected in all cows except one anovulatory cow. A representative example of the LH concentrations on d0, d3 and d10 are presented in Figure 8.1.

Anovulatory cows had a lower pulse frequency and higher pulse amplitude before and after ovariectomy than cycling cows (Figure 8.2). There was no difference in the mean LH concentration between cycling or anovulatory cows on any day (Figure 8.2). There was neither an effect of the phase of follicle development (Table 8.1) nor a phase by status interaction for any of the parameters on any day.

Table 8.1. The mean LH concentration and pulse frequency and amplitude before and after ovariectomy in cows ovariectomised when the DF was growing or had reached plateau phase.

	DF phase	Mean (ng/ml)		Frequency (pulses/8 h)		Amplitude (ng/ml)	
Day†		mean	sem	mean	sem	mean	sem
0	Growing	0.3	0.1	4.3	0.6	0.5	0.1
	Plateau	0.4	0.1	4.2	0.6	0.5	0.1
3	Growing	0.8	0.1	6.4	0.5	1.0	0.1
	Plateau	0.7	0.1	6.0	0.4	0.9	0.1
10	Growing	1.1	0.1	7.9	0.4	1.0	0.1
	Plateau	1.0	0.1	7.6	0.4	0.9	0.1

† Day relative to ovariectomy

The mean LH concentration and pulse frequency increased from d0 to d3 and again from d3 to d10 (Table 8.2). The pulse amplitude increased from d0 to d3, but not thereafter. There was no difference in the rate of these increases between the anovulatory and cycling cows or among phases of follicular development.

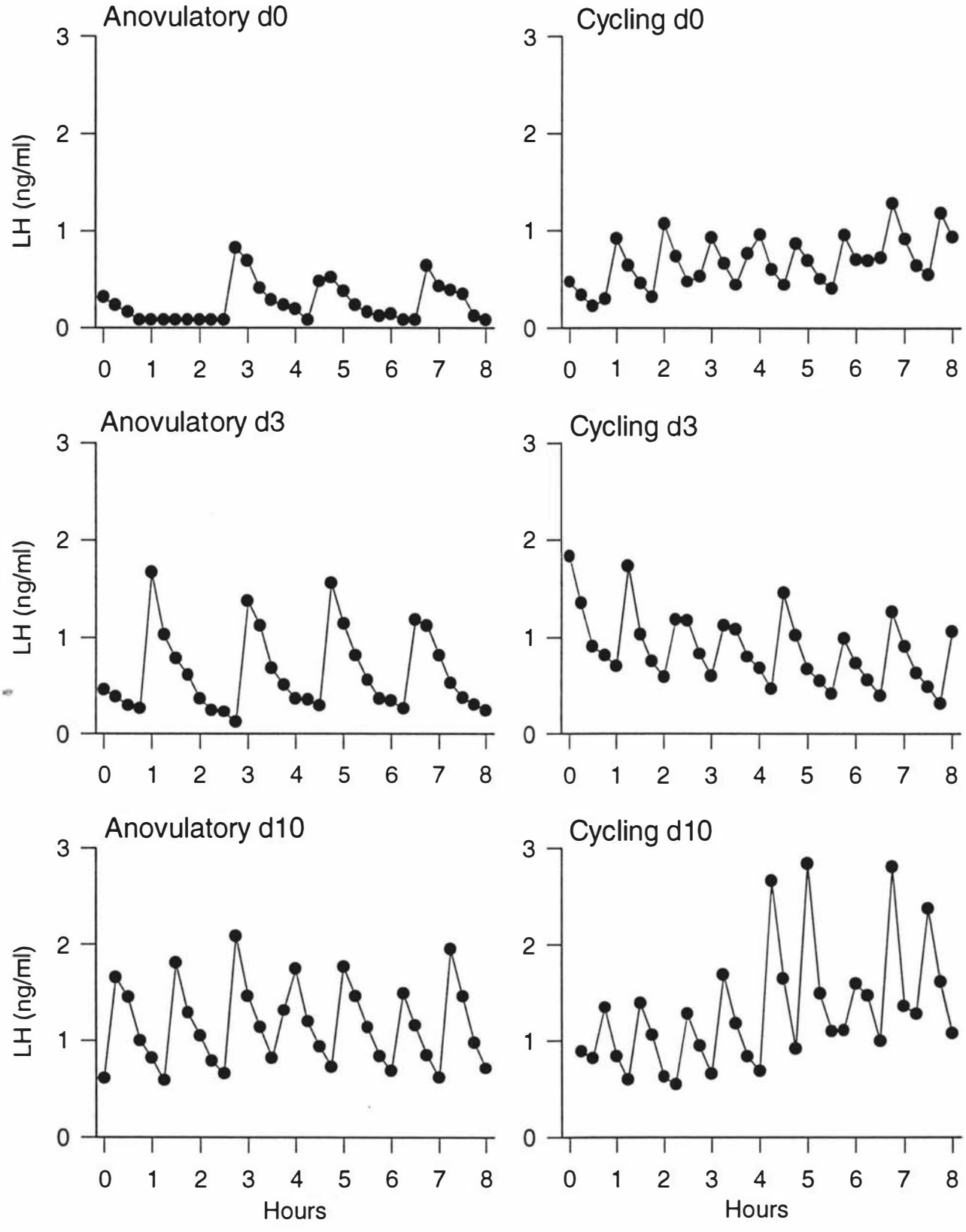

Figure 8.1. The LH concentration in plasma samples taken at 15 minute intervals before (d0), and 3 (d3) and 10 (d10) days after ovariectomy from a representative example of an anovulatory and a cycling cow.

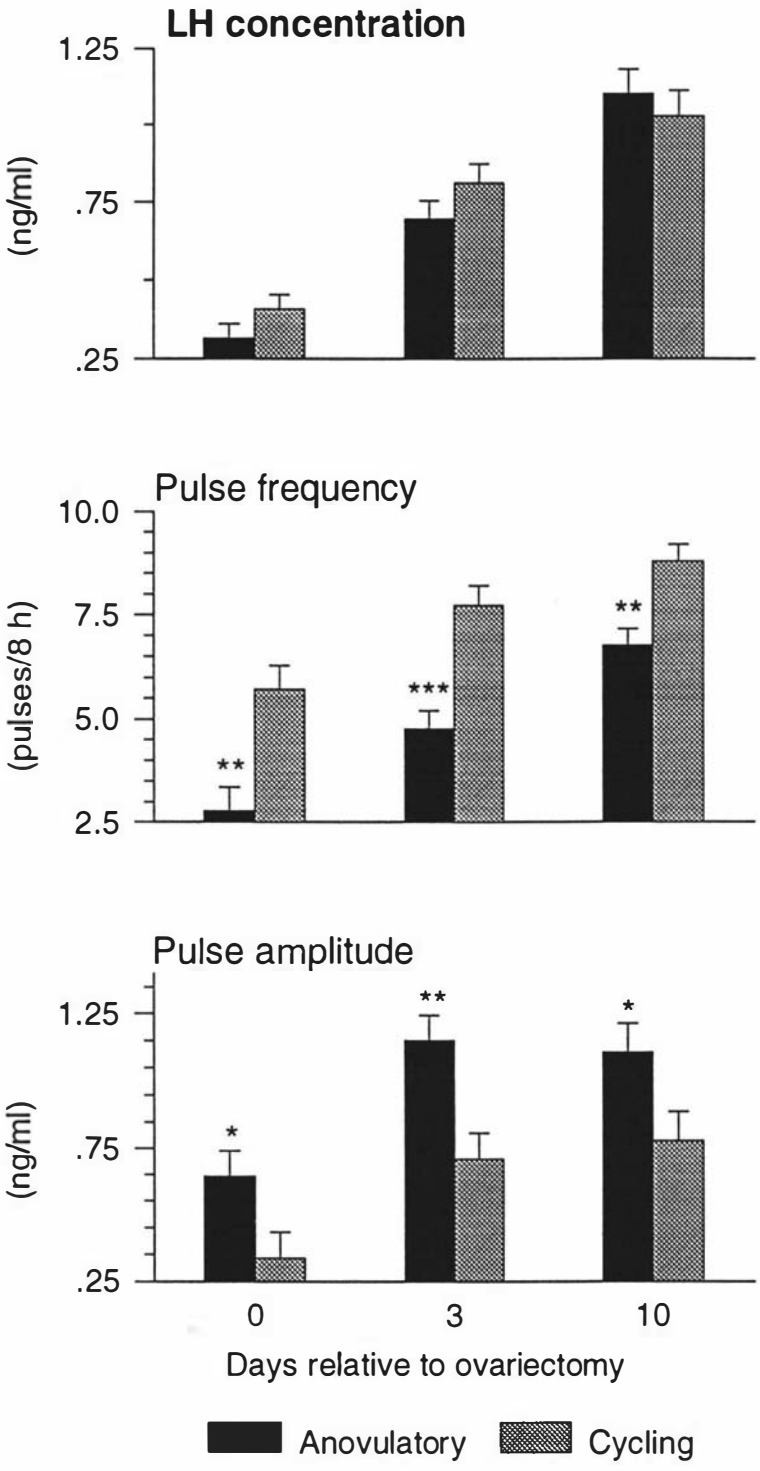

Figure 8.2. The least square (± sem) means of the mean LH concentration, the pulse frequency and amplitude before (d0), and 3 (d3) and 10 (d10) days after ovariectomy in anovulatory and cycling cows. *, P<0.05; **, P<0.01; ***, P<0.001 between status within day.

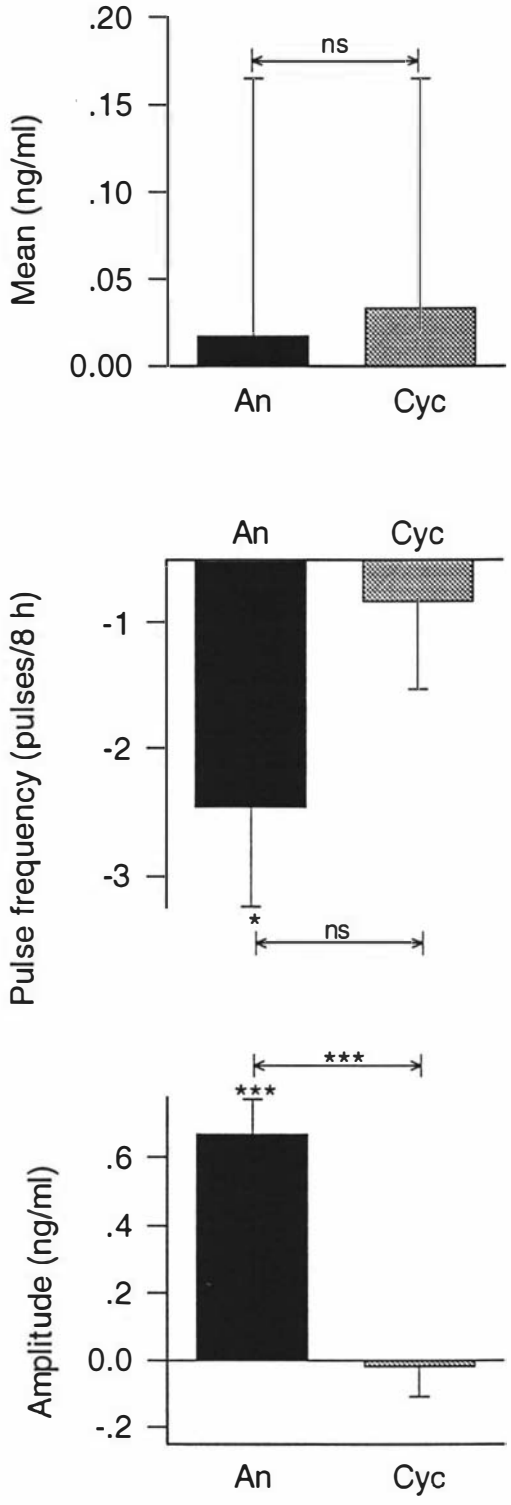

Figure 8.3. The difference (± SED) of the mean LH concentration and the pulse frequency and amplitude between d12 and d10 after treatment with two oestradiol patches on d10 after ovariectomy of cows ovariectomised while anovulatory (An) or cycling (Cyc). Symbols on top of the error bars indicate the significance of the change compared to 0, and symbols on the horizontal bars indicate differences among An and Cyc cows.

Table 8.2. The difference and standard error of the difference of mean LH concentration, pulse frequency and pulse amplitude between day 3 and day 0 and between day 10 and day 3 after ovariectomy in dairy cows.

	d3-d0‡			d10-d3‡		
Parameter	diff£	SED	P†	diff	SED	P
Mean (ng/ml)	0.39	0.06	***	0.32	0.07	***
Pulse frequency (pulses/8 h)	1.84	0.77	**	1.64	0.55	***
Pulse amplitude (ng/ml)	0.46	0.11	***	0.01	0.06	ns

‡ Day 3 - day 0 or day 10 - day 3 relative to ovariectomy

£ Change in value (diff), the standard error (SED) of this difference and significance († ns = non-significant; *, P<0.05; **, P<0.01; ***, P<0.001) of the change in LH parameters between day 3 and day 0 or day 10 and day 3 relative to ovariectomy

The E_2 patches produced a decrease in the LH pulse frequency and increase in LH pulse amplitude in the anovulatory cows, but no change in the cycling cows (Figure 8.3). There was no effect of phase nor was there a status by phase interaction.

Discussion

The cycling and anovulatory cows were ovariectomised at a time when the peripheral P_4 was low (<1.0 ng/ml) in all but one, cycling animal. Prostaglandin treatment had reduced P_4 concentrations within 16 h of treatment reducing the confounding effect of P_4 on LH parameters so that the comparison of LH parameters in anovulatory and cycling cows occurred when both groups had low P_4 concentrations. The preovulatory LH-surge occurs 44 to 60 h after prostaglandin-induced luteolysis (Ireland and Roche, 1982). In this experiment, the cycling cows were ovariectomised before the LH surge would be expected to have occurred (i.e. 24 h after prostaglandin treatment) and at a time when the concentration and pulse frequency of LH would be expected to be increasing (Peterson *et al.*, 1975; Ireland and Roche, 1982).

Long-term anovulatory cows had lower LH pulse frequencies than cows resuming cycling earlier postpartum as has been previously reported for dairy and beef cows (Fisher *et al.*, 1986; Wright *et al.*, 1990).

Following ovariectomy, LH concentration, pulse frequency and amplitude increased in both anovulatory and cycling cows. LH concentration and pulse frequency continued to rise between d3 and d10 suggesting that maximum LH release had not been achieved by this time. LH concentration continued to rise for up to 7 weeks after ovariectomy in sheep (reviewed by Martin, 1984). This increase may be due to clearance of inhibitory effects of ovarian factors, the positive effect of increasing GnRH release on the number of GnRH receptors on the pituitary, hypertrophy of the pituitary and/or increasing gonadotrophin production by the pituitary (reviewed by Martin, 1984). The increase in serum LH concentration parameters following ovariectomy was similar in anovulatory and cycling cows, suggesting that the ovarian inhibition of LH was similar in the 2 groups. However, circulating and DF E_2 concentrations in long-term anovulatory cows are lower than in cows ovulating earlier postpartum (Fisher *et al.*, 1986; Prado *et al.*, 1990; Chapter 5). As a similar degree of negative feedback was occurring, but with perhaps lower circulating E_2 concentrations, a greater sensitivity to E_2 feedback may have occurred in the anovulatory cows. The decline in LH pulse frequency and increase in the pulse amplitude following E_2 treatment in the anovulatory cows, but not the cycling cows supports this. Increased sensitivity to E_2 inhibitory feedback on LH release is the mechanism causing anovulation during seasonal anoestrus in sheep (Legan *et al.*, 1977) and has been suggested as the mechanism involved in lower LH pulse frequencies in low CS or undernourished beef cattle (Imakawa *et al.*, 1987; Wright *et al.*, 1990).

The LH pulse frequency of the anovulatory cows remained lower than in cycling cows following ovariectomy. This suggests either that stores of GnRH or LH were limiting or that there was extra-ovarian inhibition of GnRH and/or LH release following ovariectomy in anovulatory cows. The hypothalamic concentration of GnRH does not alter substantially in the postpartum period suggesting that GnRH production is not limiting resumption of postpartum cyclic activity (Moss *et al.*, 1985; Nett *et al.*, 1988). The number of GnRH

receptors in the anterior pituitary of anovulatory beef cows is reported to be lower than cycling cows in some studies (Schoenemann *et al.*, 1985), but not others (Moss *et al.*, 1985). Additionally, the pituitary LH concentration and release of LH in response to exogenous GnRH is lower at 5 than at 10 days postpartum or in mid-luteal phase cows (Moss *et al.*, 1985). However, the cows used in the present study were >60 days postpartum and exogenous GnRH elicited an LH surge sufficient to induce ovulation in cows only 2 to 3 weeks postpartum drawn from a similar population (Chapter 6). This suggests that sufficient GnRH receptors were present in the anterior pituitary gland and that pituitary stores of LH were adequate. Extra-ovarian inhibition of GnRH release, or endogenous slowing of the GnRH pulse generator may limit LH pulse frequency postpartum.

LH pulse frequency was decreased in cows with low CS at calving (Wright *et al.*, 1990) and by restriction of energy intake postpartum (Imakawa *et al.*, 1986; Butler and Smith, 1989; Richards *et al.*, 1989; Canfield and Butler, 1990). Additionally, indirect markers of nutritional status, such as glucose and NEFA concentrations, have been correlated with LH pulse frequency (Rutter and Manns, 1987; Canfield and Butler, 1990). Where ovariectomy has been followed by undernutrition, the LH pulse frequency and concentration have remained lower than in well-fed control animals (Imakawa *et al.*, 1987). Suckling inhibits LH pulse frequency (Peters *et al.*, 1981; Garcia-Winder *et al.*, 1984; 1986a). Thus, suckling, CS, metabolic, and nutritional factors may be among the extra-ovarian factors which inhibit release of GnRH and/or LH. Interactions between low CS, undernutrition and the sensitivity of the E_2 feedback mechanism have also been reported (Imakawa *et al.*, 1987; Wright *et al.*, 1990).

It was hypothesised that differences in DF diameter before ovariectomy would be associated with differences in circulating E_2 concentration. Additionally, differences in sensitivity in E_2 feedback among cycling and anovulatory cows were hypothesised. An interaction among status of the animals and phase of follicular development may have therefore been expected. However, there was no difference in any LH parameter between cows ovariectomised when there was a growing or plateau phase follicle

present before or after ovariectomy. Additionally, there was no interaction between the status of the animal and the stage of follicular development at which ovariectomy occurred indicating that anovulatory cattle responded similarly to cycling cattle. However, as plasma E_2 concentrations were not measured in the present study there may not have been differences between the groups. In ewes, the inhibitory effect of E_2 on LH appears to be constant across the physiological range of E_2 concentrations (Goodman, 1988). If true in cattle, then differences in LH parameters may not have been demonstrated even if differences in peripheral and DF E_2 concentration were present at different stages of follicular development. However, dose dependent differences in LH parameters have been demonstrated in ovariectomised cattle treated with E_2 (Price and Webb, 1988; Kinder *et al.*, 1991), suggesting that E_2 control of LH may differ between cattle and sheep.

In summary, dairy cows with extended periods (average of >60 days) of PPA had lower LH pulse frequencies and higher pulse amplitudes than cycling cows before and after ovariectomy. Anovulatory cows, but not cycling cows, had reduced LH pulse frequencies and increased pulse amplitudes following treatment with exogenous E_2. This indicates that both ovarian and extra-ovarian factors were inhibiting LH release in anovulatory cows, and that anovulatory cows were more sensitive to the inhibitory effects of E_2 on LH release than cycling cows.

CHAPTER 9:

Progesterone Enhances Oestradiol-Induced Oestrus And Ovulation During PPA In Dairy Cows

Abstract

Approximately 20% of seasonally calving New Zealand dairy cows have not been detected in oestrus by the PSM. These cows are likely to conceive later in the mating period, if at all, resulting in late calving in the subsequent year. They may be anovulatory or ovulating but not displaying behavioural oestrus. A lack of P_4 'priming' before the first postpartum ovulation may be responsible for a lack of display of behavioural oestrus. Additionally, techniques are required to induce ovulation and oestrus in these anovulatory cows.

Each of 96 mixed age (2 to 11 years), Friesian or Jersey cows which calved between 9 July and 26 August 1992 had either a controlled internal drug-releasing device containing 1.9 g of P_4, or a similar device that did not contain P_4, inserted intravaginally starting 14 to 20 days postpartum for 5 days. Two days after device removal, half of the cows in each group were injected with 0.6 mg of ODB and the remainder with saline. Milk samples for P_4 analysis were collected from calving until 30 days after ODB or saline treatment. Oestrus detection occurred twice daily from calving onwards. Any cow detected in oestrus between 5 October and 18 November was inseminated with commercially available semen and a bull was run with the cows from 19 November to 25 December.

Progesterone treatment increased the percentage of first postpartum ovulations accompanied by behavioural oestrus (83.3% vs. 37.0%, $P<0.001$). The length of the first postpartum luteal phase was longer in P_4-treated cows (9.5 ± 0.4 vs. 5.6 ± 0.9 days). Progesterone treatment reduced the intervals from calving to first ovulation, first oestrus and conception (30.7 ± 0.4 vs. 34.2 ± 1.0 days, 35.8 ± 2.6 vs. 40.0 ± 1.8 days and 85.0 ± 3.0 vs. 93.4 ± 2.3 days,

respectively). Oestradiol treatment reduced only the interval from calving to first ovulation (31.1 ± 0.7 vs. 33.6 ± 0.8 days).

Progesterone 'priming' increased the proportion of cows expressing behavioural oestrus and having a normal luteal phase length at first postpartum ovulation. Progesterone treatment reduced the intervals from calving to the start of cyclic activity and conception. Ovulation and oestrus occurred in 60% of early postpartum cows within two weeks of treatment. Routine, early postpartum treatment of cows 2 to 3 weeks postpartum may be beneficial to herd reproductive performance.

Introduction

Maintenance of a 365-day intercalving interval is an important target for the New Zealand dairy herd owner due to the highly seasonal nature of pasture growth. Uterine involution, the re-establishment of cyclic activity, expression of oestrus, ovulation, insemination and conception of the cow must occur by 86 days postpartum on average. Approximately 20% of cows in New Zealand herds are not detected in oestrus by the PSM, with over 60% of these animals apparently ovulating without expressing behavioural oestrus (Chapter 3). This can occur due to the failure of a farmer to detect expressed oestrus, or because a cow has ovulated without expressing overt behavioural signs of oestrus. Failure to express oestrus occurs in 70% to 90% of first postpartum ovulations (Lamming and Bulman, 1976; Boyd and Munro, 1979; van der Weil *et al.,* 1979; Fagan and Roche, 1986). The lack of prior P_4 'priming', provided by the CL of the preceding ovulation in cycling animals, may be the reason for this failure (Lamming and Bulman, 1976; Fagan and Roche, 1986). Progesterone treatment before injection with E_2 reduces the dose of E_2 required to induce oestrous behaviour in ovariectomised ewes supporting the concept of P_4 'priming' (Robinson *et al.,* 1956). In ovariectomised cows, pre-treatment with P_4 is reported to increase the dose of ODB required to induce oestrus (Carrick and Shelton, 1969) and reduce the proportion displaying behavioural oestrus (Davidge *et al.,* 1987). Progesterone 'priming' may not be required for expression of behavioural oestrus in cattle.

From 34 to 50% of the luteal phases after the first postpartum ovulation are shorter and have lower maximum P_4 concentrations than in subsequent cycles (Lamming *et al.,* 1981; Bloomfield *et al.,* 1986). Where first postpartum ovulation is induced by treatment with GnRH or human chorionic gonadotrophin (hCG) the resultant CL has a short lifespan and peripheral P_4 concentrations are low unless treatment is preceded by exogenous P_4 (Ramirez-Godinez *et al.,* 1981; Sheffel *et al.,* 1982; Troxel and Kesler, 1984). This indicates that a period of P_4 'priming' is necessary if a first postpartum CL of normal longevity and P_4 producing ability is to be produced.

Progesterone treatment alone (Kyle *et al.*, 1992; Stevenson and Pursley, 1994) or in combination with a gonadotrophin (Jubb *et al.*, 1989; Macmillan and Peterson, 1993) have been used to induce ovulation in PPA cows. Some trials have resulted in no reduction in the intervals from calving to first ovulation or to oestrus (Jubb *et al.*, 1989; Kyle *et al.*, 1992; Stevenson and Pursley, 1994), while in another a higher proportion of cows were detected in oestrus in the 2 weeks following treatment (Macmillan and Peterson, 1993). Oestradiol has been used to induce oestrus in postpartum dairy cows (Fielden *et al.*, 1973) and has been shown to induce an LH surge in a proportion of postpartum dairy cows (Nanda *et al.*, 1988). Failure of some P_4-treated cows to respond may be due to a failure of sufficient E_2 production by the large ovarian follicles (Prado *et al.*, 1990) to induce the required pre-ovulatory gonadotrophin surge and behavioural oestrus. Nine days of oral progestagen treatment followed a day later by an injection of 5 mg of EV resulted in a reduction of the intervals from calving to first ovulation and to first oestrus when compared to progestagen only or to non-treated beef cows (Brown *et al.*, 1972).

The aim of the present experiment was to determine if P_4 'priming' increased the proportion of cows exhibiting behavioural oestrus at the first postpartum ovulation and having a normal first postpartum cycle length. Additionally, the effect of P_4, ODB and a combined treatment on induction of cyclic activity and subsequent reproductive performance in postpartum dairy cows was examined.

Materials and Methods

Experiment 1

Twenty cows which had calved between 11 and 26 days previously, were stratified into 3 age groups (2, 3 and >3 years) and randomly assigned to 1 of 5 treatments. Cows were injected intramuscularly with either sterile 0.9% sodium chloride or 0.2, 0.4, 0.6 or 0.8 mg of ODB (Oestradiol-benzoate SA, Intervet, Sydney, Australia). A milk sample was collected on the day of treatment for P_4 assay. Each cow had tail-paint applied 3 days before ODB injection and the paint strip was oversprayed with an aerosol raddle at the time of injection to aid in oestrus detection (Williamson, 1980; Macmillan *et al.,* 1988). Cows were observed for oestrous behaviour three times daily (7.15 am, midday and 4.45 pm) for 3 days following injection. Any cow observed to stand to be ridden by another cow (a behaviour uniquely associated with oestrus; Williamson *et al.,* 1972; Glencross *et al.,* 1981), or which had more than 75% of its tail paint removed was defined as being in oestrus. The minimum dose found to induce oestrus in approximately 50% of cows was selected for use in the subsequent experiment.

Experiment 2

The experiment was a 2 by 2 factorial design with P_4 and ODB treatments as the factors. The trial involved 139 mixed age (2 to 11 years), Friesian and Jersey cows which had calved between 9 July and 26 August 1992 at an average weight of 434 $\pm$ 7 Kg and with a CS of 4.8 $\pm$ 0.1 (scale = 0 to 10; Macdonald and Macmillan, 1993). The cows were grazed entirely on ryegrass/white clover pasture and were offered a new area every 24 h. Milking occurred twice daily. At weekly intervals, for 7 weeks, those cows that were 14 to 20 days postpartum were stratified on the basis of age (2, 3, >3) and breed and then randomly assigned to treatment. One half of the cows were treated intravaginally with a controlled internal drug-releasing device containing 1.9 g of

P_4 (CIDR-B; InterAg, Hamilton, NZ) and the remainder with a similar device that contained no P_4 (Blank). At device insertion, the reproductive tract of each cow was palpated per-rectum. Any cow with pathology of its reproductive tract or a CL was removed from the experiment at this time. The device was removed after 5 days. Two days later, half of the cows in each of the CIDR and Blank group was injected with either 0.6 mg of ODB or 3 ml of 0.9% sodium chloride (Saline) i.m. The end of treatment was defined as the day of ODB or saline injection. Tail paint was applied on the day the device was removed and aerosol raddle was applied over the tail paint on the day of injection to aid in oestrus detection (Macmillan *et al.*, 1988). Visual observations for oestrous behaviour occurred twice daily as the cows moved to and from the milking yards for the duration of the experiment.

Milk samples (20 ml) were collected 3 times each week from calving to 4 weeks after the end of treatment. The P_4 concentration in each sample was estimated within 4 h of collection in unpreserved, whole milk samples using a RIA (Coat-A-Count Progesterone, DPC, Los Angeles, USA; Chapter 6). The sensitivity of the assay was 0.12 ± 0.02 ng/ml and the within- and between-assay coefficients of variation were 9.8% and 16.9% and 4.9% and 13.7%, respectively, for preserved milk samples with P_4 concentrations of 1.1 and 18.5 ng/ml, respectively (n = 33 assays, samples assayed in duplicate, 3 replicates within each assay). Cows which had samples of milk with a P_4 concentration of greater than 2.5 ng/ml before the start of treatment, had a CL at palpation or were detected in oestrus before the start of treatment were retrospectively removed from the experimental analysis. The first postpartum ovulation was defined as having occurred on the day that the P_4 concentration was first >2.5 ng/ml. The first postpartum luteal phase was categorised as 1 to 4, 5 to 10 and >10 days in length where the milk P_4 concentration was >2.5 ng/ml for 1 to 4, 5 to 10 and >10 days, respectively. Cows were defined as having continued to cycle after the first postpartum ovulation when a second rise in P_4 concentration to >2.5 ng/ml occurred within 10 days of the decline in P_4 concentration at the end of the first luteal phase and/or if the second postpartum oestrus was detected <24 days after the first one. In cows where the interval from the decline in P_4 from the first luteal phase to the end of the

milk sampling regime was <10 days, the continuity of cycling could not be determined so these cows were excluded from this analysis.

Any cow detected in behavioural oestrus after 5 October (PSM) and before 19 November 1992 was inseminated with semen available from a commercial source (Livestock Improvement Corporation, Hamilton, NZ). A bull was then run with the herd until 25 December 1992.

All oestrous events, inseminations and natural matings were recorded. Pregnancy status was assessed by rectal palpation between 35 and 50 days and again at greater than 120 days after the final insemination date. From these data, the intervals from calving to first oestrus, to first ovulation and to conception were calculated. The proportion of cows that were detected in behavioural oestrus before the PSM, the proportion of cows inseminated in the first 3 weeks of the mating period, the number of services per conception and the number of cows not pregnant at the end of the lactation were also calculated.

Statistical analyses

The response to treatment was categorised into four classes: (i) no ovulation or oestrus; (ii) ovulation but no detected oestrus; (iii) ovulation and oestrus; and (iv) oestrus without ovulation. Oestrus and/or ovulation were defined as having occurred if oestrus was seen within 14 days and/or the milk P_4 was greater than 2.5 ng/ml within 21 days of ODB or saline treatment. The proportion of ovulating cows (i.e. ovulation plus ovulation and oestrus categories) that were detected in oestrus was analysed by χ^2 analysis with age (2, 3, >3), breed (Friesian or Jersey), week of treatment (1 to 7) and treatment (P_4 vs. Blank and ODB vs. Saline) as factors.

The continuous data were ranked (Conover, 1980) and a general linear model constructed using week of treatment, age, breed, treatment, and the interaction between the two treatments as factors. Differences among least square means were compared by the least significant difference technique. These data are presented as raw means ± sem.

All analyses were performed using SAS (SAS Institute Inc., Cary, NC).

Results

Experiment 1

Milk P_4 concentration was >2.5 ng/ml in 3 cows on the day of the ODB injection; 1 in each of the 0, 0.2 and 0.4 mg ODB groups. None of these cows was detected in oestrus. None of the cows treated with less than 0.6 mg ODB displayed behavioural oestrus; but 50% of the 0.6 mg ODB group and 100% of the 0.8 mg ODB group did so. The 0.6 mg dose was selected for use in the subsequent experiment.

Experiment 2

Although 139 animals were originally included in the experiment, 43 (31%) were removed from the final analysis as they had commenced cycling before treatment (n = 31; 21%), had uterine or ovarian pathology at the time of rectal examination (n = 9; 6%), or they had a very poor CS (<4, n = 3; 2%) at the time of treatment. The remaining animals included 25 two year olds (26%), 16 three year olds (17%) and 55 older animals (57%), and there were 68 Friesians (71%), and 28 Jerseys (29%).

Progesterone treatment significantly increased milk P_4 concentrations during the time the device was in place (Figure 9.1). Jersey cows had higher P_4 concentrations than Friesian cows at 3 and 5 days after device insertion (5.5 ± 0.4 vs. 4.3 ± 0.3 and 3.8 ± 0.4 vs. 3.0 ± 0.2 ng/ml for day 3 and day 5, respectively, $P<0.05$). Two year old cows had higher milk P_4 concentrations than the older cows, with 3 year olds having intermediate levels while the device was in place (5.9 ± 0.5 vs. 5.2 ± 0.4 vs. 3.9 ± 0.2 ng/ml for 2, 3, >3 year olds, respectively, $P<0.05$).

There were significant differences in response among treatment groups ($\chi^2 = 19.9$, $P<0.05$; Table 9.1).

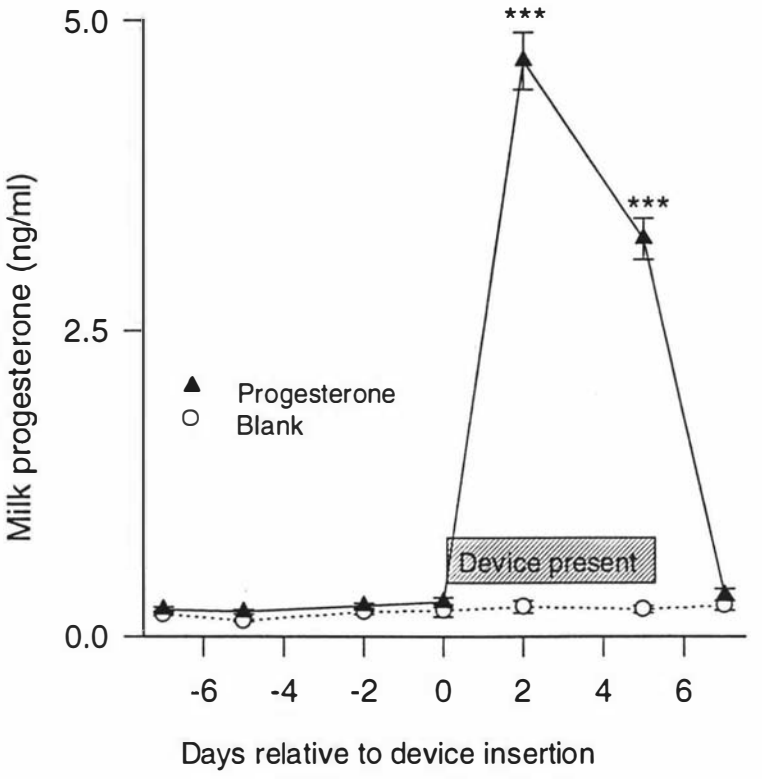

Figure 9.1. Average (± sem) milk progesterone concentrations (ng/ml) before, during and after 5 days of treatment with a CIDR device containing 1.9 g of progesterone (Progesterone, n = 49) or a blank device (Blank, n = 47).

Table 9.1. The effect of progesterone and/or oestradiol benzoate on oestrus and ovulation in anovulatory dairy cows.

Treatment	Nil[†] n (%)	Oestrus only[†] n (%)	Ovn only[†] n (%)	Oestrus + Ovn[†] n (%)	Total n
Blank Saline[‡]	6 (26.1)	4 (17.4)	8 (34.8)	5 (21.7)	23
Blank ODB[‡]	7 (29.2)	3 (12.5)	9 (37.5)	5 (20.8)	24
P_4 Saline[‡]	7 (25.9)	2 (7.4)	4 (14.3)	14 (51.9)	27
P_4 ODB[‡]	3 (13.6)	1 (4.6)	2 (9.1)	15 (72.7)	22

[†] Response within 14 days of the ODB or Saline injection coded as no oestrus or ovulation (Nil), oestrus without ovulation (Oestrus only), ovulation only (Ovn only) or oestrus and ovulation (Oestrus and Ovn)

[‡] Treatment coded as CIDR device containing progesterone (P_4) or no progesterone (Blank) followed 2 days after device removal with 0.6 mg oestradiol benzoate (ODB) or saline (Saline)

Progesterone treatment increased the proportion of ovulating cows that were detected in oestrus at first postpartum ovulation (29 of 35 (83.3%) vs. 10 of 47 (37.0%), χ^2 = 11.8, P<0.001) and Jerseys were more likely to be detected in oestrus than Friesians (17 of 22 (77.3%) vs. 23 of 41 (56.1%)).

Both P_4 and ODB treatments reduced the interval from calving to the first postpartum ovulation (30.7 ± 0.4 vs. 34.2 ± 1.0 days and 31.1 ± 0.7 vs. 33.6 ± 0.8 days for P_4 vs. Blank and ODB vs. Saline, respectively; Table 9.2). Progesterone treatment also reduced the interval from calving to first oestrus and from calving to conception (35.8 ± 2.6 vs. 40.0 ± 1.8 days and 85.0 ± 3.0 vs. 93.4 ± 2.3 days, respectively; Figure 9.2; Table 9.3) but had no significant effect on the interval from calving to first service (81.2 ± 2.1 vs. 84.7 ± 1.5 days, respectively). Oestradiol benzoate treatment had no effect on any of these intervals (Table 9.3). Jerseys had shorter intervals than Friesians from calving to first ovulation and from calving to first oestrus (30.9 ± 0.8 vs. 33.1 ± 0.7 and 32.1 ± 2.3 vs. 40.2 ± 2.0 days, respectively). Two year old animals had a longer interval from calving to first service than 3 year old and older cows (91.2 ± 2.3 vs. 78.4 ± 2.8 vs. 80.5 ± 1.6 days for 2, 3 and >3 years olds, respectively). This was partially due to the earlier mean calving date for the 2 year olds (18 July ± 2.1 vs. 25 July ± 2.9 vs. 25 July ± 1.3 for 2, 3 and >3 years olds, respectively).

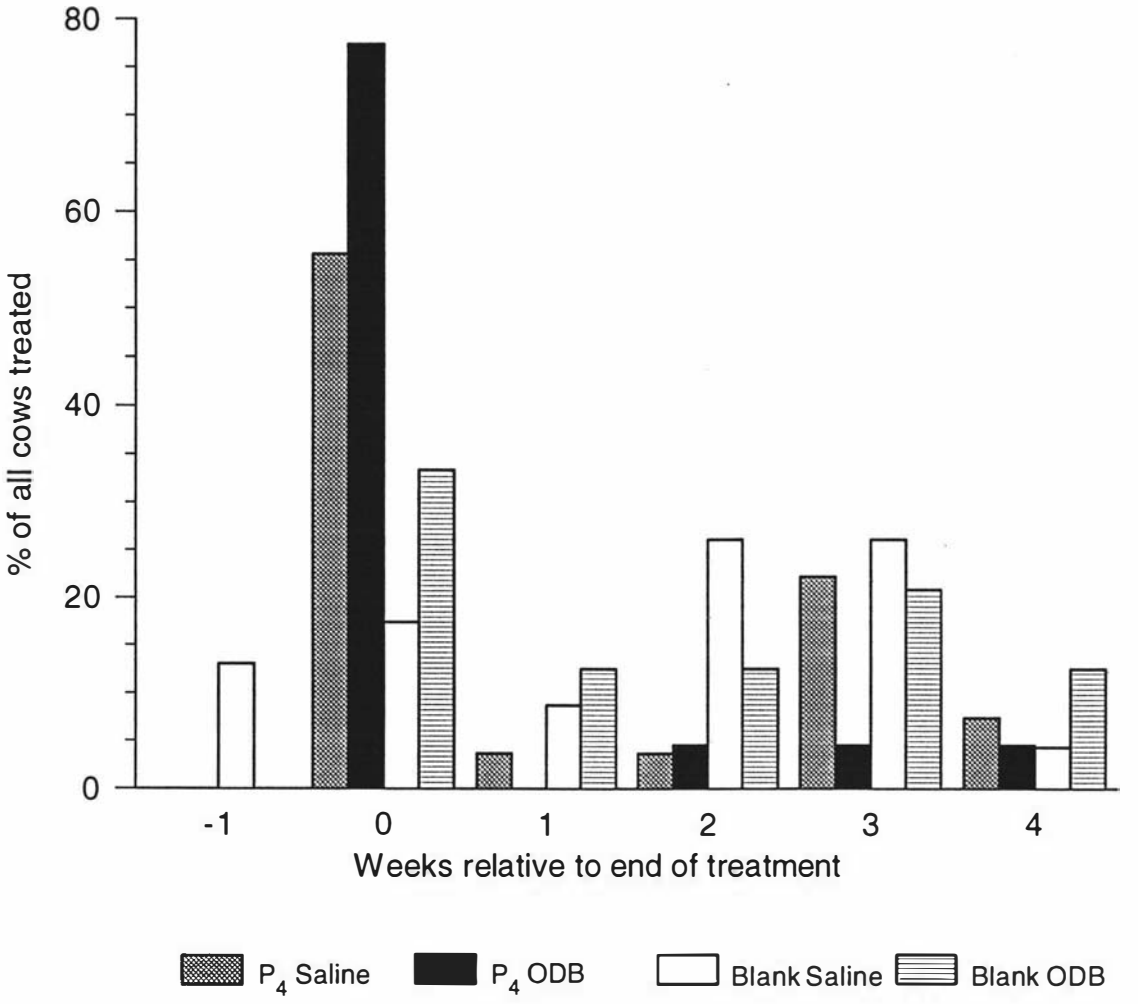

Figure 9.2. The distribution of intervals from the end of treatment to first detected oestrus for cows treated with either a CIDR device containing 1.9 g of progesterone and saline (P_4 Saline) or a CIDR device containing 1.9 g of progesterone and an injection of 0.6 mg of oestradiol-benzoate (P_4 ODB), a Blank device and saline (Blank Saline) and a Blank device and an injection of 0.6 mg of oestradiol-benzoate (Blank ODB).

Table 9.2. The effect of progesterone and/or oestradiol benzoate on the intervals from calving to end of treatment and to first ovulation and the duration of the first luteal phase.

	Calving-treatment¿			C_ovn1¿			Dur P_4¿		
	n	mean	sem	n	mean	sem	n	mean	sem
Blank Saline†	23	23.9	0.4	17	35.8^{b}	1.3	15	6.0^{ab}	1.2
Blank ODB†	24	23.8	0.4	16	32.6^{a}	1.4	13	5.2^{a}	1.3
P_4 Saline†	27	24.4	0.4	18	31.6^{ab}	0.5	18	10.5^{c}	0.5
P_4 ODB†	22	23.0	0.6	18	29.8^{a}	0.5	18	8.8^{bc}	0.6
Rx week‡		ns£			ns			ns	
Age¶		ns			ns			ns	
Breed§		ns			*			ns	
P_4 vs. Blank		ns			*			***	
ODB vs. Saline		ns			*			*	

¿ The intervals (days) from calving to the end of treatment (Calving-treatment; i.e. the day of ODB or saline injection), from calving to first ovulation (C_ovn1; i.e. 5 days before first P_4 concentration of >2.5 ng/ml) and the number of days that the P_4 concentration was >2.5 ng/ml following first ovulation (Dur P_4).

† Treatment coded as CIDR device containing progesterone (P_4) or no progesterone (Blank) followed 2 days after device removal with 0.6 mg oestradiol benzoate (ODB) or saline (Saline)

£ ns *, **, *** = not significant, P<0.05, P<0.01 and P<0.001; respectively

abc Means within a column with different superscripts differ at P<0.05

‡ Week in which treatment was commenced (1 = 15 July and 7 = 26 Aug. 1992), ¶ coded as (2, 3 and >3 years); § Friesian or Jersey

Progesterone treatment lengthened the first postpartum luteal phase (9.5 ± 0.4 vs. 5.6 ± 0.9 days, respectively; Table 9.2) and there was a greater proportion of P_4-treated cows that had first postpartum luteal phases of >5 and >10 days (χ^2 = 16.6, P<0.001, df = 2; Figure 9.3). Oestradiol treatment did not effect the length of the first luteal phase (7.3 ± 0.7 vs. 8.3 ± 0.7 days, respectively). More P_4-treated cows continued cycling after treatment than Blank-treated cows (42 of 46 (91.3%) vs. 36 of 47 (76.6%), respectively, χ^2 = 3.7, P = 0.05), but ODB treatment had no effect on this proportion (34 of 43 (79.1%) vs. 44 of 50 (88%), respectively).

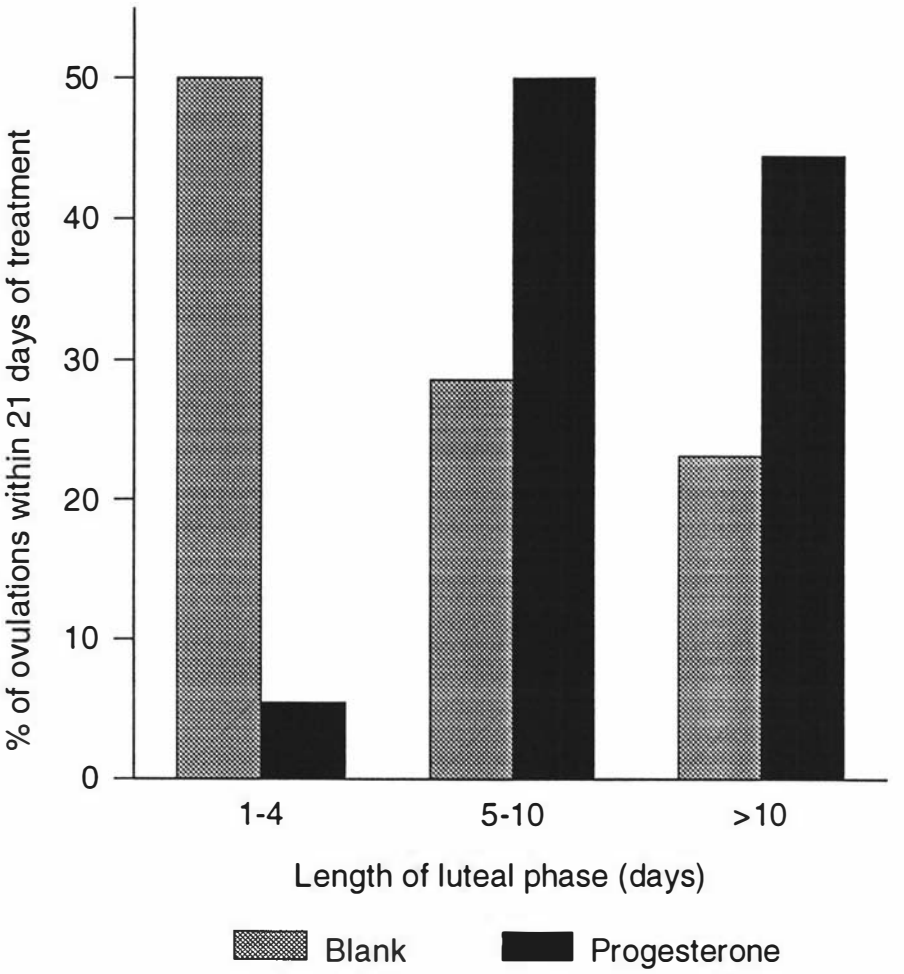

Figure 9.3. The length of the first postpartum luteal phase (i.e. days during which P_4 >2.5 ng/ml) following treatment with a CIDR-B device containing 1.9 g of progesterone (Progesterone) or a blank (Blank) device for cows ovulating within 21 days of the end of treatment.

Table 9.3. The effect of progesterone and/or oestradiol benzoate on the intervals from calving to first oestrus, to first service and to conception.

	C_h1¿			C_s1¿			C_conception¿		
	n	mean	sem	n	mean	sem	n	mean	sem
Blank Saline†	23	39.8[b]	2.5	23	85.1	2.3	22	93.0	3.7
Blank ODB†	24	40.2[b]	2.7	24	84.4	1.9	23	93.9	2.9
P_4 Saline†	27	37.5[ab]	2.8	27	82.7	2.5	25	87.3	4.4
P_4 ODB†	22	33.6[a]	4.8	22	79.4	3.5	20	82.1	3.9
Rx week‡		ns£			***			***	
Age¶		ns			*			ns	
Breed§		*			ns			ns	
P_4 vs. Blank		**			ns			*	
ODB vs. Saline		ns			ns			ns	

¿ The intervals (days) from calving to first oestrus (C_h1), from calving to first service (C_s1) and calving to conception (C_conception)

† Treatment coded as CIDR device containing progesterone (P_4) or no progesterone (Blank) followed 2 days after device removal with 0.6 mg oestradiol benzoate (ODB) or saline (Saline)

£ ns *, **, *** = not significant, P<0.05, P<0.01 and P<0.001; respectively

[abc] Indicates significant differences (P<0.05) among means within the column

‡ Week in which treatment was commenced (1 = 15 July and 7 = 26 Aug. 1992), ¶ coded as (2, 3 and >3 years); § Friesian or Jersey

Treatment, age, breed or week of treatment did not effect the proportion of cows detected in oestrus before the PSM (96.9%), the proportion of animals inseminated over the first 3 weeks of mating (92.7%), the number of non-pregnant animals following mating (6.3%) or the number of inseminations (72.9% of cows had 1 service, 23.9% had 2 services and 3.1% had 3 or more services; Table 9.4) among treatments. First service conception rate did not differ among treatments (70.8%; Table 9.4) or breeds, but there was a tendency for 2 year old cows to have higher first service conception rates than older cows (88.0% vs. 56.3% vs. 67.3% for 2, 3 and >3 year old cows, respectively, $\chi^2 = 5.5$, P = 0.06).

Table 9.4. The effect of progesterone and/or oestradiol benzoate on mating performance of anovulatory dairy cows.

	PMH¿		3-week sub¿		# insem¿			CR_s1¿		Not-preg¿	
	n	%	n	%	1	2	>2	n	%	n	%
Blank Saline‡	23	100	20	87.0	69.6	17.4	13.0	16	69.6	1	4.4
Blank ODB‡	24	100	23	95.8	58.3	29.2	12.5	14	58.3	1	4.2
P_4 Saline‡	27	100	26	96.3	85.2	11.1	3.7	23	85.2	2	7.4
P_4 ODB‡	19	86	20	90.1	72.7	27.3	0.0	15	68.2	2	9.1

¿ The number and percentage of cows detected in oestrus before the PSM (PMH), inseminated in the first 3 weeks of the mating period (3-week sub), the number of inseminations (# insem), the conception rate to first service (CR_s1) and not pregnant at the end of the mating period (Not-preg)

‡ Treatment coded as CIDR device containing progesterone (P_4) or no progesterone (Blank) followed 2 days after device removal with 0.6 mg oestradiol benzoate (ODB) or saline (Saline)

Discussion

Five day intravaginal treatment with P_4 reduced the intervals from calving to first postpartum ovulation, to first oestrus and to conception in dairy cows where treatment commenced 14 to 20 days after calving. An injection of 0.6 mg of ODB, 2 days after P_4 removal, reduced the postpartum interval to ovulation but did not effect the other intervals. More cows treated with P_4 were detected in oestrus at their first postpartum ovulation and the length of the first postpartum luteal phase was longer.

Progesterone 'priming' increased the proportion of ovulating cows expressing behavioural oestrus (83.4% vs. 37.0% for controls). Similar effects with P_4 'priming' have been demonstrated in sheep (Robinson *et al.*, 1956), but have not been previously demonstrated in cattle (Carrick and Shelton, 1969; Davidge *et al.*, 1987; Allrich *et al.*, 1989). Differences in the dose of P_4, route of delivery and vehicle for the P_4 may have contributed to the differences in results. Where P_4 was injected in an oily vehicle (Ulberg *et al.*, 1951; Melampy *et al.*, 1957; Davidge *et al.*, 1987), prolonged release may have occurred resulting in elevated P_4 concentrations at the time of the ODB injection. Plasma P_4 concentrations of greater than 0.5 ng/ml can block an ODB-induced oestrus (Nanda *et al.*, 1988).

Progesterone treatment in the present study reduced the postpartum intervals to ovulation, to oestrus and to conception. These results contrast with the studies of Kyle *et al.*, (1992) and Stevenson and Pursley (1994) using well-fed, North American cows that had intervals from calving to first ovulation of 25 to 30 days. In the present experiment, the control cows ovulated at 35.8 days postpartum (Table 9.2). Additionally, in both of the previous experiments, the plasma P_4 concentrations were between 0.5 and 2 ng/ml during the time of treatment. In the present experiment milk P_4 concentrations of nearly 5 ng/ml occurred during P_4 treatment. These differences in P_4 concentration may be associated with differences in the type of device used for P_4 administration (PRID vs. CIDR), the fluid in which the P_4 concentration was determined (serum vs. milk), the weight of the cows or the metabolic status of the cows associated with differences in milk production and feed intake. For example, increasing daily feed intake of sheep reduced plasma P_4 concentrations which was associated with increases in hepatic clearance rate (Parr *et al.*, 1993). Additionally, in the present experiment, treatments were applied on a weekly basis. This meant that there was a group of cows coming into oestrus at the same time, so that a sexually active group could form (Kilgour *et al.,* 1977) aiding in detection of oestrus.

Oestradiol benzoate treatment reduced the interval from calving to first postpartum ovulation by 2.5 days (31.1 vs. 33.6 days). This was most likely due to the ODB treatment eliciting an LH surge and hence ovulation in a proportion of animals (Nanda *et al.*, 1988). Injecting ODB following P_4 treatment produced the highest percentage of cows detected in oestrus (77.3%) and ovulating (81.8%) in the 2 weeks following treatment (Table 9.1) and the shortest intervals from calving to first ovulation (29.8 days; Table 9.2), to oestrus and to conception (33.6 and 82.1 days, respectively; Table 9.3). However, these changes were not statistically significant from P_4 treatment alone. A difference may be demonstrated between P_4 alone and P_4 and ODB treatments with larger numbers.

The ODB treatment was included in an attempt to induce both a behavioural oestrus and an LH surge sufficient to induce ovulation. However, P_4 and/or ODB treatment failed to induce ovulation (18.0%, 33.3% and 41.7%

for P_4 and ODB, for P_4 alone and ODB alone, respectively; Table 9.1) and oestrus (22.7%, 40.7% and 66.7% for P_4 and ODB, for P_4 alone and ODB alone, respectively; Table 9.1) in some cows. These failures may have occurred for a variety of reasons. An inadequate LH surge may have occurred as ODB fails to induce a surge in some postpartum cows (Smith *et al* 1981; Nanda *et al.*, 1988). Roche *et al.*, (1981) showed that although every cow had an LH surge following an exogenous P_4 treatment, only a proportion ovulated. Those that did not ovulate had a lower basal LH concentration preceding the LH surge than those that did ovulate. The authors suggested that inadequate follicle development due to insufficient gonadotrophin support, resulted in a failure of ovulation. Since follicles develop in a series of waves in the postpartum period (Savio *et al.*, 1990; Murphy *et al.*, 1990; Chapter 4), treatment at a stage of development where the follicle is unable to respond to the LH surge may also occur. Treatment with ODB (0.5 mg) when the largest follicle was in its growing phase, but only 8 mm in diameter, resulted in fewer LH surges and a lower proportion of ovulations than if the same treatment was given when the largest follicle was >10 mm in diameter and had ceased growing (Chapter 7). In the current trial, the failure of response may have been due to a failure of the LH surge or due to factors related to stage of follicle development and/or maturity.

The first postpartum luteal phase was longer in P_4-treated cows (9.7 vs. 5.7 days; Table 9.2). This agrees with previous studies in postpartum beef cows (Ramirez-Godinez *et al.,* 1981; Sheffel *et al.,* 1982; Troxel and Kesler, 1984). Five days of P_4 treatment in the present experiment was sufficient to produce behavioural oestrus at the majority of first postpartum ovulations and to induce luteal phases of normal length. The mechanism of this P_4 effect on luteal phase length is not known. The effect may be mediated by: (a) altering the hypothalamic or pituitary response to positive feedback by ODB or GnRH, respectively, consequently increasing gonadotrophin release; (b) modifying ovarian function to increase the production of endogenous ODB, increase the number of LH receptors in the pre-ovulatory follicle, or alter the pattern of follicle turnover such that more animals had a follicle at the appropriate stage of development which could ovulate in response to the LH surge; (c) preventing

premature luteolysis of the subsequently formed CL; or, (d) a combination of all of these effects (reviewed by Lishman and Inskeep, 1991).

Treatment with P_4 before the first postpartum ovulation increased the proportion of cows exhibiting behavioural oestrus at that ovulation and resulted in a higher proportion of luteal phases that were >10 days in duration. This hormone both 'primes' ODB-induced behavioural oestrus and increases the length of the luteal phase in the postpartum dairy cow. Progesterone treatment commencing between 14 and 20 days postpartum resulted in the earlier onset of ovulatory activity and a shorter interval from calving to conception. A P_4 treatment either with or without ODB treatment, induced ovulation in 77.3% and 66.7% of anovulatory cows, respectively (Table 9.1).

The application of the principle of P_4 'priming' demonstrated by this study in the reproductive management of dairy herds in New Zealand may reduce the adverse effects of anovulatory anoestrum by increasing submission rates and allowing the seasonally concentrated calving patterns to be maintained. The potentially beneficial effects of injecting ODB following P_4 'priming' justify further investigation.

CHAPTER 10:

Progesterone Treatment Followed By Equine Chorionic Gonadotrophin Shortens The Intervals To First Service And Conception In Pasture-Fed Anoestrous Dairy Cows

Abstract

Postpartum anoestrus represents a cost to herd owners due to the expenses of diagnosis, increased culling of non-pregnant cows, higher rates of induced calving and loss of production due to delayed calving in the subsequent year.

Treatment of anoestrum with P_4 and eCG has been previously reported, but with variable success. This trial examined the response to this treatment and identified factors influencing this response.

Anovulatory cows in each of 8 herds had their CS estimated, blood samples taken for analysis of a range of metabolites and had their reproductive tracts palpated per-rectum at 2 to 6 days before the planned start of mating. Cows without a CL or reproductive tract pathology were randomly assigned to two groups (Round 1). Those in the first group were treated with P_4 for 7 days using an intravaginal device and with 400 i.u. of eCG injected i.m. at device removal. The second group were treated with a blank (Nil) device for 7 days and acted as controls. Device removal occurred from 1 day before to 5 days after the PSM. Twenty-one days after device insertion, the ovaries of any cow not inseminated were re-examined and cows without a CL were re-randomised and re-treated (Round 2). A further 21 days later the process was repeated again (Round 3). A total of 172 cows were treated (85 P_4 eCG and 87 Nil) at Round 1, 48 at Round 2 and 13 at Round 3.

Cows-treated in Round 1 with P_4 and eCG were more likely to be inseminated ($P<0.05$) and conceive ($P<0.001$) than control cows. Those in lower CS (≤ 3.5) were less likely to be inseminated ($P<0.05$) and conceive than

cows in higher CS (>3.5) in Round 1. Age and breed of cow had no effect on the likelihood of service or conception. Higher serum copper concentration at the time of treatment in Round 1 was associated with a higher likelihood of conception, but no other measured metabolite was associated with reproductive performance. The interaction of CS and Round 1 treatment was not significantly associated with reproductive performance, indicating that the relative response to P_4 and eCG treatment was the same for high and low CS cows.

The probability of insemination and conception was increased by treating anovulatory cows with P_4 and eCG. Poor CS (≤3.5) delayed this response, but treatment of these animals still resulted in an increased probability of insemination and conception.

Introduction

Over 14% of pasture-fed spring-calving cows in New Zealand are not detected in oestrus and inseminated by 4 weeks into the seasonal mating period (Fielden *et al.*, 1973). These anoestrous cows impose diagnostic, treatment and replacement stock costs on the farmer.

Treatment of anoestrous cows with P_4 and eCG (Macmillan and Day, 1987), or a progestagen and EV (Galloway *et al.*, 1987) has resulted in an increased proportion of cows being inseminated and conceiving in the 14 days following treatment. In another trial using P_4 and eCG, no changes occurred in the percentage of cows in oestrus within 14 days of treatment, the treatment to conception interval or the first service conception rate (Jubb *et al.,* 1989). Even where a beneficial effect of P_4 and eCG treatment was demonstrated, 15% to 25% of treated cows were not detected in oestrus within 7 to 14 days of treatment (Macmillan and Day, 1987; Macmillan and Peterson, 1993). Factors affecting the response of anoestrous cows to hormonal induction of ovulation and oestrus have not been investigated. Extended postpartum periods of NEB (Butler *et al.*, 1981; Staples *et al.*, 1990) and factors associated with NEB such as low body CS (Grainger *et al.*, 1982), low blood glucose concentration

(McClure, 1970; Parker and Blowey, 1976), elevated NEFA concentrations, low glucose and insulin concentrations (Canfield and Butler, 1991) have been associated with extended periods of PPA. It was hypothesised that NEB and associated factors may alter the response to exogenous P_4 and eCG treatment.

The aim of the present trial was to assess the effect of P_4 and eCG treatment on the likelihood of insemination and conception. Factors that may modify the response to this treatment, such as body CS and concentrations of some blood metabolites, were also examined.

Materials and Methods

Cows (n = 1596) from 8 herds (range of 85 to 365 cows/herd) of the Central Waikato region were selected for use in this experiment if the herd managers were willing to collect the required reproductive data. Cows were observed for signs of behavioural oestrus twice daily, starting at least 4 weeks (44.0 ± 5.5 days, mean ± sem, herds range = 28 to 63 days) before the PSM. Cows not detected in behavioural oestrus by three weeks before the PSM had blood samples (10 ml) drawn from the ventral coccygeal vessels into a plain evacuated glass tube (Vacutainer, Salmond Smith Biolab, Auckland, New Zealand) at weekly intervals for 3 weeks. The blood was allowed to clot at room temperature, centrifuged at 2500 g and the serum stored at -20 ^{o}C for subsequent P_4 concentration determination.

The breed, age, calving dates, and oestrous data were collected for all cows, 10 days before the PSM. At this time, cows that had not been detected in oestrus were blocked by herd, age (2, 3 or >3 years old) and breed (Friesian, Jersey or Friesian Jersey crossbred) into two treatment groups. Between 2 and 8 days before the PSM (6.1 ± 0.8 days), all anoestrous cows had their body CS estimated (1 = thin to 10 = fat scale; Macdonald and Macmillan, 1993) and blood samples (10 ml sera, 10 ml EDTA plasma) were drawn for analysis of a range of blood metabolites and minerals.

The reproductive tract of each cow was also examined by rectal palpation by 1 of 3 experienced veterinarians. Ovarian size was assessed on a subjective scale (1 = small, 5 = large; Morris and Day, 1994), the presence of a follicle and/or of a CL was determined and any palpable reproductive tract pathology noted. Those with reproductive tract pathology or a CL were excluded at this time. Half of the retained cows each had an intravaginal device containing 1.9 g of P_4 (CIDR-B, InterAg, Hamilton, New Zealand) inserted for 7 days and then an i.m. injection of 400 i.u. of eCG (Pregnecol, Horizon Animal Health, Australia) at the time of device removal. The other half each had a CIDR device which did not contain P_4 (Nil; Round 1) inserted for 7 days. Tailpaint was applied to all cows at device insertion and spray raddle was applied over the tailpaint at device removal to aid in oestrus detection (Macmillan *et al.*, 1988) in addition to the twice daily observations for oestrous behaviour. Oestrus was regarded as having occurred if a cow was seen to stand to be ridden (Williamson *et al.*, 1972), or had greater than 50% of its tail paint removed. Any cow detected in oestrus between the PSM and the end of the artificial insemination (AI) period (37.1 ± 3.1 days; range = 29 to 51 days) was inseminated by an experienced technician using commercially available semen. Following the end of the AI period, bulls were run with each herd for an average of 59.1 ± 7.0 days (range = 39 to 81 days) to produce a total period of mating of 98.9 ± 4.4 days (range = 79 to 112 days). The dates of all inseminations and natural matings were recorded by farm staff.

The reproductive tract of any cow not inseminated by 14 days after device removal, was again examined per rectum. If a CL was detected, a $PGF_{2\alpha}$ analogue was administered i.m. (25 mg Lutalyse, Upjohn, Auckland, New Zealand). Cows not detected as having a CL were then re-randomised and treated with either a device containing P_4 and eCG, a similar device but with a capsule containing 10 mg of ODB (Cidirol, Douglas Pharmaceuticals Ltd., Auckland, New Zealand) placed in a groove on the device at the time of device insertion and with no eCG, or with a blank device (Round 2). A further 21 days later, cows not detected in oestrus were re-examined and treated with P_4 and eCG, P_4 and ODB, $PGF_{2\alpha}$ or a blank device as in Round 2 (Round 3). Pregnancy status was determined by rectal palpation of the reproductive tract

between 35 and 50 days after the last insemination and again between 3 and 6 months after the last recorded service.

Laboratory analyses

Selected metabolite and hormone concentrations were determined at the Ruakura Animal Health Laboratory (Hamilton, New Zealand) using a Hitachi 717 auto-analyser (BOH; glucose, NEFA; urea); by nitric/perchloric acid digestion followed by atomic absorbence spectrophotometry (serum copper, serum selenium) or by solid phase radioimmunoassay (total thyroxine, Quanticoat, Kallestad diagnostics, Chaska, Mn, USA). The within- and between-assay coefficients of variation were <5% for all tests.

Serum P_4 concentrations were determined using a solid phase I^{125} radioimmunoassay (Coat-a-Count, DPC, Los Angeles, Calif., USA). The sensitivity of the assay was <0.2 ng/ml and the within- and between-assay coefficients of variation were 13.0% and 10.9% and 14.7% and 16.6% for pooled samples with mean concentrations of 3.9 and 1.7 ng/ml of P_4, respectively, analysed in sextuplet in each of 4 assays. Any cow with a P_4 concentration of >1 ng/ml at any of the weekly pre-treatment samplings was retrospectively removed from the analysis. This left a final population of anovulatory anoestrous (AA) cows that had not been detected in oestrus, had no CL or reproductive tract pathology and which had no elevation in P_4 before the time of treatment.

Statistical analyses

The proportion of cows within a herd presented for veterinary examination as NDO and eventually diagnosed as AA were analysed by χ^2 analyses.

The intervals from the commencement of treatment to first service and to conception were analysed in two ways:

(1) by non-parametric survival analysis using a product limit method (Lifetest), with treatment and CS (coded as low (≤3.5) or medium (>3.5)) as the main effects. These data are presented as survival curves; and

(2) using a Coxs' proportional hazards regression procedure (Phreg). Data were coded by treatment, age (2, 3 >3 years), breed (Friesian, Jersey or crossbred), CS at treatment (≤3.5 or >3.5), and the presence of a follicle (0, 1). These coded variables and the metabolite concentrations, the CS by Round 1 treatment interaction, days postpartum at Round 1 treatment and ovarian size score were included in the analyses. Stepwise regression analyses were then run with all variables initially included with an acceptance criterion of $P<0.05$. These data are represented as the relative risk of a treatment or variable influencing the outcome (e.g. a relative risk of 1.00 indicates no effect).

Data were analysed independently for Round 1 and 2, but the number animals treated in Round 3 was too small to be analysed. The data was right-censored at 21 days (Round 1) and 42 days (Round 2) so that no confounding of subsequent treatment on outcomes occurred. Cows with a CL and treated with $PGF_{2\alpha}$ at Round 2 were removed from analysis as they were cycling rather than PPA cows. No difference in Round 2 response between cows treated with either P_4 and eCG or a Blank device in Round 1 was found, so data from these 2 groups was combined for analysis of Round 2 results.

All data analyses were performed in SAS for Windows, version 6, (SAS, SAS Institute Inc., SAS Campus drive, Cary, NC).

Results

From the 1596 cows available, 460 (28.8%) had not been detected in behavioural oestrus by 1 week before the PSM. From this group, 172 (11.2%) were finally selected as AA cows and treated (n = 87 Nil treatment; 85 P_4 eCG

Table 10.1. The number (and percentage) of cows not observed in oestrus and the number with anovulatory anoestrus in 8 herds.

		NDO[¶]		AA[†]	
Herd	size	n	(%)	n	(%)
1	355	135	38.0	39	11.0
2	170	14	8.2	11	6.5
3	103	35	34.0	14	13.6
4	218	67	30.7	35	16.1
5	95	34	35.8	14	14.7
6	226	41	18.1	14	6.2
7	146	43	29.5	17	11.6
8	283	91	32.2	28	9.9
mean*	199.5	57.5	28.3	21.5	11.2
sem			3.6		1.3

* Mean and standard error of the mean (sem) of the herd values
[¶] Not detected in oestrus by 1 week before the planned start of mating
[†] Anovulatory anoestrus

treated; Table 10.1, Table 10.2). There were significant differences among herds in the proportion of cows not detected in oestrus (χ^2 = 67.9, P<0.001) and the proportion of the herd that was AA (χ^2 = 17.3, P<0.05). Forty-eight cows (27.5% of those cows treated in Round 1) were treated in Round 2, and 13 cows (7.6% of those cows treated in Round 1) were treated in Round 3 (Table 10.2).

Cows treated with P_4 and eCG were more likely to be inseminated (relative risk = 1.63, P<0.05; Figure 10.1) and to conceive (relative risk = 2.83, P<0.001; Figure 10.1) than control cows, where treatment occurred before the PSM (Round 1). However, P_4 and eCG treatment had no effect on the probability that cows would be inseminated or conceive in Round 2 (Figure 10.1). Cows with low CS (≤3.5) were less likely to be inseminated (relative risk = 0.48, P<0.01; Figure 10.1) or to conceive (relative risk = 0.26, P<0.01; Figure 10.1) than cows with higher CS (>3.5) in Round 1, but not in Round 2. There

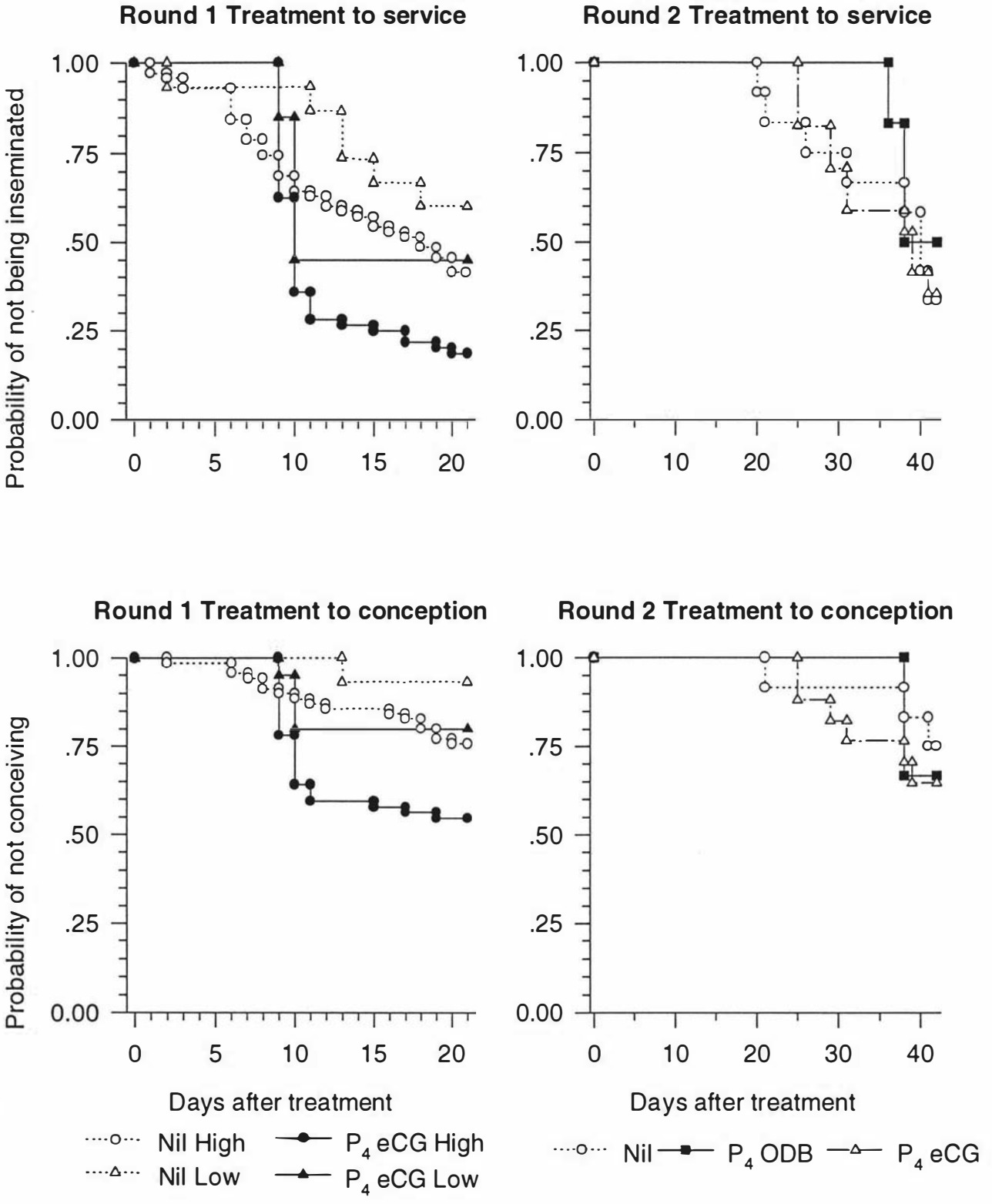

Figure 10.1. The probability of insemination and conception following treatment before (Round 1) or approximately 2 weeks after (Round 2) the PSM for cows treated with either P_4 and eCG, P_4 and ODB or a blank device (Nil). Cows were retrospectively categorised by condition score (≤3.5; Low or >3.5; High) at device insertion.

was no interaction between Round 1 treatment and CS on the probability of insemination or conception. Increased serum copper concentration was associated with an increased likelihood of conception following Round 1 treatment (relative risk = 1.12, P<0.001).

Table 10.2. The type of treatment and the number of anovulatory anoestrous cows in each treatment.

Round 1[†]		Round 2[†]		Round 3[†]	
Rx	(n)	Rx	(n)	Rx	(n)
Nil[¶]	(87)	Nil	(11)	Nil	(1)
"		"		P_4 eCG	(2)
"		PG[‡]	(8)	-	
"		P_4 eCG	(10)	PG	(3)
"		"		P_4 eCG	(1)
"		P_4 ODB ~	(4)	-	
P_4 eCG[^]	(85)	Nil	(1)	-	
"		PG	(5)	PG	(2)
"		"		P_4 eCG	(1)
"		P_4 eCG	(7)	PG	(1)
"		"		P_4 eCG	(2)
"		P_4 ODB	(2)	-	
Total	(172)		(48)		(13)

[†] Treatments occurring approximately 1 week before (Round 1) and 2 (Round 2) and 5 (Round 3) weeks after the planned start of mating

[¶] Blank CIDR device (Nil) for 7 days [^] CIDR device containing P_4 for 7 days with injection of 400 i.u. eCG at device removal [‡] 25 mg of synthetic $PGF_{2\alpha}$ ~ CIDR device containing P_4 for 7 days with inclusion of 10 mg of ODB at device insertion (P_4 ODB)

Discussion

Herds varied in the proportions of cows that were not detected in oestrus and that were AA. Large between herd differences in reproductive performance have been previously reported (Francos and Mayer, 1988). Differences in herd size, breed and age composition, timing of calving and mating and nutritional and general management may account for some of these differences (reviewed by de Kruif, 1978). A larger number of herds would be required to assess the associations producing the observed differences.

Treatment of AA cows with P_4 and eCG before PSM increased the probability that a cow would be inseminated and conceive within 3 weeks. A previous trial using a similar hormonal treatment (Jubb *et al.*, 1989), but in which treatment commenced after the start of mating, did not demonstrate a difference among P_4 and eCG-treated and untreated AA cows. This may have been due to the inhibition of expression of behavioural oestrus and ovulation which occurs where the P_4 concentration is >0.5 ng/ml (Nanda *et al.*, 1988) as would have occurred while the CIDR device was in place (Macmillan and Peterson, 1993). Thus, insemination and conception in the cows treated with P_4 and eCG during the mating period, may have been delayed relative to non-treated cows which could have been inseminated and conceived in this period. In the present trial, device removal was timed to occur soon after PSM, to minimise the time in the mating period when ovulation was inhibited. Any Round 1, P_4 and eCG-treated cow that would have spontaneously resumed ovulatory activity during treatment in the week before the start of mating was prevented from doing so until the mating period.

Cows with low CS were less likely to be inseminated or conceive. Low CS at the time of treatment may occur due to poor CS at the time of calving or due to excessive postpartum loss of CS. Condition score, both at calving (Grainger *et al.*, 1982) and just before mating (Chapter 3) is also negatively related to the PPA interval. The interactions between CS and treatment in Round 1 were not significant indicating that P_4 and eCG treatment can increase

the likelihood of cows with low and medium CS being inseminated and conceiving.

The likelihood of conception was increased in cows with higher serum copper concentrations. Copper concentration has been reported not to be correlated with measures of reproductive performance in some studies (Parker and Blowey, 1976; Larson *et al.*, 1980), but in another study it was found to be higher in cows conceiving earlier postpartum than those conceiving later postpartum (Kappel *et al.*, 1984). Copper is a component of enzymes with a wide range of activities including metabolism of energy, peroxide, connective tissue, amino acid, vitamins and lipids (Graham, 1991) and so may influence reproductive performance in a variety of ways. The range of serum copper values in this study was 2.2 to 23.0 μmol/L and 10.3% of the sampled animals fell below the recommended (Animal Health Laboratory, Ruakura) lower limit of 8 μmol/L. The first service conception rate of this group was 26.7% compared to 50.4% for those cows having normal or above normal serum copper concentrations (χ^2 = 3.0, df = 2, P = 0.07). Ensuring that dairy cows have serum copper concentrations within the normal range before the start of mating may improve reproductive performance.

The age (2, 3, >3 years), breed (Friesian, Jersey or crossbred) and time postpartum were initially included in all models, but none was included in the final models. This suggests that none of these factors affects the response to treatment.

Treatment of anovulatory cows with P_4 and eCG increased the likelihood of insemination and conception when treatment commenced before the PSM. Although low CS reduces the likelihood of insemination and conception, it was beneficial to treat these animals with P_4 and eCG. Low concentrations of serum copper were associated with a reduced likelihood of conception.

CHAPTER 11:

Some Effects Of Feeding Pasture Silage As A Supplement To Pasture On Reproductive Performance In Lactating Dairy Cows

Abstract

Supplementation of a ryegrass/white clover pasture diet with pasture silage is a common management practice in New Zealand dairy herds. The effect of this supplementation on reproductive performance has not been investigated. Five herds of 20 cows were formed before calving commenced on 1 June 1992. From 5 August to 4 September, two of these herds were fed 5 kg of dry matter/cow/day of pasture silage in addition to the ryegrass/white clover pasture offered to all herds. Pasture silage supplementation did not alter the intervals from calving to first ovulation, first oestrus, or conception. However, it reduced the first service conception rate (37.5% vs. 53.3%; difference and SED = 15.8 ± 10.0%, for cows fed pasture and silage and pasture only, respectively).

A positive effect on reproductive performance of pasture silage supplementation was not demonstrated in this trial.

Introduction

The interactions involving feed intake, milk production, body condition and reproduction are complex. A cow's nutritional requirements for milk production and maintenance exceed the nutrients supplied by feed intake in the early postpartum period, leading to mobilisation of body tissue reserves. This has been described as a period of NEB (Butler *et al.*, 1981). The degree and duration of this NEB influence the interval from calving to the resumption of cyclic ovarian activity (Butler *et al.*, 1981; Staples *et al.*, 1990; Lucy *et al.*, 1992). Increases in the quantity and quality of feed in the early postpartum period may reduce the depth or duration of NEB and hence reduce the interval

to resumption of cyclicity. This should increase the number of oestrus events before the start of the mating period and increase conception rates (Thatcher and Wilcox, 1973). However, supplementary feeding may also increase milk production (Broster *et al.,* 1969) and consequently increase NEB (Lucy *et al.,* 1992). Beef heifers, with moderate milk production, had increased conception rates when fed high levels of energy after calving (Dunn *et al.,* 1969). In contrast, dairy heifers or cows with high milk production which were fed high energy supplements had increased production, but reduced conception rates and prolonged calving to conception intervals (Ducker *et al.,* 1985; Lucy *et al.,* 1992). The factors that influence how nutrients are partitioned to milk production or to increasing body reserves are not fully understood. The genetic potential of the cow, previous feeding history, production levels before the feeding of supplements and body composition at the time of supplementation may all influence this partitioning (Broster *et al.,* 1969).

This trial investigated the effects on reproductive performance of providing pasture silage in addition to pasture, in sufficient quantities to increase milk production, body CS and liveweight.

Materials and Methods

Five herds, each comprising 18 Friesian and two Jersey cows were established on 1 June 1992 (Clark, 1993). The herds were balanced for breed, age, breeding index and expected calving date. The mean (± sem) calving date was 28 July (± 1.6; range 22 June to 25 August) and did not differ among herds.

Two herds were fed 5 kg dry matter (DM)/cow of moderate quality pasture silage (estimated ME = 10.8 MJ/kg DM and crude protein = 13.3%) daily for 30 days from 5 August, in addition to ryegrass/white clover pasture. The pasture silage was placed on the ground around the periphery of the pastures before grazing. The other herds were fed solely on ryegrass/white clover pasture. The pasture was allocated on a rotational basis with a new area of pasture being offered every 24 hours. Total dry matter (kg DM/ha) was estimated visually (Hutton and Parker, 1973) before and after grazing three

times weekly throughout the trial. Visual estimates of pasture cover were calibrated weekly against pasture cuts to ground level. The pasture dry matter intake (DMI) was calculated as the difference between the before and after grazing estimates of total DM multiplied by the area of pasture offered and divided by the number of cows per herd. Total DMI (kg/cow/day) was calculated as the sum of the pasture DMI and the supplement DM offered. The mean monthly pre-grazing DM offered, the pasture DM intake/cow and the total DM/cow were calculated by averaging the weekly estimates across each calendar month. Differences among monthly averages were tested by oneway ANOVA with herd within treatment as the main effect.

Condition score and liveweight were recorded for each animal at fortnightly intervals throughout the trial (Macdonald and Macmillan, 1993).

Milk production for each animal was estimated on a weekly basis by summing the milk volumes produced at sequential afternoon and morning milkings. Subsamples of a composite afternoon and morning sample were analysed for milkfat and milk protein composition using an infra-red scanner (Milk-o-Scan, N. Foss Electrical, Denmark). A second milk sample was collected from 10 animals selected randomly within age in each herd (three 2 year olds, three 3 year olds and four older cows), twice weekly at the morning milking. This milk sample was preserved by the addition of a potassium dichromate tablet (Merck, Darmstadt, Germany) and stored at 4 ^{0}C for a maximum of 36 hours. The samples were analysed for P_4 concentration in a validated radioimmunoassay (Coat-a-Count, DPC, Calif; Chapter 6). The within- and between-assay coefficients of variation were 6.3% and 16.6%, and 8.5% and 16.2% for two quality control sera with mean P_4 concentrations of 4.1 and 1.8 ng/ml, respectively (six replicates of each quality control serum were included in 14 assays). The sensitivity of the assay was 0.10 ± 0.01 ng/ml. Ovulation was estimated as having occurred 5 days before the first milk sample from an individual animal having a P_4 concentration of more than 1.5 ng/ml.

Oestrus detection was performed from calving onwards by twice daily observation for behaviours associated with oestrus (standing while being mounted by another cow) as cows moved to and from the yards at milking time. Removal of tail paint was also accepted as evidence for behavioural oestrus

(Macmillan and Curnow, 1977). A silent ovulation was defined as having occurred where an oestrous date was not noted within 3 days of the estimated date of ovulation.

The seasonal artificial breeding program commenced on 5 October and continued until 11 November. All inseminations were performed by one experienced technician using commercially available semen. A bull was placed with each herd from 12 November to 25 December. The pregnancy status of each cow was determined by rectal palpation from 42 days after the last recorded insemination or natural mating. Additionally, all cows were again pregnancy tested in April. The expected calving dates were confirmed by the actual calving dates for 65% of the cows (i.e. the last service date + 280 days was within 10 days of the actual calving date). Where the actual calving date was not available (i.e. the cow was removed from the herd before calving) the cow was assumed pregnant to the last recorded service date where this service date was confirmed by two rectal examinations.

The intervals from calving to first ovulation, first oestrus and conception were calculated for each cow. Submission rate was defined as the percentage of all cows in the herd inseminated in the first 21 days of the mating program. The values for the production variables (milk volume, milkfat and milk protein (kg/cow/day)) and liveweight and CS were averaged across each calendar month (July to December; n = 4 or 5 or n = 2 or 3 recordings/month for the production variables and liveweight and CS, respectively) to produce mean daily figures for further analyses. Additionally, the data for milk production, liveweight and CS of each cow at 3 weeks before and 1 week after the PSM and their difference were analysed. A General Linear Model (GLM; SAS Version 6, SAS institute Inc, Cary) with calving date, age (2, 3 or >3 years old), breed (Friesian or Jersey) and treatment (supplement or no supplement) as the main effects was fitted sequentially. A herd within treatment effect was initially included in the models but was found not to be significant and this term was removed from the final models. Initially interactions of all these factors were fitted, but only those that were significant were included in the final model.

The relationships among the mean daily milk volume, milkfat and milk protein, liveweight and CS, and the intervals to first ovulation, first oestrus and

to conception were initially examined by a stepwise regression procedure with $P<0.10$ as the acceptance level for inclusion within the model. This initial regression was done on an individual cow basis ignoring treatment, age or breed effects. Any variable that was selected by this procedure was then included in a final GLM with calving date, age, breed and treatment included as factors. Where two variables were likely to be highly correlated (e.g. milk volume and milkfat), only one of the factors was tested in the model at one time.

Discrete data were analysed by χ^2 or by logistic regression (Catmod, SAS). The relationships among the mean daily milk volume, milkfat and milk protein, liveweight and CS, and the conception rate to first service were examined by stepwise logistic regression procedure with $P<0.10$ as the acceptance level for inclusion within the model. Any factor that was significant in this procedure was then included in a final logistic regression model with calving date, age, breed and treatment included as factors. The probability of conception was estimated for each cow.

Data are presented as means ± SED unless otherwise indicated.

Results

Silage supplementation increased the total DMI and reduced pasture DMI in August and increased the pre-grazing pasture offered, the pasture DMI and the total DMI in December (Figure 11.1).

Silage supplementation increased milk production (milk volume, milkfat and milk protein) during August and September, liveweight in August and CS in August, September and October, respectively (Figure 11.2).

Silage supplementation did not affect the percentage of animals ovulating, or detected in behavioural oestrus before the PSM and did not affect the percentage of cows expressing oestrus coincident with either the first or second ovulation (Table 11.1).

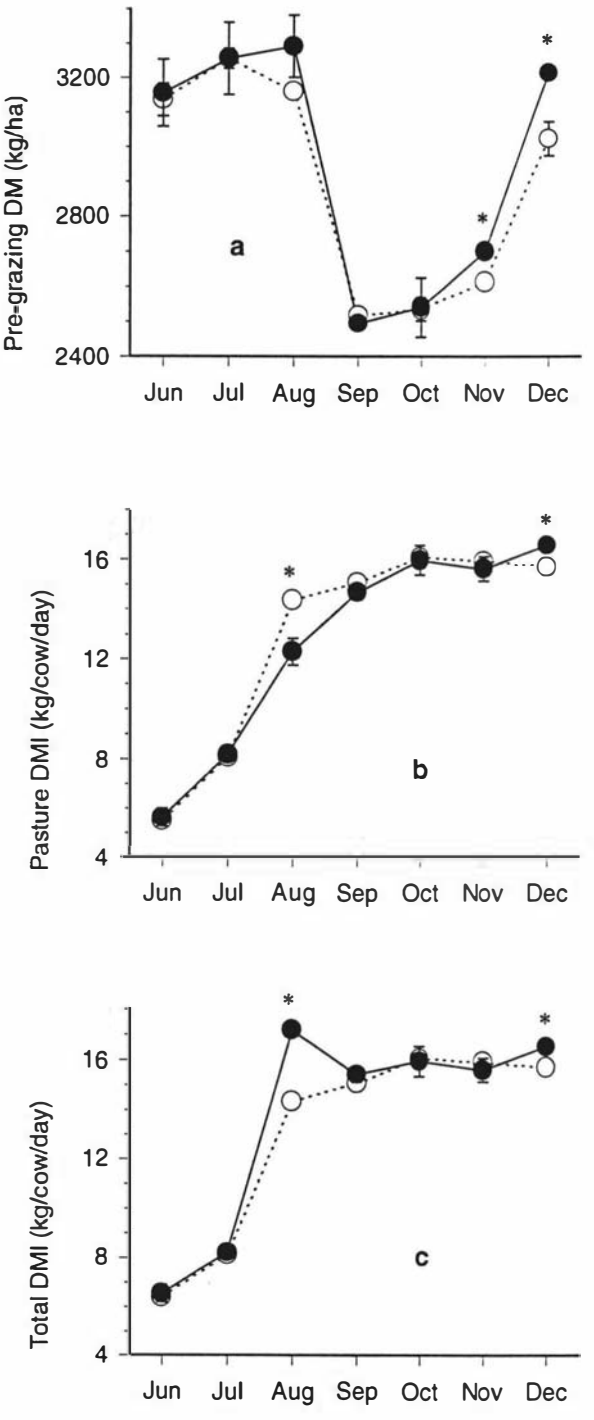

Figure 11.1. Average (± sem of herd means) daily pre-grazing pasture dry matter (a), pasture dry matter intake (b) and total dry matter intake (c) for herds fed either pasture (o; n = 3 herds) or pasture and pasture silage (•; n = 2 herds). * = $P<0.05$ within month between treatments.

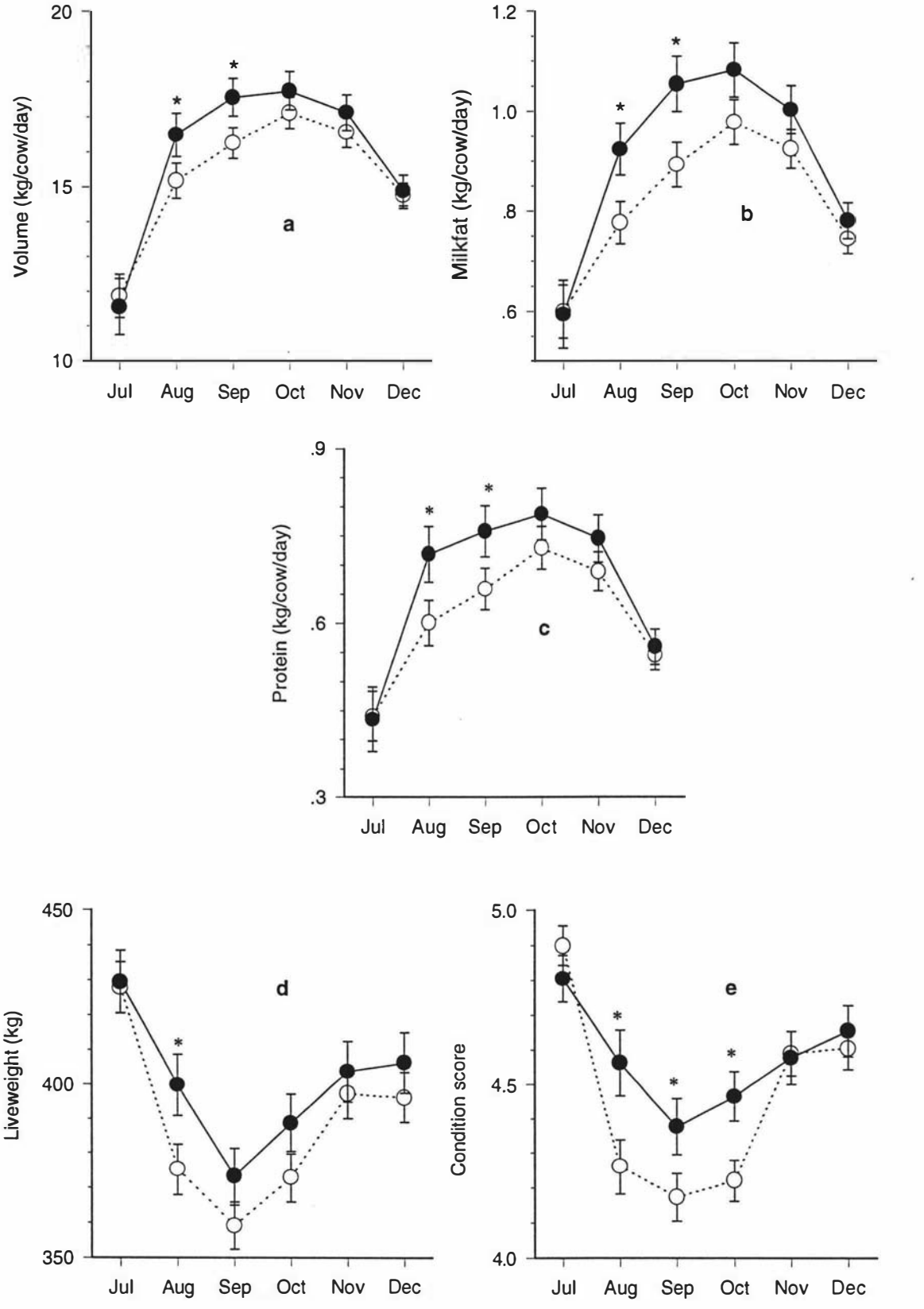

Figure 11.2. Average (± sem of herd means) daily milk volume (a), milkfat (b) and milk protein (c) production and the liveweight (d) and condition score (e) for herds fed either pasture (o; n = 3 herds) or pasture and pasture silage (•; n = 2 herds). * = $P<0.05$ within month between treatments.

Table 11.1. The reproductive performance of cows fed either pasture (n = 60) or pasture silage and pasture (n = 40)

	Pasture	Silage and Pasture	Diff	± SED
Ovulation before mating (%)	86.2	95.0	8.8	±12.1
Oestrus before mating (%)	80.0	90.0	10.0	± 7.1
Oestrus detected at 1st ovulation (%)	11.5	8.9	-2.6	± 6.1
Oestrus detected at 2nd ovulation (%)	73.9	84.2	10.3	± 8.1
Number of premating oestrus's (%)	1.5	1.7	0.2	± 1.4
21 day submission rate (%)	86.7	92.5	5.8	± 6.0
1st service conception rate (%)	53.3	37.5	-15.8†	±10.0
2nd service conception rate (%)	65.4	56.0	-9.4	±13.6
Services/conception	1.5	2.0	0.5*	± 0.2
Not pregnant at end of mating (%)	13.3	5.0	-8.3	± 5.6
Calving to 1st ovulation (days)	31.6	29.6	-2.0	± 4.0
Calving to 1st oestrus (days)	36.9	31.7	-5.2	± 4.1
Calving to conception (days)	96.6	102.3	5.7	± 4.8

† P = 0.09; * P<0.05.

Silage supplementation did not affect the intervals from calving to first postpartum ovulation, first oestrus or to conception (Tables 11.1 and 11.2).

Supplemented cows tended to achieve a lower conception rate to first service than unsupplemented cows (37.5% vs. 53.3%, ± 10.0%, supplemented vs unsupplemented, respectively; P = 0.09). The number of services per conception was higher in the supplemented (2.0) than unsupplemented cows (1.5, ± 0.2, P<0.05).

Friesian cows had a longer calving to first detected oestrus interval (44.3 days) than Jerseys (24.3 days, ± 6.7 days, respectively; P<0.05). Cows with later calving dates had shorter intervals from calving to first ovulation and to conception (Table 11.2).

The relationships among production, liveweight, CS and reproduction

Stepwise regression selected no variables as being associated with the interval from calving to first ovulation. Liveweight in August and September and CS at calving and in July were negatively associated with the interval from calving to first oestrus. The milk volume in July, liveweight and CS in August and September and the milk volume, milkfat and milk protein three weeks before the PSM were negatively associated with the interval from calving to conception. However, only the associations among the production yields three weeks before the PSM with the calving to conception interval were significant in

Table 11.2. The factors effecting the intervals from calving to first ovulation, to first oestrus, or to conception.

Factor	Regression co-efficient[¶]	C_ovn1[‡]		C_h1[‡]		Calving to conception[‡]	
				A[b]	B	C	D
Calving date	-ve	**[a]	ns	**	**	**	**
Age	-ve	†	ns	ns	†	†	†
Breed		ns	**	ns	ns	ns	ns
Rx		ns	ns	ns	ns	ns	ns
Cd x Rx[c]		ns	ns	ns	*	ns	ns
Vol_{PSM-3}[d]	-ve	-	-	na[▽]	**	na	na
Fat_{PSM-3}[d]	-ve	-	-	na	na	**	na
$Prot_{PSM-3}$[d]	-ve	-	-	na	na	na	**
R^2 (%)		34.3	11.3	34.4	40.0	40.6	39.4

[¶] The regression co-efficient of the factor in the regression analysis

[‡] The intervals (days) from calving to first ovulation (C_ovn1), first oestrous (C_h1) and to conception (calving to conception), respectively

[a] †, * and ** = P<0.10, 0.05 and 0.01 respectively, for the individual factor

[b] Model letter i.e. the basic model is A and then models with production variables fitted are B, C and D

[c] Calving date by treatment interaction

[d] Production at 3 weeks before the start of mating

[▽] Not applicable in that model

the final models (Table 11.2). Higher levels of production three weeks before the PSM were associated with shorter intervals from calving to conception on an individual animal basis (regression co-efficients = -2.1, -21.0 and -25.0, R^2 = 40.0%, 40.6% and 39.4%, respectively for milk volume, milkfat and milk protein, respectively, $P<0.05$; Table 11.2).

Higher daily milk volume and milkfat production three weeks before the PSM were positively associated with conception rate to first service (Figure 11.3, only milk volume data presented). The treatment and production effects were additive. Interactions involving treatment and milk volume or milkfat approached significance (P = 0.10 and 0.07, respectively). This was due to the lower conception rate at lower production levels in the cows fed pasture silage.

There was no relationship among the monthly liveweight or CS, or the change in liveweight or CS from three weeks before to one week after the PSM and the conception rate to first service.

Discussion

A total of 100 cows were used in this trial. Given that the coefficient of variation around the mean of the calving to first ovulation and calving to first oestrous interval is approximately 50% (Chapter 2), a difference of 35% in the mean intervals would be the smallest difference that could be detected with 90% confidence in this trial (Berndtson, 1991). Thus, it cannot be firmly stated that silage supplementation did not alter these intervals in this trial with the numbers of animals used. With a 50% coefficient of variation, approximately 250 cows per group would be needed to demonstrate a difference in mean interval of 5 days with 90% confidence (Berndtson, 1991). However, despite the small numbers of cows used, differences in first service conception rates and services per conception was demonstrated. The 37% conception rate in the silage supplemented cows is below the 50% conception rate at which investigation of herd reproductive performance is recommended (Radostits and Blood, 1985). Thus, the observed decrease in conception rate is statistically and biologically a significant result.

Pasture silage supplementation in the first month of lactation was associated with increased total daily DMI. There was an increase in milk production and a smaller loss of liveweight and CS during and following the period of supplementation. Supplementation did not alter the interval to first ovulation or oestrus, but reduced the conception rate to first service and increased the number of services/conception. Increasing production was associated with shortening of the interval from calving to conception and a higher conception rate to first service. Paradoxically, although silage supplementation increased milk production it was associated with a decreased conception rate compared to pasture fed control cows.

Milk production, liveweight, CS, and nutrient partitioning are interrelated, and conception rate may be directly or indirectly affected by changes of one or more of these factors induced by silage feeding. Alternatively, silage may be exerting its effects independently of changes in any of these factors.

A review of a large number of studies (Esslemont, 1979) found a relationship between production and reproduction in only 50% of studies and the direction of that relationship varied among studies. The calving to conception interval was positively associated with milk production in eight studies, had no relationship in seven studies and had a negative relationship in one study. The conception rate was positively associated with milk production in one study, had no relationship in four studies and had a negative relationship in three studies. Thus genetic, managemental and environmental factors, operating independently of production, influence reproductive performance.

Increases in liveweight leading up to mating have been shown to have a positive (King, 1968), no (Moller and Shannon, 1972; Broster, 1973) or a deleterious affect (Ducker *et al.*, 1985) on conception rate. No relationship among the liveweight, CS and reproductive performance was demonstrated in this study.

The variable relationships found among production, liveweight and CS and reproductive performance may be partially explained in terms of differences in energy balance and partitioning of feed intake. Cows at the same level of production may have different levels of feed intake, different partitioning of this intake and be undergoing different rates of body tissue

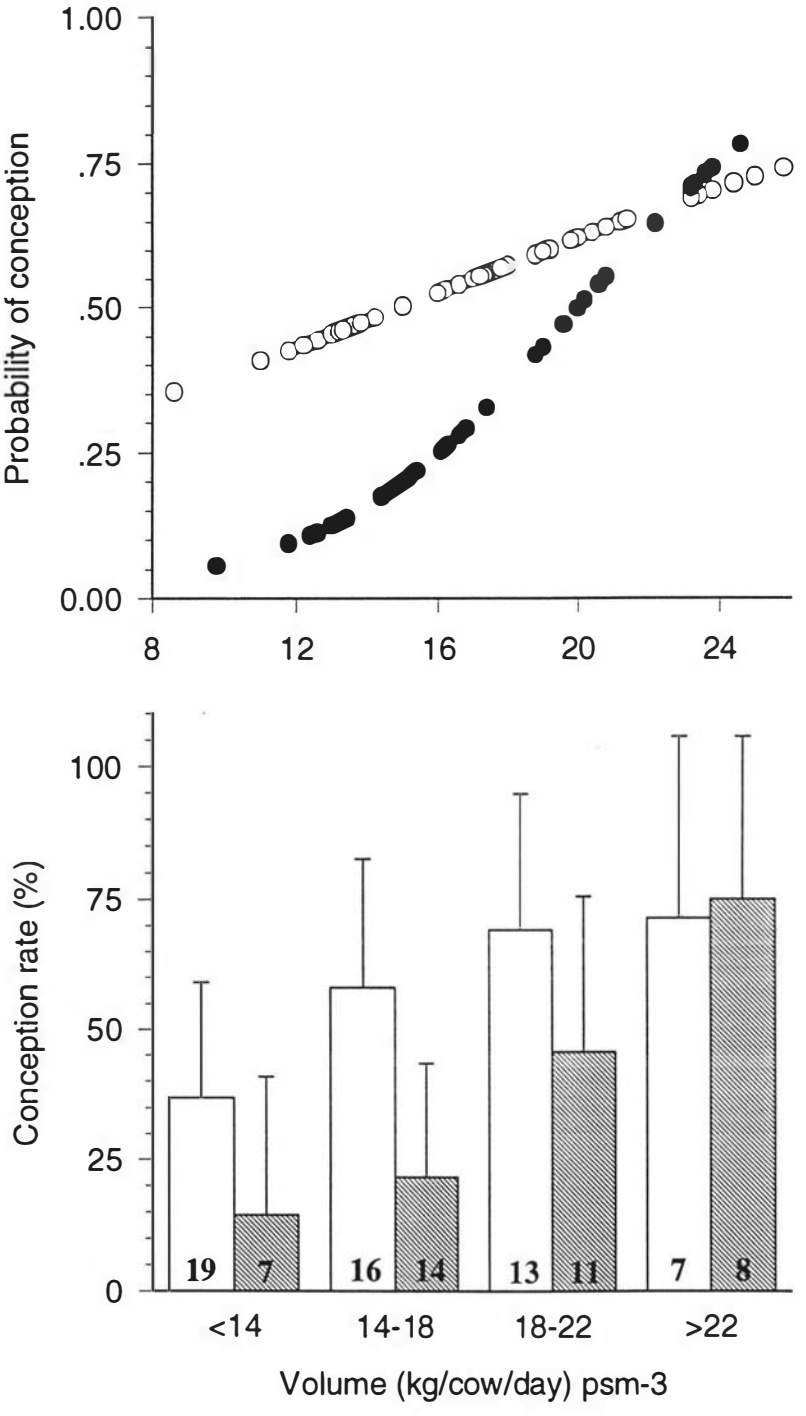

Figure 11.3. The estimated probability of conception (1.00 = conception) at first service determined by logistic regression modelling (upper) and the observed conception rate (lower) for cows fed solely pasture (o or open bars) or pasture and pasture silage (• or hatched bars) plotted against daily milk production 3 weeks before PSM (psm-3). The numbers within the bars are the number of cows in each category and the error bars are binomial.

mobilisation and hence be at widely different energy balances. Cows that have prolonged periods of NEB have extended periods of PPA (Butler *et al.,* 1981; Staples *et al.,* 1990). Extended intervals of PPA are associated with extended intervals from calving to conception. However, the relationship between conception rate and energy balance at the time of insemination has not been established. In one trial, cows with a prolonged period of NEB had higher conception rates than animals which quickly regained positive energy balance (Ducker *et al.,* 1985). However, in another trial, cows gaining weight (and presumably in positive energy balance) had higher conception rates than those losing weight approaching mating (Youdan and King, 1977).

In the present trial, individual intakes of pasture and silage were not evaluated so that no estimate of energy balance was possible. However, reduced loss of liveweight and CS and the increased production in the supplemented cows indicate that it is unlikely they were in NEB for longer or to a greater degree than the unsupplemented cows. The silage supplemented cows had higher production, liveweight and CS for at least a month after supplementation had stopped, potentially increasing the energy requirements after cessation of the supplementation. However, there was no significant difference in production or liveweight during the mating period. Nor was there any relationship among the change in production, liveweight or CS from three weeks before to one week after the PSM and conception rate to first service. This suggests that the observed reduction in conception rate was not associated with changes in body composition or milk yield, during or after the period of supplementation. The supplementation may have affected conception rate by mechanisms independent of effects on liveweight, CS or production, or by changes in these parameters too small to be detected in this experiment.

Ensiling pasture changes the chemical composition of the pasture (Ekern and Vik-Mo, 1979) and may allow the growth of undesirable bacteria and fungi. Fungal mycotoxins, for example zearalenone, have been shown to have deleterious effects on reproductive performance (Ruhr, 1986). Zearalenone has been found in pasture in New Zealand (di Menna *et al.,* 1987). Feeding zearelanone before, but not during, mating reduces ovulation

rate and fertilisation rate in sheep (Smith *et al.*, 1990) and there is a report of an association between elevated blood and urine zearalenone levels and poor reproductive performance in dairy cows in New Zealand (Towers and Sprosen, 1993). No fungal growth was observed on the pasture silage fed in this trial and there was no evidence of refusal of the pasture silage as may be expected with contaminated silage. However, no direct testing for the presence of mycotoxins was undertaken.

Pasture silage supplementation during the first month of lactation increased feed intake and milk yield as well as reducing the loss of liveweight and CS. However, no beneficial affects on reproductive performance could be demonstrated. There was a reduction in conception rate to first service that could not be explained in terms of changes in body composition or milk yield. This leaves the possibility that there was some affect of silage supplementation on conception rate not mediated by changes in body composition or milk yield.

CHAPTER 12:

General Discussion

Some New Zealand dairy herds have a high proportion of cows not detected in oestrus and inseminated in the mating period due to extended periods of PPA. This results in cows calving at inappropriate times of the year and/or being removed from the herds for infertility. The aims of this thesis were to examine managerial, endocrinological and treatment factors associated with extended PPA.

Factors influencing the prevalence of anoestrum

The comparison of Friesian and Jersey herds grazed at two stocking rates (Chapter 2) showed that some herds under pasture-grazing systems have similar PPA intervals to those reported in overseas studies (Lamming and Bulman, 1976; Fonseca *et al.*, 1983; Fagan and Roche, 1986) while others have comparatively extended periods of PPA. For example, the high stocked Friesians (Chapter 2) had an average interval from calving to first ovulation of 49.2 ± 5.0 days and to first oestrus of 52.2 ± 3.6 days. This resulted in over 50% and 62.1% of the herd not ovulating and not having been detected in oestrus by 50 days postpartum, respectively (Table 2.2). Over 20% of the cows which had calved more than 45 days had not been detected in oestrus and of these 32.7% (or 6.8% of the total number of animals in the herd calved >45 days) had no evidence of the presence of a CL (Table 3.2).

The length of PPA was found to be influenced by age, breed, stocking rate, CS in the peri-partum and pre-mating period and was weakly associated with some blood metabolite concentrations (Chapters 2, 3).

In agreement with previous studies, younger Friesian cows had longer PPA intervals than older, and Jerseys cows (Macmillan and Clayton, 1980; Fonseca *et al.*, 1983). Cows in poorer CS around calving had longer PPA periods as has been reported previously (Grainger *et al.*, 1982). This thesis

extended these findings by showing that CS assessed a week before the start of mating, stocking rate and various blood metabolites were also associated with PPA (Chapters 2, 3).

Between herd variation in the proportions of cows not detected in oestrus (NDO) and that were anovulatory anoestrus (AA) was demonstrated (Table 3.2). Differences in pre- and postpartum feed intake, stocking rate, breed and age structure of the herd and management policy on oestrus detection are some factors that may have contributed to this between herd variation. To better examine the factors associated with between herd variation in PPA intervals a larger number of herds and a wider range of measures of nutritional, reproductive and general management would need to be measured.

Increased stocking rate was associated with lower pasture intake both before and after calving (Figure 2.5). This was also associated with lower body CS around calving. Whether it was the lower mass of body fat, extended NEB or a more complex relationship involving the amount of body fat and energy balance that was affecting PPA intervals was not determined in these studies. Humans with a lack of body fat due to reduced feed intake (e.g. anorexia nervosa sufferers), or due to high levels of physical activity (e.g. sportswomen) become amenorrheic (Frisch, 1994). A combination of lower body tissue reserves due to pre-partum undernutrition combined with limitations on postpartum nutrient intake, resulted in the high stocked herds (especially the Friesian herd) falling to low body CS. This low level of body fat may have been insufficient to support early resumption of ovulatory activity following calving. Chronic undernutrition of cycling beef cattle will result in cessation of ovulatory activity when 24% of their initial bodyweight has been lost (Richards *et al.*, 1989). The amount of body weight lost by the high stocked herds, did not exceed 20% even when these weight changes included the losses associated with the liveweight of the calf (Figure 2.3). Postpartum, non-cycling cows may be more sensitive to loss of body weight than cows which have already commenced cycling. Alternatively, the cows in this experiment may have been at a lower CS initially and thus needed to shed less condition before some lower critical mass of body fat was reached.

Finally, there may be some interaction between NEB and loss of body tissue pre- and/or postpartum. The relationship between CS around calving and NEB has not been critically examined.

Several homeostatic mechanisms appear to operate in cows of low body CS. Cows with low CS at calving, fed *ad lib*, appear to have a compensatory increase in feed intake relative to animals in better condition (Garnsworthy and Topps, 1982). Additionally, cows in low CS have lower milk production than well conditioned cows (Grainger *et al.*, 1982). These two mechanisms result in cows in low CS at calving losing less CS and liveweight and hence returning to precalving liveweights and CS sooner than herdmates in better CS (Macdonald and Macmillan, 1993). As NEB is proportional to the amount of body tissue mobilised and NEB is also related to the PPA interval (Butler *et al.*, 1981), it is paradoxical that cows in low CS at calving that gain CS and liveweight more rapidly postpartum, may also have extended periods of PPA. However, cows in the high stocked Friesian herd had the lowest CS and lost as much CS as the other herds (Figure 2.3). This suggests that there was not sufficient pasture available to provide for a compensatory increase in feed intake. Lower milksolids production did occur in the high stocked herds; however, the homeorhetic drive to produce milk appears to have occurred at the expense of the body tissue reserves to such a degree as to deleteriously affect reproductive performance.

Cows selected on the basis of production will mobilise more body tissue and produce more milk as well as having higher pasture intake than unselected animals (Grainger *et al.*, 1985). Continuous selection for production may have produced animals whose homeorhetic drive to produce milk exceeds their homeostatic drive to maintain body tissue, and indirectly to maintain reproductive performance. Paradoxically, however, cows with higher production and feed intake also appeared to experience less severe NEB where *ad lib* feed was available in some trials (Staples *et al.*, 1990; Lucy *et al.*, 1992). The milk production at 4 weeks postpartum was negatively related to the interval from calving to first ovulation (Chapter 2), suggesting that the same relationship was occurring in pasture-fed cows in New Zealand herds. The relative strengths of the homeostatic and homeorhetic mechanisms in

cows at different levels of body condition and at different levels of postpartum feed intake, as well as the impact of relative differences in the strength of action of these mechanisms on reproductive performance need to be further investigated.

Strong negative relationships among CS and PPA intervals were found in several experiments (Chapters 2, 3, 10). Within the range of stocking rates, breeds and nutritional managements used in these trials, cows in satisfactory CS before calving were shown to have shorter PPA intervals than cows in poor (<3.5) CS. Increasing body tissue in the non-lactating period and then mobilising this tissue for production is regarded as an inefficient use of nutrients (Grainger *et al.*, 1982). However, the costs of extended periods of PPA in cows not having sufficient body tissue reserves may outweigh this inefficiency. The real cost/benefit may rely on the probability of reduced availability of pasture in the early postpartum period.

Some relationships among blood metabolites and reproductive performance were measured in this thesis (Chapters 2, 3, 10). Low blood glucose concentrations were negatively related to the interval from calving to first ovulation (Chapter 2) and were lower in anovulatory than cycling cows a week before the PSM (Table 3.4). Restricted feed intake lowers blood glucose (Richards *et al.*, 1989), and supplementation of pasture with hay may increase it (McClure, 1965). LH pulse frequency (Rutter and Manns, 1987; Richards *et al.*, 1989) and conception rate have been negatively related to blood glucose concentrations (McClure, 1965). NEFA concentrations, a marker of lipid mobilisation (Canfield and Butler, 1990), were negatively related to the intervals from calving to first ovulation and first oestrus (Chapter 2). NEFA concentrations have been associated with NEB in some trials (Canfield and Butler, 1990), but not others (Staples *et al.*, 1990). NEFA concentrations were also elevated during periods of controlled undernutrition (Richards *et al.*, 1989). In the present trial, NEFA concentrations were also significantly different among cows of different ages and breeds. Jersey cows had higher NEFA concentrations than Friesian cows despite losing similar amounts of bodyweight and CS. This suggests breed differences in mobilisation of adipose tissue or differences in clearance of NEFA. Urea

concentrations were higher in cows that had not ovulated by one week before the PSM in comparison with cows that had ovulated (Table 3.4). Urea concentrations have been related to dietary protein intake (Gordon and McMurray, 1979) and to increased body tissue mobilisation (Oldham and Parker, 1981). No effect of stocking rate was demonstrated on urea concentration (Table 2.5), but there was an interaction involving stocking rate, breed and time (Figure 2.4) suggesting that the lower stocked, better fed herds had higher urea concentrations, especially among the cows in the lower stocked Jersey herd. The higher urea concentrations in anovulatory cows could be related to higher pasture intake by these cows (Garnsworthy and Topps, 1982), or due to greater body protein mobilisation (Oldham and Parker, 1981). The BOH concentration is influenced by direct dietary factors, tissue mobilisation rate, and peripheral and liver use of the mobilised metabolites (Lean *et al.*, 1992). The relationship between PPA and BOH concentration was both negative and positive at different times and the temporal relationships appeared random (Figure 2.4). This suggests that the relationship between BOH and PPA may be a spurious one.

These data suggest that there are some consistent relationships among blood metabolites and reproductive performance. For example, a cow in severe NEB may be expected to have low blood glucose, to be mobilising body tissue and hence have elevated NEFA, urea and BOH concentrations and to have an extended period of PPA. However, inconsistencies are apparent. For example, younger cows were shown to have higher blood glucose and NEFA concentrations, but lower BOH and albumin concentrations, yet had significantly longer PPA intervals than older cows. The strong homeostatic controls of the concentrations of the essential metabolites within tightly defined bands which are compatible with life, and the confounding effects of feed intake and body tissue mobilisation on blood metabolite concentrations may mean simple relationships among energy balance, nutrient intake, production and reproductive performance are unlikely to be found. Adding to this problem is the lack of accurate individual cow feed intakes and estimates of NEB for pasture-fed cows. Use of measures of feed intake such as chromic oxide or alkane/alkenes may aid in estimating

individual intake and energy balance. However, problems of defining the quality or metabolisable energy and crude protein levels of cows' diets are major obstacles to accurate energy balance estimates for pasture-fed cows even where the mass of pasture intake is known.

Collectively the CS, liveweight, blood metabolite and herd pasture intake data suggest that undernutrition most probably extends the period of PPA. However, many other factors such as age, breed, and calving date affect the period of PPA, perhaps independently of the effects of undernutrition and NEB.

Oestrus detection and diagnosis of anoestrum

Over 60% of the cows presented as not having been detected in oestrus just before the start of the mating period, had actually ovulated as a CL was detected upon ovarian palpation or the serum P_4 concentration was elevated (Chapter 4). This figure is similar to those reported by Etherington *et al.*, (1991; >80%) and Williams and McCleod (1992; >70%) from North American and British studies, respectively. However, it is much higher than the New Zealand study of Fielden *et al.*, (1973; <15% for young cows). The methodology used (i.e. serial P_4 analysis vs. single, per rectum, evaluation of the ovary) or true differences among the populations may account for these differences. The cows detected as having luteal tissue may not have expressed behavioural oestrus or were not detected in oestrus by farm manager's. Only 29.3% of cows were detected in oestrus at the first postpartum ovulation, compared to 88.7% and 83.0% of second and third postpartum ovulations, respectively, using twice daily oestrus detection (Chapter 2). These figures are similar to previous reports using twice daily detection (Lamming and Bulman, 1976; Fonseca *et al.*, 1983). Continuous monitoring detected oestrus concurrent with 94% and 100% of second and third postpartum ovulations, but only 50% of first postpartum ovulations (King *et al.*, 1976). This indicates that cows that were expressing behavioural oestrus were likely not to have been detected in the present trials and in the

New Zealand dairy industry in general. With twice daily observations during daylight hours, behavioural oestrus may well be missed as there is a nocturnal peak of behavioural oestrus and the duration of oestrus is only 10 to 14 hours (Esslemont and Bryant, 1976; King *et al.*, 1976; Pennington *et al.*, 1986). Use of tail painting systems may well reduce missed oestrous events (Macmillan and Curnow, 1977; Macmillan *et al.*, 1988), but cannot be expected to achieve 100% sensitivity and specificity. Increased duration and/or frequency of observation periods have been shown to increase the proportion of cows detected in oestrus (Esslemont *et al.*, 1985; Pennington *et al.*, 1986). However, even with optimal oestrus detection systems, some cows presented for veterinary examination as having not been detected in oestrus are likely to have ovulated and commenced regular oestrous cycling.

Is differentiation of ovulating and non-ovulating anoestrous cows important and what is the cost benefit of accurate differentiation? Differentiation has traditionally been performed on the basis of a single rectal examination of the ovaries for luteal structures. Based on a single examination of the ovaries, between 11% and 30% of cows are likely to be misclassified as anovulatory and between 3% and 50% of anovulatory cows would be classified as having ovulated (Kelton, 1989). These errors may arise due to cycling cows that are in proestrus or metoestrus so that no CL is present to be palpated or due to misdiagnosis of follicular structures as luteal tissue in anovulatory animals. The effect of misdiagnosis of the ovarian status and hence treatment with inappropriate therapy has not been investigated on either an individual cow or on a herd basis.

The treatment of anoestrum

Endocrine treatments

Progesterone treatment of cows, commencing at 14 to 20 days postpartum, shortened the intervals from calving to first ovulation, to oestrus, and to conception by 3.5, 4.2 and 8.4 days, respectively, compared to non-treated control cows (Chapter 9). Treatment with P_4 and eCG a week before

the PSM increased the probability of cows being inseminated and conceiving (Figure 10.1). Progesterone treatment also increased the proportion of first postpartum ovulations associated with detected oestrus (83.3% vs. 32.0%) and increased the duration of the first postpartum luteal phase (9.5 vs. 5.6 days) compared to non-P_4 treated cows (Chapter 9). Similar positive effects of P_4 treatment on reproductive performance have been reported in some studies (Ball and Lamming, 1983; Galloway *et al.*, 1987; Macmillan and Day, 1987; Macmillan and Peterson, 1993) but not others (Jubb *et al.*, 1989; Kyle *et al.*, 1992; Stevenson and Pursley, 1994). Differences in type of progestagen used (norgestomet vs. P_4), duration of progestagen treatment (5, 7, 9 or 12 days), type of drugs used simultaneously (ODB, EV, eCG), techniques used to determine ovarian status (palpation vs. P_4 analyses), spontaneous recovery rate in control cows and variation among farms in management systems may affect response rates and patterns.

In populations where the spontaneous rate of recovery of ovulatory activity is high, P_4 treatment may delay ovulation and oestrus so that no shortening of the treatment to first ovulation or oestrous interval can be demonstrated. For example, in the study population of Jubb *et al.*, (1989), 29.3% of control animals and only 2.3% of P_4-treated animals were detected in oestrus during the first 7 days of treatment, but over a 3 week period 62.6% of control and 64.6% of treated cows were detected in oestrus. When P_4 treatment commences during the mating period, it may delay insemination where the population has a high rate of spontaneous resumption of ovulatory activity.

The diagnostic technique used to differentiate anovulatory from cycling cows may affect response to treatment. As discussed above, a proportion of cows not detected in oestrus that are presented for treatment are likely to have in fact ovulated. Where palpation alone is used, misdiagnosis of a considerable number of cows may occur (Kelton, 1989; McCleod and Williams, 1991) involving two types of error. Firstly, the truly anovulatory animal may be diagnosed as cycling; or, secondly, an ovulating animal may be diagnosed as anovulatory and be treated with P_4. The response of cycling cows to treatment with P_4 and/or eCG or ODB may depend on the stage of the

cycle at which treatment occurs, the timing of the treatment relative to the start of the insemination period and the length of the luteal phase of that cycle. Progesterone treatment in proestrus (days 1 to 4 following ovulation) will reduce the inter-ovulatory interval (Macmillan *et al.*, 1991; Burke *et al.*, 1994). Treatment for 5 or 7 days with P_4 between days 5 and 11 or 13 of the cycle will have no effect on the cycle length, as luteolysis will occur at the normal time after removal of the exogenous P_4 (Macmillan *et al.*, 1991). Treatments commencing in late dioestrus or early proestrus delay the subsequent oestrus and ovulation (Macmillan, 1993). If the treatment is instituted during a short (<10 day) interovulatory period, which commonly occur following the first postpartum ovulation (Savio *et al.*, 1990, Chapter 4), delay of the subsequent ovulation may occur.

If treatment is instituted before the start of the mating period, the inhibitory effect of P_4 may result in ovulation occurring early in the mating period rather than just before mating. This may be advantageous as it results in more cows being inseminated early in the mating period. However, when treatment commences during the mating period it may delay insemination and conception (Jubb *et al.*, 1989) even where correct diagnosis is made. The impact of misdiagnosis on herd reproductive performance may depend on the proportion of the cows misdiagnosed, the proportion of the anoestrous cows that are actually ovulating, the treatment options used on the cows diagnosed as ovulating (e.g. PGF_{2_α} or no PGF_{2_α}) and the timing of the commencement of treatment relative to the start of mating. Further physical data and modelling are required to examine these complex interactions. However, significant advantages were demonstrated where treatment commenced a week before the start of mating irrespective of the diagnostic technique used (Macmillan and Day, 1987; Chapter 10).

Early postpartum (14 to 20 days) treatment reduced the interval from treatment to first oestrus (Table 9.3). It also reduced the calving to conception interval, despite insemination occurring 4 to 11 weeks after P_4 treatment (Table 9.3). Although the reason for this is not known, an increasing number of oestrous cycles before first insemination is positively correlated with reproductive performance (Thatcher and Wilcox, 1973). Early, induced,

resumption of cyclic activity due to P_4 treatment may be having a positive effect by the same mechanism. Where P_4 treatment was followed 48 h after device removal by 0.6 mg of ODB, the intervals from calving to first oestrus, ovulation and conception were reduced by 3.9, 3.3 and 5.2 days, respectively. The small number of animals used in this trial (29 and 22) meant that these differences were not significant (Table 9.3). Field trials using larger numbers have now demonstrated that a significantly higher proportion of cows treated with P_4 and ODB than those treated with P_4 alone or with P_4 and eCG were detected in oestrus and conceived within 14 days of the end of treatment (Macmillan *et al.*, 1994).

Progesterone treatment of the anovulatory dairy cow was shown to increase the proportion of cows expressing behavioural oestrus at ovulation (83.3% vs. 32.0%; Chapter 9). This is the first time that this effect of P_4 has been illustrated in cattle, although it was earlier demonstrated in sheep (Robinson *et al.*, 1956). Progesterone also reduced the incidence of short luteal phases that are common following the first postpartum ovulation in cattle (Lamming and Bulman, 1976). An increase in the duration of the luteal phase following P_4 and gonadotrophin induced first postpartum ovulations also occurs in the suckled beef cow (Pratt *et al.*, 1982; Sheffel *et al.*, 1982). The mechanism by which P_4 produces these effects on oestrous behaviour, luteal duration and fertility are not understood (Lamming *et al.*, 1979; Lishman and Inskeep, 1991).

Ovulation in the normally cycling cow is preceded by an increasing frequency of LH pulses, with up to one pulse/h required for ovulation (Rahe *et al.*, 1980). Progesterone treatment of anovulatory cows presumably results in similar LH pulse frequencies for ovulation to result. Luteal phase concentrations of P_4 inhibit LH pulse frequency in cycling cattle (Price and Webb, 1988) and in some trials involving anovulatory suckled beef cattle (Walters *et al.*, 1982). However, norgestomet treatment resulted in increased LH pulse frequency 6 days after initiation of treatment, but not 1 day after initiation of treatment or 1, 3 or 5 days after the end of treatment in suckled beef cattle (Garcia-Winder *et al.*, 1986b). Increased intrafollicular and circulating E_2 concentrations occur following P_4 treatment of anovulatory cows

(Sheffel *et al.*, 1982; Garcia-Winder *et al.*, 1986b). Increased numbers of LH receptors in the granulosa and thecal cells of the largest follicle and increases in the size of the largest follicle also occur following norgestomet treatment (Inskeep *et al.*, 1988). These P_4 effects may occur at the hypothalamus, the pituitary and/or the ovarian level or some combination of all of these sites. Initial depression of LH pulse frequency, reducing follicular E_2 and/or inhibin concentration resulting in a rebound increase in LH pulse frequency may occur. Progesterone may reduce the sensitivity of the hypothalamus to the feedback of E_2, shown to be increased in underfed cycling (Imakawa *et al.*, 1987) or anovulatory cattle (Chapter 8) and in seasonally anoestrous sheep (Legan *et al.*, 1977; Karsch *et al.*, 1993). A final possibility is that the inhibition of GnRH pulse release may allow the pituitary concentration of LH to increase so that when P_4 is removed more and/or larger LH pulses result. Detailed studies of the LH pulse frequency before, during and after P_4 treatment in anovulatory dairy cows are required to elucidate this mechanism. Additionally, methodologies to study the effect of P_4 on the GnRH pulse generator and changes in the E_2 negative feedback mechanism need to be investigated.

Follicular growth and increased follicular E_2 production occur in the follicular phase of cycling cows (Ireland and Roche, 1983). Where luteolysis is followed by P_4 treatment which results in sub-luteal concentrations of P_4, the LH pulse frequency increases, the largest follicle grows to a larger diameter and the circulating E_2 concentration is higher than in control animals (Roberson *et al.*, 1989; Sirois *et al.*, 1989; Kojima *et al.*, 1992; Savio *et al.*, 1993). Conversely, P_4 treatment early in the metoestrus phase results in early cessation of follicular growth (Burke *et al.*, 1994), perhaps related to premature inhibition of LH pulse frequency. The effect of P_4 treatment on follicular development in anovulatory cows has not been examined. If LH pulse frequency during P_4 treatment does increase, larger follicles with higher E_2 production and more LH receptors may result. Whether the decreased conception rates associated with the large follicles in cycling cows treated with P_4 in late dioestrus (Macmillan and Peterson, 1993) also occur in anovulatory cows treated with P_4 has yet to be examined.

Management

Supplementation of pasture with silage in the first 4 weeks of lactation tended to shorten the intervals from calving to first ovulation and first oestrus, but depressed conception rates (Chapter 11). Increasing intake of pasture over the first 5 weeks of lactation has been shown to decrease the interval from calving to first oestrus by 1.2 days for each extra kg of pasture fed per day (Grainger *et al.*, 1982). Attempts to increase the energy density of diets and hence reduce the NEB, resulted in higher energy intakes, but higher production, an increase in the depth of NEB and no improvement of reproductive performance (Lucy *et al.*, 1992; Sklan *et al.*, 1994).

The inverse relationship among CS and intervals from calving to first oestrus and ovulation has been demonstrated across a wide range of body CS at calving (Chapter 2; Grainger *et al.*, 1982) and at different levels of postpartum feeding (Grainger *et al.*, 1982). This suggests that pre-calving nutrition is important, and has effects independent of postpartum nutrition.

Other managerial approaches to reducing the PPA interval include reducing milk production by milking cows once a day and separating anoestrous cows from the main herd for preferential feeding which has the added effect of removing animals from social stressors. The effectiveness of these techniques has yet to be tested.

Increasing the duration and/or frequency of oestrus detection periods may result in a higher proportion of oestrous events being detected (King *et al.*, 1976; Esslemont *et al.*, 1985; Pennington *et al.*, 1986). Herds that commenced oestrus detection earlier in the postpartum period had fewer anoestrous cows that had ovulated at the time of veterinary examination (Chapter 3).

Follicular waves and endocrine control in the postpartum period

Follicular waves in the postpartum period

Presence of large follicles in the postpartum period

Large (>10 mm) follicles were present from 10 days postpartum and underwent phases of growth, plateau and atresia (Chapter 4). This observation extends the concept of follicular turnover to include dairy cows with extended periods of PPA. Previously this pattern of follicle turnover was reported to occur in pre-pubertal dairy cattle, postpartum suckled beef cattle, cycling beef and dairy cattle and pregnant dairy cattle (Sirois and Fortune, 1988; Ginther *et al.*, 1989a; Murphy *et al.*, 1990; Hopper *et al.*, 1993). Follicular development beyond 2.5 mm in sheep (Scaramuzzi *et al.*, 1993) and beyond 8 mm in cattle (Webb *et al.*, 1994) has been shown to require gonadotrophin support. Consequently, sufficient gonadotrophins must have been present in these anovulatory cows for development of these large follicles. The diameter of the DF increased with the number of DF's postpartum in these same cows, suggesting that increasing gonadotrophin support may have occurred with increasing time postpartum. The intrafollicular steroid concentrations within these large follicles were lower in the large follicles from anovulatory than cycling cows despite there being no differences in diameter, growth rate or number of granulosa cells within the follicles (Chapter 5). Production of steroids is controlled by the gonadotrophins (McNatty *et al.*, 1984b; Fortune, 1986; 1994). Cattle with extended periods of PPA have lower LH pulse frequencies than cycling cows (Chapter 8; Wright *et al.*, 1990). No difference in the number of receptors for LH and FSH in follicles from short compared to long-term anovulatory cows has been demonstrated (Rhind *et al.*, 1992), but the intrafollicular T and E_2 concentrations do differ (Prado *et al.*, 1990). The LH pulse frequency was positively correlated with the intrafollicular T and E_2 concentration (Chapter 5). However, the E_2 to T ratio remained the same, suggesting that the aromitisation of T to E_2 (an FSH dependant function; Fortune, 1994) was occurring at a similar rate, but that LH-dependant T production by theca interna cells was limiting (McNatty *et al.*, 1984b) in the anovulatory DF. The higher P_4 concentrations in the larger (plateau phase) follicles (Chapter 5) indicates that these follicles were approaching atresia (Ireland and Roche, 1983; Voss and Fortune, 1993). However, the large DF from anovulatory cows had similar P_4 concentrations but lower T and E_2 concentrations than the

DF's from cycling cows. This suggests that the conversion of pregnenolone to P_4 by 3β-hydroxysteroid dehydrogenase/Δ^4-Δ^5-isomerase (Voss and Fortune, 1993) was less affected by the lower gonadotrophin concentrations than the cytochrome P-450 17α-hydroxylase/C-17,C-20 lyase enzyme that converts pregnenolone to dehydroepiandrosterone which is one of the androgen precursors of E_2 production (Voss and Fortune, 1993).

Endocrine control of follicle waves

The growth of the largest growing follicle is affected by exogenous P_4 (Roberson *et al.*, 1989; Sirois *et al.,* 1989; Savio *et al.*, 1993; Burke *et al.*, 1994) and E_2 (Bo *et al.*, 1993). The growth rate of the DF is also affected by GnRH treatment (Macmillan and Thatcher, 1991). Where DF growth rate is reduced due to premature atresia, the emergence of the next DF occurs earlier (Bo *et al.*, 1993). Similar changes in the timing of emergence of the next DF and cessation of follicle growth were demonstrated following treatment with GnRH (Chapter 6) and ODB (Chapter 7) in anovulatory cows.

The mechanisms for these effects are not known. It may be by direct effects on follicles or via modulation of gonadotrophin concentrations or pulse frequencies.

In-vitro treatment of cultures of granulosa or thecal cells with E_2 results in decreased steroid production (Fortune and Hansel, 1979; Henderson *et al.*, 1987) perhaps analogous to the onset of atresia *in-vivo*. However, stronger evidence exists that these steroid treatments are modulating gonadotrophin concentrations and hence effecting follicle development. Luteolysis, followed by P_4 treatment producing P_4 concentrations lower than in the luteal phase, results in continued growth of the DF, delay of emergence of the subsequent DF (Roberson *et al.*, 1989; Sirois *et al.*, 1989), and is associated with an increased LH pulse frequency (Savio *et al.*, 1993), suggesting that it is the change in LH pulse frequency that is affecting follicle development.

The mechanism for the effects of E_2 may be more complex. If P_4 concentration is <0.5 ng/ml, E_2 treatment results in a bi-phasic decrease, then increase in LH concentration (Kesner *et al.*, 1981; Schallenberger and

Prokopp, 1985). However, in the presence of >0.5 ng/ml of circulating P_4, E_2 does not induce an increase in LH concentration (Nanda *et al.*, 1988). Oestradiol treatment may result in a different LH pattern depending on whether cycling or anovulatory cows are treated. Bo *et al.*, (1993) demonstrated that EV treatment early in the cycle (i.e. less than 4 days after ovulation) resulted in cessation of growth of the DF which was accompanied by a bi-phasic change in LH concentration. However, when treatment occurred later (day 6), there was no change in growth rate and no LH surge. This coincided with both an increase in P_4 concentrations and with atresia of the first DF of the cycle. It is not clear whether the stage of follicle development or the increasing P_4 concentration affected the response to EV in the study of Bo *et al.*, (1993). In anovulatory cows, ODB treatment alone resulted in slower growth of the DF when the DF was still growing, suggesting that ODB alone is sufficient to suppress follicular growth (Chapter 7). The bi-phasic pattern of LH release occurred as there was no P_4 present to inhibit LH release (Chapter 7). When the DF was at a maximum diameter (i.e. plateau phase and approaching atresia), no change in the growth rate was apparent either in cycling (Bo *et al.*, 1993) or anovulatory cows (Chapter 7) following EV or ODB treatment, respectively. Oestradiol is therefore unable to alter the rate of decline in diameter of a DF as it approaches atresia, irrespective of the P_4 concentration and the occurrence of an LH surge. If cows are treated with E_2 when the DF is still growing, the suppression of DF growth maybe due to the decrease, increase or the bi-phasic change in LH concentration.

Reducing LH pulse frequency by increasing P_4 concentrations in cycling cows results in earlier cessation of growth of the DF and early emergence of the next DF (Savio *et al.*, 1993). However, the pre-ovulatory LH surge will reduce E_2 and increase intrafollicular P_4 concentrations in the non-ovulating follicles, indicating that atresia has occurred (Staigmiller and England, 1982; Ireland and Roche, 1983; Voss and Fortune, 1993). Treatment with 5 mg of ODB without progestagen pre-treatment did not alter the DF growth rate or the timing of subsequent DF emergence. If ODB treatment was preceded by progestagen treatment, the DF growth rate slowed and earlier emergence occurred. The progestagen pre-treatment abolished the LH and FSH surge

suggesting that the LH surge had no role in inducing DF atresia (Bo *et al.*, 1994). Treatment with ODB alone may have failed as the depression of gonadotrophin concentrations was short (approximately 6 hours) and the LH surge was maximal at 18 h (Bo *et al.*, 1994). In comparison, in the anovulatory cow treated with 0.5 mg ODB, the LH concentration was depressed for over 12 h, and the maximum LH concentration did not occur until 32 h after treatment (Chapter 7). The duration of the depression of LH and the timing of the LH surge may be critical in determining the effects of changes in gonadotrophin concentrations on the DF. Additionally, the DF of anovulatory cows may be more sensitive to changes in gonadotrophins than DF's from cycling cows.

The role of gonadotrophins in growth and atresia of follicles needs to be tested where the concentrations of P_4, E_2, LH and FSH can be individually varied. The use of cows which have been hypothalamic/pituitary disconnected, GnRH immunised or GnRH agonist down-regulated followed by treatment with LH, FSH and LH and FSH in combination, would enable the responses to individual gonadotrophins to be tested in a stable steroid hormone milieu. These models have been used extensively in the ewe (McNeilly *et al.*, 1992). Testing the direct effects of steroids on follicles is technically more difficult, as any systemic treatment with steroids results in effects on both the hypothalamic/pituitary axis and the ovary. Infusion of steroids into the ovarian artery followed by ovariectomy within a few hours and measurement of intrafollicular steroid concentrations is one approach. Alternatively, *in-vitro* culture of granulosa and thecal cells and use of co-culture systems may allow the effect of individual steroids to be tested. However, conventional granulosa cell cultures undergo luteinisation within 2 to 3 days as evidenced by increasing P_4 concentrations (Fortune and Hansel, 1979) making interpretation of the effects of E_2 and P_4 on the production of other steroids difficult. Development of a 3 dimensional granulosa cell culture systems and co-culture of theca and granulosa cells offers a more 'physiological' approach to investigating the role of steroids in follicular function (Fortune, 1986; Lavranos *et al.*, 1994).

GnRH treatment appears to induce atresia of growing follicles in cycling cows (Macmillan and Thatcher, 1991; Rettmer *et al.*, 1992) and early emergence of the next DF in anovulatory cows (Chapter 6). Early emergence may indicate removal of the inhibitory effects of a DF, as has been demonstrated by removal of the DF by electrocautery or ovariectomy (Ko *et al.*, 1991; Badinga *et al.*, 1992). GnRH treatment produces an increase in LH and FSH which is maximal 2 h after treatment, an increase in E_2 and P_4 over the 6 h following treatment, and an increase P_4 over the following days but also a decrease in E_2 concentrations for 6 to 8 days following treatment in cycling cows (Rettmer *et al.*, 1992). GnRH induces an increase in LH and FSH (Chenault *et al.*, 1990; Chapter 6), in contrast to the bi-phasic effect of E_2 on LH concentrations. This suggests that it may be an increase, rather than a decrease or bi-phasic change in gonadotrophin concentrations, that induces atresia of the growing follicle in cycling cows. The early emergence of the next DF in anovulatory cows may occur because of the removal, by ovulation, of the DF. Emergence of a new group of follicles appears to be preceded by an increase in FSH concentration (Adams *et al.*, 1992). GnRH treatment will stimulate endogenous FSH release as well as removing E_2 and inhibin inhibition of FSH by inducing ovulation of the DF. Following E_2 treatment, atresia of the DF would result in a similar decrease in inhibitory products and earlier emergence of the next follicular wave. However, Bo *et al.*, (1993) reported that emergence of the next DF occurs 4 days after EV treatment, irrespective of the effect of the EV on the extant DF. This may be due to the prolonged elevation of circulating E_2 following their treatment due to the long acting effect of EV and the large dose (5 mg) used. The use of smaller doses (0.5 mg) and a shorter acting ester (benzoate) resulted in only a 2- to 3-day delay in emergence of the DF in anovulatory cows (Chapter 7).

Applications for follicular wave control

Macmillan (1993) has advanced the concept that control of follicular growth is an essential part of synchrony systems for cycling cattle. Where synchrony systems do not account for variation in the stage of development of the largest follicle at the time of treatment, imprecise synchrony of oestrus and

ovulation may result (Macmillan and Henderson, 1983). Removal of exogenous P_4 which mimics the start of pro-oestrus may either occur at a time when the largest follicle is small and needs 3 to 4 days to mature to the pre-ovulatory stage, or at a time when the follicle is large and thus able to ovulate within 2 days of the end of treatment. If exogenous E_2 given at the beginning of a period of P_4 treatment resulted in a synchronous atresia of any follicles present and hence synchronous emergence of a new cohort of follicles, the subsequent removal of the P_4 could be timed to ensure that all animals have a large follicle present, reducing the variation in the length of pro-oestrus. This thesis has demonstrated that doses of ODB of 0.1 to 0.05 of those previously used (Bo *et al.*, 1993; 1994; Chapter 7) were effective in inducing atresia of follicles, at least in anovulatory cows. Using these reduced doses may result in shorter intervals to emergence of the subsequent follicle wave, lower costs and reduced risk of residues in the milk and meat of treated cows.

The endocrinology of postpartum anovulation

The presence of large DF's undergoing regular turnover was demonstrated in anovulatory cows (Chapter 4). However, these follicles failed to ovulate despite reaching diameters equivalent to those seen in cycling cows which do ovulate. It has been suggested (Murphy *et al.*, 1990; Roche *et al.*, 1992) that failure of the large DF in postpartum cows to ovulate, may be due to insufficient gonadotrophin support, resulting in insufficient follicular E_2 being produced to induce the pre-ovulatory GnRH and hence LH surge. The experiments in this thesis support this view.

The LH pulse frequency was lower in anovulatory cows than in cycling cows both before and after ovariectomy (Chapter 8). Similar low LH pulse frequencies have been reported in dairy (Fisher *et al.*, 1986) and beef cows (Wright *et al.*, 1990) with extended periods of PPA. The pituitary and circulating concentrations of FSH do not appear to be limiting the resumption of cyclic activity postpartum (Schallenberger *et al.*, 1982; Moss *et al.*, 1985) and will not be discussed further. Additionally, intrafollicular steroid

concentrations, which are gonadotrophin dependant, were lower in the DF's of anovulatory than in cycling cows (Chapter 5).

Why did these anovulatory cows have lower LH pulse frequencies? Pituitary LH concentration is depressed for 2 to 4 weeks following parturition (Moss *et al.*, 1985). There is also a decreased circulating LH pulse frequency following parturition, even in cows with short (<3 week) postpartum intervals (Schallenberger *et al.*, 1982; Canfield and Butler, 1990). The LH response to a set dose of GnRH increases with time postpartum (Kesler *et al.*, 1977; Fernandes *et al.*, 1978) indicating either an increase in the releasable pool of LH, or an increase in the number of GnRH receptors on the pituitary gonadotropes. GnRH treatment resulted in an LH surge in 100% of anovulatory heifers (Chapter 6) which were approximately 3 weeks postpartum at the time of treatment. This suggests that the pituitary concentration of LH and the number of GnRH receptors were not limiting factors in these heifers. There is a one-to-one relationship between the release of a GnRH pulse from the hypothalamus and an LH pulse from the pituitary in cycling sheep (Clarke and Cummins, 1982; Karsch *et al.*, 1992). The concentration of GnRH in the hypothalamus appears to be relatively constant during the postpartum period (Moss *et al.*, 1985). These data suggest that it is control of the hypothalamic release of GnRH, rather than pituitary LH deficiencies, that is limiting LH release in anovulatory postpartum cattle.

Tonic control of GnRH release from the hypothalamus

A variety of nutritional, photoperiod, opiate and steroid controls of GnRH and/or LH release have been demonstrated in cattle (Short *et al.* 1990; Peters and Lamming, 1991). These factors influence the hypothalamus via inhibitory and excitatory neuropeptides (Kalra, 1993), or via modulation of metabolic hormones or metabolites (Schillo, 1992). Hypophyseal portal sampling systems which allow direct measurement of GnRH release have not been developed in cattle. Most studies rely on the detection of LH pulses and assume that the one-to-one relationship among GnRH pulses and LH pulses occurs under a wide range of physiological and nutritional regimes.

In the postpartum period, nutrient intake in dairy cows is insufficient to meet the requirements for maintenance and lactation and hence cows are in NEB and negative protein balance (Butler and Smith, 1989). This NEB has been associated with reduced LH pulse frequency in postpartum dairy cows. Following the nadir in NEB, the LH pulse frequency increases and ovulation occurs approximately 10 days later (Butler and Smith, 1989; Canfield and Butler, 1990). Dairy cows with extended periods of PPA have lower feed intake, remain in lower NEB for longer and have lower body CS than herdmates which cycle earlier postpartum (Staples *et al.*, 1990; Lucy *et al.*, 1992).

In seasonally anoestrous ewes, there is a reduced LH pulse frequency which is at least partly due to an increased sensitivity to E_2 feedback (Legan *et al.*, 1977). A similar increase in sensitivity to E_2 feedback has been demonstrated in undernourished, suckled, beef cows (Imakawa *et al.*, 1987), and the postpartum interval to ovulation can be shortened by treatment with the E_2 antagonist, enclomiphene (Chang and Reeves, 1987). Dairy cows ovariectomised before their first postpartum ovulation had greater sensitivity to E_2 treatment than cows ovariectomised when cycling (Chapter 8). The anovulatory cows had similar mean LH concentrations, but lower LH pulse frequencies and higher LH pulse amplitudes than cycling cows, similar to the situation in anoestrous sheep (reviewed by Goodman, 1988). The LH pulse frequency, rather than the mean LH concentration, appears to be critical in controlling ovulation. The increase in sensitivity to E_2 of anovulatory animals appears to be common in a variety of physiological situations. The mechanism for this increased sensitivity is not known. However, changes in concentrations of various catecholaminergic neurotransmitters suggest they play a role in this process (Meyer and Goodman, 1985). Type B receptors for gamma-amino butyric acid only appear as sheep move from the breeding to the non-breeding season (Clarke and Scott, 1993) indicating that the increased E_2 sensitivity may be related to changes in these neurotransmitter receptors.

Ovariectomised ewes not treated with steroids also show seasonal changes in LH pulse frequency, suggesting that ovarian-independent factors

may also control GnRH release (Montgomery *et al.*, 1985). Ovary-independent control may be mediated by serotonergic neurotransmitters independent of those effected by E_2 (Meyer and Goodman, 1986). Following ovariectomy, the LH pulse frequency in the anovulatory cows remained lower than in cycling cows (Chapter 8). This is evidence that ovary-independent inhibition of LH may be occurring in these cows.

In sheep, seasonal anoestrus is associated with changes in photoperiod (reviewed by Goodman, 1988). Seasonal differences in the duration of postpartum anovulation in beef cattle can be partially explained by changes in photoperiod (reviewed by Short *et al.*, 1990). However, PPA occurs in all seasons of the year in cattle, suggesting that factors other than photoperiod may be more important regulators of inhibition of LH release in postpartum cows. Undernutrition following ovariectomy will prevent the post-ovariectomy rise in LH pulse frequency in beef cattle (Imakawa *et al.*, 1987). Similarly, ovariectomy followed by restriction of energy intake in peri-pubertal heifers results in lower LH pulse frequency, increased LH pulse magnitude and increased LH mean concentration when compared to well-fed controls (Kurz *et al.*, 1990). Pre-partum restriction of nutrient intake that resulted in depletion of body condition, resulted in reduced LH pulse frequency following ovariectomy in beef cattle (Wright *et al.*, 1990) and in sheep (Rhind *et al.*, 1989). This indicates that lack of body reserves in the postpartum period can directly inhibit LH pulse frequency.

The mechanism(s) of inhibition of LH pulse frequency by undernutrition and low body CS are not fully understood. It is likely that metabolic hormones (insulin, IGF, GH) and/or metabolites themselves are involved in the process (Schillo, 1992). Cows with extended periods of NEB have elevated circulating concentrations of NEFA (Canfield and Butler, 1991) which are associated with lipolysis. Some specific amino acids have been shown to affect LH concentrations following infusion (Schillo, 1992). It has been hypothesised that these amino acids may be involved with synthesis of neurotransmitters (Schillo, 1992). Alternatively, the availability of metabolic fuels in the central nervous system may directly affect GnRH release. Oestrus can be blocked by infusion of 2-deoxy 2-glucose, an inhibitor of glycolysis (McClure *et al.*, 1978),

and the concentration of LH is reduced following hypoglycaemia induced by phlorizin (Rutter and Manns, 1987). There also appears to be an interaction between ovarian dependant and nutritional control of LH pulse frequency. Undernourished, ovariectomised beef cows have a lower LH pulse frequency following chronic E_2 treatment than well-fed controls or underfed animals not treated with E_2 (Imakawa *et al.*, 1987).

Suckling by calves has also been shown to inhibit LH pulse frequency following ovariectomy (Garcia-Winder *et al.*, 1984; 1986a). The opiate antagonist, naloxone, increases LH pulse frequency in suckled, ovariectomised beef cattle (Rund *et al.*, 1989). Similarly, naloxone increases and morphine decreases mean LH concentrations and LH pulse frequency in ovariectomised, lactating dairy cows (Nanda *et al.*, 1989), indicating that the opiates may be involved in controlling GnRH pulse frequency both in suckled and machine milked cattle. However, infusion of naloxone did not alter any LH parameters in acutely weaned postpartum dairy cows in another experiment (Canfield and Butler, 1991).

Control of the E_2 induced LH surge

The dose of E_2 required to induce an LH surge in the seasonally anoestrous ewe is the same as that required in the breeding season. Additionally, infusion of LH over several days resulted in an increase in endogenous E_2 concentration and an LH surge in a majority of seasonally anoestrous ewes (Karsch *et al.*, 1980). This suggests that the E_2 positive feedback mechanism is not effected by season in the ewe. In contrast, only 10 of 15 and 5 of 15 anovulatory cows had an LH surge and ovulated following ODB treatment, respectively (Chapter 7). Failure of E_2 to induce an LH surge has been reported in anovulatory, ovariectomised beef cows (Richards *et al.*, 1991). The dose of E_2 and the method of delivery (injection rather than the subcutaneous implant in the case of Karsch *et al.*, 1980) may have contributed to this failure of the E_2 positive feedback in the cattle experiments. To confirm that changes in E_2 positive feedback do occur, a dose response trial involving cows ovariectomised when cycling and when

anovulatory is required, with the E_2 being delivered either as a bolus injection or as a subcutaneous implant.

Conclusions from follicular wave and endocrinology data

The anovulatory dairy cows studied in this thesis had large follicles present in their ovaries from 10 days postpartum but these follicles failed to ovulate. These cows had a lower LH pulse frequency before and after ovariectomy and lower intrafollicular steroid concentrations than cycling cows. This suggests that insufficient LH support for the developing follicles was occurring, resulting in insufficient E_2 production to induce ovulation. The mechanism for this depressed LH pulse frequency may be related to depleted body CS at calving or reduced feed intake in the postpartum period, resulting in extended periods of NEB. Increased sensitivity to E_2 feedback was demonstrated. However, failure of the positive feedback release of LH by E_2 was also demonstrated suggesting that both inhibitory and positive E_2 control of GnRH and hence LH release is dysfunctional in these cows.

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