UTERO-OVARIAN SIGNALLING IN THE POSTPARTUM COW

Thesis submitted in accordance with the requirements of the University of Liverpool

for the degree of Doctor in Philosophy by Iain Martin Sheldon

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Abstract

Utero-ovarian signalling in the postpartum cow

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The aim of the present thesis was to test the hypothesis that signalling between the ovary and the uterus is involved in the regulation of uterine involution and the resumption of cyclic ovarian activity in the postpartum cow. Fewer cows examined once between 14 and 28 days postpartum had a corpus luteum (16.9% vs. 37.0%, $P < 0.001$), or a follicle > 8 mm diameter (26.1% vs. 49.6%, $P < 0.001$) in the ovary ipsilateral to the previously gravid uterine horn, compared with the contralateral ovary. However, the presence of a follicle > 8 mm diameter in the ovary ipsilateral to the previously gravid uterine horn was associated with a shorter calving to conception interval compared with animals without such a follicle (99.0 ± 5.6 days, n = 74, vs. 112.8 ± 4.4 days, n = 210; $P < 0.05$). These observations raise an intriguing issue: how does this follicle affect subsequent fertility - does the follicle exert a local influence on the uterus, or *vice versa*?

Two studies in cattle and one in sheep examined the localised effect of large oestradiol-secreting ovarian follicles on uterine involution. Cows were administered intramuscular equine chorionic gonadotrophin (eCG) 14 days postpartum to stimulate follicular growth; oestradiol benzoate was administered into the previously gravid uterine horn lumen on Days 7 and 10; or, a 3 cm silastic implant containing oestradiol was sutured to the ovarian bursa ipsilateral to the previously gravid uterine horn after parturition in ewes. The eCG-stimulated follicular growth overcame the inhibition of follicular growth in the ovary ipsilateral to the previously gravid uterine horn, and increased oestradiol secretion compared with control animals. However, across the three studies, there was no consistent evidence that oestradiol had a systemic or localised effect on uterine involution.

To examine the effect of the regressing corpus luteum of pregnancy on folliculogenesis in the ipsilateral ovary after parturition, cows were treated with prostaglandin $F_{2\alpha}$ between 190 and 220 days of gestation to cause luteolysis, but without inducing parturition. There was no significant effect of treatment on postpartum dominant follicle ovarian location, growth, or function. Thus, an effect of the previously gravid uterine horn on ovarian function shortly after parturition should be considered.

The final study examined the effect of uterine bacterial contamination on ovarian function using swabs collected from the uterine body lumen on Day 7, 14, 21 and 28 postpartum. Bacteria were identified by aerobic and anaerobic culture; bacterial growth was scored semi-quantitatively and animals categorized into standard or high bacterial contamination based on the number of colonies detected. There was no effect of bacterial contamination on plasma FSH concentration profiles, or ovarian follicle wave emergence. However, when uterine bacterial growth scores were high on Day 7 or Day 21, fewer first (1/20 vs. 15/50, $P < 0.05$) or second dominant follicles (1/11 vs. 13/32, $P < 0.05$) were selected in the ipsilateral than the contralateral ovary, respectively. The diameter of the first dominant follicle was smaller in animals with a high Day 7 bacterial score ($P < 0.001$), dominant follicle growth was slower ($P < 0.05$) and oestradiol secretion was reduced ($P < 0.05$).

In conclusion, the present thesis provides evidence for uterine signals after parturition that affect ovarian function and subsequent fertility, and the signals are modified by the response to uterine bacterial contamination.

Acknowledgements

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Abbreviations and trivial names

The following abbreviations have been used in this thesis

CL	Corpus luteum
eCG	Equine chorionic gonadotrophin
FSH	Follicle stimulating hormone
GnRH	Gonadotrophin releasing hormone
IGF	Insulin-like growth factor
IL	Interleukin
im	Intramuscular
Ipsilateral ovary	Ovary ipsilateral to the previously gravid uterine horn
iu	International unit
iv	Intravenous
LH	Luteinising hormone
mRNA	Messenger ribonucleic acid
PGUH	Previously gravid uterine horn
PMSG	Pregnant mare serum gonadotrophin
RIA	Radioimmunoassay
Spp	Species
SEM	Standard error of the mean
TNF_{α}	Tumour necrosis factor - α

The following trivial names have been used in this thesis

Androstenedione	4-androstene-3-one
Oestradiol	oestra-1,3,5,(10)-oestratrien-17β-one
PGFM	15-keto-13,14-dihydro-prostaglandin $F_{2\alpha}$
Progesterone	4-pregene-3,20-dione
Prostaglandin $F_{2\alpha}$	9,11,15-trihydroxy-5-cis,13-trans-prostadienoic acid
Testosterone	17β-hydroxy-4-androsten-3-one

Publications from the thesis

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Chapter 1

Introduction

Ovarian function in cattle is characterised by the regular cyclic emergence and growth of waves of ovarian follicles from before puberty until death (Pierson and Ginther, 1987a; Evans *et al.*, 1994; Ginther *et al.*, 1996a). This cyclic ovarian activity is only punctuated by a matter of weeks before and after parturition when the waves are not observed (Savio *et al.*, 1990a; Ginther *et al.*, 1996a). For most of the functional life of the ovary, the relationship with the uterus would appear to be simply the source of the luteolytic signal (Ginther, 1968a). Most research into the biology of cattle reproduction and the oestrous cycle has focussed on the predictable first wave of follicular growth after ovulation in heifers (Pierson and Ginther, 1987a; Adams *et al.*, 1992; Ginther *et al.*, 1999; Rivera *et al.*, 2001). Conversely, most subfertility involves the return of ovarian cyclic activity after parturition in lactating cows (Borsberry and Dobson, 1989; Lamming and Royal, 1999; Roche *et al.*, 2000).

After parturition, the genital tract must return to a normal non-pregnant state before insemination is likely to successfully establish the next pregnancy. A short but predictable postpartum period is important because reproductive efficiency is the major determinant of lifetime productivity of farm animals and optimal fertility is essential for farm profitability (Dijkhuizen *et al.*, 1985; Stott *et al.*, 1999). The postpartum period comprises uterine involution, elimination of bacterial contamination, and resumption of normal ovarian cyclic activity.

The progression of uterine involution after parturition has been clearly described (Gier and Marion, 1968; Okano and Tomizuka, 1987). Similarly, the elimination of bacterial contamination has been widely investigated because it is ubiquitous in postpartum cattle and is associated with subfertility (Elliot *et al.*, 1968; Griffin *et al.*,

1974). Finally, with the advent of transrectal ultrasonography, observations of postpartum ovarian structures have been integrated with measurements of peripheral plasma hormone concentrations to describe the return of ovarian cyclic activity (Savio *et al.*, 1990a; Beam and Butler, 1997). Each of these individual components of the postpartum period contributes to the physiological jigsaw of this important time. However, few have investigated the integration and interaction of the systems, such as the ovary and the uterus, or the endocrine and immune systems.

An insight into how the uterus and ovary might interact after parturition, is revealed in the first four weeks, when there is an intriguing predilection for follicular growth in the ovary contralateral to the previously gravid uterine horn (Saiduddin *et al.*, 1967; Kamimura *et al.*, 1993). However, the mechanisms underlying the inhibition of folliculogenesis in the ipsilateral ovary have not been determined. Mechanisms could involve a local inhibitory effect on the ovary of the previously gravid uterine horn or its contents, or an effect within the ovary of the regressing corpus luteum of pregnancy (Dufour and Roy, 1985; Nation *et al.*, 1999).

The potential importance of this postpartum ovarian asymmetry is indicated by a small study where the unexpected presence of a larger follicle in the ipsilateral ovary on Day 26 ± 3 postpartum was associated with improved subsequent fertility (Bonnett *et al.*, 1993). One explanation might be that a large oestradiol-secreting ovarian follicle has a localised beneficial effect on the involution of the ipsilateral previously gravid uterine horn. On the other hand, the presence of such a large follicle might reflect a uterus-to-ovary signal indicating relative uterine health.

The present thesis used an integrated series of whole-animal studies to test the hypothesis that signalling between the ovary and the uterus is involved in the regulation of uterine involution and the resumption of cyclic ovarian activity in the

postpartum cow. Firstly, transrectal ultrasonography was used to examine if asymmetric ovarian folliculogenesis after parturition is a marker of fertility. Whether oestradiol has a localised effect on uterine involution, was investigated in three experiments using different strategies to deliver oestradiol. The effect of the regressing corpus luteum of pregnancy on postpartum folliculogenesis was examined by inducing premature luteolysis during the last trimester of pregnancy. In the final study, the effect of uterine bacterial contamination on ovarian function was investigated. If proven, the hypothesis will have important implications for veterinary medicine.

Chapter 2

Literature review

After parturition, the genital tract recovers and returns to a normal non-pregnant state ready for the establishment of the next pregnancy. The initial stimulus for these changes to occur is the expulsion of the fetus at parturition along with the associated membranes and fluids. The events that comprise the postpartum period are uterine involution, involving contraction of the uterus and re-modelling of the tissues; elimination of the inevitable uterine bacterial contamination; and resumption of normal ovarian cyclic activity. The postpartum period is important because delayed uterine involution, the presence of postpartum uterine infection, or abnormal ovarian function causes subfertility which results in substantial economic loss for the cattle industry (Borsberry and Dobson, 1989; Esslemont and Kossaibati, 2000).

Uterine involution

Uterine involution involves uterine contractions, physical shrinkage, necrosis and sloughing of the caruncles, and regeneration of the endometrium (Gier and Marion, 1968). The amount of uterine tissue change is illustrated by the decrease in weight from about 9.0 kg at parturition to 1.0 kg by Day 30, and 0.75 kg by Day 50 postpartum. In addition to changes in weight, uterine involution can be monitored by repeated estimation of size using palpation *per rectum* or transrectal ultrasonography (Fig. 1), reducing the between animal variation inherent in slaughter studies for monitoring uterine involution (Gier and Marion, 1968; Okano and Tomizuka, 1987). The previously gravid uterine horn is wider and longer than the non-gravid horn and this difference can be distinguished up to 30 days postpartum (Okano and Tomizuka, 1987; Tian and Noakes, 1991a; Risco *et al.*, 1994).

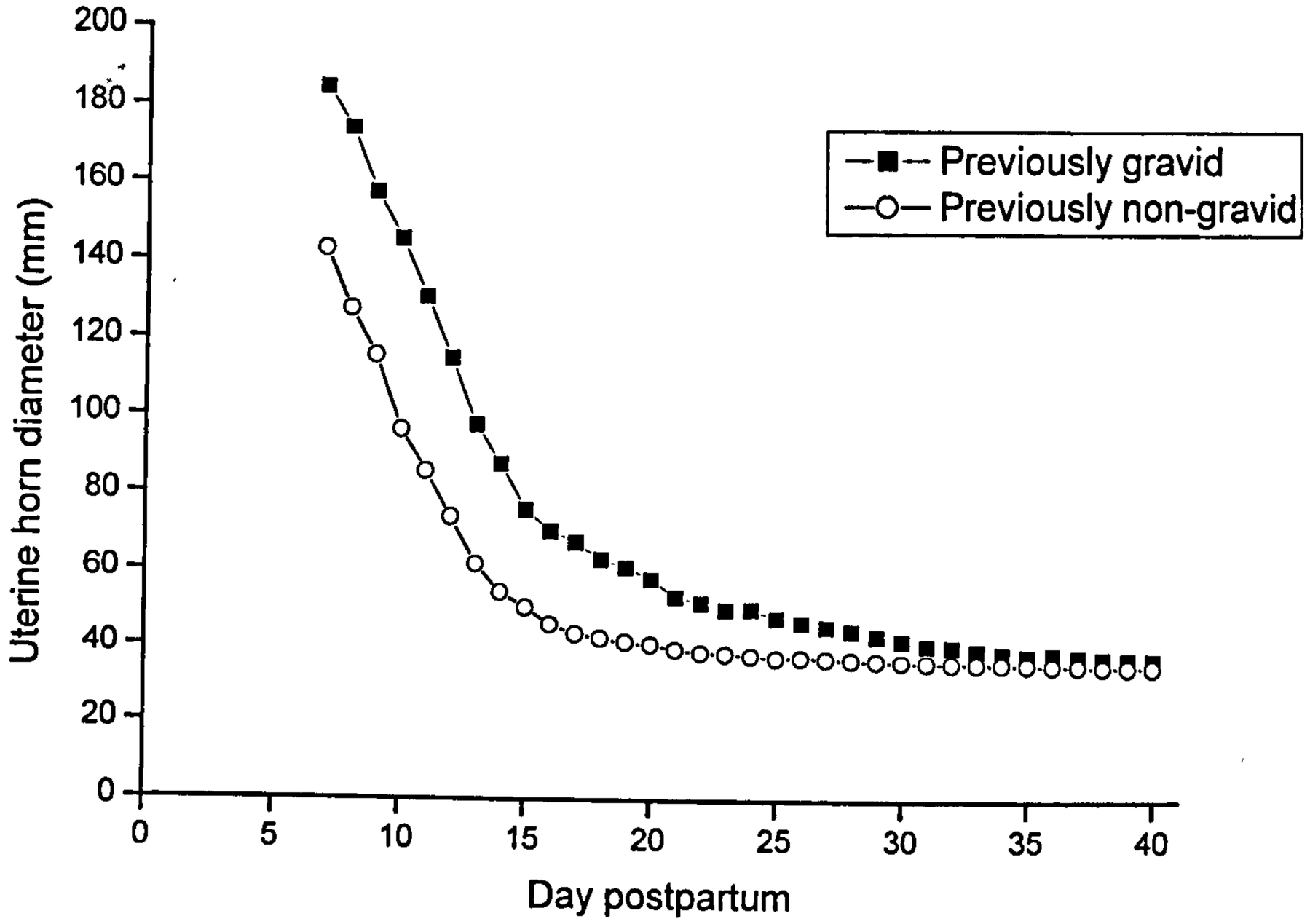

Figure 1. Progress of uterine involution between Day 7 and 40 postpartum as determined using transrectal ultrasonography to measure the diameter of the previously gravid (■) and non-gravid (○) uterine horns at the level of attachment of the intercornual ligament. Drawn from the data of Tian and Noakes (1991a, n = 25) and Sheldon (unpublished observations, n = 70).

The greatest change in uterine dimensions occur in the first few days after parturition and it is almost complete by Day 30 (Gier and Marion, 1968; Morrow *et al.*, 1969; Kaidi *et al.*, 1991a). The precise time at which involution is complete has been the subject of much debate, and in normal animals is dependent on the criteria selected for evaluation, the method of measurement, and biological variation. Estimates for completion of uterine involution vary between 23 and 50 days after

parturition (Gier and Marion, 1968; Rajamahendran and Taylor, 1990). The biological value of determining the precise day for completion of uterine involution is not clear, and physical dimensions may not fully represent the underlying cellular and biochemical changes. On the other hand, factors that delay uterine involution are important because they can cause future subfertility (Fonseca *et al.*, 1983).

Uterine involution is also reflected by changes in peripheral plasma concentrations of the prostaglandin $F_{2\alpha}$ metabolite (15-keto-13, 14-dihydro-prostaglandin $F_{2\alpha}$, PGFM), which increase at parturition and then gradually decrease to basal concentrations by about 20 days postpartum (Lindell *et al.*, 1982). As uterine involution involves significant tissue remodelling, measurement of markers of tissue catabolism such as hydroxyproline also reflect involution (Kaidi *et al.*, 1991b). However, changes in plasma concentrations of collagen metabolites are not restricted to those caused by uterine remodelling and are also influenced by other tissues such as bone, tendons and ligaments which are also affected by changes in steroid hormone concentrations (Ho and Weissberger, 1992; Peris *et al.*, 1999). Acute phase proteins are produced by heptaocytes in response to tissue damage and inflammation (Baumann and Gauldie, 1994). Plasma acute phase protein concentrations have been used to examine the process of uterine involution as the concentrations increase to maximum values between 1 and 3 days postpartum, and then decrease within two weeks to basal concentrations (Alsemgeest *et al.*, 1993; Regassa and Noakes, 1999; Sheldon *et al.*, 2001). However, one confounding factor is that uterine bacterial contamination also increases the plasma concentrations of PGFM and acute phase proteins during the postpartum period (Del Vecchio *et al.*, 1992; Sheldon *et al.*, 2001).

Factors that influence uterine involution have been investigated, and the most consistent observations are that involution is slower after postpartum disease such as dystocia, hypocalcaemia, retained fetal membranes or uterine infection (Morrow *et al.*, 1966; Oltenacu *et al.*, 1983). Involution is more rapid for primparous, than pluriparous cows (Marion *et al.*, 1968; Fonseca *et al.*, 1983); and, may differ between breeds (Larsson *et al.*, 1984). Uterine involution has been reported to be slower in winter, compared with spring or summer (Marion *et al.*, 1968).

The effect of hormonal environment on uterine involution is not clear. The interval to completion of involution was not affected by parenteral administration of oestradiol throughout the postpartum period, but was shorter in cows ovariectomized 3 to 5 days postpartum (Marion *et al.*, 1968). Furthermore, other attempts to pharmacologically hasten uterine involution, including the administrations of oxytocin, prostaglandin $F_{2\alpha}$ and GnRH have not been outstandingly successful (Leslie *et al.*, 1984; Tian and Noakes, 1991a). On the other hand, the genital tract is exquisitely sensitive to physiological doses of oestrogens administered parenterally during the puerperium (Rosenfeld, 1980). Oestradiol stimulates a several fold increased blood flow to the myometrium, endometrium and caruncles, with a greater magnitude of response with increasing interval postpartum.

During the process of uterine involution, there is a concomitant necrosis, followed by sloughing of the uterine caruncle (Archbald *et al.*, 1972). Subsequently, there is re-epithelialisation, such that the entire caruncular surface is covered with epithelium by 19 days postpartum. Subfertility has not been reported in association with problems of regeneration of the endometrium beyond Day 26 postpartum (Bonnett *et al.*, 1993).

Elimination of uterine bacterial contamination

During or shortly after parturition, when the vulva is relaxed and the cervix dilated, micro-organisms from the animal's environment, skin and faeces contaminate the uterine lumen. The postpartum uterine lumen supports the growth of a variety of aerobic and anaerobic bacteria. In one study, 93% of the uteri obtained within 15 days of calving yielded bacteria on aerobic and anaerobic culture of lumenal swabs and endometrial tissue (Elliot *et al.*, 1968). The number of uteri from which bacteria were isolated had declined to 78% by 16 to 30 days, 50% by 31 to 45 days, and 9% by 46 to 60 days postpartum. The progressive elimination of bacterial contamination is a consistent feature of several similar studies using samples collected from the uterus during the postpartum period (Fig. 2).

The proportion of animal in which uterine bacterial contamination persists for several weeks is, of course, similar to the 10 to 15% incidence of uterine bacterial disease, known as the clinical condition endometritis (Borsberry and Dobson, 1989). Endometritis is defined histologically as inflammation of the endometrium, but is characterised clinically by the presence of a mucopurulent vaginal discharge, 21 days or more after calving, and is often associated with delayed uterine involution (Sheldon and Noakes, 1998). Endometritis is an important disease because it is common, and causes considerable financial loss to the cattle industry. The total economic cost of a case of endometritis was estimated at £160 (Kossaibati and Esslemont, 1997). Thus, the cost of endometritis to the UK dairy industry is about £48 M per annum.

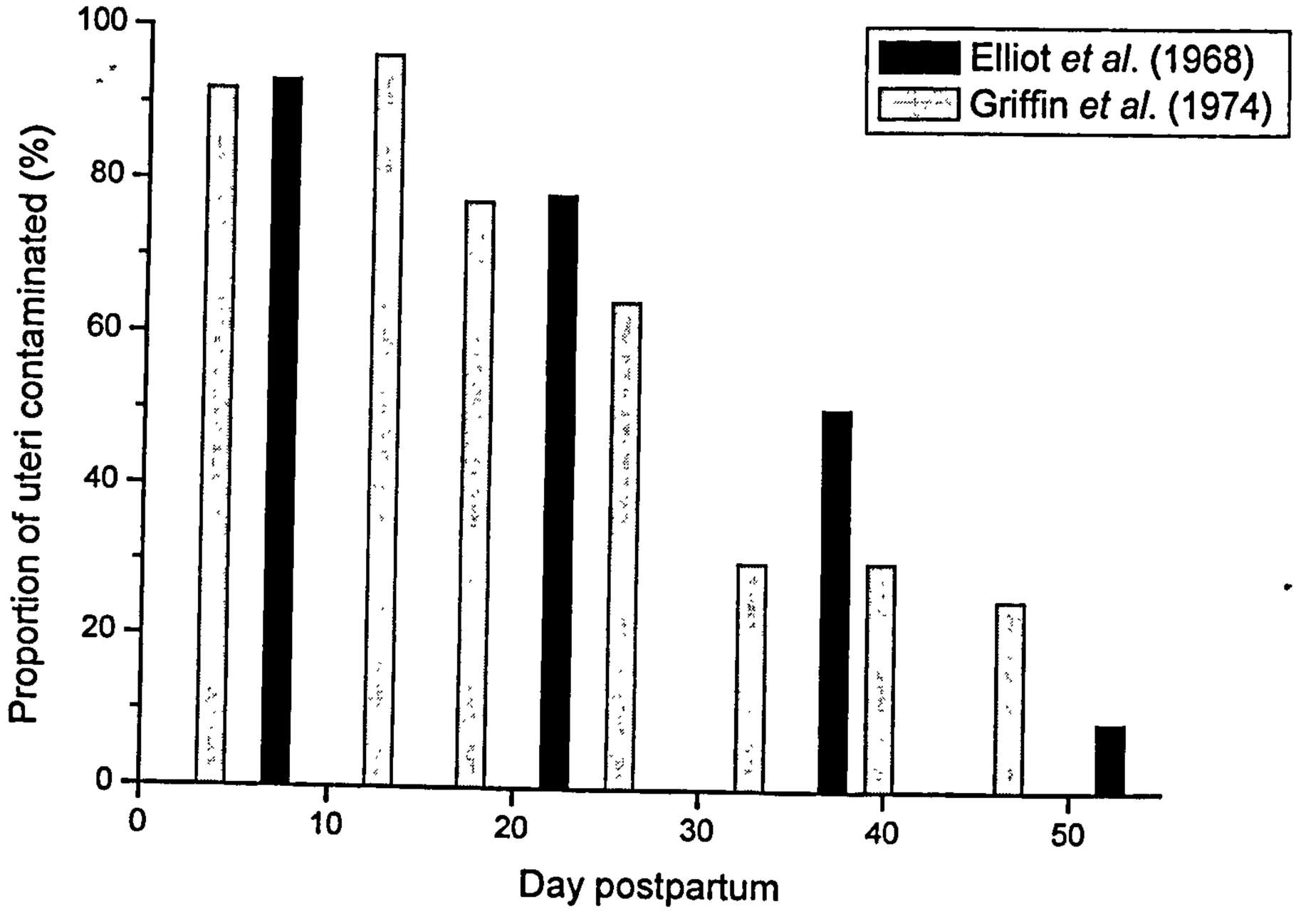

Figure 2. Proportion (%) of cattle uteri contaminated with bacteria during the postpartum period. Drawn from data of Elliot *et al.* (1968) and Griffin *et al.* (1974).

Many bacteria isolated from the uterine lumen are simple contaminants, and are removed from the uterus by a range of defence mechanisms in the live animal. Thus, there is a constantly fluctuating bacterial flora in the first 7 weeks postpartum due to spontaneous contamination, clearance and recontamination (Griffin *et al.*, 1974). The determination of which bacterial isolates are contaminants and which are potential pathogens, associated with infection, varies between studies. However, uterine infection is most commonly associated with the presence of *Escherichia coli, Arcanobacterium pyogenes, Fusobacterium necrophorum* and *Prevotella* (formerly *Bacteroides*) species. Indeed, *A. pyogenes, F. necrophorum* and *Prevotella* species

can act synergistically to enhance the likelihood of uterine disease, and increase the risk of clinical endometritis and its severity (Ruder *et al.*, 1981; Olson *et al.*, 1984). Furthermore, *A. pyogenes* is associated with severe endometritis, increased uterine tissue lesions, and subsequent infertility (Griffin *et al.*, 1974; Bonnett *et al.*, 1991).

In addition to the bacterial species present, there are several risk factors for the establishment of uterine infection (Andriamanga *et al.*, 1984; Lewis, 1997; Sheldon, 1999). The factors most frequently associated with uterine infection are those that disrupt normal parturition including stillbirth, twins, dystocia, or a caesarean section operation (Hussain *et al.*, 1990; Peeler *et al.*, 1994). The mechanisms may involve a concomitant delay in uterine involution, which delays the expulsion of lochia; disruption of neutrophil function; and, tissue damage. Retained fetal membranes are also an important risk factor for persistence of uterine bacterial infection (Markusfeld, 1984). Presumably, the presence of large amounts of necrotic tissue provides an ideal environment for the growth of bacteria. In addition, there is an association between uterine infection and ketosis or other metabolic diseases, although the specific mechanisms are not clear (Markusfeld, 1987).

The hormonal environment also affects elimination of bacterial contamination. In particular, administration of oestrogens or induction of oestrus appears to enhance the elimination of bacterial infection (Rowson *et al.*, 1953). Oestradiol at doses of 5 to 10 mg per animal is used therapeutically for postpartum endometritis and is as effective as oestrus induced using prostaglandin $F_{2\alpha}$, and both are superior to the spontaneous recovery rate of untreated animals (Pepper and Dobson, 1987; Sheldon and Noakes, 1998). Conversely, bacterial growth is facilitated in a progesterone-dominated uterine environment (Olson *et al.*, 1984; Noakes *et al.*, 1990). The mechanism underlying these observations may involve an effect on neutrophil

function; however, treatment of cows with exogenous oestradiol or progesterone did not produce consistent effects on peripheral plasma or intrauterine neutrophil function (Subandrio *et al.*, 2000).

On the other hand, uterine bacterial contamination is a key risk factor for abnormal ovarian cyclic activity after parturition (Opsomer *et al.*, 2000). Endometritis is an important contributor to bovine infertility extending the calving to conception interval, increasing the number of services per conception and the proportion of culls for failure to conceive (Tennant and Peddicord, 1968; Borsberry and Dobson, 1989). Even following successful treatment for uterine bacterial infection, these animals have a 26% lower probability of becoming pregnant (Borsberry and Dobson, 1989). Unfortunately, even the pregnancy rates for normal cattle are lower than most other species, and have declined over the last 20 years to the current 43% per oestrous cycle (Lamming and Royal, 1999).

Resumption of normal ovarian cyclic activity

The oestrous cycle

Transrectal ultrasonography and measurement of plasma and ovarian follicle hormone concentrations have been used to elucidate the events comprising the bovine oestrous cycle. Ovarian follicular development occurs in a wave pattern with 2 or 3 sequential waves during each oestrous cycle as illustrated in Fig. 3 (Pierson and Ginther, 1988; Savio *et al.*, 1988; Sirios and Fortune, 1988; Adams, 1999). Each follicular wave is preceded by an increase in plasma FSH concentration (Adams *et al.*, 1992; Sunderland *et al.*, 1994). The increase in FSH concentration that precedes the first follicular wave of an oestrous cycle has been recognised as the secondary

surge of FSH after ovulation (Dobson, 1978a). Each wave pattern starts with the contemporaneous recruitment of several follicles, which is the term given to the growth of follicles beyond the stage at which most undergo atresia (Fortune, 1994). Follicle emergence is defined by the smallest diameter used for generating growth profiles by ultrasonography, and involves three to seven 4 mm diameter follicles, which then enter a common-growth phase (Fortune *et al.*, 2001; Ginther *et al.*, 2001a). Follicle selection occurs within several days, at the end of the common-growth phase; the dominant follicle in one of the ovaries continues to grow and differentiate, whilst the remaining or subordinate follicles become atretic and regress or temporarily grow at a reduced rate before atresia and regression. This partitioning of growth during selection of the dominant follicle has been called deviation, and is coincident with the decrease in plasma FSH concentrations (Adams *et al.*, 1993; Ginther *et al.*, 2001a).

Selection of the dominant follicle results in oestradiol production, negative feedback on FSH secretion, with the follicle subsequently becoming LH dependent (Ginther *et al.*, 1996b). The fate of the dominant follicle is dependent on LH pulse frequency. Insufficient LH pulse frequency is the underlying mechanism for dominant follicle atresia (Sirois and Fortune, 1990). Luteal phase progesterone secretion suppresses LH pulse frequency such that the first or first and second dominant follicles undergo atresia in a two or three wave cycle, respectively; the next follicular wave then emerges. Functional regression of follicles is characterised by decreased steroidogenic capacity, before morphological degeneration is evident (Bao and Garverick, 1998). In contrast, after luteolysis at the end of the oestrous cycle, LH pulse frequency increases stimulating further growth of the final dominant follicle which leads to increased plasma oestradiol concentrations, positive feedback

on the hypothalamo-pituitary axis, and stimulation of an LH surge and ovulation (Moenter *et al.*, 1991; Yoshioka *et al.*, 2001). However, the dominant follicle of any follicular wave can ovulate in an appropriate endocrine environment, such as after administration of prostaglandin $F_{2\alpha}$ to cause luteolysis (Fortune *et al.*, 2001; Sartori *et al.*, 2001).

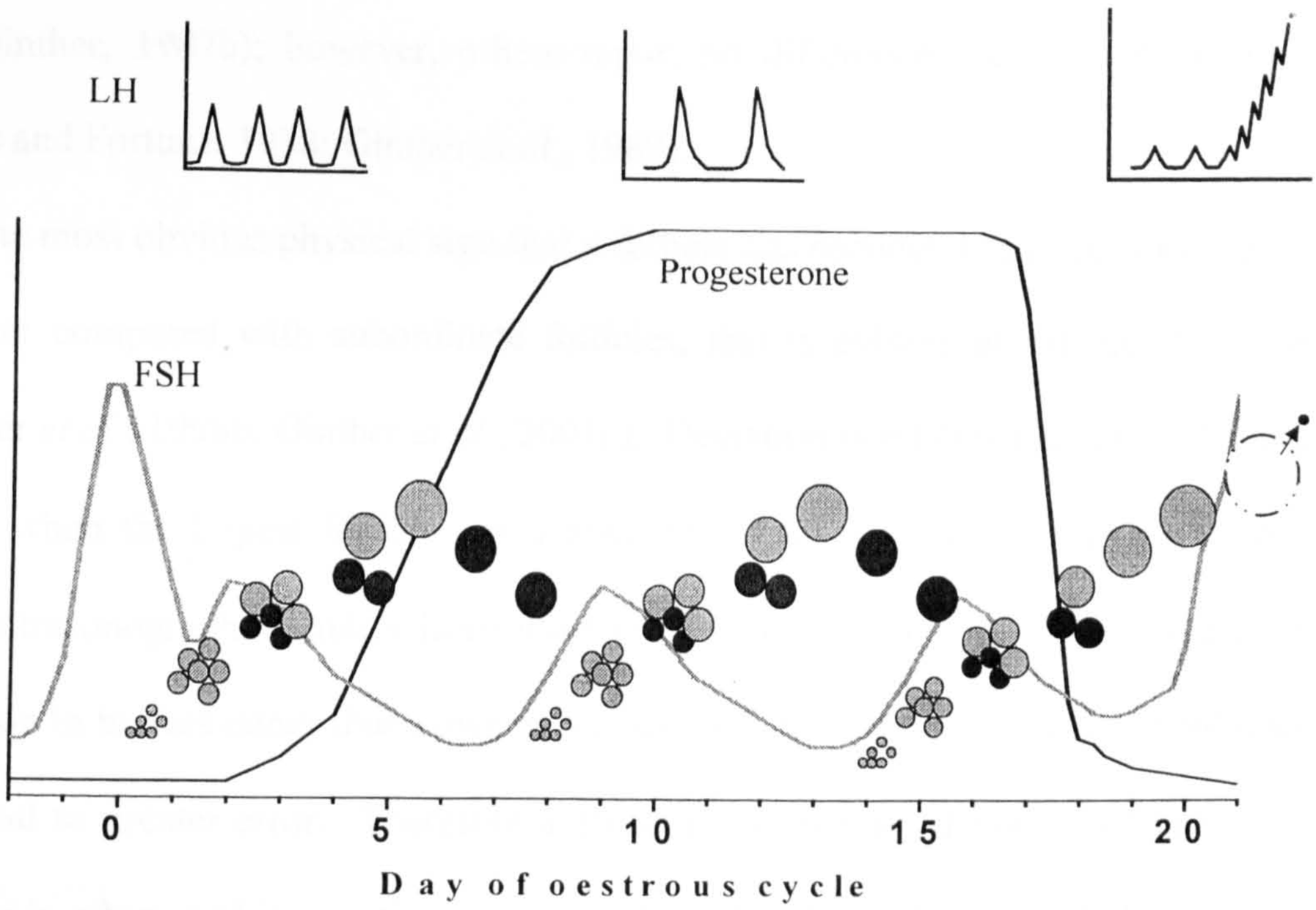

Figure 3. A model of a three follicle wave oestrous cycle. Several follicles (◉) emerge as plasma FSH concentrations increase, with a single dominant follicle selected from each wave, and the subordinate follicles undergoing atresia (●). After luteolysis, the plasma progesterone concentration falls, plasma LH concentrations increase, and the dominant follicle ovulates.

During the oestrous cycle of heifers there are more follicles, including dominant follicles, in the ovary containing the active corpus luteum, but not when the corpus luteum is regressing after luteolysis (Pierson and Ginther, 1987b; Savio *et al.*, 1988; Driancourt *et al.*, 1991). One possible explanation is that the effect of the corpus luteum on folliculogenesis could reflect increased blood flow to the ovary bearing the corpus luteum compared with the contralateral ovary (Niswender *et al.*, 1973; Wise *et al.*, 1982). Another asymmetric feature of ovarian function is that some workers have reported more ovulations are from the right than the left ovary (Pierson and Ginther, 1987b); however, others report no differences between the ovaries (Sirios and Fortune, 1988; Ginther *et al.*, 1989).

The most obvious physical sign that a follicle has become dominant is the larger diameter compared with subordinate follicles, that is evident at follicle deviation (Ginther *et al.*, 1996b; Ginther *et al.*, 2001c). Deviation is established within 8 h and begins when the largest follicle has a diameter of 8.5 mm (Ginther *et al.*, 1999). Most ultrasonographic studies have used the predictable first follicular wave after ovulation in heifers rather than cows, where the less precise measurement of follicles may lead to greater error. Therefore a 10 mm diameter for deviation of dominant follicles is often used in practice for cows when identifying dominant follicles using ultrasonography (Dobson *et al.*, 2000). However, before physical deviation is evident, a number of biochemical changes occur including the greater capacity for oestradiol production, which is a defining characteristic of the dominant follicle. Dominant follicles secrete substantially more oestradiol than other pre-dominant recruited follicles, or other subordinate follicles after selection of the dominant follicle (Badinga *et al.*, 1992; Fortune, 1994). Specifically, it is the granulosa cells of

dominant follicles that secrete more oestradiol than granulosa cells of subordinate follicles (Evans and Fortune, 1997).

The hormonal control of follicle deviation involves decreasing plasma FSH concentrations below those required by the subordinate follicles, so that only the dominant follicle continues to grow and differentiate. The termination of increasing plasma FSH concentrations and the decrease to basal concentrations is associated with secretion of oestradiol by the dominant follicle, (Ginther *et al.*, 2000a; Ginther *et al.*, 2000b), inhibin (Kaneko *et al.*, 1997; Bleach *et al.*, 2001), and possibly androgens (Evans *et al.*, 1997). However, there is a close two-way functional coupling between FSH and follicular growth; although plasma FSH concentrations decrease as the dominant follicle grows, FSH is still required to support that growth (Ginther *et al.*, 2000a). Although, these mechanisms are components of dominant follicle selection, why a particular follicle should be selected is still not clear. Ultrasonographic studies suggest that the future dominant follicle is slightly larger than others in the cohort, but the size differences are small and not always predictive of future dominance (Mihm *et al.*, 2000).

At the cellular level oestradiol synthesis involves the two-cell: two-gonadotrophin model (Sirios and Fortune, 1988). Androgen biosynthesis is supported by the action of LH on theca cells. Androgens are then transported to the granulosa and converted to oestrogens by aromatisation, initially under the influence of FSH. The mRNA for 17α-hydroxylase ($P450_{17\alpha}$), the enzyme that converts progestins to androgens (Fig. 4), is localised in thecal cells (Bao and Garverick, 1998). Whereas, FSH receptor (FSHr) and the aromatisation enzyme ($P450_{arom}$) mRNAs are localised in granulosa cells. Whilst LH receptors (LHr), the side-chain cleavage enzyme ($P450_{scc}$) and 3β hydoxysteroid dehydrogenase (3β-HSD) are found

in both theca and granulosa; their mRNAs are expressed in the different cell types during follicle growth. Recruited follicles have increased mRNA for aromatase, whereas follicle selection results in greater mRNA expression for the gonadotrophin receptors (LHr and FSHr) and the enzymes involved in androgen and progestin synthesis (Xu *et al.*, 1995; Bao and Garverick, 1998). Therefore, it has been suggested that the selected dominant follicle is the first to acquire LHr on the granulosa cells, resulting in oestradiol synthesis in response to LH as well as FSH (Ginther *et al.*, 1996b). However, the acquisition of LHr in the granulosa cells appears to occur after selection has occurred so it is not clear what pro-active role they play, if any, in achieving dominance (Evans and Fortune, 1997; Fortune *et al.*, 2001). On the other hand, after selection of the dominant follicle, growth is principally maintained by the action of LH on theca cells to provide androgen substrate, and on granulosa cells for increased aromatase activity, and the acquisition of LH responsiveness is critical for ovulation (Bao and Garverick, 1998; Ginther *et al.*, 2001b). However, pulsatile LH acts synergistically with FSH to stimulate the growth of large ovulatory follicles and both are necessary for the optimum secretion of oestradiol (Campbell *et al.*, 1998; Crowe *et al.*, 2001).

Immediately before the LH / FSH surge, mRNAs for the steroidogenic enzymes $P450_{scc}$, 3β-HSD and $P450_{17\alpha}$, but not $P450_{arom}$, increase in theca and granulosa cells and there is increased oestradiol production by granulosa cells (Bao and Garverick, 1998). After the LH surge, mRNAs for steroidogenic enzymes decrease rapidly and follicular fluid concentrations of oestradiol decrease and progesterone increase. In dominant follicles that do not ovulate, but undergo atresia, there is decreased granulosa cell mRNA for FSHr, theca cell LHr and $P450_{17\alpha}$, and $P450_{scc}$ in theca and granulosa cells. The outcome of these changes is decreased oestradiol concentrations

in atretic follicles. Similar events occur in unselected follicles that undergo atresia. Although current understanding of folliculogenesis reflects *in vivo* observations, there are also further complexities with functional differences within cellular components, such as between mural, antral, and cumulus granulosa cells (Rouillier *et al.*, 1996; Khamsi and Roberge, 2001).

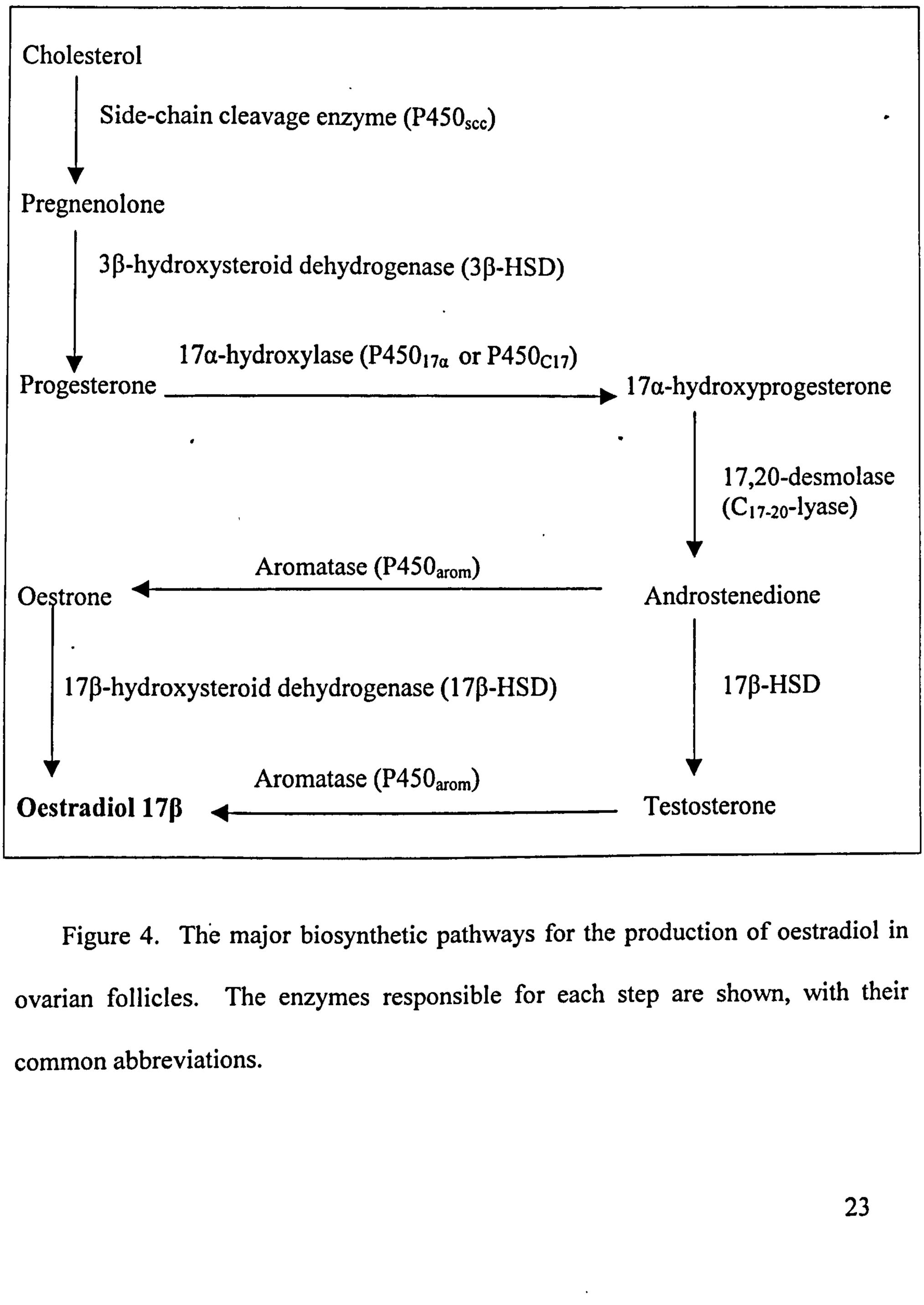

Figure 4. The major biosynthetic pathways for the production of oestradiol in ovarian follicles. The enzymes responsible for each step are shown, with their common abbreviations.

Another system critical for follicular growth and ovulation is the insulin-like growth factor (IGF) system comprising two molecules (IGF-1 and IGF-2), two receptors (Types 1 and 2), and six binding proteins (IGFBP 1 to 6) (Poretsky *et al.*, 1999). The most important ovarian component promoting follicular growth appears to be the action of IGF-1 on Type 1 receptors, with the bioavailability of IGF-1 increased by decreasing concentrations of IGFBPs. In vitro, IGF-1 acts synergistically with FSH to stimulate proliferation of granulosa cells and oestradiol secretion (Gong *et al.*, 1994; Gutierrez *et al.*, 1997). The search for factors that control IGF-1 concentrations in ovarian follicles has focussed on the binding proteins, particularly IGFBP-4. In each follicular cohort, the recruited follicle with the lowest follicular fluid IGFBP-4 concentration always became the dominant follicle (Mihm *et al.*, 2000). Further, the concentration of IGFBP-4 is regulated by the action of IGFBP-4 protease causing degradation of IGFBP-4, and the protease activity is increased in follicular fluid of dominant compared with subordinate follicles (Rivera *et al.*, 2001). In addition, experimental production of co-dominant follicles is characterised by the presence of increased IGFBP-4 protease activity in both dominant follicles, following stimulation by exogenous FSH (Rivera and Fortune, 2001). Thus, it is hypothesised that FSH induces IGFBP-4 protease in the future dominant follicle, reducing intra-follicular IGFBP-4 which increases free IGF-1 concentrations, and stimulates follicular growth and oestradiol secretion (Rivera *et al.*, 2001).

Many other molecules may affect follicle growth and function, including the Transforming Growth Factor-β (TGF-β) superfamily, Epidermal Growth Factor (EGF), and cytokines (Rouillier *et al.*, 1997; Webb *et al.*, 1999b). Most of the evidence for the role of these compounds is based on in vitro tissue culture studies,

and their mechanism of action is presumed to be principally autocrine or paracrine (Webb *et al.*, 1999a).

Changes in the peripheral plasma concentrations of oestradiol are principally derived from oestradiol secretion by the dominant follicle. Concentrations of oestradiol in the utero-ovarian vein draining the ovary containing a dominant follicle are greater than in the vein draining the contralateral ovary, and 10 to 300 times the concentration in peripheral plasma (Evans *et al.*, 1997). There are intimate relationships between both the lymphatic and blood vasculature of the uterus and ovary (Del Campo and Ginther, 1973; Staples *et al.*, 1982). The venous drainage from the ovary, the oviduct and from much of the uterine horn form a common utero-ovarian vein (Fig. 5). The ovarian artery is tortuous and is closely applied to the utero-ovarian vein (Ginther and Del Campo, 1974). The uterus-to-ovary pathway has been clearly established for the transport of prostaglandin $F_{2\alpha}$ during luteolysis (Heap *et al.*, 1985; Bonnin *et al.*, 1999). Whilst the opposite ovary to uterus pathway is probably responsible for the localised increase in progesterone concentrations in the uterine horn and uterine vasculature ipsilateral to the ovary containing the corpus luteum (Weems *et al.*, 1988; Cerbito *et al.*, 1994).

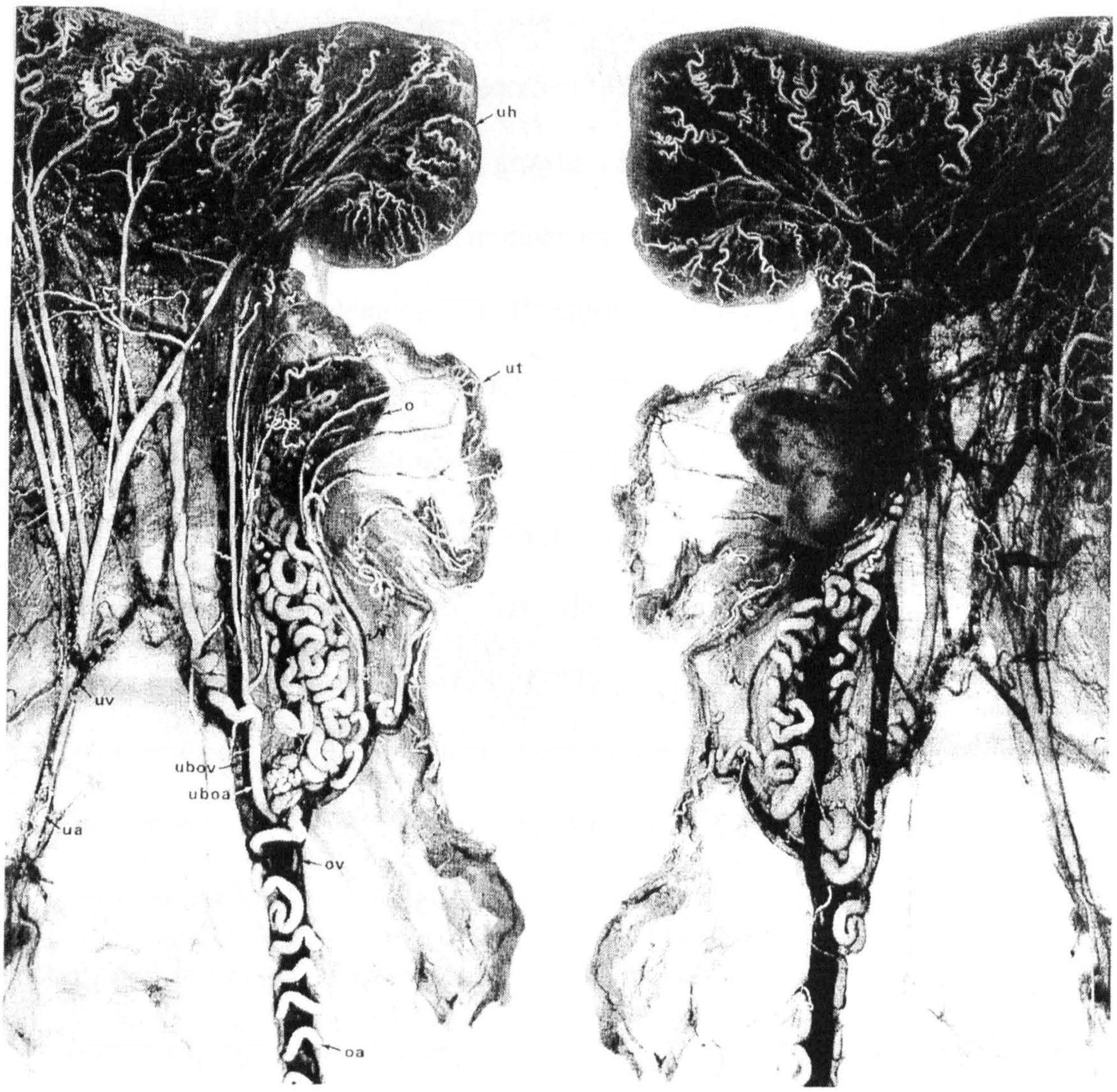

Figure 5. Ventral view (left) and dorsal view (right) of the arteries (light) and veins (dark) of a uterine horn and adjacent ovary in cattle (Ginther and Del Campo, 1974). o = ovary; oa = ovarian artery; ov = ovarian vein; ua = uterine artery; uv = uterine vein; uboa (ubov) = uterine branch of ovarian artery (vein); uh = uterine horn; ut = uterine tube

The ovary during pregnancy

Throughout pregnancy, regular periodic emergence of anovulatory follicular waves continues in response to recurrent FSH surges (Ginther *et al.*, 1996a). However, progesterone suppresses growth of the dominant follicle in a dose-dependent manner and the maximum diameter of the dominant follicle decreases as pregnancy progresses (Adams, 1999). During the last month of pregnancy, recurrent FSH surges are absent and no follicular waves emerge, probably due to very high plasma progesterone and oestrogen concentrations (Ginther *et al.*, 1996a; Crowe *et al.*, 1998). Indeed, due to the strong negative feedback of high steroid concentrations, the anterior pituitary has a decreased content of LH and FSH in late pregnancy and around parturition (Nett, 1987).

An interesting feature of follicular waves during pregnancy is an asymmetric ovarian distribution. From the time around the maternal recognition of pregnancy, there is a predilection (75 to 80%) for dominant follicles to occur in the ovary contralateral to the gravid uterine horn (Thatcher *et al.*, 1991; Bergfelt *et al.*, 1998). The source of regulatory factors could be the corpus luteum, the uterus, or the conceptus. Although, in sheep, an inhibitor of follicular aromatase activity has been isolated from corpora lutea of late pregnancy (Al-Gubory *et al.*, 1994); a number of lines of evidence suggest that the uterus or contents are more likely involved. Firstly, hysterectomy obviates the expected ovarian follicular asymmetry (Guilbault *et al.*, 1986; Thatcher *et al.*, 1991). Secondly, in an animal with a corpus luteum on each ovary, but only one conceptus, the supression of follicular growth was still evident ipsilateral to the gravid uterine horn (Thatcher *et al.*, 1991). Finally, in ewes, lutectomy during pregnancy has no effect on the ovarian follicular asymmetry (Hall *et al.*, 1993).

Postpartum ovarian activity

The ovarian follicular waves suppressed during the last month of pregnancy are re-established after parturition. Plasma steroid hormone concentrations decrease to basal values, and there is an increase in plasma FSH concentration within days of calving (Crowe *et al.*, 1998; Duffy *et al.*, 2000). Subsequent increases in plasma FSH concentrations occur regularly every 7 to 10 days and are not affected by diet, suckling, or duration of the postpartum anoestrus interval (Stagg *et al.*, 1998; Beam and Butler, 1999). Transrectal ultrasonographic examination of the ovaries is possible from 6 to 8 days after parturition when the emergence of the first follicular wave is detectable, with the first dominant follicle (diameter $\geq$ 10 mm) being identified around Day 10 postpartum (Savio *et al.*, 1990a; Stagg *et al.*, 1995; Beam and Butler, 1997). This first postpartum dominant follicle has three possible fates: ovulation and formation of the first corpus luteum (Fig. 6); atresia, with subsequent emergence of a second dominant follicle; or, persistence with continued growth, often called an ovarian cyst. However, behavioural oestrus is rarely observed if the first postpartum dominant follicle is ovulated, probably because of insufficient prior exposure to progesterone (Savio *et al.*, 1990a; Stevenson and Pursley, 1994).

The fate of the first postpartum dominant follicle is dependent on LH pulse frequency, and the re-establishment of a pulsatile LH secretion pattern sufficient for development of an ovulatory dominant follicle is the key event in the return of ovarian cyclic activity (Nett, 1987; Beam and Butler, 1999). Failure to ovulate is probably a consequence of inadequate LH pulse frequency (Beam and Butler, 1999; Duffy *et al.*, 2000). High LH pulse frequency (one / h) culminates in an LH surge and ovulation; whereas, low frequency is associated with atresia, and an intermediate frequency is associated with persistence of the dominant follicle. Those dominant

follicles destined to undergo atresia produce low plasma oestradiol concentrations, compared with ovulatory or persistent follicles (Beam and Butler, 1997). The importance of both persistent and atretic first dominant follicles is that they are associated with prolonged intervals to first postpartum ovulation of about 50 days, which is longer than the traditional voluntary waiting period before insemination of 40 days postpartum.

Postpartum recovery of pituitary LH content, and release in response to GnRH is usually complete by 10 to 14 days postpartum in dairy cattle (Lamming *et al.*, 1981; Alam and Dobson, 1987). The principal causes of low LH pulse frequency are negative energy balance and suckling or the calf-dam maternal bond. Thus, the first dominant follicle is more likely to ovulate in dairy cows, than in beef cows (Murphy *et al.*, 1990; Savio *et al.*, 1990a). In dairy cattle, LH pulse frequency is suppressed and the ovulatory competence of the first dominant follicle reduced until the nadir of negative energy balance after parturition (Canfield and Butler, 1990; Beam and Butler, 1997). Supplementation of the diet with fat reverses the effects of negative energy balance resulting in larger first dominant follicles, higher maximum oestradiol concentrations and a shorter anovulatory period. The pulse frequency of LH is most often reduced by prolonged energy deficient diets in postpartum beef cattle (Perry *et al.*, 1991). In beef cattle, suckling also sustains the reduced LH pulse frequency postpartum, although the effect is specifically mediated by the maternal bond with the calf (Silveira *et al.*, 1993). Elimination of olfaction and vision, however, precludes calf identification by the dam and eliminates the negative effect on LH secretion (Griffith and Williams, 1996).

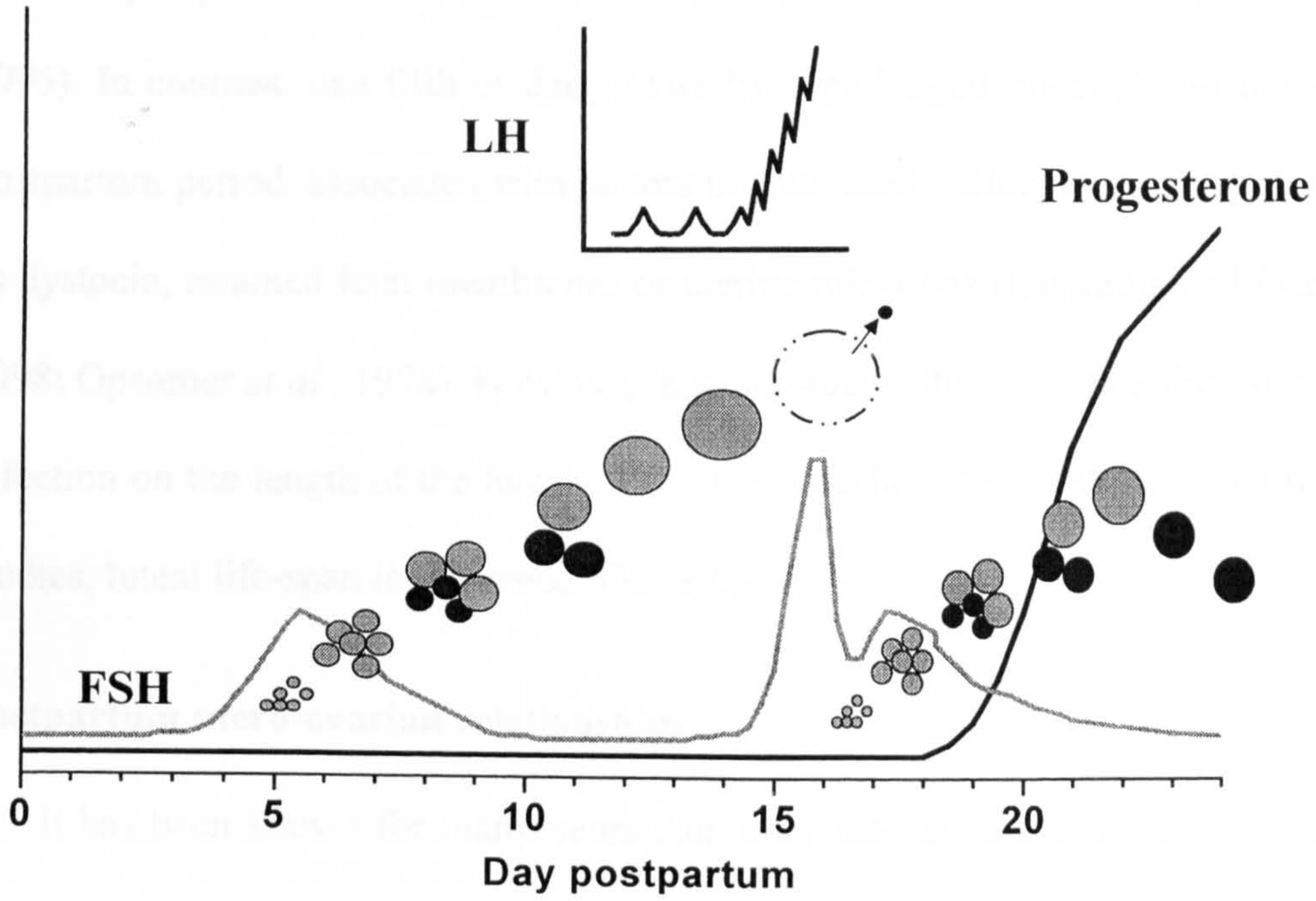

Figure 6. A model of postpartum follicular waves in which the first dominant follicle ovulates. Several follicles (●) emerge as plasma FSH concentrations increase after paruturition, with a first dominant follicle selected about Day 10, and the subordinate follicles undergoing atresia (●). Plasma LH concentrations increase, and the dominant follicle ovulates, with formation of a corpus luteum secreting progesterone, and the emergence of the first oestrous cycle follicular wave.

The first postpartum oestrous cycles are of variable duration, although the majority in dairy cattle are the usual 18 to 24 days, and associated with two or three follicular waves. Shorter oestrous cycles are associated with one or two follicular waves within a cycle, and tend to occur in animals in which the first dominant follicle is not detected before 20 days postpartum (Savio *et al.*, 1990b; Duffy *et al.*, 2000). The suggested cause of short first luteal phases is inappropriate activation of

luteolytic mechanisms, possibly due to insufficient progesterone priming preceding the first postpartum ovulation (Hunter, 1991; Inskeep, 1995; Lamming and Mann, 1995). In contrast, one fifth of dairy cows have prolonged luteal phases during the postpartum period, associated with factors that adversely affect uterine function such as dystocia, retained fetal membranes or uterine infections (Lamming and Darwash, 1998; Opsomer *et al.*, 1998). However, in some studies there are no effects of uterine infection on the length of the luteal phase (Del Vecchio *et al.*, 1992); whilst in other studies, luteal life-span is shortened (Gilbert *et al.*, 1990).

Postpartum utero-ovarian relationships

It has been known for many years that, after calving, the ovary contralateral to the previously gravid horn has greater follicular activity than the ipsilateral ovary (Casida and Venzke, 1936; Saiduddin *et al.*, 1967). Follicles in the contralateral ovary are larger and more oestrogen active than those in the ipsilateral ovary (Bellin *et al.*, 1984; Spicer *et al.*, 1986). There are also more first postpartum ovulations from the contralateral ovary, as determined by the location of the first postpartum corpus luteum (Saiduddin *et al.*, 1967; Foote and Peterson, 1968; Marion and Gier, 1968; Lewis *et al.*, 1984; Guilbault *et al.*, 1987). The majority (70 to 82%) of first dominant follicles observed using ultrasonography were in the ovary contralateral to the previously gravid horn (Kamimura *et al.*, 1993; Nation *et al.*, 1999). A similar postpartum asymmetrical distribution of follicular growth occurs in other uniparous species with a bicornuate uterus, such as sheep (Bartlewski *et al.*, 2000); and possibly horses (Allen and Newcombe, 1981). However, such observations have not been reported in species with a simplex uterus.

The asymmetric effect on folliculogenesis reduces with increasing time postpartum (Saiduddin *et al.*, 1967; Marion and Gier, 1968). Differences in

follicular growth between the ovaries were present until Day 20 to 30 postpartum (Foote and Peterson, 1968; Marion and Gier, 1968). However, in cattle that remained anovulatory, differences between ovaries were detected until Day 40 to 56 (Spicer *et al.*, 1986; Risco *et al.*, 1994). The effect of the previously gravid horn on folliculogenesis is no longer evident after the first ovulation (Dufour and Roy, 1985). In one study, the first dominant follicle was rarely observed in the left ovary, even if it was contralateral to the previously gravid uterine horn (Nation *et al.*, 1999). However, other studies found no such effect of the left or right ovary on follicle distribution after parturition (Saiduddin *et al.*, 1967; Foote and Peterson, 1968).

The mechanism suppressing folliculogenesis in the ipsilateral ovary to the previously gravid horn is not clear, although the asymmetry is more likely to be a localised, rather than a central effect. Suppression of folliculogenesis in the ipsilateral ovary decreases concurrent with uterine involution, and elimination of the ubiquitous uterine bacterial contamination after parturition (Elliot *et al.*, 1968; Marion and Gier, 1968). Furthermore, although luteolysis and regression of the corpus luteum is rapid during the oestrous cycle, following parturition luteolysis is protracted with physical remnants of luteal cells detectable for up to 35 days postpartum (Sawyer, 1995). Thus, suppression of folliculogenesis in the ipsilateral ovary could also be explained by a localised inhibitory effect of the regressing corpus luteum of pregnancy, or the previously gravid uterine horn, or uterine contents (Dufour and Roy, 1985).

Prostaglandins may be mediators of an effect of uterine involution on folliculogenesis as they are released in high concentrations during the postpartum period reflecting uterine involution (Lindell *et al.*, 1982; Guilbault *et al.*, 1987). There may be a local counter current exchange of prostaglandin $F_{2\alpha}$ from uterine

venous plasma to ovarian arterial plasma, similar to that during luteolysis (Ginther, 1968b). However, in contradiction to this hypothesis, partial suppression of endogenous prostaglandin synthesis using flunixin meglumine in the early postpartum period reduced ovarian activity of both ovaries up to 60 days. Furthermore, replacement with prostaglandin $F_{2\alpha}$ specifically increased follicular activity in the ovary ipsilateral to the previously gravid horn (Guilbault *et al.*, 1987). In addition, infusion of prostaglandin $F_{2\alpha}$ into the descending aorta between Days 2 and 13 postpartum, stimulated the growth of larger follicles in both ovaries (Villeneuve *et al.*, 1988). Other mediators that could transfer from the uterus to the ovary are tissue metabolites. There is extensive re-modelling of uterine tissue during the postpartum period, with high plasma concentrations of collagen metabolites (Tian and Noakes, 1991c). However, a uterus to ovary transfer of such metabolites has not been investigated.

The contents of the postpartum uterus could also be a source of mediators for a localised effect on the ovary. Uterine bacterial contamination has an inhibitory effect on ovarian folliculogenesis, decreasing the number and maximum diameter of ovarian follicles observed 4, 8 and 12 days postpartum (Peter and Bosu, 1988a). Furthermore, endotoxin infused into the uterus is absorbed into the peripheral circulation, reduces preovulatory follicular growth, and blunts the oestradiol-induced preovulatory LH surge (Peter *et al.*, 1989; Peter *et al.*, 1990a; Battaglia *et al.*, 1997). To explain these observations studies initially focussed on central mechanisms, demonstrating that endotoxin or various intermediary cytokines such as IL-1 or TNF_{α} block GnRH secretion and pituitary responsiveness (Rivest *et al.*, 1993; Battaglia *et al.*, 2000; Williams *et al.*, 2001). However, there is also evidence of a localised effect of uterine infection on ovarian function, as cows with retained foetal

membranes after parturition have an even greater predilection for follicular activity in the contralateral ovary, and the effect persists for longer than for normal control animals (Risco *et al.*, 1994). In addition, intrauterine or intravenous administration of endotoxin suppresses the preovulatory increase in plasma oestrogen concentration at the ovarian level in cattle and sheep, respectively (Peter *et al.*, 1990b; Battaglia *et al.*, 2000). In addition, *in vitro* endotoxin and intermediary cytokines such as interleukins (IL-1, IL-6) and tumour necrosis factor (TNF_{α}) affect ovarian function by, amongst other effects, blocking FSH-induced oestradiol secretion by granulosa cells (Alpizar and Spicer, 1994; Spicer and Alpizar, 1994; Taylor and Terranova, 1996).

The importance of the asymmetrical ovarian distribution of folliculogenesis was indicated by a small study of postpartum cattle using palpation *per rectum* to monitor ovarian status (Bonnett *et al.*, 1993). Larger follicles in the ovary ipsilateral to the previously gravid uterine horn reduced the risk of subsequent poor reproductive performance. However, the mechanisms underlying these observations have not been investigated, so it is not clear if this is an effect of follicle health or better reflects uterine health. Thus, it is to be determined if the uterus influences the ovary, or *vice versa*.

Chapter 3

The influence of ovarian activity and uterine involution determined by ultrasonography on subsequent reproductive performance of dairy cows

Introduction

The relationship between subsequent reproductive performance and postpartum changes in the uterus or ovaries of cattle has been investigated by a number of authors (Studer and Morrow, 1978; Oltenacu *et al.*, 1983). However, little attention has been paid to the local relationship between the previously gravid uterine horn and follicular growth in the ipsilateral ovary.

Results from a small study suggested that the occurrence of a larger follicle, determined by palpation *per rectum*, in the ovary ipsilateral to the previously gravid uterine horn on Day 26 ± 3 postpartum, increased the likelihood of shorter calving to conception intervals (Bonnett *et al.*, 1993). However, the accuracy of manual palpation for identifying and measuring such structures, as well as determining the degree of uterine involution, is open to criticism due to the high level of subjectivity. The monitoring of follicular growth and uterine involution in cows can be improved by the use of transrectal ultrasonography (Pierson and Ginther, 1988; Savio *et al.*, 1990a).

Greatest follicular activity after calving occurs initially in the ovary contralateral to the previously gravid uterine horn (Lewis *et al.*, 1984; Guilbault *et al.*, 1987; Risco *et al.*, 1994). Kamimura et al. (1993) reported that only 18% of first dominant follicles were identified in the ipsilateral ovary. The negative influence of the previously gravid horn on the return of ovarian cyclicity in the ipsilateral ovary

declines with increasing time postpartum (Saiduddin *et al.*, 1967; Foote and Peterson, 1968; Marion and Gier, 1968).

There are conflicting reports on the relationship between the return of ovarian cyclicity (as determined by the interval from calving to first rise in milk progesterone concentration) and calving to conception interval. An early return of cyclicity was associated with shorter calving to conception intervals in one study, whereas the reverse is claimed in another (Darwash *et al.*, 1997; Smith and Wallace, 1998). However, such studies are unable to determine in which ovary the return to cyclicity occurs in relation to the previously gravid uterine horn.

The objective of the present study was to test the hypothesis that a follicle > 8 mm diameter in the ovary ipsilateral to the previously gravid uterine horn, between 14 and 28 days postpartum, improves subsequent reproductive performance.

Materials and Methods

Animals

Three commercial Holstein-Friesian dairy farms (identified as A, C and E) were chosen for the study on the basis of accurate farm records and existing regular visits. During spring and summer, the cows were kept at pasture, whereas during the autumn and winter they were housed in freestall barns with grass silage available *ad libitum*. Concentrate feed was supplied at 0.3 kg/L milk yield. The farms had a target calving index of 365 days with a policy of inseminating cows from 45 days postpartum. All animals were bred by artificial insemination at observed oestrus by a single technician.

Each farm was visited every 2 weeks for a one year period to monitor fertility and other aspects of reproductive health. Cows that had calved 14 to 28 days earlier and without a history of peripartal problems were included in the study and examined once. Calving date, date of examination and lactation number were recorded.

Clinical Examination

The vagina of each cow was examined by direct palpation, and the lumenal contents were inspected. Purulent vaginal discharge, which is regarded as a reflection of uterine infection, was defined as the presence of mucopurulent material in the vagina (Studer and Morrow, 1978; Nakao *et al.*, 1992). Normal mucus was defined as uniform and translucent in the absence of a fetid odor. Cows with gross vaginal lacerations, inappetence or pyrexia were excluded from the study.

The previously gravid uterine horn was determined transrectally as that which was longer and of greater diameter than the contralateral horn. The genital tract was scanned *per rectum* using a SonoAce 600 ultrasound scanner with a 5-MHz linear array transrectal probe (BCF Technology Ltd., Livingston, U.K.). Follicles were defined as nonechogenic (black) spherical structures with a clear demarcation between the follicular wall and antrum. Corpora lutea (corpus luteum) were defined as grainy echogenic structures that had a well-defined border with the less echogenic ovarian stroma. In some corpora lutea there was a normal nonechogenic lacuna. The internal diameter of the largest follicular and luteal ovarian structures and the external diameter of the uterine horns at mid-point were measured using the internal calipers of the machine. When the image of the structure being scanned was not spherical, the diameter was estimated by averaging two 90° measurements. In addition, follicles > 4 mm diameter in each ovary were counted.

Ovarian activity was defined as the presence of a follicle and/or corpus luteum > 8 mm diameter. Similar definitions have been used previously (Guilbault *et al.*, 1987; Risco *et al.*, 1994). The > 8 mm measurement was selected because follicles of up to 8 mm diameter have previously been identified within 7 to 10 days of calving immediately before emergence of a dominant follicle (Peter and Bosu, 1988b; Savio *et al.*, 1990a). In addition, ovarian structures > 8 mm diameter are readily and accurately determined by ultrasonography.

Evaluation of Fertility Data

Fertility data were collected for 18 months. Cows that were culled because of failure to conceive were ascribed a calving to conception interval of 308 days, equivalent to a 305-day lactation period plus 3 days colostrum production (Shanks *et al.*, 1979). Cows culled for other reasons before conception were removed from the data. Conception date was verified by the subsequent calving date.

Statistical Analysis

The data were analyzed for the combined herds and for 3 periods of calving to examination (14 to 18, 19 to 23 and 24 to 28 days). Results are expressed as mean ± SEM. Analysis was performed using SPSS (Version 8.0, SPSS Inc, Chicago, IL). Values of $P < 0.05$ were regarded as significant.

The mean period from calving to examination was compared amongst herds using a t-test, and the mean lactation number was compared using the nonparametric Kologorov-Smirnov Z test. The calving to conception data (log_{10} transformed for analysis) were compared by analysis of covariance. The variables tested were uterine diameter, presence of a purulent vaginal discharge, presence of a follicle > 8 mm diameter, a corpus luteum, ovarian activity and their interactions. Ovarian structure

and uterine diameter comparisons were also tested separately for location: ipsilateral or contralateral to the previously gravid uterine horn.

The distribution of the number of animals with a corpus luteum or with a follicle > 8 mm diameter in the ovary ipsilateral or contralateral to the previously gravid uterine horn were compared using Chi-squared tests. Comparison of the mean number of follicles > 4 mm diameter between each ovary, the three calving to examination periods and their interactions were tested using a general linear model. Correlation between the number of follicles in each ovary and the interval from calving to examination were tested by Pearson coefficient.

Results

There were 284 cows with complete data for analysis, including 115, 120 and 49 cows in Herds A, C and E, respectively. The mean lactation number was higher for Herd A than for Herds C and E (4.2 ± 0.2 vs. 2.9 ± 0.2 and 3.0 ± 0.3, respectively; $P < 0.05$). Three cows in each herd failed to conceive. The mean interval from calving to examination for the combined data was 21.8 ± 0.3 days and was similar for each herd. The mean calving to conception interval for the combined data was 109.2 ± 3.7 days and did not differ significantly between herds. Consequently, further analyses were carried out on combined data from the 3 herds.

Ovarian Activity

Fewer cows had a corpus luteum in the ovary ipsilateral to the previously gravid uterine horn compared with the contralateral ovary (Table 1; $P < 0.001$). In addition, there were fewer follicles > 4 mm diameter in the ipsilateral ovary than in the contralateral ovary (Table 1; $P < 0.001$). The difference in the number of follicles > 4

mm diameter between the ovaries ipsilateral or contralateral to the previously gravid uterine horn declined with increasing time from calving to examination. The difference was greatest at 14 to 18 days after calving compared with 19 to 23 days and was no longer significant at 24 to 28 days. The mean number of follicles > 4 mm diameter did not differ significantly between the calving to examination periods, or for the interaction of period with ovarian location.

Fewer animals had a follicle > 8 mm diameter in the ovary ipsilateral to the previously gravid uterine horn compared with the contralateral ovary (Table 1; $P < 0.001$). The proportion of cows with a follicle > 8 mm diameter differed between the ovaries ipsilateral and contralateral to the previously gravid uterine horn at 14 to 18, 19 to 23 and 24 to 28 days from calving to examination. However, the proportion of cows with a follicle > 8 mm diameter did not differ significantly between the calving to examination periods, or for the interaction of period with ovarian location. There were significant correlations between the interval from calving to examination and the number of follicles > 4 mm diameter ($r = 0.12$, $P < 0.05$), the presence of a follicle > 4 mm ($r = 0.16$, $P < 0.01$) and the presence of a follicle > 8 mm ($r = 0.14$, $P < 0.05$) in the ovary ipsilateral to the previously gravid uterine horn. There were no significant correlations concerning the contralateral ovary.

Table 1. Ovarian activity as measured by mean ± SEM for the number of follicles > 4 mm, percent > 8 mm diameter and percent with a corpus luteum (CL) in the ovary ipsilateral or contralateral to the previously gravid uterine horn at 3 time periods after calving.

Calving to examination period (days)	Number of cows	Ipsilateral ovary			Contralateral ovary		
		No. > 4 mm	Percent > 8 mm	Percent with CL	No. > 4 mm	Percent > 8 mm	Percent with CL
14 to 18	70	0.49 ± 0.09^{a}	20.6^{a}	22.1	0.93 ± 0.11^{b}	47.1^{b}	26.5
19 to 23	105	0.72 ± 0.11^{c}	22.8^{a}	19.0^{c}	1.10 ± 0.12^{d}	54.3^{b}	34.3^{d}
24 to 28	109	0.83 ± 0.11	33.0^{e}	11.9^{a}	1.07 ± 0.10	46.8^{f}	45.9^{b}
All	284	0.69 ± 0.06^{a}	26.1^{a}	16.9^{a}	1.02 ± 0.06^{b}	49.6^{b}	37.0^{b}

Values with different superscripts are significantly different between the ipsilateral and contralateral ovary within a calving to examination period ($^{ab}P < 0.001$, $^{cd}P < 0.01$, $^{ef}P < 0.05$).

Uterine Discharge in Relation to Ovarian Activity

In the combined herds, 143/284 (50.4%) of animals had purulent vaginal mucus, and the proportion was similar in each herd. In addition, the proportion of cows with purulent vaginal mucus was similar at the different calving to examination periods.

There were fewer animals with a corpus luteum ($P < 0.01$) or a follicle > 8 mm diameter ($P < 0.01$) in the ovary ipsilateral to the previously gravid uterine horn compared with the contralateral ovary within the normal or purulent mucus groups (Table 2). More cows with normal vaginal mucus compared with purulent mucus animals had a corpus luteum in the ovary contralateral to the previously gravid uterine horn, but this was not so for the ipsilateral ovary. The number of normal compared with purulent vaginal discharge cows with a follicle > 8 mm diameter in the ipsilateral or contralateral ovary to the previously gravid uterine horn was similar. Furthermore, there was no significant difference between normal and purulent mucus cows in the mean number of follicles > 4 mm diameter in the ipsilateral ovary (0.70 ± 0.09 vs. 0.71 ± 0.08) or the contralateral ovary (1.12 ± 0.10 vs. 0.98 ± 0.08, respectively).

Subsequent Reproductive Performance

The calving to conception interval was significantly ($P < 0.01$) affected by previously gravid uterine horn diameter, the presence of a follicle > 8 mm diameter in the ipsilateral ovary, ovarian activity, purulent vaginal mucus and the interaction between ovarian activity and purulent vaginal mucus.

The calving to conception interval was shorter in those animals with a smaller diameter previously gravid uterine horn ($P < 0.01$). However, the diameter of the contralateral uterine horn was not significant.

Cows with ovarian activity on either ovary had a shorter mean calving to conception interval compared with cows with no ovarian activity (Table 3; $P < 0.05$). When each calving to examination interval was considered separately, only those examined 14 to 18 days after calving had a shorter calving to conception interval ($P < 0.01$).

The calving to conception interval was shorter for cows with a follicle > 8 mm diameter in the ovary ipsilateral to the previously gravid uterine horn compared with those without a follicle > 8 mm diameter (99.0 ± 5.6 vs. 112.8 ± 4.4 days; $P < 0.05$).

Cows with normal vaginal mucus had a shorter mean calving to conception interval than those with purulent mucus (101.4 ± 4.5 vs. 116.8 ± 5.8 days; $P < 0.05$). To investigate the influence of ovarian activity, cows with normal vaginal mucus were considered separately. Cows with normal mucus and ovarian activity had shorter mean calving to conception intervals compared with those without ovarian activity (89.1 ± 1.0 days, $n = 127$; vs. 113.1 ± 1.1 days, $n = 14$; $P < 0.05$). In animals with a purulent vaginal discharge, there was no effect of ovarian activity on the calving to conception interval.

Table 2. Number (%) of cows with normal (n = 141) or purulent (n = 143) vaginal mucus that had a corpus luteum (CL) or a follicle > 8 mm diameter in the ovary ipsilateral or contralateral to the previously gravid uterine horn.

Structure and location	Normal	Purulent
CL in the ipsilateral ovary	20 (14) [a]	28 (20) [a]
CL in the contralateral ovary	60 (43) [b c]	45 (31) [b d]
Follicle > 8 mm diameter in the ipsilateral ovary	35 (25) [a]	39 (27) [a]
Follicle > 8 mm diameter in the contralateral ovary	73 (52) [b]	68 (48) [b]

Values with different superscripts are significantly different between the ipsilateral and contralateral ovary (^{ab} $P < 0.01$) and between cows with normal and purulent vaginal mucus (^{cd} $P < 0.05$).

Table 3. Mean ± SEM calving to conception interval (days) of animals with or without ovarian activity in either ovary at 3 periods after calving.

Calving to examination interval	Ovarian activity	n	No ovarian activity	n
14 to 18 days	90.7 ± 6.2 [a]	60	139.0 ± 19.1 [b]	10
19 to 23 days	111.6 ± 6.7	91	113.9 ± 13.4	14
24 to 28 days	110.2 ± 6.7	95	139.4 ± 23.6	14
All	105.9 ± 3.9 [c]	246	130.1 ± 11.1 [d]	38

Values with different superscripts are significantly different between cows with and without ovarian activity. (^{ab} $P < 0.01$, ^{cd} $P < 0.05$)

Discussion

This study shows that the presence of a follicle > 8 mm diameter between 14 and 28 days after calving in the ovary ipsilateral to the previously gravid uterine horn is associated with shorter calving to conception intervals. In addition, there is reduced folliculogenesis in the ipsilateral ovary of cattle during this postpartum period. This association between the ovaries and uterus is further modified by the presence of purulent vaginal mucus (indicative of uterine bacterial infection), which suggests that the dialogue may be partially mediated by inflammation.

The observation that a follicle > 8 mm diameter in the ovary ipsilateral to the previously gravid uterine horn was associated with a shorter calving to conception interval confirms the results of the small study of 66 animals by Bonnet *et al.* (1993). They reported that cows with good subsequent reproductive performance had a larger diameter follicle (6.1 ± 1.3 mm) on the ipsilateral ovary, compared with a smaller follicle (2.7 ± 0.9 mm) in cows with poor performance. The shorter calving to conception interval associated with the presence of a larger follicle in the ipsilateral ovary may be due to the influence of the follicle on the uterine endometrium and/or myometrium. One hypothesis is that estradiol synthesized by a follicle > 8 mm diameter has a beneficial local effect on uterine function. Plasma oestradiol concentrations are greater within the utero-ovarian vein draining the ovary containing the ovulatory follicle (Ireland *et al.*, 1984); bearing in mind that only postpartum follicles > 8 mm diameter that eventually ovulate (not nonovulatory follicles) are associated with increased peripheral plasma oestradiol concentrations (Beam and Butler, 1997). Unfortunately, in the present study neither milk nor blood samples were taken to measure this hormone.

The current study shows that cows with ovarian activity, defined by the ultrasonographic presence of a corpus luteum and/or follicle > 8 mm diameter, had a shorter calving to conception interval compared with those without. This observation supports Shanks et al. (Lucy *et al.*, 1992), who reported shorter calving to conception intervals for cows that had an initial ovulation before, rather than after, 42 days postpartum. Similarly, Darwash *et al.* (1997) reported a significant reduction in the calving to conception interval with shorter intervals to the first postpartum increase in milk progesterone concentration, although, Smith and Wallace (1998) observed the reverse.

Fewer animals in the present study had a corpus luteum in the ovary ipsilateral to the previously gravid uterine horn, and there were fewer follicles > 4 mm diameter and fewer cows with a follicle > 8 mm diameter in the ovary ipsilateral to the previously gravid uterine horn. The mechanism could be exerted by a systemic pathway; however our observations support the suggestion that the previously gravid uterine horn has a local negative effect on ipsilateral postpartum ovarian function (Lewis *et al.*, 1984; Dufour and Roy, 1985). A local effect of the uterus on ovarian function is an established concept, for example, in relation to the transfer of prostaglandin $F_{2\alpha}$ (Ginther, 1968a), and it has been implicated in the action of interferon (Spencer *et al.*, 1999). Furthermore, in the present study there was a positive correlation between postpartum interval and number or presence of follicles in the ovary ipsilateral to the previously gravid uterine horn. These observations would support a hypothesis of a declining influence of an as yet unknown uterine-derived inhibitor of ovarian function as uterine involution progresses. Possible uterine factors could include inflammatory mediators such as prostaglandin $F_{2\alpha}$, which is produced by the postpartum uterus in high concentrations during involution

and in response to uterine infection (Lindell *et al.*, 1982; Del Vecchio *et al.*, 1994). However, Guilbault *et al.* (1987) reported that a partial inhibitor of prostaglandin synthesis administered during the postpartum period reduced ovarian activity. Furthermore, replacement with prostaglandin $F_{2\alpha}$, enhanced ovarian activity in the ovary ipsilateral to the previously gravid uterine horn. However, there are a large number of other inflammatory mediators, particularly cytokines, that participate in folliculogenesis and ovulation (Terranova and Rice, 1997). An alternative mechanism might be via immune or inflammatory cell cytokine release and/or cell-cell communication in the ovary. Neutrophils readily migrate into the uterine lumen in response to a chemotactic stimulus at all stages of the bovine reproductive cycle. Furthermore, intrauterine and peripheral neutrophil function is modified by reproductive hormonal status, retained fetal membranes and uterine infection (Cai *et al.*, 1994; Subandrio and Noakes, 1997). Perhaps immune cells could migrate from the uterine horn, or a uterine-derived chemotactic substance could attract immune cells to the ipsilateral ovary, and so influence folliculogenesis. Immune cells have been detected in ovarian follicles and luteal tissue in association with a modulation of follicular and luteal activity (Brannstrom *et al.*, 1994; Gaytan *et al.*, 1998; Penny *et al.*, 1999).

Fewer animals with a uterine infection, as reflected by the presence of purulent vaginal mucus, had a corpus luteum in the ovary contralateral to the previously gravid uterine horn. One explanation for this observation might be that increased plasma oestradiol concentrations associated with ovulatory dominant follicles may have enhanced the elimination of uterine infection in those cows that, at the time of examination, had normal vaginal mucus and a corpus luteum (Rowson *et al.*, 1953; Andriamanga *et al.*, 1984). However, in the present study, the number of corpora

lutea in the ipsilateral ovary did not differ between normal and infected animals. Therefore, an alternative hypothesis for the difference in the contralateral ovary must be sought. In cows with uterine infection, the ovarian inhibitory factor, possibly induced by inflammation, may have extended to the contralateral uterine horn in addition to its presence in the previously gravid uterine horn.

In the present study, increased previously gravid uterine horn diameter or purulent vaginal mucus increased the calving to conception interval. Similar increases in calving to conception interval have been reported previously in association with purulent vulval or vaginal discharge (Studer and Morrow, 1978; Borsberry and Dobson, 1989), or a larger uterine horn diameter (Oltenacu *et al.*, 1983; Bonnett *et al.*, 1993). However, the cows were not always differentiated into those with, or without, a purulent vaginal discharge (Shanks *et al.*, 1979).

In conclusion, the present data confirm that follicular activity is suppressed in the ovary ipsilateral to the previously gravid uterine horn between 14 and 28 days postpartum. However, this ovarian suppression declines with increasing interval after calving. In cows with a purulent vaginal discharge, there were fewer corpora lutea in the ovary contralateral to the previously gravid uterine horn, although follicle population differences were not apparent. Ovarian activity 14 to 18 days postpartum, but not later, was associated with a shorter calving to conception interval. In addition, location of ovarian structure in relation to the previously gravid uterine horn was also important. The presence of a follicle > 8 mm diameter in the ovary ipsilateral to the previously gravid uterine horn 14 to 28 days postpartum was associated with shorter calving to conception intervals. These observations raise an intriguing issue: how does this follicle affect subsequent fertility — does the follicle influence the uterus, or *vice versa*?

Chapter 4

Effect of administration of equine chorionic gonadotrophin (eCG) to postpartum cows on folliculogenesis in the ovary ipsilateral to the previously gravid uterine horn and uterine involution

Introduction

Postpartum follicular activity in the ovary ipsilateral to the previously gravid uterine horn has consistently been observed to be less than that in the contralateral ovary (Lewis *et al.*, 1984; Guilbault *et al.*, 1987; Kamimura *et al.*, 1993; Risco *et al.*, 1994; Nation *et al.*, 1999). Intriguingly, however, the presence of a follicle > 8 mm diameter in the ovary ipsilateral to the previously gravid uterine horn in cows examined between 14 and 28 days postpartum was associated with a shorter calving-to-conception interval (Sheldon *et al.*, 2000; Chapter 3), confirming a smaller study by Bonnett *et al.* (1993). One hypothesis to explain these observations is that oestrogen secreted by a dominant follicle in the ovary ipsilateral to the previously gravid uterine horn acts locally to increase the rate of involution of the ipsilateral uterine horn. Indeed plasma concentrations of oestradiol are high in the utero-ovarian vein draining the ovary containing the ovulatory dominant follicle (Ireland *et al.*, 1984). Such a hypothesis is not without precedence: treatment-by-side interactions on oviductal protein synthesis occur in cows with persistent dominant follicles, and it has been suggested that this was due to the ipsilateral increase in oestradiol concentration (Binelli *et al.*, 1999).

One method of testing the hypothesis would be to stimulate follicular growth in the ovary ipsilateral to the previously gravid uterine horn and assess uterine involution. Equine chorionic gonadotrophin (eCG) has both FSH- and LH-like activity and parenteral administration stimulates follicular growth and ovulation in

cattle (Gonzalez-Menico *et al.*, 1978; Newcomb *et al.*, 1979). Uterine involution occurs on a decreasing logarithmic scale, as reflected by changes in diameter of each uterine horn monitored accurately by transrectal ultrasonography (Okano and Tomizuka, 1987; Bekana *et al.*, 1994). In addition, involution has been characterised by the estimation of plasma concentration of 15-keto-13,14-dihydro-prostaglandin $F_{2\alpha}$ (PGFM), the metabolite of prostaglandin $F_{2\alpha}$, which is correlated with the decrease in uterine horn dimension (Lindell *et al.*, 1982; Lewis *et al.*, 1984).

The aim of the present study was to test the hypothesis that increased oestradiol secretion by large follicles in the ovary ipsilateral to the previously gravid uterine horn has a local effect to increase the rate of uterine involution. Specific objectives were: 1) to administer eCG to increase follicular growth in the ovary ipsilateral to the previously gravid uterine horn; and, 2) to determine the effect of that increased follicular growth on involution of the previously gravid uterine horn by ultrasonography and by estimation of plasma PGFM concentration.

Materials and Methods

Animals

A seasonally calving dairy herd, with 124 Holstein-Friesian cows and an annual average milk yield of ~6050 litres, was selected for the study on the basis of accurate farm records. Fifty-seven cows that had calved in a two month period were included in the study. Routine medical treatments were not used on the farm; insemination of all cows began 50 days after calving.

The farm was visited every 48 hours and generally three new cases were enrolled at each visit (range 0-6). At the first examination, 14 days after calving

(Day 0), details from the farm records of lactation number, calving date, assisted parturition, and peri-parturient disease were recorded for each cow. Cows that had intercurrent disease were excluded from the study.

Clinical examination

The side of the previously gravid uterine horn was determined transrectally by assessing which was longer and of greater diameter than the contralateral horn. The genital tract was scanned transrectally using a SonoAce 600 ultrasound scanner with a 5 MHz linear array transrectal probe (BCF Technology Ltd., Livingston, U.K.) and recorded using a video cassette recorder (Panasonic AG-5260B, Japan). Follicles were defined as non-echogenic (black) spherical structures with a clear demarcation between the follicular wall and antrum. Corpora lutea were defined as grainy echogenic structures that had a well-defined border with the less echogenic ovarian stroma; in some corpora lutea there was a non-echogenic lacuna. The diameter of the largest follicular and luteal ovarian structures, and the diameter of the uterine horns adjacent to the insertion of the free edge of the intercornual ligament, were measured using the internal callipers on the screen of the machine. When the image of the structure being scanned was not spherical, the diameter was estimated by averaging two 90° dimensions. In addition, the number of follicles > 4 mm diameter in each ovary was counted.

Animals were re-examined using the procedure outlined above, two, four and six days after the first examination (Day 0). In addition, at the time of the final examination (Day 6), the vagina of each cow was examined by direct palpation and visual inspection of the lumenal contents. The character of the vaginal mucus was recorded: abnormal mucus was defined as the presence of mucopurulent material;

normal mucus was defined as uniform, translucent material in the absence of a fetid odour.

Blood sampling and hormone assays

Blood samples were collected at each visit from the coccygeal vein or artery into evacuated heparinised tubes (Vacutainer, Becton Dickinson, Meylan, Cedex-France). Plasma was separated within 30 min by centrifugation (1000g for 10 min), harvested and stored frozen at -20°C until assayed for PGFM and oestradiol-17β.

The PGFM assay was performed in duplicate using unextracted plasma by direct radioimmunoassay using the method of Kindahl *et al.* (1976). Oestradiol-17β concentration was estimated in duplicate by radioimmunoassay using extracted plasma (Mann *et al.*, 1995). The sensitivity, intra- and inter-assay coefficient of variation was 27.0 pg/ml, 7.6% and 14.3%, respectively, for PGFM (using a pooled plasma sample of 91.2 pg/ml); and 0.24 pg/ml, 6.3% and 15.1%, respectively, for oestradiol (using a pooled plasma sample of 3.9 pg/ml).

Treatments

Treatments were administered by intramuscular injection on day 14 postpartum (Day 0 of study). A randomisation chart was used to assign cows to treatment groups. The treatments were 2ml Sterilised Water for Injection BP (Arnolds Veterinary Products, Shrewsbury) to control animals; 250 iu eCG (PMSG Folligon 1000 iu, Intervet UK, Cambridge); or, 750 iu eCG.

Statistical Analysis

Results are expressed as mean ± SEM. Significance was assigned at $P < 0.05$. The data were analysed either using all cases, or following selection of animals that had a plasma oestradiol concentration of < 1 pg/ml on Day 0. The latter cows were

selected because the response to eCG treatment is greater in the absence of a dominant follicle (Guilbault *et al.*, 1991; Huhtinen *et al.*, 1992).

The proportion of animals in each group with an assisted parturition, animals with a corpus luteum formed during the study, and animals with a follicle > 8 mm diameter in the ipsilateral ovary to the previously gravid uterine horn on Day 2, was compared by Chi-square test. The number of follicles > 4 mm or > 8 mm diameter, lactation number, and the number of inseminations per conception, were compared between treatment groups using the Kruskal-Wallis. The plasma oestradiol concentration, and log transformed values of calving-to-first insemination and calving-to-conception intervals were compared between treatment groups using analysis of variance (ANOVA). Animals with normal and abnormal vaginal mucus were compared for calving-to-first insemination and calving-to-conception intervals using *t*-test and for the number of inseminations per conception using the Mann-Whitney non-parametric test (Gibbons and Chakraborti, 1992).

Because ovarian follicular growth is not synchronous at precise times postpartum and varies between cows, the diameter of the largest follicle > 4 mm or > 8 mm diameter in each ovary was additively accumulated over the study periods. This transformation provides an estimate of the overall postpartum response to treatment for each cow (Risco *et al.*, 1994). Differences between ovarian accumulated follicular diameter ipsilateral and contralateral to the previously gravid uterine horn were compared within cow using *t*-tests within treatment groups and using ANOVA between groups. Changes in uterine horn diameter were compared within cow using *t*-tests within treatment groups and using ANOVA between groups for all cows, and between animals with or without a follicle > 8 mm diameter in the ipsilateral ovary on Day 2. The latter cows were selected to test the effect of follicles

> 8 mm diameter on uterine involution because follicles identified on Day 2, after eCG administration, were present for the remainder of the study period. Associations between the uterine horn diameter and plasma oestradiol concentration or accumulated follicular diameter were examined using Pearson correlation coefficients.

Factors that affected plasma PGFM concentration and uterine horn diameter were identified using a mixed multivariate model for repeated measures (SAS, 1997). Initially the Log_{10} transformed data were checked for normality by univariate analysis. An unstructured correlation structure provided the best fit to these data. Variables included were day of study, treatment group, abnormal vaginal mucus, lactation number, the presence of a follicle > 8 mm diameter in the ipsilateral ovary on Day 2, and animal. Variables were removed from the model, following examination for correlation with remaining variables, until those remaining were significant and results were expressed as least-square means ± SEM.

Results

There were 19, 18 and 20 cows in the control, 250 and 750 iu eCG treatment groups, respectively. The mean lactation number of the 57 cows was 3.2 ± 0.4 and did not differ significantly between the treatment groups. There was no difference in the number of animals that had a farmer-assisted parturition between treatment groups (40.4% of all cases). There were 5, 6 and 5 cows with an abnormal vaginal discharge in the control, 250 and 750 iu eCG treatment groups, respectively.

Ovarian activity

Accumulated follicular diameters of follicles > 4 mm diameter are summarised in Figure 1. There was a significant effect of Day ($P < 0.01$), and the interaction between treatment group and the relationship of the ovary to the previously gravid uterine horn ($P < 0.05$). In control cows there was a significant difference between accumulated follicular diameter in the ipsilateral and contralateral ovary on Day 0, 2, 4 and 6. However, there was no significant difference in accumulated follicular diameter between the ipsilateral and contralateral ovaries for cows administered 250 iu or 750 iu eCG. Comparison of accumulated follicular diameter was not significantly different between treatment groups, the relationship of the ovary to the previously gravid uterine horn, or interaction between treatment and Day, or ovary and Day. Similar trends in follicular growth were observed for accumulated follicular diameters of follicles > 8 mm diameter; on Day 6 the difference between the accumulated follicular diameter in the ipsilateral and contralateral ovary was greater in control cows (23.5±3.5 *vs.* 31.0±4.0 mm) compared with 250 iu (32.0±7.8 *vs.* 35.8±4.7 mm) and 750 iu eCG treated cows (33.4±7.4 *vs.* 38.9±5.3 mm); however, differences were not statistically significant.

The number of follicles > 4 mm or > 8 mm diameter did not differ significantly between treatment groups on Day 0. When data from all cows on Day 0 were combined, there were fewer follicles >4mm diameter in the ipsilateral, compared with the contralateral, ovary to the previously gravid uterine horn (0.89 ± 0.13 *vs.* 1.33 ± 0.15, $P < 0.01$), and there were fewer follicles > 8 mm diameter in the ipsilateral ovary (0.19 ± 0.05 *vs.* 0.37 ± 0.06, $P < 0.05$).

In control animals, the actual diameter of the largest follicle > 4 mm diameter in the contralateral ovary was greater in those cows with normal vaginal mucus,

compared with cows with abnormal mucus, on Day 2 (14.3 ± 1.3, n = 10 *vs.* 9.4 ± 1.0, n = 5; $P < 0.05$) and on Day 4 (15.0 ± 1.4, n = 10 *vs.* 9.7 ± 1.2, n = 3; $P < 0.05$). The presence of abnormal vaginal discharge did not affect the diameter of the largest follicle in the ipsilateral ovary of control animals, or in either ovary in the groups administered eCG.

There was a follicle > 8 mm diameter in the ipsilateral ovary to the previously gravid uterine horn on Day 2 in 5, 7 and 12 cows in the control group, 250 and 750 iu eCG treatment groups, respectively; although, differences were not statistically significant.

There were fewer corpora lutea formed during the study in the ipsilateral ovary than in the contralateral ovary (6 *vs.* 20, $P < 0.01$); however, there were no significant differences between treatments.

Plasma oestradiol concentration

Plasma oestradiol concentrations for all cows receiving each treatment during the study are summarised in Fig. 2. There were no significant differences between the treatment groups when comparing the concentration of plasma oestradiol within each Day of the study. Although plasma oestradiol concentration increased ($P < 0.05$) between Day 4 and 6 for control animals, and Day 0 and 2 for the 750 iu eCG group, there was no clear pattern of oestradiol concentration changes.

There were 12, 8 and 11 cows with a plasma oestradiol concentration < 1 pg/ml on Day 0 in the control, 250 and 750 iu eCG treatment groups, respectively. The plasma oestradiol concentrations on each Day for these animals are summarised in Figure 3. Concentrations were higher ($P < 0.05$) on Day 6 compared with Day 0, 2 and 4, for control cows; higher on Day 4 and 6, compared with Day 0 for the 250 iu cows; and, higher on Day 2 and 4, compared with Day 0, for the 750 iu cows.

Uterine horn diameter

The previously gravid and contralateral uterine horn diameters are summarised in Figure 4. There was a significant difference between uterine horn diameter between the previously gravid and contralateral horn ($P < 0.01$) and between the interaction of side of horn and Day of study ($P < 0.01$). The previously gravid uterine horn diameter was significantly ($P < 0.001$) smaller on Day 6 compared with Day 0 in control cows, 250 iu and 750 iu eCG treated cows. The contralateral uterine horn diameter was smaller ($P < 0.05$) on Day 6 compared to Day 0 for control cows and 750 iu cows, but not for 250 iu eCG animals. The change in uterine horn diameter did not differ between treatment groups, or between cows with or without a follicle > 8 mm diameter in the ipsilateral ovary on Day 2 for all cows or within treatment groups. These differences in uterine horn diameter were confirmed by multivariate models, which indicated a highly significant effect of Day of the study ($P<0.0001$), and between the previously gravid and contralateral horn ($P<0.0001$); however, eCG treatment, presence of a follicle > 8 mm diameter in the ipsilateral ovary on Day 2, presence of abnormal vaginal mucus, lactation number, cow and interactions of these terms were not significant. In addition, there was no significant correlation between the uterine horn diameter and plasma oestradiol concentration (even after selecting only for cows with < 1 pg/ml plasma oestradiol concentrations), or accumulated follicular diameter.

For cows selected on the basis of a plasma oestradiol concentration < 1pg/ml on Day 0, the previously gravid uterine horn diameter was significantly smaller on Day 6 compared with Day 0 in control cows (49.6 ± 2.8 *vs.* 38.7 ± 2.8 mm, $P < 0.01$), and 750 iu eCG cows (49.1 ± 2.9 *vs.* 40.2 ± 2.1 mm, $P < 0.01$), but not 250 iu eCG treated cows (47.4 ± 5.6 *vs.* 37.4 ± 2.1 mm). Although the contralateral uterine horn

diameter was smaller on Day 6 compared to Day 0, the differences were not statistically significant for control cows (37.1 ± 1.8 *vs*. 31.6 ± 2.5 mm), 250 iu eCG animals (33.1 ± 3.2 *vs*. 31.0 ± 1.3 mm), and 750 iu eCG treated cows (37.7 ± 2.6 *vs*. 34.5 ± 1.5 mm). The change in uterine horn diameter did not differ between treatment groups, or between cows with or without a follicle > 8 mm diameter in the ipsilateral ovary to the previously gravid uterine horn on Day 2 for all cows or within treatment groups. Multivariate models were not significant for eCG treatment in these animals.

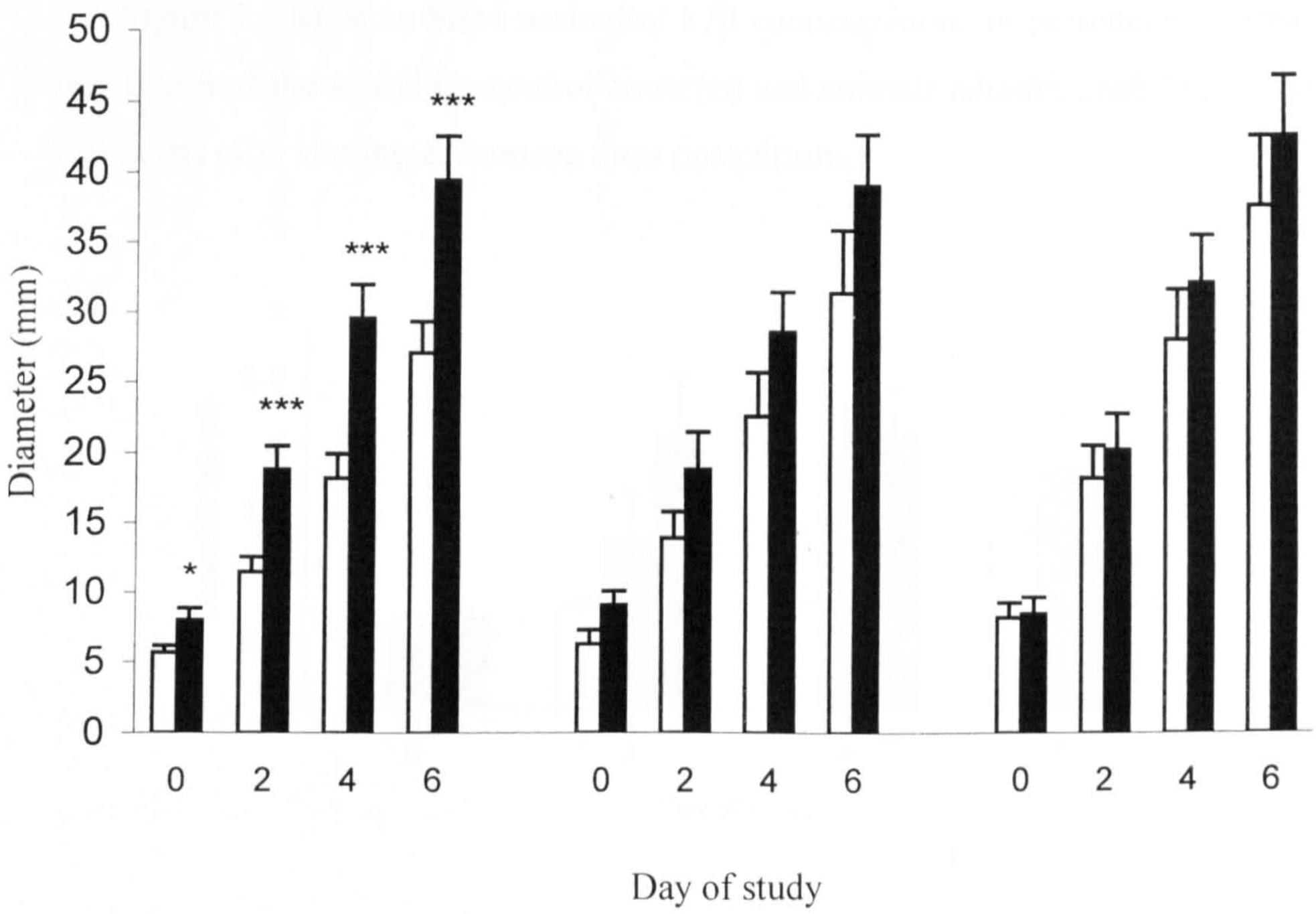

Figure 1. Mean ± SEM accumulated follicular diameter (mm) of the largest follicle > 4 mm diameter in the ovaries ipsilateral (□) and contralateral (■) to the previously gravid uterine horn on Day 0 to 6, for control cows and animals administered 250 iu or 750 iu eCG fourteen days postpartum. No significant differences were observed among data from the eCG treated cows. Values differ between columns within the control group on the same day of study. *P < 0.05; ***P < 0.001.

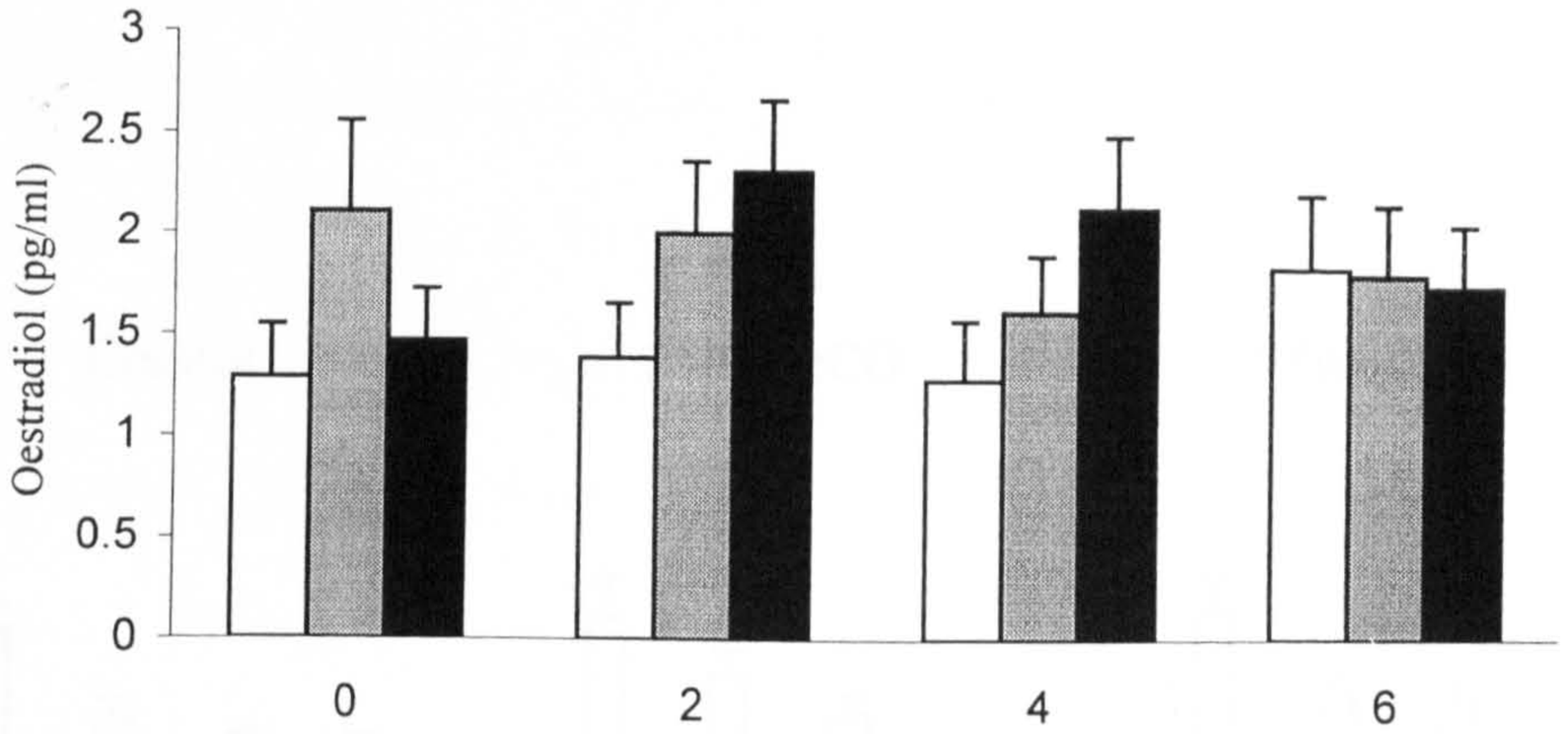

Figure 2. Mean ± SEM oestradiol 17β concentrations in peripheral plasma on Day 0 to 6 of the study, for control cows (□) and animals administered 250 iu (▩) or 750 iu (■) eCG starting at fourteen days postpartum.

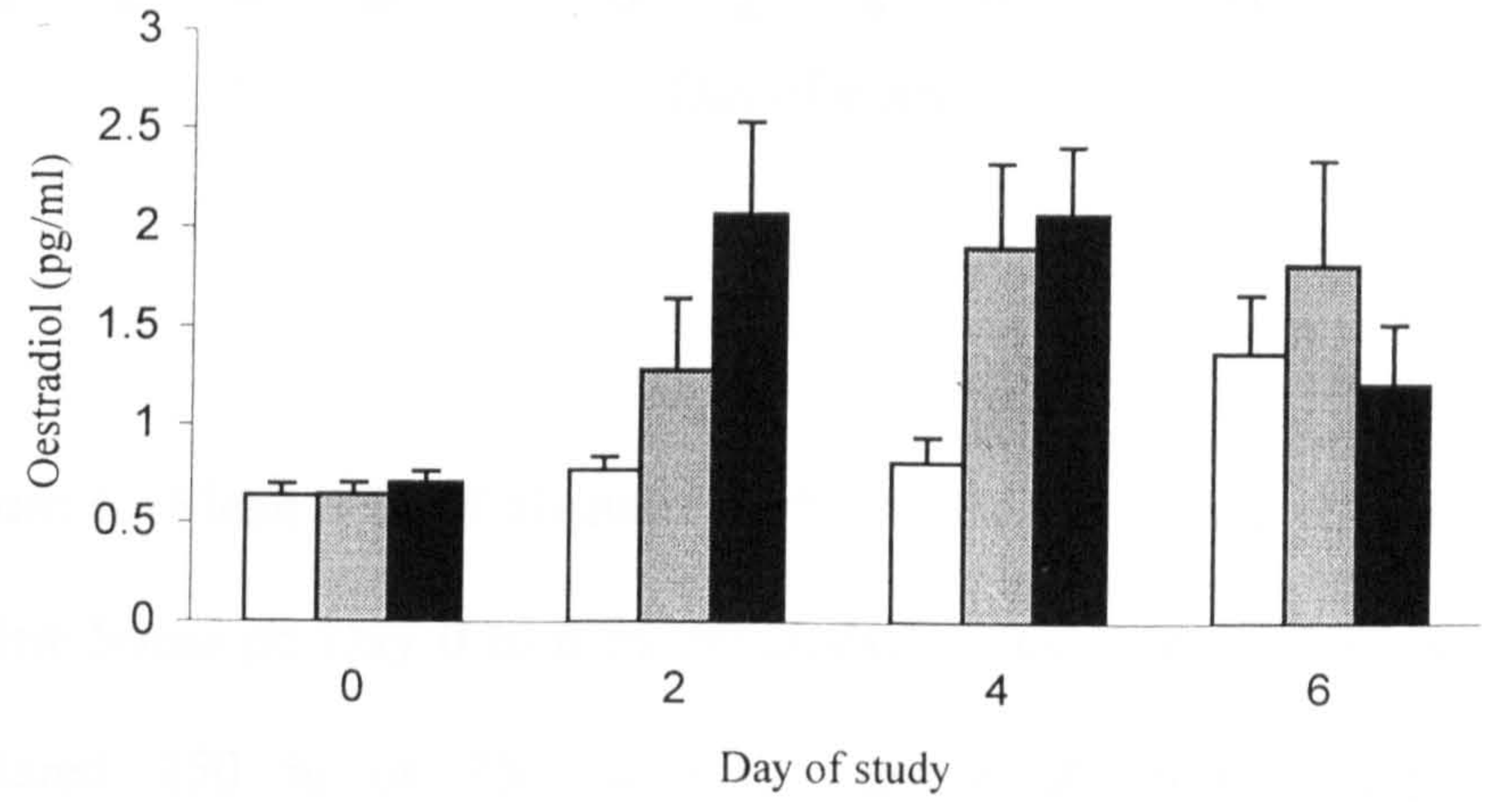

Figure 3. Mean ± SEM plasma oestradiol 17β concentrations on Day 0 to 6 of the study, for control cows (□) and animals administered 250 iu (▩) or 750 iu eCG (■) starting at fourteen days postpartum, only for cows with plasma oestradiol concentration of < 1 pg/ml on Day 0.

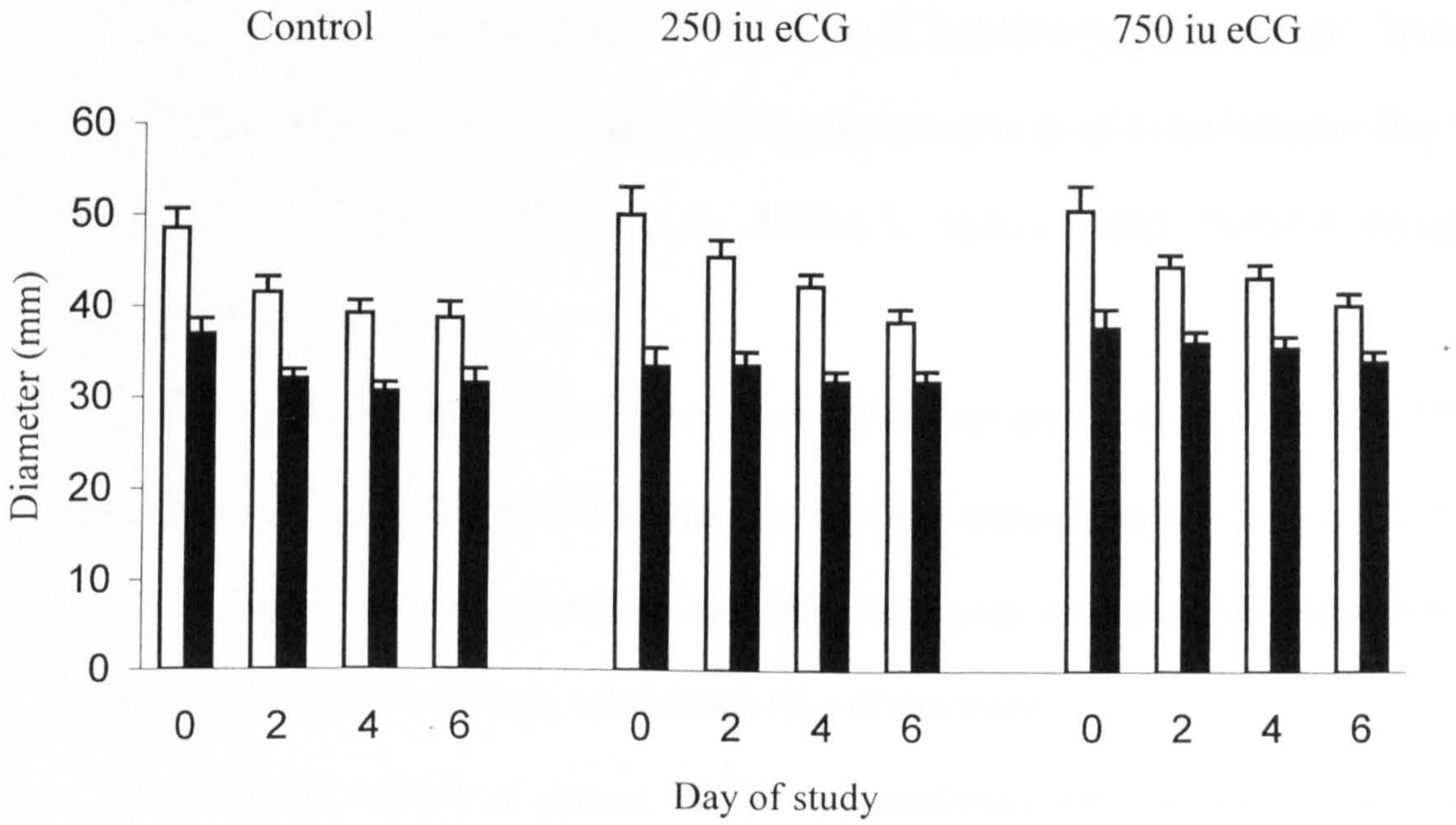

Figure 4. Mean ± SEM diameter of the previously gravid (□) and contralateral (■) uterine horns on Day 0 to 6 of the study, for all control cows and all animals administered 250 iu or 750 iu eCG starting at fourteen days postpartum.

Plasma PGFM concentration

The plasma PGFM concentration on Day 0 to 6, when subjected to multivariate modelling, showed a significant effect of Day ($P < 0.01$) and the presence of an abnormal vaginal discharge ($P < 0.01$), but not eCG treatment, presence of a follicle > 8 mm diameter in the ipsilateral ovary on Day 2, lactation number, or cow. There was a significant decrease in plasma PGFM concentration in all cows between Day 0 and Days 2, 4 and 6 (77.6±1.1 *vs.* 59.7±1.1, 56.0±1.1 and 58.6±1.1 pg/ml, respectively, $P < 0.001$).

Cows with abnormal vaginal mucus had higher concentrations of plasma PGFM throughout the study, compared with cows that had normal mucus (76.6 ± 1.1 *vs.* 51.0 ± 1.1 pg/ml, $P < 0.01$); however, the differences were not statistically significant when comparisons were made within each Day of the study.

Multivariate models of plasma PGFM concentrations for cows selected on the basis of a plasma oestradiol concentration < 1 pg/ml on Day 0, did not differ significantly between treatment groups.

Subsequent reproductive performance

The mean calving-to-first insemination interval was 84.9 ± 3.9, 85.7 ± 4.7 and 94.0 ± 7.1 days, and the mean calving-to-conception interval was 109.6 ± 13.3, 124.8 ± 13.3 and 114.2 ± 9.0 days, for the control, 250 iu and 750 iu eCG treatment groups, respectively; differences were not statistically significant. The number of inseminations per conception for the three groups was 1.4 ± 0.2, 2.1 ± 0.3 and 1.8 ± 0.3, respectively; again, differences were not statistically significant.

Cows with normal vaginal mucus had a shorter calving-to-conception interval compared with animals with abnormal mucus (106.2 ± 6.1 *vs.* 143.6 ± 18.8 days, $P < 0.05$) and fewer inseminations per conception (1.6 ± 0.2 *vs.* 2.2 ± 0.3, $P < 0.05$);

however, the calving-to-first insemination interval (88.9 ± 4.0 *vs.* 86.6 ± 4.6 days) did not differ significantly.

Discussion

Administration of 250 iu or 750 iu eCG at 14 days postpartum overcame the inhibition of folliculogenesis in the ovary ipsilateral to the previously gravid uterine horn that was evident in control animals. Although there was an associated increase in peripheral plasma oestradiol concentrations following administration of eCG, there was no local effect of greater follicular growth in the eCG treated animals on the rate of uterine involution determined by ultrasonography.

In the present study, there were fewer follicles > 4 mm or > 8 mm diameter in the ovary ipsilateral to the previously gravid uterine horn on the first day of the study, supporting previous observations (Lewis *et al.*, 1984; Guilbault *et al.*, 1987; Kamimura *et al.*, 1993; Nation *et al.*, 1999; Sheldon *et al.*, 2000; Chapter 3). Ovarian follicular activity was also estimated in the present study by additively accumulating the diameter of the largest follicle in each ovary during the study period (Risco *et al.*, 1994). In the control animals, this transformation clearly illustrated that on each day of the study there was less follicular growth in the ovary ipsilateral to the previously gravid uterine horn, confirming the negative influence exerted by the previously gravid uterine horn on folliculogenesis in the ipsilateral ovary (Lewis *et al.*, 1984; Dufour and Roy, 1985). The mechanism is unclear but may involve a response to uterine tract inflammation, possibly mediated by cytokines or immune cells; parallel immunological influences of uterine origin on ovarian corpus luteum function have already been proposed (Spencer *et al.*, 1999). Interestingly, uterine tract infection, as reflected by an abnormal vaginal discharge,

affected the rate of growth of the largest follicle in the contralateral ovary, which supports and extends previous observations that the presence of uterine infection was associated with fewer animals with a corpus luteum in the contralateral ovary (Sheldon *et al.*, 2000; Chapter 3).

Administration of 250 iu or 750 iu eCG in the current study overcame the negative influence of the previously gravid uterine horn and, specifically, increased follicular growth in the ipsilateral ovary determined using accumulated follicular diameter of follicles > 4 mm diameter; with a similar trend using accumulated follicular diameter of follicles > 8 mm diameter. These observations suggest that uterine-derived inhibitor(s) of follicular growth may inhibit the action of FSH and/or LH on follicular activity. Administration of eCG to cattle (i) leads to fewer atretic follicles, (ii) recruits more smaller follicles in which growth rate is increased, (iii) sustains the growth of larger follicles, and (iv) increases the final number of ovulations (Newcomb *et al.*, 1979; Monniaux *et al.*, 1984; Driancourt *et al.*, 1991; Gonzalez *et al.*, 1994). We suggest that a uterine-derived inhibitor of folliculogenesis, acting principally on the ovary ipsilateral to the previously gravid uterine horn, possibly blocks the action of FSH and/or LH within that ovary, and that this block can be overcome by administration of eCG. The inhibition of folliculogenesis in the ipsilateral ovary reduces the number of ovulations, reflected by fewer corpora lutea, and reduced numbers of follicles > 8 mm and > 4 mm diameter (Sheldon *et al.*, 2000; Chapter 3). Thus the principal stage at which inhibition occurs is likely to be $\leq$ 4 mm diameter. Interestingly, a variety of cytokines reduce FSH-induced oestradiol production from bovine granulosa cells collected from 1-5 mm diameter follicles, but have little effect on cells from follicles $\geq$ 8 mm diameter (Spicer and Alpizar, 1994).

The presence of a follicle > 8 to 9 mm diameter at the time of treatment, reduces the superovulatory response to eCG (Guilbault *et al.*, 1991; Huhtinen *et al.*, 1992). In the postpartum period, there is a succession of follicles growing to 5 to 9 mm diameter and then regressing, until development of the first dominant follicle (Murphy *et al.*, 1990). Plasma oestradiol concentrations are < 1 pg/ml prior to follicles reaching 8-9 mm diameter; subsequently, concentrations increase above 1 pg/ml in the presence of ovulatory, but not non-ovulatory, dominant follicles (Beam and Butler, 1997). Therefore, in the present study, cows could have been selected for analysis from the data on the basis of ovarian follicular diameter and/or plasma oestradiol concentration on Day 0; unfortunately, there were insufficient animals to select for both parameters. As our hypothesis was that the effect of a follicle on uterine involution was mediated by oestradiol, cows with plasma oestradiol concentration < 1 pg/ml on Days 0 were selected for analysis. The validity of this approach was confirmed by contrasting the plasma oestradiol concentration profile of all cows, with that of the selected animals. In the former, there was no clear pattern of plasma oestradiol concentrations throughout the study. In contrast, following selection of cases, plasma oestradiol concentration increased by Day 6 for the control animals, by Day 4 for the 250 iu group and by Day 2 for the 750 iu eCG group.

Uterine involution during the period between 14 and 28 days postpartum is characterised by a decline in uterine horn diameter (Gier and Marion, 1968). A similar decline in diameter occurred in the uterine horns of each group in the present study. However, there was no difference in the rate of previously gravid uterine horn involution between the treatment groups when all animals, or only those with a plasma oestradiol concentration < 1 pg/ml, were considered. Furthermore, the presence of a follicle > 8 mm diameter in the ipsilateral ovary on Day 2 did not

increase the rate of uterine involution in the previously gravid uterine horn, compared with cows with smaller follicles, either within treatment groups or using combined data.

An alternative method of assessing uterine involution is the estimation of plasma PGFM concentration, which is correlated with changes in the dimensions of the genital tract after calving (Lindell *et al.*, 1982; Lewis *et al.*, 1984). Plasma PGFM concentration is generally high during, and immediately after, parturition and declines to basal concentrations by Day 20 postpartum (Lewis *et al.*, 1984). In the present study plasma PGFM concentration was greater on Day 0 than on subsequent days of the study. However, there was no difference in PGFM concentration during the study period between the treatment groups when either all the animals or only those with a plasma oestradiol concentration < 1 pg/ml were considered. This observation, in addition to the absence of an effect of treatment on uterine horn diameter, indicates that eCG treatment did not affect uterine involution. Furthermore, there was no evidence that increased oestradiol secretion and/or large follicles in the ipsilateral ovary had a local effect to increase the rate of uterine involution during the study period. However, as the major changes in uterine horn diameter and plasma PGFM concentration occur between calving and day 14 postpartum, future studies could consider earlier intervention.

Although, administration of eCG failed to influence uterine involution or subsequent fertility in the present study, an explanation is required for the beneficial effect of large follicles in the ipsilateral ovary on subsequent fertility reported previously (Bonnett *et al.*, 1993; Sheldon *et al.*, 2000; Chapter 3). Possibly, the effect of such a follicle on the uterus may not be detectable by the present techniques or a longer period of study may be required. An alternative hypothesis is that the

appearance of a large follicle may reflect the early demise of the negative influence of the previously gravid uterine horn on the ipsilateral ovary. This, in turn, may reflect earlier recovery of the postpartum uterus other than changes in dimension, which may have a beneficial effect on subsequent fertility.

During the period of study, plasma PGFM concentration was higher in cows with abnormal, compared with normal, vaginal mucus which is in agreement with previous reports (Lindell *et al.*, 1982; Del Vecchio *et al.*, 1994). However, when plasma PGFM concentrations were compared on each Day of study, differences between abnormal and normal vaginal mucus cows were not significant, which agrees with reports that plasma PGFM concentration was not a reliable diagnostic indicator of endometritis (Archbald *et al.*, 1998). The presence of mucopurulent vaginal mucus was similarly associated with impaired reproductive performance in the present study and that reported by Archbald *et al.* (1998).

In conclusion, parenteral administration of 250 iu or 750 iu eCG increased follicular growth in the ipsilateral ovary, overcoming the inhibition evident in control cows. However, the increased ipsilateral ovarian follicular growth and increased plasma oestradiol concentrations did not affect uterine involution assessed by ultrasonography or by plasma PGFM concentration. Therefore, a local effect of a large follicle in the ipsilateral ovary on the previously gravid uterine horn was not demonstrated and the alternative pathway, the uterus influencing the ovary, should be considered.

Chapter 5

The effect of intrauterine administration of oestradiol on postpartum uterine involution in cattle

Introduction

There is a predilection for greater ovarian folliculogenesis in the ovary contralateral to the previously gravid uterine horn during the first four weeks after parturition in cattle (Kamimura *et al.*, 1993; Nation *et al.*, 1999; Sheldon *et al.*, 2000; Chapter 3). Fewer follicles emerge and fewer first dominant follicles are selected in the ovary ipsilateral to the previously gravid uterine horn. However, the presence of a large follicle in the ipsilateral ovary is a marker of contemporaneous and subsequent improved fertility (Bonnett *et al.*, 1993; Bridges *et al.*, 2000; Sheldon *et al.*, 2000; Chapter 3). Such improved fertility could reflect an effect of the ovary on the uterus.

After parturition, the uterus must undergo marked changes to return to the normal non-pregnant state before the next pregnancy can be established. The changes include uterine involution, regeneration of the endometrium, and elimination of the bacterial contamination that is ubiquitous after parturition in cattle. A large oestradiol-secreting follicle in the ipsilateral ovary could have a localised beneficial effect to hasten uterine involution. The principal source of increased oestradiol in peripheral plasma is via the utero-ovarian vein draining the ovary containing the dominant follicle (Ireland *et al.*, 1984). Uterine involution can be monitored directly using ultrasonography, or indirectly by estimation of the concentration of PGFM or acute phase proteins in peripheral plasma (Lindell *et al.*, 1982; Sheldon *et al.*, 2001). Increasing ovarian follicular growth and plasma oestradiol concentrations by administration of eCG on Day 14 postpartum did not affect uterine involution as

determined by plasma PGFM concentrations or changes in uterine diameter (Sheldon and Dobson, 2000; Chapter 4). However, plasma oestradiol concentrations did not increase until several days after the time for selection of the first dominant follicle, which occurs around Day 10 postpartum (Beam and Butler, 1997). Furthermore, the increased oestradiol concentration following eCG administration was not restricted to the ovary ipsilateral to the previously gravid uterine horn. Thus, the aim of the present study was to test the hypothesis that oestradiol increases the rate of uterine involution when administered directly into the previously gravid uterine horn around the expected time of selection of the first postpartum dominant follicle.

Materials and Methods

Animals

A dairy herd of 90 Holstein-Friesian cows, with an annual average milk yield of 7,500 litres, was selected for the study on the basis of accurate farm records. The vagina of each cow was examined on Day 7 postpartum by direct palpation and inspection of the lumenal contents. Cows with retained fetal membranes, gross vaginal lacerations, inappetence, or pyrexia were excluded from the study to reduce confounding effects of clinical disease. Cows were also excluded from the study if there was a dominant follicle identified on Day 7 using transrectal ultrasonography (see later). Using a randomisation chart (www.randomizer.org) animals were randomly assigned into treatment or control groups on Day 7 postpartum. Day 7 was chosen for assignment of treatments because it is 2 days before the expected ultrasonographic detection of the first dominant follicle. The interval from calving to

the day when the follicle diameter exceeded 10 mm was 9.2 ± 0.3 days in this herd (Sheldon, unpublished data).

All procedures were carried out under the Animals (Scientific Procedures) Act 1986 regulations for experiments on living animals, administered by the UK Home Office. In addition, experimental protocols were approved by the Royal Veterinary College Ethical Review Committee.

Treatments

For each animal, the side of the previously gravid uterine horn was identified by transrectal palpation as that which was longest and had the greatest diameter. Animals in the treatment group (n = 15) were infused with 10 mg oestradiol benzoate (Intervet UK Ltd, Cambridge) emulsified in 9 ml sterile 0.9 % w/v saline by vigorous shaking, into the previously gravid uterine horn using a trans-cervical catheter on Day 7 and again on Day 10. Animals in the control group (n = 14) were infused with an equal volume of saline. A dose of 10 mg oestradiol benzoate was chosen because administration of the same dose into the vagina achieves peripheral plasma concentrations similar to those during the follicular phase of the oestrous cycle (Burke *et al.*, 1999). Day 10 was selected for the administration of the second dose on the basis of a half-life for our preparation of about 16 h (DJ Sutton, Intervet UK, personal communication).

Examination

The genital tracts of all cows were examined daily by transrectal palpation and ultrasonography using a 7.5-MHz linear array transducer (Aloka SSD 210 DXII, BCF Technology, Livingstone) starting on Day 7 postpartum and continuing for 21 days. Follicles were defined as non-echogenic (black) spherical structures with a clear demarcation between the follicular wall and antrum. Corpora lutea were

defined as grainy echogenic structures that had a well-defined border with the less echogenic ovarian stroma; in some corpora lutea there was a non-echogenic lacuna. After freezing the image on the screen, the number of ovarian follicles > 4 mm internal diameter and corpora lutea in each ovary were counted, and maximum diameters measured using the internal callipers of the machine. The diameter of each uterine horn was measured at the level of attachment of the intercornual ligament. When the image of the structure being scanned was not spherical, the diameter was estimated by averaging two 90° dimensions.

A dominant follicle was defined as the largest follicle in the ovary with ≥ 10 mm internal diameter in the absence of other growing follicles (Dobson *et al.*, 2000). A dominant follicle and cohorts were defined as a follicular wave (Dobson *et al.*, 2000). The first day of dominance within a follicular wave was determined, retrospectively, as the day on which the dominant follicle was first ≥ 10 mm diameter. Day of ovulation was defined as the day when a dominant follicle was last identified ultrasonographically prior to subsequent appearance of a corpus luteum in the same location, and confirmed, retrospectively, by a subsequent increase in plasma progesterone concentration to > 1 ng/ml. A persistent follicle was defined as a follicle ≥ 10 mm internal diameter that persisted for more than 5 consecutive days and did not ovulate (Dobson *et al.*, 2000).

Uterine swab collection and bacteriology

A trans-cervical guarded swab was collected from the central uterine body before horn bifurcation from each cow on Day 7 postpartum using a previously validated method (Noakes *et al.*, 1989). The swab was transferred to a bijou bottle containing Stuart Transport Medium (Unipath, Basingstoke) and was cultured within 1 h of collection, at the on-site bacteriology laboratory. Swabs were cultured

aerobically and anaerobically on pre-equilibrated sheep blood agar (Unipath), and aerobically on MacConkey agar (Unipath). Identification of bacteria was based on the characteristics of the colony, Gram-stain, morphology, haemolysis, biochemical profile (API systems, bioMérieux, Basingstoke), and other standard tests (Barrow and Feltham, 1993). Bacterial growth on the culture plates was scored semi-quantitatively, dependent on the number of bacterial colonies detected on the plate: 0: no growth; 1: < 10 colonies; 2: 10 to 100 colonies; 3: 100 to 500 colonies; and, 4: > 500 colonies (Noakes *et al.*, 1991). The total bacterial growth score was the sum of the scores for each of the bacterial isolates and this was used to categorize cows into standard or high bacterial contamination. The standard category was defined as the lower 75% quartile total bacterial growth score and the high contamination category as the upper 25% (Sheldon and Dobson, unpublished data).

Blood Sampling and Hormone Assays

Starting on Day 7 postpartum, blood samples were collected daily for 21 days from the coccygeal vein or artery into evacuated heparinised and plain tubes (Vacutainer, Becton Dickinson, Meylan, France) and transported on ice to the laboratory. Plasma, within 30 minutes, and serum after clotting at room temperature for 1 h, were separated by centrifugation (2200g for 10 min), harvested and stored frozen at -20°C.

Oestradiol-17β concentration was measured in duplicate following diethyl ether extraction of plasma samples using a radioimmunoassay (RIA) kit (Estradiol MAIA, Serono Diagnostics Ltd, Woking) modified to increase sensitivity and validated for bovine plasma (Mann *et al.*, 1995). The mean intra-assay (n = 12 samples) and inter-assay (n = 3 assays) coefficient of variation was 8.1% and 13.1%, respectively for a 0.9 pg/ml sample, and the sensitivity was 0.24 pg/ml. Progesterone concentration

was measured in duplicate using a commercial ELISA kit (Ridgeway Science, Gloucester). The intra-assay (n = 10 samples) and inter-assay (n = 3 assays) coefficients of variation were 6.5% and 11.2%, respectively for a 1.7 ng/ml sample, and the sensitivity was 0.6 ng/ml. The concentration of FSH in plasma was measured in duplicate using a previously characterized direct RIA (Dobson, 1978b). The standard used for the FSH assay was AFP 5679C RP-1. The intra-assay (n = 20 samples) and inter-assay (n = 3 assays) coefficient of variation was 3.4% and 4.7%, respectively for a 1.2 ng/ml sample, and the sensitivity was 0.12 ng/ml. The concentration of PGFM was measured in duplicate by direct RIA using plasma in a single assay (Kindahl *et al.*, 1976). The mean intra-assay (n = 20 samples) coefficient of variation was 7.6% for a 91.2 pg/ml pooled plasma sample, and the sensitivity was 27.0 pg/ml.

The concentrations of the acute phase proteins α_1-acid glycoprotein and haptoglobin in plasma, and ceruloplasmin in serum, were measured in duplicate using methods adapted for 96 well microtitre plates (Life Technologies, Paisley, Scotland) for bovine samples (Lewis *et al.*, 1989; Sheldon *et al.*, 2001). For α_1-acid glycoprotein, the intra-assay (n = 10 samples, mean of 3 assays) and inter-assay (n = 3 assays) coefficient of variation was 9.6% and 14.8%, respectively for a 1.7 mg/ml sample, and the sensitivity was 0.2 mg/ml. For haptoglobin, the intra-assay (n = 10 samples, mean of 3 assays) and inter-assay (n = 3 assays) coefficient of variation was 10.8 % and 11.8%, respectively for a 152 μg/ml sample, and the sensitivity was 36 μg/ml. For ceruloplasmin, the intra-assay (n = 10 samples, mean of 3 assays) and inter-assay (n = 3 assays) coefficient of variation was 8.6% and 17.7%, respectively for a 20.8 units/ml sample, and the sensitivity was 3.6 units/ml.

Statistical analysis

Data analysis was performed using SAS ver 8.01 computer program (SAS Institute Inc., Cary, NC). Results are quoted as arithmetic mean ± SEM, and significance was attributed at $P < 0.05$.

The location of the first dominant follicle or first ovulation, in relation to the previously gravid uterine horn, was compared between the ipsilateral and contralateral ovaries using Fisher's exact test. Survival analysis, using the Kaplan Meier test, was used to compare between treatment groups the time intervals from calving to appearance of the first dominant follicle, or to ovulation (Kalbfleisch and Prentice, 1980).

The diameter of the largest follicle in the ovary, the first postpartum dominant follicle and uterine horn diameters, and plasma concentrations of hormones and acute phase proteins were examined using a repeated measurements ANOVA mixed model with a first order autoregressive covariance structure (SAS, 1997). Data were examined for normality, and plasma concentrations of hormones, and acute phase proteins were logarithmically transformed before analysis. The analysis of follicle diameter and plasma oestradiol and FSH concentrations were examined between the start of the study (Day 7 postpartum) and the mean day of ovulation for the first dominant follicle (Day 16); whilst, the remainder of the data were examined between Day 7 and 28. The explanatory variables were Day postpartum, treatment group, fate of the first dominant follicle, location of the first dominant follicle, and bacterial contamination category, and their interactions with Day postpartum. There were insufficient numbers of animals in which the first dominant follicle persisted for statistical analysis ($n = 2$), so analysis of the fate of the dominant follicle was restricted to animals in which the follicle ovulated or regressed.

Correlation between uterine diameters and plasma concentrations of PGFM or acute phase proteins was examined using Pearson Correlation coefficients.

Results

Ovarian ultrasonography

The mean diameter of the largest follicle on Day 7 was similar for animals assigned to control or treatment groups (7.0 ± 1.2 vs. 6.2 ± 0.6 mm). A dominant follicle was identified in each animal within two weeks of parturition. However, there were fewer dominant follicles in the ipsilateral ovary to the previously gravid uterine horn, compared with the contralateral ovary, for animals in the control group (3 vs. 11, $P < 0.05$) and the treatment group (1 vs. 14, $P < 0.001$).

For cows in the control group, the first dominant follicle ovulated, regressed, or persisted in 11, 2 and 1 animals, respectively. Treated animals had two patterns of dominant follicle growth. In 3 animals, the largest ovarian follicle present on Day 7 (n = 2, contralateral ovary) or Day 10 (n = 1, ipsilateral ovary) ovulated within 48 h of oestradiol treatment. In each of these three cases, the largest follicle was > 8 mm diameter at the time of treatment. These animals in which there was premature ovulation were excluded from further analysis. In the remaining 12 treated animals, the largest follicle on Day 7 and its cohort of smaller follicles regressed within 3 to 5 days, followed by the emergence of a new follicular wave and selection of a dominant follicle. In each case this first dominant follicle was in the contralateral ovary and ovulated, regressed, or persisted in 6, 5 and 1 cases, respectively. The calving to dominance interval for these treated animals was longer than for control animals (15.0 ± 1.0 vs. 10.1 ± 1.0 days, $P < 0.05$), and the calving to ovulation

interval was also longer (18.8 ± 1.2 vs. 14.0 ± 0.9 days, $P < 0.01$). Furthermore, there was a trend for fewer treated animals to ovulate compared with control animals (6/12 vs. 11/14, $P = 0.10$).

Changes in the diameter of the largest follicle, initially specified on Day 7, differed significantly with Day postpartum ($P < 0.001$) and between treatment groups ($P < 0.01$, Fig. 1a), reflecting the arrest of follicle growth in treated animals between Days 7 and 11. The diameter of the first dominant follicle also differed significantly with Day postpartum ($P < 0.001$) but not between treatment groups, and the interaction of Day x treatment was not significant. Similarly, the maximum follicular diameter for follicles that ovulated did not differ significantly between the control and treatment groups (14.4 ± 0.9 vs. 16.0 ± 1.0 mm).

The diameter of the first dominant follicle did not differ significantly with uterine bacterial contamination category, nor between those first dominant follicles in the ipsilateral compared with the contralateral ovary. However, there was a significant effect of the fate of the dominant follicle ($P < 0.05$) and the interaction of fate x Day was significant ($P < 0.001$). Between Days 7 and 16, the mean follicle diameter was smaller in those animals in which the first dominant follicles subsequently regressed compared with those that ovulated (7.6 ± 0.4 vs. 9.3 ± 0.3 mm, $P < 0.01$).

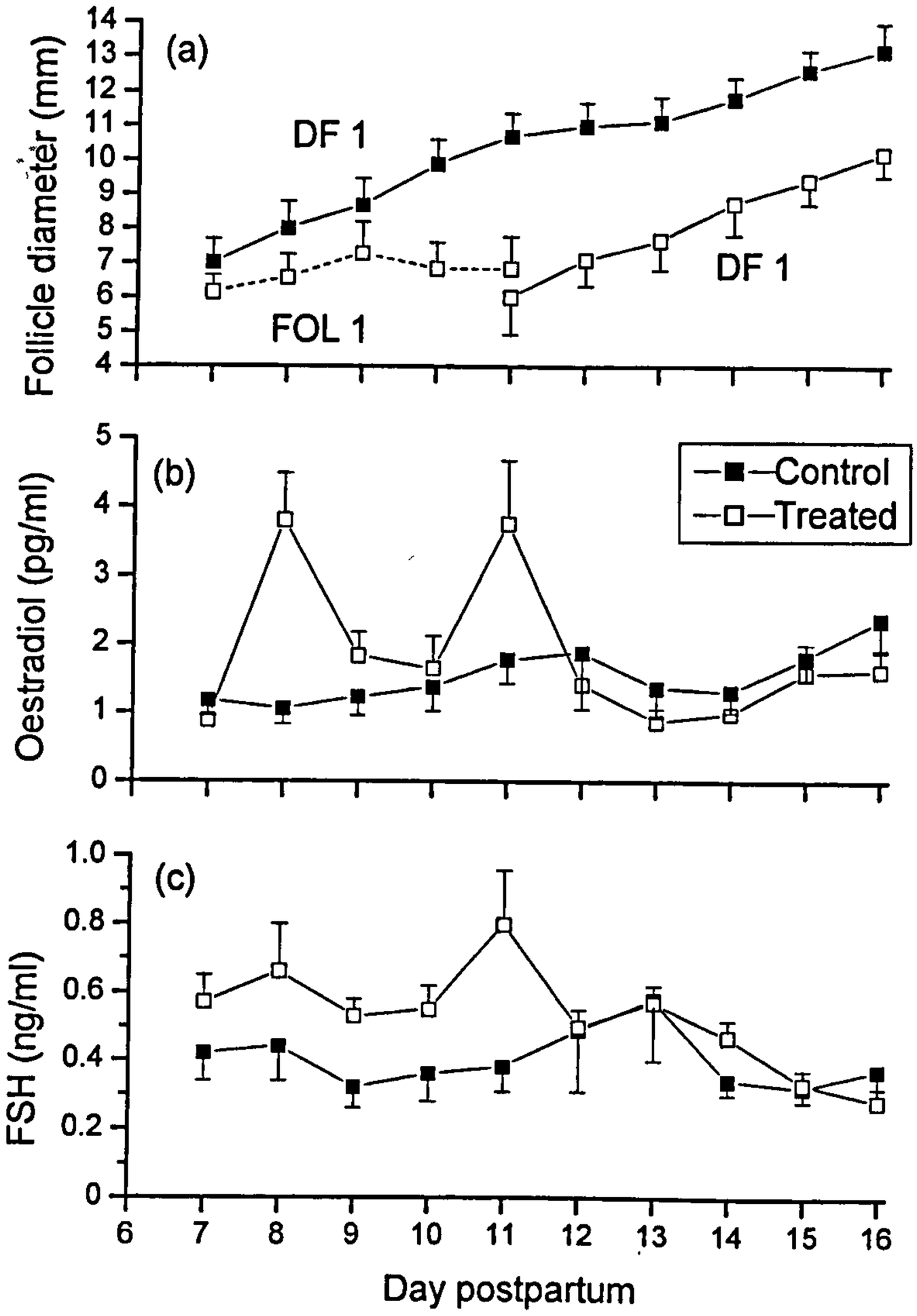

Figure 1. Mean ± SEM (a) diameter of the largest follicle initially identified on Day 7 (FOL 1) and/or the first dominant follicle (DF 1), (b) plasma oestradiol concentration, and (c) plasma FSH concentration, for 12 treated (solid symbols; 10 mg oestradiol benzoate administered into the previously gravid uterine horn on Day 7 and Day 10) or 14 control animals (open symbols). Occasionally the SEM bar was within the size of the symbol.

Plasma oestradiol and FSH concentrations

Plasma oestradiol concentrations differed significantly with Day postpartum ($P < 0.05$) and between treatment groups (Fig. 1b, $P < 0.001$), and the interaction of Day x treatment was significant ($P < 0.001$). Mean plasma oestradiol concentrations on Day 7 were similar for cows assigned to treatment or control groups. However, two peaks of plasma oestradiol concentrations were noted in the treated animals, on Days 8 and 11, following intrauterine infusion of oestradiol benzoate on Days 7 and 10. The maximum concentration of oestradiol on Day 8 was not significantly different to that on Day 11 (3.82 ± 0.68 vs. 3.75 ± 0.93 pg/ml).

Plasma oestradiol concentrations differed significantly between animals with a different fate of the first dominant follicle ($P < 0.001$), but the interaction of fate x Day was not significant. Between Day 7 and 16, mean plasma oestradiol concentrations were lower in animals in which the first dominant follicle regressed compared with cows that ovulated this follicle in the control group (0.75 ± 0.09 vs. 1.3 ± 0.10 pg/ml, $P < 0.05$), and the treated group (1.26 ± 0.26 vs. 3.08 ± 0.49 pg/ml, $P < 0.05$). Plasma oestradiol concentrations did not differ significantly between animals with the first dominant follicle in the ipsilateral or contralateral ovaries, or between uterine bacterial contamination categories.

Plasma FSH concentrations differed significantly with Day postpartum ($P < 0.05$) and between treatment groups ($P < 0.05$, Fig. 1c). For treated animals, the highest concentrations of FSH were on Days 8 and 11, coincident with peak oestradiol concentrations. Plasma FSH concentrations between Day 7 and 16 did not differ significantly between cows with different fates of the first dominant follicle, uterine bacterial contamination category, or between cows with the first dominant follicle in the ipsilateral or contralateral ovary.

Oestradiol and FSH plasma concentrations after Day 16 varied considerably because of differences in follicular growth between animals; therefore, these data precluded meaningful analysis.

Uterine ultrasonography

Between Day 7 and 28, the diameters of the previously gravid and non-gravid uterine horns progressively decreased as the postpartum interval increased ($P < 0.001$). However, uterine diameters did not differ significantly between treatment groups (Fig. 2a), or between animals in which the first dominant follicle was in the ipsilateral or the contralateral ovary. In animals with high uterine bacterial contamination, the diameters of both the previously gravid ($P < 0.001$) and the non-gravid ($P < 0.05$) uterine horns were greater than for cows with standard contamination (Fig. 2b). In addition, the diameters of the previously gravid ($P < 0.01$) and non-gravid ($P < 0.001$) uterine horns were greater for animals in which the first dominant follicle regressed, compared with cows that ovulated the first follicle (Fig. 2c). Furthermore, the differences between animals that ovulated or regressed were significant between Days 10 and 15 for both the previously gravid and non-gravid uterine horns.

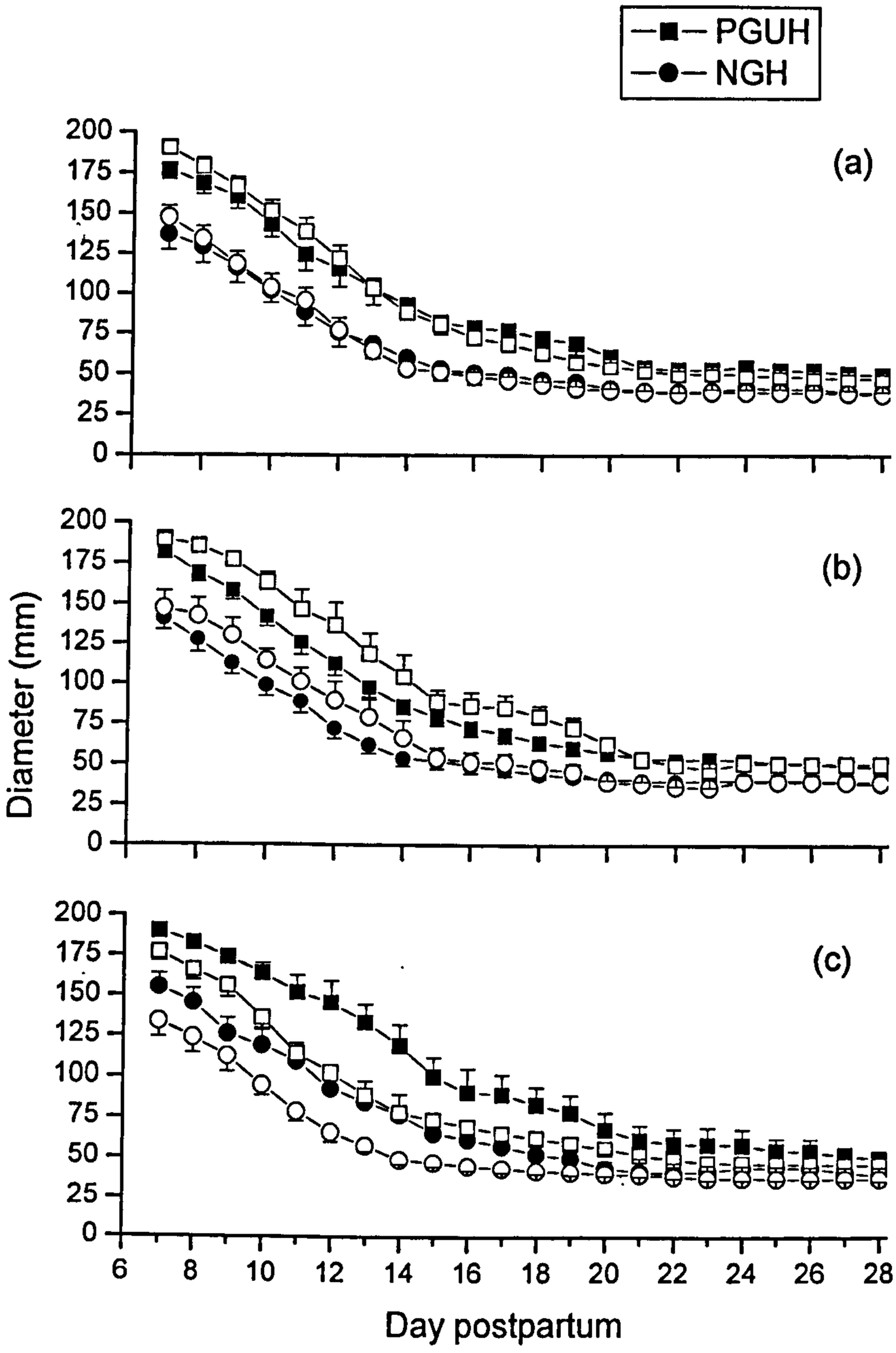

Figure 2. Mean ± SEM diameter of the previously gravid (square symbols; PGUH) and non-gravid (circular symbols; NGH) uterine horns for (a) control (solid symbols; n = 14) or treated (open symbols; n = 12; 10 mg oestradiol benzoate administered into the previously gravid uterine horn on Day 7 and Day 10) animals, (b) standard (solid symbols; n = 19) or high (open symbols; n = 7) uterine bacterial contamination, and (c) animals in which the first dominant follicle regressed (solid symbols; n = 7) or ovulated (open symbols; n = 17). SEM bars are frequently within the size of the symbol.

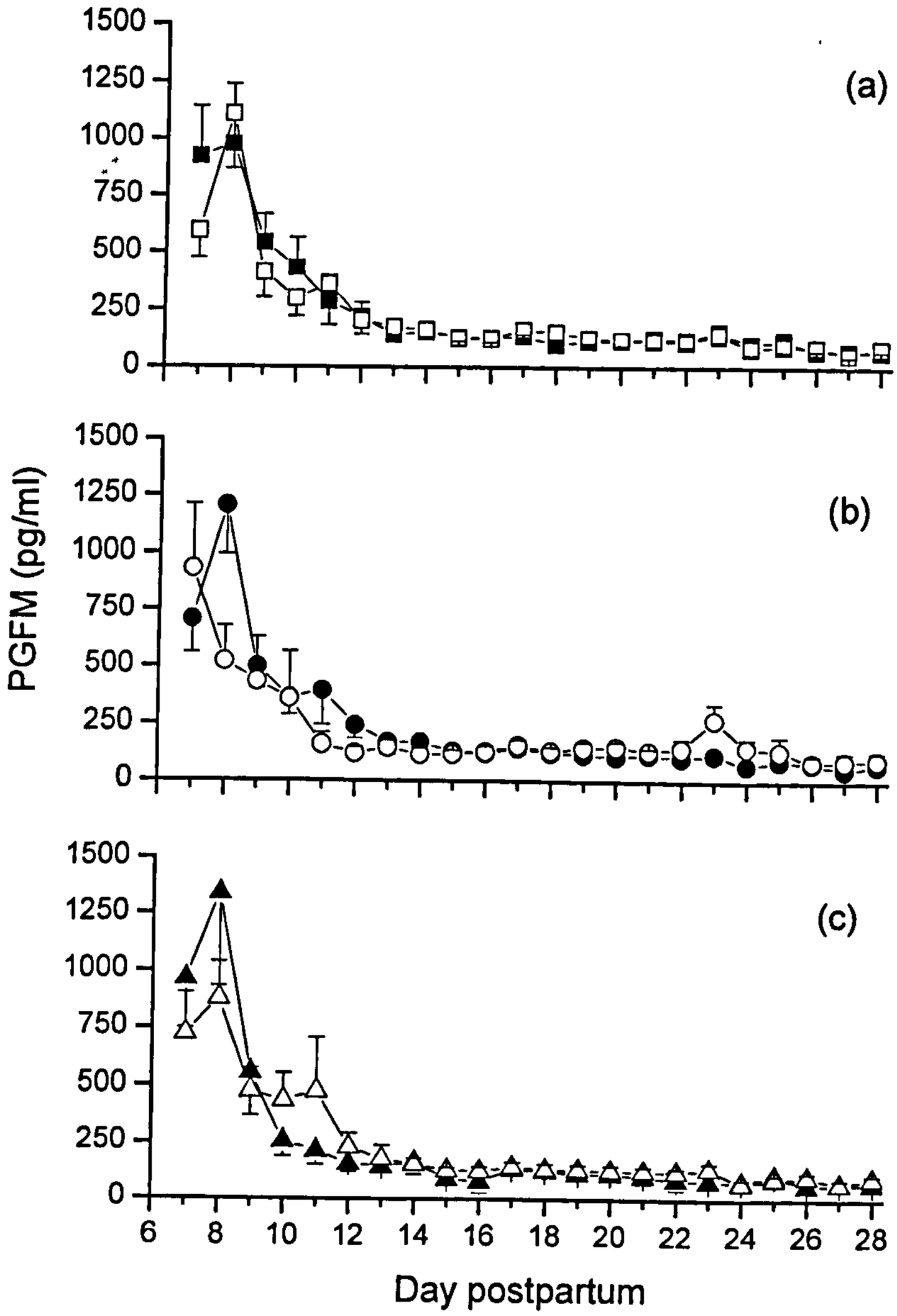

Figure 3. Mean ± SEM plasma PGFM concentration for (a) control (solid symbols; n = 14) or treated (open symbols; n = 12; 10 mg oestradiol benzoate administered into the previously gravid uterine horn on Day 7 and Day 10) animals, (b) standard (solid symbols; n = 19) or high (open symbols; n = 7) uterine bacterial contamination, and (c) animals in which the first dominant follicle regressed (solid symbols; n = 7) or ovulated (open symbols; n = 17). SEM bars are frequently within the size of the symbol.

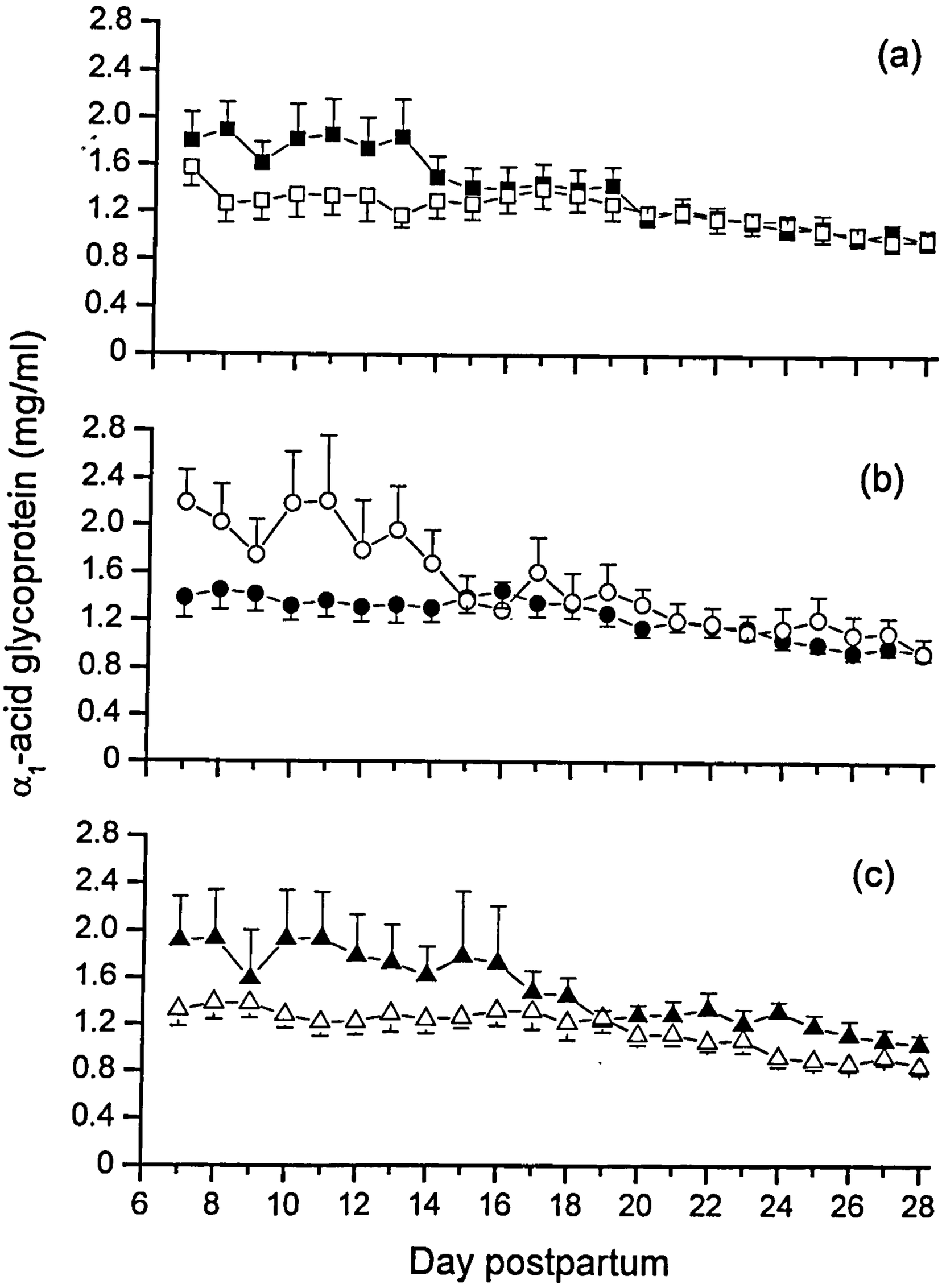

Figure 4. Mean ± SEM plasma α_1-acid glycoprotein concentration for (a) control (solid symbols; n = 14) or treated (open symbols; n = 12; 10 mg oestradiol benzoate administered into the previously gravid uterine horn on Day 7 and Day 10) animals, (b) standard (solid symbols; n = 19) or high (open symbols; n = 7) uterine bacterial contamination, and (c) animals in which the first dominant follicle regressed (solid symbols; n = 7) or ovulated (open symbols; n = 17). SEM bars are occasionally within the size of the symbol.

Plasma PGFM concentrations

Plasma PGFM concentrations were correlated with the diameter of the previously gravid ($r = 0.50$, $P < 0.001$) and non-gravid ($r = 0.53$, $P < 0.001$) uterine horns, although concentrations returned to basal values before uterine diameters reached minimum values. Plasma PGFM concentrations differed significantly with Day ($P < 0.001$) decreasing progressively from a maximum concentration of 1047 ± 174 pg/ml on Day 8 to a minimum concentration of 62 ± 12 pg/ml on Day 27. Plasma PGFM concentrations did not differ significantly between treatment groups (Fig. 3a), or between animals with standard or high uterine bacterial contamination (Fig. 3b). Furthermore, PGFM concentrations did not differ between cows with the first dominant follicle arising in the ipsilateral or contralateral ovary (data not shown), or between cows in which the first dominant follicle ovulated or regressed (Fig. 3c).

Plasma acute phase protein concentrations

There was a significant correlation between the diameter of the previously gravid or non-gravid uterine horns and the plasma concentration of α_1-acid glycoprotein ($r = 0.37$ and 0.33, respectively, $P < 0.001$), haptoglobin ($r = 0.46$ and 0.44, respectively, $P < 0.001$), and ceruloplasmin ($r = 0.50$ and 0.53, respectively, $P < 0.001$). However, plasma concentrations of acute phase proteins returned to basal values before uterine diameters reached minimum values.

Plasma α_1-acid glycoprotein concentrations differed significantly amongst Day ($P < 0.001$). Plasma concentrations progressively decreased from a maximum of 1.69 ± 0.15 mg/ml on Day 8, to a minimum of 0.93 ± 0.05 mg/ml on the last day of sampling, Day 28. Plasma α_1-acid glycoprotein concentrations were lower in treated cows than in controls (Fig. 4a, $P < 0.01$). The interaction of treatment x Day was

also significant ($P < 0.001$), with the greatest difference between the groups occurring between Day 8 and 13. Plasma α_1-acid glycoprotein concentrations were lower in animals with a standard uterine bacterial contamination compared with those highly contaminated (Fig. 4b, $P < 0.05$), and the interaction of bacterial contamination category x Day was also significant ($P < 0.001$). Plasma α_1-acid glycoprotein concentrations did not differ significantly between animals in which the first dominant follicle was in the ipsilateral of contralateral ovary (data not shown). However, in those animals in which the first dominant follicle ovulated, plasma α_1-acid glycoprotein concentrations were lower, compared with those animals in which the follicle regressed (Fig. 4c, $P < 0.01$), and the interaction of follicle fate x Day was significant ($P < 0.001$).

Plasma haptoglobin concentrations decreased progressively with increasing interval postpartum ($P < 0.001$; data not shown). Concentrations decreased from a maximum of 255.1 ± 55.9 μg/ml on Day 7 to a minimum of 9.8 ± 4.5 μg/ml on Day 28. Mean plasma haptoglobin concentrations between 7 and 28 days postpartum were higher in cows with high, compared with standard, uterine bacterial contamination (128.7 ± 20.1 vs. 68.1 ± 6.6 μg/ml, $P < 0.05$). However, plasma haptoglobin concentrations did not differ significantly between treatment groups, ovarian dominant follicle location, or fate of the first dominant follicle (data not shown).

Plasma ceruloplasmin concentrations also decreased progressively with increasing interval after parturition ($P < 0.001$), from a maximum of 40.1 ± 1.7 units/ml on Day 7 to a minimum of 26.2 ± 1.6 units/ml on Day 28. However plasma ceruloplasmin concentrations did not differ significantly between treatment groups,

uterine bacterial contamination category, ovarian dominant follicle location or the fate of the first dominant follicle (data not shown).

Discussion

The presence of an oestradiol-secreting dominant follicle in the ovary ipsilateral to the previously gravid uterine horn is a marker of contemporaneous and subsequent fertility (Bonnett *et al.*, 1993; Bridges *et al.*, 2000; Sheldon *et al.*, 2000; Chapter 3). We hypothesised that one explanation for these observations might be a localised effect of oestradiol from the dominant follicle in the ipsilateral ovary acting on the uterus to increase the rate of involution. However, in the present study, oestradiol administered into the previously gravid uterine horn around the expected time of the first postpartum dominant follicle did not influence uterine involution between Days 7 and 28 postpartum. There was no effect of oestradiol treatment on the diameter of either of the uterine horns, nor on plasma concentrations of PGFM or acute phase proteins, which are peripheral chemical markers of uterine involution. On the other hand, there was an association between smaller uterine horn diameter and subsequent ovulation of the first postpartum dominant follicle. Thus, our present hypothesis is not proven and the opposite pathway of uterus-to-ovary signalling may be more important; the uterus influences ovarian activity during the postpartum period.

The first treatment with oestradiol was administered in the present study before the expected time of selection of the first dominant follicle. This dose of oestradiol benzoate was selected on the basis of data reported by Burke *et al.* (1999). The interval between doses was selected on the basis of a half-life for this preparation of about 16 h. Administration of oestradiol benzoate into the previously gravid uterine horn lumen on Days 7 and 10 postpartum resulted in plasma oestradiol

concentrations exceeding normal follicular phase concentrations of 1.3 to 1.8 pg/ml for four to five days. The plasma concentrations in the present study were similar to those following administration of the same dose of oestradiol benzoate into the vagina (Burke *et al.*, 1999). In the latter study maximum plasma oestradiol concentrations of 6.4 pg/ml were identified 8 h after treatment, whereas in the present study peak concentrations were observed the day after each oestradiol treatment because blood samples were collected daily.

Uterine involution was monitored directly by daily measurement of uterine horn diameter using transrectal ultrasonography. The pattern of decreasing diameter of the previously gravid and non-gravid uterine horns with increasing time after parturition was similar to that of previous ultrasonographic studies (Okano and Tomizuka, 1987; Kamimura *et al.*, 1993). In the present study, localised oestradiol treatment had no effect on the progression of uterine involution. Similarly, there was no effect on the time to completion of uterine involution when 5 or 10 mg oestradiol was administered parenterally 48 h or 17 days after parturition, or in smaller doses on alternate days throughout the postpartum period (Marion *et al.*, 1968; Saiduddin *et al.*, 1968; Tian and Noakes, 1991a). Furthermore, stimulation of follicular growth and increased plasma oestradiol concentration by administration of eCG on Day 14 also had no effect on uterine involution (Sheldon and Dobson, 2000; Chapter 4). On the other hand, although there was no effect of oestradiol on gross uterine diameter, there could be an effect at the cellular or biochemical levels.

In addition to direct uterine measurements, involution in cattle can be monitored indirectly by estimation of peripheral plasma concentrations of PGFM and acute phase proteins (Lindell *et al.*, 1982; Sheldon *et al.*, 2001). Similarly, in the present study, plasma PGFM and acute phase protein concentrations decreased progressively

with increasing time after parturition and were correlated with uterine dimensions, although basal values are reached sooner. However, there was no effect of oestradiol treatment on plasma PGFM, haptoglobin, or ceruloplasmin concentrations. There was a lower plasma concentration of α_1-acid glycoprotein in treated animals, which could represent an effect of oestradiol on uterine involution. However, this appears unlikely in the absence of other similar changes in the measurement of uterine involution and in view of direct effects of oestradiol on α_1-acid glycoprotein. Oral oestrogens administered to humans have an effect on the hepatic glycosylation of α_1-acid glycoprotein which are opposite to those associated with inflammation (Brinkman-Van der Linden *et al.*, 1996). Although, the effect of oestradiol on α_1-acid glycoprotein secretion is not clear; both increased and decreased secretion rates have been reported after oestradiol treatment in rodents (Lebreton *et al.*, 1988; Deshpande *et al.*, 1997).

In the absence of a localised effect of oestradiol on uterine involution, an alternative hypothesis is required to explain how an ipsilateral first postpartum dominant follicle exerts its effect on subsequent fertility. The majority of first dominant follicles are observed in the ovary contralateral to the previously gravid uterine horn, with suppression of folliculogenesis in the ipsilateral ovary (Kamimura *et al.*, 1993; Sheldon *et al.*, 2000; Chapter 3). The reason for this suppression of folliculogenesis in the ipsilateral ovary is not clear; although it is not due to the regressing corpus luteum of pregnancy (Sheldon *et al.*, 2002; Chapter 7). The previously gravid uterine horn or its contents could cause a localised suppression of ovarian function. Thus, more rapid uterine involution, or elimination of bacterial contamination could facilitate the selection of the first dominant follicle in the ipsilateral ovary. Indeed in the present study, a smaller uterine horn diameter or

lower α_1- acid glycoprotein concentration was associated with ovulation of the first dominant follicle. Unfortunately, with few first dominant follicles in the ipsilateral ovary, statistical analysis of the effect of uterine involution on ovulation of that follicle was not possible. Another possibility is that uterine contamination may elicit a utero-ovarian signal and, as expected, uterine contamination in the present study was associated with wider uterine horns and higher concentrations of α_1-acid glycoprotein and haptoglobin.

Administration of oestradiol into the uterine lumen caused ovulation in three animals, as determined ultrasonographically and by a subsequent increase in plasma progesterone concentration. Oestradiol administered during the postpartum period, in the absence of a corpus luteum, induces increased plasma FSH and LH concentrations similar to those of the pre-ovulatory surge (Peters, 1984; Nanda *et al.*, 1988). Following the LH surge, ovulation occurs a day later, and increased plasma progesterone concentrations 6 to 7 days later (Sunderland *et al.*, 1994). In the present, study blood sample collection was probably too infrequent to detect this gonadotrophin surge precisely, although there was a significant increase in FSH concentration after oestradiol treatment and the highest concentrations of FSH and oestradiol were simultaneous. The three animals that ovulated shortly after treatment each had a follicle > 8 mm diameter at the time of treatment. This observation concurs with reports that selected dominant follicular growth deviates from subordinate follicles and physiological dominance is achieved at a diameter of 8 to 9 mm (Ginther *et al.*, 1999; Fortune *et al.*, 2001). For the remaining treated animals the first follicular wave was suppressed and a new follicular wave emerged, from which the first dominant follicle was selected. Follicular growth suppression and emergence of a new follicular wave about 4 days after treatment also occurs

following intra-vaginal administration of oestradiol benzoate (Burke *et al.*, 1999). Similarly, parenteral administration of oestradiol causes a brief decrease in plasma FSH concentration, but with a marked increase in concentration by 12 h after treatment with subsequent suppression of dominant follicle growth (Bo *et al.*, 1994).

In the control animals, most first dominant follicles ovulated, in agreement with previous studies of dairy cattle, and plasma oestradiol concentrations were lower in animals in which the first dominant follicle regressed (Roche *et al.*, 1992; Beam and Butler, 1997). The trend for fewer first dominant follicles to ovulate in the treated animals was probably caused by the exposure to oestradiol, as the presence of oestradiol at follicular phase concentrations for 2 to 4 days inhibits subsequent LH surge release (Oztürk *et al.*, 1998).

In conclusion localised administration of oestradiol into the lumen of the previously gravid uterine horn did not affect the rate of uterine involution. Thus, it is unlikely that increased fertility associated with a first dominant follicle in the ipsilateral ovary is a consequence of a localised action of ovarian oestradiol on the previously gravid uterine horn. It is therefore suggested that the presence of a first dominant follicle in the ipsilateral ovary may be a reflection of an uterus-to-ovary signal indicating more rapid uterine involution.

Chapter 6

The localised effect of oestradiol on the postpartum uterus in sheep

Introduction

After parturition in ruminants, the uterus involutes and there is regeneration of the endometrium in preparation for a subsequent pregnancy (Gier and Marion, 1968). In addition, waves of follicular growth return and, either immediately, or after a period of anoestrus, ovulation occurs and there is a return of ovarian cyclic activity (Savio *et al.*, 1990a; Bartlewski *et al.*, 1999; Bartlewski *et al.*, 2000). There is a predilection for greater folliculogenesis in the ovary contralateral to the previously gravid uterine horn during the first four weeks postpartum in cattle and sheep (Kamimura *et al.*, 1993; Bartlewski *et al.*, 2000). However in cattle, the presence of a large follicle in the ipsilateral ovary is a marker of subsequent fertility (Bonnett *et al.*, 1993; Sheldon *et al.*, 2000; Chapter 3). One explanation for this observation is that oestradiol secreted by the ipsilateral ovary has a localised effect to increase the rate of uterine involution. There is close apposition between the venous drainage of the ovary and the uterine artery (Del Campo and Ginther, 1973). Furthermore, steroid concentrations are higher in the uterine artery and the cranial portions of the uterine horn ipsilateral to the active ovary in cattle and sheep (Weems *et al.*, 1988; Weems *et al.*, 1989). Among other effects, oestradiol increases blood flow to the myometrium, endometrium and caruncles, and causes hyperplasia and hypertrophy of uterine tissues (Rosenfeld, 1980; Reynolds *et al.*, 1998).

To test the hypothesis that ovarian oestradiol increases the rate of uterine involution, follicular growth in the ipsilateral ovary was stimulated by administering eCG on Day 14 postpartum (Sheldon and Dobson, 2000; Chapter 4). In a second study, oestradiol benzoate was infused into the previously gravid uterine horn on

Days 7 and 10 postpartum (Chapter 5). However in both studies, there was no effect on uterine involution as determined by monitoring changes in uterine horn diameters, or peripheral plasma markers of uterine involution such as PGFM or acute phase proteins.

One limitation of postpartum experiments in cattle is ubiquitous contamination of the uterine lumen with bacteria. Such contamination is a confounding influence, because it delays uterine involution (Sheldon *et al.*, 2000; Chapter 3). However in sheep, postpartum uterine bacterial contamination is uncommon, and has no significant effect on uterine involution (Regassa and Noakes, 1999). Furthermore, sheep have been used previously in biochemical studies to investigate rates of uterine involution, including the collection of uterine tissues and estimation of the collagen density of tissues (Tian and Noakes, 1991b; Regassa and Noakes, 2001). Thus, to further test the hypothesis that ovarian oestradiol increases the rate of uterine involution, the present study used sheep to examine the effect on uterine involution of oestradiol administered continuously by means of a silastic implant attached to the ovarian bursa adjacent to the ovary ipsilateral to the previously gravid uterine horn.

Materials and Methods

Animal model

All procedures were carried out under the Animals (Scientific Procedures) Act 1986 regulations for experiments on living animals, administered by the UK Home Office. In addition, experimental protocols were approved by the Royal Veterinary College Ethical Review Committee. Oestrus was synchronised during the breeding season (October) in a group of 25 adult Welsh ewes using a progestagen intra-vaginal sponge inserted for 14 days (Veramix, Pharmacia). Three Welsh rams were

introduced 24 h after sponge removal, for a period of 7 days. A single fetus pregnancy was identified in 20 ewes using transabdominal ultrasonography, and these ewes were housed as a group under standard husbandry conditions for 2 months prior to parturition. Ewes were provided with concentrate feed twice daily, and had *ad libitum* access to hay and water.

On the day of parturition (Day 1; March; anoestrus at this latitude), ewes were randomly allocated to one of the following groups: oestradiol implant (n = 8), empty placebo implant (n = 7), or no implant (n = 5, un-operated controls). On the day after parturition, a left flank laparotomy was performed on ewes from the implant groups, under general anaesthesia using Thiopentone sodium (20 mg/kg i.v.; Merial Animal Health, Harlow) for induction, followed by maintenance with oxygen and Halothane (Merial Animal Health). The previously gravid uterine horn was identified as being longer and wider than the contralateral horn. Oestradiol implants were made from crystalline oestradiol packed into 3 cm lengths of medical grade silastic tubing (internal diameter 3.3 mm x external diameter 4.6 mm; Degania Silicone, Degania Bet, Israel), whilst placebo implants were empty (Karsch *et al.*, 1980; Ozturk *et al.*, 1998). Implants were pre-equilibrated in a sterile isotonic PBS solution for 24 h to prevent an initial peak of steroid release. Implants were sutured to the ovarian bursa adjacent to the ovary ipsilateral to the previously gravid uterine horn. Antibiotic (Amoxycillin Trihydrate BP, 15 mg/kg; Intervet UK Ltd., Cambridge) was administered to all animals on Day 1 after surgery, and to ewes in the control group. Animals continued to be maintained in a loose-housed group with their lambs until Day 17 when the dams were euthanased.

Blood sampling

Jugular venous blood samples were collected by venepuncture from ewes at 24 h intervals, from the day of parturition to the day of euthanasia, into evacuated heparinised and plain tubes (Vacutainer, Becton Dickinson, Meylan, France) and transported on ice to the laboratory. Plasma, within 30 minutes, and serum after clotting at room temperature for 1 h were separated by centrifugation (2200g for 10 min), harvested and stored frozen at -20°C.

Assays

Oestradiol-17β concentration was measured following diethyl ether extraction of plasma samples using a RIA kit (Estradiol MAIA, Serono Diagnostics Ltd, Woking) modified to increase sensitivity, and validated for ovine plasma (Beard *et al.*, 1994). The mean inter-assay (n = 3 assays) and intra-assay (n = 12 samples) coefficients of variation were 13.1 % and 6.3 %, respectively for a 0.9 pg/ml sample, and the sensitivity was 0.24 pg/ml. PGFM was measured in duplicate by direct radioimmunoassay using plasma samples in a single assay (Kindahl *et al.*, 1976). The mean intra-assay (n = 20 samples) coefficient of variation was 7.6 %, for a 91.2 pg/ml sample, and the sensitivity was 27.0 pg/ml. FSH concentration was measured in duplicate by radioimmunoassay using a previously validated assay (Dobson *et al.*, 2000). Inter- and intra-assay coefficients of variation were 4.7% and 3.4%, respectively (n = 3 and mean of 3 assays each including 20 samples, mean concentration 1.2 ng/ml) and the sensitivity was 0.12 ng/ml.

The acute phase proteins α_1-acid glycoprotein and haptogolobin in plasma, and ceruloplasmin in serum, were measured using methods adapted for 96 well microtitre plates (Life Technologies, Paisley, Scotland) for ovine samples (Lewis *et al.*, 1989; Regassa and Noakes, 1999). The inter and intra-assay coefficients of variation for

low, medium and high values within the effective range used in the study were all < 18% and < 12%, respectively.

Tissue collection and measurement

The ewes were killed on Day 17, and the genital tract collected immediately. Calipers (Mitutoyo, Japan) were used to measure the length and the diameter of the cervix at the midpoint, and the diameter of the previously gravid and contralateral uterine horns, 1 cm distal to the insertion of the intercornual ligament. The length of each uterine horn was measured using a nylon cord placed along the greater curvature from the intercornual ligament to the insertion of the oviduct.

The genital tract was weighed after it was cut into three sections: cervix, and each uterine horn. Full-thickness 1 cm^3 blocks of tissue were collected from the caruncular and inter-caruncular areas of each uterine horn, and the cervix. Tissue was stored in Mirskey's fixative (National Diagnostics, Atlanta, Georgia). The dry matter content of each tissue was estimated after freeze-drying for > 24 h using a lyophyliser (Edwards Plc, London).

Tissue staining and histological examination for collagen density

Blocks of uterine and cervical tissue were dehydrated using a graded series of ethanol, cleared with Citroclear (HD Supplies, Aylesbury) and embedded in wax (Ralwax, BDH, Poole). Tissue blocks were cut into six µm sections and representative sections stained by the Haematoxylin-Van Gieson method (Regassa and Noakes, 2001). The slides were stained by immersion in iron-haematoxylin for 10 min (equal parts of alcoholic haematoxylin and ferric chloride mixed immediately before use). After differentiation with 0.5 per cent acid-alcohol for 2 min, the slides were stained with Van Geison's stain (Raymond A Lamb Ltd, Eastbourne, Sussex) for 5 min.

The sections of stained tissue were examined in triplicate by measuring the optical density of an area of endometrium or myometrium using the Seescan Image Analysis System (Seescan Imaging Plc, Cambridge). Tissue collagen density was estimated from the optical density measurement using stained reference sections where collagen concentrations had been previously determined and the method verified using hydroxyproline analysis (Regassa and Noakes, 2001). The mean inter-assay (n = 3 assays) and intra-assay (n = 16 samples) coefficients of variation were 7.3 % and 1.5 %, respectively for a 16 mg/g sample, and 14.9 % and 3.8 %, respectively, for a 130 mg/g sample.

Histological technique and ovarian follicle populations

Ovaries were collected immediately after slaughter and fixed in Mirskey's fixative, until dehydrated using a graded series of ethanol, cleared with Citroclear and embedded in wax. Ovaries were serially sectioned 5 μm thick, mounted and stained with haematoxylin and eosin. Sections were examined using an ocular microscope, calibrated for measuring follicle diameter using a stage graticule (Graticules Ltd., Tonbridge, Kent). The diameters of follicles > 1.0 mm were recorded. The diameter of follicles was determined by taking the average of two measurements at right angles to each other on the section in which the area of the follicle was maximal (Al-Gubory and Abdennebi, 1996).

Statistical analysis

Data analysis was performed using SAS ver 8.01 computer program (SAS Institute Inc., Cary, NC). Results are quoted as arithmetic mean ± SEM, and significance was attributed at $P < 0.05$.

Comparisons of the collagen density, dry matter, dimensions and weights of the genital tract tissues was made using paired *t*-tests between tissues, and ANOVA between treatment groups, using Bonferonni post hoc tests where appropriate.

The number of ovarian follicles in the ipsilateral and contralateral ovary to the previously gravid horn were recorded for the following diameters: 1, 2, 3, 4, and > 4 mm. Numbers of follicles in each diameter group, the total number of follicles, and the number of follicles > 2 mm diameter, were compared between the ipsilateral and contralateral ovary using the non-parametric Wilcoxon signed rank test, and between treatment groups using the non-parametric Kruskal-Wallis test (Gibbons and Chakraborti, 1992).

Plasma concentrations of hormones and acute phase proteins were examined using a repeated measurements ANOVA mixed model with a first order autoregressive covariance structure (SAS, 1997). Data were examined for normality, and the data logarithmically transformed for analysis. The explanatory variables were Day postpartum, treatment group, and their interaction.

Results

At the time of euthanasia it was confirmed that each oestradiol implant was correctly placed adjacent to the ovary ipsilateral to the previously gravid uterine horn. Furthermore, there was no evidence of gross adhesions around the implants.

Plasma oestradiol and FSH concentrations

Plasma oestradiol concentrations differed significantly between Day postpartum ($P < 0.001$) and between treatment groups ($P < 0.001$, Fig. 1a); however, the interaction of Day x treatment was not significant. Plasma oestradiol concentrations

decreased after parturition from a maximum of 3.14 ± 0.43 pg/ml on Day 1 to a minimum of 1.41 ± 0.17 pg/ml on Day 17 in un-operated control and placebo ewes. Plasma oestradiol concentrations were higher in the treated group, compared with ewes in the un-operated control or placebo groups ($P < 0.001$). The mean plasma oestradiol concentrations between Day 1 to 17 for ewes in the treated, control, and placebo ewes was 2.61 ± 0.12, 1.40 ± 0.13, and 1.41 ± 0.09 pg/ml, respectively.

Plasma FSH concentrations differed significantly between Day postpartum ($P < 0.05$), and between treatments ($P < 0.01$, Fig. 1b); although the interaction of Day x treatment was not significant. The mean FSH concentration between Day 1 and Day 17 was persistently low in the treated ewes and lower than the control or placebo groups (2.71 ± 0.14 vs. 5.96 ± 0.45 or 6.58 ± 0.44 ng/ml, respectively, $P < 0.001$).

Plasma PGFM concentrations

After parturition, plasma PGFM concentrations decreased significantly ($P < 0.001$) from maximum values on Day 1 to basal values between Day 9 and 17 (Fig. 1c). PGFM concentrations did not differ significantly between treatments, but the interaction of Day x treatment was significant ($P < 0.01$). The effect of the Day x treatment interaction was most evident between Day 1 and 8, when the mean PGFM concentration was lower for treated animals compared with controls (0.98 ± 0.19 vs. 1.56 ± 0.28 ng/ml, $P < 0.05$); although, not significantly lower than placebo ewes (0.98 ± 0.19 vs. 1.10 ± 0.21 ng/ml).

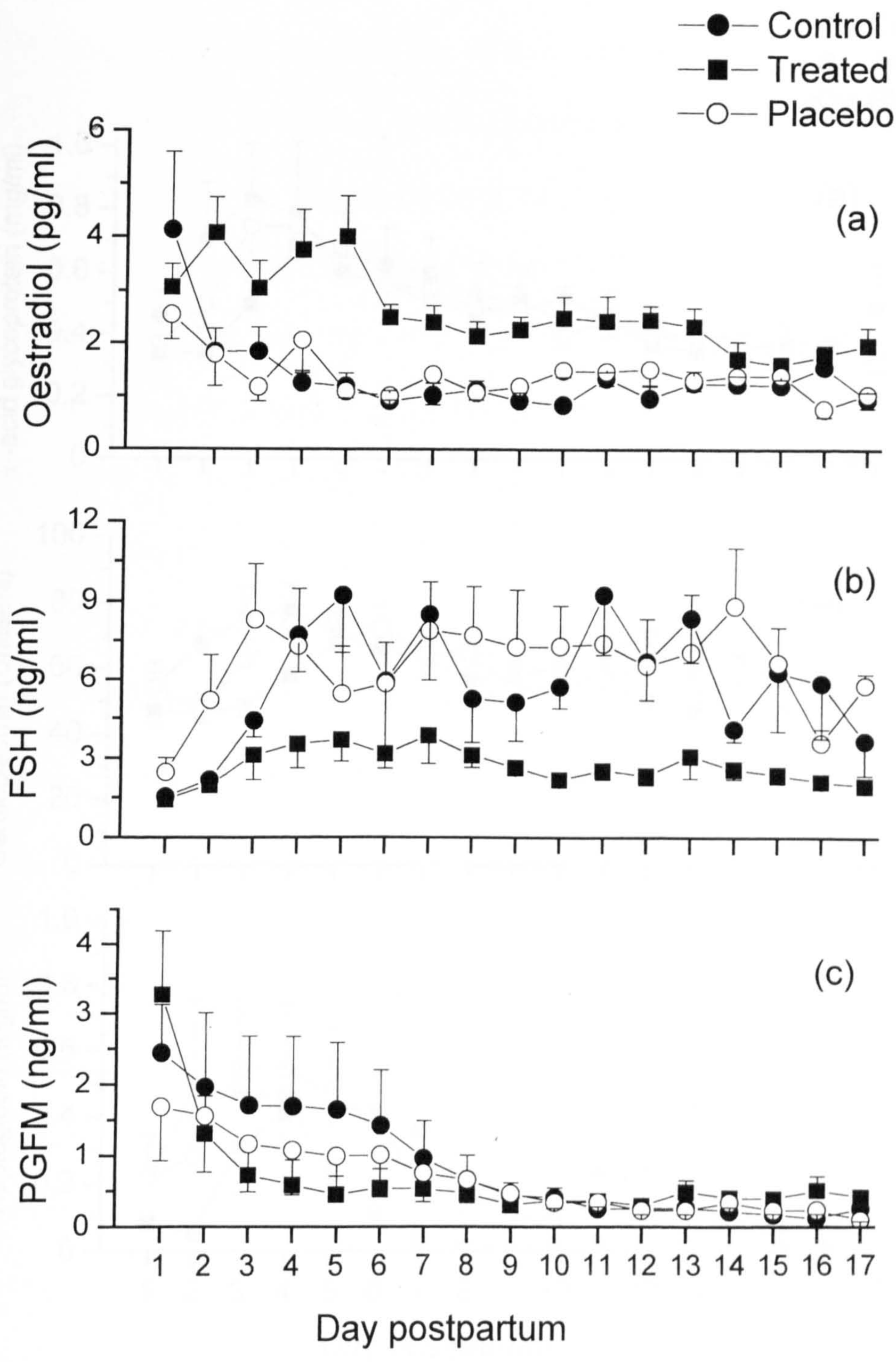

Figure 1. Mean ± SEM plasma concentrations between Day 1 to 17 postpartum of (a) oestradiol, (b) FSH, and (c) PGFM for un-operated control ewes (●), ewes administered an implant containing oestradiol (■) or an empty placebo implant (○).

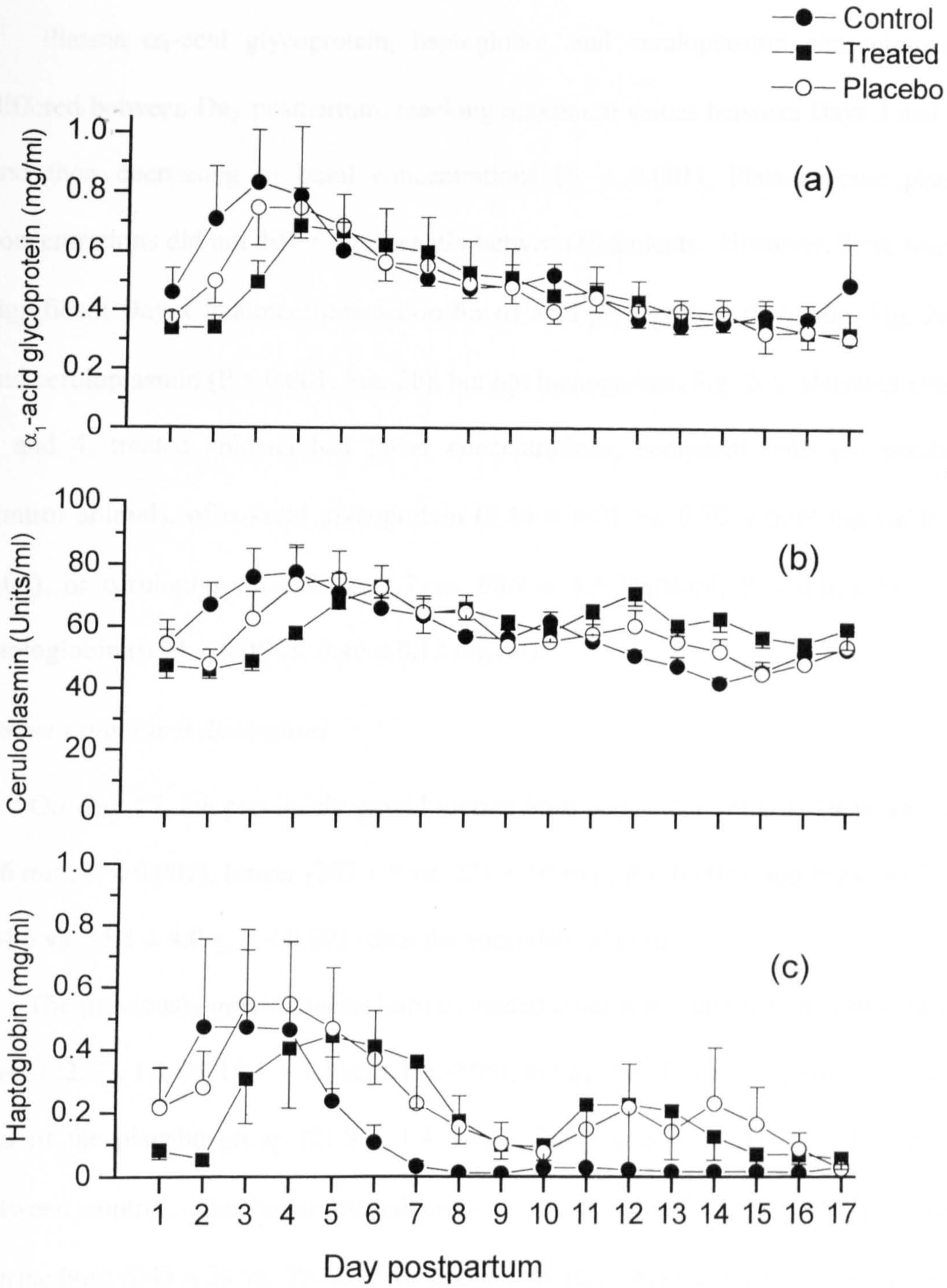

Figure 2. Mean ± SEM plasma concentrations of (a) α_1-acid glycoprotein, (b) ceruloplasmin, and (c) haptoglobin for un-operated control ewes (●), ewes administered an implant containing oestradiol (■) or an empty placebo implant (○).

Plasma acute phase protein concentrations

Plasma α_1-acid glycoprotein, haptoglobin, and ceruloplasmin concentrations differed between Day postpartum, reaching maximum values between Days 3 and 5, and then decreasing to basal concentrations ($P < 0.001$). Plasma acute phase concentrations did not differ significantly between treatments. However, there was a significant Day x treatment interaction for α_1-acid glycoprotein ($P < 0.05$, Fig. 2a), and ceruloplasmin ($P < 0.001$, Fig. 2b), but not haptoglobin (Fig. 2c). Between Days 1 and 4, treated animals had lower concentrations, compared with un-operated control animals, of α_1-acid glycoprotein (0.46 ± 0.05 vs. 0.70 ± 0.09 mg/ml, $P < 0.05$), or ceruloplasmin (50.0 ± 1.7 vs. 68.7 ± 4.3 Units/ml, $P < 0.05$), but not haptoglobin (0.21 ± 0.06 vs. 0.40 ± 0.12 mg/ml).

Tissue weight and dimensions

On Day 17, the previously gravid uterine horn was wider (21.6 ± 0.8 vs 18.6 ± 0.6 mm, $P < 0.001$), longer (257 ± 9 vs. 221 ± 10 mm, $P < 0.001$), and heavier (70.7 ± 4.6 vs. 55.5 ± 4.0 g, $P < 0.001$) than the contralateral horn.

The previously gravid uterine horn of treated ewes was wider than that of control ewes (23.5 ± 1.1 vs. 18.1 ± 1.1 mm, $P < 0.05$), but neither differed significantly from that of the placebo group (21.9 ± 1.4 mm). There was no significant difference between control, placebo or treated ewes in the length of the previously gravid uterine horn (243 ± 24 vs. 280 ± 18 vs. 245 ± 6 mm), or weight (60.3 ± 9.7 vs. 72.6 ± 9.0 vs. 75.5 ± 5.9 g). The previously non-gravid uterine horn did not differ significantly between the control, placebo, or treated ewes in diameter (16.5 ± 0.8 vs. 19.7 ± 1.9 vs. 18.8 ± 1.2 mm), length (200 ± 21 vs. 248 ± 19 vs. 211 ± 7.8 mm), or weight (46.8 ± 7.4 vs. 59.8 ± 7.6 vs. 57.3 ± 6.2 g). Similarly, there was no significant difference between control, placebo, or treated groups in cervix diameter

(11.9 ± 0.2 vs. 13.3 ± 1.3 vs. 13.2 ± 0.7 mm), length (54.2 ± 2.2 vs. 57.1 ± 4.1 vs. 53.3 ± 2.6 mm), or weight (15.9 ± 1.0 vs. 21.6 ± 1.9 vs. 19.5 ± 2.3 g).

Tissue dry matter

Dry matter did not differ significantly between treatment groups (Table 1), except for inter-caruncular tissue of the previously non-gravid uterine horn, where the dry matter was greater for treated compared with placebo ewes ($P < 0.05$).

For all animals, the dry matter content of caruncular tissue was lower than inter-caruncular uterine tissue in the previously gravid (17.2 ± 0.3 vs. 18.2 ± 0.3 %, $P < 0.01$) and the contralateral uterine horn (16.4 ± 0.2 vs. 17.9 ± 0.2 %, $P < 0.001$). The dry matter content of caruncular tissue was greater in the previously gravid compared with the contralateral uterine horn ($P < 0.05$), but inter-caruncular tissue did not differ significantly between the horns. The dry matter content of cervical tissue (20.6 ± 0.3 %) was greater than the uterine tissues ($P < 0.001$).

Tissue collagen density

Collagen density did not differ significantly between treatment groups (Table 2), except for caruncular endometrium in the non-gravid horn where the collagen concentration was higher in the control or placebo groups than in the treated ewes ($P < 0.01$).

For all animals, the caruncle collagen density of endometrium was lower than in myometrium in the previously gravid uterine horn (58.0 ± 2.7 vs. 90.5 ± 5.5 mg/g, $P < 0.001$) and the non-gravid horn (57.4 ± 3.7 vs. 90.0 ± 4.2 mg/g, $P < 0.001$). The inter-cotyledonary tissue collagen density of endometrium was lower than in myometrium in the previously non-gravid uterine horn (75.6 ± 6.1 vs. 95.3 ± 4.5 mg/g, $P < 0.001$), but not in the previously gravid horn (78.2 ± 5.2 vs. 75.8 ± 5.3 mg/g). The collagen density of endometrium, but not myometrium, was lower in

caruncles than in inter-caruncular tissue of the previously gravid ($P < 0.001$) and non-gravid ($P < 0.05$) uterine horn. The cervical collagen density was 124.0 ± 5.4 mg/g, which was greater ($P < 0.001$) than each of the uterine tissues.

Ovarian structures

There was no evidence of ovulation or new corpora lutea in the ovaries. Across treatments, the number of follicles in the 1, 2, 3, 4 or > 4 mm diameter categories did not differ significantly between the ovary ipsilateral or contralateral to the previously gravid uterine horn. Furthermore, there was no significant difference between the ipsilateral and contralateral ovary for the total number of follicles (11.2 ± 0.9 vs. 10.7 ± 0.9), or the number of follicles > 2 mm diameter (3.5 ± 0.9 vs. 3.6 ± 0.8). The maximum follicle diameter was the same in each ovary (2.8 ± 0.3 mm).

The mean number of follicles 1, 2, 3, 4 or > 4 mm diameter in the ovary ipsilateral or contralateral to the previously gravid uterine horn for the control, placebo, and treatment groups is illustrated in Fig. 3. Treated, compared with control or placebo, ewes had fewer 2 mm diameter follicles ($P < 0.01$) in the ipsilateral and fewer 2 mm ($P < 0.01$) and 3 mm ($P < 0.05$) diameter follicles in the contralateral ovary. The remaining follicle diameter groups did not differ significantly between treatment groups. Furthermore, the total number of follicles did not differ significantly between control, placebo and treated ewes for the ipsilateral ovary (11.6 ± 1.9 vs. 10.7 ± 1.4 vs. 11.4 ± 1.5) or the contralateral ovary 11.0 ± 2.0 vs. ± 12.6 ± 1.7 vs. 8.8 ± 0.9). However, the control or placebo groups had more follicles > 2 mm diameter compared with treated ewes, in the ipsilateral ovary (5.2 ± 1.5 vs. 5.8 ± 1.2 vs. 0.9 ± 0.6, $P < 0.001$), and the contralateral ovary (5.0 ± 2.2 vs. 6.0 ± 1.1 vs. 0.3 ± 0.2, $P < 0.001$).

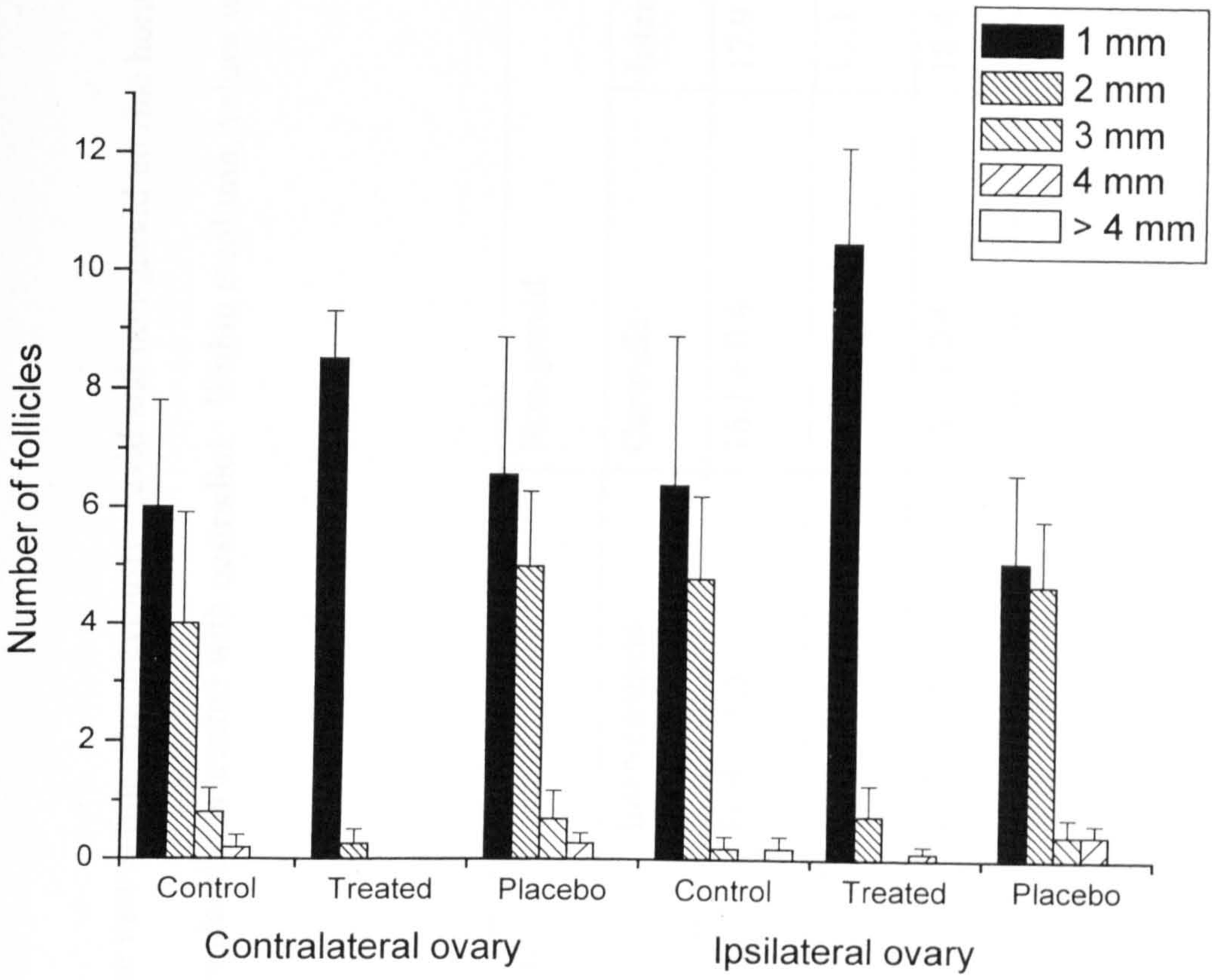

Figure 3. Mean ± SEM numbers of follicles on Day 17 in the ovary contralateral or ipsilateral to the previously gravid uterine horn for un-operated control ewes (Control), ewes administered an implant containing oestradiol (Treated) or an empty placebo implant (Placebo).

Table 1

Mean ± SEM dry matter (%) of the cervix, and of the tissues from the previously gravid and non-gravid uterine horns, on Day 17 postpartum for un-operated control ewes and animals administered a placebo or treated with oestradiol. Within a column, values with different superscripts are significantly different, ^{ab} $P < 0.05$

Group	Cervix	Previously gravid		Non-gravid	
		Caruncle	Inter-caruncle	Caruncle	Inter-caruncle
Control	19.9 ± 0.6	17.0 ± 0.5	18.4 ± 0.5	16.1 ± 0.4	17.9 ± 0.4
Placebo	20.2 ± 0.5	16.7 ± 0.4	17.8 ± 0.4	15.9 ± 0.2	17.1 ± 0.3 [a]
Treated	21.1 ± 0.4	17.7 ± 0.4	18.5 ± 0.4	16.9 ± 0.4	18.4 ± 0.3 [b]

Table 2

Mean ± SEM collagen density (mg/g) of the cervix, and of the tissues from the previously gravid and non-gravid uterine horns, on Day 17 postpartum for un-operated control ewes and animals administered a placebo or treated with oestradiol. Within a column, values with different superscripts are significantly different, ^{ab} $P < 0.01$

Group	Cervix	Previously gravid				Non-gravid			
		Caruncle		Inter-caruncle		Caruncle		Inter-caruncle	
		Endometrium	Myometrium	Endometrium	Myometrium	Endometrium	Myometrium	Endometrium	Myometrium
Control	127.8 ± 10.4	58.8 ± 5.0	86.4 ± 5.8	68.4 ± 6.0	77.9 ± 7.8	67.2 ± 5.5 [a]	102.2 ±11.2	72.8 ± 5.5	101.3 ± 3.5
Placebo	124.9 ± 9.6	63.5 ± 5.3	83.8 ±11.9	86.7 ±11.1	80.2 ±11.9	66.1 ± 3.9 [a]	91.8 ± 3.9	78.2 ±14.4	98.4 ±10.1
Treated	120.9 ± 9.3	52.6 ± 2.3	98.8 ± 6.4	77.0 ± 6.5	70.9 ± 5.1	43.8 ± 5.3 [b]	80.6 ± 7.0	75.0 ± 6.4	88.9 ± 5.2

Discussion

The presence of an oestrogen-secreting follicle in the ovary ipsilateral to the previously gravid uterine horn is a marker of subsequent fertility in cattle (Bonnett *et al.*, 1993; Sheldon *et al.*, 2000; Chapter 3). It was hypothesised that oestradiol secreted by the ipsilateral ovary has a localised effect to increase the rate of uterine involution. In the present study, postpartum sheep that had a single-lamb were used as a model, by suturing an oestradiol implant to the ovarian bursa ipsilateral to the previously gravid uterine horn. The attachment of the oestradiol implant to the ovarian bursa was an attempt to mimic the production of oestradiol by dominant follicles. Steroid hormone concentrations are higher in the uterine artery and the tissues of the cranial uterine horn ipsilateral to the active ovary in sheep and cattle (Weems *et al.*, 1988; Weems *et al.*, 1989). This is probably a result of close apposition of the venous drainage of the ovary and the ovarian bursa with the uterine artery (Del Campo and Ginther, 1973); and, because of anastomoses between ovarian and uterine lymphatic ducts (Staples *et al.*, 1982). Thus, if there is a local ovary-to-uterus pathway, the silastic implants in the present study should result in exposure of the uterus to higher oestradiol concentrations than are present in the peripheral circulation. However, ideally, oestradiol concentrations should have been measured in uterine tissues at the time of peak plasma concentrations. In the present study, there was no consistent effect of oestradiol treatment on measurements of uterine involution of the genital tract, including wet weight, size, dry matter and collagen density. Although, treated animals had lower concentrations of plasma markers of involution, including PGFM and the acute phase proteins α_1-acid glycoprotein and ceruloplasmin.

The ewes provided a good surgical model, with correct placement of each implant and no evidence of surgical inflammation, as determined by the absence of adhesions with all implants, or changes in plasma acute phase protein concentrations with the placebo implants. Plasma oestradiol concentrations increased in treated ewes to values approaching those reported after subcutaneous implantation, and remained elevated (Karsch *et al.*, 1980; Ozturk *et al.*, 1998).

Genital tracts were collected on Day 17 postpartum, in advance of the expected time for completion of uterine involution, around Day 24 to 29 (Call *et al.*, 1976; Tian and Noakes, 1991b). There was no evidence of a localised effect of oestradiol treatment on the dry matter, collagen density, wet weight, length or width of the previously gravid horn, compared with the non-gravid uterine horn. One possible explanation for the absence of a localised effect could be an unsuitable dose of oestradiol, although the peripheral plasma concentrations were within the physiological range. Another explanation may be an unsatisfactory location of the implant, which obviated a localised effect on the uterus, although each implant was adjacent to the ovary and the utero-ovarian blood vessels. On the other hand, there is increasing evidence in sheep and cattle that the effects of oestradiol on the postpartum uterus are minimal, and that effects of the uterus on the ovary are probably greater (Tian and Noakes, 1991b; Sheldon and Dobson, 2000; Chapter 4; Chapter 5).

After parturition, in sheep, decreasing plasma PGFM concentrations principally reflect uterine involution, (Lewis and Bolt, 1987; Tian and Noakes, 1991b; Regassa and Noakes, 1999). Similarly in the present study, plasma PGFM concentrations were high at parturition and decreased to basal values by Day 9. However, plasma PGFM concentrations decreased more rapidly and were lower between Days 1 to 8 in

ewes treated with oestradiol compared with the control group. Acute phase proteins are produced by hepatocytes in response to inflammation, and increased plasma concentrations after parturition also reflect uterine involution (Regassa and Noakes, 1999). In the present study, plasma acute phase protein concentrations also increased after parturition, reaching maximum values 1 to 3 days postpartum. However, in the present study, the increase in plasma α_1-acid glycoprotein, and ceruloplasmin concentrations was slower for oestradiol-treated ewes. Taken together, the effect of oestradiol on plasma PGFM, α_1-acid glycoprotein, and ceruloplasmin concentrations could reflect a beneficial effect of treatment on uterine health. Another explanation for differences between treatment groups could be a direct effect of oestradiol on PGFM and acute phase protein concentrations. Oestradiol specifically suppresses the production of the cytokines that mediate the acute phase protein response (Deshpande *et al.*, 1997). Furthermore, oestradiol has direct effects on the hepatic glycolysation of α_1-acid glycoprotein which are opposite to those associated with inflammation (Brinkman-Van der Linden *et al.*, 1996). It is unlikely that uterine bacterial contamination was a confounding factor because it is uncommon in sheep, all ewes were treated with antibiotic, and plasma haptoglobin concentrations were unaffected. In light of direct effects of oestradiol on peripheral plasma markers, and in the absence of parallel physical measurements, it is not possible to conclude that oestradiol increases the rate of uterine involution.

The pattern of plasma FSH concentrations for sheep in the control and placebo groups was similar to previous reports of postpartum and anoestrus ewes, with increasing concentrations for about 4 days, followed by a decrease, and then a rhythm of increases at 4 to 6 day intervals (Schirar *et al.*, 1990; Bartlewski *et al.*, 1998). However, oestradiol treatment was associated with consistently low FSH

concentrations throughout the study. We are not aware of other studies of continuous oestradiol administration on postpartum FSH concentrations. However, 6 h periods of administration of oestradiol to anoestrous ewes also reduced FSH concentrations (Dobson and Ward, 1977); and, oestradiol treatment of ovariectomized ewes for 4 to 32 days, suppressed the synthesis and secretion of FSH (Herring *et al.*, 1991).

In the present study, ovarian follicular populations of the ewes in the control and placebo group were consistent with those observed previously in anoestrous ewes (Bartlewski *et al.*, 1998). However, oestradiol-treated ewes had fewer follicles > 2 mm diameter, probably reflecting the reduced plasma FSH concentrations. In contrast to a previous study, there was no difference in the distribution of ovarian follicles between the ovaries ipsilateral and contralateral to the previously gravid uterine horn of control ewes (Bartlewski *et al.*, 2000). It was expected that there would be fewer follicles > 2 mm diameter in the ipsilateral ovary. The localised suppression of folliculogenesis in the ipsilateral ovary in ruminants is unlikely to be caused by the regressing corpus luteum of pregnancy (Sheldon *et al.*, 2002; Chapter 7). It is more likely that the previously gravid uterine horn or its contents, suppress folliculogenesis (Lewis and Bolt, 1987; Al-Gubory and Abdennebi, 1996); and, this suppression may be associated with uterine bacterial contamination (Peter and Bosu, 1988a). Thus, administration of antibiotic to all our animals may be an explanation for the difference between our present results and those of Bartlewski *et al.* (2000).

In conclusion, the present study examined the effect on uterine involution of continuous administration of oestradiol adjacent to the ovary ipsilateral to the previously gravid uterine horn. There were lower plasma concentrations of PGFM, α_1-acid glycoprotein, and ceruloplasmin, which might reflect a beneficial effect of

oestradiol on uterine involution or a direct effect of oestradiol on these markers. However, there was no consistent effect of treatment on the dry matter, collagen density, wet weight, length or width of the genital tract tissues. In addition, there was no evidence of a localised effect of oestradiol on the involution of the ipsilateral previously gravid uterine horn. The present results, taken together with previous studies, show no consistent localised effect of oestradiol on uterine involution (Sheldon and Dobson, 2000; Chapter 4; Chapter 5). Thus, it is unlikely that increased fertility associated with a first dominant follicle in the ipsilateral ovary is a consequence of a localised action of ovarian oestradiol to increase the rate of uterine involution, and the alternative uterus-to-ovary pathway should be considered.

Chapter 7

The effect of the regressing corpus luteum of pregnancy on ovarian folliculogenesis after parturition in cattle

Introduction

After parturition in cattle there is an increase in plasma FSH concentration which is followed by the emergence of a follicular wave and subsequent selection of a dominant follicle (Beam and Butler, 1997; Crowe *et al.*, 1998). The use of sequential transrectal ultrasonography has revealed a preference for the first postpartum dominant follicle to be selected in the ovary contralateral to the previously gravid uterine horn and, thus, the ovary bearing the corpus luteum of pregnancy (Kamimura *et al.*, 1993; Nation *et al.*, 1999). This is important, because the presence of a large follicle in the ovary ipsilateral to the previously gravid uterine horn within 4 weeks of parturition, although less frequent, is associated with improved subsequent fertility (Bonnett *et al.*, 1993; Bridges *et al.*, 2000; Sheldon *et al.*, 2000; Chapter 3).

Suppression of folliculogenesis in the ipsilateral ovary decreases as the postpartum interval advances, concurrent with disappearance of the corpus luteum of pregnancy and uterine involution (Sawyer, 1995; Sheldon *et al.*, 2000; Chapter 3). This could be explained by an inhibitory local effect of the regressing corpus luteum of pregnancy, or a regional effect of the previously gravid uterine horn (Dufour and Roy, 1985). Although luteolysis and regression of the corpus luteum is rapid during the oestrous cycle, following parturition luteolysis is protracted with physical remnants of luteal cells detectable up to 35 days postpartum (Sawyer, 1995). In addition, there is evidence for a non-steroidal aromatase inhibitor of luteal origin that can influence follicular steroidogenesis (Al-Gubory *et al.*, 1994).

The aim of the present study was to test the hypothesis that the regressing corpus luteum of pregnancy suppresses folliculogenesis in the ipsilateral ovary after parturition. Specific objectives were, firstly, to remove the corpus luteum of pregnancy without disruption of the fetus by administering prostaglandin $F_{2\alpha}$ during the last trimester of pregnancy (Conley and Ford, 1987). Then, the effect of the absence of the corpus luteum of pregnancy on ovarian follicle structure and function, after parturition, would be determined using sequential ultrasonography and plasma hormone assays.

Materials and Methods

Animals

All procedures were carried out under the Animals (Scientific Procedures) Act 1986 regulations for experiments on living animals, administered by the UK Home Office. In addition, experimental protocols were approved by the Royal Veterinary College Ethical Review Board.

A dairy herd of 90 Holstein-Friesian cows, with an annual average milk yield of 6,800 litres, was selected for the study on the basis of accurate farm records. A total of 53 cows that were due to calve within a 5-month period were included in the study. All cows had been artificially inseminated with Holstein-Friesian semen. Prostaglandin $F_{2\alpha}$ analogue (500 μg cloprostenol, Schering-Plough Animal Health, Uxbridge) was administered intramuscularly between 190 and 220 days of gestation to 17 animals selected using a randomization chart (www.randomizer.org).

Examination

The genital tracts of all cows were examined daily by transrectal palpation and ultrasonography using a 7.5-MHz linear array transducer (Aloka SSD 210 DXII,

BCF Technology, Livingstone) starting on Day 6 postpartum and continuing for 21 days. The side of the previous pregnancy was determined by assessing which uterine horn was longest and of greatest diameter on Day 6. Follicles were defined as non-echogenic (black) spherical structures with a clear demarcation between the follicular wall and antrum. Corpora lutea were defined as grainy echogenic structures that had a well-defined border with the less echogenic ovarian stroma; in some corpora lutea there was a non-echogenic lacuna. After freezing the image on the screen, the number of ovarian follicles ≥ 4 mm diameter and corpora lutea in each ovary were counted, and their maximum diameter measured using the internal calipers of the machine. When the image of the structure being scanned was not spherical, the diameter was estimated by averaging two 90° dimensions.

A dominant follicle was defined as the largest follicle in the ovary with ≥ 10 mm internal diameter in the absence of other growing follicles (Dobson *et al.*, 2000). A dominant follicle and cohorts were defined as a follicular wave (Dobson *et al.*, 2000). The first day of dominance within a follicular wave was determined, retrospectively, as the day on which the dominant follicle was first ≥ 10 mm diameter. The number of ≥ 4 mm or ≥ 10 mm follicles in a wave was based on the emergence of the follicles at the same, or consecutive, examinations (Ginther *et al.*, 1996a). Day of ovulation was defined as the day when a dominant follicle was last scanned prior to subsequent appearance of a corpus luteum in the same location, and confirmed, retrospectively, by a subsequent increase in plasma progesterone concentration > 1 ng/ml. A persistent follicle was defined as a follicle ≥ 10 mm internal diameter that persisted for more than 5 consecutive days, in the absence of new follicle growth, and did not ovulate (Dobson *et al.*, 2000).

Blood Sampling and Hormone Assays

Blood samples were collected daily for 21 days, starting on Day 6 postpartum, from the coccygeal vein or artery into evacuated heparinised tubes (Vacutainer, Becton Dickinson, Meylan, France) and transported on ice to the laboratory. Within 30 minutes, plasma was separated by centrifugation (2200g for 10 min), harvested and stored frozen at -20°C.

Oestradiol-17β concentration was estimated in duplicate using a previously characterized RIA (Estradiol MAIA, Serono Diagnostics Ltd, Woking) following diethyl ether extraction of the plasma samples (Mann *et al.*, 1995). The mean intra-assay (n = 12 samples) and inter-assay (n = 3 assays) coefficient of variation was 8.1% and 13.1%, respectively for a 0.9 pg/ml sample, and the sensitivity was 0.24 pg/ml. Progesterone concentration was estimated in duplicate using a commercial ELISA kit (Ridgeway Science, Gloucester). The intra-assay (n = 10 samples) and inter-assay (n = 3 assays) coefficients of variation were 6.5% and 11.2%, respectively for a 1.7 ng/ml sample, and the sensitivity was 0.6 ng/ml. FSH concentration was estimated in duplicate using a previously validated RIA (Dobson *et al.*, 2000). The standard used for the FSH assay was AFP 5679C RP-1. The intra-assay (n = 20 samples) and inter-assay (n = 3 assays) coefficient of variation were 3.4% and 4.7%, respectively for a 1.2 ng/ml sample, and the sensitivity was 0.12 ng/ml.

Statistical analysis

Data analysis was performed on 46 animals (14 treated and 32 control) using SAS ver 8.01 computer program (SAS Institute Inc., Cary, NC). Three treated cows were excluded from the analysis; one aborted at 243 days of gestation, one had a caesarean operation, and a third had a physical injury. Four control cows were

excluded; two had caesarean operations, and two animals had mastitis. Data are quoted as arithmetic mean ± SEM, and significance was attributed at $P < 0.05$. Continuous data were examined for normality using the Kolmogorov-Smirnoff test, and equality of variance using the Levene's test (Petrie and Watson, 1999). Plasma hormone data were Log_{10} transformed prior to statistical analysis.

The duration of gestation, time interval from insemination to PG administration, intervals between parturition and dominance or ovulation, and interval between ovulation and increase in plasma progesterone concentration were examined by Kaplan-Meier survival analysis (Kalbfleisch and Prentice, 1980). Differences between the treated and control groups were compared using the Log Rank test. The maximum diameter of follicles was compared between ovaries and between treatments using unpaired *t*-tests, and between different follicular fates using ANOVA. Comparisons of the location of ovarian structures, or the fate of ovarian structures, were tested using the Chi-square test, or Fisher's Exact test when a cell had an expected frequency of < 5. Data for the number of follicles in each ovary were compared using the non-parametric Wilcoxon signed rank test and differences between treatment groups tested using the Mann-Whitney test (Gibbons and Chakraborti, 1992).

Follicle diameters and plasma hormone concentrations were examined using ANOVA mixed models for repeated measures (SAS, 1997). Response variables tested were day postpartum, treatment group, fate of the first dominant follicle (ovulated, regressed, persistent follicle), location of the first dominant follicle (ipsilateral, contralateral ovary), and their interactions. Variables were removed from the model, following examination for correlation with remaining variables, until those remaining were significant. Model fitting and the selection of the

covariance structure were determined using Akaike's information criterion (SAS, 1997). Correlation between first dominant follicle diameter and plasma hormone concentrations were tested using Pearson's correlation coefficient.

Results

The interval from conception to administration of Prostaglandin $F_{2\alpha}$ for treated cows was 207.5 ± 3.4 days (range 190 to 220). The interval between administration of Prostaglandin $F_{2\alpha}$ and parturition was 66.4 ± 3.5 days. The gestation interval was shorter ($P < 0.001$) for treated cows (274.6 ± 2.5 days, range 255 to 289) compared with control animals (284.2 ± 0.9 days, range 276 to 296).

Location of events

The corpus luteum of pregnancy was not detected postpartum by ovarian ultrasonography in any cow administered Prostaglandin $F_{2\alpha}$, although it was observed in all control animals between Day 6 to 14. There was a wave of follicular development, with the emergence of a dominant follicle, in all cows within 14 days of parturition.

There were fewer follicles ≥ 4 mm diameter in the ipsilateral ovary compared with the contralateral ovary in the first follicular wave after parturition (2.27 ± 0.23 vs. 3.34 ± 0.20, $P < 0.001$). However, when comparisons were made between treated and control animals there were no significant differences in the numbers of follicles ≥ 4 mm diameter in the first postpartum follicular wave in the ipsilateral ovary (2.71 ± 0.37 vs. 2.03 ± 0.28) or contralateral ovary (3.36 ± 0.40 vs. 3.33 ± 0.22). There were fewer first postpartum dominant follicles in the ipsilateral ovary compared with the contralateral ovary (12 vs. 34, $P < 0.01$), but again the proportions in the ipsilateral ovary did not differ between the treated and control animals (4/14 vs.

8/32). A smaller proportion of dominant follicles, than follicles > 4 mm diameter, were observed in the ipsilateral ovary (12/46 vs. 104/257, $P < 0.05$).

The first dominant follicle ovulated in 35 out of 46 cases. There were fewer ovulations in the ipsilateral ovary compared with the contralateral ovary (11 vs. 24, $P < 0.01$). There was no statistical difference in the proportion of dominant follicles that ovulated between the ipsilateral and contralateral ovaries (11/12 vs. 24/34). There was no difference in the frequency of ovulations from the ipsilateral ovary between the treated and control groups (4/14 vs. 7/32).

A second follicular wave was detected in 36 animals; 33 animals which had ovulated and 3 where the first dominant follicle regressed. There were similar numbers of follicles $\geq$ 4 mm diameter in the ipsilateral ovary compared with the contralateral ovary (2.40 ± 0.26 vs. 2.43 ± 0.18). There was no significant difference between the number of second dominant follicles in the ipsilateral and contralateral ovaries (13 vs. 23). The frequency of second dominant follicles in the ipsilateral ovary did not differ between treated and control animals (6/14 vs. 7/22).

Timing of events

The interval between calving and achieving dominance for the first dominant follicle was similar for the treated and control animals (10.1 ± 0.4 vs. 10.7 ± 0.5 days). The interval from calving to dominance did not differ between dominant follicles located in the ipsilateral or contralateral ovaries (10.6 ± 0.5 vs. 10.2 ± 0.4 days).

For those animals in which the first dominant follicle ovulated, the interval from calving to ovulation did not differ between the treated and control groups (16.1 ± 1.0 vs. 17.0 ± 0.7 days). The interval from calving to ovulation from the ipsilateral and contralateral ovaries was also similar (16.5 ± 0.88 vs. 16.8 ± 0.8 days). After each

ovulation, an increase in plasma progesterone concentration to > 1 ng/ml was detected 3.8 ± 0.2 days later, and this interval did not differ between those animals with a first dominant follicle in the ipsilateral or the contralateral ovary (3.7 ± 0.5 vs. 3.9 ± 0.2 days). The interval from ovulation to progesterone increase for cows treated with PG more than 60 days earlier, did not differ significantly from that of control animals (4.6 ± 0.4 vs. 3.5 ± 0.2 days).

The interval from parturition to dominance of the second dominant follicle postpartum did not differ between treated and control animals (19.9 ± 1.0 vs. 20.8 ± 0.8 days) or between the ipsilateral and contralateral ovaries (20.8 ± 1.0 vs. 20.6 ± 0.8 days).

Follicular growth and function

The diameter of the first dominant follicle increased between Day 6 and 16 postpartum ($P < 0.001$), but follicle growth rates did not differ between treatment and control groups, and the interaction of group x Day was not significant (Fig. 1a). The maximum diameter of the first dominant follicle of the treated and control groups was 14.9 ± 1.1 and 16.9 ± 0.8 mm, respectively. There was no significant difference between the diameter of dominant follicles in the ipsilateral and contralateral ovaries, between Day 6 to 16 postpartum (Fig. 1b). The maximum diameter of the first dominant follicle of the ipsilateral and contralateral ovaries was 14.8 ± 1.1 and 16.8 ± 0.8 mm, respectively.

There was no significant effect of treatment group or location of the first dominant follicle on peripheral plasma oestradiol or FSH concentration, and so the combined data are illustrated in Figure 2. Plasma oestradiol concentration increased between Day 6 and 16 postpartum ($P < 0.001$) and FSH concentration decreased between Day 6 and 11 postpartum ($P < 0.001$), as follicular diameter increased.

Between Day 6 and 16, there was a significant correlation between first dominant follicle diameter and plasma oestradiol concentration ($r = 0.53$, $P < 0.001$), or FSH concentration ($r = -0.44$, $P < 0.001$). Plasma oestradiol and FSH concentration also were correlated ($r = -0.26$, $P < 0.001$).

Ultrasonography revealed three possible fates for the first dominant follicle: ovulation; regression, followed by a second follicular wave; or formation of a persistent follicle (Fig. 3). The frequency of the different fates of the first dominant follicle did not differ significantly between treated and control animals. The dominant follicle ovulated, regressed or formed a persistent follicle in 10, 3, and 1 treated animals and in 25, 3, and 4 control animals, respectively. Using pooled data across treatment groups, the interaction of fate of the first dominant follicle x Day postpartum was significant for follicle diameter ($P < 0.01$), plasma FSH concentration ($P < 0.01$), and plasma oestradiol concentration ($P < 0.05$). The diameters of first dominant follicles that regressed were smaller than for ovulatory or persistent follicles between Day 9 to 16 postpartum ($P < 0.01$), and plasma oestradiol concentrations were also lower between Day 14 and 16 ($P < 0.01$). Plasma oestradiol concentrations were correlated with diameter of the first dominant follicle for those that ovulated ($r = 0.60$, $P < 0.001$), or formed a persistent follicle ($r = 0.36$, $P < 0.01$), but not for those that regressed. Further, unlike ovulatory or persistent follicles, in those animals with a first dominant follicle that subsequently regressed, plasma oestradiol concentration did not exceed 1 pg/ml. Plasma FSH concentration was lower in animals with a persistent follicle on Day 15 postpartum, compared with animals with a first dominant follicle that ovulated or regressed (0.56 ± 0.07 vs. 0.76 ± 0.08, or 0.72 ± 0.06 ng/ml, $P < 0.05$).

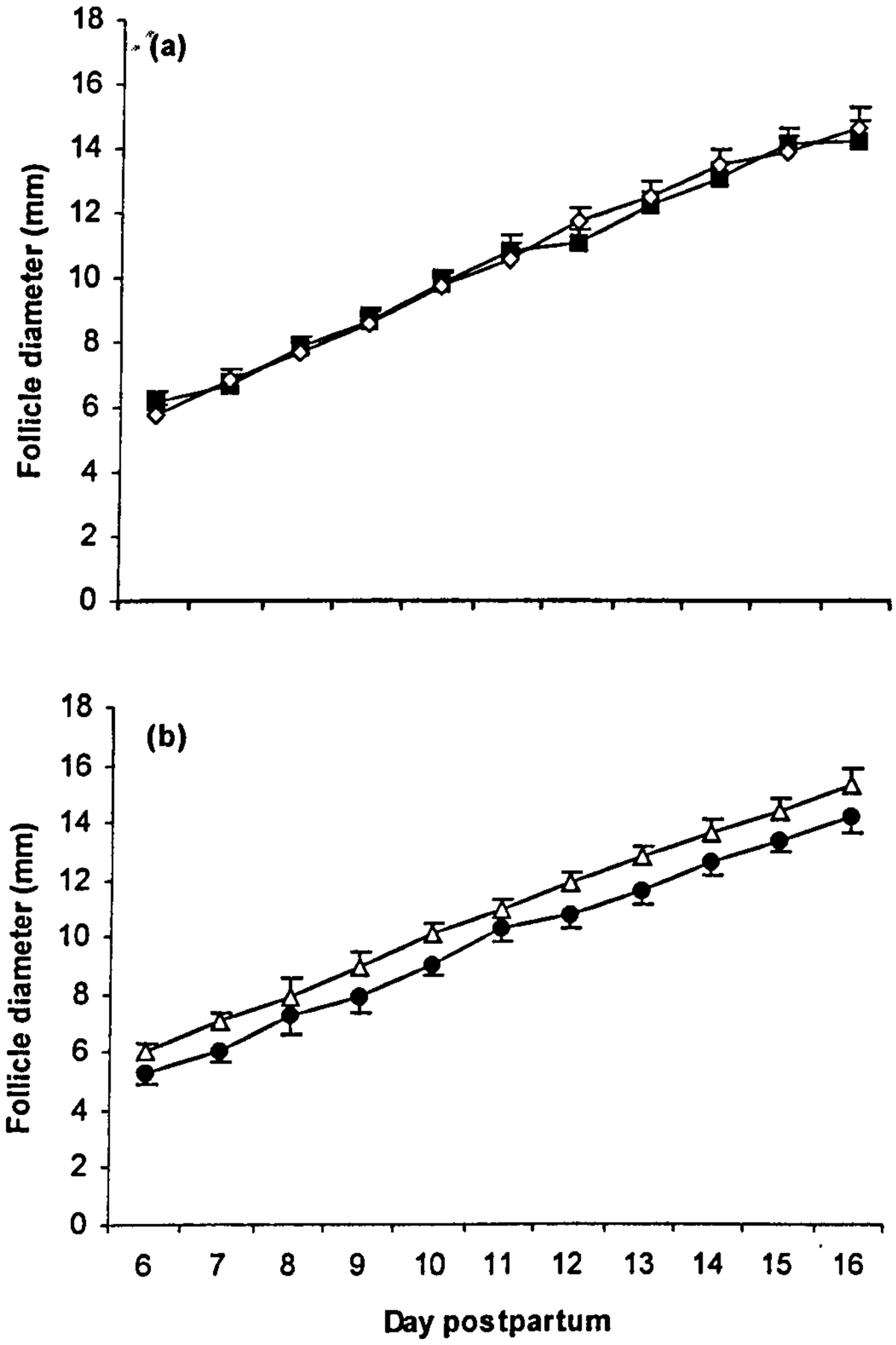

FIG. 1. Mean ± SEM diameter of the first postpartum dominant follicle (mm) between Day 6 and 16 postpartum in cows (a) administered prostaglandin $F_{2\alpha}$ around day 200 of gestation (■) and control animals (◇); and, (b) when the first postpartum dominant follicle was in the ipsilateral (●) or contralateral (△) ovary.

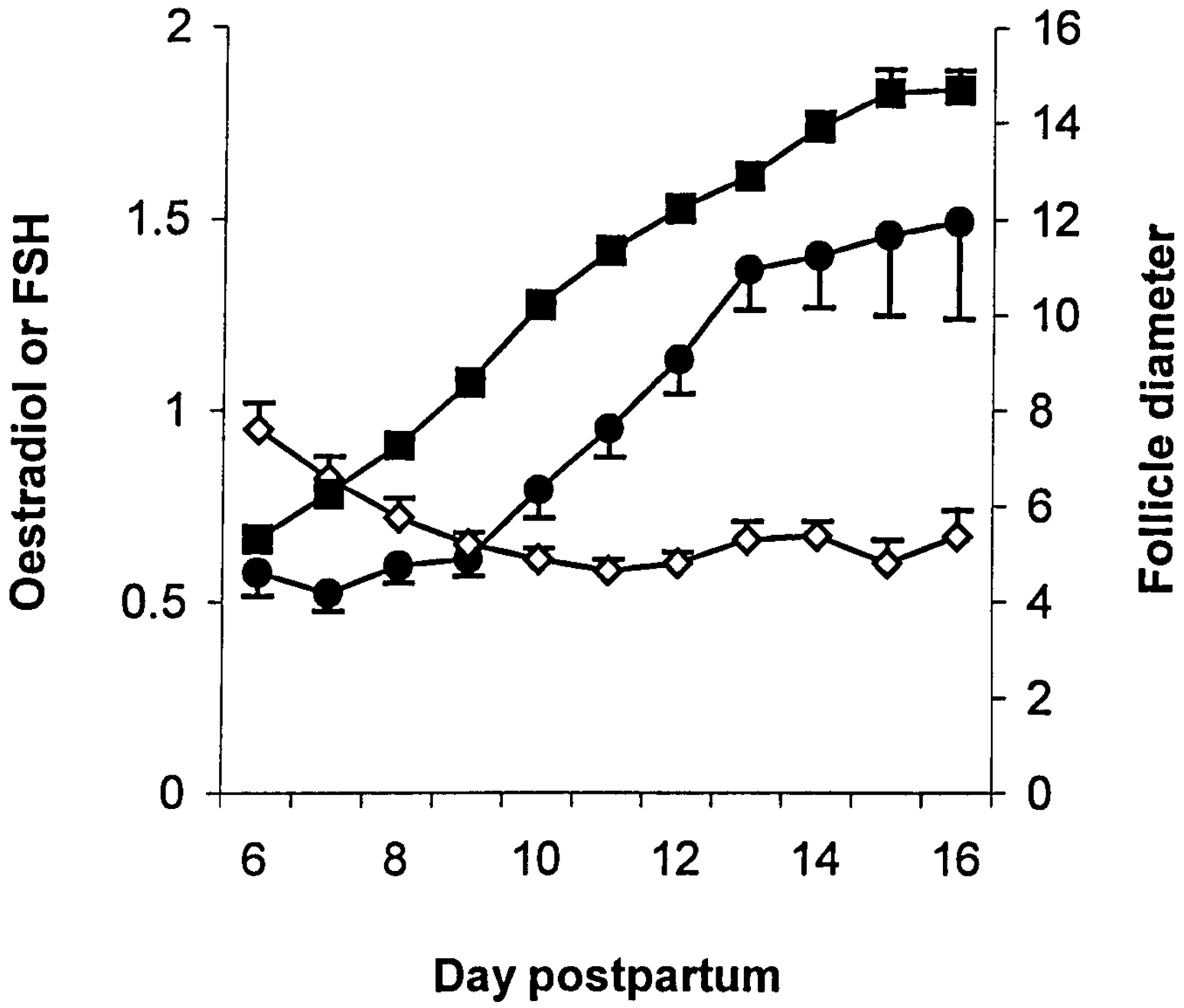

FIG. 2. Mean ± SEM diameter (■) of the first postpartum dominant follicle (mm), plasma oestradiol (●) concentration (pg/ml) and FSH (◇) concentration (ng/ml) between day 6 and 16 postpartum using data pooled from all animals

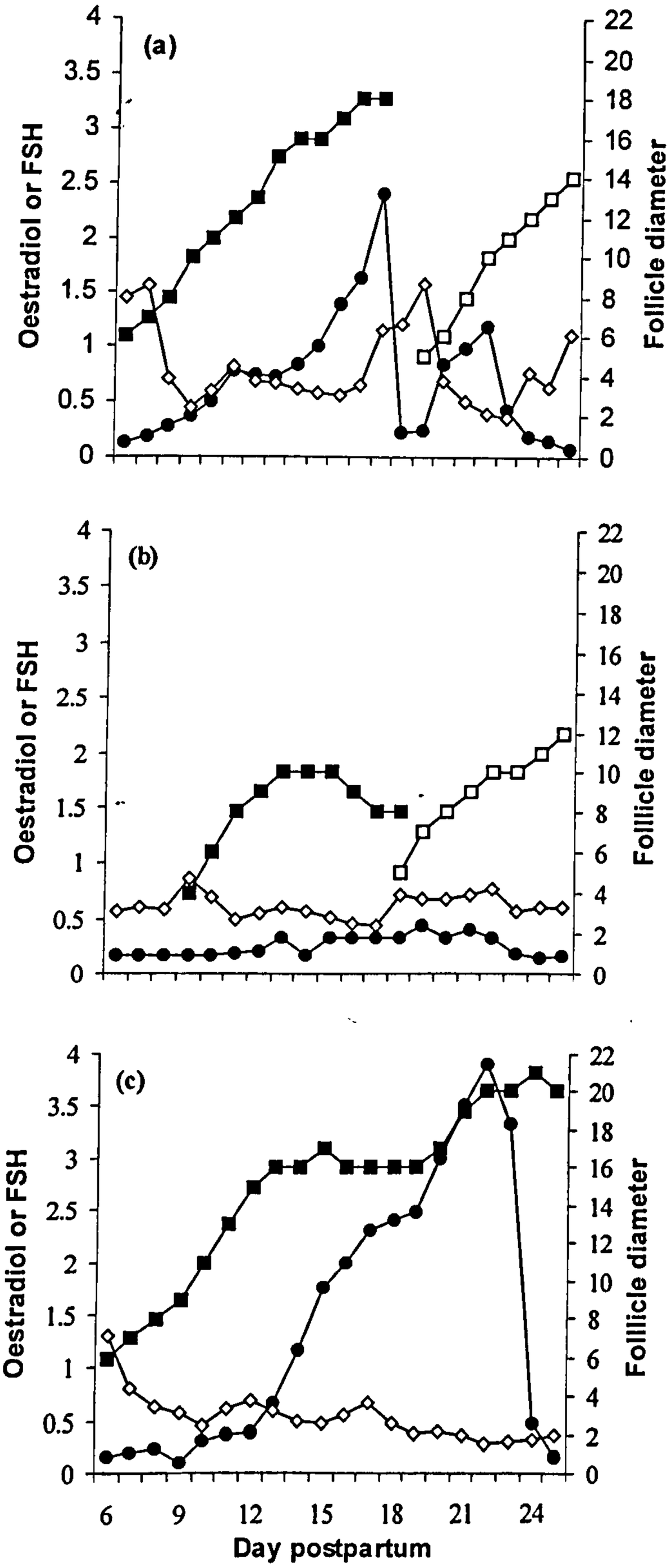

FIG. 3. Diameter of the first (■) and, where observed, the second (□) postpartum dominant follicles (mm), plasma oestradiol (●) concentration (pg/ml), and FSH (◇) concentration (ng/ml) between Day 6 and 25 postpartum for typical cases in which the first dominant follicle (a) ovulated, (b) regressed, or (c) persisted.

Discussion

Administration of a prostaglandin $F_{2\alpha}$ analogue at around 200 days of gestation caused luteolysis, without disturbance of the fetus which remained supported by extragonadal sources of progesterone (Estergreen *et al.*, 1967; Conley and Ford, 1987). The present study also confirmed that the corpus luteum of pregnancy was not detectable by ultrasonography after parturition in cows treated with prostaglandin $F_{2\alpha}$ early in the third trimester. Treated cows had a shorter gestation, although it was not possible in the present study to determine how that might affect the results. However, because the absence of the corpus luteum of pregnancy had no effect on postpartum follicular growth or function, or the timing or location of ovarian events, an effect of the previously gravid uterine horn and its contents should be considered.

A postpartum transient increase in plasma FSH concentration precedes the emergence of the first postpartum follicular wave, and subsequent selection of a dominant follicle during the following phase of decreasing FSH concentration (Adams *et al.*, 1992; Sunderland *et al.*, 1994; Beam and Butler, 1997; Crowe *et al.*, 1998). Similarly, in the present study, each animal after parturition developed a cohort of approximately six follicles $\geq$ 4 mm diameter and, as plasma FSH concentration decreased, selection of the first dominant follicle occurred. However, plasma FSH concentration did not differ between those cows in which the first dominant follicle developed in the ipsilateral or contralateral ovary, or between the treated and control animals.

The preference for the first postpartum dominant follicle to be selected in the contralateral ovary has been established by repeated transrectal ultrasonography after parturition (Kamimura *et al.*, 1993; Nation *et al.*, 1999). Additionally, in the present study there were fewer follicles $\geq$ 4 mm diameter in the ipsilateral ovary during the

first postpartum follicular wave, and there were fewer ovulations reflecting fewer first dominant follicles in the ipsilateral ovary. However, the preference for structures to occur in the contralateral ovary diminishes with increasing interval from parturition (Sheldon *et al.*, 2000; Chapter 3). Two possible explanations for these observations have been suggested. Firstly, there could be a local luteal inhibitory effect (Dufour and Roy, 1985; Nation *et al.*, 1999); however, in the present study the presence of a regressing corpus luteum of pregnancy did not affect several aspects of follicular growth. Furthermore, during normal oestrous cycles there are more dominant follicles in the corpus luteum-bearing ovary (Savio *et al.*, 1988). A second explanation is that there could be a regional effect of the involuting previously gravid uterus on folliculogenesis in the ipsilateral ovary (Lewis *et al.*, 1984; Dufour and Roy, 1985; Sheldon *et al.*, 2000; Chapter 3). A possible mechanism would be the transfer of products from the uterus to the ovary via the counter current mechanism, as established for prostaglandin $F_{2\alpha}$ during the process of luteolysis (Bonnin *et al.*, 1999).

The observation of fewer dominant follicles in the ipsilateral ovary after parturition appears to be a consequence of two components. Firstly, fewer follicles ≥ 4 mm diameter emerged in the ipsilateral ovary during the first postpartum follicular wave. Secondly, a smaller proportion of these first wave follicles were selected to achieve dominance in the ipsilateral ovary. As FSH concentration gradually decreases, there is selection of the dominant follicle, and subsequent transfer of gonadotrophin dependence from FSH to LH (Campbell *et al.*, 1999; Ginther *et al.*, 1999); LH probably plays a minor role until the point of selection (Ginther *et al.*, 2001b). After parturition, we suggest that there is further control on follicle emergence and selection exerted at the level of the ovary, acting to modulate the

response of follicles to FSH secreted by the pituitary. However, the inhibition of postpartum follicular growth in the ipsilateral ovary can be overcome by administration of eCG which has both FSH and LH-like activity (Sheldon and Dobson, 2000; Chapter 4). Interestingly, a similar multi-level control mechanism has been suggested for endotoxin disruption of the follicular phase in ewes (Battaglia *et al.*, 2000). It is possible that immune/inflammatory challenges could also be involved in the control of folliculogenesis after parturition, as uterine involution and elimination of bacterial contamination provoke an acute phase protein response (Sheldon *et al.*, 2001).

Once the location of the first dominant follicle had been determined, the timing of events was independent of location in the ipsilateral or contralateral ovary, and the presence of the corpus luteum of pregnancy. In addition, their ovarian location, or the corpus luteum of pregnancy, did not affect the growth rate of the first dominant follicles or their maximum diameter. Therefore, the mechanism responsible for imbalance between the ipsilateral and contralateral ovaries after parturition acts before and/or at the time of dominant follicle selection, and the regressing corpus luteum of pregnancy is not involved. Furthermore, first dominant follicles were as competent in the ipsilateral ovary as in the contralateral ovary, as determined by their ability to secrete oestradiol and to ovulate.

In the absence of an effect of the corpus luteum of pregnancy on postpartum follicular development, the influence of uterine involution on postpartum folliculogenesis should be considered. Postpartum folliculogenesis parallels observations of decreased follicular activity in the ovary ipsilateral to the gravid uterine horn during early and mid-pregnancy (Rexroad and Casida, 1975; Pierson and Ginther, 1987c). In addition, the suppressive effect of the pregnant uterus and/or

its contents on ovarian follicular growth is removed following hysterectomy (Thatcher *et al.*, 1991). Studies in sheep concluded that the negative effects on folliculogenesis were exerted by the gravid uterine horn, and that the corpus luteum of pregnancy had a positive effect on follicle numbers in the ipsilateral ovary (Hall *et al.*, 1993; Driancourt *et al.*, 2000).

The fates of the first dominant follicles observed in the present study and their function supported previous descriptions (Beam and Butler, 1997). In particular, first dominant follicles that regressed secreted less oestradiol, irrespective of follicle diameter; this may be a consequence of inadequate LH support (Ginther *et al.*, 2001b). The frequency with which dominant follicles ovulated, regressed or formed a persistent follicle did not differ between the treated and control cows, suggesting that fate is dependent on other factors, probably LH pulse frequency (Campbell *et al.*, 1997; Campbell *et al.*, 1998; Duffy *et al.*, 2000).

In conclusion, the removal of the corpus luteum of pregnancy by administration of prostaglandin $F_{2\alpha}$ prior to parturition did not influence first postpartum follicle wave location or timing of ovarian events, dominant follicle growth or function. Although there was greater folliculogenesis in the ovary contralateral to the previously gravid uterine horn, once the location of the future first dominant follicle was selected, the timing of events was independent of location. We suggest that the corpus luteum of pregnancy does not have a local effect on postpartum ovarian function but, instead, an effect of the previously gravid uterine horn shortly after parturition should be considered.

Chapter 8

Uterine bacterial contamination after parturition influences ovarian dominant follicle selection and inhibits follicle growth and function in cattle

Introduction

After parturition, there is an increase in plasma FSH concentration which is followed by the emergence of a wave of several 4 to 6 mm follicles, and subsequent selection of a single dominant follicle (Beam and Butler, 1997; Crowe *et al.*, 1998). The use of sequential transrectal ultrasonography has revealed that the first postpartum dominant follicle is preferentially selected in the ovary contralateral to the previously gravid uterine horn (Kamimura *et al.*, 1993; Nation *et al.*, 1999). This is important because the presence of a large follicle in the ovary ipsilateral to the previously gravid uterine horn within 4 weeks of parturition, although less frequent, is associated with improved subsequent fertility (Bonnett *et al.*, 1993; Sheldon *et al.*, 2000; Chapter 3).

Suppression of folliculogenesis in the ipsilateral ovary decreases as the postpartum interval advances, concurrent with the disappearance of the corpus luteum of pregnancy, uterine involution, and elimination of the ubiquitous uterine bacterial contamination after parturition (Elliot *et al.*, 1968; Sawyer, 1995; Sheldon *et al.*, 2000; Chapter 3). Suppression of folliculogenesis in the ipsilateral ovary could be explained by an inhibitory local effect of the regressing corpus luteum of pregnancy, or the previously gravid uterine horn, or its contents (Dufour and Roy, 1985). However, the removal of the corpus luteum of pregnancy, by administration of prostaglandin $F_{2\alpha}$ before parturition, did not influence first postpartum dominant follicle growth, function, or ovarian location (Sheldon *et al.*, 2002; Chapter 7).

Although the previously gravid uterine horn was not identified, in one study ovarian follicles were smaller after parturition in cattle with a uterine bacterial infection (Peter and Bosu, 1988a). Further, inflammatory mediators such as bacterial endotoxin, and immune mediators such as cytokines, disturb the hormonal interactions that control normal cyclic ovarian function (Rivest *et al.*, 1993; Battaglia *et al.*, 1999; Williams *et al.*, 2001). Intracerebral, or intravenous, administration of inflammatory mediators disrupts GnRH release from the hypothalamus, and LH secretion from the pituitary. However, a surprising inhibition of follicular oestradiol secretion following administration of endotoxin, in the face of adequate plasma LH concentrations, lead to the suggestion of an additional effect at the level of the ovary (Xiao *et al.*, 1998; Battaglia *et al.*, 2000). Despite these observations, little human or animal research has been focused on the impact of inflammatory mediators on the ovary, particularly under normal pathophysiological situations.

The aim of the present study was to test the hypothesis that postpartum uterine bacterial contamination alters the location of ovarian follicle emergence and selection, and inhibits dominant follicle growth and function.

Materials and Methods

Animals

All procedures were carried out under the Animals (Scientific Procedures) Act 1986 regulations for experiments on living animals, administered by the UK Home Office. In addition, experimental protocols were approved by the Royal Veterinary College Ethical Review Committee.

A dairy herd of 90 Holstein-Friesian cows, with an annual average milk yield of 6,800 litres and a rolling herd mean somatic cell count of 84 x 10^3 cells/ml, was selected for the study on the basis of accurate farm records. All cows had been pregnant to Holstein-Friesian bulls. Animals with a history of caesarean operation, or the presence of vaginal lacerations, acute mastitis, lameness, abdominal disorders or other intercurrent disease, were excluded from the study on the basis of daily clinical examination to remove any confounding influence of non-uterine bacterial infection during the study period (Skinner *et al.*, 1991; Scott *et al.*, 1992; Horadagoda *et al.*, 1999). Seventy Holstein/Friesian cows were included in the study during a one-year period. No antimicrobial treatments were administered during the study.

Clinical examination

The genital tract of each cow was examined daily from Day 7 to Day 28, using transrectal palpation and ultrasonography with a 7.5-MHz linear array transducer (Aloka SSD 210 DXII, BCF Technology, Livingstone, U.K.). The previously gravid uterine horn was identified as being longer and of greater diameter than the contralateral horn. Follicles were defined as non-echogenic (black) spherical structures with a clear demarcation between the follicular wall and antrum. A corpus luteum was defined as a grainy echogenic structure that had a well-defined border with the less echogenic ovarian stroma; in some corpora lutea there was a non-echogenic lacuna. The numbers of ovarian follicles $\geq$ 4 mm diameter and corpora lutea in each ovary were counted, and each maximum diameter was measured using the instrument's internal calipers. When the image of the structure being scanned was not spherical, the diameter was estimated by averaging two 90° dimensions.

A dominant follicle was defined as the largest follicle in the ovary with ≥ 10 mm internal diameter in the absence of other growing follicles (Dobson *et al.*, 2000). A dominant follicle and cohorts were defined as a follicular wave (Dobson *et al.*, 2000). The first day of dominance within a follicular wave was determined retrospectively as the day on which the dominant follicle initially exceeded 10 mm diameter. The number of ≥ 4 mm, or ≥ 10 mm follicles in a wave was based on emergence of the follicles at the same or consecutive examinations (Ginther *et al.*, 1996a). Day of ovulation was defined as the day when the dominant follicle was last scanned, before its sudden disappearance, and the subsequent appearance of a corpus luteum in the same location. Luteinisation was confirmed, retrospectively, by a subsequent increase in plasma progesterone concentration > 1 ng/ml. A persistent follicle (follicular cyst) was defined as a follicle ≥ 10 mm internal diameter that persisted for more than 5 consecutive days and did not ovulate (Dobson *et al.*, 2000).

Uterine swab collection and bacteriology

A trans-cervical guarded swab was collected from the uterine body of each cow on Day 7, 14, 21 and 28 postpartum using a previously validated method (Noakes *et al.*, 1989). The swab was transferred to a bijou bottle containing Stuart Transport Medium (Unipath, Basingstoke) and was cultured within 1 h of collection, at the on-site bacteriology laboratory. Swabs were cultured aerobically and anaerobically on pre-equilibrated sheep blood agar (Unipath), and aerobically on MacConkey agar (Unipath). Identification of bacteria was based on the characteristics of the colony, Gram-stain, morphology, haemolysis, biochemical profile (API systems, bioMérieux, Basingstoke), and other standard tests (Barrow and Feltham, 1993). Bacterial growth on the culture plates was scored semi-quantitatively, dependent on the number of bacterial colonies detected on the plate: 0: no growth; 1: < 10 colonies; 2: 10 to 100

colonies; 3: 100 to 500 colonies; and, 4: > 500 colonies (Noakes *et al.*, 1991). The bacterial growth score on Day 7, 14, 21, or 28, was the sum of the scores for each of the bacterial isolates, and the total bacterial growth score for each cow was the sum of the individual bacterial growth scores for all four uterine swabs. The uterine bacterial scores at each time point were used to categorise cows into categories of standard or high bacterial contamination on Day 7, 14, 21, or 28. The standard category was defined as the lower 75% quartile bacterial score and the high contamination category as the upper 25%. In addition, bacteria were categorised, based on expected pathogenic potential within the uterus (Ruder *et al.*, 1981; Olson *et al.*, 1984; Farin *et al.*, 1989; Noakes *et al.*, 1989; Noakes *et al.*, 1991; Bonnett *et al.*, 1993). The categories were (1) pathogens known to cause endometrial lesions; (2) other recognised uterine pathogens; and, (3) bacteria not recognised as uterine pathogens (see Table 1). Using these bacterial pathogenicity categories, cows were grouped for the presence of the most pathogenic bacteria on Day 7, 14, 21, or 28, for statistical analysis, with a fourth group in which no bacteria were isolated (Laven *et al.*, 2000).

Blood sampling and hormone assays

Blood samples were collected daily from Day 7 to Day 21 postpartum, from the coccygeal vein or artery into evacuated heparinised tubes (BD Vacutainer Systems, Plymouth) and transported on ice to the laboratory. Within 30 minutes, plasma was separated by centrifugation (2200g for 10 min), harvested and stored frozen at -20°C.

Oestradiol-17β concentration was estimated in duplicate by a previously characterized RIA (Estradiol MAIA, Serono Diagnostics Ltd, Woking) using diethyl ether extracted plasma (Mann *et al.*, 1995). The mean intra- (n = 12 samples) and inter-assay (n = 3 assays) coefficients of variation were 8.1% and 13.1%,

respectively, for a 0.9 pg/ml sample, and the minimum detectable quantity was 0.24 pg/ml. Progesterone concentration was estimated in duplicate using a commercial ELISA kit (Ridgeway Science, Gloucester). The intra- (n = 10 samples) and inter-assay (n = 3 assays) coefficients of variation were 6.5% and 11.2%, respectively, for a 1.7 ng/ml sample, and the minimum detectable quantity was 0.6 ng/ml. FSH concentration was estimated in duplicate by a previously characterized RIA (Dobson *et al.*, 2000). The standard used for the FSH assay was AFP 5679C RP-1. The intra- (n = 20 samples) and inter-assay (n = 3 assays) coefficients of variation were 3.4% and 4.7%, respectively for a 1.2 ng/ml sample, and the minimum detectable quantity was 0.12 ng/ml.

Statistical analysis

Data analysis was performed using SAS ver 8.01 computer program (SAS Institute Inc., Cary, NC). Results are quoted as arithmetic mean ± SEM, and significance was attributed at $P < 0.05$.

The location of ovarian structures (first dominant follicle, first ovulation, or second dominant follicle), in relation to the previously gravid uterine horn, were compared using Chi-square analysis, or Fisher's exact test if cells contained less than five observations. Logistic regression was used to examine the effect of standard or high uterine bacterial contamination, or the pathogen group, on the location of ovarian structures, and to obtain odds ratios.

Survival analysis, using Cox regression models, was used to compare time intervals from calving to appearance of the first dominant follicle, the first dominant ovulatory follicle and to ovulation, between bacterial categories, between the ovarian location of the structures, or between different bacterial pathogen groups.

Follicle numbers were compared using Mann-Whitney and Kruskall-Wallis non-parametric tests for two or more variables, respectively (Gibbons and Chakraborti, 1992). Follicle diameter, plasma oestradiol and FSH concentrations were compared by repeated measurements ANOVA using a mixed model (SAS, 1997). Data from Day 7 postpartum, the start of the study, to Day 16, the mean day of ovulation for the first dominant follicle, were included in the analysis. Data were examined for normality using the Kolmogorov-Smirnoff test, and equality of variance using the Levene's test. Where appropriate, data were Log_{10} or square root transformed to yield variance homogeneity. The explanatory variables were bacterial contamination category on Day 7, 14, 21 and 28, pathogen group, location of the first dominant follicle, fate of the first dominant follicle, and their interactions with time postpartum. A compound symmetry model best fitted the data, as determined using Akaike's information criterion. Post hoc tests were performed using Bonferonni's adjustment.

Results

Bacteriology

Bacteria were isolated from uterine swabs in each animal at least once during the study. More than 30 different bacteria were identified and categorised by their potential pathogenicity (Table 1). No bacteria were isolated from uterine swabs collected from 5, 4, 9, and 13 animals on Day 7, 14, 21, and 28, respectively. The mean ± SEM bacterial growth score for each bacterial pathogenicity group are illustrated in Fig. 1 for uterine swabs collected on Day 7, 14, 21, or 28. The ranges of total bacterial growth scores were 0 to 15, 0 to 12, 0 to 10, and 0 to 9 for Days 7,

14, 21, and 28, respectively. The 75% quartile cut-off point to categorise cows into standard or high bacterial score for uterine swabs collected on Day 7 was score 6, Day 14 was 6, Day 21 was 4, and on Day 28 was 3. The mean bacterial score of the standard categories on Days 7, 14, 21, and 28 were 2.2 ± 0.2, 2.7 ± 0.2, 1.6 ± 0.2, and 1.2 ± 0.1, respectively; and, for the high categories were 8.4 ± 0.6, 7.7 ± 0.4, 5.5 ± 0.4, and 4.2 ± 0.2, respectively. The individual animals comprising the standard or high bacterial score categories on Day 7, 14, 21, or 28 were not always the same. However, 28 animals in the standard, and 7 in the high bacterial contamination categories were identical across the four periods.

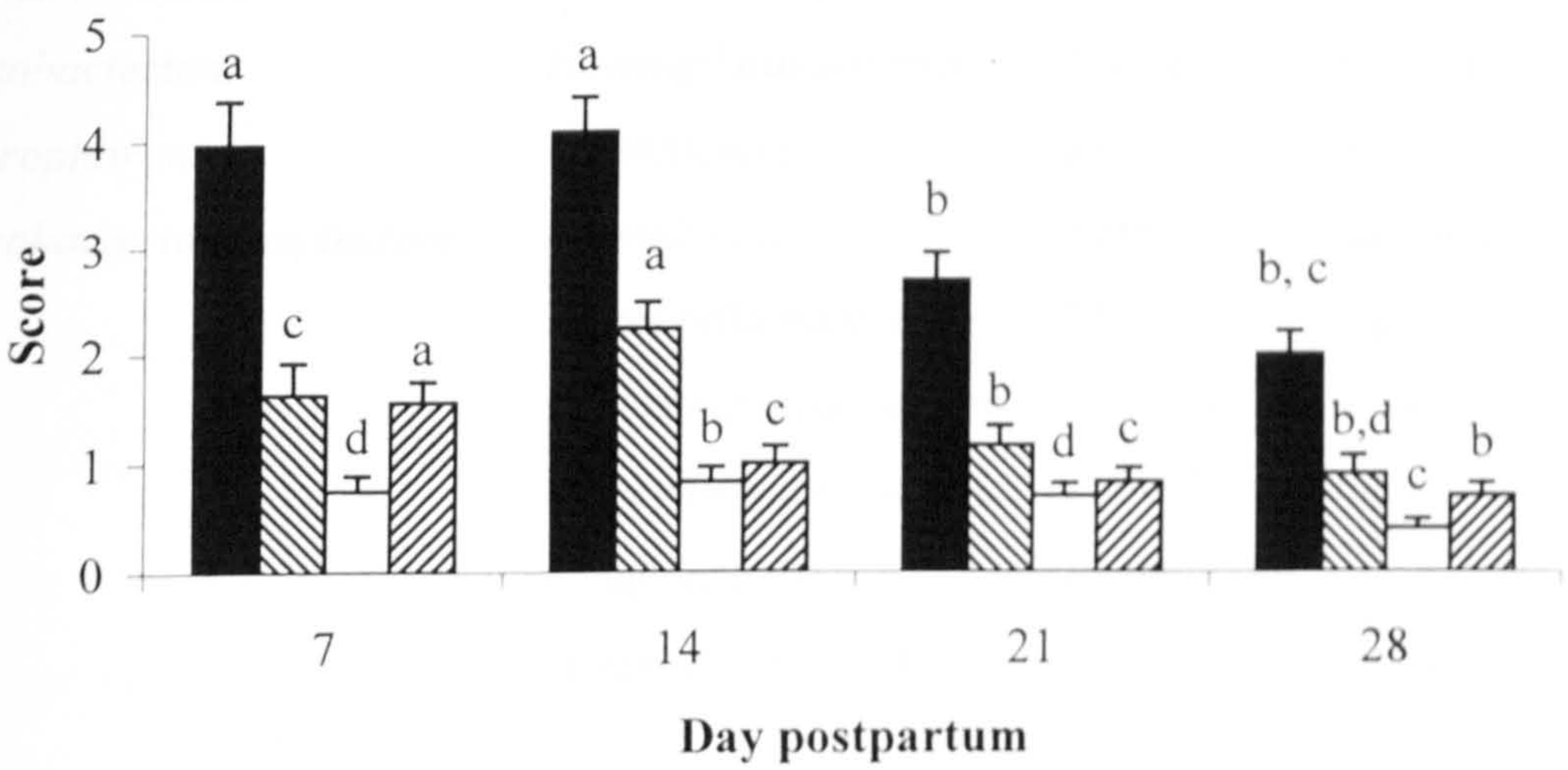

Figure 1. Mean ± SEM bacterial growth score from cows on Day 7, 14, 21 and 28 for all bacteria (■), group 1 bacteria (▧), group 2 bacteria (□), and group 3 bacteria (▨). Within bacterial group, different superscripts indicate differences between Day; $^{ab}P < 0.001$, $^{ac, bc}P < 0.01$, $^{cd}P < 0.05$.

Table 1. Categorisation of bacteria, isolated by aerobic and anaerobic culture of uterine swabs, based on their potential pathogenicity (Ruder *et al.*, 1981; Olson *et al.*, 1984; Farin *et al.*, 1989; Noakes *et al.*, 1989; Noakes *et al.*, 1991; Bonnett *et al.*, 1993). Categories: (1) pathogens known to cause endometrial lesions; (2) other recognised uterine pathogens; and, (3) bacteria not recognised as uterine pathogens

Bacterial category		
1	2	3
Arcanobacterium pyogenes	*Acinetobacter* spp	*Aerococcus viridans*
Prevotella spp	*Bacillus licheniformis*	*Clostridium butyricum*
Escherichia coli	*Enterococcus faecalis*	*Clostridium perfringens*
Fusobacterium necrophorum	*Haemophilus somnus*	*Corynebacterium* spp
	Mannhiemia haemolytica	*Enterobacter aerogenes*
Fusobacterium nucleatum	*Pasteurella multocida*	*Klebsiella pneumoniae*
	Peptostreptococcus spp	Micrococcus spp
	Staphylococcus aureus (coagulase +)	*Providencia rettgeri*
	Streptococcus uberis	*Providencia stuartii*
		Proteus spp
		Proprionobacterium granulosa
		Staphylococcus species (coagulase -)
		α-haemolyic Strctococci
		Streptococcus acidominimus

spp: species

Location of events

A wave of follicular development, with the emergence of a dominant follicle, was observed in all cows within 14 days of parturition.

There were fewer follicles ≥ 4 mm diameter in the ipsilateral ovary compared with the contralateral ovary in the first follicular wave after parturition (1.74 ± 0.15 vs. 2.89 ± 0.14, P < 0.0001). However, there were no significant differences in the numbers of follicles ≥ 4 mm diameter in the first postpartum follicular wave between high or standard Day 7 bacterial score categories, in the ipsilateral ovary (1.88 ± 0.18 vs. 1.40 ± 0.22, P = 0.14), or the contralateral ovary (2.86 ± 0.19 vs. 2.95 ± 0.17, P = 0.78). The proportion of follicles ≥ 4 mm diameter in the first follicular wave, located in the ipsilateral ovary, was greater than the proportion of first dominant follicles in the ipsilateral ovary (37.6% vs. 22.9%, P < 0.05).

There were fewer first postpartum dominant follicles in the ipsilateral ovary compared with the contralateral ovary (16 vs. 54, P < 0.0001). Logistic regression for the location of the first postpartum dominant follicles indicated that the standard or high bacterial category on Day 7 was significant (P < 0.05), but not on Day 14 (P = 0.77), Day 21 (P = 0.10), or Day 28 (P = 0.87). The high Day 7 bacterial score category was 14.4 (odds ratio) times less likely to have a first dominant follicle in the ipsilateral ovary, compared with the standard category (Fig. 2a). However, the location of the first dominant follicle did not differ significantly between animals in different bacterial pathogenicity groups on Day 7 postpartum (P = 0.65).

Ultrasonography revealed three possible fates for the first dominant follicle: ovulation (n = 48), regression followed by a second follicular wave (n = 10), or formation of a persistent follicle (n = 12). The frequency of the first dominant

follicles that ovulated, regressed or persisted did not differ significantly between the standard and high bacterial categories on Day 7 (37, 6 and 10 vs. 11, 4 and 2, respectively, P = 0.41), Day 14 (33, 6 and 7 vs. 15, 4 and 5, respectively, P = 0.73), Day 21 (36, 6 and 11 vs. 12, 4 and 1, P = 0.22), or Day 28 (40, 6 and 11 vs. 8 , 4 and 1, P = 0.14).

There were fewer ovulations in the ipsilateral ovary compared with the contralateral ovary (11 vs. 37, P < 0.001). However, a similar proportion of dominant follicles in the ipsilateral ovary ovulated compared with the contralateral ovary (11/16 vs. 37/54, P = 1.00).

A second follicular wave was detected in 43 animals. There was a similar number of follicles ≥ 4 mm diameter in the ipsilateral ovary compared with the contralateral ovary (2.29 ± 0.20 vs. 2.54 ± 0.24, P = 0.46). There were no significant differences in the numbers of follicles ≥ 4 mm diameter in the second postpartum follicular wave between high or standard Day 21 bacterial score categories, in the ipsilateral ovary (2.11 ± 0.54 vs. 2.34 ± 0.21, P = 0.52), or the contralateral ovary (2.89 ± 0.45 vs. 2.44 ± 0.28, P = 0.31). There were fewer second dominant follicles in the ipsilateral ovary compared with the contralateral ovary (14 vs. 29, P < 0.05). Logistic regression for the location of the second postpartum dominant follicle indicated that the bacterial category on Day 21 was significant (P < 0.05), but not on Day 7 (P = 0.66), Day 14 (P = 0.70), or Day 28 (P = 0.36). The high Day 21 bacterial score category was 6.0 (odds ratio) times less likely to have a first dominant follicles in the ipsilateral ovary, compared with the standard category (Fig. 2b). However, the location of the second dominant follicle did not differ significantly between uterine pathogenicity group (P = 0.35).

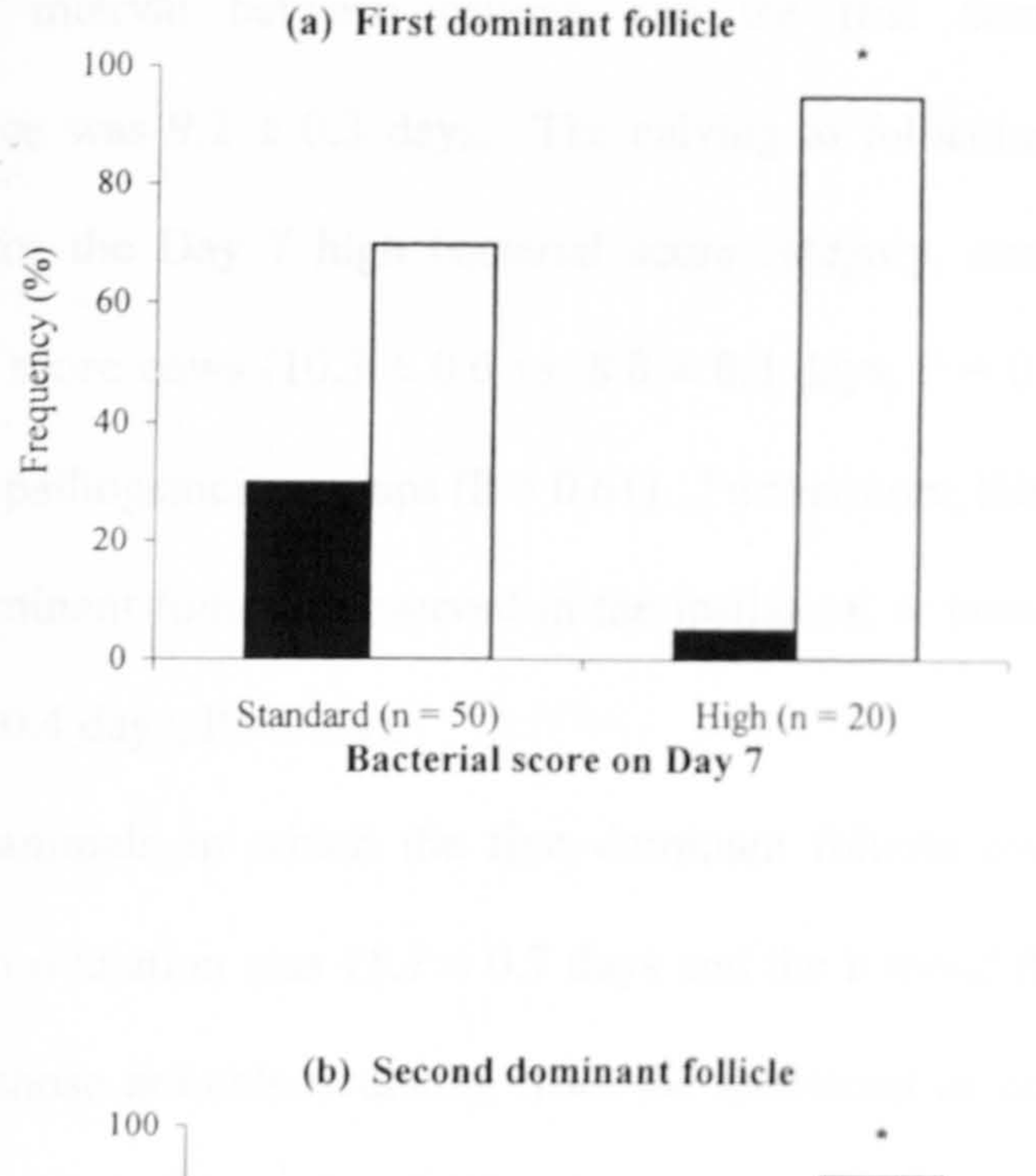

Fig. 2. The proportion (%) of (a) first postpartum dominant follicles, and (b) where observed, second dominant follicles, in the ipsilateral (■) or contralateral ovary (□), for cows with standard or high bacterial scores on (a) Day 7, and (b) Day 21. Within bacterial score category, proportions differ between ovaries, $^{*}P < 0.05$.

Timing of events

The interval between calving and the first dominant follicle achieving dominance was 9.2 ± 0.3 days. The calving to follicular dominance interval was similar for the Day 7 high bacterial score category, compared with the standard bacterial score cows (10.3 ± 0.6 vs. 8.8 ± 0.3 days, P = 0.44), and for the different bacterial pathogenicity groups (P = 0.61). Furthermore, the interval did not differ for those dominant follicles observed in the ipsilateral or contralateral ovary (8.9 ± 0.6 vs. 9.3 ± 0.4 days, P = 0.64).

For animals in which the first dominant follicle ovulated, the interval from calving to ovulation was 15.7 ± 0.7 days and the interval did not differ significantly between those animals ovulating from the ipsilateral or contralateral ovary (14.0 ± 1.2 vs. 16.2 ± 0.8 days, P = 0.12). Furthermore, there was no significant difference in the calving to ovulation interval between cows with a high or standard bacterial score on Day 7 (16.4 ± 1.4 vs. 15.4 ± 0.8 days, P = 0.33), or on Day 14 (17.3 ± 1.6 vs. 15.2 ± 0.8 days, P = 0.11).

In each case after ovulation an increase in plasma progesterone concentration to > 1 ng/ml was detected 3.2 ± 0.2 days later, and the interval did not differ between those animals with a first dominant follicle in the ipsilateral or the contralateral ovary (3.3 ± 0.5 vs. 3.1 ± 0.2 days, P = 0.50). In addition, bacterial score on Day 7, 14 or 21 was not a significant variable for the interval from ovulation to progesterone values > 1 ng/ml.

The interval from parturition to dominance of the second dominant follicle was 19.7 ± 0.7 days and, again, did not differ between the ipsilateral or contralateral ovarian location of the follicle (20.0 ± 1.4 vs 19.6 ± 0.9 days, P = 0.64). Furthermore,

bacterial score on Day 7, 14, or 21 was not a significant variable for the interval from calving to dominance of the second follicle.

Follicular growth and function

Follicle diameter

The mean internal diameter of first dominant follicles increased daily between Day 7 and 15 postpartum ($P < 0.0001$). However, uterine bacterial score, ovarian location, and the fate of the dominant follicle significantly influenced follicle diameter. The diameter of the first dominant follicle was smaller in animals with a high Day 7 bacterial score compared with standard score cows ($P < 0.001$), and there was a significant interaction of bacterial score category with time ($P < 0.05$, Fig. 3). However, the influence of the bacterial pathogenicity group was not significant. The diameter of the first dominant follicle differed between those located in the ipsilateral and those in the contralateral ovary ($P < 0.05$), and the interaction of location with time was also significant ($P < 0.05$, Fig 4). There was no difference in follicle diameter between follicles that persisted and those that ovulated or regressed. However, between Day 9 and 13 postpartum, the first dominant follicle was smaller before regression, compared with those that ovulated ($P < 0.05$, Fig. 5). The interaction of the fate of the dominant follicle and time was not significant.

Plasma oestradiol concentration

Plasma oestradiol concentration increased between Day 7 and 16 postpartum ($P < 0.0001$). In addition, there were significant interactions of Day x Day 7 bacterial category ($P < 0.05$, Fig. 3), and Day x fate of the first dominant follicle ($P < 0.05$, Fig. 5). There was no significant effect of the bacterial pathogen group, or the

location of the first dominant follicle (Fig. 4). On Day 15 and Day 16, plasma oestradiol concentrations were lower in animals with a high Day 7 bacterial score (Fig. 3). Oestradiol was also lower between Day 12 to Day 15, for animals in which the first dominant follicle regressed, compared with animals in which the follicle ovulated, or persisted (Fig. 5).

Plasma FSH concentration

Plasma FSH concentration between Day 7 and 16 postpartum differed significantly between Days postpartum ($P < 0.05$, Figs. 3-5). FSH concentration was highest on Day 7, decreased between Days 8 to 12, and subsequently increased between Days 13 to 16. Additional significant variables were the fate of the first dominant follicle ($P < 0.01$, Fig. 5), and the interaction of Day x dominant follicle fate ($P < 0.01$). However, plasma FSH concentration did not differ significantly between animals with standard or high bacterial score on Day 7 (Fig. 3); between bacterial pathogenicity group; or, the location of the first dominant follicle between the ipsilateral or contralateral ovary (Fig. 4).

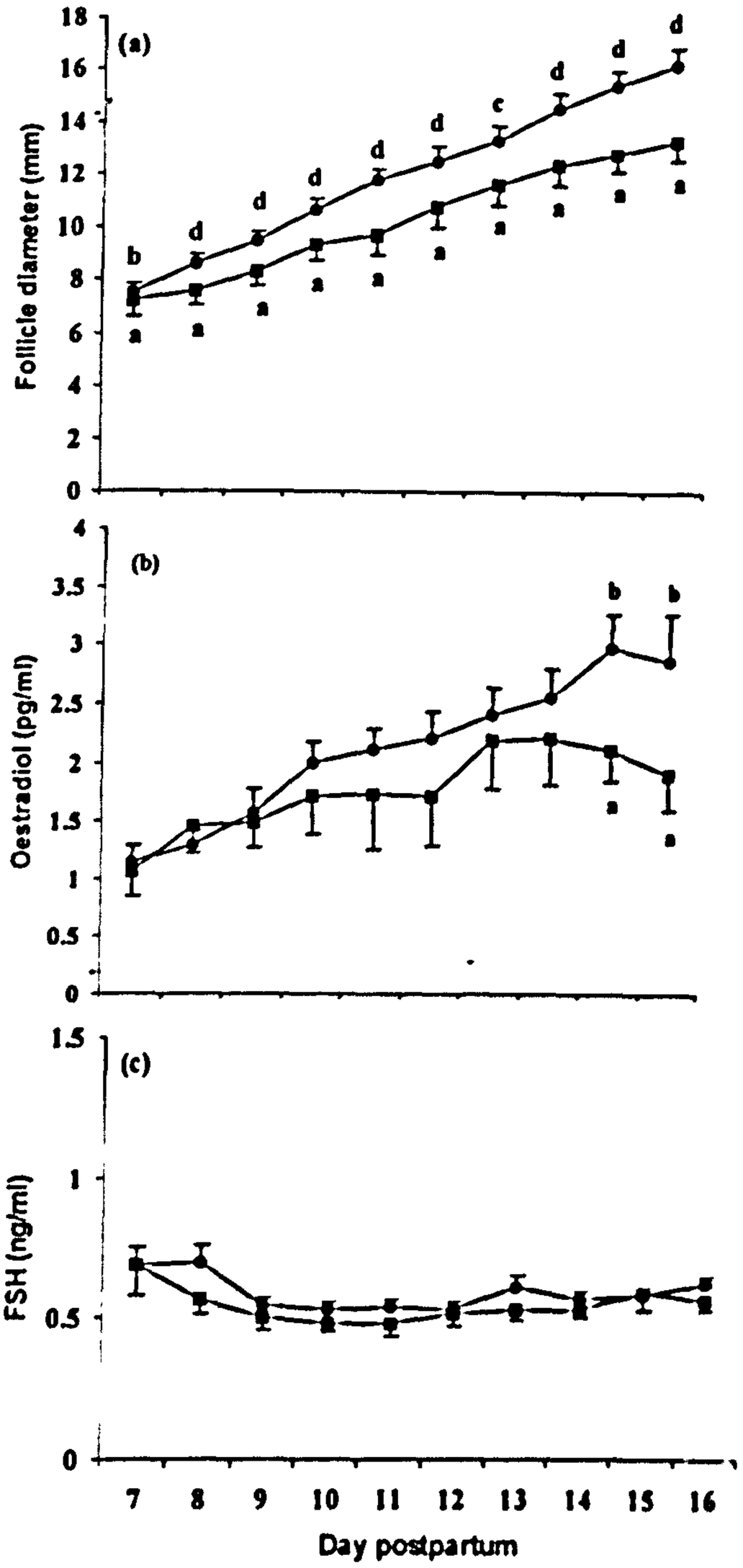

Fig. 3. Mean ± SEM (a) first dominant follicle diameter, (b) plasma oestradiol concentration, and (c) plasma FSH concentration between Day 7 and 16 postpartum for cows in which there was a standard (●, n = 50) or high (■, n = 20) uterine bacterial contamination on Day 7. Within a Day, values differ between bacterial contamination category $^{ab}P < 0.05$, $^{ac}P < 0.01$, $^{ad}P < 0.001$.

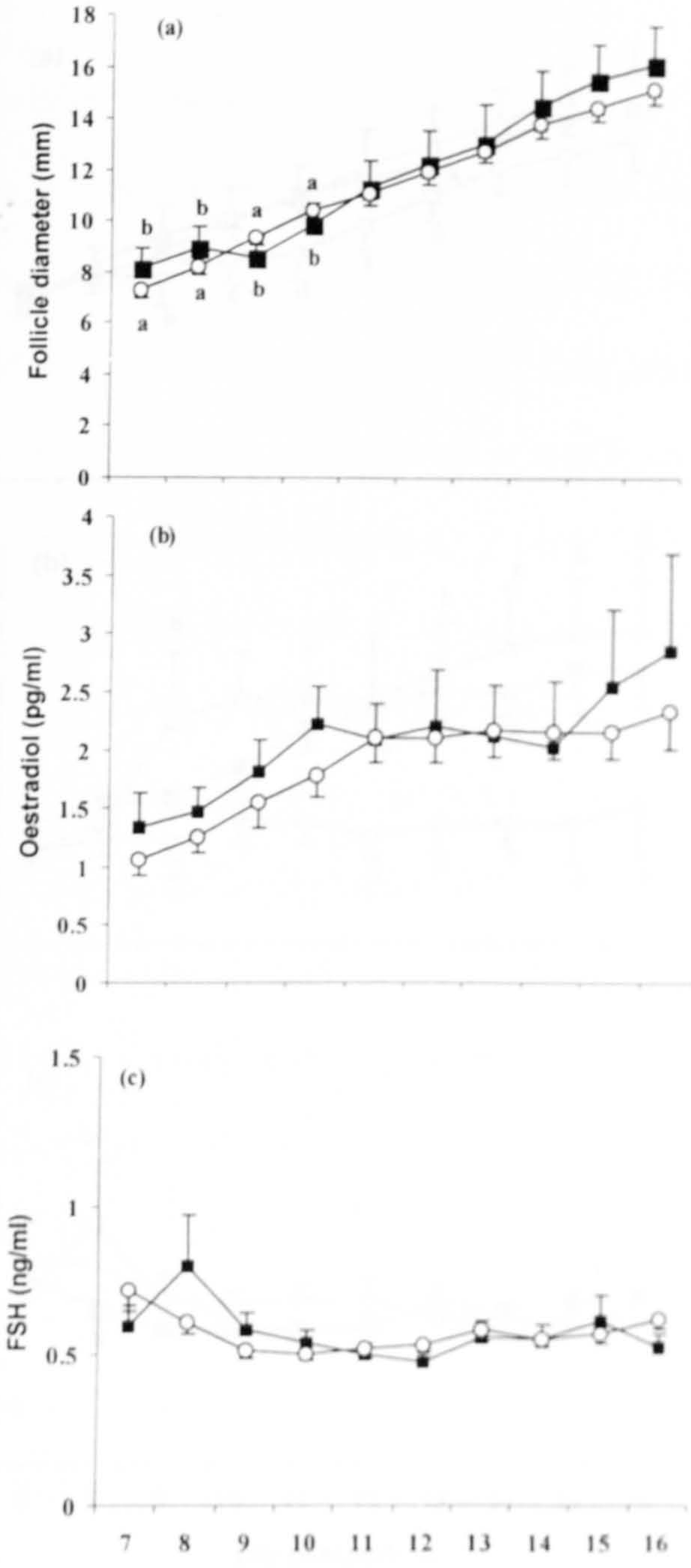

Fig. 4. Mean ± SEM (a) first dominant follicle diameter, (b) plasma oestradiol concentration, and (c) plasma FSH concentration between Day 7 and 16 postpartum for cows in which the dominant follicle was located in the ipsilateral (■, n = 16) or contralateral (○, n = 54) ovary. Within a Day, values differ between ovary ^{ab} $P < 0.05$.

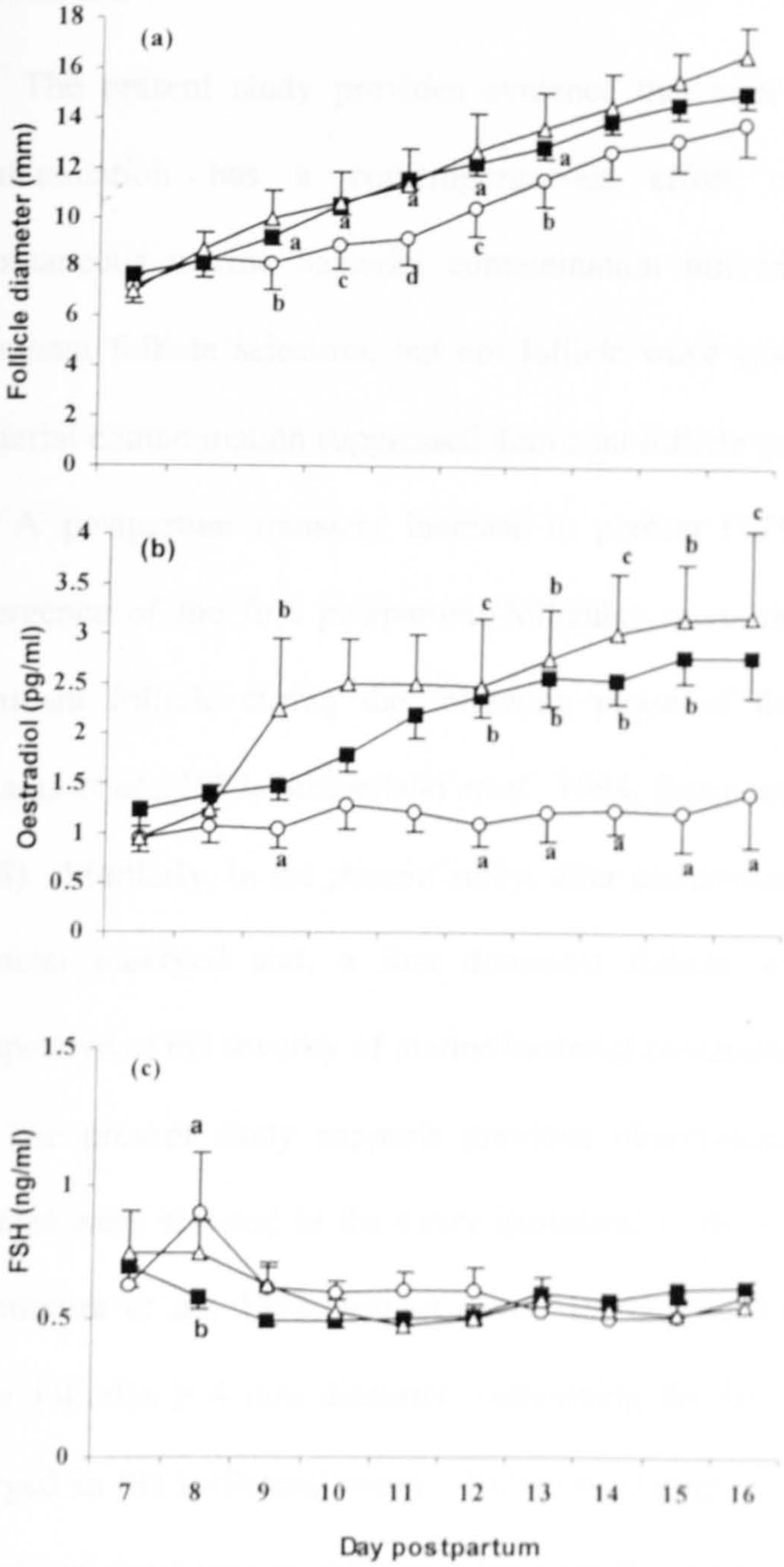

Fig. 5. Mean ± SEM (a) first dominant follicle diameter, (b) plasma oestradiol concentration, and (c) plasma FSH concentration between Day 7 and 16 postpartum for cows in which the dominant follicle ovulated (■, n = 48), regressed (○, n = 10), or persisted (Δ, n = 12). Within a Day, values differ between follicle group ^{ab} $P < 0.05$, ^{ac} $P < 0.01$, ^{ad} $P < 0.001$.

Discussion

The present study provides evidence that after parturition, uterine bacterial contamination has a contemporaneous effect on ovarian folliculogenesis. Spontaneous uterine bacterial contamination influenced the location of ovarian dominant follicle selection, but not follicle wave emergence. In addition, uterine bacterial contamination suppressed dominant follicle growth and function.

A postpartum transient increase in plasma FSH concentration preceded the emergence of the first postpartum follicular wave, and subsequent selection of a dominant follicle during the following phase of decreasing FSH concentration (Adams *et al.*, 1992; Sunderland *et al.*, 1994; Beam and Butler, 1997; Crowe *et al.*, 1998). Similarly, in the present study, after parturition, a wave of follicles $\geq$ 4 mm diameter emerged and, a first dominant follicle was selected in each animal irrespective of the severity of uterine bacterial contamination.

The present study supports previous observations that fewer first dominant follicles were selected in the ovary ipsilateral to the previously gravid uterine horn (Kamimura *et al.*, 1993; Nation *et al.*, 1999). Furthermore in the present study, fewer follicles $\geq$ 4 mm diameter, comprising the first postpartum follicular wave, emerged in the ipsilateral ovary. However, during the second follicular wave after parturition, there was no difference between the ovaries for the number of follicles $\geq$ 4 mm diameter emerging, although fewer second dominant follicles were selected in the ipsilateral ovary. These observations support our previous findings that the effect on folliculogenesis in the ipsilateral ovary declines with increasing time after parturition, in parallel with uterine involution and disappearance of the corpus luteum of pregnancy (Sawyer, 1995; Sheldon *et al.*, 2000; Chapter 3). However, removal of the corpus luteum of pregnancy, before parturition, did not affect the

asymmetric distribution of dominant follicles between the ovaries (Sheldon *et al.*, 2002; Chapter 7). Thus, it is suggested that uterine bacterial contamination after parturition determines the location of dominant follicle selection.

In the present study, when uterine bacterial growth scores were high on Day 7 or Day 21, few first or second dominant follicles were selected in the ipsilateral ovary, respectively. These observations suggest that uterine bacterial contamination had a localised effect preventing dominant follicle selection in the ipsilateral ovary. Similarly, cows with retained fetal membranes, which are likely to be associated with greater uterine bacterial contamination, had less follicular activity in the ipsilateral ovary, although the presence of dominant follicles was not identified (Risco *et al.*, 1994). The effect of uterine bacterial contamination appeared to be short-term. The uterine bacterial scores on Day 7 and Day 21, were contemporaneous with the time of first and second dominant follicle selection on ~ Day 9 and ~ Day 20, respectively. Bacterial scores at other times were not significant. Furthermore, the individual animals comprising the high score categories on Day 7 and 21 were not exactly the same cows.

There are two potential mechanisms whereby uterine bacterial contamination could have had a localised effect on the ipsilateral ovary. Firstly, bacterial load, or the induced inflammatory response, may differ between the two uterine horns. Alternatively, the concentration of inflammatory mediators reaching the ipsilateral ovary may be greater than the contralateral ovary because of the greater blood flow to and from the gravid uterine horn (Ford *et al.*, 1979). Most animals had bacterial contamination of the uterus within 2 weeks of parturition, so it was not possible to determine if the inflammatory response was the sole mechanism for the disparity in the pattern of folliculogenesis between the two ovaries. On the other hand, the

inflammatory response to uterine bacterial contamination may be modulated by an existing effect of the involuting previously gravid horn on the ipsilateral ovary. In cattle, uterine involution provokes an inflammatory response, independent of uterine bacterial contamination (Sheldon *et al.*, 2001).

The precise mechanism by which inflammatory mediators could influence ovarian events has not been determined. However, intravenous or intracerebral infusion of endotoxin, or the cytokine IL-1, disrupts the follicular phase of the oestrous cycle in several species (Peter *et al.*, 1989; Rivest *et al.*, 1993; Battaglia *et al.*, 1999; Puder *et al.*, 2000). As in the present study, the effects of inflammatory mediators on ovarian function were rapid, and did not carry over into subsequent oestrous cycles (Xiao *et al.*, 1998). A contemporaneous effect of inflammatory mediators was to be expected, as there is acute inhibition of hypothalamic GnRH release and pituitary LH secretion (Rivest *et al.*, 1993; Williams *et al.*, 2001). However, these neuro-endocrine mechanisms do not fully explain the present observations of a localised effect on the ipsilateral ovary. Furthermore, evidence has been provided for a direct effect of inflammatory mediators at the ovarian level by the suppression of oestradiol secretion, in the presence of adequate plasma LH concentration (Xiao *et al.*, 1998; Battaglia *et al.*, 2000). Dominant follicle selection involves a close functional coupling with changes in plasma FSH concentration (Ginther *et al.*, 2000a; Ginther *et al.*, 2001b). However, plasma FSH concentrations did not differ between standard or high bacterial score categories, and there was no effect on the number of follicles $\geq$ 4 mm diameter that emerged in each ovary.

In addition to the effect of the high Day 7 uterine bacterial score on dominant follicle location, follicles grew slower, and produced lower plasma oestradiol concentrations. A significant difference in plasma oestradiol concentration between

dominant follicles in the standard or high Day 7 bacterial score categories was not detected until Day 15 to 16, when the difference in follicle diameter was maximal. The effect of uterine bacterial contamination on follicle growth and function could be a centralised effect mediated by disruption of LH secretion, or a direct effect on the ovary (Battaglia *et al.*, 2000). Decreased plasma LH concentration reduced the growth rate and oestradiol secretion of dominant follicles, after follicle selection (Ginther *et al.*, 2001b). However, in the present study, the effect on follicle growth rate was evident at a diameter of < 8.5 mm, the diameter for deviation between dominant and subordinate follicles (Ginther *et al.*, 1999). Furthermore, although inflammatory challenge disrupts ovulation by perturbing the LH surge, the proportion of dominant follicles ovulating was not affected by spontaneous uterine bacterial contamination, in the present study.

Although there were small differences in diameter between dominant follicles in the ipsilateral and contralateral ovaries before dominance, subsequent follicle diameters were similar. In addition, follicle function as determined by plasma oestradiol concentrations was similar for both ovaries, and the interval from parturition to ovulation was similar. These observations suggest that dominant follicles in the ipsilateral ovary were at least as functionally competent as in the contralateral ovary.

Important uterine pathogenic bacteria are associated with more severe clinical disease, increased endometrial inflammation, and reduced fertility (Farin *et al.*, 1989; Bonnett *et al.*, 1993). In particular, *A. pyogenes, F. necrophorum* and Prevotella spp. act synergistically to cause more severe clinical signs (Ruder *et al.*, 1981; Olson *et al.*, 1984). In the present study, whilst there was an effect of bacterial load on folliculogenesis, the importance of uterine bacterial pathogens was not significant.

This suggests that the inflammatory response to the amount of bacterial contamination is more important than the presence of particular bacterial species. That response is likely to involve several mediators, rather than single mediators used in many intervention studies. Indeed, intrauterine infusion of *E. coli* shortened the oestrous cycle, whereas intrauterine or intravenous administration of endotoxin, did not (Gilbert *et al.*, 1990).

The fate and function of the first dominant follicles observed in the present study supported previous descriptions (Beam and Butler, 1997). In particular, the first dominant follicles that regressed, secreted less oestradiol irrespective of follicle diameter, possibly as a consequence of inadequate LH support (Ginther *et al.*, 2001b). The time interval from parturition to ovulation, and from ovulation to increased plasma progesterone concentration, did not differ between ovaries, and was not affected by uterine bacterial contamination. The frequency with which dominant follicles ovulated, regressed, or became persistent, was also unaffected by ovarian location or uterine bacterial contamination. These observations suggest that follicle fate was dependent on other factors such as LH pulse frequency (Duffy *et al.*, 2000).

In conclusion, there was no effect of uterine bacterial contamination on plasma FSH concentration, and ovarian follicle wave emergence. However, spontaneous bacterial contamination of the postpartum uterus influenced the ovarian location for dominant follicle selection. Contemporaneous, high bacterial contamination was associated with fewer first and second dominant follicles in the ipsilateral ovary to the previously gravid uterine horn. In addition, dominant follicle growth was slower, and oestradiol secretion reduced, in the presence of high bacterial contamination. The present study provides evidence for a localised effect of uterine bacterial contamination on the ovary after parturition.

Chapter 9

General discussion

The present thesis tested the hypothesis that utero-ovarian signalling is involved in the regulation of uterine involution and the resumption of cyclic ovarian activity in the postpartum cow. The formulation of this hypothesis was stimulated by three pieces of evidence. Firstly, there is a predilection for folliculogenesis in the ovary contralateral to the previously gravid uterine horn (Lewis *et al.*, 1984; Kamimura *et al.*, 1993). Secondly, a small epidemiological study noted that a larger follicle in the ipsilateral ovary around Day 26 postpartum was associated with improved subsequent fertility (Bonnett *et al.*, 1993). Finally, the anatomical structure of the ruminant reproductive tract lends itself to such localised effects of the ovary on the uterus, or *vice versa*. There are intimate relationships between both the lymphatic and blood vasculature of the uterus and ovary (Del Campo and Ginther, 1973; Staples *et al.*, 1982). The uterus-to-ovary pathway has been clearly established for the transport of prostaglandin $F_{2\alpha}$ during luteolysis (Heap *et al.*, 1985; Bonnin *et al.*, 1999). Whilst the opposite ovary-to-uterus pathway is probably responsible for the localised increase in progesterone concentrations in the uterine horn and vasculature ipsilateral to the ovary containing the corpus luteum (Weems *et al.*, 1988; Cerbito *et al.*, 1994)

The first study in the present thesis used transrectal ultrasonography to confirm the postpartum suppression of folliculogenesis in the ovary ipsilateral to the previously gravid uterine horn (Sheldon *et al.*, 2000; Chapter 3). This suppression of follicular growth decreased with increasing time postpartum, until the ovarian asymmetry was no longer significant by 24 to 28 days postpartum, in agreement with previous studies using palpation *per rectum* (Foote and Peterson, 1968; Marion and

Gier, 1968). Despite this limited period of ovarian asymmetry, the presence of a follicle > 8 mm diameter in the ipsilateral ovary between 14 and 28 days postpartum, in the present study, was associated with improved subsequent fertility, as determined by shorter calving-to-conception intervals. In the meantime, it was also reported that conception rates tended to be higher if cows ovulate from the ipsilateral ovary 25 or 39 days postpartum (Bridges *et al.*, 2000). We suggest that our observations, taken together with those of Bonnett *et al.* (1993) and Bridges *et al.* (2000), indicate that the presence of a dominant follicle in the ipsilateral ovary within four weeks of parturition is a marker of subsequent fertility.

Results from the first study stimulated several questions about how a dominant follicle in the ipsilateral ovary could act to improve fertility, and the mechanism controlling asymmetric ovarian folliculogenesis. On the one hand, there may be ovary-to-uterus signalling, whereby a large oestradiol-secreting follicle in the ovary has a localised effect to increase the rate of involution of the ipsilateral previously gravid uterine horn. Alternatively, there could by uterus-to-ovary signalling, whereby a relatively healthy previously gravid uterine horn is reflected by the presence of a large follicle within the ipsilateral ovary. In addition, the decreasing asymmetry between ovaries with increasing time after parturition could reflect an effect of the parallel regression of the corpus luteum of pregnancy or changes in the uterus (Elliot *et al.*, 1968; Sawyer, 1995). To answer these questions, an integrated series of whole-animal experiments were designed.

Oestradiol enhances uterine defence mechanisms and is an effective treatment for postpartum uterine infection in cattle (Rowson *et al.*, 1953; Pepper and Dobson, 1987; Sheldon and Noakes, 1998). Furthermore, oestradiol treatment after parturition increases blood flow to all uterine tissues several fold (Rosenfeld, 1980).

However, there is no information about the localised effect of a large oestrogen-secreting follicle in the ipsilateral ovary on uterine involution. In the first of three studies to examine for a localised effect of oestradiol on uterine horn involution, eCG was administered 14 days postpartum to increase ovarian follicular growth (Sheldon and Dobson, 2000; Chapter 4). There was no discernible effect on uterine diameter that could be attributed to a localised increase in ovarian follicular growth and function, nor a dose response effect of eCG treatment. However, a possible weakness of the study was that uterine dimensions were measured for only six days. Nevertheless, repeated measures of uterine dimensions do provide a sensitive method of examining uterine involution when examined using a mixed model for repeated measures ANOVA (SAS, 1997). This analysis technique was also used to examine the concentrations of PGFM or acute phase proteins in peripheral plasma. The plasma concentration of PGFM and the acute phase proteins α_1-acid glycoprotein, haptoglobin and ceruloplasmin are increased around the time of parturition in ruminants, and decrease gradually during the first two to three weeks postpartum (Lindell *et al.*, 1982; Regassa and Noakes, 1999; Sheldon *et al.*, 2001). These changes in plasma concentrations, at least in part, reflect the progress of uterine involution and were additional measurements used to compare treatments in two further studies.

To more precisely mimic a localised effect, oestradiol benzoate was infused into the uterine lumen of cattle on Days 7 and 10 postpartum, and in a second study, attaching an implant to the ovarian bursa ipsilateral to the previously gravid uterine horn prolonged the period of oestradiol administration. In the latter study, sheep were used as a model to reduce the possible confounding effect of the ubiquitous postpartum uterine bacterial contamination, which is present in cattle but to a lesser

extent in sheep (Elliot *et al.*, 1968; Regassa and Noakes, 1999). In addition, the use of ewes permitted the evaluation of uterine involution by estimating the dry matter and collagen content of the genital tract tissues after slaughter on Day 17 postpartum. There was no consistent evidence in either study of a localised effect on uterine involution, as determined by changes in uterine dimensions, wet weight, dry matter or collagen content. However, there was evidence that oestradiol decreased the concentration of inflammatory mediators in the peripheral circulation. Although, it was not clear whether this suppression was mediated via an effect on uterine involution; or, directly on the metabolism and secretion of the acute phase proteins and PGFM (Brinkman-Van der Linden *et al.*, 1996; Mann and Haresign, 2001). Taken together, the three studies that examined an ovary-to-uterus pathway provide increasing evidence that oestradiol secretion from the ovary has a minimal localised effect on the uterus after parturition. A possible reason for not detecting an effect could be inappropriate doses of oestradiol in relation to the concentration of uterine oestradiol receptors present at the time. However, each experiment produced peripheral plasma concentrations within the expected physiological range. In addition, although oestrogen receptor immunoreactivity decreases after parturition, concentrations are above basal values and the concentration of oestrogen receptors would be expected to be high in the presence of follicular phase concentrations of oestradiol (Boos *et al.*, 2000; Robinson *et al.*, 2001).

An intriguing feature of one of the experiments testing the ovary-to-uterus signalling was the effect of eCG to overcome the expected suppression of folliculogenesis in the ipsilateral ovary (Sheldon and Dobson, 2000; Chapter 4). Administration of eCG, which mimics FSH and LH activity in cattle (i) leads to fewer atretic follicles, (ii) recruits more smaller follicles in which growth rate is

increased, (iii) sustains the growth of larger follicles, and (iv) increases the final number of ovulations (Newcomb *et al.*, 1979; Monniaux *et al.*, 1984; Driancourt *et al.*, 1991; Gonzalez *et al.*, 1994). Possibly, an inhibitor of folliculogenesis, acting principally on the ovary ipsilateral to the previously gravid uterine horn, blocks the action of FSH and/or LH within that ovary, and this block can be overcome by administration of eCG. Mechanisms that have been suggested to cause such a localised inhibition include the regressing corpus luteum of pregnancy, the uterus, or its contents (Dufour and Roy, 1985; Nation *et al.*, 1999).

To examine the effect of the regressing corpus luteum of pregnancy on postpartum folliculogenesis cows were treated with prostaglandin $F_{2\alpha}$ between 190 and 220 days of gestation to cause luteolysis, but without inducing parturition (Sheldon *et al.*, 2002; Chapter 7). However, the proportion of first dominant follicles in the ipsilateral ovary was similar for treated and control animals, as was the time interval between calving and establishment of a dominant follicle. Further, there was no significant effect of treatment on dominant follicle growth, or function as determined by plasma hormone concentrations. Thus, it is suggested that the corpus luteum of pregnancy does not have a local effect on postpartum ovarian folliculogenesis and that, instead, an influence exerted by the previously gravid uterine horn should be considered. This suggestion is also supported by the observation that ovarian postpartum follicular asymmetry occurs in uniparous species with a bicornuate uterus, but not in multiparous species or those with a simplex uterus (Allen and Newcombe, 1981; Kamimura *et al.*, 1993; Bartlewski *et al.*, 2000).

Additional indications of uterus-to-ovary signalling include the observations that animals in which the first dominant follicle did not ovulate have larger diameter uterine horns than animals in which the follicle ovulated (Chapter 5); and ovulation

is less likely if there is a purulent vaginal discharge (Sheldon *et al.*, 2000; Chapter 3). Therefore, the effect of the postpartum uterus on the location of ovarian follicle emergence, selection, growth and function was examined. Swabs were collected from the uterine body lumen of cattle on Days 7, 14, 21 and 28 postpartum. Bacteria were identified by aerobic and anaerobic culture; bacterial growth was scored semi-quantitatively and animals categorised into standard or high bacterial contamination based on the number of colonies detected. There was no effect of bacterial contamination on plasma FSH concentration profiles, or ovarian follicle wave emergence. However, when uterine bacterial growth scores were high on Day 7 or Day 21, fewer first or second dominant follicles were selected in the ipsilateral than the contralateral ovary, respectively. In addition, the diameter of the first dominant follicle was smaller in animals with a high Day 7 bacterial score, dominant follicle growth was slower and oestradiol secretion was reduced. Thus, uterine bacteria have a contemporaneous localised effect on ovarian follicle selection, and subsequent growth and function, but not initial emergence. This uterus-to-ovary signalling could also be an explanation for a first dominant follicle in the ipsilateral ovary being a marker of fertility; in this case by indicating relative uterine health. This hypothesis would agree with previous observations that animals with endometritis are subfertile (Borsberry and Dobson, 1989).

In conclusion, signalling between the uterus and ovary is a key feature of the postpartum reproductive biology of the cow. There is a predilection for folliculogenesis and first dominant follicle selection in the ovary contralateral to the previously gravid uterine horn. However, this is not a local effect of the regressing corpus luteum of pregnancy. Although less frequent, the presence of a first dominant follicle in the ipsilateral ovary is a marker of subsequent fertility. There was little

evidence from three studies testing ovary-to-uterus signalling, that oestradiol from a dominant follicle affects the involution of the ipsilateral previously gravid uterine horn. The mechanism underlying both the asymmetry of folliculogenesis postpartum and its relationship to fertility is likely to involve uterus-to-ovary signalling, probably involving uterine bacterial contamination. The future challenge is to elucidate the details of this pathway by examining the relative contributions of immune mediators and products of uterine involution such as metabolites of collagen. In addition, as pelvic inflammatory disease is an important cause of subfertility in humans, which may involve parallel mechanisms, the signalling between uterus and ovary in the postpartum cow has important implications for both human and veterinary medicine.

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Appendix

Copies of published papers

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Sheldon IM, Noakes DE and Dobson H (2002) Effect of the Regressing Corpus Luteum of Pregnancy on Ovarian Folliculogenesis after Parturition in Cattle *Biology of Reproduction* **66** 266-271

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Effect of administration of eCG to postpartum cows on folliculogenesis in the ovary ipsilateral to the previously gravid uterine horn and uterine involution

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This study tested the hypothesis that increased oestradiol secretion by large follicles in the ovary ipsilateral to the previously gravid uterine horn has a local effect to increase the rate of uterine involution. Cows were administered an i.m. water placebo ($n = 19$), 250 iu equine chorionic gonadotrophin (eCG) ($n = 18$) or 750 iu eCG ($n = 20$) 14 days post partum (day 0). Transrectal ultrasonography at the time of treatment and 2, 4 and 6 days later monitored uterine horn diameter and ovarian structures. Blood samples collected contemporaneously were assayed for 15-keto-13,14-dihydro-prostaglandin $F_{2\alpha}$ and oestradiol concentration. For control cows, accumulated diameter of the largest follicle in the ovary ipsilateral to the previously gravid uterine horn, compared with the contralateral ovary, was smaller on day 0 ($P < 0.05$) and days 2, 4 and 6 ($P < 0.001$). There were no significant differences in the eCG-treated animals. There were 12, 8 and 11 cows with a plasma oestradiol concentration < 1 pg ml^{-1} on day 0 in the control, 250 and 750 iu eCG treatment groups, respectively. For control cows, the peripheral oestradiol concentrations were higher on day 6 compared with days 0, 2 and 4 ($P < 0.05$); for cows treated with 250 iu eCG, concentrations were higher on days 4 and 6 compared with day 0 ($P < 0.05$); and for cows treated with 750 iu eCG, concentrations were higher on days 2 and 4 compared with day 0 ($P < 0.01$). Treatment with eCG, or the presence of a follicle > 8 mm in diameter in the ovary ipsilateral to the previously gravid uterine horn, did not affect the rate of uterine involution or plasma 15-keto-13,14-dihydro-prostaglandin $F_{2\alpha}$ concentration. In conclusion, administration of eCG to increase follicular growth and oestradiol production overcame the inhibition of follicular growth in the ovary ipsilateral to the previously gravid uterine horn, but did not affect uterine involution.

Introduction

Postpartum follicular activity in the ovary ipsilateral to the previously gravid uterine horn is lower than that in the contralateral ovary (Lewis *et al.*, 1984; Guilbault *et al.*, 1987; Kamimura *et al.*, 1993; Risco *et al.*, 1994; Nation *et al.*, 1999). However, the presence of a follicle > 8 mm in diameter in the ovary ipsilateral to the previously gravid uterine horn in cows examined between 14 and 28 days post partum was associated with a shorter calving-to-conception interval (I. M. Sheldon and H. Dobson, unpublished), confirming the results of a smaller study by Bonnet *et al.* (1993). One hypothesis to explain these observations is that oestrogen secreted by a dominant follicle in the ovary ipsilateral to the previously gravid uterine horn acts locally to increase the rate of involution of the ipsilateral uterine horn. Plasma concentrations of oestradiol are high in the utero-ovarian vein draining the ovary containing the ovulatory dominant follicle (Ireland *et al.*, 1984). Such a hypothesis is not without precedence: treatment-by-side interactions on oviductal protein synthesis occur in cows with persistent dominant follicles, and it has been suggested that this is due to the ipsilateral increase in oestradiol concentration (Binelli *et al.*, 1999).

One method of testing the hypothesis would be to stimulate follicular growth in the ovary ipsilateral to the previously gravid uterine horn and assess uterine involution. Equine chorionic gonadotrophin (eCG) has both FSH- and LH-like activity and parenteral administration stimulates follicular growth and ovulation in cattle (Gonzalez-Menico *et al.*, 1978; Newcomb *et al.*, 1979). Uterine involution occurs on a decreasing logarithmic scale, as reflected by changes in diameter of each uterine horn monitored accurately by transrectal ultrasonography (Okano and Tomizuka, 1987; Bekana *et al.*, 1994). In addition, involution has been characterized by the estimation of plasma concentration of 15-keto-13,14-dihydro-prostaglandin $F_{2\alpha}$ (PGFM), the metabolite of prostaglandin $F_{2\alpha}$, which is correlated with the decrease in uterine horn dimension (Lindell *et al.*, 1982; Lewis *et al.*, 1984).

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The aim of the present study was to test the hypothesis that increased oestradiol secretion by large follicles in the ovary ipsilateral to the previously gravid uterine horn has a local effect to increase the rate of uterine involution. Specific objectives were: (i) to administer eCG to increase follicular growth in the ovary ipsilateral to the previously gravid uterine horn; and (ii) to determine the effect of the increased follicular growth on involution of the previously gravid uterine horn by ultrasonography and by estimation of plasma PGFM concentration.

Materials and Methods

Animals

A seasonally calving dairy herd of 124 Holstein–Friesian cows with an annual average milk yield of ~6050 l was selected for the study on the basis of accurate farm records. A total of 57 cows that had calved in a 2 month period were included in the study. Routine medical treatments were not used on the farm; insemination of all cows began 50 days after calving.

The farm was visited every 48 h and generally three new cases were enrolled at each visit (range 0–6). At the first examination, 14 days after calving (day 0), details from the farm records of lactation number, calving date, assisted parturition and peri-parturient disease were recorded for each cow. Cows that had intercurrent disease were excluded from the study.

Clinical examination

The side of the previously gravid uterine horn was determined transrectally by assessing which was longer and of greater diameter than the contralateral horn. The genital tract was scanned transrectally using a SonoAce 600 ultrasound scanner with a 5 MHz linear array transrectal probe (BCF Technology Ltd, Livingstone) and recorded using a video cassette recorder (Panasonic AG-5260B). Follicles were defined as non-echogenic (black) spherical structures with a clear demarcation between the follicular wall and antrum. Corpora lutea were defined as grainy echogenic structures that had a well-defined border with the less echogenic ovarian stroma; in some corpora lutea there was a non-echogenic lacuna. The diameter of the largest follicular and luteal ovarian structures, and the diameter of the uterine horns adjacent to the insertion of the free edge of the intercornual ligament, were measured using the internal callipers on the screen of the machine. When the image of the structure being scanned was not spherical, the diameter was estimated by averaging two 90° dimensions. In addition, the number of follicles > 4 mm in diameter in each ovary was counted.

Animals were re-examined using the procedure outlined above at 2, 4 and 6 days after the first examination (day 0). In addition, at the final examination (day 6), the vagina of each cow was examined by direct palpation and visual inspection of the luminal contents. The character of the vaginal mucus was recorded: abnormal mucus was defined as the presence of mucopurulent material; normal mucus was defined as uniform translucent material in the absence of a fetid odour.

Blood sampling and hormone assays

At each visit blood samples were collected from the coccygeal vein or artery into evacuated heparinized tubes (Vacutainer, Becton Dickinson, Meylan). Plasma was separated within 30 min by centrifugation (1000 *g* for 10 min), harvested and stored frozen at –20°C until assayed for PGFM and oestradiol.

The PGFM assay was performed in duplicate using unextracted plasma by direct radioimmunoassay using the method of Kindahl *et al.* (1976). Oestradiol concentration was estimated in duplicate by radioimmunoassay using extracted plasma (Mann *et al.*, 1995). The sensitivity, intra- and interassay coefficients of variation were 27.0 pg ml^{-1}, 7.6% and 14.3%, respectively, for PGFM (using a pooled plasma sample of 91.2 pg ml^{-1}); and 0.24 pg ml^{-1}, 6.3% and 15.1%, respectively, for oestradiol (using a pooled plasma sample of 3.9 pg ml^{-1}).

Treatments

Treatments were administered by i.m. injection on day 14 post partum (day 0 of the study). A randomization chart was used to assign cows to treatment groups. The treatments were 2 ml sterilized water for injection BP (Arnolds Veterinary Products, Shrewsbury) to control animals or 250 or 750 iu eCG (PMSG Folligon 1000 iu, Intervet UK, Cambridge).

Statistical analysis

Results are expressed as mean ± SFM. Significance was assigned at $P < 0.05$. The data were analysed either using all cases, or after selection of animals that had a plasma oestradiol concentration of < 1 pg ml^{-1} on day 0. The animals chosen on the basis of plasma oestradiol were selected because the response to eCG treatment is greater in the absence of a dominant follicle (Guilbault *et al.*, 1991; Huhtinen *et al.*, 1992).

The proportion of animals in each group with an assisted parturition, animals with a corpus luteum formed during the study, and animals with a follicle > 8 mm in diameter in the ovary ipsilateral to the previously gravid uterine horn on day 2 was compared by the chi-squared test. The number of follicles > 4 mm or > 8 mm in diameter, lactation number and the number of inseminations per conception were compared between treatment groups using a non-parametric test (Kruskal–Wallis). The plasma oestradiol concentration, and log transformed values of calving-to-first insemination and calving-to-conception intervals were compared between treatment groups using ANOVA. Animals with normal and abnormal vaginal mucus were compared for calving-to-first insemination and calving-to-conception intervals using *t*

tests and for the number of inseminations per conception using a non-parametric test (Mann–Whitney).

Because ovarian follicular growth is not synchronous at precise times post partum and varies among individuals, the diameter of the largest follicle > 4 mm or > 8 mm in diameter in each ovary was accumulated over the study periods. This transformation provides an estimate of the overall postpartum response to treatment for each cow (Risco *et al.*, 1994). Differences between ovarian accumulated follicular diameter ipsilateral and contralateral to the previously gravid uterine horn were compared within cows using *t* tests within treatment groups and using ANOVA between groups. Changes in the diameter of the uterine horn were compared within cows using *t* tests within treatment groups and using ANOVA between groups for all cows, and between animals with or without a follicle > 8 mm in diameter in the ovary ipsilateral to the previously gravid uterine horn on day 2. These cows were selected to test the effect of follicles > 8 mm in diameter on uterine involution because follicles identified on day 2, after eCG administration, were present for the remainder of the study period. Associations between the diameter of the uterine horn and plasma oestradiol concentration or accumulated follicular diameter were examined using Pearson correlation coefficients.

Factors that affected plasma PGFM concentration and uterine horn diameter were identified using a mixed multivariate model for repeated measures (SAS Inc., 1997). Initially, the log transformed data were checked for normality by univariate analysis. An unstructured correlation structure provided the best fit to these data. Variables included were day of study, treatment group, abnormal vaginal mucus, lactation number, the presence of a follicle > 8 mm in diameter in the ovary ipsilateral to the previously gravid uterine horn on day 2, and animal. Variables were removed from the model, after examination for correlation with remaining variables, until those remaining were significant and results were expressed as least-square means ± SEM.

Results

There were 19, 18 and 20 cows in the control, 250 and 750 iu eCG treatment groups, respectively. The mean lactation number of the 57 cows was 3.2 ± 0.4 and did not differ significantly among the treatment groups. There was no difference in the number of animals that had a farmer-assisted parturition among treatment groups (40.4% of all cases). There were 5, 6 and 5 cows with an abnormal vaginal discharge in the control, 250 and 750 iu eCG treatment groups, respectively.

Ovarian activity

Accumulated follicular diameters of follicles > 4 mm in diameter are summarized (Fig. 1). There was a significant effect of day ($P < 0.01$) and the interaction between treatment group and the relationship of the ovary to the previously gravid uterine horn ($P < 0.05$). In control cows, there was a significant difference between accumulated follicular diameter in the ipsilateral and contralateral ovary on days 0, 2, 4 and 6. However, there was no significant difference in accumulated follicular diameter between the ipsilateral and contralateral ovaries for cows administered 250 or 750 iu eCG. Comparison of accumulated follicular diameter was

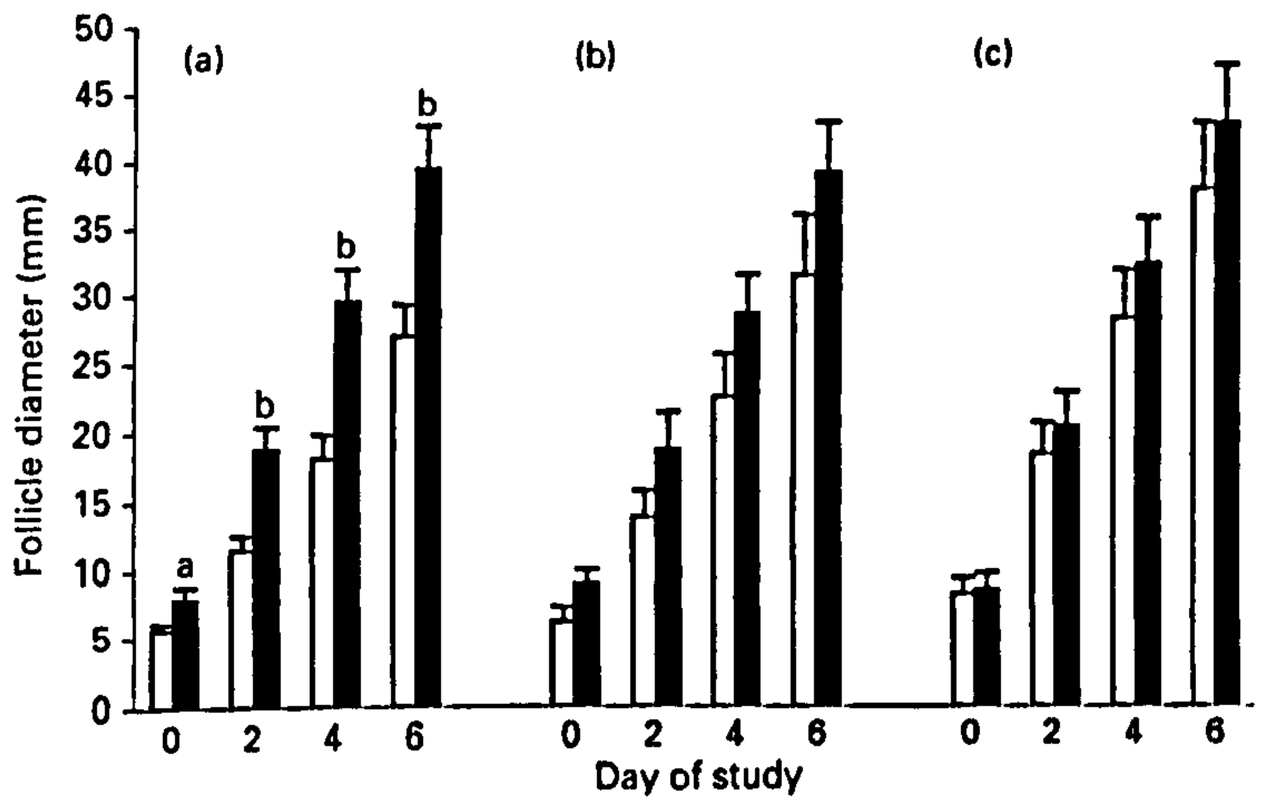

Fig. 1. Mean ± SEM accumulated follicular diameter (mm) of the largest follicle > 4 mm in diameter in the ovaries ipsilateral (□) and contralateral (■) to the previously gravid uterine horn on day 0 to day 6 for control cows (a) and cows administered 250 (b) or 750 (c) iu eCG at 14 days post partum. No significant differences were observed among eCG treated cows. Within the control group, values differ between columns on the same day of study. $^{a}P < 0.05$; $^{b}P < 0.001$.

not significantly different among treatment groups, the relationship of the ovary to the previously gravid uterine horn, or interaction between treatment and day, or ovary and day. Similar trends in follicular growth were observed for accumulated follicular diameters of follicles > 8 mm in diameter. On day 6, the difference between the accumulated follicular diameter in the ipsilateral and contralateral ovary tended to be greater in control cows (23.5 ± 3.5 versus 31.0 ± 4.0 mm) compared with cows treated with 250 (32.0 ± 7.8 versus 35.8 ± 4.7 mm) or 750 iu eCG (33.4 ± 7.4 versus 38.9 ± 5.3 mm); however, the differences were not statistically significant.

The number of follicles > 4 mm or > 8 mm in diameter did not differ significantly among treatment groups on day 0. When data from all cows on day 0 were combined, there were fewer follicles > 4 mm in diameter in the ipsilateral compared with the contralateral ovary to the previously gravid uterine horn (0.89 ± 0.13 versus 1.33 ± 0.15; $P < 0.01$), and there were fewer follicles > 8 mm in diameter in the ipsilateral ovary (0.19 ± 0.05 versus 0.37 ± 0.06; $P < 0.05$).

In control animals, the actual diameter of the largest follicle > 4 mm in diameter in the contralateral ovary was greater in those cows with normal vaginal mucus compared with cows with abnormal mucus, on day 2 (14.3 ± 1.3, $n = 10$ versus 9.4 ± 1.0, $n = 5$; $P < 0.05$) and on day 4 (15.0 ± 1.4, $n = 10$ versus 9.7 ± 1.2, $n = 3$; $P < 0.05$). The presence of abnormal vaginal discharge did not affect the diameter of the largest follicle in the ipsilateral ovary of control animals, or in either ovary in the groups administered eCG.

There was a follicle > 8 mm in diameter in the ipsilateral ovary to the previously gravid uterine horn on day 2 in 5, 7 and 12 cows in the control, 250 and 750 iu eCG treatment groups, respectively, although differences were not statistically significant.

There were fewer corpora lutea formed during the study in the ipsilateral ovary to the previously gravid uterine horn than in the contralateral ovary (6 versus 20, $P < 0.01$); however, there were no significant differences among treatments.

Plasma oestradiol concentration

Plasma oestradiol concentrations for all cows receiving each treatment during the study are summarized (Fig. 2). There were no significant differences among the treatment groups when the concentrations of plasma oestradiol within each day of the study were compared. Although plasma oestradiol concentration increased ($P < 0.05$) between day 4 and day 6 for control animals, and day 0 and day 2 for the 750 iu eCG group, there was no clear pattern of changes in oestradiol concentration.

There were 12, 8 and 11 cows with a plasma oestradiol concentration < 1 pg ml^{-1} on day 0 in the control, 250 and 750 iu eCG treatment groups, respectively. The plasma oestradiol concentrations on each day for these animals are summarized (Fig. 3). Concentrations were higher ($P < 0.05$) on day 6 compared with days 0, 2 and 4 for control cows; higher on days 4 and 6 compared with day 0 for the cows treated with 250 iu eCG; and higher on days 2 and 4 compared with day 0 for cows treated with 750 iu eCG.

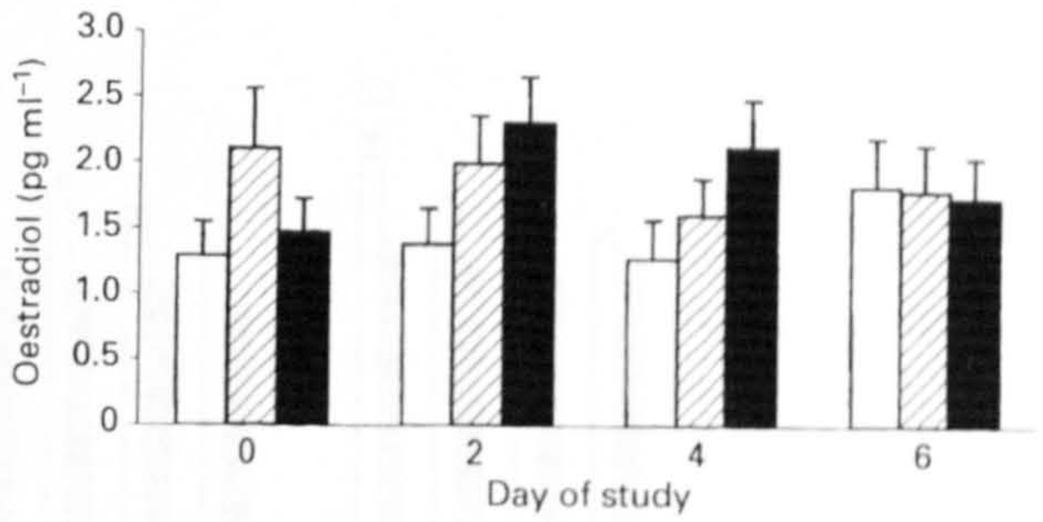

Fig. 2. Mean ± SEM oestradiol concentrations in peripheral plasma on day 0 to day 6 of the study in control cows (□) and cows administered 250 (▨) or 750 (■) iu eCG starting at 14 days post partum.

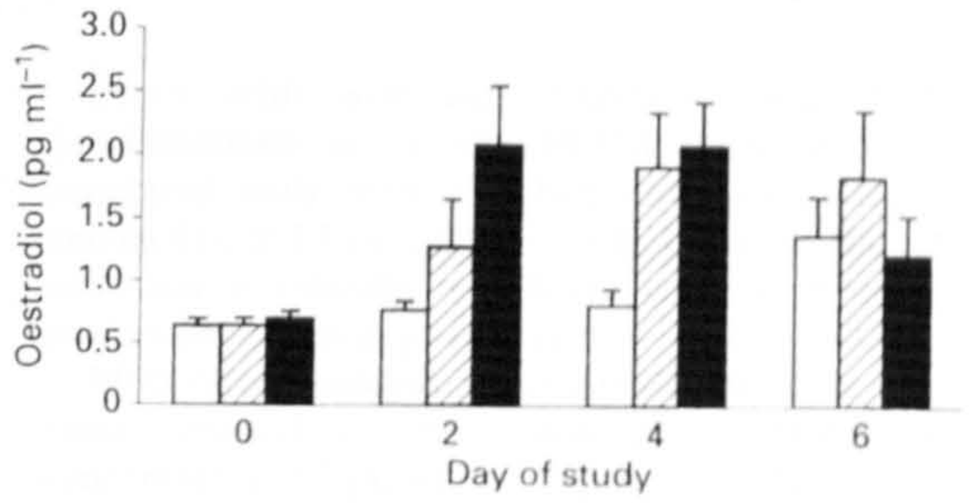

Fig. 3. Mean ± SEM plasma oestradiol concentrations on day 0 to day 6 of the study in control cows (□) and cows administered 250 (▨) or 750 (■) iu eCG starting at 14 days post partum only for cows with plasma oestradiol concentration < 1 pg ml^{-1} on day 0.

Uterine horn diameter

The diameters of the previously gravid uterine horn and contralateral horn are summarized (Fig. 4). There was a significant difference in uterine horn diameter between the previously gravid and contralateral horn ($P < 0.01$) and between the interaction of side of horn and day of study ($P < 0.01$). The diameter of the previously gravid uterine horn was significantly ($P < 0.001$) smaller on day 6 compared with day 0 in control cows and cows treated with 250 or 750 iu eCG. The diameter of the contralateral uterine horn was smaller ($P < 0.05$) on day 6 compared with day 0 for control cows and cows treated with 750 iu eCG, but not for animals treated with 250 iu eCG. The change in diameter of the uterine horn did not differ among treatment groups, or between cows with or without a follicle > 8 mm in diameter in the ipsilateral ovary to the previously gravid uterine horn on day 2 for all cows or within treatment groups. The differences in uterine horn diameter were confirmed by multivariate models, which indicated a highly significant effect of day of the study ($P < 0.0001$), and between the previously gravid and contralateral horn ($P < 0.0001$). However, eCG treatment, presence of a follicle > 8 mm in

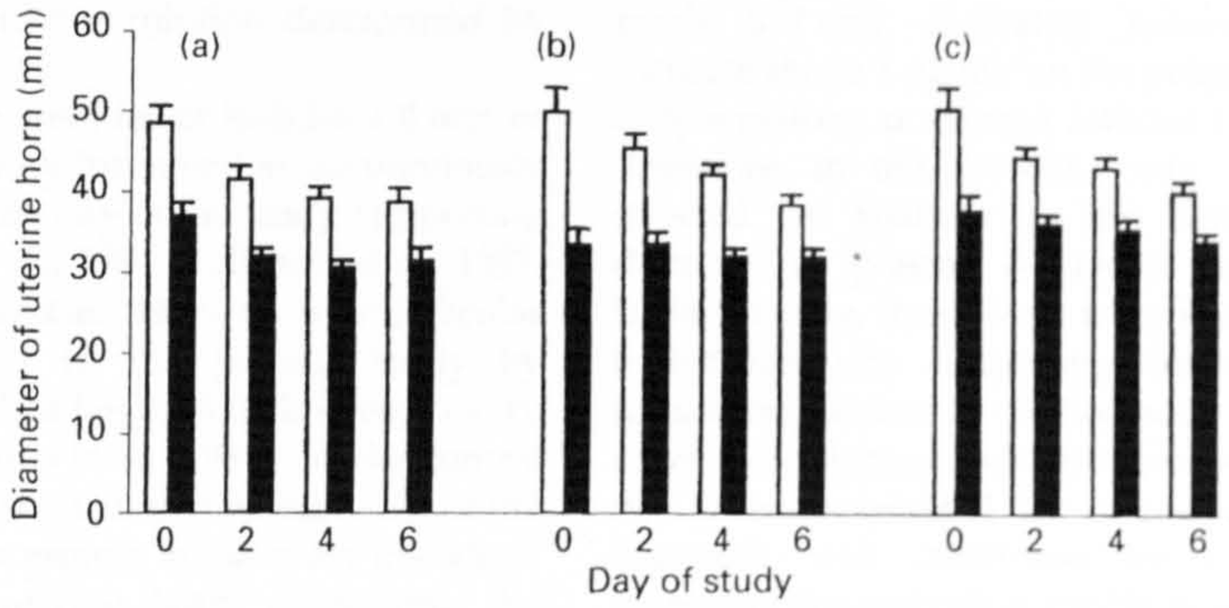

Fig. 4. Mean ± SEM diameter of the previously gravid (□) and contralateral (■) uterine horns on day 0 to day 6 of the study for all control cows (a) and all cows administered 250 (b) or 750 (c) iu eCG starting at 14 days post partum.

diameter in the ipsilateral ovary to the previously gravid uterine horn on day 2, presence of abnormal vaginal mucus, lactation number, cow and interactions of these terms were not significant. In addition, there was no significant relationship between the uterine horn diameter and plasma oestradiol concentration (even after selecting only for cows with plasma oestradiol concentrations < 1 pg ml^{-1}), or accumulated follicular diameter.

For cows selected on the basis of a plasma oestradiol concentration < 1pg ml^{-1} on day 0, the diameter of the previously gravid uterine horn was significantly smaller on day 6 compared with day 0 in control cows (49.6 ± 2.8 versus 38.7 ± 2.8 mm; $P < 0.01$) and in cows treated with 750 iu eCG (49.1 ± 2.9 versus 40.2 ± 2.1 mm; $P < 0.01$), but not in cows treated with 250 iu eCG (47.4 ± 5.6 versus 37.4 ± 2.1 mm). Although the diameter of the contralateral uterine horn diameter was smaller on day 6 compared with day 0, the differences were not statistically significant for control cows (37.1 ± 1.8 versus 31.6 ± 2.5 mm), or cows treated with 250 (33.1 ± 3.2 versus 31.0 ± 1.3 mm) or 750 iu eCG treated cows (37.7 ± 2.6 versus 34.5 ± 1.5 mm). The change in diameter of the uterine horn did not differ among treatment groups, or between cows with or without a follicle > 8 mm in diameter in the ipsilateral ovary to the previously gravid uterine horn on day 2 for all cows or within treatment groups. Multivariate models were not significant for eCG treatment in these animals.

Plasma PGFM concentration

The plasma PGFM concentration on days 0–6, when subjected to multivariate modelling, showed a significant effect of day ($P < 0.01$) and the presence of an abnormal vaginal discharge ($P < 0.01$), but not eCG treatment, presence of a follicle > 8 mm in diameter in the ipsilateral ovary to the previously gravid uterine horn on day 2, lactation number, or cow. There was a significant decrease in plasma PGFM concentration in all cows between day 0 and days 2, 4 and 6 (77.6 ± 1.1 versus 59.7 ± 1.1, 56.0 ± 1.1 and 58.6 ± 1.1 pg ml^{-1}, respectively; $P < 0.001$).

Cows with abnormal vaginal mucus had higher concentrations of plasma PGFM throughout the study compared with cows that had normal mucus (76.6 ± 1.1 versus 51.0 ± 1.1 pg ml^{-1}; $P < 0.01$); however, the differences were not statistically significant when comparisons were made within each day of the study.

Multivariate models of plasma PGFM concentrations for cows selected on the basis of a plasma oestradiol concentration < 1 pg ml^{-1} on day 0 did not differ significantly among treatment groups.

Subsequent reproductive performance

The mean calving-to-first insemination interval was 84.9 ± 3.9, 85.7 ± 4.7 and 94.0 ± 7.1 days, and the mean calving-to-conception interval was 109.6 ± 13.3, 124.8 ± 13.3 and 114.2 ± 9.0 days, for the control, 250 iu and 750 iu eCG treatment groups, respectively; differences were not statistically significant. The number of inseminations per conception for the three groups was 1.4 ± 0.2, 2.1 ± 0.3 and 1.8 ± 0.3, respectively; again, differences were not statistically significant.

Cows with normal vaginal mucus had a shorter calving-to-conception interval compared with animals with abnormal mucus (106.2 ± 6.1 versus 143.6 ± 18.8 days; $P < 0.05$) and fewer inseminations per conception (1.6 ± 0.2 versus 2.2 ± 0.3; $P < 0.05$); however, the calving-to-first insemination interval (88.9 ± 4.0 versus 86.6 ± 4.6 days) did not differ significantly.

Discussion

Administration of 250 or 750 iu eCG at 14 days post partum overcame the inhibition of folliculogenesis in the ovary ipsilateral to the previously gravid uterine horn that was apparent in control animals. Although there was an associated increase in peripheral plasma oestradiol concentrations after administration of eCG, there was no local effect of greater follicular growth in the eCG treated

animals on the rate of uterine involution determined by ultrasonography.

In the present study, there were fewer follicles > 4 mm or > 8 mm in diameter in the ovary ipsilateral to the previously gravid uterine horn on the first day of the study, supporting other observations (Lewis *et al.*, 1984; Guilbault *et al.*, 1987; Kamimura *et al.*, 1993; Nation *et al.*, 1999). Ovarian follicular activity was also estimated in the present study by accumulating the diameter of the largest follicle in each ovary during the study period (Risco *et al.*, 1994). In the control animals, this transformation showed that on each day of the study there was less follicular growth in the ovary ipsilateral to the previously gravid uterine horn, confirming the negative influence exerted by the previously gravid uterine horn on folliculogenesis in the ipsilateral ovary (Lewis *et al.*, 1984; Dufour and Roy, 1985). The mechanism is unclear but may involve a response to uterine tract inflammation, possibly mediated by cytokines or immune cells; parallel immunological influences of uterine origin on ovarian corpus luteum function were proposed by Spencer *et al.* (1999). Uterine tract infection, as reflected by an abnormal vaginal discharge, affected the rate of growth of the largest follicle in the contralateral ovary, which supports the observation that the presence of uterine infection is associated with fewer animals with a corpus luteum in the contralateral ovary (I. M. Sheldon and H. Dobson, unpublished).

In the present study, administration of 250 or 750 iu eCG overcame the negative influence of the previously gravid uterine horn and, specifically, increased follicular growth in the ipsilateral ovary determined using accumulated follicular diameter of follicles > 4 mm in diameter; a similar trend was found using accumulated follicular diameter of follicles > 8 mm in diameter. These observations indicate that uterine-derived inhibitors of follicular growth may inhibit the action of FSH or LH on follicular activity. Administration of eCG to cattle leads to fewer atretic follicles, recruits more smaller follicles in which growth rate is increased, sustains the growth of larger follicles, and increases the final number of ovulations (Newcomb *et al.*, 1979; Monniaux *et al.*, 1984; Driancourt *et al.*, 1991; Gonzalez *et al.*, 1994). It is possible that a uterine-derived inhibitor of folliculogenesis, acting principally on the ovary ipsilateral to the previously gravid uterine horn, blocks the action of FSH or LH within that ovary, and this block can be overcome by administration of eCG. The inhibition of folliculogenesis in the ipsilateral ovary reduces the number of ovulations, reflected by fewer corpora lutea and fewer follicles > 8 mm and > 4 mm in diameter (I. M. Sheldon and H. Dobson, unpublished). Thus the principal stage at which inhibition occurs is likely to be < 4 mm diameter. A variety of cytokines reduce FSH-induced oestradiol production from bovine granulosa cells collected from 1–5 mm diameter follicles, but have little effect on cells from follicles ⩾ 8 mm in diameter (Spicer and Alpizar, 1994).

The presence of a follicle > 8–9 mm in diameter at the time of treatment reduces the superovulatory response to eCG (Guilbault *et al.*, 1991; Huhtinen *et al.*, 1992). In the post-partum period, there is a succession of follicles growing to 5–9 mm diameter and then regressing until development of the first dominant follicle (Murphy *et al.*, 1990). Plasma oestradiol concentrations are < 1 pg ml^{-1} before follicles reach 8–9 mm diameter; subsequently, concentrations increase above 1 pg ml^{-1} in the presence of ovulatory, but not non-ovulatory, dominant follicles (Beam and Butler, 1997). Therefore, in the present study, cows could have been selected for analysis on the basis of ovarian follicular diameter or plasma oestradiol concentration on day 0. Unfortunately, there were insufficient animals to select for both parameters. As the hypothesis was that the effect of a follicle on uterine involution was mediated by oestradiol, cows with plasma oestradiol concentrations < 1 pg ml^{-1} on day 0 were selected for analysis. The validity of this approach was confirmed by contrasting the plasma oestradiol concentration profile of all cows with that of the selected animals. Before selection there was no clear pattern of plasma oestradiol concentrations throughout the study. In contrast, after selection of cases, plasma oestradiol concentration increased by day 6 for the control animals, by day 4 for the 250 iu eCG group and by day 2 for the 750 iu eCG group.

Uterine involution during the period between 14 and 28 days post partum is characterized by a decrease in uterine horn diameter (Gier and Marion, 1968). A similar decrease in diameter occurred in the uterine horns of each group in the present study. However, there was no difference in the rate of involution of the previously gravid uterine horn between the treatment groups when all animals, or only those with a plasma oestradiol concentration < 1 pg ml^{-1}, were considered. Furthermore, the presence of a follicle > 8 mm in diameter in the ovary ipsilateral to the previously gravid uterine horn on day 2 did not increase the rate of uterine involution in the previously gravid uterine horn compared with cows with smaller follicles, either within treatment groups or using combined data.

An alternative method of assessing uterine involution is the estimation of plasma PGFM concentration, which is correlated with changes in the dimensions of the genital tract after calving (Lindell *et al.*, 1982; Lewis *et al.*, 1984). Plasma PGFM concentration is generally high during, and immediately after, parturition and decreases to basal concentrations by day 20 post partum (Lewis *et al.*, 1984). In the present study, plasma PGFM concentration was greater on day 0 than on subsequent days of the study. However, there was no difference in PGFM concentrations between the treatment groups when either all the animals or only those with a plasma oestradiol concentration < 1 pg ml^{-1} were considered. This observation, in addition to the absence of an effect of treatment on uterine horn diameter, indicates that eCG treatment did not affect uterine involution. Furthermore, there was no evidence that increased oestradiol secretion or large follicles in the ovary ipsilateral to the previously gravid uterine horn had a local effect to increase the rate of uterine involution during the study period. However, as the major changes in uterine horn diameter and plasma PGFM concentration occur between calving and day 14 post partum, future studies could consider earlier intervention.

Although administration of eCG failed to influence uterine involution or subsequent fertility in the present study, an explanation is required for the beneficial effect on subsequent fertility of large follicles in the ipsilateral ovary (Bonnet *et al.*, 1993; I. M. Sheldon and H. Dobson,

unpublished). It is possible that the effect of such a follicle on the uterus may not be detectable by the techniques used in the present study or a longer period of study may be required. An alternative hypothesis is that the appearance of a large follicle may reflect the early demise of the negative influence of the previously gravid uterine horn on the ipsilateral ovary. This, in turn, may reflect earlier recovery of the postpartum uterus, other than changes in dimension, which may have a beneficial effect on subsequent fertility.

During the period of study, plasma PGFM concentration was higher in cows with abnormal, compared with normal, vaginal mucus. This finding is in agreement with other reports (Lindell *et al.*, 1982; Del Vecchio *et al.*, 1994). However, when plasma PGFM concentrations were compared on each day of the study, differences between cows with abnormal and normal vaginal mucus were not significant, which supports the finding that plasma PGFM concentration is not a reliable diagnostic indicator of endometritis (Archbald *et al.*, 1998). The presence of mucopurulent vaginal mucus was similarly associated with impaired reproductive performance in the present study and in that reported by Archbald *et al.* (1998).

In conclusion, parenteral administration of 250 or 750 iu eCG increased follicular growth in the ovary ipsilateral to the previously gravid uterine horn, overcoming the inhibition apparent in control cows. However, the increased ipsilateral ovarian follicular growth and increased plasma oestradiol concentrations did not affect uterine involution as assessed by ultrasonography or by plasma PGFM concentration. Therefore, a local effect of a large follicle in the ipsilateral ovary on the previously gravid uterine horn was not demonstrated and the alternative pathway, the uterus influencing the ovary, should be considered.

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ELSEVIER

THE INFLUENCE OF OVARIAN ACTIVITY AND UTERINE INVOLUTION DETERMINED BY ULTRASONOGRAPHY ON SUBSEQUENT REPRODUCTIVE PERFORMANCE OF DAIRY COWS

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ABSTRACT

The objective of this study was to test the hypothesis that a follicle >8 mm diameter in the ovary ipsilateral to the previously gravid uterine horn (PGUH), between 14 and 28 days postpartum, improves subsequent reproductive performance. Lactating Holstein-Friesian cows (n=284) in 3 commercial herds were examined using transrectal ultrasonography once between 14 and 28 days postpartum to determine associations between uterine and ovarian measurements and subsequent fertility. There were fewer cows with a corpus luteum in the ovary ipsilateral to the PGUH compared with the contralateral ovary (16.9% vs. 37.0%; P<0.001). In addition, in the ovary ipsilateral to the PGUH there were fewer follicles >5 mm diameter (mean ± SEM; 0.69 ± 0.06 vs. 1.02 ± 0.06; P<0.001) and fewer animals with a follicle >8 mm diameter (26.1% vs. 49.6%; P<0.001). These differences between the ovaries ipsilateral or contralateral to the PGUH declined with increasing time between 14 and 28 days postpartum. The presence of a purulent vaginal discharge decreased the number of animals with a corpus luteum in the ovary contralateral to the PGUH (45/143 vs. 60/141; P<0.05), but not in the ovary ipsilateral to the PGUH. The presence of a follicle >8 mm diameter in the ovary ipsilateral to the PGUH was associated with a shorter calving to conception interval compared with animals without such a follicle (99.0 ± 5.6 days, n=74, vs. 112.8 ± 4.4 days, n=210; P<0.05). These observations raise an intriguing issue: how does this follicle affect subsequent fertility — does the follicle exert a local influence on the uterus, or vice versa?

Key words: postpartum, follicle, fertility, ipsilateral ovary, uterus

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INTRODUCTION

The relationship between subsequent reproductive performance and postpartum changes in the uterus or ovaries of cattle has been investigated by a number of authors (21, 32). However, little attention has been paid to the local relationship between the previously gravid uterine horn (PGUH) and follicular growth in the ipsilateral ovary.

Results from a small study suggested that the occurrence of a larger follicle, determined by palpation per rectum, in the ovary ipsilateral to the PGUH on Day 26 ± 3 postpartum, increased the likelihood of shorter calving to conception intervals (3). However, the accuracy of manual palpation in accurately identifying and measuring such structures, as well as determining the degree of uterine involution, is open to criticism due to the high level of subjectivity. The monitoring of follicular growth and uterine involution in cows can be improved by the use of transrectal ultrasonography (24, 28).

Greatest follicular activity after calving occurs initially in the ovary contralateral to the PGUH (13, 16, 25). Kamimura et al. (15) reported that only 18% of first dominant follicles were identified in the ovary ipsilateral to the PGUH. The negative influence of the previously gravid horn on the return of ovarian cyclicity in the ovary ipsilateral to the PGUH declines with increasing time postpartum (10, 19, 27).

There are conflicting reports on the relationship between the return of ovarian cyclicity (as determined by the interval from calving to first rise in milk progesterone concentration) and calving to conception interval. Darwash et al. (7) claim that early return of cyclicity is associated with shorter calving to conception intervals, whereas Smith and Wallace (30) claim the reverse. However, such studies are unable to determine in which ovary the return to cyclicity occurs in relation to the PGUH.

The objective of the present study was to test the hypothesis that a follicle >8 mm diameter in the ovary ipsilateral to the PGUH, between 14 and 28 days postpartum, improves subsequent reproductive performance.

MATERIALS AND METHODS

Animals

Three commercial Holstein-Friesian dairy farms (identified as A, C and E) were chosen for the study on the basis of accurate farm records and existing regular visits. During spring and summer, the cows were kept at pasture, whereas during the autumn and winter they were housed in freestall barns with grass silage available ad libitum. Concentrate feed was supplied at 0.3 kg/L milk yield. The farms had a target calving index of 365 days with a policy of inseminating cows from 45 days postpartum. All animals were bred by artificial insemination at observed estrus by a single technician.

The same veterinarian (I.M. Sheldon) visited each farm every 2 weeks for one year to monitor fertility and other aspects of reproductive health. Cows that had calved 14 to 28 days earlier and without a history of peripartal problems were included in the study and examined once. Calving date, date of examination and lactation number were recorded.

Clinical Examination

The vagina of each cow was examined by direct palpation, and the lumenal contents were inspected. Purulent vaginal discharge, which is regarded as a reflection of uterine infection, was defined as the presence of mucopurulent material in the vagina (20, 32). Normal mucus was defined as uniform and translucent in the absence of a fetid odor. Cows with gross vaginal lacerations, inappetence or pyrexia were excluded from the study.

The previously gravid uterine horn was determined transrectally as that which was longer and of greater diameter than the contralateral horn. The genital tract was scanned per rectum using a SonoAce 600 ultrasound scanner with a 5-MHz linear array transrectal probe (BCF Technology Ltd., Livingston, U.K.). Follicles were defined as nonechogenic (black) spherical structures with a clear demarcation between the follicular wall and antrum. Corpora lutea (CL) were defined as grainy echogenic structures that had a well-defined border with the less echogenic ovarian stroma. In some corpora lutea there was a normal nonechogenic lacuna. The internal diameter of the largest follicular and luteal ovarian structures and the external diameter of the uterine horns at mid-point were measured using the internal calipers of the machine. When the image of the structure being scanned was not spherical, the diameter was estimated by averaging two 90° measurements. In addition, follicles >5 mm diameter in each ovary were counted.

Ovarian activity was defined as the presence of a follicle and/or corpus luteum >8 mm diameter. Similar definitions have been used previously (13, 25). The >8 mm measurement was selected because follicles of up to 8 mm diameter have previously been identified within 7 to 10 days of calving immediately before emergence of a dominant follicle (23, 28). In addition, ovarian structures >8 mm diameter are readily and accurately determined by ultrasonography.

Evaluation of Fertility Data

Fertility data were collected for 18 months. Cows that were culled because of failure to conceive were ascribed a calving to conception interval of 308 days, equivalent to a 305-day lactation period plus 3 days colostrum production (29). Cows culled for other reasons before conception were removed from the data. Conception date was verified by the subsequent calving date.

Statistical Analysis

The data were analyzed for the combined herds and for 3 periods of calving to examination (14 to 18, 19 to 23 and 24 to 28 days). Results are expressed as mean ± SEM. Analysis was

performed using SPSS (Version 8.0, SPSS Inc, Chicago, IL). Values of $P<0.05$ were regarded as significant.

The mean period from calving to examination was compared amongst herds using a t-test, and the mean lactation number was compared using the nonparametric Kologorov-Smirnov Z test. The calving to conception data (log_{10} transformed for analysis) were compared by analysis of covariance. The variables tested were uterine diameter, presence of a purulent vaginal discharge, presence of a follicle >8 mm diameter, a CL, ovarian activity and their interactions. Ovarian structure and uterine diameter comparisons were also tested separately for location: ipsilateral or contralateral to the PGUH.

The distribution of the number of animals with a CL or with a follicle >8 mm diameter in the ovary ipsilateral or contralateral to the PGUH were compared using Chi-squared tests. Comparison of the mean number of follicles >5 mm diameter between each ovary, the three calving to examination periods and their interactions were tested using a general linear model. Correlation between the number of follicles in each ovary and the interval from calving to examination were tested by Pearson coefficient.

RESULTS

There were 284 cows with complete data for analysis, including 115, 120 and 49 cows in Herds A, C and E, respectively. The mean lactation number was higher for Herd A than for Herds C and E (4.2 ± 0.2 vs. 2.9 ± 0.2 and 3.0 ± 0.3, respectively; $P<0.05$). Three cows in each herd failed to conceive. The mean interval from calving to examination for the combined data was 21.8 ± 0.3 days and was similar for each herd. The mean calving to conception interval for the combined data was 109.2 ± 3.7 days and did not differ significantly between herds. Consequently, further analyses were carried out on combined data from the 3 herds.

Ovarian Activity

Fewer cows had a CL in the ovary ipsilateral to the PGUH compared with the contralateral ovary (Table 1; $P<0.001$). In addition, there were fewer follicles >5 mm diameter in the ovary ipsilateral to the PGUH than in the contralateral ovary (Table 1; $P<0.001$). The difference in the number of follicles >5 mm diameter between the ovaries ipsilateral or contralateral to the PGUH declined with increasing time from calving to examination. The difference was greatest at 14 to 18 days after calving compared with 19 to 23 days and was no longer significant at 24 to 28 days. The mean number of follicles >5 mm diameter did not differ significantly between the calving to examination periods, or for the interaction of period with ovarian location.

Fewer animals had a follicle >8 mm diameter in the ovary ipsilateral to the PGUH compared with the contralateral ovary (Table 1; $P<0.001$). The proportion of cows with a follicle >8 mm diameter differed between the ovaries ipsilateral and contralateral to the PGUH at 14 to 18, 19 to 23 and 24 to 28 days from calving to examination. However, the proportion of cows with a follicle >8

mm diameter did not differ significantly between the calving to examination periods, or for the interaction of period with ovarian location. There were significant correlations between the interval from calving to examination and the number of follicles >5 mm diameter ($r=0.12$, $P<0.05$), the presence of a follicle >5 mm ($r=0.16$, $P<0.01$) and the presence of a follicle >8 mm ($r=0.14$, $P<0.05$) in the ovary ipsilateral to the PGUH. There were no significant correlations concerning the ovary contralateral to the PGUH.

Table 1. Ovarian activity as measured by mean ± SEM for the number of follicles >5 mm, percent >8 mm diameter and percent with a CL in the ovary ipsilateral or contralateral to the previously gravid uterine horn at 3 time periods after calving.

Calving to examination period (days)	Number of cows	Ipsilateral ovary			Contralateral ovary		
		No. >5 mm	Percent >8 mm	Percent with CL	No. >5 mm	Percent >8 mm	Percent with CL
14 to 18	70	0.49 ± 0.09^{a}	20.6^{a}	22.1	0.93 ± 0.11^{b}	47.1^{b}	26.5
19 to 23	105	0.72 ± 0.11^{c}	22.8^{a}	19.0^{c}	1.10 ± 0.12^{d}	54.3^{b}	34.3^{d}
24 to 28	109	0.83 ± 0.11	33.0^{e}	11.9^{a}	1.07 ± 0.10	46.8^{f}	45.9^{b}
All	284	0.69 ± 0.06^{a}	26.1^{a}	16.9^{a}	1.02 ± 0.06^{b}	49.6^{b}	37.0^{b}

Values with different superscripts are significantly different between the ipsilateral and contralateral ovary within a calving to examination period ($^{ab}P<0.001$, $^{cd}P<0.01$, $^{ef}P<0.05$).

Uterine Discharge in Relation to Ovarian Activity

In the combined herds, 143/284 (50.4%) of animals had purulent vaginal mucus, and the proportion was similar in each herd. In addition, the proportion of cows with purulent vaginal mucus was similar at the different calving to examination periods.

There were fewer animals with a CL ($P<0.01$) or a follicle >8 mm diameter ($P<0.01$) in the ovary ipsilateral to the PGUH compared with the contralateral ovary within the normal or purulent mucus groups (Table 2). More cows with normal vaginal mucus compared with purulent mucus animals had a CL in the ovary contralateral to the PGUH, but this was not so for the ovary

ipsilateral to the PGUH. The number of normal compared with purulent vaginal discharge cows with a follicle >8 mm diameter in the ipsilateral or contralateral ovary to the PGUH was similar. Furthermore, there was no significant difference between normal and purulent mucus cows in the mean number of follicles >5 mm diameter in the ovary ipsilateral to the PGUH (0.70 ± 0.09 vs. 0.71 ± 0.08) or the contralateral ovary (1.12 ± 0.10 vs. 0.98 ± 0.08, respectively).

Table 2. Number (%) of cows with normal and purulent vaginal mucus with a CL or a follicle >8 mm diameter in the ovary ipsilateral or contralateral to the previously gravid uterine horn.

Structure and location	Normal n=141	Purulent n=143
CL in the ipsilateral ovary	20 (14) [a]	28 (20) [a]
CL in the contralateral ovary	60 (43) [b c]	45 (31) [b d]
Follicle >8 mm diameter in the ipsilateral ovary	35 (25) [a]	39 (27) [a]
Follicle >8 mm diameter in the contralateral ovary	73 (52) [b]	68 (48) [b]

Values with different superscripts are significantly different between the ipislateral and contralateral ovary ([ab] P<0.01) and between cows with normal and purulent vaginal mucus ([cd] P<0.05).

Subsequent Reproductive Performance

The calving to conception interval was significantly (P<0.01) affected by PGUH diameter, the presence of a follicle >8 mm diameter in the ipsilateral ovary, ovarian activity, purulent vaginal mucus and the interaction between ovarian activity and purulent vaginal mucus.

The calving to conception interval was shorter in those animals with a smaller diameter previously gravid uterine horn (P<0.01). However, the diameter of the contralateral uterine horn was not significant.

Cows with ovarian activity on either ovary had a shorter mean calving to conception interval compared with cows with no ovarian activity (Table 3; P<0.05). When each calving to examination interval was considered separately, only those examined 14 to 18 days after calving had a shorter calving to conception interval (P<0.01).

The calving to conception interval was shorter for cows with a follicle >8 mm diameter in the ovary ipsilateral to the PGUH compared with those without a follicle >8 mm diameter (99.0 ± 5.6 vs. 112.8 ± 4.4 days; P<0.05).

Cows with normal vaginal mucus had a shorter mean calving to conception interval than those with purulent mucus (101.4 ± 4.5 vs. 116.8 ± 5.8 days; P<0.05). To investigate the influence of ovarian activity, cows with normal vaginal mucus were considered separately. Cows with normal mucus and ovarian activity had shorter mean calving to conception intervals compared with those without ovarian activity (89.1 ± 1.0 days, n=127; vs. 113.1 ± 1.1 days, n=14; P<0.05). In animals with a purulent vaginal discharge, there was no effect of ovarian activity on the calving to conception interval.

Table 3. Mean ± SEM calving to conception interval (days) of animals with or without ovarian activity in either ovary at 3 periods after calving.

Calving to examination interval (days)	Ovarian activity	n	No ovarian activity	n
14 to 18	90.7 ± 6.2 [a]	60	139.0 ± 19.1 [b]	10
19 to 23	111.6 ± 6.7	91	113.9 ± 13.4	14
24 to 28	110.2 ± 6.7	95	139.4 ± 23.6	14
All	105.9 ± 3.9 [c]	246	130.1 ± 11.1 [d]	38

Values with different superscripts are significantly different between cows with and without ovarian activity. ([ab] P<0.01, [cd] P<0.05)

DISCUSSION

This study shows that the presence of a follicle >8 mm diameter between 14 and 28 days after calving in the ovary ipsilateral to the PGUH is associated with shorter calving to conception intervals. In addition, there is reduced folliculogenesis in the ovary ipsilateral to the PGUH of cattle during this postpartum period. This association between the 2 organs is further modified by the presence of purulent vaginal mucus (indicative of uterine bacterial infection), which suggests that the dialogue may be partially mediated by inflammation.

The observation that a follicle >8 mm diameter in the ovary ipsilateral to the PGUH was associated with a shorter calving to conception interval confirms the results of the small study of 66 animals by Bonnet et al. (3). They reported that cows with good subsequent reproductive performance had a larger diameter follicle (6.1 ± 1.3 mm) on the ovary ipsilateral to the PGUH, compared with a smaller follicle (2.7 ± 0.9 mm) in cows with poor performance. The shorter calving to conception interval associated with the presence of a larger follicle in the ovary ipsilateral to the PGUH may be due to the influence of the follicle on the uterine endometrium and/or

myometrium. One hypothesis is that estradiol synthesized by a follicle >8 mm diameter has a beneficial local effect on uterine function. Plasma estradiol concentrations are greater within the utero-ovarian vein draining the ovary containing the ovulatory follicle (14); bearing in mind that only postpartum follicles >8 mm diameter that eventually ovulate (not nonovulatory follicles) are associated with increased peripheral plasma estradiol concentrations (2). Unfortunately, in the present study neither milk nor blood samples were taken to measure this hormone.

The current study shows that cows with ovarian activity, defined by the ultrasonographic presence of a CL and/or follicle >8 mm diameter, had a shorter calving to conception interval compared with those without. This observation supports Shanks et al. (18), who reported shorter calving to conception intervals for cows that had an initial ovulation before, rather than after, 42 days postpartum. Similarly, Darwash et al. (7) reported a significant reduction in the calving to conception interval with shorter intervals to the first postpartum increase in milk progesterone concentration, although, Smith and Wallace (30) observed the reverse.

Fewer animals in the present study had a CL in the ovary ipsilateral to the PGUH, and there were fewer follicles >5 mm diameter and fewer cows with a follicle >8 mm diameter in the ovary ipsilateral to the PGUH. .The mechanism could be exerted by a systemic pathway; however our observations support the suggestion that the PGUH has a local negative effect on ipsilateral postpartum ovarian function (9, 16). A local effect of the uterus on ovarian function is an established concept, for example, in relation to the transfer of luteolysins (12), and it has been implicated in the action of interferon (31). Furthermore, in the present study there was a positive correlation between postpartum interval and number or presence of follicles in the ovary ipsilateral to the PGUH. These observations would support a hypothesis of a declining influence of an as yet unknown uterine-derived inhibitor of ovarian function as uterine involution progresses. Possible uterine factors could include inflammatory mediators such as $PGF2_{\alpha}$, which is produced by the postpartum uterus in high concentrations during involution and in response to uterine infection (8, 17). However, Guilbault et al. (13) reported that a partial inhibitor of prostaglandin synthesis administered during the postpartum period reduced ovarian activity. Furthermore, replacement with $PGF2_{\alpha}$ enhanced ovarian activity in the ovary ipsilateral to the PGUH. However, there are a large number of other inflammatory mediators, particularly cytokines, that participate in folliculogenesis and ovulation (34). An alternative mechanism might be via immune or inflammatory cell cytokine release and/or cell-cell communication in the ovary. Neutrophils readily migrate into the uterine lumen in response to a chemotactic stimulus at all stages of the bovine reproductive cycle. Furthermore, intrauterine and peripheral neutrophil function is modified by reproductive hormonal status, retained fetal membranes and uterine infection (6, 33). Perhaps immune cells could migrate from the uterine horn, or a uterine-derived chemotactic substance could attract immune cells to the ipsilateral ovary, and so influence folliculogenesis. Immune cells have been detected in ovarian follicles and luteal tissue in association with a modulation of follicular and luteal activity (5, 11, 22).

Fewer animals with a uterine infection, as reflected by the presence of purulent vaginal mucus, had a CL in the ovary contralateral to the PGUH. One explanation for this observation might be that increased plasma-estradiol concentrations associated with ovulatory dominant follicles may have

enhanced the elimination of uterine infection in those cows that, at the time of examination, had normal vaginal mucus and a CL (1, 26). However, in the present study, the number of corpora lutea in the contralateral ovary did not differ between normal and infected animals. Therefore, an alternative hypothesis for the difference in the ovary contralateral to the PGUH must be sought. In cows with uterine infection, the ovarian inhibitory factor, possibly induced by inflammation, may have extended to the contralateral uterine horn in addition to its presence in the PGUH.

In the present study, increased PGUH diameter or purulent vaginal mucus increased the calving to conception interval. Similar increases in calving to conception interval have been reported previously in association with purulent vulval or vaginal discharge (4, 32), or a larger PGUH diameter (3, 21). However, the cows were not always differentiated into those with, or without, a purulent vaginal discharge (29).

In summary, the present data confirm that follicular activity is suppressed in the ovary ipsilateral to the PGUH between 14 and 28 days postpartum. However, this ovarian suppression declines with increasing interval after calving. In cows with a purulent vaginal discharge, there were fewer corpora lutea in the ovary contalateral to the PGUH, although follicle population differences were not apparent. Ovarian activity 14 to 18 days postpartum, but not later, was associated with a shorter calving to conception interval. In addition, location of ovarian structure in relation to the PGUH was also important. The presence of a follicle >8 mm diameter in the ovary ipsilateral to the PGUH 14 to 28 days postpartum was associated with shorter calving to conception intervals. These observations raise an intriguing issue: how does this follicle affect subsequent fertility — does the follicle influence the uterus, or vice versa?

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Acute phase protein responses to uterine bacterial contamination in cattle after calving

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Repeated ultrasonographic examinations and collections of blood samples and uterine lumenal swabs between seven and 28 days after calving were used to examine the relative effects of bacterial contamination and involution of the uterus on the concentrations of acute phase proteins in the blood of 26 dairy cows. The severity of bacterial contamination, as determined by the total bacterial growth score, was a significant variable for the concentrations of the acute phase proteins α_1-acid glycoprotein ($P<0.0001$), haptoglobin ($P<0.05$) and ceruloplasmin ($P<0.0001$). In addition, the concentrations of α_1-acid glycoprotein and ceruloplasmin were increased in the cows from which *Escherichia coli* ($P<0.0001$) and *Arcanobacterium pyogenes* ($P<0.05$), respectively, were isolated from the uterine lumen. Uterine involution, as determined by the decreasing diameter of the previously gravid uterine horn, was associated with a decrease in the concentrations of α_1-acid glycoprotein ($P<0.005$), haptoglobin ($P<0.05$) and ceruloplasmin ($P<0.01$). However, the response of the acute phase proteins to bacterial contamination was independent of the day on which the samples were collected, indicating that their concentrations were increased by bacterial contamination in addition to the changes associated with uterine involution.

THE uterus of most cows is contaminated with a wide range of bacteria at the time of calving or immediately afterwards (Elliot and others 1968), but by seven weeks after calving it is usually sterile and provides an environment suitable for supporting a normal pregnancy. However, in about 10 per cent of dairy cattle the bacteria persist and cause endometritis, which is associated with longer intervals from calving to conception and lower fertility (Borsberry and Dobson 1989).

At parturition and during the period immediately afterwards, when uterine involution is occurring and the bacterial contaminants are being eliminated, there are increased concentrations of acute phase proteins in the peripheral circulation (Alsemgeest and others 1993, Regassa and Noakes 1999). Because these hepatocyte-derived substances limit tissue damage and promote tissue repair (Baumann and Gauldie 1994, Steel and Whitehead 1994), it has been suggested that their synthesis and release are a consequence of normal uterine involution, endometrial degeneration and tissue remodelling (Regassa and Noakes 1999). However, the bacterial contaminants may also increase the concentrations of the acute phase proteins because bacterial infections are considered to stimulate their production (Whicher and Dieppe 1985, Miller and others 1997, Gayle and others 1999).

The main objective of this study was to test the hypothesis that the concentration of acute phase proteins may be increased by the contamination of the uterus with bacteria, as well as by the changes associated with uterine involution. The effects of specific pathogenic bacteria on the acute phase protein response were also examined.

MATERIALS AND METHODS

Animals

Cows from a dairy herd of 90 Holstein-Friesian cows with an annual average milk yield of approximately 7200 litres, were used over a period of four months. They were housed in straw yards and fed a diet of grass and maize silage and concentrate. Any cows that had dystocia, or diseases such as retained fetal membranes, lameness or mastitis which might have provoked an acute phase protein response were excluded from the study on the basis of a clinical examination made before the collection of each blood sample (Skinner and others 1991, Scott and others 1992, Horadagoda and others 1999). The herd's rolling mean somatic cell count was 135×10^3 cells/ml and the mean (sd) cell count of the 26 cows included in the study was $53{\cdot}7$ ($7{\cdot}5$) $\times 10^3$ cells/ml. When the cows were first examined, seven days after calving, details of their lactation number and calving date were obtained from the farm records.

Blood sampling

Blood samples were collected on day 7 after calving and then three times a week until day 28, from a coccygeal vein or artery into evacuated heparinised and plain tubes (Vacutainer; Becton Dickinson), and transported on ice to the laboratory. Plasma and serum were separated by centrifugation at 1000 *g* for 10 minutes, and stored frozen at –20°C until assayed.

Acute phase proteins

The concentrations of the acute phase proteins α_1-acid glycoprotein and haptoglobin in plasma, and of ceruloplasmin in serum, were measured by the methods of Lewis and others (1989), adapted for 96-well microtitre plates (Life Technologies) by Regassa and Noakes (1999). The intra- and interassay coefficients of variation for low, medium and high values within the effective ranges used in the study were all less than 12 per cent and less than 18 per cent, respectively.

Ultrasonography

The genital tract of each cow was scanned transrectally, on the days when blood samples were collected, with an Aloka SSD 210 DX II ultrasound machine (BCF Technology) with a 7·5 MHz linear array transducer. The previously gravid uterine horn was identified as being the larger, and its diameter was measured at the level of insertion of the intercornual ligament by using the internal calipers on the screen of the scanner. When the image of the structure being scanned was not circular, its diameter was estimated by averaging two dimensions measured at right angles.

Uterine swabs and bacteriology

A transcervical guarded swab was collected from the body of the uterus on days 7, 14, 21 and 28 after calving by the method of Noakes and others (1989). The swab was transferred to a bijou bottle containing Stuart Transport Medium (Oxoid) and taken to the laboratory within one hour of collection. The swabs were cultured aerobically and anaerobically on pre-equilibrated blood agar plates, and aerobically on MacConkey agar (Oxoid). Bacteria were identified on the basis of the characteristics of the colony, Gram-stain morphology, haemolysis, and appropriate tests including motility, catalase, oxidase,

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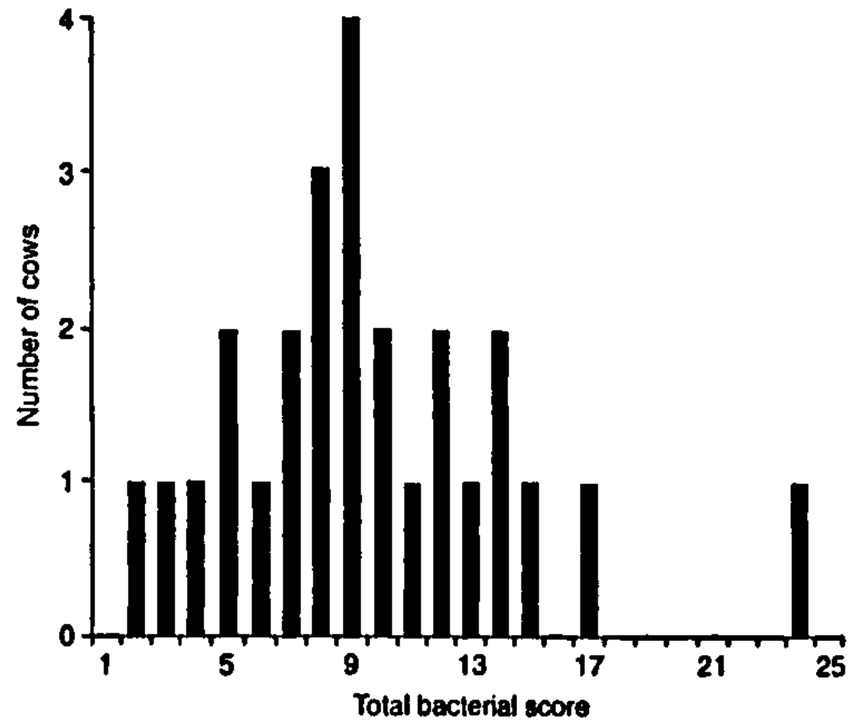

FIG 1: Numbers of cows with different total bacterial growth scores (the sum of the scores of the four weekly samples) after calving

nitrate, oxidation-fermentation, methyl red, Voges Proskauer, β-galactosidase, lysine decarboxylase, arginine dihydrolase, indole, urease and citrate (Barrow and Feltham 1993). The growth of the principal pathogens on the culture plates was scored on a scale from 0 to 4 on the basis of the number of bacterial colonies detected on the plate: 0, no growth; 1, less than 10 colonies; 2, 10 to 100 colonies; 3, 100 to 500 colonies; 4, more than 500 colonies (Noakes and others 1991). The bacterial growth score for each uterine swab was the sum of the scores for the principal pathogens, and the total score for a cow was the sum of the scores for its four swabs.

Statistical analysis

The concentrations of the acute phase proteins were $\log_{10}$ transformed and analysed by using the mixed procedure for repeated samples (SAS 1997); they were best described by an autoregressive model. The explanatory variables included were the bacterial growth score for each swab, the total bacterial growth score, the day of collection of the blood sample, and interactions between these variables. In addition, when the bacterial growth score was a significant variable, the effect of the scores for *Arcanobacterium pyogenes* and *Escherichia coli* were tested, and their interaction with day of sample collection. The results are expressed as arithmetic least square means (se). The data for the diameter of the uterus were analysed by using repeated measures analysis of variance, and the results are expressed as estimated marginal means (se). Significance was accepted at $P<0.05$.

RESULTS

Data from 26 cows were eligible for analysis. None of them had a sterile uterus throughout the study and the frequency distribution of their total bacterial growth scores is shown in Fig 1. *A pyogenes* was isolated from 16 of the cows and *E coli* from 24. Other bacteria identified included *Bacteroides* species (two cases), α-haemolytic streptococci (22), *Staphylococcus aureus* (six), *Proteus* (four), and *Pasteurella* species (five). The diameter of the uterus decreased with time (Fig 2); most of the reduction had occurred by day 16 and after day 21 there was no further significant reduction.

α_1-acid glycoprotein

The concentration of α_1-acid glycoprotein in plasma was affected by the day of collection of the blood sample ($P<0.005$) and by the total bacterial growth score ($P<0.0001$). The interaction between day of sampling and bacterial score was not significant. Its concentration decreased gradually after calving until day 21 (Fig 2). Its concentration was highest (2·39 [0·23] mg/ml) in the cow with a total bacterial growth score of 24 (Fig 3) and lowest (0·60 [0·27] mg/ml) in the cow with a score of 4. When bacterial pathogens were examined separately, the significant variables were *E coli* ($P<0.0001$) and the day of blood sampling ($P<0.05$), but not their interaction. The concentration of α_1-acid glycoprotein was higher in the cows with *E coli* than in those without (1·30 [0·04] v 0·79 [0·09] mg/ml).

Haptoglobin

The concentration of haptoglobin in plasma was affected by the day of collection of the blood sample ($P<0.05$) and by the total bacterial growth score ($P<0.05$). The interaction between the day of sampling and the bacterial score was not significant. The plasma concentrations were very variable, but on average they decreased with time after calving (Fig 2), and reached a minimum on day 14, seven days earlier than the concentration of α_1-acid glycoprotein. The haptoglobin concentration was highest (101 [45] μg/ml) in the cow with a total bacterial growth score of 15, and lowest (5·6 [4·5] μg/ml) in the cow with a score of 2. There were no significant variables when the bacterial pathogens were examined separately.

Ceruloplasmin

The serum concentration of ceruloplasmin was affected by the day of collection of the blood sample ($P<0.01$) and by the total bacterial growth score ($P<0.0001$), but the interaction between these variables was not significant. Its concentration decreased only slowly after calving (Fig 2). The cow with a total bacterial growth score of 12 had the highest ceruloplasmin concentration (31·2 [2·2] units/ml) and the cow with a score of 2 had the lowest (17·4 [2·2] units/ml) (Fig 3). When bacterial pathogens were examined separately, the significant variables were *A pyogenes* ($P<0.05$) and the day of blood sampling ($P<0.0001$), but not the interaction. The concentration of ceruloplasmin was higher in the cows with *A pyogenes* than in those without (29·1 [0·9] v 25·1 [1·1] units/ml).

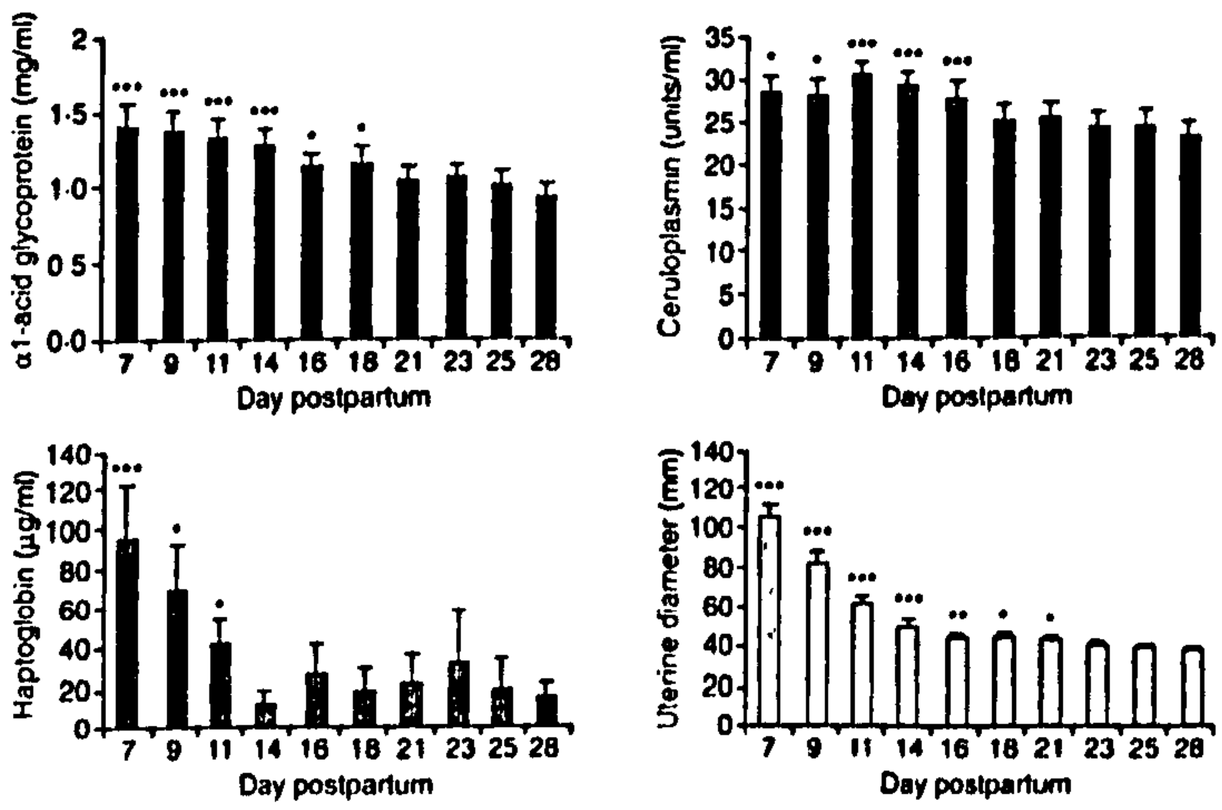

FIG 2: Mean (se) concentrations of α_1-acid glycoprotein, haptoglobin and ceruloplasmin and the diameter of the previously gravid uterine horn in 26 cows between seven and 28 days after calving. *P<0·05, **P<0·01, ***P<0·001; values different from value on day 28

DISCUSSION

The results of these studies indicate that the plasma concentrations of the acute phase proteins α_1-acid glycoprotein, haptoglobin and ceruloplasmin were different in cows with

different degrees of uterine bacterial contamination. These observations agree with observations in ewes, in that ewes with contaminated uteri had higher concentrations of α_1-acid glycoprotein and haptoglobin than ewes with a sterile uterus, although the concentration of ceruloplasmin was not significantly different (Regassa and Noakes 1999). Acute and chronic infections of other body systems in cattle have similarly been detected by changes in the plasma concentration of α_1-acid glycoprotein, and, to a smaller extent, of haptoglobin (Horadagoda and others 1999). The concentrations of α_1-acid glycoprotein, haptoglobin and ceruloplasmin decreased as uterine involution progressed. However, the relationship between the acute phase protein response and the uterine bacterial growth score was independent of the day of collection of the samples, supporting the hypothesis that the concentrations of the acute phase proteins may increase in association with uterine bacterial contamination independently of the changes associated with uterine involution.

The uteri of all the cows were contaminated with bacteria, the predominant pathogens being *A pyogenes* and *E coli*, in agreement with previous reports (Elliot and others 1968, Peter and Bosu 1987, Bonnett and others 1991). The presence of *A pyogenes* and *E coli* was associated with increased concentrations of ceruloplasmin and α_1-acid glycoprotein in the blood. The isolation of *A pyogenes* from the uterine lumen has been associated with the most extensive tissue inflammation in dairy cows after calving (Bonnett and others 1991); and *E coli* produces a lipopolysaccharide endotoxin which induces the release of the cytokines interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor-α (TNFα) (Miller and others 1997, Gayle and others 1999). These cytokines, produced in response to bacterial infection, induce hepatocytes to secrete acute phase proteins (Andus and others 1988, Marinkovic and others 1989, Henderson and Wilson 1996). Because assays for IL-1, IL-6 and TNFα are not widely available, the concentrations of acute phase proteins in blood could possibly be used as a convenient measure of the degree of bacterial contamination of the uterus. Furthermore, the concentrations of cytokines in local tissues, rather than in the blood, are more reliable measures of biological activity and the severity of a bacterial challenge (Miller and others 1997). However, the concentrations of acute phase proteins should be used to assess the degree of uterine bacterial contamination with caution, because increases can also indicate bacterial infections in other parts of the body (Skinner and others 1991, Horadagoda and others 1999).

Uterine involution was estimated by measuring the diameter of the larger uterine horn ultrasonographically. Most of the reduction in the diameter of the uterine horn had occurred by day 16 and there was no significant decrease after day 21, in agreement with observations by Gier and Marion (1968) and Tian and Noakes (1991). In parallel with these observations, the concentrations of α_1-acid glycoprotein and ceruloplasmin decreased to basal values by day 18 to 21 after calving, whereas the concentration of haptoglobin had decreased to near basal concentrations by day 14. Similar trends in the concentrations of acute phase proteins were observed in sheep by Regassa and Noakes (1999).

The differences between the responses of the acute phase proteins to the bacterial contamination and involution of the uterus may be due to the responses of hepatocytes to different cytokines. Studies in rats indicate that different acute phase proteins are regulated differently by various cytokines (Andus and others 1988). For example, the synthesis of haptoglobin is stimulated by IL-6 and TNF-α, but not by IL-1 (Nakagawa-Tosa and others 1995). The variability of the concentration of haptoglobin observed in this study agrees with the results of previous studies in sheep and cattle (Scott and others 1992, Regassa and Noakes 1999). As a result, haptoglobin may not be as useful for monitoring uterine involution or bacterial contamination as α_1-acid glycoprotein and ceruloplasmin. In one study, a number of animals with acute metritis had low serum concentrations of haptoglobin (Smith and others 1998).

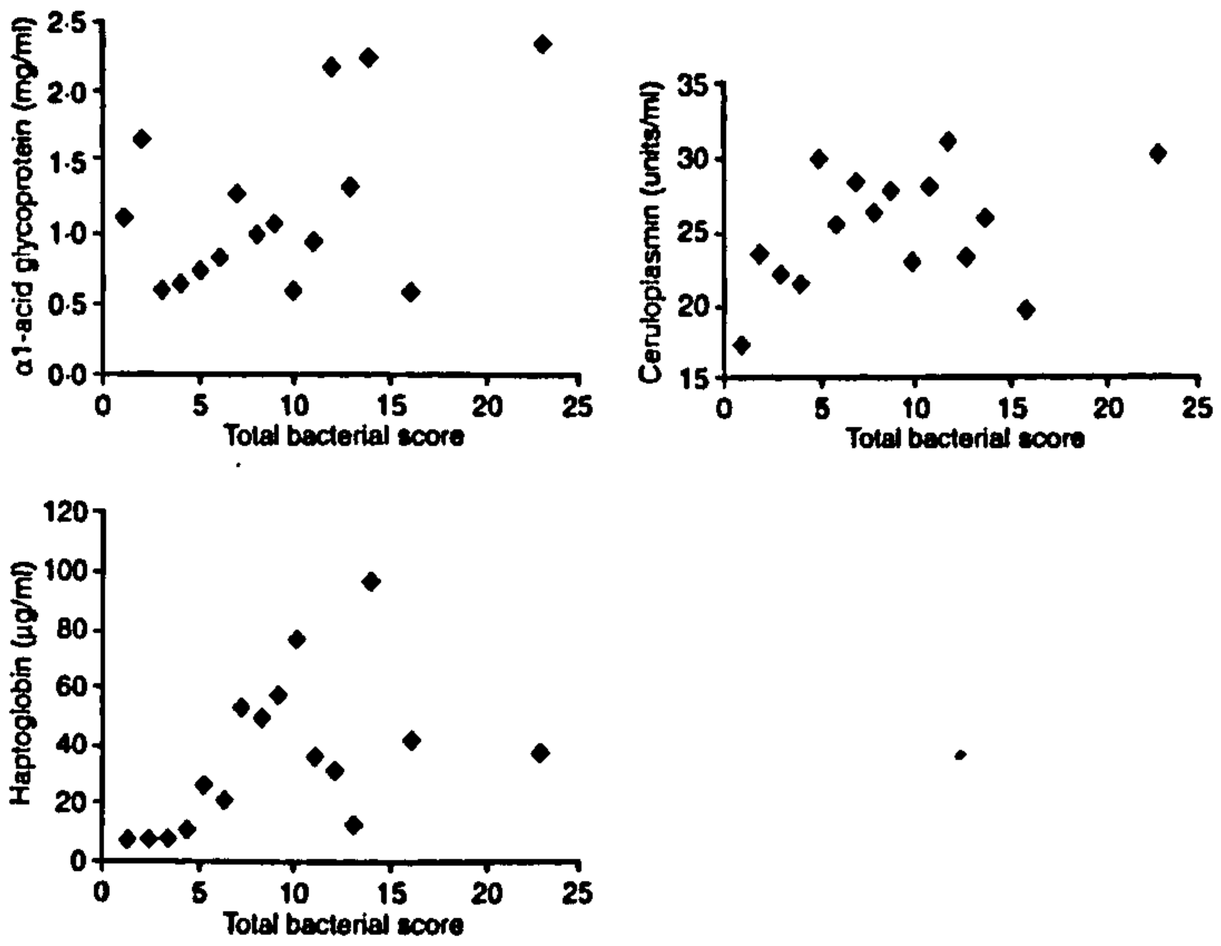

FIG 3: Relationships between the mean concentrations of α_1-acid glycoprotein, haptoglobin and ceruloplasmin in 26 cows, and their total bacterial scores

The results of this study indicate that the concentrations of acute phase proteins in the blood of cattle were increased by uterine bacterial contamination. In addition, the isolation of *A pyogenes* and *E coli* from the uterus, was associated with increased concentrations of the acute phase proteins. The concentrations of α_1-acid glycoprotein, haptoglobin and ceruloplasmin decreased as uterine involution progressed. However, the response of the acute phase proteins to the bacterial contamination of the uterus was independent of the day on which the samples were collected, supporting the hypothesis that acute phase proteins may be increased by bacterial contamination in addition to the changes associated with uterine involution.

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Scapulohumeral osteoarthritis in 20 Shetland ponies, miniature horses and falabella ponies

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This paper describes the clinical and diagnostic features of 20 cases of scapulohumeral osteoarthritis in Shetland ponies, miniature horses and falabella ponies. The history and clinical signs were similar in all the cases. Radiographically they all had consistent changes which consisted predominantly of articular osteophytes and periarticular enthesiophytes. Six of the cases had radiographic evidence of dysplasia of the scapulohumeral joint, although it was uncertain whether this was a primary or a secondary finding. No specific treatment appeared to be advantageous. At follow up, six of the ponies had to be euthanased owing to continuing severe lameness; the other 14 ponies remained lame, but were maintained at pasture by the occasional use of oral non-steroidal anti-inflammatory drugs. No definitive aetiology for the condition was identified, but it is proposed that an underlying dysplasia, or lack of collateral support may predispose the scapulohumeral joint of miniature horse breeds to the disease.

OSTEOARTHRITIS of the scapulohumeral joint in horses is relatively rare, and when it does occur it is usually secondary to a primary disease or injury. Such primary diseases include fracture, subchondral bone cysts, osteochondritis dissecans, articular sepsis and luxation (Dyson 1986a, b). There has been only one previous report of a miniature horse with the condition (Arighi and others 1987), and in this case no obvious aetiology for it was recorded. In a study of a large number of horses with lameness localised to the shoulder, there were no cases in Shetland ponies or other miniature breeds (Dyson 1986b). A recent study has demonstrated that Shetland ponies have a shallower glenoid cavity of the scapula than horses, and it was postulated that this difference may predispose them to osteoarthritis as a result of a primary dysplasia of the scapulohumeral joint (Boswell and others 1999).

This paper describes the clinical and diagnostic findings in 20 Shetland ponies, falabella ponies and miniature horses which became lame as a result of osteoarthritis of the scapulohumeral joint. The condition appears to be specific to miniature horse breeds, and although no definitive aetiology was identified, several possible causes are proposed.

MATERIALS AND METHODS

Over a period of seven years, 17 Shetland ponies, two falabella ponies and one miniature horse were referred to the authors for the investigation of forelimb lameness and were diagnosed with osteoarthritis of the scapulohumeral joint. Eleven of the cases were lame on the left forelimb, seven were lame on the

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Effect of the Regressing Corpus Luteum of Pregnancy on Ovarian Folliculogenesis after Parturition in Cattle[1]

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ABSTRACT

In cattle, the first postpartum dominant follicle has a predilection for the ovary contralateral to the previously gravid uterine horn, possibly due to a local inhibitory effect of the regressing corpus luteum of pregnancy in the ipsilateral ovary. The aim of the present study was to test the hypothesis that the regressing corpus luteum of pregnancy suppresses folliculogenesis in the ipsilateral ovary after parturition. Dairy cows were treated with prostaglandin $F_{2\alpha}$ between 190 and 220 days of gestation to cause luteolysis without inducing parturition (n = 14) or were untreated controls (n = 32). Follicular growth and function were monitored by daily transrectal ultrasonography and collection of plasma samples for estimation of FSH, estradiol, and progesterone concentrations. The proportion of first dominant follicles in the ipsilateral ovary was similar for treated and control animals (4/14 vs. 8/32), as was the time interval between calving and establishment of a dominant follicle (mean ± SEM, 10.1 ± 0.4 vs. 10.7 ± 0.5 days). Furthermore, no significant effect of treatment on dominant follicle growth or function was found as determined by plasma hormone concentrations. Although greater folliculogenesis was found in the ovary contralateral to the previously gravid uterine horn, once the location of the future first dominant follicle was selected, the timing of events was independent of location. We suggest that the corpus luteum of pregnancy does not have a local effect on postpartum ovarian folliculogenesis and that, instead, an effect of the previously gravid uterine horn shortly after parturition should be considered.

corpus luteum, follicular development, ovary, parturition, uterus

INTRODUCTION

After parturition in cattle, an increase in plasma FSH concentration occurs, which is followed by the emergence of a follicular wave and subsequent selection of a dominant follicle [1, 2]. Use of sequential transrectal ultrasonography has revealed a preference for the first postpartum dominant follicle to be selected in the ovary contralateral to the previously gravid uterine horn and, thus, the ovary bearing the corpus luteum (CL) of pregnancy [3–5]. This is important, because the presence of a large follicle in the ovary ipsilateral to the previously gravid uterine horn within 4 wk of parturition, although less frequent, is associated with improved subsequent fertility [6–8].

Suppression of folliculogenesis in the ipsilateral ovary decreases as the postpartum interval advances, concurrent with disappearance of the CL of pregnancy and uterine involution [6, 9]. This could be explained by a local inhibitory effect of the regressing CL of pregnancy or by a regional effect of the previously gravid uterine horn [10]. Although luteolysis and regression of the CL is rapid during the estrous cycle, luteolysis following parturition is protracted, with physical remnants of luteal cells being detectable up to 35 days postpartum [9]. In addition, evidence for a nonsteroidal aromatase inhibitor of luteal origin that can influence follicular steroidogenesis has been found [11].

The aim of the present study was to test the hypothesis that the regressing CL of pregnancy suppresses folliculogenesis in the ipsilateral ovary after parturition. Specific objectives were, first, to remove the CL of pregnancy without disruption of the fetus by administering prostaglandin $F_{2\alpha}$ (PG) during the last trimester of pregnancy [12]. Then, the effect of the absence of the CL of pregnancy on ovarian follicle structure and function, after parturition, would be determined using sequential ultrasonography and plasma hormone assays.

MATERIALS AND METHODS

Animals

All procedures were carried out under the Animals (Scientific Procedures) Act 1986 regulations for experiments on living animals, administered by the UK Home Office. In addition, experimental protocols were approved by the Royal Veterinary College Ethical Review Board.

A dairy herd of 90 Holstein-Friesian cows, with an annual average milk yield of 6800 L, was selected for the study on the basis of accurate farm records. A total of 53 cows that were due to calve within a 5-mo period were included in the study. All cows had been artificially inseminated with Holstein-Friesian semen. The PG analogue (500 μg of cloprostenol; Schering-Plough Animal Health, Uxbridge, UK) was administered i.m. between 190 and 220 days of gestation to 17 animals selected using a randomization chart (http://www.randomizer.org).

Examination

The genital tracts of all cows were examined daily by transrectal palpation and ultrasonography using a 7.5-MHz, linear-array transducer (Aloka SSD 210 DXII; BCF Technology, Livingstone, UK) starting on Postpartum Day 6 and continuing for 21 days. The side of the previous pregnancy was determined by assessing which uterine horn was longest and of greatest diameter on Postpartum Day 6. Follicles were defined as nonechogenic (black), spherical structures with a clear demarcation between the follicular wall and antrum. Corpora lutea were defined as grainy, echogenic structures having a well-defined border with the less echogenic ovarian stroma; in some CL, a nonechogenic lacuna was observed. After freezing the image on the screen, the number of ovarian follicles ≥4 mm in

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diameter and of CL in each ovary were counted, and their maximum diameter was measured using the internal calipers of the machine. When the image of the structure being scanned was not spherical, the diameter was estimated by averaging two 90° dimensions.

A dominant follicle was defined as the largest follicle in the ovary with an internal diameter of ≥10 mm in the absence of other growing follicles [13]. A dominant follicle and cohorts were defined as a follicular wave [13]. The first day of dominance within a follicular wave was determined, retrospectively, as the first day on which the dominant follicle was ≥10 mm in diameter. The number of follicles ≥4 mm or ≥10 mm in a wave was based on the emergence of the follicles at the same, or consecutive, examinations [14]. Day of ovulation was defined as the day when a dominant follicle was last scanned before subsequent appearance of a CL in the same location and was confirmed, retrospectively, by a subsequent increase in plasma progesterone to a concentration of >1 ng/ml. A persistent follicle was defined as a follicle with an internal diameter of ≥10 mm that persisted for more than 5 consecutive days in the absence of new follicular growth and that did not ovulate [13].

Blood Sampling and Hormone Assays

Blood samples were collected daily for 21 days, starting on Postpartum Day 6, from the coccygeal vein or artery into evacuated, heparinized tubes (Vacutainer; Becton Dickinson, Meylan, France) and transported on ice to the laboratory. Within 30 min, plasma was separated by centrifugation (2200 × *g* for 10 min), harvested, and stored frozen at −20°C.

Estradiol-17β concentration was estimated in duplicate using a previously characterized RIA (Estradiol MAIA; Serono Diagnostics Ltd., Woking, UK) following diethyl ether extraction of the plasma samples [15]. The mean intraassay (n = 12 samples) and interassay (n = 3 assays) coefficients of variation were 8.1% and 13.1%, respectively, for a sample of 0.9 pg/ml, and the sensitivity was 0.24 pg/ml. Progesterone concentration was estimated in duplicate using a commercial ELISA kit (Ridgeway Science, Gloucester, UK). The intraassay (n = 10 samples) and interassay (n = 3 assays) coefficients of variation were 6.5% and 11.2%, respectively, for a sample of 1.7 ng/ml, and the sensitivity was 0.6 ng/ml. The FSH concentration was estimated in duplicate using a previously validated RIA [13]. The standard used for the FSH assay was AFP 5679C RP-1. The intraassay (n = 20 samples) and interassay (n = 3 assays) coefficients of variation were 3.4% and 4.7%, respectively, for a sample of 1.2 ng/ml, and the sensitivity was 0.12 ng/ml.

Statistical Analysis

Data analysis was performed on 46 animals (14 treated and 32 control) using the SAS version 8.01 computer program (SAS Institute Inc., Cary, NC). Three treated cows were excluded from the analysis: one aborted at 243 days of gestation, one had a cesarean operation, and one had a physical injury. Four control cows were excluded: 2 had cesarean operations, and 2 had mastitis. Data are quoted as the arithmetic mean ± SEM, and significance was attributed at $P < 0.05$. Continuous data were examined for normality using the Kolmogorov-Smirnoff test and for equality of variance using Levene test [16]. Plasma hormone data were $\log_{10}$ transformed before statistical analysis.

The duration of gestation, time interval from insemination to PG administration, intervals between parturition and dominance or ovulation, and interval between ovulation and increase in plasma progesterone concentration were examined by Kaplan-Meier survival analysis [17]. Differences between the treated and control groups were compared using the log rank test [17]. The maximum diameter of follicles was compared between ovaries and between treatments using unpaired *t*-tests and between different follicular fates using ANOVA. Comparisons of the location or the fate of ovarian structures were tested using the chi-square test or, when a cell had an expected frequency of less than five, Fisher Exact test. Data for the number of follicles in each ovary were compared using the nonparametric Wilcoxon signed rank test, and differences between treatment groups were tested using the Mann-Whitney test [18].

Follicle diameters and plasma hormone concentrations were examined using ANOVA mixed models for repeated measures [19]. Response variables tested were day postpartum, treatment group, fate of the first dominant follicle (ovulated, regressed, persistent follicle), location of the first dominant follicle (ipsilateral, contralateral ovary), and their interactions. Variables were removed from the model following examination for correlation with remaining variables until those variables remaining were significant. Model fitting and selection of the covariance structure were determined using Akaike information criterion [19]. Correlation between diameter of the first dominant follicle and plasma hormone concentrations were tested using the Pearson correlation coefficient.

RESULTS

The interval from conception to administration of PG for treated cows was 207.5 ± 3.4 days (range, 190–220 days). The interval between administration of PG and parturition was 66.4 ± 3.5 days (range, 48–82 days). The gestation interval was shorter ($P < 0.001$) for treated cows (274.6 ± 2.5 days; range, 255–289 days) compared with control animals (284.2 ± 0.9 days; range, 276–296 days).

Location of Events

The CL of pregnancy was not detected postpartum by ovarian ultrasonography in any cow administered PG, although it was observed in all control animals between Postpartum Days 6 and 14. A wave of follicular development, with the emergence of a dominant follicle, was observed in all cows within 14 days of parturition.

Fewer follicles of ≥4 mm in diameter were found in the ipsilateral ovary compared with the contralateral ovary in the first follicular wave after parturition (2.27 ± 0.23 vs. 3.34 ± 0.20 mm, $P < 0.001$). However, when comparisons were made between treated and control animals, no significant differences were found in the numbers of follicles ≥4 mm in diameter in the first postpartum follicular wave in the ipsilateral ovary (2.71 ± 0.37 vs. 2.03 ± 0.28 mm) or contralateral ovary (3.36 ± 0.40 vs. 3.33 ± 0.22 mm). Fewer first postpartum dominant follicles were observed in the ipsilateral ovary compared with the contralateral ovary (12 vs. 34, $P < 0.01$), but again, the proportions in the ipsilateral ovary did not differ between treated and control animals (4/14 vs. 8/32). A smaller proportion of dominant follicles, compared to follicles of ≥4 mm in diameter, was observed in the ipsilateral ovary (12/46 vs. 104/257, $P < 0.05$).

The first dominant follicle ovulated in 35 of 46 cases. Fewer ovulations occurred in the ipsilateral ovary compared with the contralateral ovary (11 vs. 24, $P < 0.01$). No statistical difference was found in the proportion of dominant follicles that ovulated between the ipsilateral and contralateral ovaries (11/12 vs. 24/34), and no statistical difference was found in the frequency of ovulations from the ipsilateral ovary between the treated and control groups (4/14 vs. 7/32).

A second follicular wave was detected in 36 animals, 33 of which had ovulated and 3 in which the first dominant follicle had regressed. Similar numbers of follicles of ≥4 mm in diameter were found in the ipsilateral ovary compared with the contralateral ovary (2.40 ± 0.26 vs. 2.43 ± 0.18). No significant difference was observed between the number of second dominant follicles in the ipsilateral and contralateral ovaries (13 vs. 23), and the frequency of second dominant follicles in the ipsilateral ovary did not differ between treated and control animals (6/14 vs. 7/22).

Timing of Events

The interval between calving and achieving dominance for the first dominant follicle was similar for the treated and control animals (10.1 ± 0.4 vs. 10.7 ± 0.5 days). The interval from calving to dominance did not differ between dominant follicles located in the ipsilateral or contralateral ovaries (10.6 ± 0.5 vs. 10.2 ± 0.4 days).

For those animals in which the first dominant follicle ovulated, the interval from calving to ovulation did not dif-

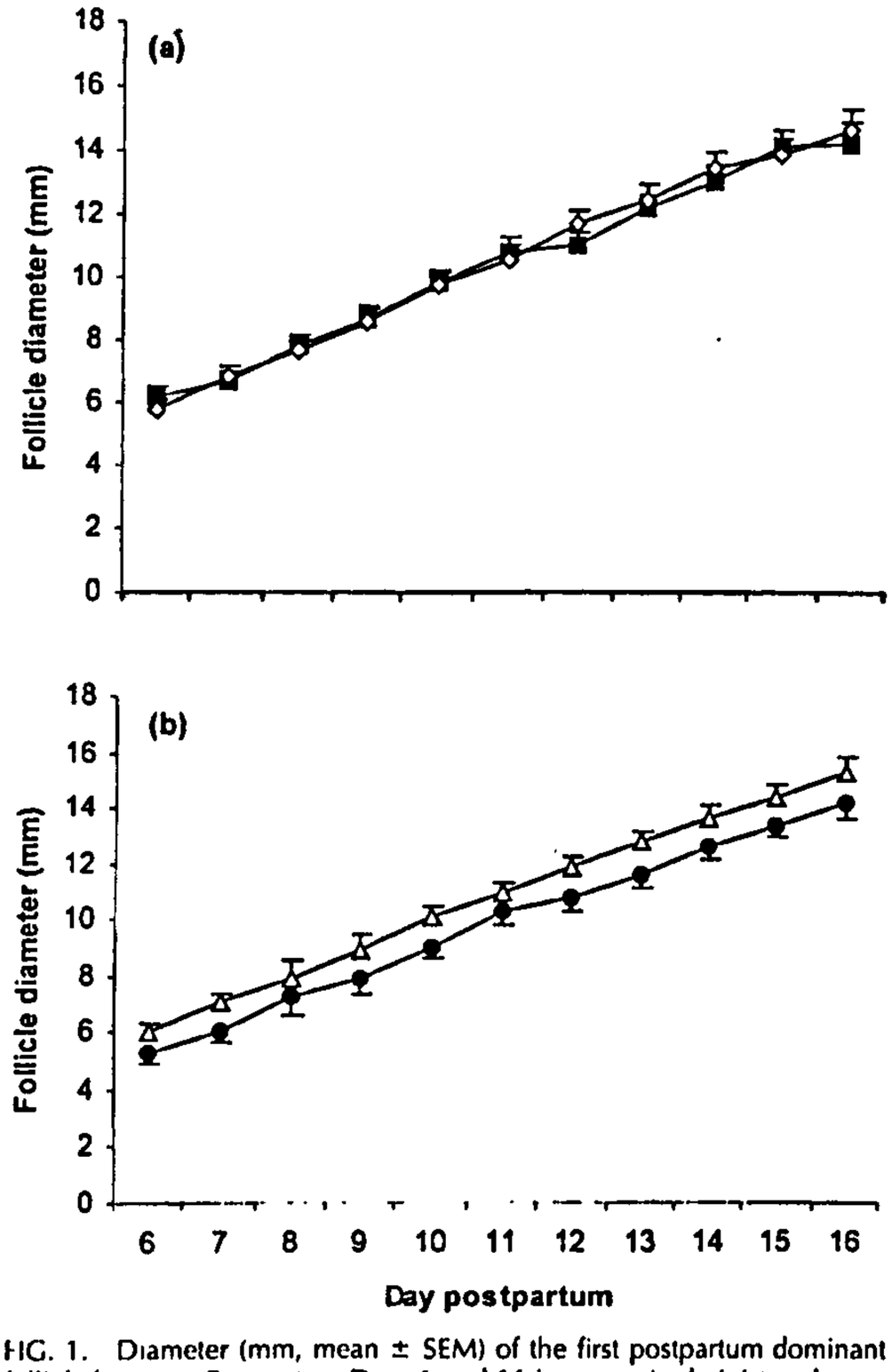

FIG. 1. Diameter (mm, mean ± SEM) of the first postpartum dominant follicle between Postpartum Days 6 and 16 in cows a) administered prostaglandin $F_{2\alpha}$ at approximately 200 days of gestation (■) and control animals (◊) and b) when the first postpartum dominant follicle was in the ipsilateral (●) or contralateral (Δ) ovary.

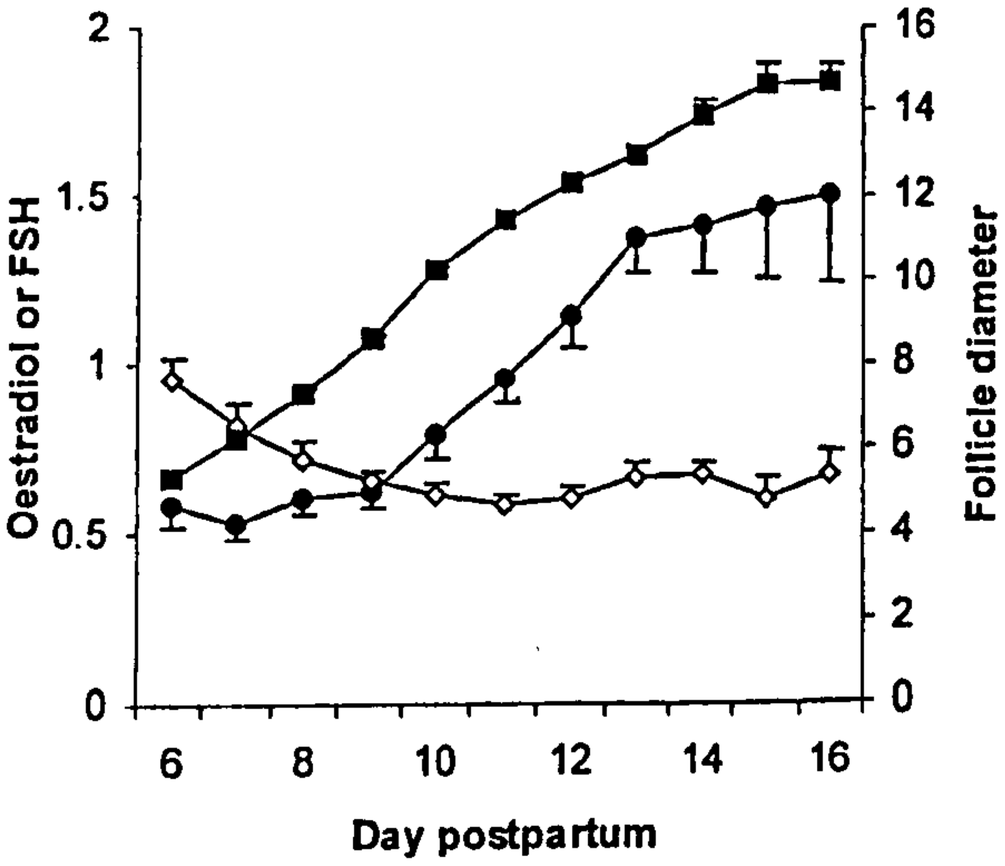

FIG. 2. Diameter (mm, mean ± SEM) of the first postpartum dominant follicle (■), plasma estradiol (●) concentration (pg/ml) and FSH (◊) concentration (ng/ml) between Postpartum Days 6 and 16 using data pooled from all animals.

fer between the treated and control groups (16.1 ± 1.0 vs. 17.0 ± 0.7 days). The interval from calving to ovulation from the ipsilateral and contralateral ovaries was also similar (16.5 ± 0.88 vs. 16.8 ± 0.8 days). After each ovulation, an increase in plasma progesterone concentration to >1 ng/ml was detected 3.8 ± 0.2 days later, and this interval did not differ between those animals with a first dominant follicle in the ipsilateral or the contralateral ovary (3.7 ± 0.5 vs. 3.9 ± 0.2 days). The interval from ovulation to progesterone increase for cows treated with PG more than 60 days earlier did not differ significantly from that of control animals (4.6 ± 0.4 vs. 3.5 ± 0.2 days).

The interval from parturition to dominance of the second dominant follicle postpartum did not differ between treated and control animals (19.9 ± 1.0 vs. 20.8 ± 0.8 days) or between the ipsilateral and contralateral ovaries (20.8 ± 1.0 vs. 20.6 ± 0.8 days).

Follicular Growth and Function

The diameter of the first dominant follicle increased between Postpartum Days 6 and 16 ($P < 0.001$). However, follicular growth rates did not differ between treatment and control groups, and the interaction of group × day postpartum was not significant (Fig. 1a). The maximum diameter of the first dominant follicle of the treated and control groups was 14.9 ± 1.1 and 16.9 ± 0.8 mm, respectively. No significant difference was found between the diameter of dominant follicles in the ipsilateral and contralateral ovaries between Postpartum Days 6 and 16 (Fig. 1b). The maximum diameter of the first dominant follicle of the ipsilateral and contralateral ovaries was 14.8 ± 1.1 and 16.8 ± 0.8 mm, respectively.

No significant effect of treatment group or location of the first dominant follicle was observed on plasma estradiol or FSH concentration, so the combined data are illustrated in Figure 2. Plasma estradiol concentration increased between Postpartum Days 6 and 16 ($P < 0.001$), and FSH concentration decreased between Postpartum Days 6 and 11 ($P < 0.001$), as follicular diameter increased. Between Postpartum Days 6 and 16, a significant correlation was observed between first dominant follicle diameter and plasma estradiol concentration ($r = 0.53$, $P < 0.001$) or FSH concentration ($r = -0.44$, $P < 0.001$). Plasma estradiol and FSH concentration also were correlated ($r = -0.26$, $P < 0.001$).

Ultrasonography revealed three possible fates for the first dominant follicle: ovulation; regression, followed by a second follicular wave; or formation of a persistent follicle (Fig. 3). The frequencies of these different fates of the first dominant follicle did not differ significantly between the treated and control animals. The dominant follicle ovulated, regressed, or formed a persistent follicle in 10, 3, and 1 treated animals and in 25, 3, and 4 control animals, respectively. Using pooled data across treatment groups, the interaction of fate of the first dominant follicle × day postpartum was significant for follicle diameter ($P < 0.01$), plasma FSH concentration ($P < 0.01$), and plasma estradiol concentration ($P < 0.05$). The diameters of first dominant follicles before regression were smaller than those of ovulatory or persistent follicles between Postpartum Days 9 and 16 ($P < 0.01$), and plasma estradiol concentrations were also lower between Postpartum Days 14 and 16 ($P < 0.01$). Plasma estradiol concentrations were correlated with diameter of the first dominant follicle for those that ovulated ($r = 0.60$, $P < 0.001$) or formed a persistent follicle

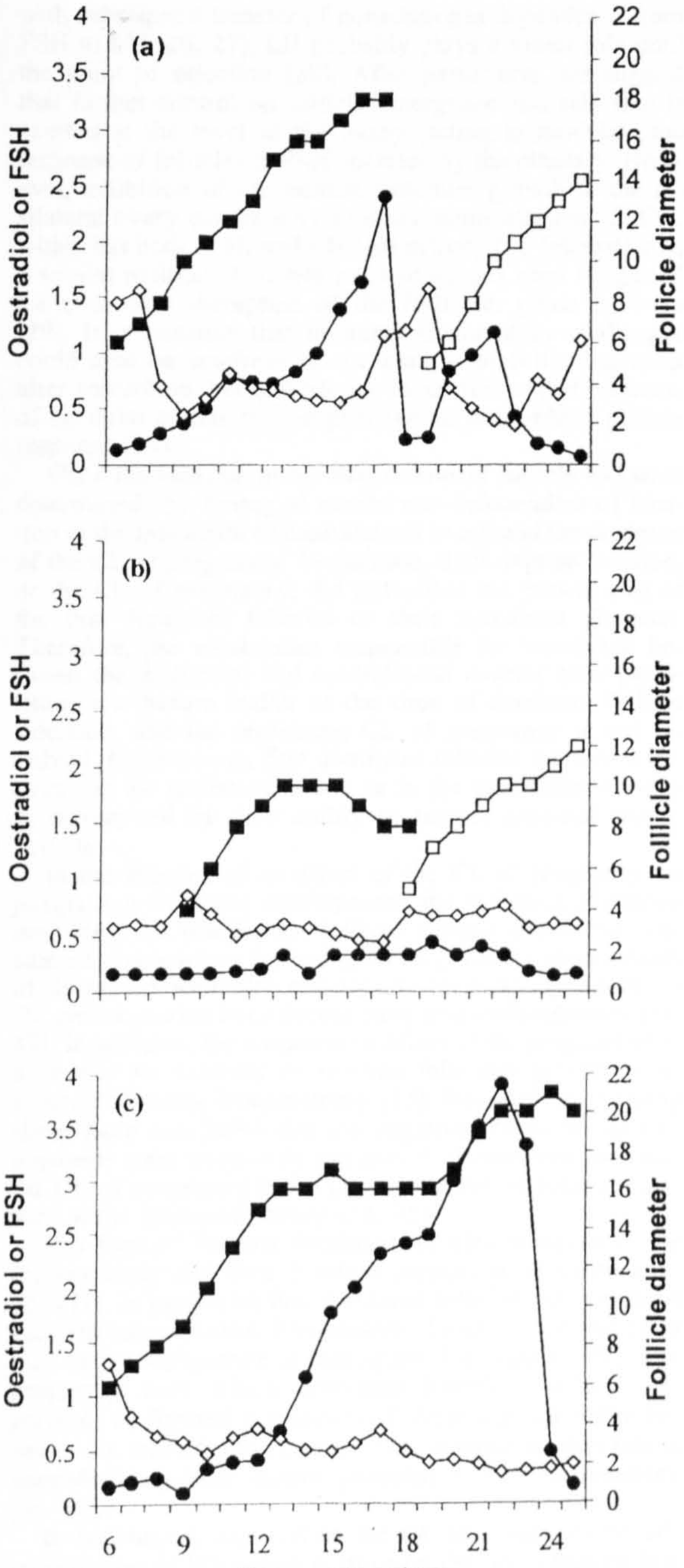

FIG. 3. Diameter (mm) of the first (■) and, where observed, the second (□) postpartum dominant follicles, plasma estradiol (●) concentration (pg/ml), and FSH (◇) concentration (ng/ml) between Postpartum Days 6 and 25 for typical cases in which the first dominant follicle **a**) ovulated, **b**) regressed, or **c**) persisted.

($r = 0.36$, $P < 0.01$), but not for those that regressed. Furthermore, unlike ovulatory or persistent follicles, plasma estradiol concentration in those animals with a first dominant follicle that subsequently regressed did not exceed 1 pg/ml. Plasma FSH concentration was lower in animals with a persistent follicle on Postpartum Day 15 compared to animals with a first dominant follicle that ovulated or regressed (0.56 ± 0.07 vs. 0.76 ± 0.08 or 0.72 ± 0.06 ng/ml, $P < 0.05$).

DISCUSSION

Administration of PG at approximately 200 days of gestation caused luteolysis without disturbance of the fetus, which remained supported by extragonadal sources of progesterone [12, 20]. The present study also confirmed that the CL of pregnancy was not detectable by ultrasonography after parturition in cows treated with PG early in the third trimester. Treated cows had a shorter gestation, although it was not possible in the present study to determine how this might affect the results. However, because the absence of the CL of pregnancy had no effect on postpartum follicular growth or function, or on the timing or location of ovarian events, an effect of the previously gravid uterine horn and its contents should be considered.

A postpartum, transient increase in plasma FSH concentration precedes emergence of the first postpartum follicular wave and subsequent selection of a dominant follicle during the following phase of decreasing FSH concentration [1, 2, 21, 22]. Similarly, in the present study, each animal after parturition developed a cohort of approximately 6 follicles of ≥4 mm in diameter, and as plasma FSH concentration decreased, selection of the first dominant follicle occurred. However, plasma FSH concentration did not differ between those cows in which the first dominant follicle developed in the ipsilateral or contralateral ovary or between the treated and control animals.

The preference for the first postpartum dominant follicle to be selected in the contralateral ovary has been established by repeated transrectal ultrasonography after parturition [3, 4]. Additionally, in the present study, fewer follicles of ≥4 mm in diameter were found in the ipsilateral ovary during the first postpartum follicular wave, and fewer ovulations were observed, reflecting fewer first dominant follicles in the ipsilateral ovary. However, the preference for structures to occur in the contralateral ovary diminishes with increasing interval from parturition [6]. Two possible explanations for these observations have been suggested. First, there could be a local luteal inhibitory effect [4, 10]; however, in the present study, the presence of a regressing CL of pregnancy did not affect several aspects of follicular growth. Furthermore, during normal estrous cycles, more dominant follicles are found in the CL-bearing ovary [23]. A second explanation is that there could be a regional effect of the involuting, previously gravid uterus on folliculogenesis in the ipsilateral ovary [6, 10, 24]. A possible mechanism would be the transfer of products from the uterus to the ovary via the countercurrent mechanism, as established for PG during the process of luteolysis [25].

The observation of fewer dominant follicles in the ipsilateral ovary after parturition appears to be a consequence of two components. First, fewer follicles of ≥4 mm in diameter emerged in the ipsilateral ovary during the first postpartum follicular wave. Second, a smaller proportion of these first-wave follicles were selected to achieve dominance in the ipsilateral ovary. As FSH concentration gradually decreases, selection of the dominant follicle occurs,

with subsequent transfer of gonadotropin dependence from FSH to LH [26, 27]; LH probably plays a minor role until the point of selection [28]. After parturition, we suggest that further control on follicle emergence and selection is exerted at the level of the ovary, acting to modulate the response of follicles to FSH secreted by the pituitary. However, inhibition of postpartum follicular growth in the ipsilateral ovary can be overcome by administration of eCG, which has both FSH- and LH-like activity [5]. Interestingly, a similar multilevel control mechanism has been suggested for endotoxin disruption of the follicular phase in ewes [29]. It is possible that immune/inflammatory challenges could also be involved in the control of folliculogenesis after parturition, because uterine involution and elimination of bacterial contamination provoke an acute-phase protein response [30].

Once the location of the first dominant follicle had been determined, the timing of events was independent of location in the ipsilateral or contralateral ovary and the presence of the CL of pregnancy. In addition, their ovarian location, or the CL of pregnancy, did not affect the growth rate of the first dominant follicles or their maximum diameter. Therefore, the mechanism responsible for imbalance between the ipsilateral and contralateral ovaries after parturition acts before and/or at the time of dominant follicle selection, and the regressing CL of pregnancy is not involved. Furthermore, first dominant follicles were as competent in the ipsilateral ovary as in the contralateral ovary as determined by their ability to secrete estradiol and to ovulate.

In the absence of an effect of the CL of pregnancy on postpartum follicular development, the influence of uterine involution on postpartum folliculogenesis should be considered. Postpartum folliculogenesis parallels observations of decreased follicular activity in the ovary ipsilateral to the gravid uterine horn during early and midpregnancy [31, 32]. In addition, the suppressive effect of the pregnant uterus and/or its contents on ovarian follicular growth is removed following hysterectomy [33]. Researchers studying sheep have concluded that the negative effects on folliculogenesis were exerted by the gravid uterine horn, and that the CL of pregnancy had a positive effect on follicle numbers in the ipsilateral ovary [34, 35].

The fates of the first dominant follicles observed in the present study and their function support previous descriptions [1]. In particular, first dominant follicles that regressed secreted less estradiol, irrespective of follicle diameter; this may be a consequence of inadequate LH support [28]. The frequency with which dominant follicles ovulated, regressed, or formed a persistent follicle did not differ between the treated and control cows, suggesting that fate is dependent on other factors, probably LH pulse frequency [36–38].

In conclusion, removal of the CL of pregnancy by administration of PG before parturition did not influence first postpartum follicular wave location, timing of ovarian events, or dominant follicle growth or function. Although greater folliculogenesis was observed in the ovary contralateral to the previously gravid uterine horn, once the location of the future first dominant follicle was selected, the timing of events was independent of location. We suggest that the CL of pregnancy does not have a local effect on postpartum ovarian function; instead, an effect of the previously gravid uterine horn shortly after parturition should be considered.

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