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**Does peripheral concentration of progesterone affect ovulation and lambing rates
in Barbados Blackbelly ewes?**

Ezra Devonish

**Thesis
Submitted to the College of Agriculture and Forestry
At West Virginia University
In partial fulfillment of the requirements for
The degree of**

**Master of Science
In
Reproductive Physiology**

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College of Agriculture, Forestry and Consumer Affairs

**Morgantown, West Virginia
2005**

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Abstract

Does peripheral concentration of progesterone affect ovulation and lambing rates in Barbados Blackbelly ewes?

Ezra Devonish

The hypothesis that concentration of progesterone influences number of follicles that mature during estrus, and thus ovulation rate, was tested in cycling ewes. Ewes were assigned at random to three treatments, and bred at estrus after treatment. Ewes in low (LP) and high (HP) groups received exogenous progesterone from day 4 to 14; control ewes (MP) were untreated. The LP ewes were given PGF₂ α on day 6 to regress corpora lutea (CL). Numbers of follicles and CL were observed by transrectal ultrasonography. Fewer follicles disappeared at estrus in ewes on HP than in MP or LP. Ewes on LP had a greater number of CL compared with MP or HP, which did not differ. Number of CL increased, but lambs born per CL decreased linearly with decreasing concentrations of progesterone; therefore litter size was not changed. In conclusion, LP increased ovulation rates, but did not increase prolificacy.

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Dedication

My journey to a successful graduate career began with this advice from Dr Inskeep, my mentor, "A good researcher is unbiased and a dispassionate observer". His constant quest for me to reach my full potential was ascertained through constructive criticism, appreciation of experimental design and statistical analysis. My challenge was to develop proficiency in verbal and written communication. However, the task was made easier, but at times intimidating, by his incisive literary skills gained from over 40 years experience of writing manuscripts or editing journals. I was deeply moved, that even during his illness, he was still concerned with the well being of all his students. His unending zeal and support of each student's career objectives and to maximize their knowledge in order that each may reach their full potential. I was blessed to be one of those students. As I continue my journey to follow in the huge footsteps of my mentor, who has succeeded in giving me some of the tools, which I will need to work in a challenging environment such as a small Caribbean island (Barbados). "I would like to thank my mentor Dr Inskeep for his patience, unending generosity and guidance, which allowed me to acquire the skills required to become a scientist.

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Introduction

The estrous cycle and its components have been characterized in many breeds of sheep. Studies conducted mainly in temperate regions of North America, Europe, Australia and New Zealand with breeds of sheep that originated within those regions have resulted in the identification of breeds with differing levels of prolificacy. Prolific breeds, in contrast to non-prolific breeds, have smaller ovarian follicles (Driancourt et al., 1990, 1996; Bartlewski et al., 1999; Gibbons et al., 1999), with fewer layers of granulosa cells per follicle (Driancourt et al., 1990, 1996), reduced numbers of atretic follicles (Turnbull et al., 1978; Mandiki et al., 2000), and lower peripheral concentrations of progesterone and estradiol (Avdi et al., 1997; Reynaud et al., 1999; Bartlewski et al., 2003). Thus, lower circulating concentrations of progesterone are associated with higher ovulation rates.

The evolution of breeds has resulted in a diverse gene pool from which to select traits (carcass, wool and milk) for genetic improvement. Breeding programs have been successful in increasing fertility. However, an increase in prolificacy through genetic selection is a slow process. For example, development of the relatively high prolificacy in the Barbados Blackbelly sheep started in the year 1657, upon the introduction of parent stock onto the island of Barbados (Ligon R., 1657), and evolved over 300 years. Regardless of the production traits of interest, the ability to have multiple offspring depends on reproductive management techniques to facilitate increased ovulation and lambing rates (prolificacy).

An ovulation rate of one or two follicles is the usual species specific number for sheep. A reproductive management technique to increase prolificacy quickly will be

advantageous to producers, by providing increased revenue from lambs sold and more breeding stock. Attempts to manipulate ovulation rate through the use of exogenous gonadotropins have had limited success, partly due to differences in breed responsiveness (Saumande et al., 1978), FSH/LH ratio of the gonadotropin used (Chupin et al., 1984), and variation in the response based on the day of the cycle when stimulation was begun (Sreenan et al., 1978).

Although pregnancy rates, percent ewes lambing, and lambing rates to first service were not different, anestrus ewes treated with progesterone had higher overall lambing rates than the controls, and ewes treated with progesterone and FSH tended to have greater prolificacy than those treated with progesterone only (Knights et al., 2001). In another study, Knights et al. (2003) treated anestrus ewes with progesterone inserts (controlled internal drug-releasing device; CIDR-G[®]), for induction of estrus, for 5 days; at the removal of the insert, rams were introduced. A single injection of FSH at 12 h before removal of the insert resulted in a small increase in ovulation rate, but no overall effect on pregnancy rate or prolificacy. However, the highest dosage used in the study would have elicited a superovulatory response when given in the breeding season. Interestingly, from both studies, the authors suggested that the combination of progesterone and ram introduction was as effective as adding low dosages of FSH to those treatments in increasing prolificacy during the anestrus season.

Use of gonadotropins often has resulted in release of an unpredictable number of ova, at different stages of follicular development, leading to decreased fertility and high embryonic loss (Lunstra and Christenson, 1981). One possible alternative might be to use exogenous progesterone/progestogen to increase ovulation rate. Subluteal

concentrations of progesterone were shown to influence the recruitment of follicles, endocrine patterns and follicular waves during the luteal phase of the ewe (Johnson et al., 1996; Viñoles et al., 1999), and the number of follicles ovulated (Bartlewski et al., 1999).

Concentration of progesterone during the luteal phase was relatively high in a non-prolific breed (Western white-face) and low in a prolific breed (Finn ewes) (Bartlewski et al., 1999). Studies of follicular dynamics in less-prolific breeds, for example, the Western white-faced (Bartlewski et al., 1999; Duggavathi et al., 2001; Bartlewski et al., 2003) and Suffolk (Bister et al., 1999), have shown that ovulatory follicles arose predominantly in the follicular wave prior to estrus. In contrast, Bartlewski et al. (1999) found that, in prolific Finn ewes, follicles were ovulated from the penultimate wave of follicular development as well. In addition, Bartlewski et al. (1999) found that ovulation rate was increased by 50% in Western white-faced ewes treated with medroxyprogesterone acetate (MAP), to simulate low concentrations of progesterone after regression of the corpora lutea. They postulated that lifespan of the largest follicles in the penultimate wave was increased, which allowed them to ovulate with those in the final wave (Bartlewski et al., 1999). Johnson et al. (1996) observed ovulatory follicles emerging as early as day 9 or as late as day 15 in ewes of mixed breeding that had been selected for twinning, supporting the hypothesis that follicles from different cohorts could survive to ovulate. Effects of experimental manipulations of concentrations of progesterone during the breeding season on ovulation rate and prolificacy in ewes with naturally greater ovulation rates have not been reported.

Embryonic loss can negate or reduce any improvement in reproductive efficiency

induced by an increased ovulation rate. Hulet et al. (1956) estimated embryonic and fetal loss from day 18 to term to be 9%; other authors have stated that embryonic losses after day 30 were low (Robinson, 1951; Quinlivan et al., 1966). In contrast, fetal loss after day 50 was estimated to be approximately 78% for ewes with ovulation rates that exceeded 6 oocytes (Quinlivan et al., 1966) and 19% for those that had ovulation rates of 5 or fewer oocytes (Rhind et al., 1980). Nevertheless, if low progesterone before breeding increases ovulation rate by a nominal amount, it might be possible to increase litter size.

Review of Literature

1. Characterization and regulation of the events in the estrous cycle

The length of the estrous cycle is approximately 17 days in ewes. As summarized by Goodman (1994), regulation of length of the estrous cycle is dependent upon inter-relationships among the hypothalamus, the anterior pituitary, the ovary and the uterus. The estrous cycle is divided into four phases; proestrus, estrus, metestrus, and diestrus.

Proestrus and estrus

During proestrus, the decline of progesterone releases the hypothalamo-hypophyseal axis from negative feedback inhibition (Baird and Scaramuzzi, 1976; Karsch et al., 1979; 1980) on tonic secretion of GnRH, which results in a concomitant increase in LH, which in turn increases secretion of estradiol from the ovulatory follicle (Scaramuzzi et al., 1977). However, progesterone 'priming' is a prerequisite for the expression of estrus and the subsequent LH surge, which is required to cause

ovulation. Even though estradiol might induce estrus in the absence of progesterone 'priming, a higher dosage is required. Progesterone pretreatment in ovariectomized ewes reduced the interval from estrogen treatment to the onset of estrus, in addition to which it increased the number of animals that responded to estrogen and subsequent repeated treatments with estrogen (Robinson, 1954b; 1955). Interestingly, repeated use of estradiol under circumstances of a short time period (intervals of days) has resulted in a reduction of the estrous response (Robinson, 1954a; Scaramuzzi et al., 1972).

The decline of progesterone is due to the regression of the CL (luteolysis). The regression of the CL (and alternatively the maintenance of pregnancy) is regulated by the timing and changes in amplitude of the secretion of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) by the uterus. Even though the uterus secretes $PGF_{2\alpha}$ continuously throughout the estrous cycle, its episodic secretion increases beginning on day 11 to 12 (Ottobre et al., 1984). These episodes of $PGF_{2\alpha}$ initiate a decline in progesterone. The removal of the main source of estrogen (follicles) by X-irradiation of the ovaries on days 5 or 6 or cauterization of follicles on day 13 delayed luteolysis (Karsch et al., 1970). Gengenbach et al. (1977) found that estrogen and $PGF_{2\alpha}$ given simultaneously, were more effective in luteolysis than either separately in hysterectomized ewes with X-irradiated ovaries. Thus, the CL is regressed by $PGF_{2\alpha}$ and estradiol, both functionally and structurally (Gengenbach et al., 1977; Griffeth et al., 2002).

The CL will regress in response to $PGF_{2\alpha}$ by day 5 (Houghton et al., 1995). Acritopoulou et al. (1980) treated ewes on days 3, 5, 8, 11, 14 or 16 of the estrous cycle and concluded that the corpus luteum of the ewe was responsive to $PGF_{2\alpha}$ only between days 5 through 14 of the estrous cycle. They found that intervals to the onset

of estrus were 33, 40 and 46 h for ewes treated with PGF₂α on days 5, 8, or 11 post-estrus, respectively, and that the LH surge occurred approximately 10 to 11 h after onset of estrus. Deaver et al. (1986) postulated two mechanisms through which the interval from luteolysis to estrus can be influenced. First, the variability in response could be attributed to differences in sensitivity of the hypothalamic-pituitary axis to the positive effects of estradiol. This effect might be mediated by either the concentration of progesterone or the ratio of progesterone to estradiol on the days of the cycle when the ewes were treated. For example, Reeves et al. (1970) showed that highly purified porcine GnRH induced the release of LH from the pituitary in the female and castrated male sheep. Estradiol sensitizes the pituitary to GnRH and mediates the release of LH by a positive feedback to the hypothalamus (Goodman and Karsch, 1980). GnRH is released from the median eminence of the hypothalamus and regulates the pituitary release of LH in both tonic and acute patterns. The maximal response in a diestrous ewe or a ram given 81 µg GnRH was 12 ng/mL above pre-treatment concentrations of LH, but when 27 µg GnRH was given to wethers, it resulted in a peak concentration of 160 ng/mL LH. The lack of a significant dose response in the ewe was due to the inhibition by progesterone. In addition, the intact ram produces testosterone, which has a negative feedback at the pituitary, unlike the wether, that has no progesterone or testosterone to inhibit the response (Reeves et al., 1970). This finding supports the inhibitory effects of progesterone (ewe) and testosterone (ram) on the pituitary responsiveness to GnRH.

The pre-ovulatory rise in estradiol might mediate a change in pituitary responsiveness to GnRH. Arimura et al. (1971) showed an increase in pituitary

responsiveness to exogenous GnRH in the intact female rat pretreated with estradiol benzoate. The responsiveness of the pituitary to GnRH was greatest on the day of estrus, presumably because estradiol had been elevated (Reeves et al., 1971). This was attributed to an increased pituitary sensitivity to GnRH, due to both an absence of inhibiting effect of progesterone and a stimulating effect of estrogen. Padmanabhan et al. (1978) incubated bovine pituitary cells with estradiol-17 β for 4 or 28 h and exposed them to GnRH (0 and 100 ng/ml) during the final 2 h of incubation. Incubation of pituitary cells with E₂ for 2 h followed by GnRH did not affect LH secretion. In contrast, all concentrations of GnRH tested stimulated LH secretion in cells pre-treated with E₂ for 26 hours. The authors concluded that an increased concentration of E₂ for a period of time was required for the GnRH induced-release of basal LH. Similar findings were later reported by Moss et al. (1980) and helped to explain the positive feedback by E₂ that triggers the LH surge. In contrast, pulsatile frequency of tonic LH is regulated by estradiol negative feedback on GnRH in anestrus ewes and by progesterone negative feedback in cyclic ewes.

LH stimulates those follicles that have acquired LH receptors and are responsive to increased tonic LH secretions. As a result, follicular secretion of estradiol-17 β rises and eventually peaks. However, despite major influences of estradiol on the duration of estrus and timing of the LH surge, it has little effect on the intensity of estrous behavior (Fabre-Nys and Martin, 1991).

Behavioral estrus, which lasts for about a day, is influenced mainly by the time of progesterone withdrawal and rate of change and timing of concentrations of estradiol. During estrus, concentration of estradiol falls abruptly, immediately after the LH surge,

which induces ovulation.

Based upon the above observations, estrus, ovulation and timing of the LH surge (Dutt and Casida, 1948; Karsch et al., 1980) are determined not only by estrogen production by the ovulatory follicle (Scaramuzzi et al., 1971), but also by the initial concentrations of progesterone when luteolysis is initiated (Acritopolou et al., 1977; Deaver et al., 1983).

The luteal phase (metestrus and diestrus)

The luteal phase (metestrus and diestrus) is the longest period of the cycle, approximately 13 to 14 days. Metestrus follows estrus and lasts for one to two days. During this period, concentrations of both estrogen and progesterone are relatively low. The ovulated follicle undergoes luteinization of thecal and granulosa cells, which results in the formation of the CL (Niswender et al., 1985). This process results in a gradual increase in concentration of progesterone, which occurs as the CL forms over a period of approximately four days (Britt, 1989). The first four days after estrus are characterized by a small rise in estradiol, attributed to the first cohort of follicles, until the gradual rise in progesterone suppresses LH pulse frequency.

The remainder of diestrus is characterized by high concentrations of progesterone and low concentrations of estrogen. Peripheral concentrations of progesterone begin to increase around day 3 after ovulation, which coincides with the activation of the newly-formed CL. Maximum concentrations of progesterone occur around day 10 to 12 and are maintained through day 14 to 15 (Scaramuzzi et al., 1993). The rise in progesterone sets the timing of increased uterine secretion of $\text{PGF}_2\alpha$, which begins around day 12 or 13 and initiates luteal regression by day 15, which signals

entrance into pro-estrus and the start of a new estrous cycle. In a mated ewe, a viable conceptus produces interferon-tau and other factors that prevent the regression of the CL, such that pregnancy is recognized and luteal function is maintained (Spencer and Bazer, 2002).

Follicular growth and development during the estrous cycle

Role of follicle stimulating hormone (FSH) in follicular growth and ovulation rate.

The resting pool. At any one time, it has been suggested that there are 50 antral follicles in the ovaries of adult ewes (Cahill et al., 1979; McNatty et al., 1982; Driancourt et al., 1986). Growth of the selected follicles during a wave initiated by an increase of FSH is maintained by LH, once they have developed LH receptors (Scaramuzzi et al., 1993). However, breeds with high ovulation rates have more growing follicles than breeds with low ovulation rates (Cahill et al., 1979). The number of primordial follicles laid down at birth constitutes a resting pool from which the first growing follicles are recruited at about 70 days of gestation (Mariana et al., 1991). These follicles undergo maturational changes in both morphology and size and are classified as primary, secondary and tertiary. However, even after puberty, relatively few follicles progress to the tertiary stage and even fewer to ovulation; many undergo atresia during the primary and secondary stages. The development of follicles from the resting stage to preovulatory stage requires 34 to 43 days (Turnbull et al., 1977; Cahill and Mauleon, 1980). The mechanism that determines which follicles are recruited from the resting pool is not understood.

Follicular recruitment, follicular waves and dominance. The proposed mechanism of preovulatory folliculogenesis begins with recruitment of small antral follicles from the

resting pool. One or more of the antral follicles are eventually selected to continue maturation, ultimately being ovulated or becoming atretic (diZerega and Hodgen, 1981; Driancourt et al., 1984). All follicles in a cohort in the ewe arose within a span of 2.3 to 3.0 days (Ginther and Kot 1994; Bartlewski et al., 1999).

Follicle stimulating hormone is required for the recruitment of antral follicles into a growing pool (Picton et al., 1990). In hypophysectomized and unilaterally ovariectomized ewes, Dufour et al. (1979) found that the population of preantral follicles (0.1 mm diameter) was regulated by gonadotropins. In contrast, McNatty et al. (1990) found a significant reduction in the weight of the ovary, but no reduction in the total number of antral follicles (2 mm diameter) for as long as 70 days after ewes were hypophysectomized.

The existence of a wave-like pattern of growth of follicles in sheep is equivocal. For example, Turnbull et al. (1977) postulated that continual growth and regression of follicles occurred during the estrous cycle, an observation supported by (1) the presence of normal and atretic follicles of various sizes on each day of the cycle, (2) the unpredictable time of appearance of very large diameter follicles during the luteal phase of different ewes, and (3) growth of the ovulatory follicle can occur at any stage of the estrous cycle upon removal of the corpus luteum. The latter finding corroborated the earlier results of Smeaton et al. (1971) and was supported later by Lahlou-Kassi et al. (1984), and by Schrick et al. (1993) and Ravindra et al. (1994), who followed follicles with ultrasonography.

In ultrasonographic studies that were restricted to only larger follicles (3 to ≥ 5 mm in diameter), a wave-like pattern emerged (Ginther et al., 1994; Leyva et al., 1998b;

Souza et al., 1998). With this restriction, follicles appeared to be recruited and undergo growth and atresia in patterns of two, three (Noel et al., 1993) or four waves during the luteal phase (Ginther et al., 1994). Duggavathi et al. (2005) treated ewes with FSH between expected endogenous peaks of FSH and found that recruitment of additional cohorts of follicles into the growing phase, did not modify either the endogenous peaks of FSH or the effect of those peaks on recruiting cohorts of follicles. Thus the regulatory mechanism that initiates follicular recruitment in sheep may be different from that in cattle.

The term follicular dominance is used to define the inherent capacity of a follicle to prevent the growth of other follicles on both ovaries without compromising its own growth (Driancourt, 1991, 1994; Webb et al., 1999). Follicular dominance in ewes might be dependent on the stage of the cycle. Duggavathi et al. (2003) suggested that the demise of the ovine CL might be a contributory factor. In their study, the total number of small follicles began to increase two days prior to ovulation and after luteolysis, in agreement with Baird et al. (1978) and Bartlewski et al. (1999). There is other evidence for dominance of ovulatory follicle in the follicular phase as reported by Ravindra et al. (1994) and in later studies (Lopez-Sebastian et al., 1997; Evans et al. 2000; Gonzalez-Bulnes et al., 2001).

Evans et al. (2000) observed that the larger follicle has more estradiol and a higher estradiol: progesterone ratio than the other follicles. In a later study, Evans et al. (2002) postulated that their previous observations might be attributed to follicular dominance. They found that the ablation of the largest follicles on day 4.5 post-estrus resulted in continued growth and increased life span of the second largest follicle, in

agreement with the earlier studies by Rubianes et al. (1997).

In the luteal phase, Ginther et al. (1994) and Rubianes et al. (1997) found that the largest follicle may prevent the development of other follicles. In sheep, recruitment of follicles ≥ 2 mm was high at the beginning of the wave and remained low during the growing phase of the largest follicle, but growth of another follicle might occur in the presence of the largest follicle (Viñoles et al., 1999). Other authors also have observed the growth and emergence of follicles in the presence of larger follicles from a previous wave (Schrick et al., 1993; Johnson et al., 1996; Leyva et al., 1998b; Bartlewski et al., 1999; Flynn et al., 2000). Bartlewski et al. (1999) observed that such follicles not only were able to grow, but also to ovulate.

In contrast to the ewe, the evidence for follicular dominance in cattle is more definitive. In cattle, follicular development during an estrous cycle occurs in 2 or 3 waves that involve follicular dominance, which occurs at the point of divergence in size between two largest follicles, along with the suppression of the total number of growing follicles (Ginther et al., 1996). Sufficient dominance exists in cattle that recruitment of new follicles into the growing pool occurs only after the dominant follicle loses its dominance (Adams et al., 1992; Evans et al., 1997). The role of dominance in recruitment of follicles and continued folliculogenesis in the ewe is uncertain and is still debated.

Follicular maturation. Both duration of exposure to secretion of FSH and sensitivity to FSH can affect ovulation rate. For example, gonadotropin-responsive follicles have an absolute requirement for FSH to continue to develop. As these follicles grow, they become gonadotropin-dependent and require increasing concentrations of

FSH. Once they acquire LH receptors, they need increasing concentrations of LH, which is controlled by progesterone, as discussed earlier. If they remain viable, a recruited cohort of follicles secretes increasing amounts of estradiol and inhibin, which exert a negative feedback that reduces the secretion of FSH, thus limiting further follicular recruitment. Therefore, the numbers of ovulatory follicles are dependent on sensitivity of the hypothalamo-pituitary axis to the inhibitory effects of estradiol and inhibin on FSH and progesterone on LH (Scaramuzzi et al., 1993).

Effects of progesterone on follicular development.

Progesterone inhibits tonic LH secretion (Baird et al., 1976), thus affecting follicular growth. High concentrations of progesterone limit the growth of the largest non-ovulatory follicles in follicular waves during the luteal phase. Those follicles were found by Sirois and Fortune (1988) in cattle, and Noel et al. (1993) in sheep, to be smaller than the largest follicles in the ovulatory wave. These observations were made during the early and late luteal phase when concentrations of progesterone might be low due to early stages of luteinization or the decline in the progesterone output from the CL. Similarly, Brand and de Jong (1973) found that more follicles were recruited at the beginning and end of the estrous cycle, periods of low concentrations of progesterone. That result was supported later by Schrick et al. (1993), based on ultrasonographic observations. All of these findings support the hypothesis that high concentrations of progesterone limit the development of follicles during the luteal phase.

Approaches to increasing ovulation rate

Breeds and genetic mechanisms involved in prolific and non-prolific sheep. Breed is a

major factor that determines the ovulation rate (Davis et al., 2001; Montgomery et al., 2001; Bodin et al., 2002; Galloway et al., 2002). McNatty et al. (2003, 2004) postulated that the oocyte played a key role in both follicular development and ovulation rate. They suggested that the oocyte controlled-processes that differed between species of low and high ovulation rate phenotypes and that control was mediated by oocyte-secreted factors. This concept may explain why some mammals are more predisposed to multiple or single offspring. The discovery of bone morphogenic protein 15 (BMP 15) and its receptors (BMP 15 is expressed only in the oocytes; reviewed by McNatty et al., 2003) has provided an explanation as to why some breeds are prolific. For example, the Booroola gene involves a mutation in the BMP 15 receptor in oocytes, granulosa cells and luteal cells. Alternatively, genetic changes in sensitivity to FSH have been postulated to influence ovulation rate (Scaramuzzi et al., 1993).

In prolific ewes, two alternative mechanisms have been postulated to account for the occurrence of multiple ovulations. Scaramuzzi et al. (1993) suggested that recruitment of follicles was based on increased sensitivity to FSH and that support of their continued growth shifted to LH, which stimulates the production of androgen by the theca that is aromatized to estradiol in the granulosa. In this concept, a “window of opportunity” to ovulate is a period during which follicles are sensitive to FSH. In mechanism A, multiple ovulations occur as a result of increased numbers of follicles that retain the ability to respond to gonadotropin (FSH) for a longer period of time (window) and resist atresia. In mechanism B, a greater number of small antral follicles become gonadotropin-responsive in a narrower window of opportunity. Each mechanism is dependent upon the acquisition of LH receptors by developing follicles, and the two are not mutually exclusive, even though mechanism A is

dependent on duration of gonadotrophin sensitivity and mechanism B on greater numbers of recruited follicles.

In the prolific Booroola ewe, which carries the F gene, recruited follicles were protected from atresia (Driancourt et al., 1985), and a longer period of recruitment resulted in a greater number of follicles available for ovulation. In contrast, rapid turnover of the largest follicles was observed in monovulatory Merino del Pais ewes (Lopez-Sebastian et al., 1997). In prolific breeds of sheep, such as the Romanov, Booroola, and Finnish Landrace, mechanism A might be responsible for high ovulation rates. There is corroborating evidence that the recruited follicles were maintained longer on the ovary, allowing for a larger number of follicles to be ovulated (Driancourt et al., 1986).

Webb et al. (1989) and Driancourt et al. (1990) reported that the total number of large follicles, on both ovaries combined, at days 13 and 15 of the estrous cycle was similar to the ovulation rate. They attributed the differences in the ovulation rate between the Finn sheep, which were selected for high ovulation rates, and the Merino, to changes in growth and differentiation of the ovulatory follicles. However, these findings were contradicted by Noel et al. (1993), who found that the number of large follicles in Suffolk ewes was 2 to 3 times the ovulation rate. Interestingly, Duggavathi et al. (2003) postulated that ovulation rate was dependent on a stable small follicular recruitment pool that did not change throughout the estrous cycle except around the periovulatory period; these more recent observations were supportive of the previous findings of Webb et al. (1989) and Driancourt et al. (1990).

Hormonal approaches: Use of FSH.

Follicle stimulating hormone regulates the ovulation rate through the number of

follicles recruited (Driancourt et al., 1991). However, the variability in concentrations of FSH among breeds has created some doubt as to the full extent that it is involved in ovulation rate. Even though both the Romanov and Finn are prolific breeds, the high concentrations of FSH in the Romanov (Cahill et al., 1981) are in direct contrast to the low concentrations in the Finn (Adams et al., 1988). Furthermore, despite the high concentrations of FSH in the prolific Booroola Merino (McNatty et al., 1987), the ovulation rate is dependent on the presence of a major gene, which regulates the increased concentrations of progesterone secreted from an increased number of CL (luteal tissue; McNatty et al., 1990). Thresholds in responsiveness to FSH among follicles and within ewes might explain some of the high variability in ovulatory response to FSH. Fry et al. (1996) observed three groups of ewes; group 1 had three successive estrous cycles in which ovulation rates were the same, group 2 had three different ovulation rates during three cycles and group 3 had the same ovulation rate in two of the three estrous cycles. However, they were unable to identify thresholds in FSH concentrations associated with changes in ovulation rate; they attributed the inability to determine a threshold to variation in response of the individual follicles within a ewe.

The direct effect of gonadotropin preparations on the follicle was greater for the less acidic isoforms of FSH, even though the number of women who became pregnant, with respect to different isoforms in preparations used for treatment, did not differ (Andersen et al., 2004). Therefore, responsiveness within ewes could be confounded by isoform (whether more or less acidic), when ewes are treated with exogenous gonadotropins that might contain different isoforms of FSH. Exogenous gonadotropins have improved prolificacy in some cases, but their high cost and unpredictable

responses have resulted in limited adoption in general reproductive management of livestock.

Hormonal approaches: Exogenous progesterone. Ovulation rates are influenced by concentrations of progestogen (Robinson et al., 1968; Allison et al., 1970). In comparing ewes on high and low dosages of progesterone, larger follicles were found in ewes treated with low progesterone (Dutt and Casida, 1948). Synchronization of estrus in ewes with a single sponge containing progestogen (60 mg medroxyprogesterone acetate) from days 5 to 19 (long-duration) produced fewer medium-sized follicles at the time sponges were removed (day 19) than in ewes treated with multiple progestogen sponges (day 5 and replaced on days 10 and 15; Flynn et al., 2000). They postulated that low concentrations of progesterone caused the development of aged follicles (dominant), which prevented further recruitment of small follicles, thus fewer small follicles were available to progress into medium follicles, and the number of large did not change. However, three of the eight ewes studied grew follicles from 3 mm to 4 mm even though a large follicle was present. In another study, ewes had their endogenous source of progesterone removed (Johnson et al., 1996) and were implanted with subcutaneous packets of progesterone in silastic. Ewes exposed to low concentrations of progesterone had greater LH pulse frequency and increased concentrations of estradiol, and they ovulated older (larger) follicles than control ewes. Similar results were found by Levya et al. (1998) and Viñoles et al. (1999). These findings might explain the reduction in pregnancy rate observed with ewes on low concentrations of progesterone (Johnson et al., 1996).

Effects of age and nutrition on ovulation rate and litter size.

The plane of nutrition of ewes prior to mating can influence ovulation rate. Bramley et al. (1976) observed that ewes, fed either on a low nutritional plane for six weeks prior to mating and for four weeks postmating (LL), or on a low nutritional plane for three weeks and then on a high nutritional plane (LH), or on a high nutritional plane throughout (HH), had significant differences in ovulation and lambing rates. There were linear increases in body weight, body condition score, ovulation rate and lambing rate for LL, LH and HH, respectively. This phenomenon was referred to as the flushing response, which occurred in ewes on an increased plane of nutrition before mating. The flushing response might be due to an induced increase in hepatic concentration of mixed function oxidase enzymes, which could increase the metabolism of steroids, reducing the inhibition to the hypothalamic-pituitary axis, and result in the secretion of more gonadotropins and increased numbers of ovulations and lambs (Thomas et al., 1987). In a later study, Nottle et al. (1997) observed a significant interaction between previous nutrition imposed 6 months prior to ovulation and lupin supplementation during the estrous cycle immediately before ovulation. Supplementation with lupins increased ovulation rate by 0.22 in ewes on a low plane, but not in ewes on a high plane of nutrition. Ewes on the high plane of nutrition exhibited higher ovulation rates but a lower conception rate to synchronized estrus than ewes on the low plane. Interestingly, the body condition score also influenced embryo survival. Ewes on the low plane with two or three ovulations had fewer lambs than ewes on the high plane. The authors concluded that uterine efficiency among ewes with multiple ovulations was higher for ewes with good body condition scores at mating regardless of post-mating nutrition.

Age at first breeding can affect the reproductive efficiency of the ewe. Quirke et al. (1985) observed that ovulation rates (CL count) in each of two consecutive estrous cycles were greater for ewes two years of age and over compared to yearlings. Body size and uterine capacity might be the first limiting factors for the reduction in lambs born to yearlings. In conclusion, flushing is a mechanism that can be utilized to increase ovulation rate and litter size, but there are limits to increases in litter size other than ovulation rate.

Immunological approaches: antibodies. Active immunization of sheep against steroids and inhibin increased ovulation rate. Estrogen is a negative feedback inhibitor of LH and FSH, thus immunization against estradiol would result in an increase of both of these gonadotropins (Scaramuzzi, 1994). In contrast, immunization against androstenedione increased LH secretion but had little or no effect on FSH (McNatty et al., 1988). The ovaries of androstenedione-immunized ewes usually contained two follicles, which doubled the production of inhibin (Campbell et al., 1990a). The relative lack of androstenedione substrate prevented its aromatization to estradiol (Campbell et al., 1990c). Additionally, the negative feedback effect is partially blocked due to antibodies that bind estradiol and progesterone; thus, plasma LH is increased, while FSH remains the same. The mechanism of how an increase in the ovulation rate occurs is not fully understood.

Immunization against either inhibin or inhibin-enriched follicular fluid immunoneutralized the biological activity of endogenous inhibin (Price et al., 1987). Consequently concentrations of FSH were increased (O'Shea et al., 1989). As a result of increased concentrations of FSH, more follicles were recruited. The use of

immunization techniques has had limited application on sheep farms; immunogen has been available commercially only in New Zealand and Australia.

Pre and post-ovulatory factors that affect lambing rate.

Ram introduction. The sudden introduction of rams to a group of previously-isolated ewes can interrupt anestrus; this is known as the ram effect. The ram induces physiological changes within the ewe that affect hormonal patterns, time to estrus, ovulation rate and fertility. The physiological effects are mediated largely by pheromones (Cohen-Tannoudji et al., 1989; Rekwot et al., 2001) and to a lesser degree through other non-olfactory cues (Cohen-Tannoudji et al., 1989; Pearce and Oldham, 1988). At ram introduction, increased secretion of LH occurred within minutes (Martin et al., 1980; Atkinson and Williamson, 1985; O'Callaghan et al., 1994). Oldham et al. (1978) found that exposure of ewes to a ram resulted in an ovulatory surge that occurred within 6 to 52 h, premature ovulations that varied between 1 to 3 days, and the formation of inadequate or short-lived corpora lutea.

Evans et al. (2004) observed that ram exposure, during the last three days of treatment with 30 mg fluorogestone acetate pessaries for 13 days, advanced the timing of the onset and end of estrus, the LH surge, and ovulation by 2 to 6 h in crossbred ewes and decreased the duration of estrus compared to the control. However, the ewes exposed to rams before withdrawal of progestogen in one experiment had lower fertility, possibly due to some ewes not being bred during the early-induced estrus. Raddled, intact rams were introduced 48 h after sponge removal. Fewer ewes were bred in ram-exposed versus the control, and of those bred, fewer lambed. Ram-induced LH

secretion, follicular development, estradiol production, and early onset of estrus might have led to ovulation of aged oocytes and lower fertility, in agreement with the previous findings of Martin et al. (1983). In a second experiment (Evans et al., 2004), ewes received an intravaginal sponge for 14 days and on the 11th day were divided into control and ram-exposed ewes. Rams used during treatment were separated from ewes at sponge withdrawal and breeding rams were introduced 24 h later.

The responses to ram introduction at progesterone withdrawal were altered from introduction of rams alone. The length of time to onset of estrus was directly related to dosage of progesterone; a larger dose of progesterone resulted in a longer latency period (Robinson and Smith, 1967; Lewis and Inskeep, 1973; Pearce and Robinson, 1985). Parsons and Hunter, (1967^a) found that estrous periods synchronized by injections of progesterone were shortened by the continuous presence of the rams by 30 to 50%. In another study, Parsons and Hunter, (1967^b) found that in ewes teased at 4-h intervals (control), ovulation occurred on average shortly after the end of estrus, but the ewes that remained continuously with the rams had a longer interval to ovulation. The authors concluded that there was a correlation between length of estrus and time of ovulation in ewes that were continuously with rams, but not in controls.

Hormonal and environmental factors and age of ovulatory follicle. Embryonic loss can be caused by environmental factors, in particular high ambient temperatures prior to pregnancy recognition (Alliston and Ulberg, 1961; Thwaites et al., 1967) or by low concentrations of progesterone secreted by the CL and placenta that are required for the maintenance of pregnancy. Embryonic loss was correlated with low concentrations of progesterone (1 ng/ml), particularly during the period immediately before and after

maternal recognition of pregnancy (Henricks et al., 1971; Lukaszewska and Hansel, 1980; Lamming et al., 1989; Kastelic and Ginther, 1991). Even though conception rates between 70 to 80% sometimes have been achieved with synchronization protocols using progestagens (Gordon, 1997), one possible disadvantage is a reduction in the productivity of farming enterprises due to embryonic loss and, consequently a lower weaning rate.

Ovulation of aged follicles can reduce fertility due to a poor quality oocyte, as observed in rats (Butcher, 1972) and in cattle (Stock and Fortune, 1993; Mihm et al., 1999). Induction of luteolysis at the beginning of progestogen synchronization resulted in ewes ovulating old (7 to 14 days) or young (4 to 5 days) follicles, unlike those that were exposed to high concentrations of progesterone continuously, which ovulated young follicles only (3 to 6 days; Flynn et al., 2000). However, in the study by Evans et al. (2001) the age of the follicles had no deleterious effects on birth of live animals. Elevated concentrations of LH, estradiol or both might reduce fertility by the disruption of signaling mechanisms in the oocyte; primarily premature activation of the oocyte (Revah and Butler, 1996; Mihm et al., 1999) or ovulation of an aged oocyte (Roche et al., 1999) in cattle. Research to determine whether estradiol or LH might be responsible for the demise of the early embryo is yet to be done, except in rats. In that species, Butcher and Pope (1979) showed direct effects of estradiol on embryonic development and congenital anomalies.

Actions of progesterone on follicles, the uterus and embryogenesis

A successful pregnancy is dependent on successful embryogenesis

(development of the conceptus), which is influenced by extrinsic and intrinsic factors, including endocrine patterns prior to conception. Progesterone might directly (oviduct or uterus) or indirectly (gonadotropins) influence gamete transport (Allison and Robinson, 1972), fertilization (Soede et al., 1994), cleavage, the formation of a morula and a blastocyst (Oussaid et al., 1999), hatching of the blastocyst from the zona pellucida, elongation of the blastocyst and the initiation of attachment during maternal recognition of pregnancy (Lukaszewska and Hansel, 1980; Lee and Ax, 1984; Lamming et al 1989).

Asdell et al. (1949) found that progesterone influenced the remodeling of the myometrium and endometrium of the bovine uterus in preparation for implantation and their findings were corroborated in ewes by Miller and Moore (1983). The latter authors removed explants over the first 10 days of the estrous cycle from the endometrium of untreated ewes and from (pregnant) ovariectomized ewes treated with estradiol and progesterone. When progesterone was given before estradiol, it inhibited the tissue RNA:DNA and protein:DNA ratios in intercaruncular and caruncular endometrium at about 4 to 7 days after estrus. They concluded that embryonic development was impaired in bred ewes that were ovariectomized and did not receive supplemental progesterone.

Ottobre et al. (1980) found that ewes treated with 40 mg progesterone at 8 and 32 h after onset of estrus (day 0) had short cycles (11.0 ± 1 days) compared to the average cycle length (17.0 ± 0.3 days) in untreated controls. The treatment advanced timing of greater secretion of $\text{PGF}_2\alpha$, which began on day 8, compared to day 12 for the control, and led to early luteal regression and shortened the cycle. The elevation of estrogen at estrus increases the concentration of progesterone receptors; as a result,

increased progesterone sets the subsequent timing of the secretion of $\text{PGF}_2\alpha$ (ewe - Ottobre et al., 1980, 1984; postpartum beef cow - Zollers et al., 1993). Short luteal phases in cattle (Cooper et al., 1991) and sheep (Southee et al., 1988) were associated with premature release of $\text{PGF}_2\alpha$ from the uterus. Kieborz-Loos et al. (2003) found that oxytocin-induced release of PGFM was inhibited on day 6 after a second exposure to progesterone treatment in postpartum ovariectomized cows, which were treated previously with estradiol-17 β . These authors concluded that both estradiol-17 β and progesterone were involved in timing secretion of $\text{PGF}_2\alpha$ in the postpartum cow.

Vincent et al. (1986) treated ewes with daily injections of progesterone on days 1 through 3 post estrus and transferred a day-10 blastocyst into the uterus on day-6 postestrus. One group of control ewes was treated with vehicle and a day-10 blastocyst was transferred into the uterus on day-6 postestrus. Concentration of PGE_2 was elevated beginning on day 8 in progesterone-treated ewes that received a day-10 blastocyst on day-6 postestrus but did not increase until day 12 in mated pregnant ewes. Interestingly, the authors observed that the uterus in progesterone treated-ewes was able to support a day-10 embryo but not a day-6 embryo transferred on day-6. Untreated ewes supported a day-6 but not a day-10 embryo transferred on day-6. These observations support the suggestion that progesterone is involved in the coordinated temporal regulation of uterine development. Garrett et al. (1988) observed that 14-day-old conceptuses from beef cows treated with progesterone on days 1 through 4 were more developed with the capacity to produce the maternal recognition protein bTP-1, unlike the untreated controls. In a later study, Albiñ et al. (1991) found a correlation between concentrations of progesterone and the development of 17-day-old

embryos.

Progesterone is involved in the quality of the embryo. Henricks et al. (1971) observed an inverse relationship between plasma concentrations of progesterone during the 2-day period before estrus in the cow and the number of blastomeres in the conceptus 3 days after mating. The interaction of concentrations of progesterone and estrogen can impact the embryo directly or indirectly (CL). The dosage of progesterone required to maintain pregnancy in beef heifers that were ovariectomized, allowed pregnancy loss when given to unilaterally ovariectomized heifers, due to higher concentrations of estrogen in the unilaterally ovariectomized animals (Zimbelman and Smith, 1966). From this observation, it was concluded that elevated concentrations of estradiol inhibited embryo maintenance (Butcher et al., 1992; Maurer and Echterkamp, 1982).

Low progesterone might have a negative impact on embryogenesis indirectly through increased LH following inadequate formation of the CL. The increase in LH and the concomitant rise in estradiol might have decreased embryo survival in the sow (Blair et al., 1993). In sheep, Oussaid et al. (1999) found that cleavage and development to the 16-cell stage were impaired by suppressed estradiol during the follicular phase, which was indirectly inhibited by GnRH antagonist. This might have resulted in reduced implantation and accounted for increased embryonic loss.

2. The origin of hair sheep and breed characteristics

Hair sheep comprise 10% of the world sheep population (Bradford and Fitzhugh, 1983). Their origins and current geographical locations are mainly in South America,

South Africa and the Caribbean. They are prolific breeders throughout the year (Bradford and Fitzhugh, 1983), with an apparent resistance to internal parasites (Yazwinski et al., 1979), and with lower rectal temperatures (Horton et al., 1991), respiration rates (Ross et al., 1985), dry matter intakes (Warren et al., 1984; Quick and Dehority, 1986) and water intakes than wool breeds (Quick and Dehority, 1986). One breed of hair sheep is the Barbados Blackbelly (BB). The BB coat color ranges from a light fawn to a deep reddish-brown with a black belly, and black markings extending from around the eye to its polled forehead, the nose and under the chin, and black stockings extending from the inner thigh to the hoof. The BB is a non-seasonal breeder, with a litter size of approximately two lambs (Patterson, 1983) and a 78.5 % lamb survival to weaning (Fitzhugh and Bradford, 1983). The BB evolved from crosses of parent stocks of African hair sheep and European woolled breeds. Ligon (1657) suggested that the origin of the breed was an evolution from these parent stocks, which gave rise to a highly prolific and tropically-adapted breed in Barbados during the first quarter-century of colonization.

3. Application to the Barbados Blackbelly ewe

The goal of sheep producers is to maximize efficiency of production through reduction in feed costs, improvements in average daily gain, reduction in morbidity and mortality, and increased reproductive efficiency. A sheep selection program (Greenland Livestock Research Station) has been implemented in Barbados to improve the current average daily gain of 0.31 kg.

General management strategies ensure that nutritional and health care needs

are met. However, reproductive efficiency and further genetic improvement can be enhanced through the use of artificial insemination (AI) with frozen semen and an efficient synchronization protocol. Synchronized lambing allows producers to be present at lambing and enhances chances to reduce mortality, which occurs at parturition due to hypoxia.

The livestock industry in Barbados consists mainly of small producers with fewer than 25 ewes, and 27 major sheep breeders with approximately 100 ewes each. There is an emerging niche market in the tourism and restaurant sector for fresh Barbados Blackbelly lamb and international requests for frozen semen. The main constraint in development of these markets is the inability to develop production systems that will maximize the inherent prolificacy of the breed, increase production efficiency, and minimize cost of production.

The goal (Barbados Sheep Producers Association) is to have three lambings in two years using timed artificial insemination, thus providing a supply of high quality lamb and sires continuously throughout the year. To help sheep producers achieve these objectives, an efficient method of estrous synchronization must be developed.

Statement of the Problem

Some protocols for estrous synchronization reduce conception rates through the production of aged oocytes or inadequate estrogen, coupled with the distribution of interval to estrus being too great for timed AI. The effects of progesterone on ovulation and lambing rates were investigated within a breed at a location in which seasonality is not expressed, by varying concentrations of progesterone. Therefore, the confounding factors in many of the studies outlined previously with respect to breed and season

were removed in this study. The aim was to compare effects of three concentrations of progesterone on ovulation and lambing rates. Controlled internal drug releasing devices (CIDR) impregnated with progesterone were used to create low and high concentrations of progesterone. Van Cleeff et al., (1998) measured concentrations of progesterone of 3.3 ± 0.2 ng/ml (range, 1.0 to 5.9) and 2.7 ± 0.3 ng/ml (range 1.4 to 8.5 ng/ml) at CIDR insertion on approximately day 8 of the estrous cycle (10 days after PGF₂α), and removal (8 days after insertion), respectively. In the present study, high concentrations of progesterone were achieved by insertion of a CIDR in ewes with intact CL. Low concentrations of progesterone were brought about by insertion of a CIDR, followed by removal of the CL by PGF₂α, and medium concentrations were expected in ewes with an intact CL only. In a subset of ewes within each treatment group, the follicles were followed from recruitment to ovulation by ultrasonography. Embryonic and fetal survivability was determined by the number of lambs born per CL counted at 7 days after post-treatment estrus.

Materials and Methods

The experiment was conducted in Barbados (latitude 10⁰N) with 168 Barbados Blackbelly ewes in two replicates. Sixty nine ewes were studied in the dry season (December, 2003) and 99 in the wet season (July, 2004). The ewes had average ages of 2.5 years, parities of 1.8 and body weights of 43 kg, which did not differ among treatments or between replicates. The ewes were penned in an enclosed barn and received a daily maintenance ration of 2 kg of concentrates (corn, soybean meal and minerals formulated to contain 18% crude protein), with water and pangola hay ad libitum.

Ewes were randomized to three treatment groups (low, medium, or high progesterone) of 23 or 33 ewes each for replicates 1 and 2, respectively. All ewes were treated twice, 8 days apart, to synchronize estrus before the study. Each time they were given two injections of 5 mg PGF₂α (Lutalyse®, Pfizer Animal Health) 3 hours apart, which mimics the natural episodic secretion of PGF₂α by the uterus (and by the CL in response to uterine PGF₂α). Ewes that were between estrus and the 4th day of their cycle would have non-responsive CL at first treatment, however, at the second treatment, CL were expected to be 6 to 12 days old and synchronous luteolysis should occur. Each ewe assigned to low progesterone received a progesterone-containing insert (Controlled Internal Drug Releasing Device [CIDR] containing 0.3 mg of progesterone) on day 4 after estrus and was given PGF₂α on d 6 (as in the synchronization protocol) to regress the CL and remove endogenous progesterone. These ewes were expected to have low circulating concentrations of progesterone. Ewes in the high progesterone group received a CIDR on day 4 to provide additional progesterone to that produced by the CL. In each group, the CIDR was removed on day 14. Untreated ewes were used as controls, and were expected to have medium circulating concentrations of progesterone.

Breeding soundness examination, ram introduction and observation for estrus

A breeding soundness examination (testicular size, sperm concentration and motility; Salamon, 1976) was performed on six Barbados Blackbelly rams, each of which was found to be in breeding condition. On day 7 after estrus, the rams were raddled and allotted to six pens, each of which contained five or six ewes from each of the low, medium, and high treatment groups. A maximum ewe-to-ram ratio of 17:1 was

maintained. Ewes were observed on days 7 through 21 at 6 am and 6 pm for raddle marks. Time and date of observed estrus were recorded to determine the interval from CIDR removal to post-treatment estrus (marked by a ram; 0.5 d increments). After each observation for estrus, briskets of the rams were repainted with different colors to distinguish ewes that subsequently came into estrus and to ensure that raddle marks were visible.

Observations of follicular development

Follicular development and ovulation were observed by transrectal ultrasonography using an Aloka 500 SSD (Corometrics) equipped with a 7.5 MHz linear-array transducer, as described by Schrick et al. (1993). Ultrasonographic observations were done by two operators in replicate 1, but by only one of them in replicate 2. Ovaries of 10 ewes per treatment in replicate 1 and of 8 ewes per treatment in replicate 2 were scanned daily from day 5 through subsequent estrus and ovulation. Ovarian follicular diameters were measured in three categories; small (2 to 3 mm), medium (4 to 5 mm), and large (≥ 6 mm), and their relative positions and those of CL were recorded on an ovarian map. This allowed determination of the sequential appearance, growth, atresia and disappearance (ovulation) of follicles. Data recorded included the day of emergence of each ovulatory follicle, the numbers of small, medium and large follicles, and maximum size of each ovulatory follicle prior to ovulation. Growth rates of follicles were determined by retrospective comparisons of the follicle sizes recorded up to the point of plateau in diameter or ovulation. The interval from day of appearance (at 2 or 3 mm) to disappearance was termed the life span of the follicle. Follicles that were first detected at 4 mm were recorded as 3 mm one day earlier, because average growth rate

approximates 1 mm per day (Schrack et al., 1993). After estrus, follicles ≥ 4 mm were recorded as ovulated if not observed on the ovary at the subsequent scanning (Schrack et al., 1993). The interval from CIDR removal to ovulation was measured from these observations. Ovulation rate was confirmed by the number of CL observed by ultrasonography seven days after ovulation.

Lambing dates and numbers of lambs born were recorded. Conception rate was determined by ewes lambing as a percentage of ewes in estrus. Pregnancy rate was measured as the percentage of ewes treated that lambed. Prolificacy was expressed as the number of lambs born per ewe that lambed.

Sampling and assay for progesterone

To determine circulating concentrations of progesterone, 10 mL of blood was collected by jugular venipuncture at 5:30 am, prior to transrectal ultrasonography, from a subset of eight ewes per treatment in each replicate on selected days. Collections were made at pre-treatment estrus (day 0), and on days 4 (CIDR insertion), 6 (PGF₂ α), 8, 11, 12, 14 (CIDR removal), and 16 and post-treatment estrus. At each bleeding, the sample was collected into a heparinized vial and centrifuged immediately. The plasma was transferred to glass ampules and stored at -20 C until assayed for progesterone. Estrus was confirmed by low concentrations of progesterone, and patterns of concentrations during treatment were used to validate treatments.

The DSL-10-3900 ACTIVE[®] Progesterone Enzyme Immunoassay (EIA) kit, which was developed for measurement of progesterone in human beings, was used according to instructions by the manufacturer (Diagnostic Systems Laboratories, Inc., Corporate Headquarters, 445 Medical Center Blvd. Webster, Texas 77598-4217. USA) for assay

of progesterone. The sensitivity of the assay was 0.13 ng/mL. In this study, the EIA values (mean 8.7 ng/ml) were similar to the range obtained using ELISA (Godfrey et al., 1997; OVUCHECK, Cambridge Veterinary Sciences Ltd) and RIA (Van Cleeff et al. 1998). However, the reagents in the kit were not tested for cross reactivity with lipids or other components of sheep plasma, and the samples were tested without extraction.

As expected, concentrations of progesterone increased after insertion of the CIDRS on day 4 (Figure 1). In ewes treated with PGF₂ α on day 6 (low progesterone group), concentrations of progesterone were about 1 ng/ml lower than in the high group from days 6 through 14, but remained above the ewes expected to have medium progesterone until day 12. Higher concentrations of progesterone were maintained in the high compared to the medium and low groups until day 14 when the CIDRS were removed; then progesterone declined precipitously in each group.

Figure 1 Concentrations of progesterone (ng/mL) relative to day 0 in ewes treated to

have high (◆), medium (■) or low (▲) progesterone.

Statistical Analysis

Significant differences were set at $p < 0.05$ and $p < 0.01$. The intervals from CIDR removal to estrus and ovulation and the growth rate of follicles in the final and penultimate waves were analyzed by GENMOD procedure. A square root transformation on counts of follicles was performed. Differences in the average diameter of all ovulatory follicles and growth rate of follicles with both final and penultimate waves combined were compared as least squares means, by analysis of variance with PROC MIXED GLM with a square root transformation, using version 8.1 of Statistical Analysis Systems Institute (2002). The number of follicles per day with ewe as a repeated autoregressive measure and number of ovulatory follicles were compared by GENMOD using Poisson regression. The data were normalized to day 0 for the scanned period and the effects of treatment on the numbers of small (2 to 3 mm), medium (4 to 5 mm) and large (≥ 6 mm) follicles were compared, with ewe nested within treatment and day and day by treatment interaction within ewe. Conception rate and pregnancy rate were examined by Chi-square. Lambs born per CL, per ewe lambing (prolificacy), and partial embryonic loss were compared by GENMOD procedure using logistic regression. The number of follicles and the interval (days) that each follicle was observed in the final and penultimate waves, and lambing patterns were analyzed by PROC MIXED GLM. Correlation coefficients was calculated to examine the relationship between follicles that disappeared and CL formed.

Results

Interval to estrus

Time to estrus was determined as the interval from removal of the CIDRS until the ewes were marked by raddled rams. The percentages of ewes marked were 94.6, 91.0 and 83.9 for low, medium, and high progesterone, respectively. Days to estrus were fewer for ewes treated to have low (1.5 ± 0.1) than medium (2.2 ± 0.2 ; $p < 0.0001$) or high (1.9 ± 0.2 ; $p < 0.01$) concentrations of progesterone. The interval (days) from CIDR removal to ovulation (Table 1) was less for ewes with low (2.0 ± 0.3) than with high (3.2 ± 0.2 ; $p < 0.05$) progesterone and tended to be less than for ewes with medium (2.9 ± 0.6 ; $p < 0.09$) progesterone.

Table 1. Occurrence of estrus and ovulation rates in Barbados Blackbelly ewes in response to low, medium or high circulating concentrations of progesterone during the previous cycle.

Variable	Concentration of Progesterone		
	Low	Medium	High
Number of ewes	56	56	56
Ewes marked by rams (%)	53 (94.6)	51 (91.0)	47 (83.9)
Interval from CIDR removal to estrus ^a	1.5 ± 0.1	2.2 ± 0.2	1.9 ± 0.1
Number of ewes observed for ovulation ^b	18	18	18
Ewes observed to ovulate %	88.8	83.3	83.3
Interval from CIDR removal to ovulation ^c	2.0 ± 0.3	2.9 ± 0.6	3.2 ± 0.2

a) Low vs High, $p < 0.01$; Low vs Medium ($p < 0.0001$).

b) Time of ovulation was estimated by disappearance of follicles at twice daily observations by transrectal ultrasonography.

c) Low vs High, $p < 0.05$; Low vs Medium ($p < 0.09$).

Follicle numbers, growth, ovulation rates and days to emergence

The numbers of large (≥ 6 mm in diameter) and medium (4 to 5 mm) follicles present each day during days 6 to 14 varied with day and day by treatment interaction ($p < 0.0001$), but number of small (2 to 3 mm) follicles did not differ (Figure 2). The total number of follicles on the ovary varied with day by treatment interaction ($p < 0.001$; figure 2). Ewes with low progesterone had fewer ($p < 0.01$) medium and large follicles prior to ovulation than ewes with high and medium concentrations of progesterone (Figure 3). One ewe with medium concentrations of progesterone in replicate 1, showed a cystic follicle prior to treatment, which remained on the ovary throughout treatment and did not ovulate, although the ewe came into estrus and ovulated two follicles on the opposite ovary. The disappearing follicles in the penultimate wave grew more slowly (0.7 mm/day) when compared to those of the final wave (0.9, mm / d). The ewes with high (0.7; mm/d), medium (0.8) and low (0.6) concentrations of progesterone did not differ in mean follicular growth rate in the final or penultimate wave. The effect of treatment (expected concentration of progesterone) on ovulation rate was linear ($p < 0.01$), regardless of whether ovulation rate was estimated by disappearance of follicles at estrus or number of CL on day 7 after ovulation. The number of follicles (≥ 4 mm) that disappeared in relation to estrus increased linearly ($p < 0.02$) with decreasing concentrations of progesterone. Disappearance of follicles was apparently an overestimate of ovulation rate. Ewes with low progesterone (2.6 ± 0.2) had a higher number of CL than ewes with medium progesterone (1.9 ± 0.2 ; $p < 0.05$) or high (1.7 ± 0.1 ; $p < 0.005$), which did not differ. The number of follicles that disappeared was correlated to the number of CL formed ($r = 0.53$; $p < 0.0001$).

Last diameters of ovulatory follicles did not differ among ewes on low (4.9 ± 0.5), medium (4.6 ± 0.7) or high (4.9 ± 0.5) concentrations of progesterone. The mean interval (days) from emergence at 2 or 3 mm to ovulation in the final or penultimate follicular wave did not differ (Table 2). The mean number of days that ovulatory follicles were present prior to ovulation was 5.7 ± 0.6 , 7.6 ± 0.6 and 8.1 ± 0.2 for ewes with high, medium and low concentrations of progesterone, respectively. The mean numbers of days from emergence to ovulation for ovulatory follicles were 9.0 ± 0.2 and 5.0 ± 0.2 for the penultimate and final waves, respectively.

Figure 2. Numbers of total follicles (height of each bar at each day) and numbers in each size class (Diagonal-2 and 3 mm; horizontal-4 and 5 mm; and cross hatched ≥ 6 mm) on days 4 through 16 in ewes on high (A), medium (B), or low (C) progesterone summed over both ovaries.

Figure 3. Numbers of follicles in each size class on the day prior to ovulation (Diagonal-high; solid-medium; horizontal-low). Numbers of medium and large follicles for the low progesterone group were greater than in the high and medium groups ($p < 0.01$).

Figure 4. The number of disappearing follicles in ewes with high, medium or low concentrations of progesterone. The number of disappearing follicles increased linearly with decreasing concentrations of progesterone ($p < 0.02$).

Figure 5 Mean growth rates of disappearing follicles in the final (▲) or penultimate (■) waves in low, medium and high progesterone groups. The follicular growth rates in the final or penultimate waves did not differ among treatment groups.

Table 2. Time from emergence to disappearance of presumed ovulatory follicles from the final or penultimate waves.

	Concentration of progesterone			All Groups
	High	Medium	Low	
Time from emergence to ovulation:	n=15	n=15	n=16	
Final follicular wave				
Total number of follicles observed	21	19	13	53
Interval (days)	4.6± 0.5 ^a	5.2 ± 0.3 ^a	5.5 ± 0.3 ^a	5.0± 0.3 ^c
Penultimate follicular wave				
Total number of follicles observed:	12	22	43	77
Interval (days)	8.2± 0.3 ^a	9.0 ± 0.3 ^a	9.22 ± 0.2 ^a	9.0 ± 0.2 ^d
Both waves				
Total number of follicles observed:	33	41	56	130
Interval (days)	5.7 ± 0.6 ^a	7.6 ± 0.6 ^b	8.1 ± 0.2 ^b	7.2± 0.4

Means in the same row with different superscripts (a,b,) differed among treatments(p<0.05; Duncan's multiple range test)

Means of waves differed (p < 0.05; designated by superscripts c and d).

Conception and pregnancy rates

Conception and pregnancy rates averaged 75.8 and 73.8%, respectively, and did not differ among treatment groups. Partial embryonic or fetal loss (CL not represented by lambs born) was greater (1.3 ± 0.2 ; $p < 0.006$) for ewes with low concentrations of progesterone than for either ewes on medium (0.9 ± 0.2) or high (0.8 ± 0.2) concentrations of progesterone.

Lambs born in relation to ovulation rate; prolificacy and lambing rate per ewe treated

Number of CL (figure 6A) increased linearly ($p < 0.001$) with decreasing concentrations of progesterone. Births per CL decreased linearly ($p < 0.001$) with decreasing concentrations of progesterone (figure 6B). As a result, the greater ovulation rates (Figure 6A) observed with lower concentrations of progesterone did not produce a corresponding increase in the number of lambs born per ewe lambing (Figure 6C). Lambs born per ewe lambing (prolificacy) averaged 2.00 ± 0.14 , 1.86 ± 0.11 and 1.90 ± 0.12 for low, medium and high progesterone, respectively, which did not differ. Lambs born per ewe treated (Lambing rate) did not differ among ewes on low (1.5 ± 0.2), medium (1.5 ± 0.1) or high (1.3 ± 0.1) concentrations of progesterone. Quintuplets were born to one ewe with high and one ewe with low concentrations of progesterone and quadruplets were born to one ewe with medium concentrations of progesterone.

6A

6B

6C

Figure 6 A Means ($\pm$ SEM) number of CL in ewes that lambd to first service with high (n= 39), medium (n= 44), or low (n= 41) concentrations of progesterone. Treatment effect was linear ($p < 0.001$). B. The treatment effect on lambs born per CL was linear ($p < 0.01$). C. Number of lambs born per ewe lambing (prolificacy) did not differ.

Lambing pattern

The mean intervals from estrus to lambing were 148 ± 0.4 , 147.7 ± 0.5 , and 147.5 ± 0.4 days for ewes on high, medium and low concentrations of progesterone, respectively, and did not differ (Figure 7).

Figure 7 Lambing patterns of ewes with high (♦), medium (■) or low (▲) progesterone concentrations of progesterone.

Discussion

Ovulation rate, pregnancy rate and prolificacy. Ovulation rate, as represented by either the number of follicles that disappeared or the number of CL observed on day 7 after estrus, increased linearly as the expected concentration of progesterone decreased. The increase in ovulation rate in ewes with lower concentrations of progesterone was due to retention and ovulation of more follicles from the penultimate wave of follicular development. This finding is in agreement with Johnson et al. (1996) and Bartlewski et al. (1999; 2003), who reported that follicles of varying ages, or originating from both the penultimate and final follicular waves, ovulated in the cycling ewe. Additionally, it extends to prolific ewes the finding by Bartlewski et al. (2003) in non-prolific ewes that exposure to low concentrations of progestogens during the luteal phase can increase

ovulation rate. In contrast, low concentrations of progesterone prolonged the growth phase of the dominant follicle, which inhibited other follicles from growing in heifers (Adams et al., 1992). Furthermore, multiple ovulations in cattle were attributed to the effect of co-dominance, which occurs during the first follicular wave when two follicles are maintained on the ovary. This phenomenon is a result of low concentrations of progesterone, which permits frequent pulses of LH; greater concentrations of estradiol are increased due to the increased number of follicles (Kulick et al., 2001). This mechanism might occur early in the estrous cycle in the ewe, but unlike the cow, other follicles can be recruited while the other largest follicles are present on the ovary of the ewe throughout the luteal phase.

The number of follicles that disappeared and was presumed to have ovulated was an overestimate of the number of CL observed on day 7 after estrus, which was used as a confirming and final measure of ovulation rate. Similarly, Bartlewski et al. (2003) observed that not all follicles that disappeared at estrus formed CL. The accuracy of observation of disappearance of follicles as a measure of ovulation rate might have been reduced, because some follicles ≥ 4 mm did not ovulate but regressed in size and might have been assumed to be new 2 or 3 mm follicles. Alternatively, some ovulated follicles might not form a CL. In earlier work, Murdoch et al. (1983) observed that ewes injected with LH or FSH on d 15 ovulated, but were unable to develop sufficient luteal function. They suggested that the pre-ovulatory follicle was forced to ovulate prematurely and lacked gonadotropin receptors on follicular cells, which is measure of follicular maturity and the inherent ability to undergo luteinization to form a CL. When follicular synthesis of estrogen was reduced by treatment with an aromatase inhibitor and follicles were ovulated/luteinized with an injection of hCG (Benoit et al., 1992), estrus did not occur and the onset of luteal function was delayed. However, ewes showed estrus naturally after withdrawal of progesterone in the present study, and pregnancy rates were in the normal range, so function of CL that were formed must have been adequate.

Pregnancy rates did not differ with concentrations of progesterone in the present study. Evans et al. (2001) found that lower dosages of progestogen had no deleterious effects on embryo quality or fertility and concluded that aged follicles in sheep were less critical than in cattle. In contrast, lower luteal phase concentrations of progesterone or

progestogens and the ovulation of older follicles was associated with reduced conception and pregnancy rates in ewes (Johnson et al., 1996; Ungerfeld and Rubianes 1999; Viñoles et al., 2001). Similarly, cows with low concentrations of progesterone during the luteal phase ovulated an older follicle. and had decreased pregnancy rate (Cooperative Regional Research Project NE-161, 1996) due to death of embryos during the 2 to 16 cell stage (Ahmad et al., 1995; Oussaid et al. 1999).

The number of lambs born per CL observed on day seven after estrus declined linearly with decreasing concentrations of progesterone during the luteal phase preceding ovulation. As a result, despite the linear increase in the ovulation rate with decreasing concentrations of progesterone, there was no net improvement in prolificacy of the ewe. Because pregnancy rates did not differ with concentrations of progesterone in the present study, decreasing progesterone resulted in an increase in partial rather than total pregnancy loss. The greater reproductive wastage in ewes with lower luteal phase concentrations of progesterone, which ovulated more follicles from the penultimate wave, might have been due to reduced fertilization rate, greater embryonic death, greater fetal death, or a combination of those factors.

Fertilization rates were reduced at the start of the breeding season (Hulet et al., 1956) or the late breeding season, as a possible consequence of seasonal shifts in LH secretion and (or) associated effects on follicular function (Mitchell et al., 2002). However, fertilization loss can be complete (Hulet et al., 1956) or partial (Wilkins, 1997; Kleemann et al., 2005). Early embryonic death losses were greater in early season breeding and accounted for the complete loss of the pregnancy (Hulet et al., 1956). Negative relationships between ovulation rate and embryonic mortality in sheep have been reported previously (Meyer, 1985, cited by Dixon 2003; Dixon, 2003; Knights et al., 2003). Losses might well be expected to occur in the early embryonic period. Older ovulatory follicles originating from the penultimate wave may not be as healthy and may be a causative factor of the higher reproductive wastage.

However, uterine capacity might have influenced litter size indirectly through the inability to sustain additional embryos (Meyer, 1985, cited by Dixon, 2003). In addition, complete embryonic loss was greater in ewes with lower progesterone at days 26 to 30 of gestation (Dixon, 2003). However, partial embryonic loss of pregnancy was not related to concentrations of progesterone at that time. In a later study, Dixon (2003)

observed that ewes with a higher number of embryos were more prone to have complete or partial embryonic loss. The majority of ewes lost one embryo and 22% of embryos were lost before term. Three to four percent of embryos present at day 25 subsequently were lost during each 20-day interval of pregnancy. In contrast, in dairy cattle, the entire pregnancy might be lost between days 30 and 60 in cows that had twin ovulations (Starbuck et al., 2004). Interestingly, the occurrence of double ovulations was minimally different between cows and heifers, yet twinning occurred more frequently in cows than in heifers. In previous work, Vandeplasseche et al. (1979) postulated that uterine capacity was the limiting factor in heifers.

Mechanism of effects of low progesterone on follicles. The mechanism through which low luteal phase concentrations of progesterone might alter the pattern of follicular development and increase ovulation rate in the ewe is not clear. In cows, lower progesterone led to increased pulse frequency of LH, maintenance of dominant follicles and greater secretion of estradiol (Stock and Fortune, 1993; Kinder et al., 1996). Taft et al. (1996) found that treatment of cows with frequent injections of bovine LH during normal luteal phases maintained the largest (dominant) follicle and suppressed recruitment of other follicles. This mechanism might not apply to the ewe, in which follicles are recruited despite the presence of other large follicles (Duggavathi et al., 2003). However, low concentrations of progesterone reduced atresia of recruited follicles in ewes (Turnbull et al., 1978; Avdi et al., 1997; Reynaud et al., 1999; Mandiki et al., 2000). Follicles in the penultimate wave might have been protected from atresia because of greater frequency of secretion of pulses of LH in ewes on lower concentrations of progesterone, as observed by Johnson et al. (1996) and Van Cleeff et al. (1998).

Growth rate of follicles was slower in the penultimate than in the final wave. As a result, average maximum diameter of the ovulatory follicles did not differ among treatment groups. In contrast, Johnson et al. (1996) observed that the diameter of the ovulatory follicle was greater in ewes that had low concentrations of progesterone. Concentrations of progesterone in the “low” group were below both the “medium” and “high” groups for only the last 2 to 3 days of the luteal phase of the estrous cycle before breeding in the present study. Johnson et al. (1996) treated with lower concentrations of progesterone (and thus ewes were subjected to a greater frequency of LH pulses) for a

longer period of time, stimulating a greater increase in diameter of follicles. Thus, rate of growth of follicles in the penultimate wave might have been limited by relatively greater concentrations of progesterone during the mid- to late luteal phase of the cycle. In comparison, most growth of ovulatory follicles from the ultimate wave occurred during the very late luteal and follicular phases of the estrous cycle, when progesterone concentrations were waning and pulsatile secretion of LH was increased.

The average ovulatory follicle was retained on the ovary for about one day longer in ewes with low concentrations of progesterone than in ewes with high or medium concentrations of progesterone. Follicles from the penultimate wave were an average of three days older at ovulation than follicles from the ultimate wave. Similarly, the lifespan of the largest follicles in the penultimate wave (Bartlewski et al., 1999) or that arose earlier (Johnson et al. 1996) was increased, which allowed them to ovulate with follicles from the final wave.

Other considerations. Rams were introduced to the ewes in this study seven days before withdrawal of progesterone or regression of corpora lutea. Based upon a study by Evans et al. (2004), exposure to rams for three days before progestogen withdrawal might have been expected to limit fertility or prolificacy. They found 14 and 9 percentage point reductions in ewes lambing to first service in two trials and 0.23 fewer lambs born per ewe lambing in one of those trials, which they attributed to increased LH pulse frequency in response to ram introduction during treatment with progestogen. However, their results might have been influenced in part by the fact that they delayed introduction of intact rams for breeding until 48 hours after sponge withdrawal. In the present study, the same intact rams remained with the ewes throughout and overall pregnancy rate was a quite acceptable 74%, similar to that reported for hair sheep in the tropics (74%; Godfrey et al., 1997) and greater than that observed in ewes treated with intravaginal inserts (CIDRS) in the breeding season (57%; Rhodes and Nathanielsz, 1988).

Conclusions

In summary, the removal of endogenous progesterone and the use of intravaginal inserts induced low circulating concentrations of progesterone, which increased the ovulation rate through more follicles being maintained from the penultimate wave, but did not result in an increased litter size. The increase in ovulation rate might be due to greater LH pulse frequency, which regulates follicular and oocyte

maturation. Older oocytes, ovulated from follicles of the penultimate wave, might have been incapable of either fertilization or embryonic development, the latter being more likely based upon the literature in the cow. Furthermore, uterine capacity might have been a factor that limited litter size.

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