FOLLICULAR DEVELOPMENT AND FUNCTION OF INDUCED CORPORA LUTEA IN UNDERFED POSTPARTUM ANESTROUS BEEF COWS

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SUMMARY

Effects of energy restriction during late gestation and early lactation on pituitary and ovarian responses to FSH and GnRH, and the effects of exogenous FSH or estradiol on follicular growth and luteal function after GnRH were examined in three experiments. In experiment I, 43 2-year-old Hereford heifers were assigned randomly 30 days prior to expected calving to be fed either 115% or 60% of the NRC recommended energy requirements. Although cows on the low plane lost more weight (P<.01) through the first 30 days after parturition, level of feeding had no effect on size of the largest ovarian follicle or concentration of plasma estradiol-17β at 30 days postpartum.

At 30 days postpartum cows received injections twice daily of 0.125 or 0.25 mg FSH for 3 days and were then treated with 300 µg GnRH. Dose of FSH did not affect estradiol-17β in daily plasma samples. Concentrations of LH in jugular blood samples taken every 30 min for 7 hr after GnRH were affected by plane of nutrition (P<.01). There was a positive association between follicle size and both the pattern of release of LH (P<.01) and the area under the LH response curve (P<.05). Occurrence of CL after GnRH was not related to body weight postpartum, change in weight during lactation, amount of LH released, follicle size, nutrition or dose of FSH. In 22 of 23 cows the CL palpated on day 7 had regressed by day 14 and progesterone in plasma at 3- or 4-day intervals after GnRH reflected a shortened life span of the induced CL.

In experiment II, 17 cows that did not have CL in experiment I were injected twice daily for 3 days with either 0 or 2 mg FSH followed by injection 300 µg GnRH. Concentrations of estradiol-17β in plasma collected prior to FSH were correlated with body weights at 48 days postpartum (r = .70). Estradiol prior to GnRH, was correlated with size of the largest ovarian follicle (r = .53) at that time. Treatment with FSH (4 mg/day) increased mean diameter of the largest follicle (P<.05), but not estradiol-17β prior to GnRH. The proportion of cows that formed CL was not higher in cows pre-treated with FSH (88%) than in controls (56%). Progesterone in plasma at 14 days after GnRH indicated that induced CL were short-lived.

In experiment III, 18 3-year-old cows were treated twice daily from day 34 to 38 postpartum as follows: (1) corn oil for 1 day followed by saline for 3.5 days, (2) corn oil for 1 day plus increasing doses of FSH for 3.5 days or (3) estradiol benzoate (75 µg) for 1 day plus FSH for 3.5 days. GnRH (300 µg) was administered 4 days after initiation of treatments. FSH or estradiol plus FSH did not affect follicular development, profiles of estrogen or LH, or occurrence of lifespan of induced CL. Sixty-one percent of the cows had CL on day 7 and progesterone in plasma of those cows averaged only 1.4 ng/ml on day 7 and 45% of these CL were short-lived.

(Key Words: Postpartum Anestrus, Lactating Beef Cows, LH, GnRH, Corpora Lutea, Plane of Nutrition.)

INTRODUCTION

In many beef herds a significant proportion of lactating cows have not ovulated by the beginning of the mating period (Wiltbank, 1974). Conception rates are poorer in cows mated at first estrus.
following parturition than in those bred after several estrous cycles (Perkins and Kidder, 1963; Casida et al., 1968; Short et al., 1972; Whitmore et al., 1974). There is increasing evidence that luteal function may be deficient during early estrous cycles improving only gradually during subsequent cycles (Short et al., 1972; Edgerton and Hafs, 1973; Dickey et al., 1975).

Inadequate steroid secretion by the ovary during early lactation could contribute to the high incidence of ovulation without overt estrus during this time (Casida et al., 1968; Whitmore et al., 1974; Castenson et al., 1976). After injection of gonadotropin releasing hormone (GnRH) between 20 and 40 days postpartum, corpora lutea (CL) were detected in a limited number of anestrous lactating beef cows (Britt et al., 1975; Webb et al., 1975). Many induced CL were short-lived (<14 days) and peripheral concentrations of progesterone were low (<1 ng/ml) relative to those observed during a normal estrous cycle. Priming with pregnant mare serum gonadotropin (PMSG) has improved the ovulatory response to GnRH in prepuberal rats (Steger et al., 1975) and gilts (Baker et al., 1973).

Follicular development may be deficient in postpartum anestrous beef cows. Antral formation in ovarian follicles requires follicle-stimulating hormone (FSH). Increased binding of FSH to granulosal cells after treatment of rats with estrogen (Goldenberg et al., 1972) follows the increase in mitosis of granulosal cells due to estrogen (Louvet and Vaitukaitis, 1976). While FSH alone increased receptors for LH in granulosal cells (Richards et al., 1976), the increase was greater in immature hypophysectomized rats treated with estradiol plus FSH. Both LH and FSH are necessary for synthesis of estradiol-17β (Fortune and Armstrong, 1978). Increased numbers of receptors for gonadotropins in preovulatory follicles brought about by interactions of steroid and protein hormones might improve ovulatory response to GnRH and/or the performance of CL induced by GnRH.

Underfeeding, either pre- or postpartum, can delay onset of estrous cycles following calving (Dunn et al., 1969; Whitman et al., 1975). Research into the mechanisms whereby undernutrition reduces fertility has produced conflicting results regarding the release of LH and luteal activity (Dunn et al., 1974; Aggar et al., 1975; Beal et al., 1975; Spitzer et al., 1975). It therefore appeared pertinent to test the suggestion of Gombe and Hansel (1973) that the ability of the ovary to respond to gonadotropins may be reduced by inadequate feeding.

Objectives of these studies were to examine: 1) effects of energy restriction during late gestation and early lactation on pituitary and ovarian responses to treatment with FSH and GnRH, and 2) the effects of exogenous FSH or estradiol on follicular growth and on the formation and life span of CL following injections of GnRH.

MATERIALS AND METHODS

Experiment I. Thirty days prior to expected calving, 43 2-year-old Hereford heifers (bred at a synchronized estrus) were weighed and divided randomly into groups of 20 and 23 which were fed 115% (high) or 60% (low) of the recommended requirements for energy, respectively (NRC, 1970). All animals were fed alfalfa hay and corn silage; those on the high plane received corn grain in addition. After parturition the low level yielded 50% of NRC, while the high level was increased to maintain 115%. After approximately 48 days postpartum, all cows were fed at the high level. The cows were weighed again shortly before and after calving and at 30 and 48 days postpartum.

At 29 days postpartum ovarian activity was assessed by palpation per rectum. Commencing on day-30 postpartum, the cows were divided randomly within feeding groups and injected (s.c.) twice daily (6:00 am and 5:00 pm) for 3 days with either 0.125 or .25 mg FSH in saline. These doses were in a range expected to induce growth of follicles and estrogen syntheses, but not ovulation. Thus, the treatment was expected to 1) provide a measure of the sensitivity of the ovary in terms of steroid secretion and 2) induce follicular growth prior to a GnRH-induced release of LH. Blood samples (20 ml) were drawn (venipuncture) daily from 29 through 33 days postpartum to quantify estradiol-17β. The morning after the last injection of FSH (day 0; 34 days postpartum) each cow received (i.m.) 300 μg GnRH and during the ensuing 7 hr blood was collected every 30 min by jugular cannulae for measurement of LH. Blood was drawn every 3 or 4 days for 18 days after GnRH for quantification of progesterone. Rectal palpations were performed on day 7 and again on day 14 after GnRH to detect CL.

Experiment II. In view of the possibility that doses of FSH in experiment I had been insufficient to stimulate the ovaries, a second experiment was conducted utilizing only those cows which apparently had not ovulated following treatment

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1FSH-P. Armour-Baldwin Laboratories, Omaha, NE.  
GnRH. Abbott Laboratories.
with GnRH in experiment I. The ovaries of 17 cows were re-examined on day-56 postpartum to confirm the absence of CL and assess follicular activity. The cows were divided randomly and injected twice daily for 3 days with either 0 or 2 mg FSH (s.c.). Follicular development was estimated by palpating the ovaries again subsequent to FSH, the second examination being followed by an injection (i.m.) of 300 μg GnRH in all cows. Blood was drawn prior to FSH and again prior to GnRH for quantification of estradiol-17β and 7 and 14 days after GnRH for measurement of progesterone. Corpora lutea were detected by rectal palpation 7 and 14 days after GnRH.

Experiment III. Eighteen lactating, anovulatory 3-year-old Hereford cows were assigned at random to receive one of three treatments. These cows from the same herd used in experiments I and II, received 100% of NRC recommended energy and were studied 1 year later. On days 30, 34 and 37 postpartum the ovaries were palpated to assess ovarian activity. Twice daily (0600 and 1800 hr) hormonal treatments were made beginning on day 34 postpartum as follows: (1) corn oil (i.m.) on day -4 and saline (s.c.) on days -3,-2 and -1 (5 cows); (2) corn oil on day -4, and FSH on days -3 (.25 mg), -2 (.375 mg) and -1 (.5 mg) (6 cows); (3) estradiol benzoate (75 μg) on day -4, and FSH on days -3 (.25 mg), -2 (.375 mg) and -1 (.5 mg) (7 cows). The treatments given on day -1 also were given at 0600 hr on day 0, immediately prior to injection of 300 μg GnRH (i.m.).

Jugular venous blood collected by cannulae just prior to (0 hr) and every 30 min for 6 hr after GnRH were used to quantify plasma concentrations of LH. Samples taken by jugular venipuncture on day -4,-3,-2 and -1 and at 0, 8, 12 and 24 hr after GnRH on day 0 were assayed to quantify plasma concentrations of LH and estradiol-17β. Progesterone was measured in plasma from samples taken on days -8,-4 and 0 before GnRH and every 3 days from day 1 to 16 after GnRH. Cows were palpated per rectum on days 7 and 14 and again on day 30 to determine if new CL were formed spontaneously or cows had returned to anestrus subsequent to regression of induced CL.

Quantification of Hormones. Estradiol-17β, progesterone and LH were measured in plasma by radioimmunoassays (Butcher et al., 1974; Butcher, 1977) validated for bovine plasma (Fogwell et al., 1978). LH is reported as ng equivalents NIH-LH-B9 (.7 × NIH-LH-S1)/ml.

Statistical Analyses. Data for experiments I and II were examined by least squares analysis of variance using models with terms for “nutritional level,” “weight postpartum,” “weight pre-GnRH,” “weight change postpartum to pre-GnRH,” “weight change postpartum to post-GnRH,” “FSH treatment,” and interactions. Data on follicular development from experiment III were examined by analysis of variance and least significant difference. Chi-square was used to examine the data on occurrence of CL. Profiles of concentrations of hormones in all experiments were examined for differences among groups by analysis of variance using time as an independent continuous variable and the partitioned linear, quadratic and cubic regressions of hormonal concentration on time in the manner described by Fogwell et al. (1978).

RESULTS AND DISCUSSION

Experiment I. Animals fed the low energy ration initially weighed an average of 6.8 kg more than those on the high plane diet. Just prior to parturition this weight difference had decreased to a mean of 2.0 kg (379.1 vs 377.1). From parturition to 30 days postpartum, cows on the low plane lost 31.5 kg while those on the high plane lost 10.7 kg (P<.1). Thereafter they gained 1.1 (low) and 9.6 kg (high; P<.05) until 48 days postpartum.

The level of feeding had no effect on size of the largest follicle (high 11.6±4.4 mm, low 10.9±3.3) or the proportion of cows with a follicle ≥12 mm in diameter (12/20 high vs 11/23 low) prior to injection of FSH. The proportion of cows that had CL on day 7 after GnRH was not affected by treatment with FSH or plane of nutrition (table 1). Overall, neither body weight postpartum nor weight change after parturition appeared to determine whether or not a cow would produce a CL. Among the low-plane animals, nine of the 12 that lost the most weight had CL on day 7 as compared to four of the 11 that lost the least weight. Only seven of 21 cows in which the largest follicle in the ovaries, four days prior to GnRH, was less than 12 mm in diameter had CL on day 7 after GnRH in contrast to 16 of 22 of those with a follicle ≥12 mm.

Neither level of feeding nor FSH affected concentrations of estradiol-17β prior to (daily samples) or 8 hr after GnRH (overall mean 1.2±.1 pg/ml), so it was not possible to determine whether the level of feeding had any influence on ovarian response to FSH. Pretreatment with FSH did not alter basal concentrations of LH (.2 ng/ml) or the pattern of release or the maximum concentration of LH (table 1) in response to GnRH. One cow (no. 38) on the high plane that did not receive FSH exhibited maximum LH of 252 ng/ml, which was 111 ng/ml
TABLE 1. EFFECTS OF PLANE OF NUTRITION AND FSH ON RESPONSES OF POSTPARTUM ANESTROUS COWS TO GnRH, EXPERIMENT I

<table>
<thead>
<tr>
<th>Level of feeding</th>
<th>Dose of FSH (mg/day)</th>
<th>No. of cows</th>
<th>Cows with CL on day 7 after GnRH</th>
<th>Maximum concentration of LH±SE (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>0</td>
<td>7</td>
<td>4</td>
<td>82.2±32.6*</td>
</tr>
<tr>
<td></td>
<td>.25</td>
<td>7</td>
<td>3</td>
<td>55.0±9.9</td>
</tr>
<tr>
<td></td>
<td>.5</td>
<td>6</td>
<td>3</td>
<td>59.4±18.6</td>
</tr>
<tr>
<td>Low</td>
<td>0</td>
<td>8</td>
<td>4</td>
<td>49.0±14.8</td>
</tr>
<tr>
<td></td>
<td>.25</td>
<td>8</td>
<td>3</td>
<td>49.6±8.0</td>
</tr>
<tr>
<td></td>
<td>.5</td>
<td>7</td>
<td>6</td>
<td>60.6±16.9</td>
</tr>
</tbody>
</table>

*Cow no. 38 had an extremely high value of 252. When that value is deleted, the mean for the remaining six cows is 53.9 ng/ml.

higher than the next highest animal in this treatment group. If values for this cow are omitted, then the high dosage of FSH may have increased the maximum level of LH.

The pattern of release of LH (figure 1) was altered (P<.05) by plane of nutrition. The high plane of feeding increased the slope of the rise in LH so that the maximum occurred 30 min earlier than in underfed animals. The mechanism by which level of feeding and possibly FSH influenced release of LH may be through their effects on follicular development, even though effects of level of feeding on size of follicles were not detected here. (The number of degrees of freedom available to test for this effect was considerably fewer than for the pattern of release (P<.01) and in the area under the profile of LH (P<.05). The maximum value for LH was not correlated significantly with the concentration of estradiol, either 24 hr before or at the time of injection of GnRH (pooled within r = .1 and -.1, respectively).

The profiles of plasma progesterone in cows with CL for each plane of nutrition and dose of FSH are shown in figure 3. There was a significant FSH×time interaction (P<.05) and a trend towards an FSH×plane of nutrition interaction (P<.08). Apparently FSH led to an increase in progesterone 7 days after GnRH, except in the high plane animals that received the higher dose of FSH. Cows that lost more weight from parturition prior to GnRH released considerably more LH than those with follicles < 10 mm (figure 2). The effects of size of follicle were reflected both in the pattern of release (P<.01) and in the area under the profile of LH (P<.05). The maximum value for LH was not correlated significantly with the concentration of estradiol, either 24 hr before or at the time of injection of GnRH (pooled within r = .1 and -.1, respectively).

Figure 1. Effects of plane of nutrition on profiles of LH induced by GnRH in postpartum anestrous beef cows.

Figure 2. Effects of sizes of follicles on profiles of LH induced by GnRH in postpartum anestrous beef cows.
TABLE 2. CHANGES IN OVARIAN FOLLICLES AND SECRETION OF STEROIDS IN COWS TREATED WITH FSH PRIOR TO RE-INJECTION WITH GnRH. EXPERIMENT II

<table>
<thead>
<tr>
<th>FSH pre-treatment</th>
<th>Days post-partum</th>
<th>Diameter (mm) of largest follicle</th>
<th>Plasma estradiol-17(\beta) (pg/ml)</th>
<th>Plasma progesterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pre-FSH</td>
<td>Post-FSH</td>
<td>Pre-FSH</td>
</tr>
<tr>
<td>4.0 mg/day</td>
<td>8</td>
<td>55.1</td>
<td>9.7±.6</td>
<td>13.5±.5*</td>
</tr>
<tr>
<td>Control</td>
<td>9</td>
<td>56.7</td>
<td>10.0±.7</td>
<td>11.8±.6</td>
</tr>
</tbody>
</table>

*P<.05.

Experiment II. A positive correlation (r = .70; P<.01) was observed between body weights of these 17 cows at 48 days postpartum and concentration of estradiol-17\(\beta\) (table 2) prior to commencement of treatment with FSH at 56 days postpartum. Concentration of estradiol-17\(\beta\) and size of follicle were not associated at that time, but were associated immediately prior to injection of GnRH on day 59 (r = .53; P<.05). Mean estradiol (pg/ml) ranged from .7 in one animal with an 8 mm follicle to 1.8, 2.4, 2.2 and 15.0 in cows with 10, 12, 14 and 16 mm follicles, respectively.

Level of feeding did not influence the diameter of the largest follicle immediately prior to GnRH. Treatment with FSH (4 mg/day) for 3 days resulted in a greater (P<.05) mean diameter of the largest follicle than in the control animals (table 2). Despite this effect and the association of estradiol with size of follicle, plasma estradiol-17\(\beta\) in cows treated with FSH was not greater than in untreated controls (table 2).

Although a greater proportion of cows that were injected with FSH (88%) than control cows (56%) had CL after GnRH, the difference was not significant (table 2) and concentrations of progesterone were not affected by treatment.

According to palpation and progesterone measured on day 14 (table 2), the induced CL were short-lived.

Experiment III. Size of ovaries, number of follicles \(\geq 10\) mm, sizes of follicles per ovary and the size of the largest follicle on day 37 did not vary with hormonal treatments or over time (table 3). Similarly the profiles of estradiol-17\(\beta\) were parallel over time, averaging 4 pg/ml, except for an increase to nearly 14 pg/ml on day -3 in cows receiving exogenous estradiol on day -4 (day 34 postpartum). Clearly, follicular activity as measured by several end points was not affected by either treatment.

Profiles of concentrations of LH after GnRH were parallel among all three groups of cows. In addition, the peak concentrations and areas under the curves did not differ among groups.

Figure 3. Effects of plane of nutrition and FSH treatment on profiles of progesterone after GnRH in postpartum anestrous beef cows.
Sixty-one percent of all cows treated with GnRH had palpable CL in the ovaries on day 7. There was a tendency for fewer cows treated with estradiol plus FSH to have CL on day 7 than in the other two groups (2/7 vs 9/11; P<.1). At least one cow in each group and five in total had a CL with a very short life span, as judged by its absence from the ovaries on day 13. Nine of the 11 (82%) cows with CL on day 7 had ovulated spontaneously by day 30 (68 days postpartum). Fewer cows treated with estradiol plus FSH had CL on day 30 (1/7) than in the other groups (8/14; P<.05). The location of CL on day 30 (same or opposite ovary as day 7), did not vary with treatment.

Progesterone (ng/ml) in cows with induced CL averaged only 1.4±.3 on day 7 and .9±.4 on day 30 compared to usually reported values of 4 to 5 at those stages of a normal cycle. Profiles of progesterone did not vary among the three groups of hormonally-treated cows.

**Discussion**

The question arises as to why there was an apparent response in occurrence of CL to FSH in the energy-restricted animals but not among cows fed the high energy ration in experiment I. Examination of the results does not support the idea that the high-plane cows tended to be above a threshold and therefore could not respond to small doses of FSH. The most likely explanation appears to be that although the cows were allocated randomly to treatments the high-plane cows assigned to the control group possessed an advantage since the population of follicles within the ovaries reflected a more active condition than cows in the remaining groups. This contention is supported by the tendency for a CL occurring in response to GnRH to be greater when the diameter of the largest ovarian follicle was 12 mm or greater. Because of this finding and the increase in diameter of the largest follicle under the influence of exogenous FSH in experiment II, it is suggested that attention should be given to the ovarian situation when selecting the dose of FSH and the duration of treatment. In view of the lack of association between the peak concentration of LH and the formation of CL, it is doubtful whether the reduction in release of LH associated with inadequate follicular development warrants adjustment of the dose of GnRH according to the follicular population.

When estrous cycles resume spontaneously after parturition in cows the first overt estrus is preceded usually by a 4- to 5-day period during which plasma levels of progesterone are elevated (Donaldson et al., 1970; Henricks et al., 1972;
Dickey et al., 1975; Castenson et al., 1976; Humphrey et al., 1976. Treatment of lactating, anestrous beef cows with GnRH can induce luteal function for a number of days (Britt et al., 1975; Webb et al., 1975; present experiments). Questions arise as to why (1) the luteal function is usually of short duration and (2) the elevation in progesterone levels is not followed by further spontaneous ovulations. Since it has been demonstrated that the major luteotropin in the bovine is LH (Hansel and Siefart, 1967), it is tempting to consider the possibility of quantitative deficiencies (concentration and/or duration) in the LH needed for sustained luteal function. Since there was great variation in the peak concentration, but induced CL were always short-lived, the deficiency probably does not rest in this area. The only common factor regarding release of LH was the short duration of some 6 hr compared to 10 hr or more in the cycling cow (Lemon et al., 1975). This appears a more fertile ground for exploration; indeed when the duration of the peak has been prolonged in ewes by employing divided doses of GnRH (Restall et al., 1977), the abnormal CL were reported to be eliminated.

A further possibility for the premature regression of the CL is that the follicle which ovulated was incompetent or deficient in some receptor for gonadotropin. This contention is supported by the finding that treatment with FSH prior to GnRH tended to increase progesterone one week after formation of the CL (figure 3, table 2). Although FSH affects both size of follicle and secretion of estrogen, the latter, via its effect on the pituitary and hypothalamus, may be the more important aspect regarding luteal function. Consequently, ovarian follicles may reflect the hypothalamic-pituitary situation rather than ovarian readiness as such.

When attention is focused on the failure to initiate regular ovulations, the case of cow no. 38 is of considerable interest. During the 22-day period from prior to GnRH until the end of experiment I this animal gained 34.5 kg, whereas her calf weighed 13.7 kg (19.3%) less than the mean for the group. This cow was the only animal that re-ovulated after regression of the GnRH-induced CL and her release of LH after GnRH reached an exceptionally high peak value. It is tempting to attribute the atypical gain in weight to some change in the nutritional status of the cow, but the possibility of a reduced frequency of suckling by the calf, perhaps due to illness, cannot be eliminated in this particular case.

In sheep, pituitary reserves of LH are low during early lactation (Restall and Starr, 1977) and in beef heifers the feeding of a low energy diet reduced the LH reserves (Beal et al., 1975). However, differences in the level of nutrition during pregnancy did not influence the peak level of LH following GnRH in heifers (Cummins et al., 1975). It remains to be demonstrated therefore, whether the delayed and reduced response of LH to GnRH in the underfed, lactating cows (figure 1) was simply the result of deficient pituitary reserves or whether inadequate sensitization by ovarian steroids was also involved. Estradiol did not vary with plane of nutrition and, in contrast to the report of Fernandes et al. (1978) in dairy cattle, maximum LH was not correlated with concentrations of estradiol prior to GnRH.

Ovarian follicular populations and secretion of estradiol-17β were not affected by exogenous FSH or estradiol and FSH in experiment III. Also, the formation and life-span of induced CL were not affected by these treatments. Dose of FSH may have been too low since follicular development was increased in postpartum cows with 4 mg of FSH per day (experiment II). Similarly, the dose the estradiol used may not have accomplished physiological concentrations of estradiol within the follicle; microgram concentrations are known to occur in follicular-fluid (England et al., 1973); thus potential effects of estradiol on receptors for FSH may not have been obtained. Possibly, the anestrous ovaries require low level stimulation by FSH over longer periods rather than higher or graded doses administered acutely (4 days). Because of these questions, these data do not clarify whether ovarian insensitivity is a possible explanation for low occurrence or short-life of CL induced by GnRH. It may be important to note that in the 3-year-old cows (experiment III) six of 11 induced CL were palpable on day 13 in contrast to one of 35 in the 2-year-old cows in the previous experiments. Even so, progesterone had decreased by day 13.

The presence of CL on day 30 after GnRH, particularly in those cows which had induced CL of apparently nearer normal life span indicates possible resumption of estrous cycles. This hypothesis deserves more critical investigation because of the possibility of a short intervening period of anestrus.

From these data, it appears that investigations of postpartum anestrous in beef cows should consider the mechanism by which increases in progesterone prior to first estrus affect follicular development.
LITERATURE CITED


Leshman ET AL.


