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Role of Prolactin and Luteinizing Hormone in Regulating Timing of Implantation in the Spotted Skunk[1]

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ABSTRACT

The western spotted skunk exhibits an obligate delay of implantation lasting 200–220 days. The pituitary is essential for luteal activation. The corpora lutea, in turn, secrete the hormones necessary for blastocyst implantation. Two experiments were designed to determine which pituitary hormones are responsible for increasing luteal activity and induction of implantation. Forty-two pregnant skunks with delayed implanting blastocysts were treated as follows: 13 served as untreated controls, 6 received 0.5 mg prolactin (PRL) daily, and 5 received diluent beginning in January. Four received 1.5 mg bromocriptine (CB-154) daily, 3 received both CB-154 and PRL, 3 received diluent, 5 received a gonadotropin-releasing hormone agonist (GnRHa) dispensed from osmotic minipumps, and 3 received diluent dispensed from osmotic minipumps starting in April. The skunks were subjected to a natural photoperiod. Duration of preimplantation and blood levels of progesterone and luteinizing hormone were measured. PRL significantly ($p<0.05$) shortened and CB-154 significantly ($p<0.05$) prolonged the duration of preimplantation when compared to controls (148 ± 33.6 vs. 251 ± 3.2 vs. 199 ± 5.1 days, respectively). PRL was able to reverse the inhibitory effect of CB-154 when both were administered simultaneously (195 ± 4.0 vs. 251 ± 3.2 days). GnRHa had no significant ($p>0.05$) effect on duration of preimplantation (199 ± 5.1 days) when compared to controls (203 ± 3.2 days). These results indicate that PRL is the primary pituitary hormone responsible for increased luteal activity and subsequent blastocyst implantation in the spotted skunk.

INTRODUCTION

Western spotted skunks breed in late September and early October. However, unlike most mammals, the blastocysts of the skunk enter a prolonged period of arrested development lasting 200 to 220 days (Mead, 1968). During this period of embryonic diapause, ovarian activity is reduced as suggested by the inactive appearance of the corpora lutea and relatively low plasma progesterone levels (Mead and Eik-Nes, 1969; Sinha and Mead, 1975). However, in response to increased daylength, the corpora lutea enlarge, plasma progesterone levels increase, and the blastocysts resume their development and implant in late April (Mead, 1971, 1981; Enders et al., 1986). The pituitary is essential for both increased luteal activity and blastocyst implantation, as hypophysectomy abolishes both events (Mead, 1975). The precise pituitary hormone(s) responsible for initiating increased luteal activity and nidation in the spotted skunk remains unknown (Mead, 1981). Luteinizing hormone (LH) levels tend to parallel increases in daylength in the spring (Foresman and Mead, 1974); however, continuous infusion of ovine LH into pregnant skunks with diapausing blastocysts for up to 13 days or 5 daily injections of gonadotropin-releasing hormone (GnRH) for 21 consecutive days had no significant effect on plasma progesterone levels and did not hasten implantation (Mead, unpublished results). Other investigators have demonstrated that prolactin (PRL) will induce increased luteal activity and implantation in other mustelids such as the ferret

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(Murphy, 1979) and mink (Papke et al., 1980; Martinet et al., 1981; Murphy et al., 1981). However, there is some evidence that LH may also be part of a luteotropic complex in the mink (Martinet et al., 1981) and ferret (Agu et al., 1986). The following experiments were designed to determine whether PRL and/or LH are required for increased luteal activity and blastocyst implantation in the spotted skunk.

MATERIALS AND METHODS

Pregnant western spotted skunks (*Spilogale putorius latifrons*), which had bred in the wild, were purchased from a trapper in Oregon and received between 12 December 1986 and 28 January 1987. All animals were individually housed and exposed to a lighting schedule controlled by a Tork Model 7122Z light timer (Tork Inc., Mt. Vernon, NY) that precisely simulated annual changes in the natural photoperiod at a latitude of 46° 43′ 57″ N. Food and water were available ad libitum, and the diet was supplemented weekly with liver.

Experiment 1

The first experiment was designed to test the hypothesis that PRL is responsible for luteal activation and subsequent implantation of the blastocysts. In the first part of this experiment, 13 skunks served as untreated controls, 6 received 0.5 mg ovine PRL (Sigma, St. Louis, MO) in 5% beeswax in oil s.c., and 5 received beeswax in oil s.c. Injections were made daily starting 19 January 1987 and were continued for 47 days (Fig. 1). In the second part of this experiment (and in Experiment 2), groups initially containing 5–7 skunks were subsequently discovered to contain animals in which implantation occurred prior to initiation of the treatments (6 April 1987). Consequently, 8 females were omitted for this reason and 3 others were omitted because they were not pregnant. In the resulting groups, 7 skunks served as untreated controls; 4 animals received 1.5 mg bromocriptine, a dopamine agonist, (CB-154; Sandoz Inc., Basel, Switzerland) in acidified saline s.c.; 3 skunks received injections of 0.5 mg PRL plus 1.5 mg CB-154 s.c.; and 3 skunks received injections of the diluents. Injections were given daily starting 6 April and continued through 10 May (Fig. 1). Blood samples were taken from skunks anesthetized with ketamine hydrochloride (40 mg/kg, i.m.) by cardiac puncture at biweekly intervals in part 1 and weekly intervals in part 2. Blood was collected in heparinized vacutainers and centrifuged; plasma was stored at −20°C until the time of assay.

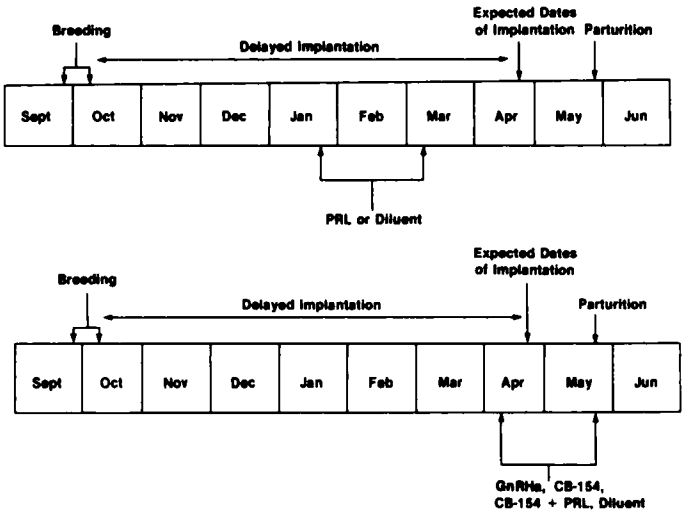

FIG. 1. Experimental design used to determine whether prolactin and/or luteinizing hormone are required for luteal activation and subsequent blastocyst implantation in the spotted skunk. *Arrows* below dates indicate beginning and ending of treatments. *PRL*, prolactin; *CB-154*, bromocriptine; *GnRHa*, gonadotropin-releasing hormone agonist.

Experiment 2

This experiment tested the hypothesis that LH is responsible for luteal activation and subsequent implantation of the blastocysts. Seven skunks served as untreated controls, 3 animals received osmotic minipumps (Alzet, Model 2001, Palo Alto, CA), inserted s.c. in the interscapular region, containing acidified saline, and 5 received osmotic minipumps containing 150 μg GnRH agonist (GnRHa), D-Ala^6-LHRH (Sigma) starting 6 April. Blood samples were collected weekly as described above from 6 April through 21 May. The minipumps were changed at the time of bleeding, from 6 April through 10 May. These dates correspond to the peri- and postimplantation periods (Fig. 1).

Detection of Blastocyst Implantation

Nidation, in both experiments, was first visually detected by vulval and mammary gland enlargement, and subsequently was confirmed in all skunks by laparotomy performed under sodium pentobarbital anesthesia (40 mg/kg, i.p.). The time of implantation was determined according to the method described by Foresman and Mead (1973). Timing of implanta-

tion is unrelated to the time of breeding but is, however, triggered by increasing daylength (Mead, 1971). Therefore, 1 October was used as the date of conception for computing the duration of preimplantation since most skunks are bred by the end of the first week in October (Mead, 1968).

Radioimmunoassays

Progesterone concentrations in plasma were determined by radioimmunoassay using a commercial [^{125}I]progesterone kit (Diagnostic Products Corporation, Los Angeles, CA). The interassay coefficient of variation (mean ± SD) for all assays was 11.9 ± 1.6% and the intraassay coefficient of variation, calculated between duplicates, was 6.7 ± 5.6%. The antibody was reported by the manufacturer to have less than 2.4% cross-reactivity with 16 other steroids. Sensitivity of the assay, defined as the minimum concentration that significantly displaced [^{125}I]-progesterone, was 50 pg/ml.

Plasma LH concentrations were determined in GnRHa-treated and control animals as described by Foresman et al. (1974) in a single assay. The intraassay coefficient of variation between triplicates was 8.6 ± 4.6%, with 0.6 ng/ml being the lowest detectable concentration of LH.

Statistical Analysis

The results for duration of preimplantation are expressed as the mean ± the standard deviation (SD). These data were subjected to analysis of variance, using the Statistical Analysis System (SAS, 1985) General Linear Models Procedure. If the F statistic was significant, Duncan's new multiple range test was performed to determine significant differences among group means for duration of preimplantation. The hormonal data were subjected to analysis of variance for repeated measures to determine differences in mean hormone concentrations. Individual comparisons were made using Duncan's new multiple range test to determine differences between mean plasma concentrations at each bleeding.

RESULTS

Experiment 1

Daily administration of 0.5 mg PRL resulted in a significant ($p<0.05$) shortening of the preimplantation period when compared to skunks receiving diluent or untreated controls (Table 1). One animal receiving PRL was unresponsive to this treatment, since implantation was not hastened (216 days). However, in the remaining 5 skunks, blastocysts were implanted 24 ± 5.0 days after treatment was initiated whereas in females in the control group, blastocysts were implanted 82 ± 8.6 days after treatment began.

TABLE 1. Effect of prolactin (PRL) on duration of preimplantation.

Treatment	n	Duration of preimplantation from: Beginning of treatment $\bar{X}$ ± SD (Days)	Breeding $\bar{X}$ ± SD (Days)
Untreated	13	83 ± 14.5	194 ± 14.5
Diluent	5	82 ± 8.6	193 ± 8.6
PRL	6	37 ± 33.6*	148 ± 33.6*

*Differs significantly from other values in column ($p<0.05$).

Exogenous PRL significantly ($p<0.05$) hastened the rise in progesterone concentration when compared to untreated controls and skunks receiving the diluent (Fig. 2). A significant ($p<0.05$) increase in plasma progesterone levels was detectable after 14 days of treatment. Progesterone levels in the 2 control groups were not significantly different ($p>0.05$) from each other and reached peak concentrations approximately 60 days after the PRL-treated group (Fig. 2).

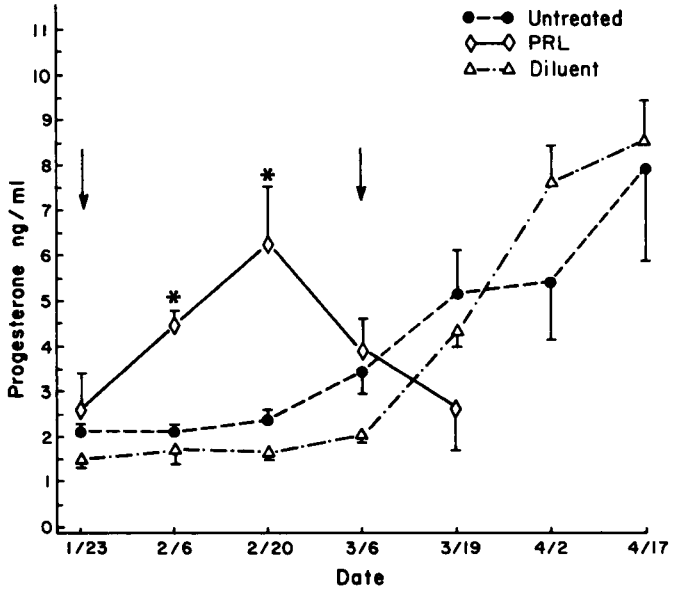

FIG. 2. Plasma progesterone levels (mean ± SEM) in skunks treated daily with prolactin (PRL), diluent, or that were left untreated. *Arrows* indicate beginning and ending of treatment. *, Values are significantly ($p<0.05$) greater than other values in that sampling period.

TABLE 2. Effect of CB-154 on duration of preimplantation.

Treatment	n	Duration of preimplantation from: Beginning of treatment $\bar{X}$ ± SD (Days)	Breeding $\bar{X}$ ± SD (Days)
Untreated	7	17 ± 10.4	205 ± 10.4
CB-154	4	63 ± 3.2*	251 ± 3.2*
Diluent	3	11 ± 5.1	199 ± 5.1
CB-154 + prolactin	3	7 ± 4.0	195 ± 4.0

*Differs significantly from other values in column ($p<0.05$). One skunk in this group died prior to implantation of its blastocysts. However, two healthy-appearing blastocysts were recovered after the uterus was flushed.

CB-154 injected daily for 31 days significantly ($p<0.05$) prolonged the preimplantation period when compared to skunks receiving injections of diluent or no treatment (251 ± 3.2 vs. 199 ± 5.1 vs. 205 ± 10.4 days; Table 2). Blastocyst implantation was further delayed for 63 ± 3.2 days after treatment with CB-154 began, whereas blastocysts in the diluent-treated control group implanted in 11 ± 5.1 days. Implantation of the blastocysts occurred an average of 29 ± 3.2 days after CB-154 treatment was terminated. Ovine PRL was able to significantly ($p<0.05$) reverse the inhibitory effect of CB-154, because in skunks receiving both compounds, blastocysts were implanted about the same time as in skunks receiving the diluent and untreated controls (195 ± 4.0 vs. 199 ± 5.1 vs. 205 ± 10.4 days; Table 2).

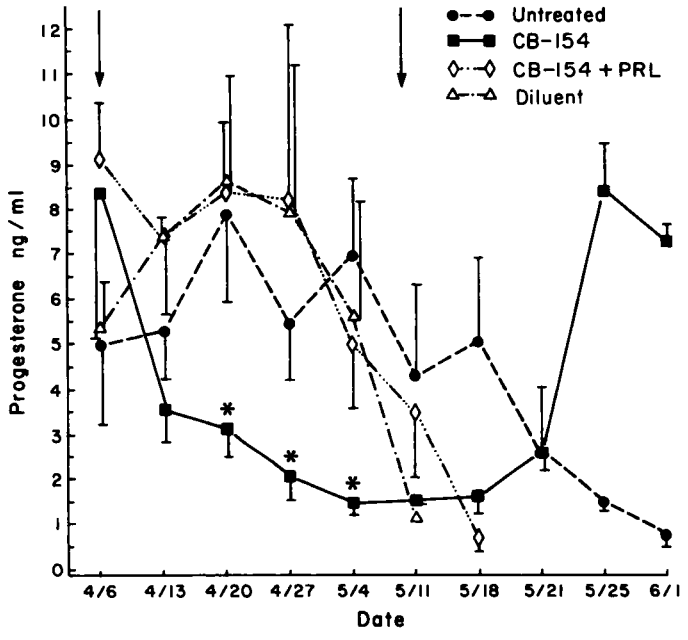

FIG. 3. Plasma progesterone levels (mean ± SEM) in skunks treated daily with bromocriptine (CB-154), CB-154 + prolactin (PRL), diluent, or that were left untreated. *Arrows* indicate beginning and ending of treatment. *, Values are significantly ($p<0.05$) less than other values in that sample period.

CB-154 significantly ($p<0.05$) suppressed progesterone levels after the third week of treatment when compared to skunks receiving CB-154 plus PRL, the diluent, or no treatment (Fig. 3). Progesterone profiles in these 3 groups were not significantly ($p>0.05$) different from each other. Progesterone levels in skunks treated with CB-154 did not begin to rise until treatment was stopped, at which time progesterone concentrations increased within 3 wk to levels normally seen just prior to nidation (Fig. 3).

Experiment 2

Continuous infusion of GnRHa via osmotic minipumps had no significant ($p>0.05$) effect on the duration of preimplantation when compared to skunks receiving the diluent only or no treatment (199 ± 5.1 vs. 203 ± 3.2 vs. 205 ± 10.4 days; Table 3). In skunks receiving GnRHa, blastocysts implanted after 11 ± 5.1 days from the time treatment was initiated, which did not significantly ($p>0.05$) differ from animals receiving minipumps plus diluent (15 ± 3.2 days) or from untreated controls (17 ± 10.4 days; Table 3).

No significant ($p>0.05$) difference between the various treatment groups in progesterone or LH concentrations was found throughout the peri- or postimplantation periods (Figs. 4 and 5). However, LH levels were nondetectable in the skunks receiving GnRHa at the third, fifth, and sixth blood sampling periods, while the untreated control and diluent-treated skunks consistently had detectable LH levels (Fig. 5).

TABLE 3. Effect of gonadotropin-releasing hormone agonist (GnRHa) on duration of preimplantation.

Treatment	n	Duration of preimplantation from: Beginning of treatment $\bar{X}$ ± SD (Days)	Breeding $\bar{X}$ ± SD (Days)
Untreated	7	17 ± 10.4	205 ± 10.4
Minipump + diluent	3	15 ± 3.2	203 ± 3.2
Minipump + GnRHa	5	11 ± 5.1	199 ± 5.1

*No significant difference exist within columns ($p>0.05$).

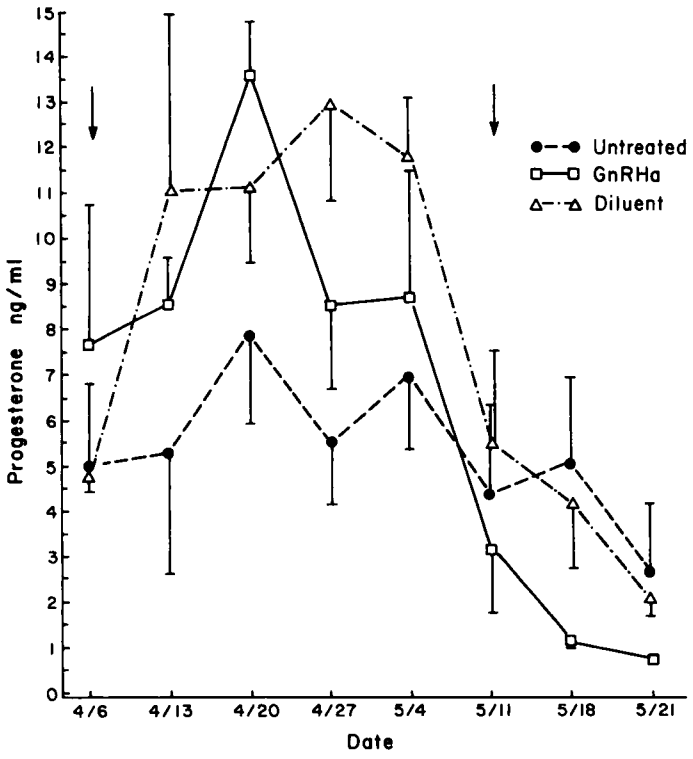

FIG. 4. Plasma progesterone levels (mean ± SEM) in skunks continuously infused with gonadotropin-releasing hormone agonist (GnRHa), diluent, or that were left untreated. *Arrows* indicate the first time osmotic minipumps were inserted and the time they were removed. No significant ($p>0.05$) difference in progesterone levels were found at any time during the treatment.

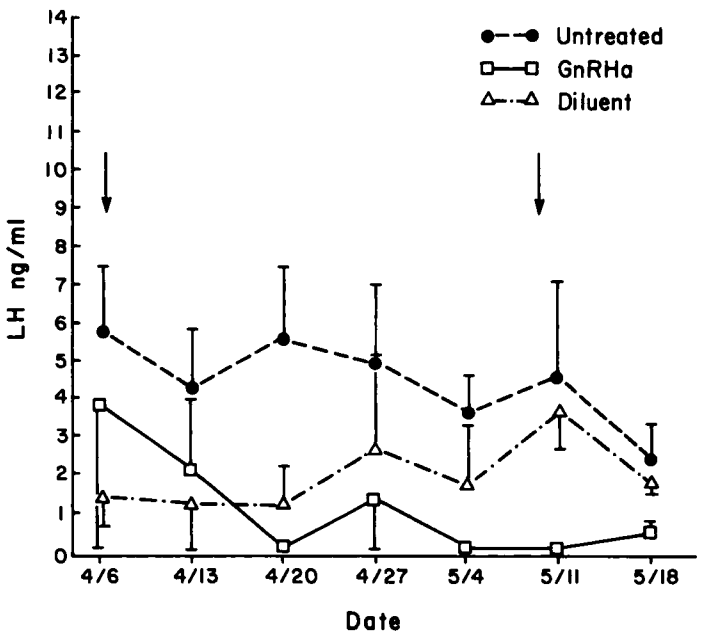

FIG. 5. Plasma luteinizing hormone (LH) levels (mean ± SEM) in skunks continuously infused with gonadotropin-releasing hormone agonist (GnRHa), diluent, or that were left untreated. *Arrows* indicate the first time minipumps were inserted and the time they were removed. No significant ($p>0.05$) difference in LH levels were found at any time during the treatment. However, LH was nondetectable at 4/20, 4/27, and 5/11 in the GnRHa-treated skunks.

DISCUSSION

Corpora lutea of the western spotted skunk are incompletely developed for most of the preimplantation period in that the vast majority of luteal cells are small (Sinha and Mead, 1975) and peripheral plasma levels of progesterone range between 2 and 6 ng/ml. These small luteal cells do not become completely differentiated until the time of implantation, at which time they double in size and plasma progesterone levels significantly increase (Mead and Eik-Nes, 1969). These changes in luteal morphology and function, referred to as luteal activation, always precede nidation. Results of our experiments indicate that PRL is the primary pituitary hormone responsible for activation of the corpora lutea and subsequent initiation of blastocyst implantation. This interpretation is supported by the fact that progesterone levels were significantly elevated above those of controls within 14 days after initiating PRL treatment, and implantation occurred after an average of 24 days of PRL treatment in the 5 skunks that responded to exogenous PRL. One skunk failed to respond to exogenous PRL treatment and her blastocysts implanted within the same time frame as the controls. This could psosibly have been due to the development of antibodies to ovine PRL. Moreover, CB-154, which is a dopamine agonist and therefore inhibits PRL release (Fluckiger, 1978), significantly suppressed plasma progesterone levels and further delayed implantation. The inhibitory effects of CB-154 on luteal function and nidation were reversible as plasma levels of progesterone increased and implantation occurred when exogenous PRL was simultaneously administered with CB-154. Implantation also occurred within 29 ± 3.2 days after CB-154 treatment ceased.

Plasma levels of LH in the spotted skunk gradually increase as daylength increases and the time of implantation approaches (Foresman and Mead, 1974). However, the results of these experiments suggest that LH plays a minor role if any in initiating or maintaining luteal function during the peri- or postimplantation periods in the western spotted skunk. This interpretation is supported by our finding that CB-154-treated skunks with presumably normal endogenous levels of LH were unable to induce luteal

activation at the expected time. Moreover, progesterone levels did not increase until CB-154 treatment ceased. It is possible that CB-154 also suppressed plasma LH in addition to PRL, as has been demonstrated in immature rats and humans (Shaban and Terranova, 1986; Lachelin et al., 1977). If LH is part of a luteotropic complex and CB-154 reduced LH levels, one would predict that plasma progesterone levels in skunks treated with both CB-154 and PRL would be somewhat less than those of controls. However, this was not the case, as skunks receiving both CB-154 and PRL had the same progesterone profile as untreated and diluent-treated controls (Fig. 3). In addition, the GnRH agonist we used suppressed LH to nondetectable levels within 14 days after treatment was initiated, yet this had no significant effect on postimplantation levels of plasma progesterone and did not further delay implantation, which occurred an average of 11 days after initiation of treatment. In addition, both skunks in the GnRHa treatment group that were allowed to complete pregnancy gave birth to live kits. This provides further confirmation that the GnRHa did not sufficiently suppress progesterone levels to terminate pregnancy. It is, however, conceivable that the GnRHa may have initially elevated LH levels before suppressing them and thus somewhat hastened luteal activation and implantation. This view is supported by the fact that plasma progesterone levels were slightly higher by the end of the first week and the preimplantation period was slightly but not significantly shorter in the GnRHa-treated group. However, we believe that the renewed blastocyst development, which is presumed to begin approximately 20 days prior to implantation (Mead and Rourke, 1985; Enders et al., 1986), and luteal activation may have already begun prior to initiation of GnRHa treatment. This interpretation is supported by the fact that plasma progesterone levels were somewhat higher prior to initiation of treatment in this group and implantation occurred somewhat earlier than in the other groups. If this is indeed the case, then one cannot argue that the GnRHa and, thus, LH had a stimulatory effect on luteal function. Consequently, the data do not support the hypothesis that LH plays a major role in luteal activation or postimplantation luteal function. However, the data do not rule out a less important role for LH in maintenance of luteal function in the skunk. In fact, LH may have been the primary hormone maintaining basal progesterone levels during CB-154 treatment. Recent experiments with pseudopregnant ferrets have demonstrated that both LH and PRL are required for luteal maintenance (Agu et al., 1986), but PRL alone was sufficient to sustain luteal function and support blastocyst implantation in ferrets hypophysectomized on the fifth or sixth day of pregnancy (Murphy, 1979). It has also been suggested that LH may form part of a luteotropic complex prior to implantation in the mink (Martinet et al., 1981). However, hypophysectomy of mink during the postimplantation period and constant infusion of 0.5 μg of LH/day resulted in progesterone profiles that did not differ significantly from those of mink receiving both PRL and LH (Murphy et al., 1980).

Our conclusion that PRL is the primary pituitary hormone required for luteal activation and initiation of blastocyst implantation is consistent with results reported for other mustelid carnivores (Donovan, 1963, 1967; Murphy, 1979). PRL has been demonstrated to induce increased luteal function and hasten implantation in intact mink whereas bromocriptine suppressed progesterone levels and further delayed implantation (Papke et al., 1980; Martinet et al., 1981). PRL also induced luteal activation and blastocyst implantation in mink hypophysectomized during delayed implantation (Murphy et al., 1981). Moreover, luteal activation and nidation were significantly hastened in intact mink treated with Pimozide, a dopamine antagonist, which presumably enhanced endogenous prolactin secretion (Murphy, 1983). Consequently, most of the published data for the ferret, mink, and western spotted skunk are consistent with the hypothesis that prolactin is the primary pituitary hormone responsible for luteal activation and termination of embryonic diapause. The latter is not, however, due to progesterone alone (Mead, 1981; Mead et al., 1981) but rather to another luteal product that is now believed to be a protein (Kintner and Mead, 1983; Ravindra et al., 1984; Mead, 1986; Mead et al., 1988). It has long been known that increased daylength will hasten implantation whereas exposure to constant darkness or melatonin generally delays implantation in mustelids (Mead, 1981). It has been reported that increased daylength enhances plasma prolactin levels whereas short day photoperiods or melatonin decrease them in mink (Martinet et al., 1983; Rose et al., 1985). Consequently, results of previous experiments in which the photoperiod has been manipulated to alter the timing of implantation

in the spotted skunk and mink can now be explained on the basis of enhanced PRL secretion.

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