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Levels of Luteinizing Hormone, Estradiol and Progesterone in Serum During the Estrous Cycle and Pregnancy in the Beagle Bitch[1] (38491)

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(Introduced by M. L. Hopwood)

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There is a need for effective alternatives to surgery for controlling canine reproduction. This need is based on the costs of controlling millions of free-roaming dogs, including dollars, hazards to public health and damage to the ecosystem and human sensibilities (1). A prerequistite for developing effective agents to control canine reproduction is a basic knowledge of the hormonal interrelationships in reproductive processes in the bitch.

The estrous cycle of the bitch is characterized by extended periods of proestrus and estrus, each lasting up to 12 days, and in the absence of pregnancy, a pseudopregnancy of 30–90 days duration (2).

The following study was designed to determine levels of luteinizing hormone (LH), progesterone and estradiol-17β during proestrus, estrus, pregnancy and pseudopregnancy in serum collected from Beagle bitches.

Materials and Methods. Animals. Male and female Beagle dogs (1.6–3.3 yr of age) from the Collaborative Radiological Health Laboratory were housed in outdoor pens and the bitches were examined twice weekly for signs of proestrus (vulvular swelling and bleeding). The appearance of a sanguineous vulvar discharge was considered the first day of proestrus. Seven bitches in proestrus were tested daily with a fertile male and were allowed to breed. Four other bitches included in the study were not bred. The first day of acceptance was designated day 1 of estrus. A sharp decline in the number of superficial epithelial cells in the vaginal smear was defined as day 1 of diestrus (3).

Daily blood samples were obtained from 11 bitches (five pregnant and six nonpregnant) during proestrus, estrus and early diestrus. To determine hormonal levels during mid and late pregnancy and pseudopregnancy blood was also collected from pregnant bitches on day 28, 1 day prior to whelping and on the day of whelping and from nonpregnant bitches on days 28 and 117 after the onset of diestrus. In addition, blood samples were collected at 20 min intervals for 3 hr on the second day of acceptance from five bitches. All blood samples were collected from the jugular vein, allowed to clot at 4° and centrifuged at 1200 *g* for 30 min. The serum was decanted and stored at −20° until assayed.

Bioassay. Luteinizing hormone activity of the canine gonadotropin preparations was determined by the ovarian ascorbic acid depletion assay (4, 5) and expressed as NIH-LH-S1 equivalents. Follicle-stimulating hormone (FSH) activity was determined by the HCG-augmentation method (6) and expressed as NIH-FSH-S1 equivalents. Thyroid-stimulating hormone (TSH) was determined by the thyroid-^{32}P uptake method in 2-day-old chicks and expressed as USP unit/mg, using USP thyrotropin (bovine) as the reference standard.

Radioimmunoassay. The details of the radioiodination and radioimmunoassay procedures have been described previously (7, 8). The assay was partially characterized by determining that the inhibition curves produced by the canine LH standard (LER-1685-1), other canine gonadotropin prep-

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arations, crude pituitary extract and serum were parallel. To determine the homogeneity of immunologically active material, crude canine pituitary extract and radioiodinated ovine LH were subjected to electrophoresis on polyacrylamide gels as described previously (9). The gels were cut into 3 mm segments and the segments were eluted overnight in 2 ml phosphate-buffered saline (pH 7.0) containing 0.1% gelatin (gel-PBS). Eluates were subjected to radioimmunoassay to determine LH content or counted directly to determine the location of radioiodinated LH.

Preliminary results suggested that canine TSH may cross react in the LH radioimmunoassay. Therefore, three normal male dogs were administered 100 μg synthetic thyrotropin-releasing hormone (TRH) intravenously to elevate serum levels of TSH. This treatment should not influence serum levels of LH. Blood samples were collected at 30-min intervals for 4-hr, beginning 1 hr prior to injection of TRH. Serum from these samples was assayed for thyroxine (T_4) and LH by radioimmunoassay. The T_4 determinations were performed using a kit obtained from Abbott Laboratories.

Concentrations of progesterone in sera were determined by a specific radioimmunoassay described previously (10). One ml of serum was extracted twice with 5 ml petroleum ether and the extract taken to dryness. The dried extract was reconstituted in 1.0 ml gel-PBS and duplicate 200 μl aliquots were assayed for progesterone. Recoveries were monitored by addition of 10,000 dpm ^{3}H-progesterone (specific activity 50.3 Ci/mmole) prior to extraction.

The radioimmunoassay for estradiol-17β was similar to that described by England *et al.* (11). Three ml of serum were extracted three times with 10 ml anhydrous diethyl ether, and the extract was taken to dryness. The dried extract was redissolved in 1 ml gel-PBS, and duplicate 300 and 30 μl aliquots were assayed for estradiol-17β. Recoveries were monitored by addition of 4000 dpm ^{3}H-2,4,6,7-estradiol-17β (specific activity 106 Ci/mmole) prior to extraction.

Results. Inhibition curves obtained with crude pituitary extract and sera from normal male dogs and from bitches during different

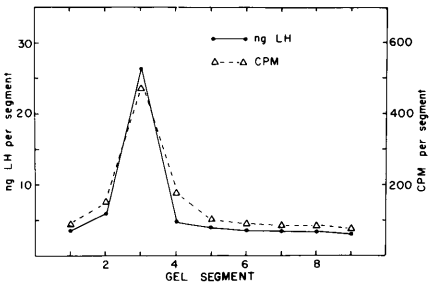

FIG. 1. Luteinizing hormone content of eluates from segments following polyacrylamide gel electrophoresis of canine pituitary extract. Each segment was 3 mm in length. Radioiodinated ovine LH was added to a separate gel and treated in a similar manner. The counts per minute from segments of that gel are also shown.

physiological states were parallel to those obtained with the standard (LER-1685-1). The sensitivity of the assay, defined as the least amount of standard LH which significantly ($P < 0.05$) inhibited the binding of radioiodinated ovine LH to the antiserum was 200 pg. Electrophoresis of the canine pituitary extract and radioiodinated ovine LH on polyacrylamide gel suggested that the immunological activities associated with these preparations were electrophoretically similar (Fig. 1). The relative LH, FSH and TSH contents of five canine gonadotropin preparations are listed in Table I. The mean values obtained by radioimmunoassay include duplicate estimates from at least four points on the inhibition curve. In all but one preparation the estimates of LH potency by radioimmunoassay are in close agreement with bioassay estimates, even though large differences in LH to FSH ratios existed. That the high level of TSH present in LER-1685-1 was not responsible for the significant ($P < 0.05$) difference observed in the radioimmunological and biological estimates of the LH content of this preparation was suggested by the results following injection of TRH into male dogs. Levels of T_4 in serum were increased by 90 min following the injection of TRH indicating that TSH had been released previously. Serum LH levels following administration of TRH did not increase suggesting that TSH does not interfere in the canine LH radioimmunoassay (Table II). Quantitative recovery

TABLE I. GLYCOPROTEIN HORMONE CONTENT OF CANINE PITUITARY PREPARATIONS.

Preparation	TSH by Bioassay USP unit/mg	FSH by Bioassay NIH-FSH-S1 unit/mg	LH Content by Bioassay NIH-LH-S1 unit/mg	LH Content by RIA NIH-LH-S1 unit/mg	RIA/BIO
LER-1685-3A	—	.54	0.0024	0.0024	
LER-1685-1	0.59	0.045	0.025 (0.021–0.030)[a]	0.068 (0.0057–0.0076)	2.72
LER-1685-2	—	—	0.0045 (0.0029–0.0065)	0.0042 (0.0009–0.0075)	1.07
LER-1685-3B	—	.38	0.0011 (0.00061–0.0017)	0.0015 (0.0013–0.0015)	0.73
LER-1685-4	—	0.09	0.0011 (0.00074–0.0016)	0.0010 (0.0008–0.0011)	1.10

[a] Mean ± 95% confidence intervals.

of various amounts of exogenous canine LH added to 200 μl of serum from normal male dogs and estrous bitches suggests that serum components other than LH do not interfere with the assay. Regression analysis of these data resulted in a slope of the regression line of 0.99 and a correlation coefficient of 1.00. The within assay error, estimated from 15 replicates of 200 μl of serum, was 3.2% (coefficient of variation), and the between assay error (determined by assaying the same serum sample in duplicate in six different assays) was 16%.

Levels of LH, estradiol-17β and progesterone in serum during proestrus, estrus and early diestrus are depicted in Fig. 2. There were no significant differences during proestrus and estrus in the levels of these hormones in sera of bitches mated to fertile males and those not mated. Therefore, the data were pooled to obtain means for 11 females. During proestrus, mean levels of estradiol-17β and LH were 56.7 ± 6.5 pg/ml and 2.8 ± 0.1 ng/ml, respectively. A sharp increase in levels of LH to 35.5 ± 10.0 ng/ml was found to coincide with the onset of estrus. The levels of estradiol-17β were maximal (68.9 ± 11.0 pg/ml) about 24 hr prior to the LH peak. Following the peak, levels of LH declined rapidly and remained low for the duration of estrus. Levels of estradiol-17β decreased gradually reaching a nadir approximately 5 days after the LH peak and remained low for the duration of estrus. During proestrus and early estrus progesterone levels remained below 2 ng/ml. The levels of progesterone began to increase approximately 2 days after the LH peak, reaching a maximum of 24.2 ± 3.3 ng/ml 7 days following the LH peak.

TABLE II. SERUM LEVELS OF THYROXINE AND LUTEINIZING HORMONE IN NORMAL MALE DOGS TREATED WITH THYROTROPIN-RELEASING HORMONE.[a]

Time (minutes)	Thyroxine μg/100 ml	LH ng/ml
−60	1.35 ± 0.05	8.4 ± 7.1
−30	1.49 ± 0.19	23.0 ± 14.4
0	1.47 ± 0.26	19.4 ± 12.0
30	1.66 ± 0.08	12.8 ± 6.3
60	1.89 ± 0.16	9.8 ± 9.2
90	2.44 ± 0.37	14.0 ± 7.8
120	2.14 ± 0.28	15.1 ± 11.9
150	2.27 ± 0.40	13.4 ± 9.1
180	2.67 ± 0.49	13.2 ± 8.7

[a] One-hundred μg of TRH was administered to each of three dogs at time 0. The values listed are mean levels, ± S.E.

On day 28 following a sharp decline in the number of superficial epithelial cells in the vaginal smears, there were no significant differences ($P > 0.05$) in the levels of LH, estradiol-17β and progesterone between pregnant and nonpregnant bitches (Table III). The levels of estradiol-17β tended to be higher ($P < 0.10$) in nonpregnant bitches (23.4 ± 7.3 pg/ml) than in pregnant bitches (14.3 ± 1.6 pg/ml).

Levels of progesterone declined significantly ($P < 0.05$) to 3.3 ± 1.2 ng/ml one day prior to whelping in the pregnant bitches. A further decrease to 1.2 ± 0.2 ng/ml was observed on the day of whelping. In contrast, levels of estradiol-17β were 14.6 ± 6.4 pg/ml and 13.0 ± 7.4 pg/ml 1 day prior to whelping and on the day of whelping, respectively, in the same animals. Levels of LH were not different at all times follow-

ing the LH peak in both pregnant and nonpregnant bitches (Table III).

Analyses of the samples collected at 20-min intervals suggests that LH is not released in episodic bursts but rather is secreted almost continuously. Some variation was noted in levels of estradiol-17β and progesterone in the same samples, but neither appeared to be secreted in a definite pattern. Values from a representative bitch are listed in Table IV.

Discussion. That FSH and TSH do not interfere in the radioimmunoassay for canine LH is strongly suggested from the analysis of samples following polyacrylamide gel electrophoresis of canine pituitary extract and following injection of TRH into dogs. The rigorous chemical purification of LER-1685-1 may have partially destroyed the biological activity of the LH in this preparation without affecting its immunological activity accounting for the significant difference between estimates of LH content by bioassay and radioimmunoassay for this preparation. Inhibition curves obtained with crude pituitary extract, other canine LH pituitary preparations and sera were parallel to the standard, further indicating the immunologic similarity between the inhibiting material present in these preparations and standard canine LH. Quantitative recovery of LH added to serum suggests that serum components *per se* do not interfere with the measurement of LH by radioimmunoassay.

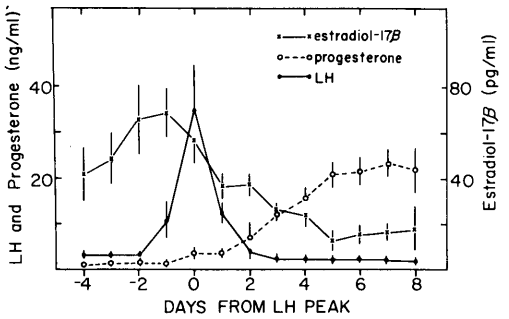

FIG. 2. Serum levels of luteinizing hormone, estradiol-17β and progesterone in Beagle bitches. All data are normalized to the occurrence of the LH peak.

The increase in levels of estradiol-17β prior to the peak of LH in Beagle bitches is similar to that observed in ewes (12), cows (13, 14), pigs (15) and women (16). Levels of estradiol-17β during proestrus were nearly three times greater than levels observed during diestrus. Proestrous levels reached a maximum approximately 24 hr prior to the LH peak. These results are consistent with those of Jones *et al.* (17) in the bitch, Korenman and Sherman (18) in women and Henricks *et al.* (19) in the cow. In contrast, Bell *et al.* (20) and Phemister *et al.* (21) did not find an increase in estrogen before the peak of LH in Beagle bitches. However, these investigators measured total estrogens rather than estradiol-17β. Levels of progesterone in serum were lowest prior to the LH peak and began to increase rapidly 48 hr following the peak of LH. Levels of progesterone continued to increase through-

TABLE III. SERUM LEVELS OF LUTEINIZING HORMONE, ESTRADIOL AND PROGESTERONE IN NONPREGNANT AND PREGNANT BITCHES.

			Whelping	
	Diestrus day 28	Anestrus	one day before whelping	on the day of whelping
Nonpregnant				
LH (ng/ml)	4.17 ± 1.10	2.75 ± 0.29[a]	—	—
Estradiol (pg/ml)	23.41 ± 7.28	32.54 ± 15.07	—	—
Progesterone (ng/ml)	18.20 ± 6.06	0.55 ± 0.08[a]	—	—
Pregnant				
LH (ng/ml)	2.97 ± 0.24	—	2.46 ± 0.31	1.86 ± 0.30
Estradiol (pg/ml)	14.28 ± 1.60	—	14.56 ± 6.39	12.98 ± 7.38
Progesterone (ng/ml)	14.88 ± 10.32	—	3.34 ± 1.16	1.15 ± 0.22

[a] Includes bitches that whelped after the last estrous period and those not bred at the last estrous period.

TABLE IV. SERUM LUTEINIZING HORMONE, ESTRADIOL AND PROGESTERONE IN THE BLOOD SAMPLES COLLECTED AT 20-MIN INTERVALS FROM A BEAGLE BITCH ON DAY 2 OF ESTRUS.

Sample #	LH (ng/ml)	Estradiol (pg/ml)	Progesterone (ng/ml)
1	7.3	104.9	3.4
2	11.8	79.1	3.6
3	7.9	96.1	3.5
4	10.9	58.4	4.5
5	10.0	45.8	4.7
6	11.8	58.8	5.0
7	8.1	47.5	1.6
8	7.4	58.7	2.2
9	9.1	36.4	2.2

out estrus and were more than 10 times greater than the levels found during proestrus by the onset of diestrus. These data are consistent with previous reports (17, 22, 23). The bitch is unique when compared to other domestic animals in that estrus is continued in the presence of high levels of progesterone.

Elevated levels of progesterone on day 28 of diestrus and of pregnancy suggests that luteal activity is high for at least 28 days in this species. No differences in the concentrations of LH, estradiol-17β nor progesterone were found between pregnant and nonpregnant bitches on day 28 after the decline in superficial epithelial cells in the vaginal smear. Serum progesterone had decreased significantly by 117 days after the onset of diestrus in nonpregnant bitches. In pregnant bitches progesterone levels were low one day before whelping with a further decrease noted on the day of whelping. Levels of estradiol-17β and LH were not different at midpregnancy nor prior to whelping in the pregnant bitches. These findings are consistent with previous reports (22). Similarly, the levels of progesterone in other species have been noted to decline prior to parturition (24, 25). In contrast to the bitch, levels of estradiol-17β increase dramatically prior to parturition in most species (24, 25). An inverse relationship between levels of estradiol-17β and progesterone in serum was observed during proestrus, estrus and diestrus in pregnant and nonpregnant bitches.

Analysis of LH, progesterone and estradiol-17β in serum samples collected at 20 min intervals for 3 hr on the second day of acceptance suggested that these hormones were not released in episodic bursts in the bitch. Episodic bursts of LH release have been reported in ewes (26), monkeys (27) and women (28).

Summary. Levels of luteinizing hormone (LH), estradiol-17β and progesterone were determined by specific radioimmunoassays in sera obtained from Beagle bitches during proestrus, estrus and diestrus. Concentrations of LH (expressed as NIH-LH-Sl equivalents) were: 2.8 $\pm$ 0.1 ng/ml in proestrus, 35.5 $\pm$ 10.0 ng/ml during early estrus and 2.2 $\pm$ 0.1 ng/ml in early diestrus. Peak levels of estradiol-17β (68.9 $\pm$ 11.0 ng/ml) were detected 24 hr prior to the LH peak, declined rapidly and reached basal levels (17.8 $\pm$ 6.3 ng/ml) by five days following the LH peak. Levels of progesterone were 1.7 $\pm$ 0.3 ng/ml during proestrus, 3.5 $\pm$ 0.3 ng/ml during early estrus and 23.3 $\pm$ 2.8 ng/ml on day 5 after the LH peak. Progesterone levels remained elevated through day 28 of diestrus and pregnancy. A significant decrease ($P < 0.05$) in levels of progesterone occurred between day 28 of pregnancy and one day prior to whelping (3.3 $\pm$ 1.2 ng/ml), with a further decrease on the day of whelping (1.1 $\pm$ 0.2 ng/ml). Levels of estradiol-17β and LH did not change significantly ($P > 0.05$) during diestrus or pregnancy.

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