Serotonin antagonist-induced lowering of prolactin secretion does not affect the pattern of pulsatile secretion of follicle-stimulating hormone and luteinizing hormone in the bitch

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Abstract

Dopamine agonists decrease plasma prolactin concentration and shorten the duration of anoestrus in the bitch. In order to determine whether this shortening results from decreased prolactin release or is due to another dopamine agonistic effect on the pulsatile release of FSH and LH, eight anoestrous beagle bitches were treated with a low dose of the serotonin antagonist metergoline (0.1 mg per kg body weight, twice daily) starting 100 days after ovulation. Six-hour plasma profiles of LH and FSH were obtained 7 days before, immediately before, 1 week after, and then at 2-week intervals after the start of the treatment with the serotonin antagonist until signs of pro-oestrus appeared. Plasma prolactin concentration was measured three times weekly from 75 to 142 days after ovulation and thereafter once weekly until the next ovulation, and was observed to decrease significantly after the start of treatment. The length of the interoestrous interval in the treated dogs was, however, not different from that in the preceding pretreatment cycle or from that in a group of untreated bitches. During the first weeks of treatment no changes were observed in the pulsatile plasma profiles of FSH and LH. Four weeks after the start of the treatment with the serotonin antagonist there was an increase in the mean basal plasma FSH concentration and the mean area under the curve for FSH, without a concurrent increase in LH secretion. The increase in FSH secretion continued until late anoestrus. In conclusion, the serotonin antagonist-induced lowering of plasma prolactin concentration was not associated with shortening of the interoestrous interval. The plasma profiles of LH and FSH were similar to those observed during physiological anoestrus, but different from those observed during anoestrus shortened by treatment with a dopamine agonist. Hence the prematurely induced oestrus observed during administration of dopamine agonists cannot be explained by a decreased plasma prolactin concentration but must be due to some other dopamine agonistic effect, probably increased FSH secretion. The observations in this study further strengthen the hypothesis that an increase in circulating FSH is essential for ovarian folliculogenesis and consequently the termination of anoestrus in the bitch.


Introduction

The oestrous cycle in the dog is considerably longer than that in most other domestic animals. The follicular phase and spontaneous ovulations are followed by a luteal phase having an average duration of about 75 days and a non-seasonal anoestrus of 2–10 months (Bouchard et al. 1991, Concannon 1993, Schaefers-Okkens 1996). Although several of the hormonal changes during the progression of anoestrus and the start of a new follicular phase are known, the exact mechanism controlling the transition from anoestrus to the follicular phase is still not completely elucidated in the bitch.

In the bitch, progression from early to late anoestrus is characterized by increased release of gonadotrophin-releasing hormone (GnRH) by the hypothalamus (Tani et al. 1996). There is also enhanced hypothalamic expression of the genes encoding for the oestrogen receptor (Tani et al. 1997) and the P450 aromatase that catalyses oestrogen biosynthesis (Inaba et al. 2002). During the course of anoestrus, there is an increase in the sensitivity of the pituitary to GnRH (Van Haften et al. 1994) and in ovarian
responsiveness to gonadotrophins (Jeffcoate 1993). A rise in the basal plasma follicle-stimulating hormone (FSH) concentration (Kooistra et al. 1999a, Onclin et al. 2001) and increased luteinizing hormone (LH) pulsatility shortly before the onset of pro-oestrus (Concannon et al. 1986, Kooistra et al. 1999a,b, Tani et al. 1999) appear to be important determinants of the initiation of a new follicular phase leading to ovulation in the bitch.

In addition to these changes in the hypothalamic–pituitary–ovarian axis, dopaminergic influences appear to be involved in the initiation of a new follicular phase in the bitch. Dopamine agonists such as bromocriptine and cabergoline decrease plasma prolactin concentration and shorten the interoestrous interval (Okkens et al. 1985a, Onclin et al. 1995), suggesting that the latter effect is due to the former. Shortening of the luteal phase may indeed be ascribed to the prolactin-lowering effect of dopamine agonists (Beijerink et al. 2003), for prolactin is the main luteotrophic factor in the bitch (Okkens et al. 1990). However, the role of dopamine agonist-induced lowering of plasma prolactin concentration in the shortening of anoestrus is questionable. Metergoline, a serotonin antagonist when given in a low dose, also appears to suppress prolactin secretion, but does not shorten anoestrus (Okkens et al. 1997a). Furthermore, low-dose bromocriptine administration shortens anoestrus without suppressing plasma prolactin concentration (Beijerink et al. 2003), while low plasma prolactin concentrations have been found during anoestrus under physiological conditions (Kooistra & Okkens 2002). Finally, no obvious changes in plasma prolactin concentration have been observed in the bitch during the transition from anoestrus to the follicular phase (Olson et al. 1982). These observations indicate that dopamine agonists do not induce a follicular phase by suppressing prolactin secretion but rather by other direct or indirect dopaminergic effects.

The bromocriptine-induced shortening of anoestrus in the bitch is also associated with an increase in basal FSH secretion without a concurrent rise in LH secretion (Kooistra et al. 1999b). Based on this and the observation that FSH concentration rises late in physiological anoestrus (Kooistra et al. 1999a), an increase in circulating FSH should be considered essential for ovarian folliculogenesis in this species. The observation that serotonin antagonists, in contrast to dopamine agonists, do not shorten the interoestrous interval despite decreased prolactin secretion prompted us to investigate the effects of a low dose of the serotonin antagonist metergoline on the pulsatile secretion patterns of FSH and LH.

Materials and Methods

Animals, treatment and collection of blood samples

Eight healthy beagle bitches, aged 1.5 to 7 years, weighing 9.6 to 15.2 kg and ovulating at different times of the year, were used in this study. All were whelped and raised in the Department of Clinical Sciences of Companion Animals and were accustomed to the laboratory environment and procedures such as collection of blood samples. They were housed in pairs in indoor-outdoor runs, fed a standard commercial dog food once daily, and water was available ad libitum.

Each dog was examined thrice weekly for swelling of the vulva and the presence of a serosanguinous vaginal discharge, which were considered to signify the onset of pro-oestrus. Ovulation (day 1) was estimated by measuring the plasma progesterone concentration three times weekly from the start of pro-oestrus onwards using a 125I radioimmunoassay (RIA) previously validated for fertility breeding management (Okkens et al. 2001). The intra-assay and interassay coefficients of variation were 6% and 10.8% respectively, and the limit of quantitation was 0.13 nmol l\(^{-1}\). Blood samples were collected via jugular venipuncture.

Each dog received 0.1 mg of the serotonin antagonist metergoline (Contralac, generously provided by Virbac, Barneveld, The Netherlands) per kg body weight orally twice daily, at 0900 and 2100 h daily, starting 100 ± 2 (mean±s.d.) days after ovulation, immediately after the sampling for the second plasma profile.

Measurements of the 6-h plasma profiles of LH and FSH were made 7 days and 1 day before treatment with metergoline (days 93 and 100), then after 7 and 14 days of treatment and subsequently every 2 weeks until signs of pro-oestrus appeared. In two bitches the plasma profiles were measured six times (until day 142), in four bitches seven times (until day 156), in one bitch eight times (until day 170), and in one bitch 11 times (until day 212). Blood samples were collected at 15-min intervals between 0800 and 1400 h, placed immediately in chilled EDTA-coated tubes, and centrifuged at 4°C for 10 min at 1500 g; plasma was stored at −25°C until analysis.

Plasma prolactin concentration was measured thrice weekly from day 75 to day 142 and once weekly thereafter until the next ovulation. To ascertain that ovulation was not missed during treatment with the serotonin antagonist, plasma progesterone concentration was measured once weekly from day 75 until the next ovulation.

The interoestrous interval was defined as the number of days between ovulations. In four of the dogs the mean duration of the preceding interoestrous interval was 214 ± 20 days, while the fifth was treated after the first ovulation and the remaining three had whelped during the preceding cycle. The mean duration of the interoestrous interval in 10 bitches in the same colony during the period of these experiments was 195 ± 11 days.

Hormone measurements

From 75 days after ovulation until the next ovulation, plasma progesterone was measured by a previously validated 3H-RIA (Dieleman & Schoenmakers 1979, Okkens et al. 1985b). The intra-assay and interassay
coefficients of variation were 11% and 14% respectively, and the lower limit of measurement was 0.13 nmol l\(^{-1}\).

Plasma FSH was measured by a homologous canine immunoradiometrical assay (IRMA; AHCOO4, Biocode SA, Liège, Belgium) using monoclonal antibodies to canine FSH, a canine FSH standard, and \(^{125}\)I-labelled monoclonal anti-canine FSH antibodies. The intra-assay and interassay coefficients of variation for values above 1.6 µg l\(^{-1}\) were 3.2% and 15% respectively. The limit of measurement was set at the lowest standard point, i.e. 1.50 µg l\(^{-1}\).

Plasma LH concentration was measured by a heterologous RIA described previously by Nett et al. (1975), with a few modifications. A rabbit antiserum raised against ovine LH (CSU-204, kindly supplied by G D Niswender, Colorado State University, CO, USA), radio-iodinated bovine LH-7981, and canine pituitary standard LER 1685-1 (a gift of Dr L E Reichert, Albany Medical College, NY, USA) were used in this assay. The intra-assay and interassay coefficients of variation for values above 0.5 µg l\(^{-1}\) were 2.3% and 10.5% respectively, and the lower limit of measurement was 0.3 µg l\(^{-1}\).

Plasma prolactin concentration was determined by a previously validated heterologous RIA (O¨kkens et al. 1985 b). The intra-assay and interassay coefficients of variation were 3.5% and 11.5% respectively, and the lower limit of measurement was 0.8 µg l\(^{-1}\).

Data analysis

The 6-h pulsatile profiles of plasma FSH and LH were analysed by means of the Pulsar programme developed by Merriam and Wachter (1982). The programme identifies secretory peaks by height and duration from a smoothed baseline, using the assay s.d. as a scale factor. The cut-off parameters G1-G5 of the Pulsar programme were set at 3.98, 2.40, 1.68, 1.24 and 0.93 times the assay s.d., as criteria for accepting peaks that were 1, 2, 3, 4 and 5 points wide respectively. The smoothing time, a window used to calculate a running mean value omitting peaks, was set at 4 h. The splitting cut-off parameter was set at 2.7 and the weight assigned to peaks was 0.05. The A, B and C values of the Pulsar programme, used to calculate the variance of the assay, were set at A = 0, B = 5 and C = 0 for the FSH assay and at A = 0, B = 9.5 and C = 20 for the LH assay. The values extracted from the Pulsar analyses included: the mean of the smoothed baseline (basal plasma hormone concentration), the mean peak amplitude, the pulse frequency, and the area under the curve above the zero line (AUC).

Differences in the mean duration of the interoestrous intervals were analysed by unpaired or, if appropriate, paired Student’s t-test. Differences in prolactin secretion were analysed using a linear model with treatment effect, day effect and treatment-day interaction as factors, using logarithmic transformation to normalize the prolactin values. The model included a AR(1) (first order autoregressive process) correlation process and different variances before treatment compared with during treatment. According to Akaike Information Criterion this model could not be reduced. Changes in the characteristics of the pulsatile secretion patterns of LH and FSH were evaluated by ANOVA for repeated measures on the following time points: mean of the values before treatment (days 93 and 100), days 107, 114, 128 during treatment, and during late anoestrus (26±3 days before the next ovulation). Subsequently, multiple comparisons were performed using the Student-Newman-Keuls test. Differences in pulse frequency were determined by non-parametric analysis using the Friedman test, and multiple comparisons were performed using Dunnett’s test. P < 0.05 was considered significant. Results are presented as means±s.e.m.

Ethics of experimentation

This study was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Utrecht University.

Results

The mean interoestrous interval in the eight bitches treated with the serotonin antagonist metergoline (183±8 days) did not differ significantly from that in 10 untreated beagle bitches in the same colony during the same period (195±11 days). In addition, the mean interoestrous interval in four treated bitches (192±8 days) did not differ significantly from that in the cycle preceding treatment (214±20 days). The mean plasma progesterone concentration on day 97 was 2.58±0.60 nmol l\(^{-1}\). After the start of the treatment the plasma progesterone concentration remained low in all dogs until the next pro-oestrus. Plasma prolactin concentrations in individual dogs are shown in Fig. 1. Before treatment the average prolactin concentration decreased slightly with a negative day effect.

Figure 1 Plasma prolactin concentrations in eight beagle bitches before and during treatment with the serotonin antagonist metergoline. In each dog, plasma prolactin concentration was measured three times weekly from day 75 to day 142 and once weekly thereafter until the next ovulation. Treatment with 0.1 mg metergoline per kg body weight twice daily was started 100±2 days after ovulation and was continued until the next ovulation.
A marked significant decreasing treatment effect on the plasma prolactin concentration from the time of start of treatment was observed ($P = 0.001$). Throughout treatment there was no significant treatment-day interaction effect. Furthermore, the variance in the data before treatment was significantly higher than during treatment.

Both LH and FSH were secreted in a pulsatile fashion (Figs 2 and 3). In the 6-h profiles of the eight bitches, 65 significant LH pulses were identified by the Pulsar programme, 56 of which coincided with a significant FSH pulse. Six of the nine LH pulses lacking a concurrent significant FSH pulse were observed during late anoestrus. There were no significant FSH pulses without a concurrent LH pulse. Plasma LH pulses rose much higher above the basal level than did FSH pulses (Figs 2 and 3). Both were characterized by an abrupt and rapid rise.

**Figure 2** The 6-h plasma profiles of FSH (black squares) and LH (grey diamonds) in a 5-year-old beagle bitch at 93, 100, 107, 114 and 128 days after ovulation and during late anoestrus (day 170). Blood samples were collected at 15-min intervals. Treatment with the serotonin antagonist metergoline (0.1 mg per kg body weight twice daily) was started 100 days after ovulation and was continued until the next ovulation. Pro-oestrus was observed on day 184 and ovulation occurred on day 197. * Significant pulses of both FSH and LH identified by the Pulsar programme.
rise followed by a slow decline, slower for FSH than for LH.

Compared with pre-treatment values, during the first 2 weeks of treatment with metergoline there were no significant changes for either FSH or LH in the mean value of the smoothed baseline, the mean AUC above the zero line, the pulse frequency, or the mean peak amplitude of the plasma profile (Table 1). On day 128 both the mean basal plasma concentration and the mean AUC of FSH were significantly higher than the mean pre-treatment values (Fig. 4). The mean basal plasma concentration and the mean AUC of FSH during late anoestrus (26 ± 3 days before the next ovulation) were significantly higher than before treatment or at 1, 2 and 4 weeks (days 107, 114 and 128 respectively) after the start of metergoline treatment (Fig. 4). The mean basal
plasma LH concentration, the mean AUC of LH, the mean pulse frequencies of LH and FSH, and the mean peak amplitudes of LH and FSH did not change significantly with time (Table 1).

In two bitches there were frequent short pulses in the last 6-h plasma profile of LH, a few days before the onset of pro-oestrus and approximately 14 days before the assumed day of ovulation (Fig. 3).

**Discussion**

Metergoline, of which the prolactin-lowering effect of a low dose is due to its serotonin antagonistic properties (Müller et al. 1983), significantly suppressed both prolactin secretion and the variance in prolactin secretion. These findings are consistent with observations in previous studies that the same dose of this serotonin antagonist decreased plasma prolactin concentrations and their variance in non-pregnant anoestrous bitches (Okkens et al. 1997a) and suppressed the high plasma prolactin concentrations in pseudopregnant Afghan hounds within 2 h (Okkens et al. 1997b). After the onset of treatment there was no significant treatment-day interaction, indicating that the rate of suppression of prolactin secretion was the same throughout the whole treatment period. The slight decrease in the plasma prolactin concentrations before treatment could be related to the progression of early anoestrus (Kooistra & Okkens 2002). This slight decrease in plasma prolactin concentration before treatment was followed by a marked decrease concurrent with the start of the treatment with the serotonin antagonist. Seasonal influence on circulating prolactin concentrations, described by Kreeger and Seal (1992) and Corrada et al. (2003), can be ruled out, because the bitches ovulated at different times of the year.

Dopamine agonists shorten the length of anoestrus in the bitch (Okkens et al. 1985a, Onclin et al. 1995, Kooistra et al. 1999b). Taking into account the prolactin-lowering effects of dopamine agonists, it was hypothesized that the premature oestrus after treatment with dopamine agonists was due to a decreased prolactin level. However, in accordance with the results of an earlier study (Okkens et al. 1997a), the serotonin antagonist-induced lowering of the plasma prolactin concentration in the present study did not lead to premature oestrus. These findings and the observation that low dosage bromocriptine shortens the interoestrous interval without suppressing plasma prolactin concentration (Beijerink et al. 2003) provide further evidence that other effects of dopamine agonists must be responsible for the induction of premature oestrus.

The dopamine agonist-induced shortening of anoestrus in the bitch is associated with increased secretion of FSH

**Table 1** Pulse characteristics for 6-h plasma profiles of FSH and LH in eight healthy beagle bitches treated with the serotonin antagonist metergoline, starting 100 days after ovulation (day 100). The plasma profiles were determined just before treatment (Before), on days 107, 114 and 128 during treatment, and during late anoestrus (26 ± 3 days before the next ovulation). The values are expressed as means ± s.e.m. or median and range, and n = number of bitches in which a parameter could be determined.

<table>
<thead>
<tr>
<th></th>
<th>Before (n = 8)</th>
<th>Day 107 (n = 8)</th>
<th>Day 114 (n = 8)</th>
<th>Day 128 (n = 8)</th>
<th>Late anoestrus (n = 8)</th>
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<tr>
<td>LH peak amplitude (µg/l)</td>
<td>9.0 ± 2.0 (n = 8)</td>
<td>9.0 ± 1.9 (n = 7)</td>
<td>17.0 ± 2.3 (n = 5)</td>
<td>14.9 ± 3.8 (n = 6)</td>
<td>10.0 ± 1.7 (n = 8)</td>
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<td>FSH peak amplitude (µg/l)</td>
<td>4.5 ± 0.5 (n = 7)</td>
<td>5.1 ± 1.1 (n = 7)</td>
<td>8.1 ± 1.9 (n = 5)</td>
<td>6.3 ± 0.9 (n = 6)</td>
<td>5.5 ± 1.0 (n = 6)</td>
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<td>LH pulse frequency (peaks/6 h)</td>
<td>1 (0–3)</td>
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<td>FSH pulse frequency (peaks/6 h)</td>
<td>1 (0–3)</td>
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<td>1 (1–4)</td>
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<td>LH basal (µg/l)</td>
<td>1.3 ± 0.1</td>
<td>1.3 ± 0.2</td>
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<td>FSH basal (µg/l)</td>
<td>3.7 ± 1.0</td>
<td>4.2 ± 0.8</td>
<td>4.1 ± 0.6</td>
<td>5.2 ± 0.5</td>
<td>6.7 ± 0.9</td>
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<td>AUC for LH (µg/l*6 h)</td>
<td>12.7 ± 2.0</td>
<td>11.1 ± 1.3</td>
<td>12.3 ± 2.3</td>
<td>12.4 ± 1.4</td>
<td>13.8 ± 2.0</td>
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<tr>
<td>AUC for FSH (µg/l*6 h)</td>
<td>26.3 ± 7.0</td>
<td>28.5 ± 6.0</td>
<td>29.5 ± 3.9</td>
<td>35.7 ± 3.6</td>
<td>43.1 ± 4.6</td>
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**Figure 4** Mean (± s.e.m.) basal plasma FSH (left panel) and LH (right panel) concentrations in eight beagle bitches before (days (d) 93 + 100) and during serotonin antagonist treatment (days (d) 107, 114 and 128), and during late anoestrus. Treatment with the serotonin antagonist metergoline (0.1 mg per kg body weight twice daily) was started about 100 days after ovulation (day 100) and was continued until the next ovulation.

*Significant difference (P < 0.05).
but not LH (Kooistra et al. 1999b). The increase in FSH secretion occurred 2 weeks after the start of bromocriptine administration. In contrast, during the first weeks of treatment with the serotonin antagonist metergoline there were no significant changes in the pulsatile plasma profiles of FSH or LH. These findings indicate that serotonin antagonist-induced lowering of plasma prolactin does not lead to increased secretion of FSH.

Four weeks after the start of treatment with the serotonin antagonist, mean basal plasma FSH concentrations and the mean AUC for FSH increased without a concurrent change in the pulsatile plasma profiles of LH. The increase in FSH secretion continued until late anoestrus. These changes in secretion of the gonadotrophins are very similar to those observed during physiological anoestrus (Kooistra et al. 1999a). In most mammals studied, FSH is considered to be the most important factor in the early stages of follicular development (Monniaux et al. 1997). There are similarities in women, in whom observations during gonadotrophin-induced ovulation have emphasized that plasma FSH concentrations must exceed a certain level before preantral follicles reaching the FSH-dependent stage can progress to maturation (Brown 1978, Schoemaker et al. 1993). It can be hypothesized that in dogs dopamine agonists raise plasma FSH concentration above that level, with consequent shortening of anoestrus. Because the serotonin antagonist metergoline does not induce an increase in FSH secretion, premature oestrus does not occur.

The mean plasma progesterone concentration on day 97 was 2.58 ± 0.60 nmol l\(^{-1}\). After the start of the treatment plasma progesterone concentration remained low in all the dogs until the start of the next pro-oestrus. This indicates that the dogs were in anoestrus at the start of the treatment with the serotonin antagonist and that no oestrus was missed during the experiment.

In two bitches the 6-h plasma profile of LH during late anoestrus revealed frequent brief pulses of LH without concurrent increases in FSH. This pattern of LH secretion shortly before the start of pro-oestrus has been reported previously and has been associated with termination of anoestrus (Concannon et al. 1986, Concannon 1993, Kooistra et al. 1999a,b, Tani et al. 1999), as it was in these dogs, occurring within 14 days of ovulation. According to Concannon et al. (1986), the period of increased frequency of LH pulses is brief, perhaps only 4–8 days, and it may not be continuous during that period. The exact role of increased LH secretion in the termination of anoestrus in the bitch remains elusive. One of the main effects of the rising FSH level is the acquisition of LH receptors in the granulosa cells. Beyond this stage, LH is progressively able to replace FSH in supporting follicular maturation (Monniaux et al. 1997). It is therefore possible that the increase in LH pulsatility at the end of anoestrus provides a stimulus to follicles which are no longer receptive to FSH but have acquired enough LH receptors. There are similarities in the ewe, in which the increased frequency of low amplitude LH pulses is thought to be an effective means of follicle selection. After transferring their gonadotrophic requirement from FSH to LH, the follicles become critically dependent on LH support. Follicles in which the FSH threshold has not yet been surpassed and consequently do not yet have enough LH receptors are not stimulated to develop. Transference of gonadotrophic dependence from FSH to LH allows the preovulatory follicles to withstand the fall in FSH that occurs at the onset of the follicular phase (Picton et al. 1990, McNeilly et al. 1992, Campbell et al. 1995). A similar rise in LH secretion concurrent with a fall in FSH secretion takes place during the progressing follicular phase in the bitch (Kooistra et al. 1999a). The FSH-induced acquisition of LH receptors may also explain why the administration of pharmacological doses of porcine LH during anoestrus can cause follicle growth (Verstegen et al. 1997). Another explanation may be that LH modulates the FSH threshold. It is well known that regulatory substances of thecal cell origin modulate sensitivity to FSH. Since LH regulates thecal cell function, stimulation by LH might indirectly sensitize granulosa cells to FSH, i.e. modulate the FSH threshold (Hillier 1996).

In conclusion, the results of this study have shown that administration of the serotonin antagonist metergoline does not shorten the interoestrous interval, despite decreased plasma prolactin levels after the start of the treatment. The plasma profiles of LH and FSH were similar to those observed during physiological anoestrus, but different from those observed during anoestrus shortened by a dopamine agonist. Therefore, the premature onset of oestrus brought about by a dopamine agonist cannot be a consequence of a decreased plasma prolactin level but must be due to some other dopamine-agonistic effect, probably increased secretion of FSH. The findings of this study further strengthen the hypothesis that an increase in circulating FSH is essential for ovarian folliculogenesis and consequently the termination of anoestrus in the bitch.

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