Endocrinology of physiological and progestin-induced canine anoestrus

Niek Beijerink

Voor Maureen

Endocrinology of physiological and progestin-induced canine anoestrus

Endocrinologie van de fysiologische en de door progestagenen geïnduceerde anoestrus bij de hond
(met een samenvatting in het Nederlands)

Proefschrift

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CHAPTER 1

Aims and scope of the thesis

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The oestrous cycle of the domestic bitch 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 2 months, irrespective of pregnancy. A non-seasonal anoestrus, with a duration that may last from 2 to 10 months, follows each oestrous cycle. Information regarding endocrine changes during the canine anoestrus is scarce. The general aim of **Part I** of this thesis was to get further insight into the endocrinology of the physiological anoestrus.

Anoestrus can be prolonged by oestrus-preventing drugs such as progestins. The endocrine mechanisms by which progestins exert their contraceptive action are unclear. **Part II** of this thesis concentrates on the endocrinology of the progestin-induced canine anoestrus.

In the general introduction of this thesis (**Chapter 2**) an overview is given of 1) the hypothalamus-pituitary system, 2) the canine oestrous cycle, with a focus on the anoestrus, and 3) the effects of progestins, used to prolong the duration of anoestrus.

The endocrine changes that lead to termination of anoestrus, and thus to the start of a new oestrous cycle, are not completely understood in the bitch. Progression from early to late anoestrus is associated with an increase in the basal plasma follicle-stimulating hormone (FSH) concentration, suggesting that an increase in circulating FSH levels is a critical event in the initiation of ovarian folliculogenesis. Also, an increasing hypothalamic release of gonadotrophic releasing hormone (GnRH) and an increase in the sensitivity of the pituitary to GnRH from early to late anoestrus have been observed in this species. In addition, an increase in ovarian responsiveness to gonadotrophins, increased basal plasma luteinizing hormone (LH) concentrations, and a brief period of increased LH pulsatility at the end of anoestrus have been reported as important determinants of the initiation of a new follicular phase. Apart from these changes in the hypothalamus-pituitary-gonad axis, there are indications of involvement of prolactin release and/or dopaminergic influences. Administration of dopamine-2 receptor agonists results in shortening of the anoestrus and lowering of the plasma prolactin concentration. The goal of the study reported in **Chapter 3** was to get more evidence that the anoestrus-shortening effect of dopamine agonists is due to another dopaminergic effect than their prolactin-lowering properties. In addition, we investigated (**Chapter 4**) whether lowering of the plasma prolactin concentration by a serotonin receptor antagonist is associated with changes in the pulsatile plasma profiles of LH and/or FSH.

Differentiation between bitches in anoestrus and neutered bitches is not easy when information about the reproductive status is lacking. Provocative testing of the pituitary-ovarian axis using GnRH may be helpful in this respect. To learn more about the effects of GnRH administration on the plasma concentrations of LH, oestradiol-17β, progesterone, and testosterone in anoestrous bitches and the differences with the effects in ovariectomized bitches, the study reported in **Chapter 5** describes the results of a GnRH-stimulation test in both anoestrous and ovariectomized bitches.

Because of the important role of FSH in ovarian folliculogenesis in the bitch, a canine FSH IRMA, which recently has been developed, was validated. Using this FSH IRMA, the effects of GnRH administration on the plasma FSH concentrations were investigated in both anoestrous and ovariectomized bitches (**Chapter 6**).

Part II of this thesis comprises reports on the endocrinology of the progestin-induced canine anoestrus. Progestins, such as medroxyprogesterone acetate (MPA), are commonly used to prevent oestrus in the bitch. Whether the oestrus-preventing properties of MPA in the bitch are due to effects on the hypothalamus, on the pituitary gland, and/or at the ovarian level is not clear. To obtain an integral picture of the effects of MPA on the function of the canine adenohypophysis, the effects of suprapituitary stimulation on adenohypophyseal hormone release both before and during MPA treatment were studied (**Chapter 7**). To further characterize FSH and LH release during MPA treatment, the secretion pattern of the gonadotrophins were investigated in more detail, i.e., the pulsatile plasma profiles of FSH and LH before and during MPA treatment were studied (**Chapter 8**).

In **Chapter 9** the results of the studies are summarized and discussed.

CHAPTER 2

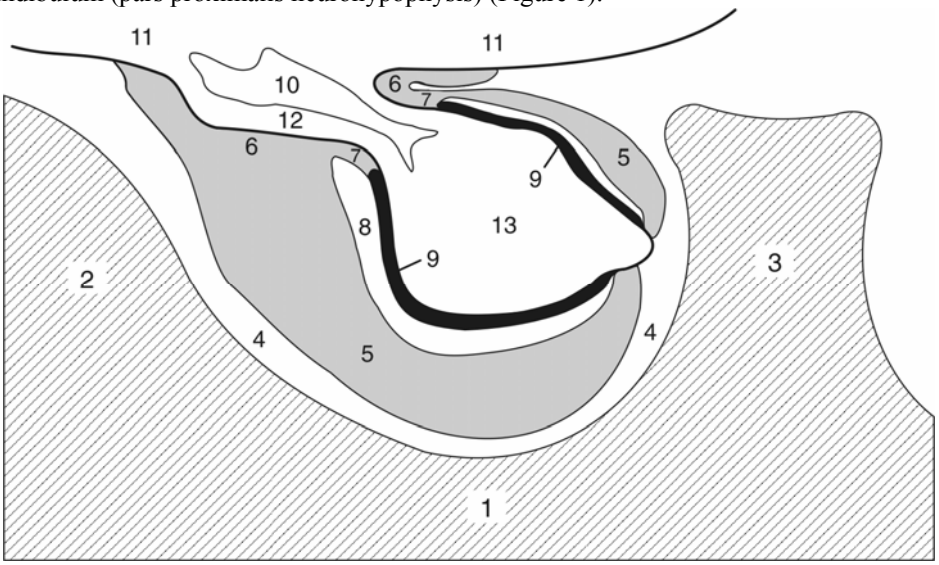
General Introduction

1. The hypothalamus-pituitary system

Reproductive endocrinology includes the study of the endocrine glands involved in reproduction and their secretory products, the reproductive hormones. In the female, the hypothalamus, pituitary, and ovaries play a primary role in controlling reproduction. Other endocrine glands such as the adrenals and thyroid glands also have some influence on reproductive function.

The hypothalamus-pituitary system constitutes the main axis of the neuroendocrine system of the body. In this axis the pituitary is an essential regulatory interface integrating signals from the periphery and brain to control vital functions such as growth, reproduction, lactation, basal metabolism, and stress response (Treier and Rosenfeld 1996).

According to the Nomina Anatomica Veterinaria, the canine pituitary gland is composed of two main parts 1) the adenohypophysis and 2) the neurohypophysis (Hullinger 1993). The pituitary gland is suspended from the midline of the hypothalamus by a cylindrical stalk. This stalk is an extension of the median eminence of the hypothalamus, and is called the infundibulum (pars proximalis neurohypophysis) (Figure 1).



1 = sphenoid bone
2 = tuberculum sellae
3 = dorsum sellae
4 = pituitary fossa
5 = pars distalis adenohypophysis
6 = pars infundibularis adenohypophysis
7 = transitional zone
8 = hypophyseal cleft or cavity
9 = pars intermedia adenohypophysis
10 = third ventricle
11 = hypothalamus (median eminence)
12 = pars proximalis neurohypophysis
13 = pars distalis neurohypophysis

Figure 1. Schematic illustration of the median sagittal section through the canine pituitary gland (Modified from Meij 1997). Left is rostral, right is caudal.

The adenohypophysis can be divided into two functional units 1) the anterior lobe and 2) the pars intermedia. The pars intermedia (PI) is in direct contact with the neurohypophysis and is separated from the anterior lobe (AL) by the hypophyseal cleft or cavity, which is a remnant of the embryonic Rathke's pouch. The AL is populated by at least five highly differentiated types of endocrine cells, which are classified according to the trophic hormones they produce: somatotrophic cells secreting growth hormone (GH), lactotrophic cells secreting prolactin (PRL), thyrotrophic cells secreting thyroid-stimulating hormone (TSH), gonadotrophic cells secreting luteinizing hormone (LH) and follicle stimulating hormone (FSH), and corticotrophic cells synthesizing the precursor molecule pro-opiomelanocortin, which gives rise to adrenocorticotrophic hormone (ACTH) and related peptides. Somatotrophic cells account for 50% or more of the endocrine AL cells, with the other cell types each representing about 5-15% of the AL cell population (Rijnberk 1996, Meij 1997).

The hypothalamus-pituitary axis consists of three major systems: 1) a neuroendocrine system connected to an endocrine system by a portal circulation, 2) a neurosecretory pathway, and 3) a direct neural regulation of endocrine secretion. The neuroendocrine system connects clusters of peptide- and monoamine-secreting cells in the anterior and middle portion of the ventral hypothalamus to the AL (Swanson 1987). Their products, being releasing and inhibiting factors such as GH-releasing hormone (GHRH), somatostatin, thyrotrophin-releasing hormone (TRH), corticotrophin-releasing hormone (CRH), and gonadotrophin-releasing hormone (GnRH), are transported along nerve fibres to the median eminence. From the median eminence they are released into the capillary vessels of the hypothalamic-pituitary portal system and transported to the pituitary to regulate the secretion of hormones from the AL (Figure 2). Specificity is achieved by the presence of specific receptors on individual types of AL cells. For example, the interaction between hypothalamic GnRH and the GnRH receptor on the gonadotrophic cells of the AL may result in LH and/or FSH secretion. In addition to the hypothalamic hypophysiotrophic hormones, secretion of AL hormones is regulated by feedback from target organs such as the thyroids, adrenals, and gonads (Figure 2).

The neurosecretory pathway is involved in osmoregulation through the production and release of vasopressin, and in parturition and nursing through the secretion of oxytocin. The two neurohypophyseal hormones are synthesized by populations of magnocellular neurons, grouped in the paraventricular and supraoptic nuclei in the hypothalamus (Swanson 1987), from which axons extend through the pituitary stalk and terminate in the neurohypophysis on fenestrated blood vessels. Here they are stored in secretory granules within these nerve terminals and are released by exocytosis into the bloodstream in response to appropriate stimuli.

The pituitary PI is a poorly-vascularized structure that is directly innervated by predominantly dopaminergic nerve fibres from the hypothalamus. This direct neural control is largely a tonic inhibitory influence. The main hormone secreted by the PI is α-melanocyte stimulating hormone (α-MSH) (Figure 2).

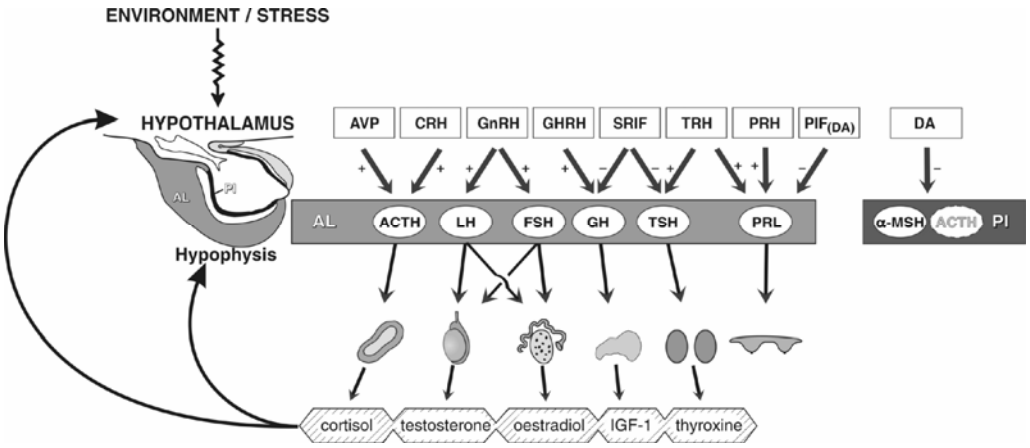


Figure 2. Simplified diagram of the hypophysiotrophic regulation of the secretion of hormones in the adenohypophysis. Modified from Meij (1997).

Pulsatile secretion

In the dog, all adenohypophyseal hormones are secreted in a pulsatile fashion (Kooistra 2000). This pattern of secretion represents an important component of neuroendocrine signaling mechanisms (Negro-Vilar et al. 1987, Brabant et al. 1992). The presumed advantages of pulsatile hormone secretion are 1) prevention of down-regulation of target tissue responses, 2) stimulation of target cells with the optimal pulse frequency for a distinct intracellular signaling pathway reduces the net amount of hormone required to elicit a certain level of response, and 3) time-dependent processing of cellular information could result in differential activation of specific cellular responses simply by changes in the pulse frequency and pulse amplitude of a single hormone.

The pulsatile plasma profiles of adenohypophyseal hormones can be determined by collecting blood samples serially over a period of hours or days. After determination of the plasma hormone concentrations in the samples, the plasma hormone profile has to be analysed by a computer programme. The pulsatile plasma hormone profiles in this thesis were analysed using the Pulsar programme. The Pulsar programme, developed by Merriam and Wachter (1982), identifies adenohypophyseal hormone pulses based on their amplitude and duration with respect to a smoothed baseline (using a weighted linear regression of hormone level versus time within a certain time window), taking into account the standard deviation of the assay at a given hormone concentration. First, the programme uses a robust smoothing technique to generate a smoothed baseline that omits peaks or trends with time constants less than 6-12 h. The smoothed baseline is subtracted from the original data and then the difference, the residual series, is examined for the presence of peaks. The standard deviation of the assay is

calculated at each point and the residuals are rescaled in terms of this unit. Peaks are identified as individual series of n hormone levels above the smoothed baseline, in which all points must have a magnitude of at least G(n). The values of G are cut-off criteria based on the width of the peak. The narrower the peak the higher the residual hormone level should be in order to be identified as a significant peak. A point in the scaled residual series is accepted as forming part of a peak if it alone is higher than a certain cut-off value G(1), or if it belongs to a pair of adjacent points both higher than a second lower cut-off G(2), or to a triplet all higher than G(3), and so on, up to G(5). Thus the algorithm selects both narrow high peaks and broader peaks that may be lower (Figure 3). The user selects the G(n) for each hormone based on theoretical considerations or a set of calibration data series. In addition, G(n) values have been reported that are chosen in such a way that the probability of falsely claiming that there is a peak in a series without peaks is below a given error rate, say 5%. To assure that the significant peaks do not influence the calculation of the smoothed baseline, the smoothing technique is repeated, whereby the residual hormone levels of significant peaks are assigned a reduced weight, and the subsequent residual series is again examined for the presence of peaks. This process is repeated until there are no further changes. Next, each peak is examined once more to determine whether it can be resolved into two or more overlapping peaks (Merriam and Wachter 1982). Values extracted from Pulsar analysis include the smoothed baseline, the pulse frequency, the peak amplitude, the pulse duration, and the area under the curve (AUC).

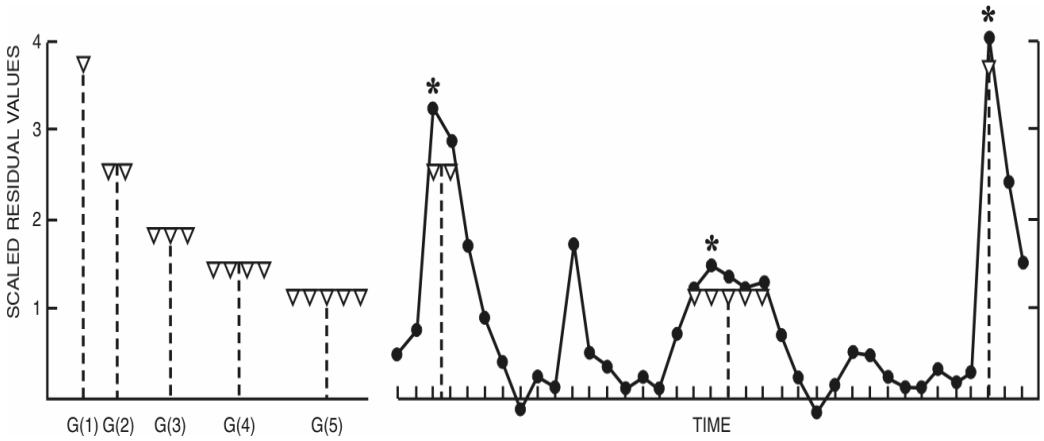


Figure 3: Computer-assisted analysis of pulsatile secretion by comparison of possible peaks against cut-off criteria G(1)-G(5). Before testing, series were stripped of long-term trends and scaled in units of assay standard deviation. Three significant peaks, having 2 successive points greater than G(2), 5 successive points greater than G(5), and 1 point greater than G(1), respectively, meet the cut-off criteria and are marked with asterisk.

Characterization of the pulsatile plasma profile of adenohypophyseal hormones reflects hypothalamic-pituitary dynamics. The pulse frequency of the pituitary hormones can

be used indirectly as an indication of the pulsatile release of hypothalamic releasing hormone. For instance, GnRH and LH pulses have been found to correspond in about a one-to-one ratio in ewes (Clarke and Cummins 1982) and rhesus monkeys (Xia et al. 1992). The inaccessibility of the portal blood stream, the rapid degradation of GnRH due to its short half life time of only 2-4 minutes (Speroff and Fritz 2005), and the enormous dilution of GnRH on entry into the peripheral circulation, prohibit routine measurement of circulating GnRH concentration. Therefore, the collection of frequent blood samples from the peripheral blood stream is used to define the pulsatile nature of pituitary LH secretion, and the pulsatile plasma LH profile is used as an indirect measure of the activity of the GnRH secretory system.

Secretion of LH and FSH

Unlike the other hormones of the pituitary AL, each secreted by a specific cell type, LH and FSH are secreted in a pulsatile fashion by the same type of pituitary cell, the gonadotroph. The primary stimulator of the synthesis and pulsatile secretion of both LH and FSH is the decapeptide GnRH. It might therefore be expected that FSH and LH molecules are discharged concomitantly by the gonadotrophs. However, in most mammalian species differential secretion of LH and FSH has also been demonstrated, indicating the presence of regulatory mechanisms that allow independent secretion of LH and FSH. One mechanism for differential control of LH and FSH is gonadal feedback. For example, both oestradiol and inhibin can specifically suppress FSH synthesis and secretion (Shupnik 1996). Other important factors in gonadotrophin control are the frequency and amplitude of GnRH pulses, which have been shown to differentially alter LH and FSH gene expression as well as the secretion of these hormones (Vizcarra et al. 1997). In addition, a specific hypothalamic FSH-releasing decapeptide has been demonstrated in the rat (Yu et al. 1997).

Gonadotrophins act on the gonads to stimulate gonadal hormone secretion. In the female animal, granulose, theca, interstitial, and luteal cells are capable of secreting hormones in response to LH and FSH. The type and amount of hormones released vary according to the morphological status of the follicle and corpus luteum. The patterns of secretion of LH and FSH together with the morphologic and secretory changes in the ovaries determine the species-specific configuration of the oestrous cycle.

Secretion of prolactin

The pulsatile secretion of prolactin by the pituitary is governed jointly by hypothalamic inhibitory and stimulatory signals. The biogenic amine dopamine has been recognized as the main inhibitory neural signal in the regulation of prolactin release (Ben-Jonathan 1985). Besides the inhibitory dopaminergic tone, several substances are known to have prolactin-releasing activity, such as serotonin, vasoactive intestinal peptide, opioid peptides, oxytocin, nitric oxide and TRH (Lamberts and MacLeod 1990, Mol and Rijnberk

1997). A specific prolactin-release-promoting peptide in the hypothalamus has also been identified and characterized (Hinuma et al. 1998).

Neurogenic factors also influence prolactin secretion. Suckling and milking are almost immediately followed by prolactin release. Oestrogens rapidly induce an enhanced prolactin response to TRH in dogs (Rutteman et al. 1987). Also progesterone has a modulating role on prolactin secretion in the dog (Kooistra and Okkens 2002).

Determination of circulating reproductive hormone concentrations

Because plasma hormone measurements of hormones presented in this thesis have for a large part been analyzed by radioimmunoassay (RIA) or immunoradiometric assay (IRMA), some discussion of these methods is appropriate. The use of immunoassay techniques has dramatically increased our knowledge and understanding of reproductive endocrinology in most species investigated. Hormones such as LH and FSH, which are glycoproteins with molecular weights of around 30,000, are antigenic because of their size and chemical composition. Immunoassay techniques utilize antibodies as binding proteins. Various techniques, including radioactive labelling, enzyme reactions and chemiluminescence, can be used. Since for the studies described in this thesis, only techniques with radioactive labelling are used, this paragraph will focus on this technique. The RIA technique was originally introduced for the measurement of insulin in plasma (Yalow and Berson 1959). The principle of the RIA technique is based on the ability of nonlabelled hormone to compete with a fixed amount of isotopically labelled hormone for the binding sites on a fixed amount of protein. The nonlabelled hormone reduces the number of free binding sites on the protein, thus decreasing the availability of the binding sites to the labelled hormone. At equilibrium, the free hormone is separated from the protein-bound hormone, and the reaction is quantified by the determination of the amount of labelled hormone that is antibody-bound or free. The degree of inhibition of binding of the labeled hormone to the binding protein is a function of the concentration of nonlabelled hormone present in the solution. As a basis for the quantification, a standard curve is developed with fixed amounts of labelled hormone and binding protein incubated together in the presence of a known and graded concentration of unlabelled hormone.

A variation to the RIA is the IRMA, which is a double antibody sandwich method. In this assay system, tubes or wells are coated with antibody. The sample is then added, and its hormone binds to the antibody coated tube. A second isotopically labelled antibody directed against another epitope of the hormone is then added. The amount of isotope-labeled antibody bound is directly proportional to the amount of hormone in the sample.

The reliability of immunoassay analyses depends on specificity, sensitivity, accuracy, and precision. The specificity of an immunoassay, or its freedom from interference by substances other than the one to be measured, is dependent on several different factors, the most important being the specificity of the antibodies used. Demonstration of specificity for the immunoassay of large protein hormones such as LH and FSH is relatively difficult and relies upon indirect criteria. Because it is not possible to synthesize these hormones, they must be

isolated and purified from biological material, for example pituitary gland material. The purity of such preparations is variable, and the most common cause of nonspecificity for these hormones is impurity of the immunizing material. Antibody specificity is usually demonstrated by testing the binding of hormones other than the one intended to be measured to the antibody. If, for example, cLH significantly inhibits the binding of cFSH to an antibody to cFSH, this indicates that the antiserum used is nonspecific or that the cLH preparation contains cFSH. If the inhibition curves are parallel, the latter explanation is likely because the parallelism indicates the same binding kinetics. It should be noted that parallelism, in itself, is not adequate proof of specificity. Both LH and FSH are composed of two subunits, an α-subunit that is identical for the two hormones and a β-subunit that is unique for each hormone. It is possible that an antiserum could contain binding sites that will react only with the α-subunit. In such a case, the dose-response curve of LH and FSH utilizing such an antisera will be parallel, the assay system will not be hormone specific, and thus the system will be invalid for the measurement of LH or FSH. Double antibody sandwich methods, such as the IRMA, utilizing monoclonal and/or polyclonal antisera can partly reduce the problem.

In case of immunoassay techniques for steroid hormones, the same proof of specificity has to be undertaken. Here the situation is simpler, because lower-molecular-weight hormones can easily be purified and, in most cases, produced synthetically.

The sensitivity of an immunoassay is defined as the smallest quantity of hormone that the assay can detect reliably. The most meaningful sensitivity to establish is the smallest amount of hormone that can be measured per unit of biological fluid, for example per ml of plasma.

The accuracy of an assay is defined as the extent to which the measurement of a hormone agrees with the exact amount of the hormone. Accuracy is often determined by comparing immunoassay data with values determined by other procedures such as gravimetry, gas-liquid chromatography, and mass spectrometry. Accuracy is also often determined by recovery experiments in which different amounts of hormones are added to a biological fluid, such as plasma, that contains low concentrations of the hormone. The amount of hormone measured in the assay is then compared with the amount of hormone added.

Two types of precision are usually evaluated. The intra-assay precision (inter-assay coefficient of variation) is determined from duplicate measurements of the same sample within the same assay. The interassay precision (interassay coefficient of variation) is determined from replicate analyses of the same sample in different assays. Usually the interassay variance is greater than the intra-assay variance. Assay variance should be checked continuously with each assay of a certain hormone by use of plasma pools containing set amounts of the hormone. Usually three different plasma sets containing low, medium, and high hormone concentrations are used.

With regard to the interpretation of hormone measurements some methodological aspects should be taken into account. First, high precision and sensitivity of the detection methods are crucial for the analysis of pulsatile secretion patterns. A high intra-assay coefficient of variation will make it quite difficult to distinguish significant hormone pulses

form assay noise. Secondly, hormone-specific responses are determined by the bioactivity of the hormone. However, plasma hormone concentrations are measured by immunoassays rather than by bioassays. Thirdly, hormone secretion patterns are measured as a relatively small number of points, because “on-line” measurements of hormones are not yet available. The shorter the sampling interval, the higher the chance that significant hormone pulses are indeed detected.

2. The canine oestrous cycle and anoestrus

Onset of puberty

In the healthy bitch the onset of puberty is between 6 and 18 months of age (Schaefers-Okkens 1996). Breed has an effect on the timing of a dog's first oestrus. Generally, bitches exhibit their first cycle several months after they achieve adult height and body weight. There is, however, considerable variation within a breed. This natural variability, coupled with cycles referred to as silent heats, adds to the inability to precisely predict the timing of a first oestrus (Johnston et al. 2001, Feldman and Nelson 2004).

Interoestrous intervals and seasonality of the oestrous cycle

The oestrous cycle of the bitch is considerably longer than in most other domestic animals. The common interoestrous interval (the period from the start of the pro-oestrus to the beginning of the following pro-oestrus) is 5 to 11 months. Differences in the duration of the interoestrous interval between dog breeds and strains within a breed indicate a genetic basis for the variation in interoestrous intervals, i.e. differences in the periodicity of an endogenous circannual cycle. Especially the anoestrus varies considerably in duration among bitches, is non-seasonal, and can vary from 2 to 10 months (Bouchard et al. 1991, Concannon 1993). In the Collie, for instance, the mean interoestrous interval is 36 weeks, and in the German shepherd dog this interval is only approximately 20–22 weeks (Christie and Bell 1972, Sokolowski et al. 1977, Christiansen 1984). Individuals and strains within breeds can also have exceptionally short cycle intervals relative to the breed average. Some dog breeds such as the Basenji and Tibetan Mastiff, yet, have a single annual oestrous cycle, which may perhaps be influenced by a photoperiod (Christiansen 1984). Environmental factors can also affect the interoestrous interval: an anoestrous bitch placed in close proximity to a bitch in oestrus can show an advance of the onset of pro-oestrus by several weeks. Furthermore, bitches housed together often have synchronous oestrous cycles.

Phases of the oestrous cycle

Using a behaviour-orientated classification, the oestrous cycle comprises of four components: pro-oestrus, oestrus, metoestrus (or dioestrus), and anoestrus (Figure 4).

Pro-oestrus is most reliable defined as the period from onset of vaginal bleeding and vulvar swelling to the first willingness to accept copulation, and has an average duration of 9 days. However, variations can be tremendous, i.e., as brief as 3 days to as long as 17 days. On average the oestrus has also a duration of 9 days, with a range of 3 to 21 days, in which period the bitch accepts mating. During oestrus the vulva starts to shrink and soften. The discharge usually persists, and may remain sanguineous or turn straw-coloured. The metoestrus begins

when the bitch is no longer willing to accept the male dog. It is associated with the presence of active corpora lutea, and has an average duration of about 70 days, if we assume that it ends when the plasma progesterone concentration declines for the first time to a level of ≤ 3 nmol/l. Anoestrus is the time between the end of the metoestrus and the beginning of the next pro-oestrus. In addition to this behaviour-orientated classification, it is also possible to classify according to ovarian function and to distinguish the follicular phase, the phase of preovulatory luteinization and ovulation, the luteal phase, and the anoestrus (Schaefers-Okkens 2005).

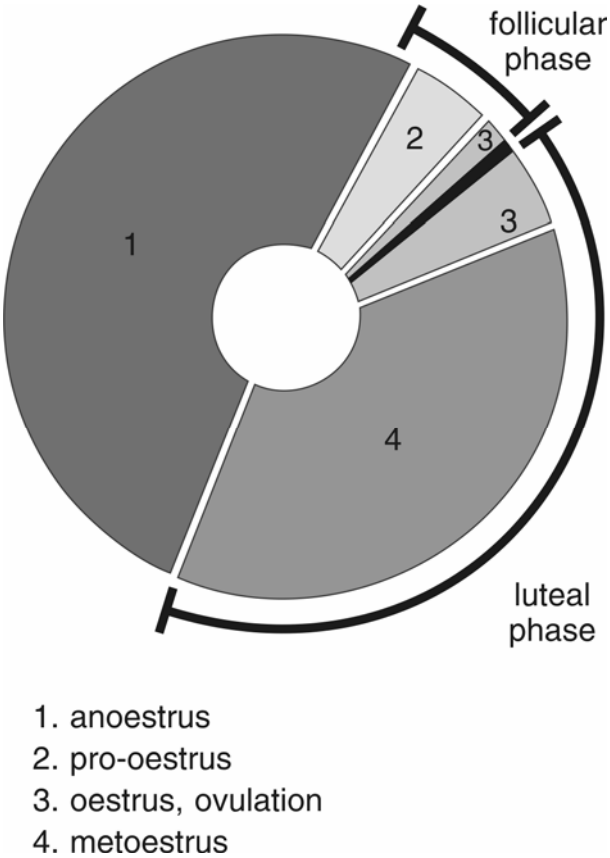


Figure 4: Diagram of the oestrous cycle and anoestrus in the dog. Modified from Schaefers-Okkens, 1996.

The follicular phase

As tertiary follicles develop in the ovaries they produce oestradiol, leading to peak plasma levels of 180-370 pmol/l in late pro-oestrus (Figure 5). The external signs of pro-oestrus, such as hyperemia and oedema of the vulva and bloody vaginal discharge, are caused by these increased plasma levels of oestradiol. The high plasma oestradiol levels also cause lengthening and hyperemia of the uterine horns, enlargement of the cervix, and thickening of the vaginal wall. During early onset of the follicular phase, parabasal and intermediate vaginal epithelial cells are the predominant cells on vaginal cytology. Oestrogen effect during the progression of the follicular phase causes the vaginal epithelial cells to proliferate and mature and leads to (1) disappearance of neutrophils, (2) appearance of erythrocytes, (3) increasing percentage of superficial cells, and (4) decreasing percentage of parabasal and small intermediate cells. During the progression of the follicular phase, ovulation and fertilization phase, superficial cells, with or without a pycnotic nucleus, and large intermediate cells account for a large part of the epithelial cells. Therefore, oestrogenic influence can be monitored by vaginal cytology. However, it should be realized that although vaginal cytology gives an indication of the stage of the cycle it is not reliable for timing the preovulatory LH surge or ovulation (Hiemstra et al. 2001). With vaginoscopy it can be observed that the vaginal mucosal folds are swollen, are pale, and have smooth, rounded surfaces (balloons). The increased plasma concentration of oestradiol frequently causes hypertrophy of the floor of the posterior vagina, just cranial to the urethral orifice and therefore folding over and covering the urethral orifice. At the end of the follicular phase, which is during the decline in plasma oestradiol concentration and the rise in plasma progesterone concentration, shrinkage begins in response to reduced oestradiol-dependent water retention. These cyclic changes are most obvious in the dorsal median fold and precede those of the mid vaginal mucosa. During the early start of the follicular phase the basal plasma FSH concentration is elevated, but relatively low during the progression of the follicular phase, whereas the basal plasma LH concentration is higher than in different phases of the anoestrus (De Gier et al. 2006). The secretory pattern of LH is characterized by frequent increases of short duration (Kooistra et al. 1999a). Plasma progesterone concentrations initially remain low but fluctuate and increase during the second half of the follicular phase as a result of partial luteinization of the granulosa cells.

Preovulatory luteinization and ovulation

Concannon et al. (1979) reported that the start of the pre-ovulatory LH surge in the bitch is associated with a decrease in the plasma oestradiol-17β concentration. De Gier et al. (2006) however, demonstrated during a study in which frequent blood sampling was performed, that also in the bitch, similar to most mammals (Liu and Yen 1983, Evans et al. 1997, Karsch et al. 1997), increasing or high plasma oestradiol concentrations during the late follicular phase have a positive feedback effect on LH release leading to the pre-ovulatory LH surge. Unlike in most other species, in dogs the duration of the pre-ovulatory LH surge is

relatively long, ranging from 1 to 5 days (Wildt et al. 1978, Concannon 1993, Onclin et al. 2002, De Gier et al. 2006). Coinciding with the start of the pre-ovulatory LH surge granulosa cells begin to luteinize and secrete progesterone (De Gier et al. 2006). This rapid and extensive luteinization continues during the preovulatory LH surge. Ovulating follicles therefore have many of the characteristics of rapidly developing corpora lutea. Most ova in the dog are ovulated in an immature state as primary oocytes. The plasma progesterone concentration ranges from 6 to 13 nmol/l at the time of the LH peak, and 15 to 25 nmol/l at the time of ovulation, 36-48 hours later (Figure 5). The process of ovulation can take up to 24 hours. In the first 2 to 3 days after ovulation the oocytes mature; that is, they undergo the first meiotic division and the extrusion of the first polar body, after which fertilization can occur (Phemister et al. 1973). Concurrent with the LH peak, a pre-ovulatory surge in FSH occurs that lasts about three times longer than the pre-ovulatory LH peak (De Gier et al. 2006). Oestrus behaviour usually starts synchronously with the pre-ovulatory LH peak, but some bitches initially demonstrate oestrus behaviour days before or after the LH peak (Concannon 1986). Shrinkage of the vaginal mucosa continues through the phase of pre-ovulatory luteinization and ovulation, when many longitudinal folds can be observed. With vaginal cytology, superficial cells account for a large part of the epithelial cells during the phase of preovulatory luteinization and ovulation.

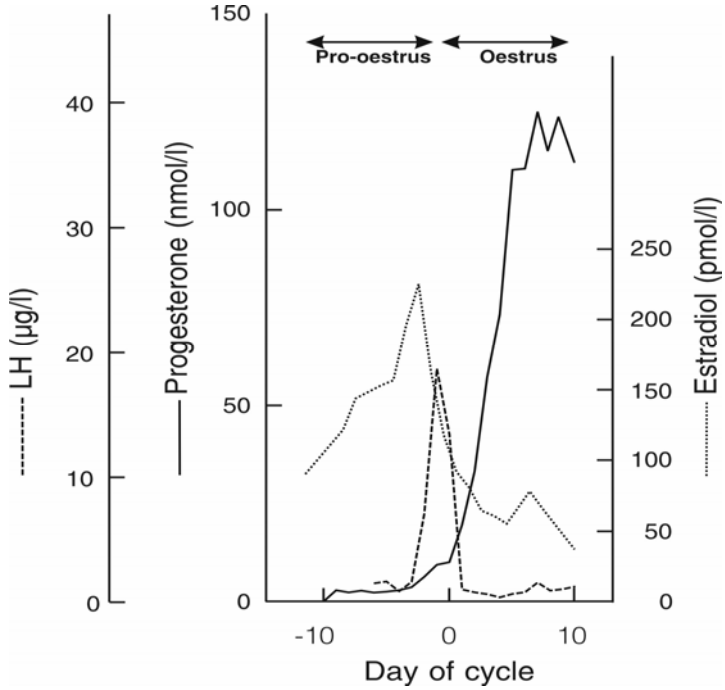


Figure 5. Concentrations of oestradiol, LH, and progesterone in plasma in relation to oestrus behaviour of the bitch. Modified from Schaefers-Okkens 1996.

Luteal phase

After ovulation the luteal phase starts. In the behaviour-orientated classification, the plasma concentration of progesterone, originating from the corpora lutea, increases during the remainder of the oestrus and during the onset of metoestrus. Oestrus behaviour is thus seen during a rising plasma progesterone concentration. The plasma progesterone concentration plateaus at 10 to 30 days after ovulation. Thereafter, in non-pregnant bitches, the progesterone secretion declines slowly and reaches a basal level of 3 nmol/l for the first time at about 75 days after the start of the luteal phase (Figure 6). During the initial part of the luteal phase, at about 4-8 days after ovulation, the transition from oestrus to metoestrus takes place. In this period, the cytology of the vaginal mucosa changes from primarily superficial cells to mainly intermediate and parabasal cells and leukocytes. This change is an indication that the fertile period has expired. During oocyte maturation, shrinkage of the vaginal mucosa continues and increasing numbers of sharp edged summit profiles appear. In the transition period from oestrus to metoestrus, the mucosa thins and profiles become round. At the start of metoestrus, a patchwork of red and white areas can be seen.

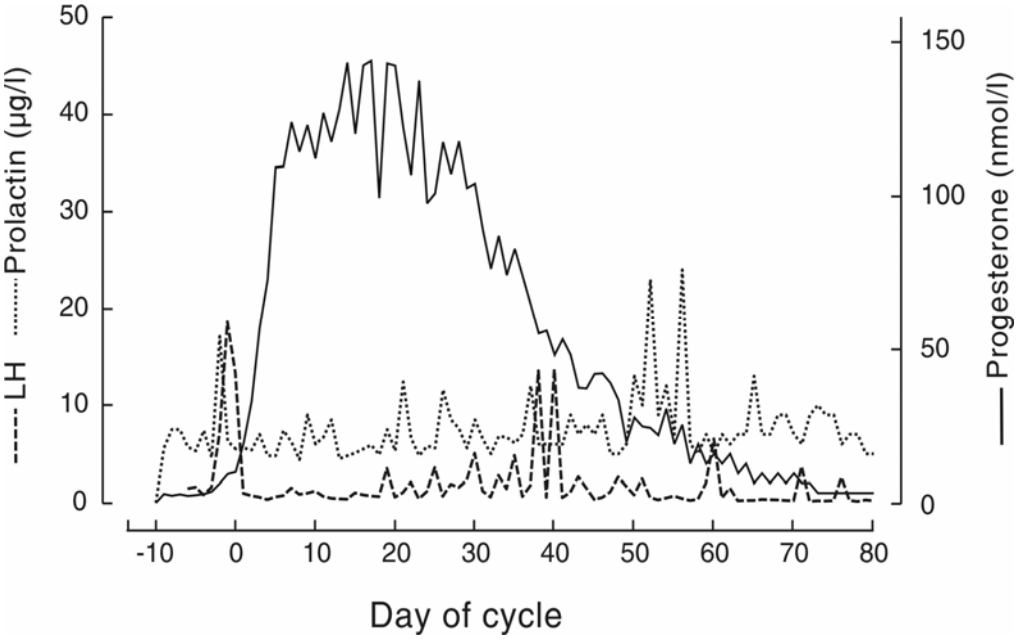


Figure 6. Mean LH, progesterone, and prolactin levels in plasma of three dogs during the follicular and luteal phase. The data have been synchronized on day 1, the day after the onset of the follicular phase, on which the progesterone concentration in the peripheral blood had reached 16 nmol/l. Modified from Okkens et al. 1990.

The factors that are responsible for initiating the regression of the corpora lutea are still unknown in the dog. Prostaglandin $F_{2\alpha}$ originating from the endometrium is not the causative factor in the bitch, as it is in the cow, ewe and mare. Removal of the uterus from the latter species during the luteal phase results in a longer lifespan of the corpus luteum and prolongation of luteal activity. It is now well established that the uterus in these species synthesizes and releases $PGF_{2\alpha}$, which causes the corpus luteum to regress (McCracken et al. 1972). In the dog however, hysterectomy does not influence the length of the luteal phase (Okkens et al. 1985a, Hoffmann et al. 1992).

Prolactin acts as a luteotrophic factor in the second half of the luteal phase (Okkens et al. 1985b, Okkens et al. 1990). During the first half of the luteal phase the corpus luteum functions independently of pituitary support (Okkens et al. 1986). Thereafter, maintenance of the *corpora lutea* during the second half of the luteal phase or of pregnancy mainly is a function of prolactin. Inhibition of prolactin secretion causes a sharp decrease in progesterone secretion (Figure 7).

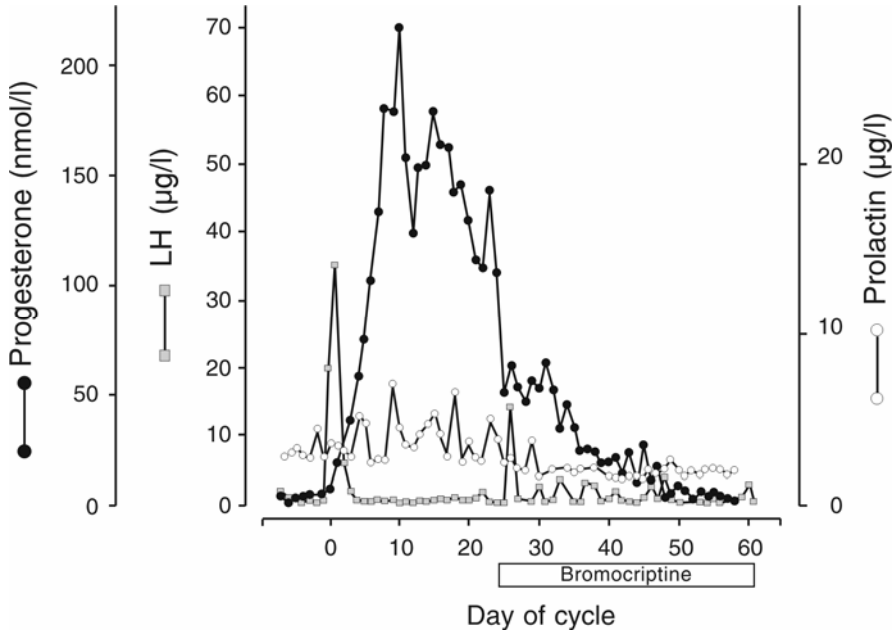


Figure 7. Mean progesterone, prolactin, and LH levels in the plasma of four dogs, treated with bromocriptine, 20 µg/kg body-weight, twice daily, orally from day 20-24 after the onset of the luteal phase until the end of the luteal period (bar). The data have been synchronized on day 1, the day after the onset of the follicular phase, on which the progesterone concentration in the peripheral blood had reached 16 nmol/l. Modified from Okkens et al. 1990.

Plasma LH concentrations change little during the luteal phase, with the exception of a slight increase in the second half of the luteal phase (Olson et al. 1982, Fernandes et al. 1987). There is no proof of direct luteotrophic properties for LH in the cyclic bitch (Okkens et al. 1990, Onclin and Verstegen 1997, Hori et al. 2004).

Anoestrus

In the non-pregnant bitch, the transition from the luteal phase into anoestrus is gradual. The time of the onset of anoestrus depends on which criteria are being used to define the end of the luteal phase. These can be (1) after 2 to 3 months when mammary development subsides, (2) the first time that the plasma progesterone concentration reaches a level below 3 nmol/l, or (3) the moment that the influence of progesterone on the endometrium is no longer evident. The end of the luteal phase may be best defined when the plasma progesterone concentration reaches a plasma level below 3 nmol/l for the first time. The vaginal cytology of anoestrus is quite acellular; it contains primarily parabasal cells, small intermediate and basal cells. Because there are no external signs associated with anoestrus, this phase has been described erroneously as a period of sexual quiescence. In fact, studies performed during recent years have made clear that during anoestrus many changes take place at the hypothalamic, pituitary and ovarian level in the dog. The next paragraphs focus on changes in the hypothalamus-pituitary-ovary axis during anoestrus of the dog, and the role of prolactin and the dopaminergic system in the initiation of ovarian folliculogenesis.

Hypothalamus-pituitary-ovary axis during the progression of anoestrus

FSH and LH play an essential role in the induction of folliculogenesis and ovulation. The main regulator of FSH and LH secretion is GnRH. The spontaneous GnRH release from excised hypothalamic fragments, that include the 'mediobasal hypothalamic-preoptic area-suprachiasmatic nucleus units' derived from Beagle bitches at different stages of the oestrous cycle, is pulsatile throughout all stages of the oestrous cycle. Progression from early to late anoestrus in the bitch is characterized by an increased release of GnRH by the hypothalamus. Especially during late anoestrus GnRH pulse frequency is significantly increased (Tani et al. 1996). The sensitivity of the pituitary to GnRH and the indirect response of the ovary to GnRH of bitches in early and late anoestrus have been investigated by intravenous administration of graded doses of GnRH (0, 0.01, 0.1, 1, 10 and 100 μg/kg). The responses, expressed as circulating LH and oestradiol concentration profiles over time, were significantly dose-dependent and significantly higher in late anoestrus than in early anoestrus. In addition, GnRH-induced LH and oestradiol profiles were positively correlated. These results indicate that during the course of anoestrus, there is an increase in the sensitivity of the pituitary to GnRH (Van Haaften et al. 1994) and in ovarian responsiveness to gonadotrophins (Jeffcoate 1993a, Van Haaften et al. 1994).

In another study, the pulsatile plasma profiles of LH and FSH were determined during early-, mid- and late anoestrus in Beagle bitches (Kooistra et al. 1999a). During anoestrus each FSH pulse coincided with a LH pulse. The gonadotrophin pulses were characterized by a very abrupt and rapid increase and a slow decrease in plasma LH and, even more pronounced, plasma FSH concentrations. The mean plasma LH concentration of the smoothed baseline and the mean AUC for LH did not differ significantly when the different phases of anoestrus were compared. In contrast, the mean plasma FSH concentration of the smoothed baseline and the AUC for FSH during late anoestrus was significantly higher than those during mid- and early anoestrus (Kooistra et al. 1999a, Onclin et al. 2001). These observations suggest that an increase in circulating FSH levels is a key event in ovarian folliculogenesis in the dog and consequently for the termination of anoestrus. Indeed, in most mammals studied, FSH is regarded as the most important factor in the early stages of follicular development, whereas LH is regarded as the primary regulatory factor in the more mature follicles (Moyle and Campbell 1995, Monniaux et al. 1997). Consequently, it may be hypothesized that at a certain moment during anoestrus the rising plasma FSH concentration will exceed the threshold value of the most sensitive follicles of the canine ovarian antral follicle pool, leading to an enhancement of the development of these follicles. One of the main effects of FSH 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). This may also explain why administration of pharmacological doses of LH alone during anoestrus can cause follicle growth and induce pro-oestrus in the bitch (Concannon 1993, Verstegen et al. 1997). Another explanation may be that LH modulates the FSH threshold. It is well known that regulatory substances of theca cell origin modulate sensitivity to FSH. As LH regulates theca cell function, stimulation by LH might indirectly sensitize granulosa cells to FSH, i.e. modulate the FSH threshold (Hillier 1996).

Although FSH pulses appear to occur concomitantly with LH pulses in all stages of the canine oestrous cycle and anoestrus (Kooistra et al. 1999a), differential regulation of FSH and LH has also been reported in this species, both during anoestrus and during the follicular phase, and during the period of ovulation and fertilization (Kooistra et al. 1999a, De Gier et al. 2006). Differential regulation of FSH and LH secretion can at least partly be explained by the frequency and amplitude of GnRH pulses (Haisenleder et al. 1991, Vizcarra et al. 1997). In addition, gonadal feedback (Mann et al. 1992, Shupnik 1996) and a specific hypothalamic FSH-releasing factor (Yu et al. 1997) may play a role in the differential or non-parallel secretion of FSH and LH. Furthermore, the intracellular mechanisms for the storage and release differ for FSH and LH (Chowdhury and Steinberger 1975, Moyle and Campbell 1995).

Besides a rise in hypothalamic GnRH release, an increase in basal plasma FSH concentration, and increased sensitivity of the pituitary and the ovaries during progression of anoestrus, several other factors that may be involved in the initiation of folliculogenesis and termination of anoestrus have been reported. For example, in some bitches an increased LH pulsatility has been observed (Concannon et al. 1986, Kooistra et al. 1999b, Tani et al. 1999). This pattern of LH secretion shortly before the start of pro-oestrus has been associated with

termination of anoestrus. 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 (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).

In addition, during anoestrus there is enhanced expression of the genes encoding for the oestrogen receptor (Tani et al. 1997) and P450 aromatase that catalyses oestrogen biosynthesis in the canine hypothalamus (Inaba et al. 2002). Although sporadic elevations are observed, plasma oestradiol concentrations are usually low and do not begin to rise until about a month before the LH peak (Jeffcoate 1993a). Furthermore, there is little doubt that there are no manifestations of oestrogen on the reproductive tract or on sexual behaviour during anoestrus, and vaginal endoscopy reveals no evidence of oestrogenic stimulation of the tract until late anoestrus.

Induction of a follicular phase may take place by pulsatile administration of GnRH. In a study, in which GnRH was administered in pulses of 15 to 500 ng/kg every 90 minutes for 7 to 9 days to 36 anoestrous bitches, GnRH pulses resulted in pro-oestrus, oestrus, ovulation, and pregnancy in 26, 20, 16, and 12 bitches, respectively. Efficacy was dose-dependent (Concannon et al. 1997). A fertile oestrus could also be induced by administering a timed-release GnRH agonist, followed by a GnRH analogue on the first day of induced oestrus (Inaba et al. 1998). Anoestrus can also be terminated by treating bitches with porcine LH. In one study pro-oestrus was induced in all bitches (n=16). Twelve bitches came in oestrus, from which seven ovulated. Bitches, in which pro-oestrus but not oestrus occurred, were all treated in early anoestrus (Verstegen et al. 1997). The observed rapid increase of plasma oestradiol concentration after LH treatment suggests that increased follicle steroidogenesis is a primary effect of LH. On the other hand, the insufficient reaction to porcine LH of bitches in early anoestrus may be due to a deficiency of FSH or LH receptors in this stage of anoestrus.

Furthermore, there is some evidence that factors causing a decrease in opioidergic activity promote LH release and the termination of anoestrus (Concannon 1993). Treatment with 1 mg/kg naloxone, an opioid antagonist, stimulated LH release at nearly all stages of the oestrous cycle (Concannon and Temple 1988). The responsiveness to naloxone was high during the late luteal phase and throughout anoestrus, including the month before pro-oestrus, and was reduced during pro-oestrus and oestrus. These results suggest that, in dogs, there is

endogenous opioid tone inhibitory to LH release during the luteal phase, which persists during anoestrus.

Finally, no evidence of a change in the expression of the FSH receptor specific to anoestrus has been identified. This demonstrated that anoestrus in bitches is due neither to the failure of expression of the canine FSH receptor nor to a change in splicing pattern to an inactive form (McBride et al. 2001).

Dopamine-agonists during the progression of anoestrus

Apart from the changes in the hypothalamus-pituitary-ovary axis, there is evidence of involvement of dopaminergic influences in the initiation of a new follicular phase in the bitch. Administration of the dopamine-agonists bromocriptine and cabergoline is associated with inhibition of prolactin release and shortening of the interoestrous interval in bitches (Okkens et al. 1985b, Van Haaften et al. 1989, Concannon 1993, Onclin et al. 1995, Kooistra et al. 1999b, Verstegen et al. 1999, Gobello et al. 2002). If bromocriptine treatment is started during the luteal phase, shortening of the interoestrous interval is primarily the result of shortening of anoestrus (Okkens et al. 1985b), but is also due to shortening of the luteal phase (Okkens et al. 1985b, 1990) (Figure 8). The shortening of the luteal phase is probably caused by a decrease in the secretion of prolactin, the main luteotrophic factor in the bitch (Okkens et al. 1990; Onclin and Verstegen 1997).

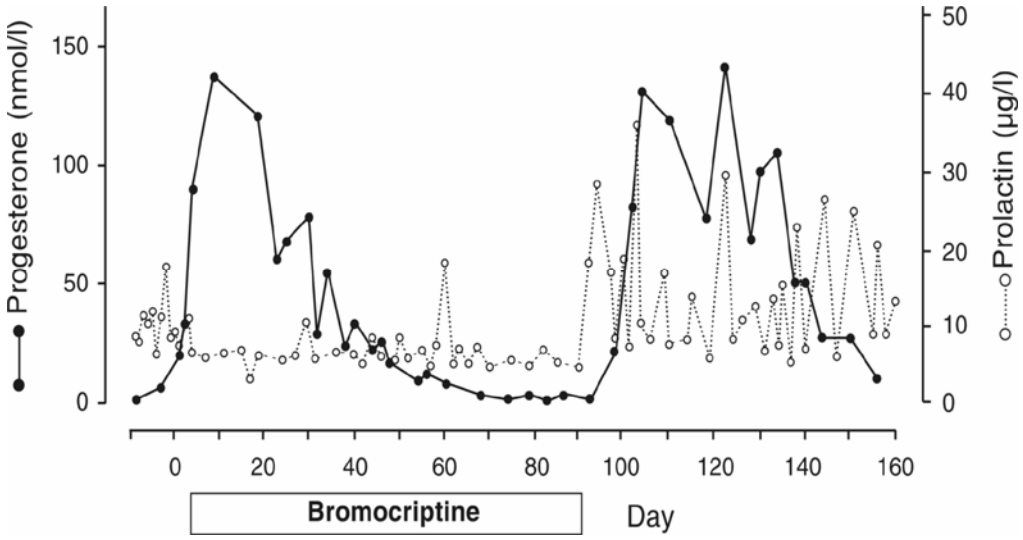


Figure 8. Progesterone and prolactin levels in the peripheral blood of a dog treated with bromocriptine (bar) from the time of ovulation in the first cycle until the onset of the next follicular phase. The luteal phase and especially anoestrus are considerably shortened. Modified from Okkens et al. 1985b.

It has been hypothesized that also the shortening of the anoestrus by dopamine-agonists is the result of the suppression of prolactin secretion, as prolactin may inhibit gonadotrophin release. Indeed, it has been demonstrated in various mammalian species that high circulating levels of prolactin in different pathological situations inhibit LH pulsatility (Sauder et al. 1984, Yazigi et al. 1997) or are associated with decreased LH secretion (Park et al. 1993). In addition, decreased plasma LH levels were observed during physiological hyperprolactinaemia in lactating sows, while lowering of the plasma prolactin concentration by bromocriptine administration led to a rise in plasma LH levels in these animals (Bevers et al. 1983). Yet, under physiological conditions plasma prolactin concentrations are low during canine anoestrus (Olson et al. 1982, Kooistra and Okkens 2001) and no obvious changes in plasma prolactin concentration have been observed during the transition from anoestrus to the follicular phase in the bitch (Olson et al. 1982). Furthermore, anoestrus was not shortened in dogs treated with low dosages of the serotonin receptor antagonist metergoline (Okkens et al. 1997), of which the prolactin-lowering effect of a low dose is due to its serotonin antagonistic properties (Müller et al. 1983), while the plasma prolactin levels were even lower than in bromocriptine-treated dogs. The results of the latter study suggest that the induction of the follicular phase is not initiated by suppression of prolactin secretion. This raises the question whether administration of a dopamine agonist in a dosage that is too low to suppress prolactin secretion still will result in a shortening of anoestrus in the bitch. If so, it is proven that the shortening of anoestrus in the bitch is not due to suppression of prolactin secretion but is due to another dopaminergic effect.

Besides an inhibition of prolactin release, the bromocriptine-induced shortening of anoestrus is also associated with a quick rise in the basal plasma FSH concentration without a concomitant increase in the basal plasma LH concentration (Kooistra et al. 1999b). In all dogs in the latter study, both basal plasma FSH concentration and the AUC for FSH increased shortly after the start of the bromocriptine treatment. The mean basal plasma FSH concentrations at 14, 28 and 42 days after the start of the treatment were significantly higher than that at the day before the onset of treatment. The mean AUC for FSH at 14 days after the start of the bromocriptine treatment was significantly higher than that before treatment. Differences in mean basal plasma LH concentration and mean AUC for LH between the day before and 14, 28 and 42 days after the start of the bromocriptine treatment were not significant. These results demonstrate that the shortened anoestrus was associated with an increase in both basal plasma FSH concentration and AUC for FSH, without a concomitant rise in basal plasma LH concentration and AUC for LH, which gives further support to the notion that in the bitch an increase in circulating FSH should be considered to be a critical event required for ovarian folliculogenesis. In contrast to dopamine agonists, low dosages of the serotonin receptor antagonist metergoline do not result in shortening of anoestrus (Okkens et al. 1997). Therefore, it may be expected that the changes in gonadotrophin release associated with dopamine agonist-induced shortening of anoestrus will not be observed during treatment with low dosages of the serotonin receptor antagonist metergoline. Unfortunately, information

on the pulsatile plasma profiles of LH and FSH during treatment with a serotonin receptor antagonist is lacking.

A role for dopamine in the control of reproduction has been demonstrated in different mammalian species, although the effects of dopamine in other species differ from those in the bitch. In the ewe, dopamine appears to play a major role in the inhibition of gonadotrophin secretion during seasonal anoestrus (Meyer and Goodman 1986, Kao et al. 1992, Le Corre and Chemineau 1993, Gayrard et al. 1994, Havern et al. 1994, Sakurai et al. 1995) and administration of the dopamine-agonist bromocriptine does not affect plasma FSH concentration and the time of ovulation (Land et al. 1980). In the mare, bromocriptine treatment has no effect during seasonal anoestrus (Bennett-Wimbush et al. 1998), while treatment with dopamine-antagonists induces an early-spring transitional period and subsequent ovulation (Besognet et al. 1997). In ovariectomized and prepubertal female rats, dopamine inhibits the release of LH (Beck et al. 1978, Lacau de Mengido et al. 1987). Furthermore, dopamine-antagonists induce puberty prematurely. In adult rats, dopamine-antagonists increase LH secretion (Advis et al. 1981, Sarkar and Fink 1981). Taken together, these data result in a fascinating picture. In species other than the dog, dopamine-agonists may inhibit gonadotrophin secretion during anoestrus and dopamine-antagonists may induce reproductive activity, whereas in the bitch dopamine-agonists induce the onset of oestrus.

Differentiation between intact anoestrous and OVX bitches

No obvious clinical difference can be seen between the anoestrous bitch and one that has been ovariectomized or ovariohysterectomized. Consequently, it can be difficult to verify the neuter status of dogs with an unknown reproductive status. In addition, it is occasionally difficult to differentiate ovariectomized (OVX) dogs having remnant ovarian tissue from completely OVX dogs. The presence of ovarian tissue can be confirmed by cytological evaluation of a vaginal smear and vaginoscopy during the follicular phase, measurement of the plasma progesterone concentration during the progression to the late follicular phase, ovulation and luteal phase, abdominal ultrasonography and exploratory laparotomy (Wallace 1991, Root and Spaulding 1994, Okkens et al. 1981).

During the follicular phase, demonstration of the presence of ovarian tissue is straightforward in the bitch. The concentration of oestradiol, which is mainly synthesized and secreted by granulosa cells in developing ovarian follicles, rises during this phase and reaches peak levels around the pre-ovulatory LH surge (Olson et al. 1982, Schaefers-Okkens 2005, De Gier et al. 2006). Vaginoscopy and vaginal cytology can be used to recognize the influence of oestrogens (Schutte 1976, Schaefers-Okkens 2005). The use of cytological evaluation of a vaginal smear as a bioassay for oestrogen influence is even more reliable than a single plasma oestradiol determination (Shille and Olson 1989). The plasma concentration of progesterone, which is secreted by partially luteinizing granulosa cells before ovulation and by mature luteal cells after ovulation, is increased in the progression to the late follicular phase, ovulation and

during the luteal phase (Schaefers-Okkens 2005). Clearly elevated plasma progesterone concentrations provide evidence for the presence of ovarian tissue (Okkens et al. 1981).

During anoestrus the plasma progesterone concentration is in general below 3 nmol/l (Okkens et al. 1985a). The plasma oestradiol concentration is also usually low and does not begin to rise again until late anoestrus, although sporadic elevations have been reported (Olson et al. 1982). During anoestrus, LH is secreted in a pulsatile fashion but the basal plasma LH concentration is low (Olson et al. 1982, Kooistra et al. 1999a). Plasma testosterone concentrations are also low during anoestrus in the bitch. The plasma testosterone concentration begins to rise near the end of anoestrus and reaches peak levels near the time of the pre-ovulatory LH surge (Olson et al. 1984, Concannon and Castracane 1985). There are no obvious clinical or behavioural differences between anoestrous and OVX bitches. Vaginal cytology also has no diagnostic value in this differentiation and ultrasonographic visualization of ovarian tissue in anoestrous bitches is sometimes difficult (England and Allen 1989, Root and Spaulding 1994). Production of the ovarian hormones oestradiol and progesterone ceases with OVX, but the plasma concentrations of these hormones overlap in anoestrous and OVX bitches (Jeffcoate 1993a, Frank et al. 2003). The loss of negative feedback of ovarian steroids causes a rapid increase in the concentration of circulating gonadotrophins (Chaffaux et al. 1981, Olson et al. 1992, Concannon 1993, Jeffcoate 1993b, Löfstedt and Vanleeuwen 2002, Reichler et al. 2004), while their secretion pattern remains pulsatile (Concannon 1993). Baseline gonadotrophin levels may provide some useful information, but as a result of the pulsatile secretion pattern a considerable overlap in the plasma concentrations may be expected between intact and OVX animals and thus the diagnostic value of a single LH measurement is questionable (Jeffcoate 1993b, Löfstedt and Vanleeuwen 2002). The potential use of a single FSH measurement in verification of neuter status has not been investigated in the bitch.

To differentiate between bitches with and without ovarian tissue, a provocative test of the pituitary-ovary axis using GnRH may be helpful. In the intact bitch, GnRH administration during anoestrus causes an increment in the plasma concentrations of LH and oestradiol (Van Haaften et al. 1994). The GnRH induced plasma LH and oestradiol responses are higher in late anoestrus than in early anoestrus (Van Haaften et al. 1994). Information on GnRH-induced secretion of FSH, testosterone, and progesterone in anoestrous bitches is lacking.

Information regarding the response to exogenous GnRH after ovariectomy is limited to only a few studies. One study found a rise in LH after GnRH administration in OVX bitches (Chaffaux et al. 1981), while another found an unpredictable response (Jeffcoate 1993b). The discrepancy may be because of differences in sampling times and doses of GnRH that were used. Only one study investigated the effect of a GnRH challenge on plasma oestradiol concentration in OVX bitches (Jeffcoate 1993b). The low dose of GnRH that was used in the latter study produced no increase in plasma oestradiol concentration. Information on GnRH-induced secretion of FSH, testosterone, and progesterone in OVX bitches is not available.

3. Progestin-induced canine anoestrus

Progesterone and synthetic progestins

In the bitch, natural progesterone is biosynthesized and secreted by partial luteinized granulose cells during the preovulatory LH peak and subsequently the corpus luteum during the luteal phase of the oestrous cycle. Although the main source of progesterone in the intact bitch is the ovary, small amounts of progesterone can also be secreted by the adrenal cortex (Frank et al. 2004). In general, in mammals progesterone plays a major role in regulating the growth, development and function of female reproductive tissues (Clarke and Sutherland 1990, Graham and Clarke 1997) Apart from the genital tract and ovaries, also brain, mammary gland, bone, blood vessels, thymus, pancreas, bone and lung (Graham and Clarke 1997, Lantinga van Leeuwen et al. 2000) express progesterone receptors, and are therefore target organs for progesterone action.

The terms progestin, progestagen, and progestogen are used interchangeably to refer to any of the manufactured steroids with progestational activity and derived from progesterone or related steroids. Progestins are widely used in companion animal medicine and the main use involves the control of the reproductive cycle (Schaefers-Okkens 1996, Romagnoli and Concannon 2003). The progestins most frequently used for oestrus prevention in the dog are proligestone and medroxyprogesterone acetate (MPA). In veterinary medicine, depot-injectable MPA rapidly became, and remained for a few decades, the most widely used progestin in Europe. In the United States, however, it was quickly withdrawn from the veterinary market because of a high incidence of uterine disease reported in dogs administered MPA. Other progestins that were developed as potential human contraceptives have also been marketed for contraceptive use in dogs and/or cats e.g. oral megestrol acetate, oral MPA, oral delmadinone acetate, oral chlormadinone acetate, and depot-injectable proligestone (Romagnoli and Concannon 2003).

When used as a depot preparation, the drug cannot be rapidly withdrawn after injection of the progestin, and care must be taken to use the lowest possible effective dosing regimen. The single injection dosage recommended by the manufacturer for proligestone ranges from 10 mg/kg for a dog of about 60 kg, to 30 mg/kg for one of 3 kg, s.c., and for MPA the single injection dose is 2 mg/kg (maximum 60 mg), s.c. (Schaefers-Okkens 1996). They should be administered during anoestrus about one month before the expected follicular phase. The first oestrus after the use of proligestone in the majority of bitches can be expected within 9-12 months; after MPA administration it may take up to 2-3 years. MPA can also be administered orally, 5 mg once daily (10 mg for large dogs during the first 5 days) for as long as oestrus prevention is wanted or for a maximum of 21 days. The recurrence of oestrus may vary from 2-9 months (Schaefers-Okkens 1996).

Mechanism of action of progestins

Synthetic progestins act in the same manner as endogenous progesterone. In most if not all target tissues, progesterone and synthetic progestins diffuse through the cell membrane, and bind to intracellular receptors. In most mammalian species two progesterone receptor (PR) variants are known, the 51-94 kDa PR-A and the larger 116-120 kDa PR-B form. PR-A and PR-B share the same hormone- and DNA-binding domains and differ only in the length of the amino terminus. Cellular responsiveness to progestins may be modulated via alterations in the ratio of PR-A and PR-B (Graham and Clarke 1997). After binding of progesterone, the progesterone-PR complex binds to a progesterone-response-element in the nuclear genome, resulting in suppression or activation of transcription and eventual translation of various genes. The translation products include structural and secretory proteins, enzymes, and other regulatory proteins. It is also likely that in some tissues, progesterone, similar to oestradiol, can bind to membrane receptors and has cellular effects in response to binding to membrane receptors (Graham and Clarke 1997).

Whether the oestrus-preventing properties of progestins in the bitch are due to effects on the hypothalamus, on the pituitary gland, and/or at the ovarian level is not clear. Originally, it was assumed that the oestrus-preventing action of progestins in the bitch primarily involved inhibition of the pituitary gonadotrophin secretion with consequent prevention of oestrus, as is the case with the combined oestrogen-progestagen oral contraceptives in women (Kafrissen and Adashi 2003). However, McCann et al. (1987) and Colon et al. (1993) reported that basal plasma levels of LH and FSH do not change during progestin treatment in the bitch, indicating a lack of suppression of the hypothalamus-pituitary system in this species. Information about the effect of GnRH on gonadotrophin secretion during progestin treatment is conflicting in bitches. GnRH-induced increases in plasma LH concentrations in progestin-treated dogs did not differ from those in control dogs in one study (Colon et al. 1993), while in another study the GnRH-induced LH levels were reduced (McCann et al. 1987). In addition, in the male dog there are indications that the effect of progestins are the result of direct action on the epididymal phase of sperm development, without suppression of the plasma LH concentration (England 1997). There is no information on the effect of progestins on GnRH-stimulated FSH secretion in the dog.

In women, progestins are known to prevent ovulation by inhibiting the mid-cycle surges of FSH and LH, whereas the tonic release of these gonadotrophins continues at luteal phase levels (Mishell 1996, Jain et al. 2004), indicating also a lack of complete suppression of the hypothalamus-pituitary system. Especially the suppression of FSH is not as intense as with the combination oral contraceptives; thus follicular growth is maintained sufficiently to produce oestrogen levels comparable to those in the early follicular phase of the normal menstrual cycle (Fraser and Weisberg 1981). In women, MPA may inhibit the positive feedback effect of oestradiol on the hypothalamus-pituitary system, which in the absence of MPA would stimulate the midcycle release of LH (Ortiz et al. 1977). Long-term use of depot preparations of MPA (DMPA) in women does not affect the pituitary responsiveness of LH

and FSH to GnRH administration, suggesting that the pituitary is not the primary site for ovulation inhibition in women (Ismael et al. 1987). In addition to the inhibition of ovulation, other mechanisms of action of DMPA in women have been recognized. First, the endometrium becomes thin and does not secrete sufficient glycogen to provide nutrition for a blastocyst entering the endometrial cavity. Second, DMPA keeps cervical mucus thick and viscous, so sperm cells are unlikely to reach the oviduct and fertilize an egg. With these multiple mechanisms of action, DMPA is one of the most effective reversible methods of contraception currently available (Mishell 1999).

In addition to an effect at the hypothalamus-pituitary-ovary axis, prolonged treatment with progestins is associated with alterations in the release of pituitary hormones other than gonadotrophins in bitches. Progestin administration leads to a decrease in the pituitary responsiveness of GH to GHRH (Watson et al. 1987, Selman et al. 1991, Selman et al. 1994a). This change is due to progestin-induced GH release from foci of hyperplastic ductular mammary epithelium (Selman et al. 1994b, Van Garderen et al. 1997), leading to elevated plasma GH levels that do not have a pulsatile plasma profile (Watson et al. 1987). The progestin-induced GH excess may give rise to acromegaly and glucose intolerance, which may lead to exhaustion of the pancreatic β-cells and subsequently diabetes mellitus (Eigenmann et al. 1983). Diabetes mellitus is a common finding in acromegaly in humans, cats and dogs and can be explained by the diabetogenic properties of GH, leading to insulin resistance (Eigenmann and Rijnberk 1981).

The hypothalamic–pituitary–adrenocortical (HPA) axis is suppressed by progestins (McCann et al. 1987, Selman et al. 1997, Rutteman et al. 1987), due to the intrinsic glucocorticoid properties of progestins (Selman et al. 1997, Guthrie and John 1980, Selman et al. 1996). While basal plasma concentrations of ACTH are only moderately affected (Selman et al. 1997), the basal plasma concentrations of cortisol are markedly decreased (McCann et al. 1987, Selman et al. 1997, Concannon et al. 1980, Rutteman et al. 1989). In addition, the response of ACTH and cortisol to stimulation with CRH may be reduced (McCann et al. 1987, Selman et al. 1997). Also, in women the administration of MPA causes suppression of the HPA axis (Jones et al. 1974).

With regard to other anterior pituitary hormones, such as prolactin and TSH, there is little information about the effect of progestin treatment on their release. In the bitch, progestin treatment does not seem to affect mean prolactin (Frank et al. 1979, Concannon et al. 1980) and TSH concentrations (Frank et al. 1979). These results are surprising, since in the second half of the luteal phase of the canine oestrous cycle there is a clear negative correlation between plasma progesterone and prolactin concentrations, which may be explained by a clear-cut decrease in progestational activity, which is known to be a trigger of prolactin release (Galac et al. 2000).

Information with regard to pituitary responsiveness of prolactin to suprapituitary stimulation is limited to one study, in which MPA administration did not change prolactin response to TRH in ovariohysterectomized, oestradiol-primed bitches (Rutteman et al. 1987).

On the other hand, there is evidence that treatment with MPA increases the pituitary prolactin responsiveness to TRH in women (Mishell et al. 1977).

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CHAPTER 3

Low doses of bromocriptine shorten the interoestrous interval in the bitch without lowering plasma prolactin concentration

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Abstract

In order to investigate the effect of different doses of bromocriptine on plasma prolactin concentration and the interoestrous interval, Beagle bitches were treated twice daily with 5 μg (5-group), 20 μg (20-group), or 50 μg (50-group) bromocriptine per kg body weight orally, starting 28 days after ovulation.

In the 5-group, the difference between the mean plasma prolactin concentration before and that during bromocriptine treatment was not significant. In contrast, mean plasma prolactin concentration decreased significantly after the start of bromocriptine treatment in the 20- and 50-groups. The mean interoestrous interval was significantly shorter in all three groups than in untreated bitches in the same colony. The mean interoestrous interval in the 20-group and that in the 50-group were similar, but both were significantly shorter than that in the 5-group.

The results of this study indicate that bromocriptine shortens the interoestrous interval in the bitch even when the dose is so low that it does not lower plasma prolactin concentration. Induction of oestrus in the bitch by bromocriptine therefore involves a mechanism other than via the lowering of plasma prolactin concentration. Furthermore, this study shows that the extent of shortening of the interoestrous interval by bromocriptine is dose dependent.

Introduction

The nonseasonal oestrous cycle is considerably longer in the dog than in most other domestic animals. The follicular phase and spontaneous ovulation are followed by a luteal phase having an average duration of about 75 days (Schaefers-Okkens, 1996). An anoestrus of variable duration (2–10 months) follows each oestrous cycle (Bouchard et al. 1991, Concannon 1993). Endocrine changes leading to the termination of anoestrus and the start of a new follicular phase in the bitch are still not completely understood and need further elucidation.

In bitches, administration of the dopamine agonists bromocriptine and cabergoline is associated with inhibition of prolactin release and shortening of the interoestrous interval (Okkens et al. 1985a, Van Haaften et al. 1989, Concannon 1993, Onclin et al. 1995, Kooistra et al. 1999a, Verstegen et al. 1999, Gobello et al. 2002). If bromocriptine treatment is started during the luteal phase, shortening of the interoestrous interval is mainly the result of shortening of anoestrus (Okkens et al. 1985a) but is also due to shortening of the luteal phase (Okkens et al. 1985a, 1990). The shortening of the luteal phase is caused by a decrease in the secretion of prolactin, the main luteotrophic factor in the bitch (Okkens et al. 1990, Onclin and Verstegen 1997a). It has been hypothesized that the induction of oestrus in dogs by dopamine agonists is the result of the suppression of prolactin secretion, since prolactin may inhibit gonadotrophin release. Indeed, it has been clearly demonstrated in various mammalian species that high circulating levels of prolactin in different pathological situations inhibit LH pulsatility (Sauder et al. 1984, Yagizi et al. 1997) or are associated with decreased LH secretion (Park et al. 1993). In addition, decreased plasma LH levels were observed in the physiological hyperprolactinemia during lactation in sows, while bromocriptine treatment decreased plasma prolactin concentration, leading to increased plasma LH levels (Bevers et al. 1983). However, the interoestrous interval was not shortened in dogs treated with metergoline, a drug that in low dosage lowers plasma prolactin concentration via a serotonin-antagonistic pathway (Okkens et al. 1997). Furthermore, low plasma prolactin concentrations were found during anoestrus under physiological conditions (Kooistra and Okkens 2001) and 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 findings raise the question whether a reduction in plasma prolactin concentration or some other dopamine-agonistic influence during bromocriptine treatment is the critical factor in the shortening of the oestrous cycle. This study therefore investigated the effects of different doses of bromocriptine on plasma prolactin concentration and the initiation of oestrus.

Materials and methods

Animals, treatment and collection of blood samples

Thirteen healthy Beagle bitches, 2–6 years of age, were used for this study. Some were used twice, but an oestrous cycle without treatment was always allowed to occur between the two cycles in which treatment was given. The results of the treatments were therefore considered to be independent. Body weight ranged from 9.3 to 18.3 kg. All dogs were born and raised in the Department of Clinical Sciences of Companion Animals and were accustomed to the laboratory environment and procedures, such as the collection of blood. They were housed in pairs in indoor–outdoor runs, fed a standard commercial dog food once daily, and provided with water ad libitum.

All dogs were examined thrice weekly for the presence of swelling of the vulva and serosanguinous vaginal discharge, changes signifying the onset of pro-oestrus. Plasma progesterone concentration was measured three times per week from the start of pro-oestrus until the day on which it exceeded 16.0 nmol/l, when ovulation is assumed to occur (Concannon et al. 1977, Wildt et al. 1979, Okkens et al. 1985a). The interestrous interval was defined as the number of days between ovulations.

Blood samples were collected from the jugular vein three times per week at 09:00 h. Plasma was separated by centrifugation at 4 °C for 10 min at 1500×*g* and was stored at −25 °C until used for hormone assay. Plasma prolactin concentration was measured three times weekly from 14 ± 1 days after ovulation until the next ovulation. Plasma progesterone concentration was measured once weekly from 30 ± 2 days after ovulation until the next ovulation. Starting 28 ± 1 days after ovulation, six bitches (5-group), six bitches (20-group), and eight bitches (50-group) were treated twice daily at 09.00 and 21.00 h with 5, 20, and 50 µg, respectively, bromocriptine (Lactafal®, generously supplied by Eurovet, Bladel, The Netherlands) per kg body weight orally. The mean control interoestrous interval was 216 ± 9 (mean ± S.E.M.) days in the same colony (11 bitches; 19 cycles) during the period of these experiments.

Hormone measurements

Plasma progesterone concentration was measured by a previously validated RIA (Dieleman and Schoenmakers 1979, Okkens et al. 1985b). The intra- and interassay coefficients of variation were 11 and 14%, respectively. The lower limit of quantitation was 0.13 nmol/l.

Plasma prolactin concentration was measured by a previously validated heterologous RIA (Okkens et al. 1985b). The intra- and interassay coefficients of variation were 3.5 and 11.5%, respectively. The lower limit of quantitation was 0.8 µg/l.

Data analysis

In one dog some plasma prolactin concentrations were below the limit of quantitation and were assigned a value of 0.8 µg/l. Statistical analysis was performed using SPSS for Windows 10.1 (SPSS Inc.; Chicago, IL, USA). For each bitch the mean plasma prolactin concentration before and during treatment was calculated. The average plasma prolactin concentration per group was calculated as the average of the mean values in the individual dogs. The mean plasma prolactin concentrations before and during treatment were compared using a one-tailed Student's *t*-test for paired observations. Differences among the three groups in the duration of the interoestrous interval and in plasma progesterone concentration were analysed using one-way analysis of variance (ANOVA) followed by multiple comparisons using the Student–Newman–Keuls test for data with significant main effect. Differences in plasma progesterone concentrations among the three groups were not analysed after week 6 of treatment; from week 7 onward, statistical analysis was not useful because oestrus had begun in many dogs, leaving too few per group. $P<0.05$ was considered significant. Results are presented as mean ± S.E.M.

Ethics of experimentation

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

Results

In the bitches receiving 5 µg bromocriptine per kg body weight twice daily (5-group), the difference between the average plasma prolactin concentration before and during treatment, 3.3 ± 0.3 µg/l and 3.0 ± 0.3 µg/l, respectively, was not significant. In the 20- and the 50-groups the average plasma prolactin concentration before bromocriptine treatment was significantly higher than that during treatment, 6.6 ± 2.1 µg/l versus 4.7 ± 1.4 µg/l and 5.2 ± 0.8 µg/l versus 3.2 ± 0.5 µg/l, respectively. The mean interoestrous interval was 136 ± 16 days in the 5-group, 96 ± 6 days in the 20-group, and 92 ± 11 days in the 50-group. Each of these intervals was significantly shorter than the mean interoestrous interval in control cycles, 216 ± 9 days. The mean interoestrous intervals in the 20- and 50-groups were similar and significantly shorter than that in the 5-group (Figure 1).

The mean plasma progesterone concentration was significantly higher in the 5-group than in the 20- and 50-groups from the second week of treatment onward (Figure 2). During the seventh week of treatment, i.e. 70–77 days after ovulation, the mean plasma progesterone concentration in the 5-group was 9.2 ± 0.6 nmol/l (n=6), while in five bitches of the 20-group and two bitches of the 50-group the mean plasma progesterone concentrations were 2.1 ±

1.3 nmol/l and 3.6 ± 1.3 nmol/l, respectively. The remaining dogs in the 20-group (*n*=1) and in the 50-group (*n*=6) had at this point already ovulated or were in pro-oestrus.

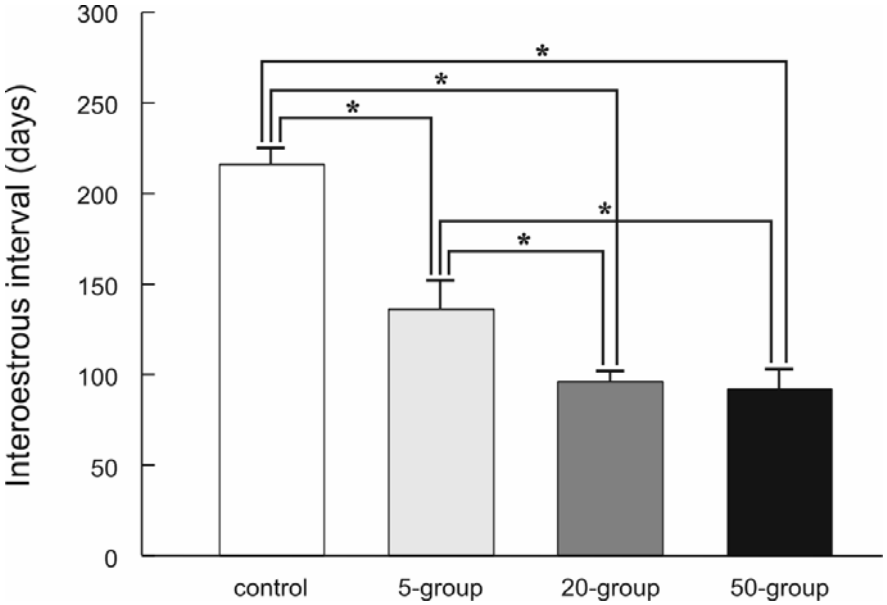

Figure 1. Mean (±S.E.M.) interoestrous interval in control bitches and in bitches treated with different doses of bromocriptine. The bitches in the 5-, 20-, and 50-groups (n=6, 6, and 8, respectively) were treated with 5, 20, and 50 µg bromocriptine per kg body weight, respectively, twice daily from 28 days after ovulation until the next ovulation. (*) P<0.05.

Discussion

Okkens et al. (1997) reported that the interoestrous interval was shortened and plasma prolactin concentration was decreased during treatment with the dopamine agonist bromocriptine. However, treatment with the serotonin antagonist metergoline did not result in shortening of the interoestrous interval, although there was a similar decrease in plasma prolactin concentration. These observations suggest that the initiation of oestrus is not triggered by a decline in plasma prolactin concentration but is probably due to some other action of bromocriptine. The present study provides further evidence for this hypothesis, since in bitches receiving a low dose of bromocriptine (5-group) there was significant shortening of the interoestrous interval without a decrease in plasma prolactin concentration. Treatment with bromocriptine may cause an increase in plasma FSH concentration to a level that enhances

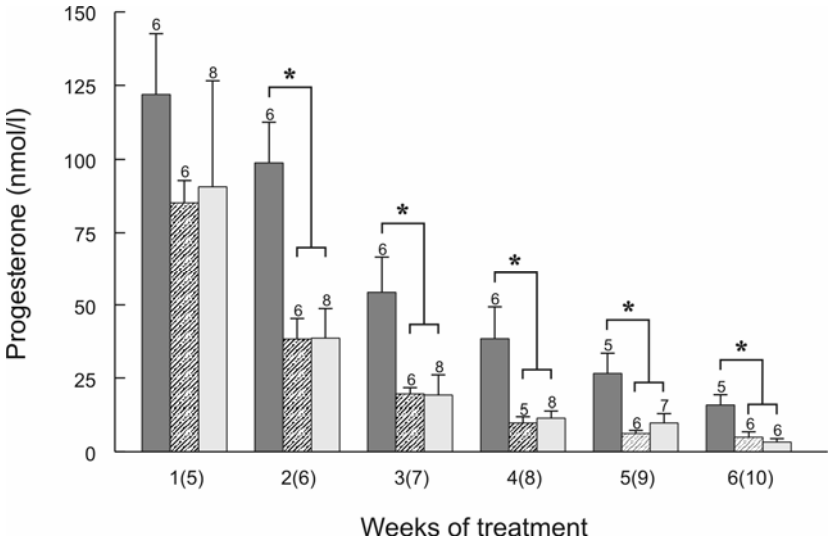

*Figure 2. Mean (±S.E.M.) plasma concentration of progesterone in bitches treated with different doses of bromocriptine. Plasma progesterone concentration was measured once weekly from day 30.4 ± 1.6 days after ovulation until the next ovulation. The bitches of the 5-group (open bars), 20-group (gray bars), and 50-group (black bars) were treated with 5, 20, and 50 µg bromocriptine per kg body weight, respectively, twice daily from 28 days after ovulation until the next ovulation. Numbers above the bars denote the number of observations; numbers in parentheses denote weeks after ovulation. * indicates P<0.05 between the 5-group and the 20- and 50-groups.*

development of follicles. Shortening of the interoestrous interval by bromocriptine in a dose that also lowers plasma prolactin concentration is associated with an increase in plasma FSH concentration without a concomitant increase in plasma LH concentration (Kooistra et al. 1999a). This is similar to the endocrine events during late anoestrus in nontreated bitches (Kooistra et al. 1999b).

A role for dopamine in the control of reproduction has been demonstrated in different species, although the effects of dopamine in other species differ from those in the bitch. In the ewe, dopamine appears to play a major role in the inhibition of gonadotrophin secretion during seasonal anoestrus (Meyer and Goodman 1986, Kao et al. 1992, Le Corre and Chemineau 1993, Gayrard et al. 1994, Havern et al. 1994, Sakurai et al. 1995) and administration of the dopamine agonist bromocriptine does not affect plasma FSH concentration and the time of ovulation (Land et al. 1980). In the mare, bromocriptine treatment has no effect during seasonal anoestrus (Bennett-Wimbush et al. 1998), while treatment with dopamine antagonists induces an early-spring transitional period and subsequent ovulation (Besognet et al. 1997). In ovariectomized and prepubertal female rats, dopamine inhibits the release of LH (Beck et al.

1978, Lacau de Mengido et al. 1987), and dopamine antagonists induce puberty prematurely; in adult rats, dopamine antagonists increase LH secretion (Sarkar and Fink 1981, Advis et al. 1981). In species other than the dog, dopamine agonists may inhibit gonadotrophin secretion during anoestrus and dopamine antagonists may induce reproductive activity, while in the bitch dopamine agonists induce the onset of oestrus.

The effect of bromocriptine on the interoestrous interval observed in this study appears to be dose dependent, for the interoestrous interval was significantly shorter in the 20- and 50-groups than in the 5-group. The difference in the interval between the 20- and the 50-groups was not significant, possibly because there is a maximum effective dopamine-agonist dose, above which no further shortening of the interoestrous interval occurs.

In the 5-group the luteal phase was not shortened. In the 7th week of treatment, i.e. 70–77 days after ovulation, all bitches in this group had a plasma progesterone concentration above 3.2 nmol/l and were therefore still in the luteal phase, which was of normal duration (Okkens et al. 1985b, Onclin and Verstegen 1997b). Shortening of the interoestrous interval in this group was thus exclusively due to shortening of anoestrus. In the 20- and 50-groups, plasma progesterone concentration was decreased earlier, probably due to a decreased plasma prolactin concentration, which is the main luteotrophic factor in the bitch (Okkens et al. 1985a, 1990, Onclin and Verstegen 1997a).

In summary, this study provides further evidence that the bromocriptine-induced shortening of anoestrus in the bitch is due to an action of bromocriptine other than its lowering of plasma prolactin concentration. Furthermore, this study shows that the extent of shortening of the interoestrous interval by bromocriptine is partly dose dependent.

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CHAPTER 4

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 Haaften 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 and 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 ^{125}I 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. 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 ± SD) days after ovulation, immediately after the blood 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 ± 1 days.

Hormone measurements

From 75 days after ovulation until the next ovulation, plasma progesterone was measured by a previously validated ^{3}H-RIA (Dieleman and 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.

Plasma FSH concentration was measured by a heterologous canine immunoradiometric assay (IRMA) (AHC004, Biocode SA, Liège, Belgium) described by Beijerink et al. (2007). The intra-assay and interassay CV for values above 1.6 µg/l were 3.2% and 15%, respectively. The limit of quantitation was 1.5 µg/l.

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 GD Niswender, Colorado State University, CO, USA), radio-iodinated bovine LH-7981, and canine pituitary standard LER 1685-1 (a gift of Dr LE 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 were 2.3% and 10.5% respectively, and the lower limit of measurement was 0.3 µg/l.

Plasma prolactin concentration was determined by a previously validated heterologous RIA (Okkens et al. 1985b). 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.

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 SD 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 SD, 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 ± SEM.

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 ± 18 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. 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 Figure 1.

Before treatment the average prolactin concentration decreased slightly with a negative day effect ($P = 0.04$). 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 (Figures 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 (Figures 2 and 3). Both were characterized by an abrupt and rapid rise followed by a slow decline, slower for FSH than for LH.

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).

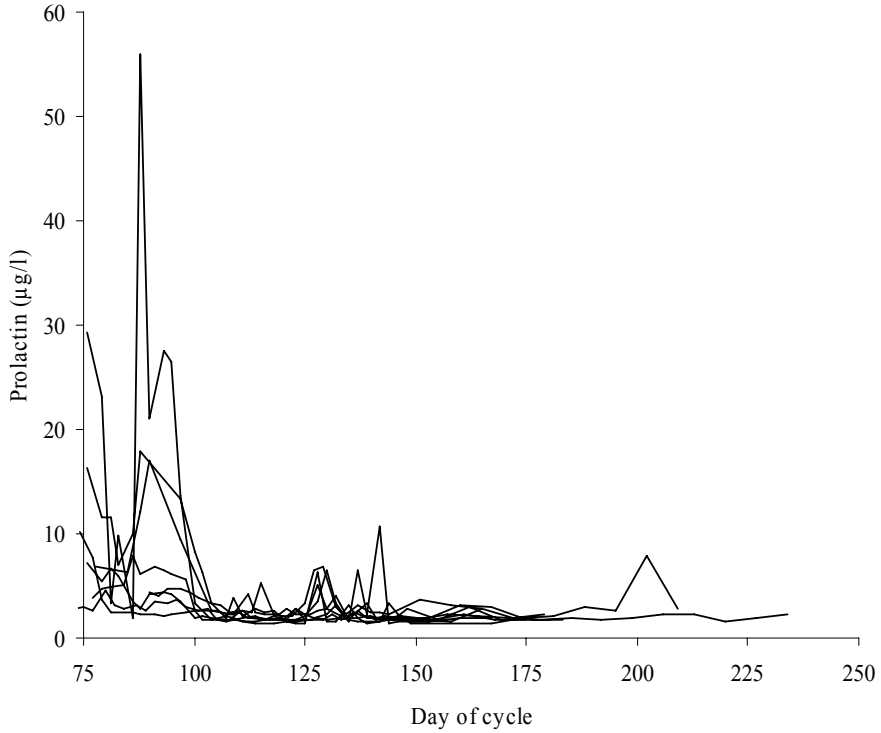

Figure 1. Plasma prolactin concentrations in eight Beagle bitches before and during treatment with the serotonin antagonist metergoline. In each dog, plasma prolactin concentration was measured three times weekly from day 75 to day 142 and once weekly thereafter until the next ovulation. Treatment with 0.1 mg metergoline per kg body weight twice daily was started 100 ± 2 days after ovulation and was continued until the next ovulation.

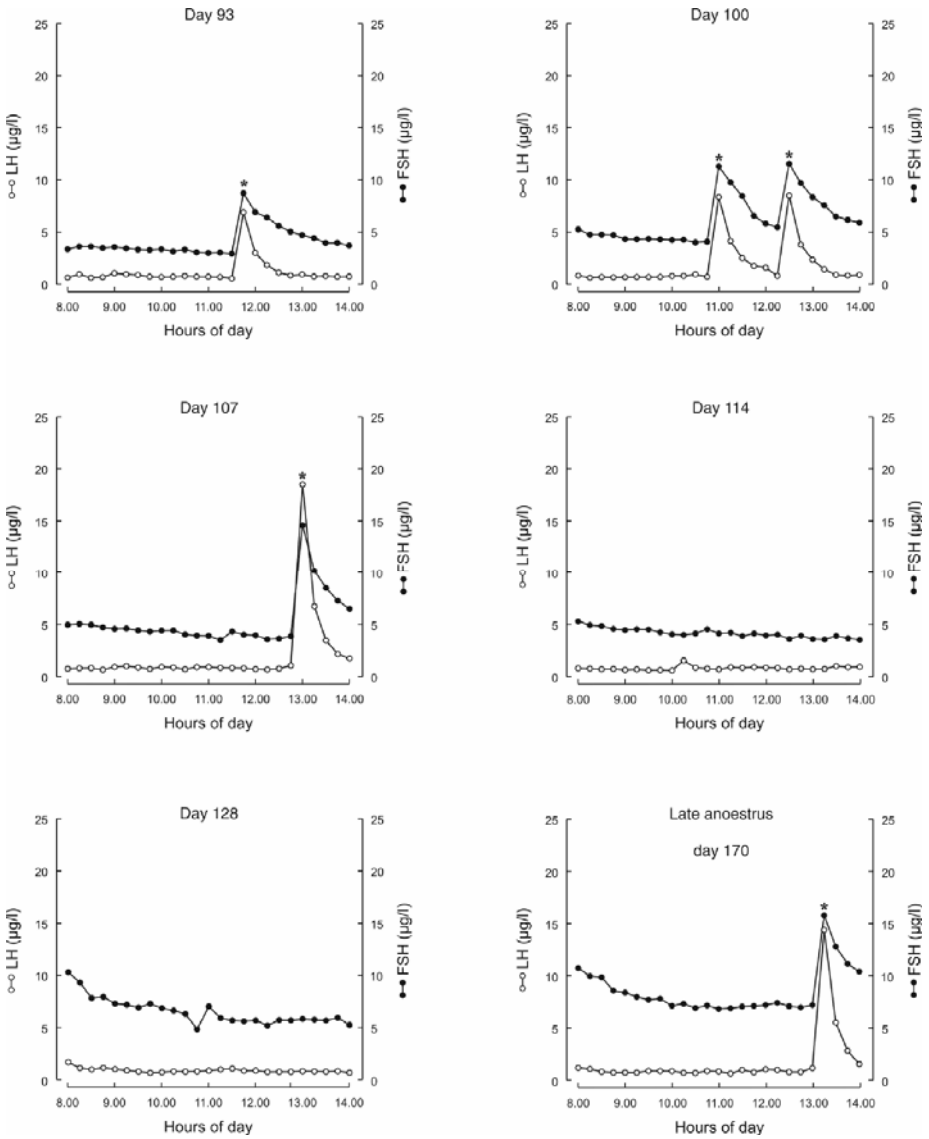

*Figure 2. The 6-h plasma profiles of FSH (black squares) and LH (grey diamonds) in a 5-year-old Beagle bitch at 93, 100, 107, 114 and 128 days after ovulation and during late anoestrus (day 170). Blood samples were collected at 15-min intervals. Treatment with the serotonin antagonist metergoline (0.1 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.*

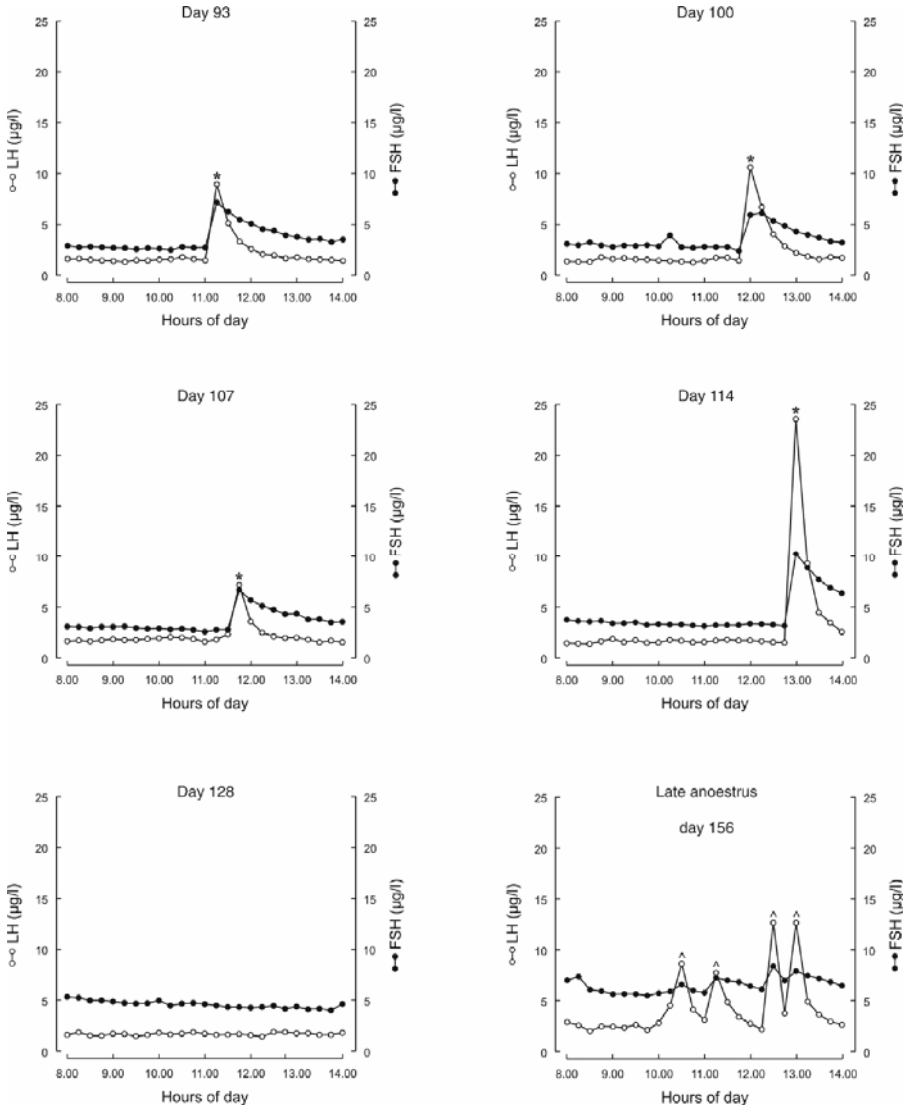

*Figure 3. The 6-h plasma profiles of FSH (black squares) and LH (grey diamonds) in a 2-year-old Beagle bitch at 93, 100, 107, 114 and 128 days after ovulation and during late anoestrus (day 156). Blood samples were collected at 15-min intervals. Treatment with the serotonin antagonist metergoline (0.1 mg per kg body weight twice daily) was started 100 days after ovulation and was continued until the next ovulation. The plasma profile of LH on day 156 is characterized by frequent brief pulses. Ovulation occurred on day 169. *Significant pulses of both FSH and LH identified by the Pulsar programme; ^ significant pulse of LH without a concurrent significant pulse of FSH.*

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).

	Before (n=8)	Day 107 (n=8)	Day 114 (n=8)	Day 128 (n=8)	late anoestrus (n=8)
LH peak amplitude (µg/l)	9.0 ± 2.0 (n=8)	9.0 ± 1.9 (n=7)	17.0 ± 2.3 (n=5)	14.9 ± 3.8 (n=6)	10.0 ± 1.7 (n=8)
FSH peak amplitude (µg/l)	4.5 ± 0.5 (n=7)	5.1 ± 1.1 (n=7)	8.1 ± 1.9 (n=5)	6.3 ± 0.9 (n=6)	5.5 ± 1.0 (n=6)
LH pulse frequency (peaks/6h)	1 (0 - 3)	1 (0 - 2)	1 (0 - 2)	1 (0 - 2)	1 (1 - 4)
FSH pulse frequency (peaks/6h)	1 (0 - 3)	1 (1 - 2)	1 (0 - 2)	1 (0 - 2)	1 (0 - 1)
LH basal (µg/l)	1.3 ± 0.1	1.3 ± 0.2	1.2 ± 0.2	1.3 ± 0.1	1.5 ± 0.2
FSH basal (µg/l)	3.7 ± 1.0	4.2 ± 0.8	4.1 ± 0.6	5.2 ± 0.5	6.7 ± 0.9
AUC for LH (µg/l * 6h)	12.7 ± 2.0	11.1 ± 1.3	12.3 ± 2.3	12.4 ± 1.4	13.8 ± 2.0
AUC for FSH (µg/l * 6h)	26.3 ± 7.0	28.5 ± 6.0	29.5 ± 3.9	35.7 ± 3.6	43.1 ± 4.6

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.

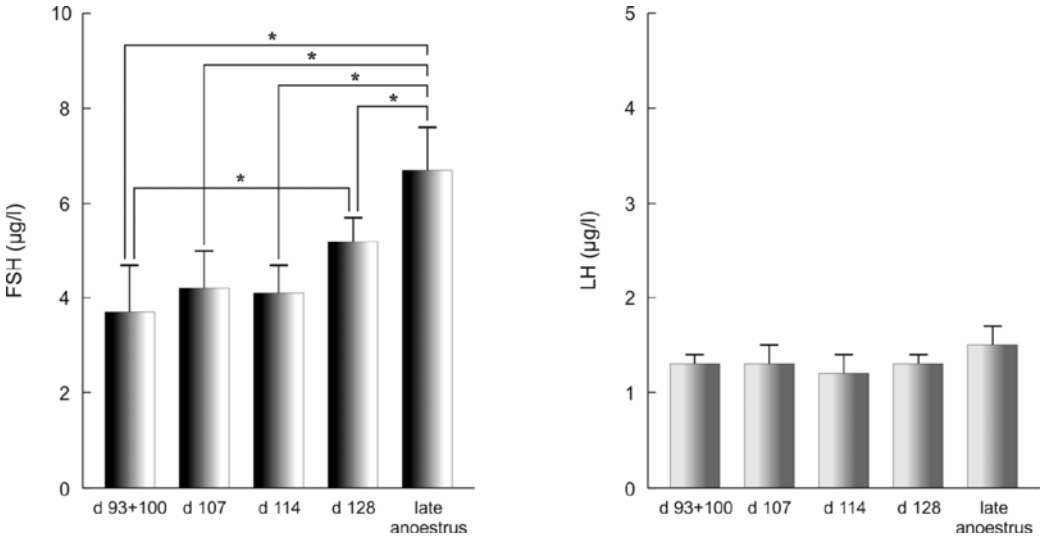

*Figure 4. Mean (± S.E.M.) basal plasma FSH (left panel) and LH (right panel) concentrations in eight Beagle bitches before (days (d) 93 + 100) and during serotonin antagonist treatment (days (d) 107, 114 and 128), and during late anoestrus. Treatment with the serotonin antagonist metergoline (0.1 mg 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).*

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 and 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 antagoninst-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 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. 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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CHAPTER 5

Effects of gonadotrophin releasing hormone administration on the pituitary-ovarian axis in anoestrous versus ovariectomized bitches

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Abstract

The aim of this study was to determine the effects of gonadotrophin releasing hormone (GnRH) administration on the plasma concentrations of reproductive hormones in intact and ovariectomized (OVX) bitches. Therefore, blood samples were collected at multiple times before and after the administration of 10 μg/kg GnRH (Fertagyl®) for the determination of the plasma concentrations of luteinizing hormone (LH), oestradiol, progesterone, and testosterone in 6 anoestrous and in 6 OVX bitches.

The mean plasma LH concentrations before and 60 min after GnRH administration were significantly lower in the anoestrous bitches than in the OVX bitches. In both groups GnRH administration resulted in a significant increase in the plasma LH concentration. The highest plasma LH concentrations were found at 10 min after GnRH administration and these values did not differ significantly between the 2 groups. Only in the anoestrous bitches a significant increase in plasma oestradiol concentrations was found after GnRH administration and these values were significantly higher than those in the OVX bitches. The plasma concentrations of progesterone and testosterone were low (close to or below the limit of quantitation) both before and after GnRH administration and the differences between anoestrous and OVX bitches were not significant.

It can be concluded that 1) basal plasma LH concentration is significantly higher in OVX bitches than in anoestrous bitches, 2) plasma LH concentration increases after GnRH administration in both anoestrous and OVX bitches, 3) GnRH administration causes a significant rise in plasma oestradiol concentration only if ovarian tissue is present, and 4) measurement of plasma progesterone and testosterone concentrations before and after GnRH administration does not aid in distinguishing between anoestrous and OVX bitches. The results of this study may provide a basis for the diagnosis of remnant ovarian tissue and verification of neuter status in the bitch.

Introduction

It can be difficult to verify the neuter status of dogs with an unknown reproductive status. In addition, it is occasionally difficult to differentiate ovariectomized (OVX) dogs having remnant ovarian tissue from completely OVX dogs. The presence of ovarian tissue can be confirmed by cytological evaluation of a vaginal smear and vaginoscopy during the follicular phase, measurement of the plasma progesterone concentration during the progression to the late follicular phase, ovulation and luteal phase, abdominal ultrasonography and exploratory laparotomy (Wallace 1991, Root and Spaulding 1994, Schaefers-Okkens 2005).

In the intact bitch, the stages of the oestrous cycle can be classified on the basis of ovarian function as the follicular phase, the phase of preovulatory luteinization and ovulation, the luteal phase, and non-seasonal anoestrus (Schaefers-Okkens 2005). During the follicular phase, demonstration of the presence of ovarian tissue is straightforward. The concentration of oestradiol, which is mainly synthesized and secreted by granulosa cells in developing ovarian follicles, rises during this phase and reaches peak levels around the pre-ovulatory luteinizing hormone (LH) surge (Olson et al. 1982, Schaefers-Okkens 2005, De Gier et al. 2006). Vaginoscopy and vaginal cytology can be used to recognize the influence of oestrogens (Schutte 1976, Schaefers-Okkens 2005). The use of cytological evaluation of a vaginal smear as a bioassay for oestrogen influence is even more reliable than a single plasma oestradiol determination (Schille and Olson 1989). The plasma concentration of progesterone, which is secreted by partially luteinizing granulosa cells before ovulation and by mature luteal cells after ovulation, is increased in the progression to the late follicular phase, ovulation, and during the luteal phase (Schaefers-Okkens 2005). Elevated plasma progesterone concentrations provide evidence for the presence of ovarian tissue (Okkens et al. 1981).

During anoestrus, which lasts from 2 to 10 months, the plasma progesterone concentration is in general below 3 nmol/l (Okkens et al. 1985a). Plasma oestradiol concentration is also usually low and does not begin to rise again until late anoestrus, although sporadic elevations have been reported (Olson et al. 1982). During anoestrus, LH is secreted in a pulsatile fashion but the basal plasma LH concentration is low (Olson et al. 1982, Kooistra et al. 1999). Plasma testosterone concentrations are also low during anoestrus in the bitch. Plasma testosterone concentration begins to rise near the end of anoestrus and reaches peak levels near the time of the preovulatory LH surge (Olson et al. 1984, Concannon and Castracane 1985).

There are no obvious clinical or behavioural differences between the anoestrous bitch and the OVX bitch. Vaginal cytology also has no diagnostic value in this differentiation and ultrasonographic visualisation of ovarian tissue in anoestrous bitches is sometimes difficult (England and Allen 1989, Root and Spaulding 1994). Production of the ovarian-derived hormones oestradiol and progesterone ceases with OVX, but their plasma concentrations in anoestrous and OVX bitches overlap (Jeffcoate 1993a, Frank et al. 2003). The loss of negative feedback of ovarian steroids causes a rapid increase in the concentration of circulating gonadotrophins (Chaffaux et al. 1981, Olson et al. 1992, Jeffcoate 1993a, Concannon 1993, Löfstedt and Vanleeuwen 2002, Reichler et al. 2004), while their secretion pattern remains

pulsatile (Concannon 1993). Baseline gonadotrophin levels may provide some useful information, but due to the pulsatile secretion pattern a considerable overlap in the plasma concentrations may be expected between intact and OVX animals and thus the diagnostic value of a single hormone measurement is questionable (Jeffcoate 1993a, Löfstedt and Vanleeuwen 2002).

To differentiate between bitches with and without ovarian tissue, a provocative test of the pituitary-ovarian axis using GnRH may be helpful. In the intact bitch, GnRH administration during anoestrus causes an increment in the circulating plasma concentrations of LH and oestradiol (Van Haaften et al. 1994). The GnRH induced plasma LH and oestradiol responses are higher in late anoestrus than in early anoestrus (Van Haaften et al. 1994). Information regarding the response to exogenous GnRH after ovariectomy is limited to a few studies. One study found a rise in LH after GnRH administration in OVX bitches (Chaffaux et al. 1981), while another found an unpredictable response (Jeffcoate 1993a). The discrepancy may be due to differences in sampling times and doses of GnRH that were used. Only one study investigated the effect of a GnRH challenge on plasma oestradiol concentration in OVX bitches (Jeffcoate 1993a). The low dose of GnRH that was used in the latter study produced no increase in plasma oestradiol concentration. No data have been reported on the response of the plasma testosterone concentration to GnRH stimulation in intact and OVX bitches.

The aim of this study was to determine the effects of gonadotrophin releasing hormone (GnRH) administration on the plasma concentrations of reproductive hormones in intact and OVX bitches. Therefore, blood samples were collected at multiple times before and after the administration of GnRH iv for the determination of the plasma concentrations of LH, oestradiol, progesterone and testosterone in anoestrous and in OVX bitches.

Materials and methods

Animals, treatment and collection of blood samples

Twelve bitches, consisting of ten Beagles, one Border Collie, and one mongrel, were used in this study. Six of them, 3 to 7 years of age and weighing 13.6 to 16.9 kg, were sexually intact. The other six, 5 to 12 years of age and weighing 13.2 to 27.0 kg, were ovariectomized. The Beagles were whelped and raised in the Department of Clinical Sciences of Companion Animals and were accustomed to the laboratory environment and handling such as collection of blood samples. They were housed in pairs in indoor-outdoor runs, fed a standard commercial dog food once daily, and given water ad libitum. The Border Collie and the mongrel dog were owned by veterinarians working at the Department of Clinical Sciences of Companion Animals and were also accustomed to the collection of blood samples.

The six intact Beagles were examined three times weekly for swelling of the vulva and a serosanguinous vaginal discharge, which were considered to signify the onset of pro-oestrus. Plasma concentrations of progesterone were determined three times weekly from the

start of pro-oestrus until plasma progesterone concentration exceeded 16 nmol/l, at which time ovulation was assumed to occur (Concannon et al. 1977, Wildt et al. 1979, Okkens et al. 1985b). During this study the 6 Beagle bitches were in anoestrus (123–203 days after ovulation), as confirmed by plasma progesterone concentrations below 3 nmol/L (Okkens et al. 1985a). Six dogs had been ovariectomized at the Department of Clinical Sciences of Companion Animals at Utrecht University at least one year before the start of the experiment and there had been no subsequent signs of oestrus as demonstrated by the absence of swelling of the vulva, vaginal discharge and attractiveness of male dogs.

Blood samples were collected for measurement of the plasma concentrations of LH (at -40, 0, 10, and 60 min), oestradiol (at -40, 0, 60, and 120 min), progesterone (at -40, 0, 60, and 120 min), and testosterone (at 0, 60, and 90 min) after the iv administration of 10 µg GnRH (Fertagyl®; Intervet, Boxmeer, The Netherlands) per kg body weight at 0 min. GnRH was administered via the cephalic vein. Blood samples were collected from the jugular vein, placed immediately in chilled heparin-coated tubes, and centrifuged at 4 ºC for 10 min at 1500 *g*. Plasma was stored at –25 ºC until analysis.

Hormone measurements

Plasma LH concentration was measured by use of a heterologous radioimmunoassay (RIA) as described previously (Nett et al. 1975) with the following modifications. A rabbit antiserum raised against ovine LH (CSU-204, kindly supplied by G.D. Niswender, Colorado State University, CO, USA), radioiodinated bLH-7981 as prepared for our bovine LH assay (Dieleman and Bevers 1987) and canine pituitary standard LER 1685-1 (a gift from Dr. L.E. Reichert, Albany Medical College, NY, USA) were used in this assay. The intra-assay and interassay coefficients of variation (CV) for values above 0.5 µg/l were 2.3% and 10.5%, respectively. The lower limit of quantitation was 0.3 µg/l.

Plasma oestradiol concentration was measured by use of a solid-phase RIA (Count-A-Count TKE; Diagnostic Products Corporation, Los Angeles, CA) according to the manufacturer's instructions with modifications as described previously (Dieleman and Schoenmakers 1979) and validated for the dog (Van Haaften et al. 1994). The intra-assay and interassay CV were 14% and 11.8%, respectively. The lower limit of quantitation was 7 pmol/l.

Plasma progesterone concentration was measured by a previously validated RIA (Dieleman and Schoenmakers 1979, Okkens et al 1985a). The intra-assay and interassay CV were 11% and 14%, respectively. The lower limit of quantitation was 0.13 nmol/l.

Plasma testosterone concentration was measured by RIA (Coat-A-Count® Total Testosterone, Diagnostic Product Corporation) according to the manufacturer's protocol with the following two modifications to increase the sensitivity. First, three extra standard points were included corresponding with 51, 255 and 1020 pmol/l producing standard curves with an average estimated dose of 1643 (CV 11.4%, n=10 RIAs), 230 (7.1%) and 37 (6.8%) pmol/tube at 20, 50 and 80 % relative binding, respectively. Second, aliquots of 1 ml plasma were extracted with 2.5 ml diethylether. The residue after evaporating under nitrogen was dissolved

in 125 µl A-serum (Diagnostic Products Corporation) of which 50 µL duplicates were used for RIA. The average extraction efficiency was 85% (CV 1.8%, n=10 RIAs) as determined on the basis of 3H-testosterone added to parallel series of plasma samples (n=6 per RIA). Values of the samples obtained in pmol/tube were calculated into pmol/l by correction for volume and extraction efficiency. The intra-assay and interassay CV were 5 and 6%, respectively. The lower limit of quantitation was 51 pmol/l.

Data analysis

Statistical analysis was performed using SPSS® for Windows, version 12.0.1 (SPSS Inc., Chicago, IL, USA). When the plasma concentration of oestradiol, progesterone, or testosterone was below the limit of quantitation, the respective lower limit value was assigned to the sample. For each bitch the mean basal plasma hormone concentration was calculated from the hormone concentrations before GnRH administration. The average plasma hormone concentration per group was calculated as the average of the mean values of the individual dogs. The plasma LH, oestradiol, progesterone, and testosterone data were made normally distributed by converting them to their natural (or neperian) logarithms (ln) and these were analysed using a multivariate repeated measures model, with 'time' as the within-subject variable and 'group' as the between-subject variable. Subsequently, a contrast study was performed to evaluate time effects within each group in comparison with the mean basal level. $P<0.05$ was considered significant. Results are presented as mean ± SEM and range.

Ethics of experimentation

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

Results

No adverse effects of GnRH administration on health or behaviour were observed in any of the dogs. Mean plasma LH concentrations before and 60 min after GnRH administration were significantly lower ($P<0.001$ and $P<0.01$, respectively) in the intact bitches than in the OVX bitches. Moreover, there was no overlap between the two groups when these values were compared (Table 1). In both groups GnRH administration resulted in a significant increase in the plasma LH concentration (Figure 1). In the protocol used, the highest plasma LH concentrations were found at 10 min after GnRH and these values did not differ significantly between the two groups. The mean plasma LH concentration at 10 min after GnRH was significantly higher than those before ($P<0.001$) and 60 min after GnRH ($P<0.001$) in the anoestrous dogs. In the OVX dogs, the mean plasma LH concentration at 10 min after GnRH was significantly higher ($P<0.05$) than the mean basal plasma LH concentration.

	Anoestrous bitches		Ovariectomized bitches	
	Mean ± SEM	Range	Mean ± SEM	Range
Basal LH (µg/l)	0.64 ± 0.04*,a	0.48 – 0.93	20.2 ± 3.6*,a	6.2 – 41.0
LH 10 min after GnRH(µg/l)	58.0 ± 11.1b	25.4 – 101	63.9 ± 15.3b	30.3 – 113
LH 60 min after GnRH (µg/l)	4.9 ± 0.5*,a	2.8 – 6.2	28.4 ± 8.7*,ab	10.9 -68.9
Basal oestradiol (pmol/l)	NCa	< 7 – 14.1	NC	< 7
Oestradiol 60 min after GnRH (pmol/l)	23.0 ± 4.0*,b	14.1 – 35.7	NC *	< 7
Oestradiol 120 min after GnRH (pmol/l)	24.4 ± 3.5*,b	12.3 – 32.4	NC *	< 7
Basal progesterone (nmol/l)	NC	<0.13 – 2.2	NC	<0.13 – 0.95
Progesterone 60 min after GnRH (nmol/l)	NC	<0.13 – 1.9	NC	< 0.13
Progesterone 120 min after GnRH (nmol/l)	NC	<0.13 – 1.6	NC	< 0.13
Basal testosterone (pmol/l)	NC	< 51 – 75.6	NC	< 51.0 – 70.0
Testosterone 60 min after GnRH (pmol/l)	74.7 ± 7.8	56.2 – 102.5	NC	< 51.0 – 55.7
Testosterone 90 min after GnRH (pmol/l)	80.4 ± 7.8	56.7 – 101.8	NC	< 51.0

*Table 1. Mean ± SEM and range of plasma concentrations of LH, oestradiol, progesterone and testosterone in 6 anoestrous and 6 ovariectomized (OVX) bitches before and after intravenous administration of 10 µg per kg body weight of GnRH (Fertagyl®). * indicates a significant difference in mean hormone concentration between the anoestrous and the OVX bitches.*
a, b, ab: Different letters within a column per hormone indicate significant differences. NC: mean not calculated because not all plasma hormone concentrations were above the detection limit. When a plasma hormone concentration was below the limit of quantitation, in order to perform statistical analysis, the respective lower limit value was assigned to the sample.

Basal plasma oestradiol concentration in all dogs except one anoestrous bitch was below the detection limit of the assay. The difference in the basal plasma oestradiol concentration between the two groups was not significant. After GnRH stimulation, plasma oestradiol concentration was below the detection limit in all OVX dogs but above this level in all anoestrous bitches. In the protocol used, the maximum plasma oestradiol concentration after GnRH administration was observed at 60 min in three of the anoestrous bitches and at 120 min

in the other three. At 60 and 120 min after GnRH stimulation, plasma oestradiol concentrations were significantly higher in the anoestrous bitches than in the OVX bitches ($P<0.05$ in both cases) and the increase above baseline in the anoestrous bitches was also significant ($P<0.02$ and $P<0.01$, respectively).

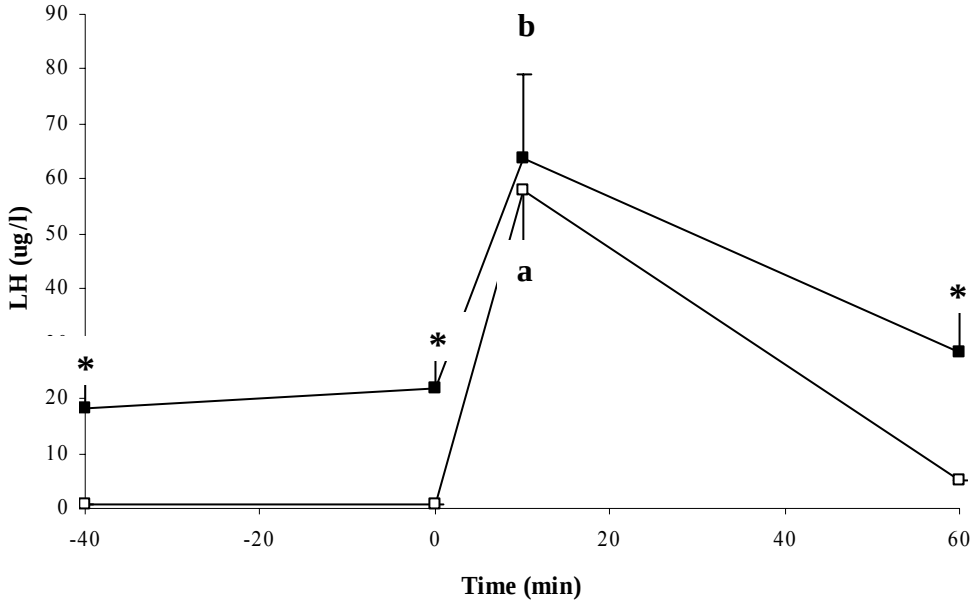

Figure 1: Mean (± SEM) plasma LH concentration after intravenous administration of GnRH in a dose of 10 μg per kg body weight at t = 0 min in six anoestrous (□) and six ovariectomized (OVX) (■) bitches. (*) indicates a significant difference in mean plasma LH concentration between the anoestrous and the OVX bitches. (a) indicates a significantly higher mean plasma LH concentration at t = 10 min in the anoestrous dogs compared with the values before and 60 min after GnRH administration. (b) indicates a significantly higher mean plasma LH concentration at t = 10 min in the OVX bitches compared with the values before GnRH administration.

Basal plasma progesterone concentration was above the detection limit of the assay in all but three samples from the anoestrous bitches, whereas it was below the detection limit in all but one sample from the OVX bitches. In both groups, no change in plasma progesterone concentration after GnRH administration was significant. Differences in mean plasma progesterone concentration between the two groups before and after GnRH administration were not significant (Table 1).

Basal plasma testosterone concentration was above the detection limit of the assay in three anoestrous bitches and one OVX bitch. After GnRH administration, plasma testosterone concentration was detectable in all anoestrous bitches but only one OVX bitch. Plasma testosterone concentration did not change significantly after GnRH administration and the difference in mean plasma testosterone concentration between the anoestrous dogs and the OVX dogs was not significant.

Discussion

The plasma concentrations of reproductive hormones were measured before and after GnRH administration in OVX bitches and bitches in anoestrus, to evaluate the applicability of such measurements for ascertaining the presence or absence of ovarian tissue. The anoestrous period was chosen because during this phase neither vaginoscopy nor vaginal cytology nor the basal plasma progesterone concentration are of value in differentiating between intact and OVX bitches.

The different sampling times for LH and oestradiol were based upon information from previous experiments (Van Haaften et al. 1994, Meij et al. 1996). No data have been reported on the response of the plasma testosterone concentration to GnRH stimulation in the bitch. In intact male dogs administration of 10 μg/kg GnRH results in a maximum testosterone concentration at 60 min after injection (Knol et al. 1993), and therefore we assumed that a GnRH-induced increase in plasma testosterone concentration will be found in blood samples at 60 and 90 min after GnRH administration. Because circulating testosterone concentrations in bitches are described to be low or undetectable (Nickel 1996), and no studies could be found that report diurnal variation of plasma testosterone concentrations in bitches, only one pre-GnRH trial sample for testosterone was collected.

The results of this study demonstrate that mean basal plasma LH concentrations were significantly higher in OVX bitches than in anoestrous bitches. Moreover, there was no overlap in basal plasma LH concentrations between the two groups. The higher basal plasma LH concentrations in the OVX dogs are in agreement with the results of other studies (Chaffaux et al. 1981, Olson et al. 1992, Jeffcoate 1993a, Concannon 1993, Löfstedt and Vanleeuwen 2002, Reichler et al. 2004) and can be explained by the loss of negative feedback of the ovarian hormones. However, taking into account the pulsatile nature of LH release (Concannon 1993, Kooistra et al. 1999), overlapping of plasma LH concentrations between larger groups of intact and OVX bitches may be expected, and therefore one single LH result should be interpreted with caution. Indeed, a single measurement of LH has been shown not to be a reliable means of determining whether or not a bitch was intact (Löfsteds and Vanleeuwen 2002). In addition, circulating LH concentrations of < 1 μg/l 10 to 16 weeks after ovariectomy in some bitches have been found (Reichler et al. 2004). Consequently, the presence or absence of ovarian tissue should not be based solely upon the results of a single basal plasma LH measurement.

In both anoestrous and OVX bitches, GnRH administration provoked a significant increase in the plasma LH concentration. Due to the complete loss of negative feedback of ovarian hormones a maximal stimulation of pituitary gonadotrophin release may have been expected. However, in postmenopausal women the secretion pattern of LH is still pulsatile, and GnRH administration provokes an increase in the plasma LH concentration (Rossmanith et al. 1991), indicating that stimulation of pituitary LH release is still occurring in ovarian hormone-deprived women. Moreover, pulsatile LH release has also been reported in OVX bitches (Concannon 1993).

In agreement with previous studies (Van Haaften et al. 1994, Meij et al. 1996), in the protocol used plasma LH concentration reached its maximum level at 10 min after GnRH administration in both groups. It also reached its maximum in OVX bitches at 10 min after intramuscular administration of GnRH (Chaffaux et al. 1981). The overlap between anoestrous OVX bitches in plasma LH values 10 min after GnRH administration makes this measurement an unreliable method for distinguishing between the two groups.

Plasma oestradiol concentration was below the detection limit of the assay in all OVX dogs and 5 of the 6 anoestrous dogs before GnRH administration and in the OVX dogs after GnRH administration. There was a significant increase in plasma oestradiol concentration following GnRH stimulation in all the intact bitches but none of the OVX bitches. As found in a previous study (Van Haaften et al. 1994), the dose of GnRH (Fertagyl®) of 10 µg/kg was sufficient to induce an increase in plasma oestradiol concentration in the intact bitches. In other studies (Jeffcoate 1992, Jeffcoate 1993b), a dose of 0.16 µg GnRH (Receptal®) per dog, which is, although Receptal® is more efficient than Fertagyl® (Chenault 1990), a much lower dose than used in this protocol, failed to induce a clear increase in plasma oestradiol concentration in intact dogs. LH and oestradiol responses after GnRH administration in anoestrous bitches are significantly dose dependent; a dose of 0.01 µg/kg produced little or no response (Van Haaften et al. 1994). The results of the present study suggest that the plasma oestradiol response to an adequate dose of GnRH (Fertagyl®; 10 µg/kg) may be a useful and reliable test with which to distinguish between bitches with and without ovarian tissue. The time at which the plasma oestradiol concentration is maximal after GnRH administration could not been determined in this study. Also in another study, it has been observed that GnRH doses of 0.1 to 100 µg/kg raise plasma oestradiol concentration for 160 min (Van Haaften et al. 1994).

In women, oestradiol is also produced in a number of extra-ovarian sites. These sites include the mesengial cells of adipose tissue including that of the breast, osteoblasts and chondrocytes of bone, vascular endothelium and aortic smooth muscle cells and numerous sites in the brain. However, at these sites oestradiol acts locally as a paracrine or intracrine factor, rather than that it is secreted into the circulation (Simpson 2003). Most probably this also accounts for the dog, and therefore a significant rise in plasma oestradiol concentration after GnRH administration can be expected only if ovarian tissue is present. In addition, Frank et al. (2003) performed an ACTH stimulation test and no increase in circulating oestradiol could be evoked in either OVX or intact bitches, indicating that the adrenal glands do not contribute to the circulating oestradiol concentration.

The difference in plasma progesterone concentration between anoestrous and OVX bitches was not significant and the ranges overlapped both before and after GnRH administration. This is in agreement with the findings of others (Jeffcoate 1993a, Frank et al. 2003). Although the main source of progesterone in the intact bitch is the ovary, progesterone can also be secreted by the adrenal cortex (Frank et al. 2004), which is the most logical explanation for the detectable plasma progesterone level in one of the OVX dogs (Concannon 1986). This could be related to stress, since progesterone increases have been reported to occur as a reaction to fear provocations in dogs (Hydbring-Sandberg et al. 2004). On the other hand, in the male dog LH and testosterone values are not influenced by blood sampling (Knol et al. 1992), implying that under the experimental conditions used the validity of results will not be affected by blood collection. Since it is not completely clear to what extent stress might influence the results, the potential effect of stress has to be considered when examining the function of the hypothalamic-pituitary-ovarian axis.

The differences in plasma testosterone concentrations before and after GnRH administration between intact and OVX bitches were not significant. For baseline testosterone concentrations this is in agreement with the results in another study in the dog (Frank et al. 2003). Although mean plasma testosterone concentration in the anoestrous bitches appeared to reflect the increase in mean plasma oestradiol concentration, the change was not significant ($P=0.15$). As oestradiol is formed from testosterone by the action of the aromatase system (Edqvist and Forsberg 1997), this non-significant increase in the plasma testosterone concentration most probably reflects oestradiol synthesis in the follicles. Different findings have been reported in women. The basal plasma testosterone concentration in oophorectomized women is significantly lower than that in intact women. Furthermore, stimulation with human chorionic gonadotrophin (hCG) raises plasma testosterone concentration in intact women but not in oophorectomized women (Burger 2002, Piltonen et al. 2002).

It is uncertain whether the plasma LH and oestradiol responses observed in intact bitches can be expected to be completely the same in bitches with remnant ovarian tissue. In a previous study (Okkens et al. 1981) 12 of 47 bitches with remnant ovarian tissue were found to have macroscopic cystic structures in the residual ovarian tissue. The effect of these cystic remnants on the results of the GnRH stimulation test is unknown and the successful use of this test in the detection of remnant ovarian tissue has not yet been reported in animals, as it has in humans (Scott et al. 1995).

It can be concluded that 1) basal plasma LH concentration is significantly higher in OVX bitches than in anoestrous bitches, 2) plasma LH concentration increases after GnRH administration in both anoestrous and OVX bitches, 3) GnRH administration causes a significant rise in plasma oestradiol concentration only if ovarian tissue is present, and 4) measurement of plasma progesterone and testosterone concentrations before and after GnRH administration does not aid in distinguishing between anoestrous and OVX bitches. The results of this study provide a basis for the diagnosis of remnant ovarian tissue and verification of neuter status in the bitch. However, further studies remain to be applied in bitches suspected of remnant ovarian tissue.

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CHAPTER 6

Basal and GnRH-induced secretion of FSH and LH in anoestrous versus ovariectomized bitches

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Abstract

The basal and gonadotrophin releasing hormone (GnRH)-induced plasma concentrations of follicle stimulating hormone (FSH) and luteinizing hormone (LH) were studied in four anoestrous and four ovariectomized (OVX) bitches. Blood samples were obtained via jugular venipuncture 40 min before and 0, 10, 20, 30, 60, 90, and 120 min after the i.v. administration of synthetic GnRH in a dose of 10 μg/kg body weight.

The basal plasma FSH and LH concentrations were significantly higher in the OVX bitches than in the anoestrous bitches. In the anoestrous bitches the plasma FSH concentration was significantly higher than the pretreatment level at 10, 20, and 30 min, whereas the plasma LH concentration was significantly elevated at 10 and 20 min. The maximal GnRH-induced plasma FSH concentration in the anoestrous bitches did not surpass the lowest plasma FSH concentration in the OVX bitches, whereas the GnRH-induced plasma LH concentrations in the anoestrous bitches overlapped with the basal plasma LH concentrations in the OVX bitches. In the OVX bitches GnRH administration did not induce a significant change in the plasma FSH concentration, whereas the plasma LH concentration increased significantly at 10 and 20 min.

In conclusion, the results of the present study indicate that in anoestrous bitches GnRH challenge results in increased plasma levels of both FSH and LH, whereas in the OVX bitches, in which the basal plasma FSH and LH concentrations are higher, only a rise in the plasma LH concentration is present after GnRH stimulation. The results also suggest that a test to measure plasma concentration of FSH in single samples appears to have potential in verification of neuter status in bitches.

Introduction

The reproductive cycle of the bitch is under endocrine control exerted by interactions among the hypothalamus, anterior pituitary, and ovary – the so-called hypothalamic-pituitary-ovarian axis. The neuroendocrinological mechanisms responsible for the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) by the pituitary are very complex. It is generally accepted that these gonadotrophins are secreted in a pulsatile fashion (Kooistra et al. 1999) in response to the similarly pulsatile release of gonadotrophin releasing hormone (GnRH). The gonadotrophins stimulate ovarian hormone secretion, which in turn regulates the gonadotrophins via a negative feedback mechanism (long-loop negative feedback). The extent of GnRH release and subsequently gonadotrophin release seems to be dependent upon sensitivity of the hypothalamus to oestradiol. During progression of anoestrus, there is gradual reestablishment of positive feedback of oestradiol on the hypothalamus (Klein et al. 2003), and GnRH secretion (Tani et al. 1996) and the responsiveness of the pituitary to GnRH increase (Van Haaften et al. 1994). In addition, a large amount of LH is released prior to ovulation, due to this positive feedback elicited by the rapidly rising plasma oestradiol level (De Gier et al. 2006). Apart from the feedback of the gonadal hormones, two additional feedback loops have been identified. These are the gonadotrophin-dependent inhibition of GnRH production (short-loop negative feedback) and the GnRH-dependent inhibition of GnRH production, known as ultrashort-loop negative feedback (Speroff and Fritz, 2005).

The measurement of plasma FSH and LH concentrations before and after administration of a GnRH analogue during different reproductive conditions, such as during anoestrus and after ovariectomy, permits assessment of the capacity of the pituitary to secrete gonadotrophins. Knowledge about the characteristics of gonadotrophin secretion after intravenous administration of a GnRH analogue in the anoestrous bitch is mainly limited to LH (Buijtels et al. 2006), the response of which is dosage-dependent (Van Haaften et al. 1994; Chakraborty and Fletcher 1977). Furthermore, an increasing dosage-dependent response of LH to stimulation with GnRH was found in the bitch during the progression of anoestrus (Van Haaften et al. 1994), but another study revealed no apparent correlation between stage of anoestrus and LH response (Jeffcoate 1992). However, the used GnRH dosage in the latter study was minimal. Only one study was concerned with the concomitant secretion of the gonadotrophins in the bitch after GnRH administration and showed that in cyclic bitches FSH and LH levels increased within 15 min after GnRH stimulation (Reimers et al. 1978).

Ovariectomy (OVX) influences the long-loop feedback. The loss of inhibitory influence of ovarian steroids and peptides on the hypothalamic-pituitary axis causes a rapid increase in the plasma concentration of both LH (Chaffaux et al. 1981; Olson et al. 1992; Concannon 1993; Jeffcoate 1993; Löfstedt and Vanleeuwen 2002; Reichler et al. 2004; Buijtels et al. 2006) and FSH (Olson et al. 1992; Concannon 1993; Reichler et al. 2004), while there are indications that the secretion pattern of both remains pulsatile (Concannon 1993). Little is known about the characteristics of gonadotrophin secretory response to exogenous GnRH in the OVX bitch. Both a rise (Chaffaux et al. 1981; Buijtels et al. 2006) and an unpredictable

response (Jeffcoate, 1993) in the plasma LH concentration have been reported. So far, in OVX bitches there is neither information on GnRH-induced FSH secretion nor on the concomitant secretion of FSH and LH after GnRH administration.

The present study was designed to compare the basal and GnRH-induced plasma concentrations of FSH and LH in OVX bitches with those in anoestrous bitches.

Materials and methods

Animals, treatment and collection of blood samples

Four intact Beagle bitches (weighing 11.3 to 14.1 kg and aged 2 to 5 years), in which the previous oestrous cycle had been monitored and characterized, and four OVX Beagle bitches (weighing 12.8 to 16.0 kg and aged 9 to 10 years) were included in this study. All dogs were whelped and raised in the Department of Clinical Sciences of Companion Animals and were accustomed to the laboratory environment and procedures, such as the collection of blood samples. They were housed in pairs in indoor-outdoor runs, fed a standard commercial dog food once daily, and provided with water ad libitum. The four intact Beagles were examined three times per week for the presence of swelling of the vulva and a serosanguinous vaginal discharge, which were considered to signify the onset of pro-oestrus. Plasma concentrations of progesterone were determined three times per week from the start of pro-oestrus until the day on which the plasma progesterone concentration exceeded 16 nmol/l, which is when ovulation was assumed to occur (Concannon et al. 1977; Wildt et al. 1979; Okkens et al. 1985a). During this study all bitches were in anoestrus (104 to 118 days after ovulation), as confirmed by plasma progesterone concentrations below 3 nmol/l (Okkens et al. 1985b). The four remaining dogs had been ovariectomized at the Department of Clinical Sciences of Companion Animals at Utrecht University at least one year before the start of the experiment and had exhibited no signs of recurrent oestrus.

At 09.00 h (time is 0 min) 10 µg/kg synthetic GnRH (Fertagyl® batch 20105B; Intervet International BV, Boxmeer, The Netherlands) was administered via the cephalic vein in all Beagle bitches. Blood samples were taken 40 min before and 0, 10, 20, 30, 60, 90, and 120 min after the administration of GnRH.

Blood samples were collected from the jugular vein, immediately placed in chilled EDTA-coated tubes, and centrifuged at 4 ºC for 10 min at 1500 *g*. Plasma was stored at –25 ºC until analysis. All samples from each dog were measured in the same FSH or LH assay.

Hormone measurements

Plasma FSH concentration was measured by the canine immunoradiometric assay (IRMA) of Biocode SA (Liège, Belgium) coded AHCOO4. This IRMA uses a first and a second monoclonal mouse anti-FSH antibody. These antibodies were raised against rat-FSH

and have cross-species reactivity (rat, dog, horse) (personal communication, Biocode SA). Briefly, the procedure used highly purified canine FSH diluted in horse serum provided by the manufacturer to produce a standard curve ranging from 1.5 to 240 µg/l. An excess of a first monoclonal mouse anti-rat FSH antibody bound to polystyrene tubes captured the canine FSH present in 100 µl standards or samples. Thereafter, a second [^{125}I]-labeled monoclonal mouse anti-rat FSH antibody (50 µl), directed to another determinant on the FSH molecule, was added to the tubes, which were incubated at room temperature for 90 min. According to the manufacturer, the cFSH used as standard was derived from extracts of pituitary glands of dogs collected over several years in Belgium. Pituitary extracts were purified by ion exchange and gel filtration. The monoclonal antibodies were obtained by injecting highly purified rat FSH in mice after which splenocytes of the mice were fused with Sp2O myeloma cells. Finally, diluted positive clones were tested for binding to iodinated cFSH to obtain highly stabilized monoclonal cells. The reported purity of the antibodies (IgG class) is >95%. More detailed information on the reagents is not available.

To validate the FSH IRMA for parallelism, accuracy, and precision, plasma from an OVX bitch 10 min after the administration of 10 µg/kg GnRH was diluted with plasma from an anoestrous bitch before GnRH stimulation (dilutions 1:2 to 1:32). Each level of dilution was measured six times. As shown in Table 1, the precision and accuracy are within commonly accepted ranges. In addition, the intra-assay coefficient of variation (CV) was measured in 12 replicates of each of four samples in a single assay. These four quality controls samples were included in duplicate in all assays (n=12) to determine the interassay CV. The intra-assay and the interassay CV for values above 1.60 µg/l were 3.5% and 15.1%, respectively. Cross-reactivity of canine LH (LER 1685-1; Dr. L.E. Reichert, Albany Medical College, NY, USA) with the first mouse anti-rat FSH monoclonal antibodies bound to the tube was determined by adding the preparation to PBS buffer containing 0.29 % (w/v) human immunoglobulin (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) and 0.36 % (w/v) bovine serum albumin (BSA; Povite, Organon, Oss, The Netherlands) at pH of 7.4. Maximal binding in the FSH IRMA was 36.8 ± 2.2%. At 50% of the maximal binding the cross-reactivity of canine LH (LER 1685-1) in this assay was 1.8%. The limit of quantitation of canine FSH was arbitrarily defined as the value of the lowest standard, 1.50 µg/l, and FSH values below this were arbitrarily assigned the value of one-third of this limit, i.e., 0.5 µg/l.

Plasma LH concentration was measured by a heterologous RIA as described previously (Nett et al. 1975; Van Haaften et al. 1994). Briefly, a rabbit antiserum raised against ovine LH (CSU-204, kindly supplied by GD Niswender, Colorado State University, CO, USA), radioiodinated bLH-7981 as prepared for our bovine LH assay (Dieleman and Bevers, 1987), and canine pituitary standard LER 1685-1 (a gift from Dr. LE Reichert) were used in this assay. The intra-assay and interassay CV for values above 0.5 µg/L were 2.3% and 10.5%, respectively, and the limit of quantitation was 0.3 µg/l.

Plasma progesterone concentration was measured by a previously validated ^{125}I RIA (Okkens et al. 2001). The intra-assay and interassay CV were 6% and 10.8%, respectively, and the limit of quantitation was 0.13 nmol/l.

Dilution	Added dose µg/l *[1]	Estimated dose µg/l *[2]	SD µg/l *[3]	CV % *[4]	Recovery % *[5]
Undiluted	47.76	47.76	0.99	2.07	100
1:2	23.88	22.25	0.95	4.26	93.2
1:4	11.94	10.59	0.41	3.88	88.7
1:8	5.97	5.24	0.31	5.90	87.8
1:16	2.98	2.65	0.13	4.95	88.9
1:32	1.49	1.32	0.13	9.88	88.6

*Table 1. Accuracy and precision in the estimation of follicle stimulating hormone (FSH) by immunoradiometric assay. Each dilution was measured six times. All samples were measured in the same assay. [*1] Canine FSH was added by diluting plasma collected from an ovariectomized bitch 10 minutes after intravenous administration of a GnRH analogue (10 µg/kg) with plasma from an anoestrous bitch. [*2] Values were corrected for the FSH responses in the IRMA of 1.57 µg/l of the plasma of the anoestrous bitch. [*3] SD: standard deviation. [*4]CV: coefficient of variation, this value indicates the precision of the assay. [*5] Accuracy (recovery) is defined as estimated dose relative to added dose (E/A * 100%).*

Data analysis

A Kolmogorov Smirnov test showed that, even after transformation to natural logarithms, the plasma profile data were not normally distributed. Hence the untransformed plasma profile data for FSH and LH were compared per group by the nonparametric Friedman test, followed by a multiple comparisons test using Dunnett's procedure. This test evaluated the changes in the GnRH stimulation test compared with the mean of the two pretreatment values. Differences in the pretreatment values, the peak concentration, the relative peak compared with the pretreatment value (i.e. peak value/basal value) and the area under the curve (AUC) above the pretreatment concentration between the two groups were assessed for statistical significance by the nonparametric Wilcoxon-Mann-Whitney test. The AUC of the hormone concentrations after the GnRH challenge test was calculated by the trapezoidal method after subtraction of the mean pretreatment level per dog. Results are presented as median and range or mean ± SEM. The level of significance was $P < 0.05$.

Ethics of experimentation

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

Results

The basal (pre-GnRH) plasma concentrations of FSH and LH were significantly higher in the OVX dogs than in the anoestrous dogs (Table 2). In the anoestrous dogs, plasma concentrations of both FSH and LH increased after GnRH administration. The plasma FSH concentration was significantly elevated at 10, 20, and 30 min, whereas the plasma LH concentration was significantly higher than the basal level at 10 and 20 min (Figure 1). In the anoestrous dogs, GnRH-induced plasma profiles of both FSH and LH were characterized by an abrupt and rapid rise followed by a slow decline; slower for FSH than for LH. The GnRH-induced increase in plasma LH concentration above basal levels was much higher than that of FSH. In the OVX bitches, plasma FSH concentration remained unchanged after GnRH administration. In contrast, plasma LH concentration was significantly increased at 10 and 20 min (Figure 2). The median peak concentration of LH was significantly higher in the OVX dogs than in the anoestrous dogs, but the relative increase in LH was significantly greater in the anoestrous dogs than in the OVX dogs. The median AUC of the LH response did not differ between the anoestrous and the OVX dogs (Table 2). The maximal GnRH-induced plasma FSH concentration in the anoestrous bitches did not exceed the lowest plasma FSH concentration in the OVX bitches, whereas the GnRH-induced plasma LH concentrations in the anoestrous bitches overlapped with the basal plasma LH concentrations in the OVX bitches (Table 2).

Discussion

Pituitary gonadotrophin release plays a central role in the development and function of the canine reproductive system. The measurement of plasma LH and FSH concentrations before and after the administration of a GnRH analogue permits assessment of the capacity of the pituitary to secrete gonadotrophins under different reproductive conditions. In agreement with the findings of others, GnRH administration elicited a distinct release of LH (Chakraborty and Fletcher 1977; Reimers et al. 1978; Jeffcoate 1992; Van Haaften et al. 1994; Buijtels et al. 2006) and FSH (Reimers et al. 1978). The course of the GnRH-induced surges in FSH and LH in the anoestrous bitches was comparable to what has been reported previously for spontaneous FSH and LH pulses in anoestrous bitches (Kooistra et al. 1999). The GnRH-induced plasma LH increments above the pre-GnRH levels were much greater than the corresponding FSH

	Anoestrous bitches (n=4)	Ovariectomized bitches (n=4)
FSH pre-GnRH concentration (µg/l)	5.6* 2.8 – 13.9	81.7* 40.7 - 107.5
FSH peak amplitude (µg/l)	25.4 18.4 - 27.0	_
FSH peak amplitude relative to basal	3.9 2.0 - 9.5	_
FSH time of peak amplitude (min)	20 10 - 20	_
FSH AUC above baseline (µg*min/l)	1475 333 - 1952	_
LH pre-GnRH concentration (µg/l)	1.02* 0.81 - 5.4	31.4* 6.4 - 64.3
LH peak amplitude (µg/l)	34.9* 26.5 - 38.4	60.7* 48.3 - 359
LH peak amplitude relative to basal	30.7* 9.7 - 41.4	3.8* 1.3 - 6.4
LH time of peak amplitude (min)	10 10 - 10	10 10 - 10
LH AUC above baseline (µg*min/l)	1038 862 - 1237	2032 688 - 5546

*Table 2: Characteristics of the GnRH-induced surges of follicle stimulating hormone (FSH) and luteinizing hormone (LH) in four anoestrous and four ovariectomized (OVX) bitches (median and range). * indicates significant difference between anoestrous and OVX dogs.*

increments. Subsequently, plasma FSH concentration declined more slowly than plasma LH concentration in the anoestrous bitches. As in other mammals (Schwartz, 1995), this may be explained by a different pattern of glycosylation of FSH and LH, resulting in a longer half-life for FSH than LH.

The loss of ovarian feedback after ovariectomy resulted in increased plasma concentrations of FSH and LH in all OVX bitches, as has been reported earlier (Chaffaux et al. 1981; Olson et al. 1992; Concannon 1993; Jeffcoate 1993; Löfstedt and Vanleeuwen 2002; Reichler et al. 2004). In the OVX bitches, GnRH only elicited a significant response in the plasma LH concentration. Since there was no significant rise in plasma FSH, it can be concluded that the pituitary response to GnRH in OVX bitches differs from that in anoestrous bitches. It may be hypothesized that this is the consequence of different intracellular mechanisms for storage and release for FSH and LH. This view is supported by in vitro studies

with cells of other mammalian species, which have shown that although FSH and LH are produced in the same cell type, they are stored in different secretory granules (Moyle and Campbell 1995), newly synthesized FSH is secreted at a greater rate than LH (Chowdhury and Steinberger 1975), and that the magnitude of the FSH response to secretagogues is smaller than that of LH (Chowdhury and Steinberger 1975; Muyan et al. 1994).

Occasionally, it can be difficult to verify the neuter status of dogs with an unknown reproductive status (Buijtels et al. 2006). Although all pre-GnRH plasma LH concentrations were higher in OVX dogs than in sexually intact dogs, measurement of circulating LH concentration in a single sample has limited potential for distinguishing between OVX and sexually intact bitches. Taking into account the strong pulsatile nature of LH release (Kooistra et al. 1999; Concannon 1993), an overlap in the plasma LH concentration between larger groups of intact and OVX bitches may be expected. Indeed, in the current study GnRH-induced LH increments in the intact bitches overlapped pre-GnRH plasma LH concentrations in the OVX bitches. A single high LH value measured by a commercial test kit was not reliable in indicating whether a bitch was intact (Löfstedt and Vanleeuwen, 2002).

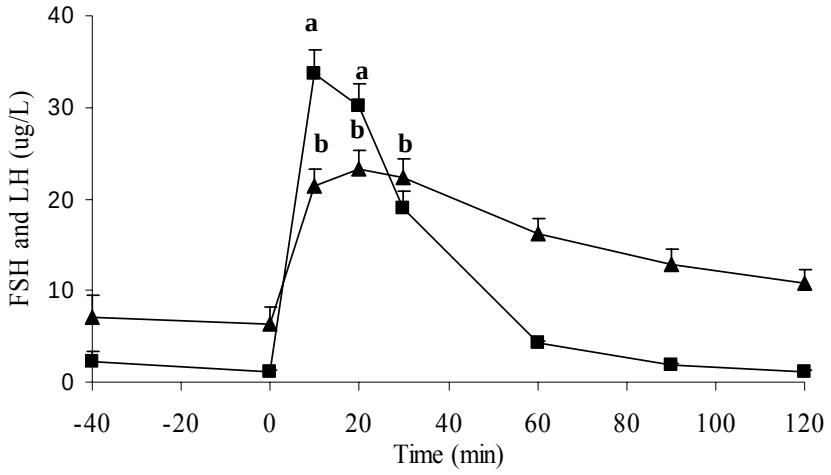

Figure 1. Mean (± SEM) plasma concentrations of follicle stimulating hormone (FSH) and luteinizing hormone (LH) in four intact anoestrous bitches after administration of synthetic GnRH (gonadotrophin releasing hormone) (10 μg/kg) via the cephalic vein at t = 0 min. ▲= FSH, ■ = LH. The letters (a, b) indicate significant difference compared with the mean of the pre-GnRH values for the plasma concentrations of LH and FSH, respectively. The bars indicating the SEM are shown only when they exceed the size of the symbols.

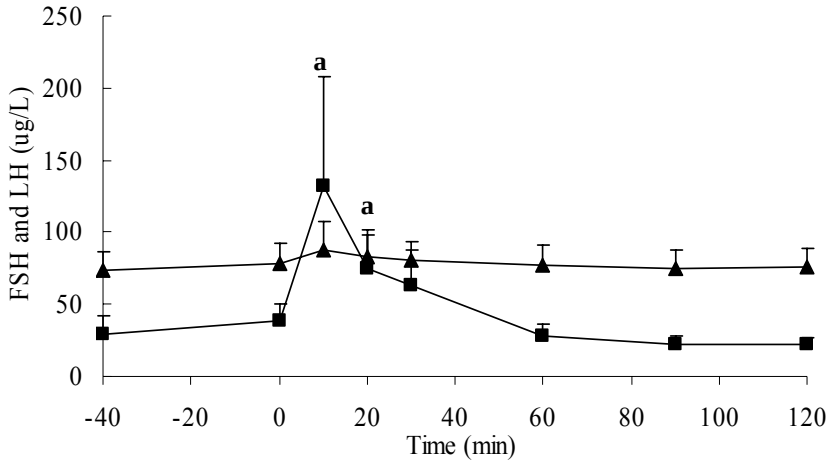

Figure 2. Mean (± SEM) plasma concentrations of follicle stimulating hormone (FSH) and luteinizing hormone (LH) in four ovariectomized bitches after administration of synthetic GnRH (gonadotrophin releasing hormone) (10 µg/kg) via the cephalic vein at t = 0 min. ▲= FSH, ■ = LH. Letter (a) indicates significant difference compared with the mean of the pre-GnRH values for the plasma LH concentration. The bars indicating the SEM are shown only when they exceed the size of the symbols.

In contrast, the results reported here indicate that measurement of plasma FSH concentration in a single sample may well prove reliable for determination of neuter status. Even after GnRH administration the maximum plasma FSH level in the intact bitches did not exceed the lowest plasma FSH level during the entire GnRH-stimulation test in the OVX bitches. Other investigators have also suggested that the pituitary capacity to secrete FSH in the absence of ovarian negative feedback is far greater than that ever observed in the intact bitch (Olson et al. 1992; Concannon 1993). Such a difference could be expected if there is a specific inhibition of FSH as opposed to LH secretion in intact bitches, i.e., due to ovarian derived inhibin (Shupnik 1996). Furthermore, plasma FSH levels in women usually increase more than do LH levels when the ovaries are functionally inactive or surgically removed (Bulun and Adashi 2003, Genuth 2004). This indicates that differences in circulating FSH concentration between intact and OVX bitches could be greater than within-group variability. Another study with a greater number of animals is needed to evaluate the measurement of plasma FSH concentration as a potential diagnostic marker of neuter status.

In conclusion, the results of the present study indicate that in anoestrous bitches GnRH challenge results in increased plasma levels of both FSH and LH, whereas in the OVX bitches, in which the basal plasma FSH and LH concentrations are higher, only a rise in the plasma LH concentration is present after GnRH stimulation. The results also suggest that a test to measure

plasma concentration of FSH in single samples appears to have potential in verification of neuter status in bitches.

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CHAPTER 7

Adenohypophyseal function in bitches treated with medroxyprogesterone acetate

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Abstract

The aim of this study was to investigate the effects of treatment with medroxyprogesterone acetate (MPA) on canine adenohypophyseal function. Five Beagle bitches were treated with MPA (10 mg/kg, every 4 weeks) and their adenohypophyseal function was assessed in a combined adenohypophyseal function test. Four hypophysiotrophic hormones (CRH, GHRH, GnRH, and TRH) were administered before and 2, 5, 8, and 11 months after the start of MPA treatment, and blood samples for determination of the plasma concentrations of ACTH, cortisol, GH, IGF-1, LH, FSH, prolactin, α-MSH, and TSH were collected at -15, 0, 5, 10, 20, 30, and 45 min after suprapituitary stimulation.

MPA successfully prevented the occurrence of oestrus, ovulation, and a subsequent luteal phase. MPA treatment did not affect basal and GnRH-induced plasma LH concentrations. The basal plasma FSH concentration was significantly higher at 2 months after the start of MPA treatment than before or at 5, 8, and 11 months after the start of treatment. The maximal FSH increment and the AUC for FSH after suprapituitary stimulation were significantly higher before treatment than at 5, 8, and 11 months of MPA treatment. Differences in mean basal plasma GH concentrations before and during treatment were not significant, but MPA treatment resulted in significantly elevated basal plasma IGF-1 concentrations at 8 and 11 months. MPA treatment did not affect basal and stimulated plasma ACTH concentrations, with the exception of a decreased AUC for ACTH at 11 months. In contrast, the maximal cortisol increment and the AUC for cortisol after suprapituitary stimulation were significantly lower during MPA treatment than prior to treatment. MPA treatment did not affect basal plasma concentrations of prolactin, TSH, and α-MSH, with the exception of slightly increased basal plasma TSH concentrations at 8 months of treatment. MPA treatment did not affect TRH-induced plasma concentrations of prolactin and TSH.

In conclusion, the effects of chronic MPA treatment on adenohypophyseal function included increased FSH secretion, unaffected LH secretion, activation of the mammary GH-induced IGF-I secretion, slightly activated TSH secretion, suppression of the hypothalamic-pituitary-adrenocortical axis, and unaffected secretion of prolactin and α-MSH.

Introduction

Progestins, such as medroxyprogesterone acetate (MPA), are commonly used to prevent oestrus in the bitch (Schaefers-Okkens 1996, Romagnoli and Concannon 2003). Whether the oestrus-preventing properties of progestins in the bitch are due to effects on the hypothalamus, on the pituitary gland, or at the ovarian level is not clear. McCann et al. (1987) and Colon et al. (1993) reported that basal plasma levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) do not change during progestin treatment. Information about the effect of gonadotrophin-releasing hormone (GnRH) on the secretion of LH during progestin treatment is conflicting. GnRH-induced increases in plasma LH concentrations in progestin-treated dogs did not differ from those in control dogs in one study (Colon et al. 1993), while in another study the GnRH-induced LH levels were reduced (McCann et al. 1987). In dogs there is no information on the effect of progestins on GnRH-stimulated FSH concentrations.

In women, progestins are known to prevent ovulation by inhibiting the mid-cycle surges of FSH and LH, whereas the tonic release of these gonadotrophins continues at luteal phase levels (Mishell 1996; Jain et al. 2004). Long-term use of depot MPA in women does not affect the pituitary responsiveness of LH and FSH to GnRH administration, suggesting that the pituitary is not the primary site for ovulation inhibition in women (Ismael et al. 1987).

Prolonged treatment with progestins in bitches is associated with alterations in the release of pituitary hormones other than gonadotrophins. Progestin administration leads to a decrease in the pituitary responsiveness of growth hormone (GH) to growth hormone releasing hormone (GHRH) (Watson et al. 1987; Selman et al. 1991, 1994a). This change is due to GH release from foci of hyperplastic ductular mammary epithelium (Selman et al. 1994b; Van Garderen et al. 1997), leading to elevated plasma GH levels that do not have a pulsatile plasma profile (Watson et al. 1987). The hypothalamic-pituitary-adrenocortical (HPA) axis is suppressed by progestins (McCann et al. 1987; Rutteman et al. 1987; Selman et al. 1997), due to the intrinsic glucocorticoid properties of progestins (Guthrie and John 1980; Selman et al. 1996; Selman et al. 1997). While basal plasma concentrations of adrenocorticotrophic hormone (ACTH) are only moderately affected (Selman et al. 1997), the basal plasma concentrations of cortisol are markedly decreased (Concannon et al. 1980; McCann et al. 1987; Rutteman et al. 1989; Selman et al. 1997). In addition, the response of ACTH and cortisol to stimulation with corticotrophin-releasing hormone (CRH) may be reduced (McCann et al. 1987; Selman et al. 1997). Also, in women the administration of MPA causes suppression of the HPA axis (Jones et al. 1974).

With regard to other anterior pituitary hormones, such as prolactin and TSH, there is little information about the effect of progestin treatment on their release. In the bitch, progestin treatment does not seem to affect mean prolactin (Concannon et al. 1980) and TSH concentrations (Frank et al. 1979). Information with regard to pituitary responsiveness of prolactin to suprapituitary stimulation is limited to one study, in which MPA administration did not change prolactin response to TRH in ovariohysterectomized, oestradiol-primed bitches

(Rutteman et al. 1987). On the other hand, there is evidence that treatment with MPA increases the pituitary prolactin responsiveness to TRH in women (Mishell et al. 1977). Finally, there are no reports on the effect of progestins on the pituitary release of α-melanocyte-stimulating hormone (α-MSH).

The aim of the present study was to obtain an integral picture of the effect of progestins on the function of the adenohypophysis in the bitch. For this purpose, the effects of suprapituitary stimulation on the release of seven adenohypophyseal hormones were studied before and several times during MPA treatment in Beagle bitches by means of a combined anterior pituitary function test (Meij et al. 1996a,b).

Materials and methods

Animals, treatment and collection of blood samples

Studies were carried out in 5 healthy intact Beagle bitches, aged 3 to 9 years and weighing 9.0 to 10.3 kg, that never had been treated with progestins. They were housed in pens with outdoor access, fed a commercial diet once daily, and given water *ad libitum*. They were accustomed to the laboratory environment and procedures such as collection of blood samples. Throughout the study the general condition of the dogs was monitored by physical examination and routine clinical chemistry.

In the dogs used in this study, the tip of the right uterine horn and the corresponding ovary had been excised to serve as control tissues in another study. This surgical procedure had been performed 245 ± 42 days (mean ± SD) before the start of the treatment with MPA. After the surgery, all of the dogs had had one complete oestrous cycle.

Treatment with the synthetic progestin depot preparation MPA (Depo-Promone®, Pharmacia Animal Health, Puurs, Belgium) was begun during anoestrus in a dose of 10 mg/kg body weight subcutaneously at intervals of 4 weeks, for a total of 13 injections. Three days before the start of the treatment with MPA, the mean plasma progesterone concentration was 0.9 ± 0.3 nmol/l (mean ± SD).

Before and at 2, 5, 8, and 11 months after the start of the treatment with MPA, a combined anterior pituitary function test was performed using four releasing hormones (4RH test) according to methods described previously (Meij et al. 1996a,b). Briefly, an intravenous catheter was placed in the cephalic vein of each dog to facilitate rapid sequential injection. Immediately after the collection of the zero blood sample from the jugular vein, four releasing hormones were injected intravenously within 30 seconds, in the following order and doses per kg body weight: 1 µg oCRH (Peninsula Laboratories Inc., Belmont, CA, USA), 1 µg hGHRH (hGHRF; Peninsula Laboratories Inc., Belmont, CA, USA), 10 µg GnRH (Fertagyl®; Intervet, Boxmeer, The Netherlands), and 10 µg TRH (Hoffman-La Roche, Basel, Switzerland). During progestin treatment, the 4RH tests were always performed immediately before the next 4-weekly administration of MPA. The clock for blood sampling was started immediately after the

administration of the last releasing hormone. Blood samples were collected at -15, 0, 5, 10, 20, 30, and 45 min from the jugular vein and transferred to ice-chilled EDTA-coated and heparinized (for TSH) tubes. Samples were centrifuged at 4º C for 10 min. Plasma was stored at -25º C until assayed for ACTH, cortisol, GH, FSH, LH, prolactin, and TSH. Plasma concentrations of α-MSH and IGF-1 were determined in the -15 and 0 min samples only.

Hormone measurements

Plasma progesterone concentration was measured by a previously validated radioimmunoassay (RIA) (Henry et al. 1987). The sensitivity of the assay was 0.005 ng. The intra-assay and interassay coefficients of variation (CV) were 7.05% and 8.75%, respectively.

Plasma ACTH concentration was measured by use of a two-site immunoradiometric assay (IRMA) (Nichols Institute, Wijchen, The Netherlands) as described previously (Bosje et al. 2002). The antiserum is highly specific for $ACTH_{1-39}$. The intra-assay and interassay CV were 3.2% and 7.8%, respectively, and the lower limit of quantitation was 0.22 pmol/l. The antiserum cross-reacts with neither α-MSH nor ACTH precursors (Hodgkinson et al. 1984; Findling et al. 1990).

Plasma cortisol concentration was measured by an RIA validated for the dog (Coat-A-Count® Cortisol, Diagnostic Product Corporation, Los Angeles, CA, USA) as described previously (Galac et al. 2005). Intra-assay and interassay CV ranged from 3.0 to 5.1 and from 4.0 to 6.4%, respectively. The lower limit of quantitation was 1 nmol/l.

Plasma FSH concentration was measured by a heterologous canine immunoradiometric assay (IRMA) (AHC004, Biocode SA, Liège, Belgium), validated previously (Beijerink et al. 2007). The intra-assay and interassay CV for values above 1.6 μg/l were 3.2% and 15%, respectively. The limit of quantitation was 1.5 μg/l.

Plasma GH concentration was measured by a commercially available RIA for porcine and canine GH according to the manufacturer's protocol (PGH-46HK; Linco Research, St. Charles, MS, USA). The interassay CV was 7.6% at a plasma concentration of 4.4 μg/l. Cross reactivity of porcine prolactin and human GH was less than 0.5%. Recovery of porcine GH, which is identical to canine GH, in canine serum averaged 104 ± 14 % (mean ± SD). Dilution of a canine plasma sample containing high endogenous GH concentrations revealed parallelism to the standard curve. The lower limit of quantitation was 1.0 μg/l.

Plasma total IGF-1 concentration was measured by a heterologous RIA, validated for the dog, after acid–ethanol extraction to remove interfering IGF binding proteins (IGFBPs). Plasma IGF was extracted using a mixture of 87.5% (v/v) ethanol and 12.5% 2 M formic acid. Tubes containing 100 μl plasma and 400 μl of the ethanol–formic acid mixture were mixed thoroughly and incubated for 30 min at room temperature. After centrifugation for 30 min at 5500 *g* at 4°C, a 50 μl aliquot of the supernatant was diluted 1:50 with assay buffer containing 63 mM Na_2HPO_4 (pH 7·4), 13 mM Na_2EDTA, and 0·25% (w/v) BSA. The extraction efficiency was 92.5 ± 5.7 % (Favier et al. 2001). The intra-assay and interassay CV were 8.6% and 13.4% at a plasma concentration of 100 and 73 μg/l, respectively. The lower limit of

quantitation was 10 µg/l. IGF-1 antiserum AFP4892898 and human IGF-1 for iodination were obtained from the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance CA).

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 GD Niswender, Colorado State University, CO, USA), radio-iodinated bovine LH-7981 as prepared for our bovine LH assay (Dieleman and Bevers, 1987), and canine pituitary standard LER 1685-1 (a gift of Dr LE Reichert, Albany Medical College, NY, USA) were used in this assay. The intra-assay and interassay CV for values above 0.5 µg/l were 2.3% and 10.5% respectively. The lower limit of quantitation was 0.3 µg/l.

Plasma α-MSH concentration was measured by RIA without extraction according to methods described previously (Mol et al. 1987). The intra-assay and interassay CV were less than 8% and 23%, respectively. The lower limit of quantitation was 5 pmol/l.

Plasma prolactin concentration was measured by a previously validated heterologous RIA (Okkens et al. 1985). The intra-assay and interassay CV were 3.5% and 11.5%, respectively. The lower limit of quantitation was 0.8 µg/l.

Plasma TSH concentration was determined by a homologous solid-phase, two-site chemiluminescent enzyme immunometric assay (Immulite canine TSH Diagnostic Products Corporation (DPC), Los Angeles, CA) according to the instructions of the manufacturer as described previously (Kooistra et al. 2000). The intra-assay CV were 5.0, 4, and 3.8% at TSH levels of 0.20, 0.50, and 2.6 µg/l, respectively. The interassay CV were 6.3 and 8.2% at TSH levels of 0.16 and 2.8 µg/l, respectively. The lowest detectable amount of TSH was 0.03 µg/L. Cross-reactivity with FSH and LH was negligible. The upper limit of the reference range for the plasma TSH concentration in euthyroid dogs is 0.6 µg/l.

Data analysis

Analyses were performed with SAS version 9.1 for Windows (Insightful Corp., Seattle, WA, USA). Differences in body weight before and after the study were assessed in a paired Student's t-test. Plasma α-MSH concentrations below the limit of quantitation were assigned a value of 5 pmol/l. The following response variables were considered: basal hormone value, maximal increment from basal level, and area under the curve above the basal hormone level (AUC). The basal concentration was defined as the mean of the hormone concentrations at -15 and 0 min. The AUC for hormone concentration following stimulation was calculated by the trapezoidal method. Basal hormone concentration, maximal increment, and AUC before and after MPA treatment were compared for the different hormone concentrations using a mixed model with dog as random effect and period (0, 2, 5, 8, and 11 months after MPA treatment) as categorical fixed effects factor. The periods were compared in pairs, applying Tukey's multiple comparisons technique at a global significance level of 5% to obtain adjusted P values.

Additionally, a mixed model was used with dog as random effect and period, time (minutes after stimulation) and the period by time interaction as categorical fixed effects to investigate whether the time evolution after stimulation differed from period to period.

Ethics of experimentation

The study protocol was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium.

Results

No signs of oestrus were detected during the 12 months of MPA treatment. In addition, plasma progesterone concentrations were low at 5 months (0.6 ± 0.6 nmol/l; mean ± SD) and 12 months (0.6 ± 0.3 nmol/l) after the start of the treatment. The mean body weight of the dogs on the day of the last injection of MPA (12.4 ± 1.6 kg, mean ± SD) was significantly higher (P=0.02) than that on the day of the first injection of MPA (9.5 ± 0.7 kg).

Basal plasma LH concentrations did not change significantly. In each sampling period, suprapituitary stimulation resulted in a significant rise (P<0.001) in plasma LH concentration. The maximal increment and the AUC for LH did not vary significantly with time (Table 1, Fig. 1).

Basal plasma FSH concentration was significantly higher at 2 months after the start of treatment with MPA than before treatment (P=0.004) or at 5 (P=0.004), 8 (P=0.002), or 11 months (P<0.001) after the start of the treatment. In each sampling period, suprapituitary stimulation resulted in a significant rise (P<0.001) in the plasma FSH concentration. The maximal increment was significantly higher before treatment than after 5 (P=0.01) or 11 months (P=0.01) of treatment with MPA, while it tended to be higher (P=0.07) at 8 months. The AUC for FSH was significantly higher before MPA treatment than after 5 (P<0.001), 8 (P=0.03), or 11 (P=0.02) months of treatment with MPA (Table 1, Fig. 1).

Differences in basal plasma GH concentration before and during treatment with MPA were not significant. At 8 months after the start of treatment with MPA suprapituitary stimulation resulted in a significant rise (P<0.001) in plasma GH concentration, while no significant effect was noted during the other periods. Differences in the maximal increment and the AUC for GH before and during the treatment with MPA were not significant (Table 2, Fig. 2). Basal plasma IGF-1 concentration was significantly higher at 8 months (P=0.02) and 11 months (P<0.001) of treatment with MPA than before treatment. In addition, basal plasma IGF-1 concentration was significantly higher at 11 months of treatment than at 2 months (P=0.02) (Table 2).

Differences in basal plasma ACTH concentration before and during treatment with MPA were not significant. In each sampling period, suprapituitary stimulation resulted in a significant rise (P<0.001) in plasma ACTH concentration. Differences in the maximal

increment before and during the treatment with MPA were not significant. The AUC for ACTH after 11 months of MPA treatment was significantly lower (P=0.05) than at 2 and 8 months after the start of the MPA treatment (Table 3, Fig. 3). Differences in basal plasma cortisol concentration before and during treatment with MPA were not significant. In each sampling period, suprapituitary stimulation resulted in a significant rise (P<0.001) in plasma cortisol concentration. The maximal increments decreased significantly during treatment (P=0.003, P=0.01, P=0.002, and P=0.002, respectively). The AUC for cortisol in the 4 periods of treatment with MPA was significantly lower than that before MPA treatment (P=0.002, P=0.003, P=0.002, and P=0.002, respectively) (Table 3, Fig. 3).

	Before treatment	Months after starting treatment with MPA			
		2	5	8	11
LH basal (µg/l)	3.0 ± 1.2	4.6 ± 1.9	2.9 ± 0.6	3.9 ± 2.1	2.5 ± 1.1
LH increment (µg/l)	57.4 ± 9.3	58.3 ± 8.9	62.3 ± 10.6	82.9 ± 21.3	82.0 ± 19.3
LH Tmax (min)	10 5 – 20	10 10 - 20	10 10 - 20	10 10 - 20	10 10 – 20
LH AUC (µg/l*45min)	1633 ± 262	1801 ± 363	1797 ± 302	2019 ± 558	2024 ± 386
FSH basal (µg/l)	7.4 ± 0.9	14.2 ± 2.1[a]	7.5 ± 1.2	6.9 ± 1.2	5.6 ± 0.9
FSH increment (µg/l)	27.2 ± 4.3	22.2 ± 3.8	17.6 ± 3.2[b]	20.2 ± 4.1	17.5 ± 3.6[b]
FSH Tmax (min)	20 10 - 20	20 20 - 30	20 20 - 45	20 10 - 20	20 10 – 20
FSH AUC (µg/l*45min)	932 ± 137	756 ± 121	599 ± 116[b]	645 ± 135[b]	608 ± 118[b]

Table 1. Characteristics of LH and FSH secretion in 5 Beagle bitches in a combined anterior pituitary function test (Meij et al. 1996a,b) before and 2, 5, 8, and 11 months after starting treatment with MPA. The values are expressed as mean ± SEM or median and range. Tmax indicates the time at which maximal suprapituitary stimulation was observed. [a] significantly different from before treatment and at 5, 8, and 11 months after starting treatment with MPA; [b] significantly different from before MPA treatment.

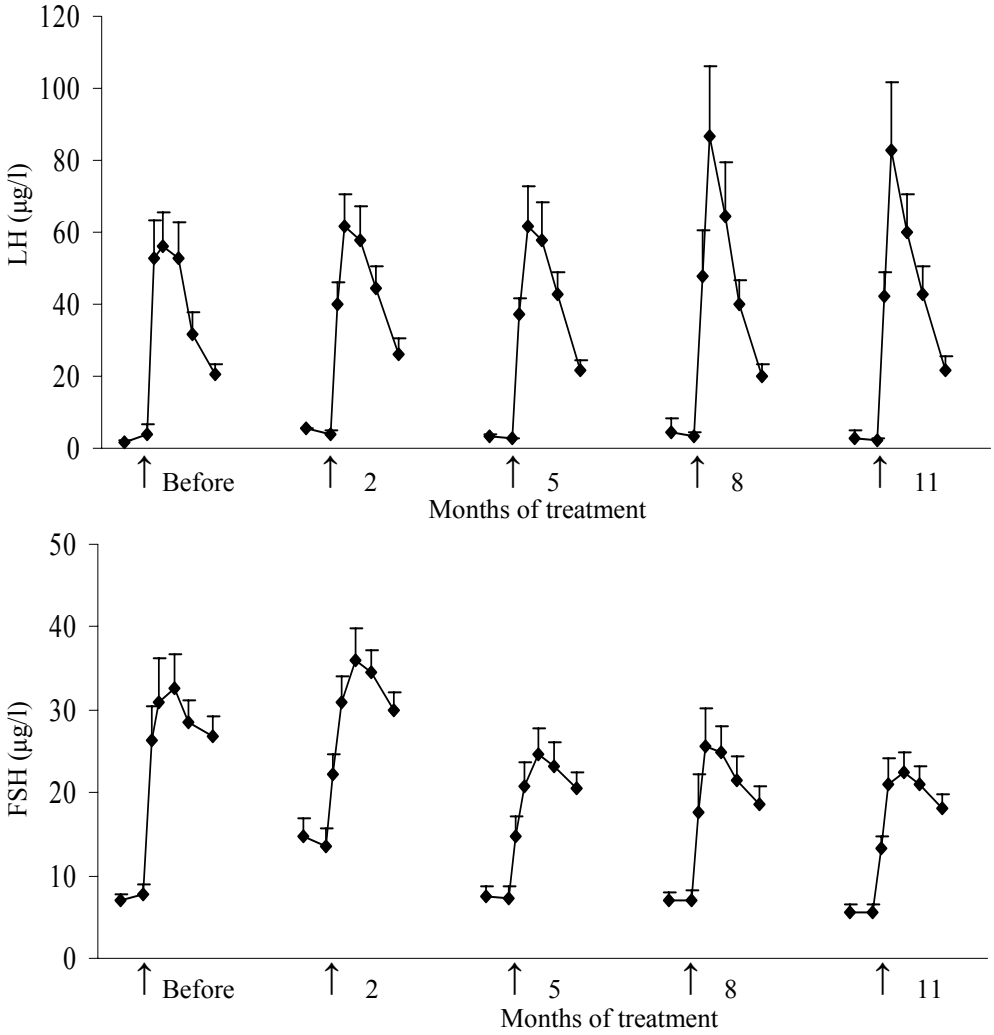

Figure 1. Plasma LH and FSH responses (mean ± SEM) in 5 Beagle bitches in a combined anterior pituitary function test (Meij et al. 1996a,b) before and 2, 5, 8, and 11 months after starting treatment with MPA. Blood samples were collected at -15, 0, 5, 10, 20, 30, and 45 min following the injection of the releasing hormones at 0 min (arrow).

	Before treatment	Months after starting treatment with MPA			
		2	5	8	11
GH basal (µg/l)	2.7 ± 0.9	2.3 ± 0.3	2.5 ± 0.3	2.7 ± 0.4	3.0 ± 0.5
GH increment (µg/l)	1.8 ± 1.5	0.6 ± 0.3	0.9 ± 0.4	1.1 ± 0.2	0.6 ± 0.4
GH Tmax (min)	5 -15 - 45	20 10 - 30	10 -15 - 20	20 5 - 45	10 0 – 20
GH AUC (µg/l*45min)	5.1 ± 44.4	13.1 ± 9.5	23.3 ± 17.6	34.5 ± 7.4	12.4 ± 13.7
IGF-I basal (µg/l)	45.3 ± 5.6	108 ± 24.6	135 ± 35.4	159 ± 29.8[a]	224 ± 53.3[a,b]

Table 2. Characteristics of GH and IGF-I secretion in 5 Beagle bitches in a combined anterior pituitary function test (see also legend to Table 1).[a] significantly different from before MPA treatment; [b] significantly different from 2 months after starting treatment with MPA.

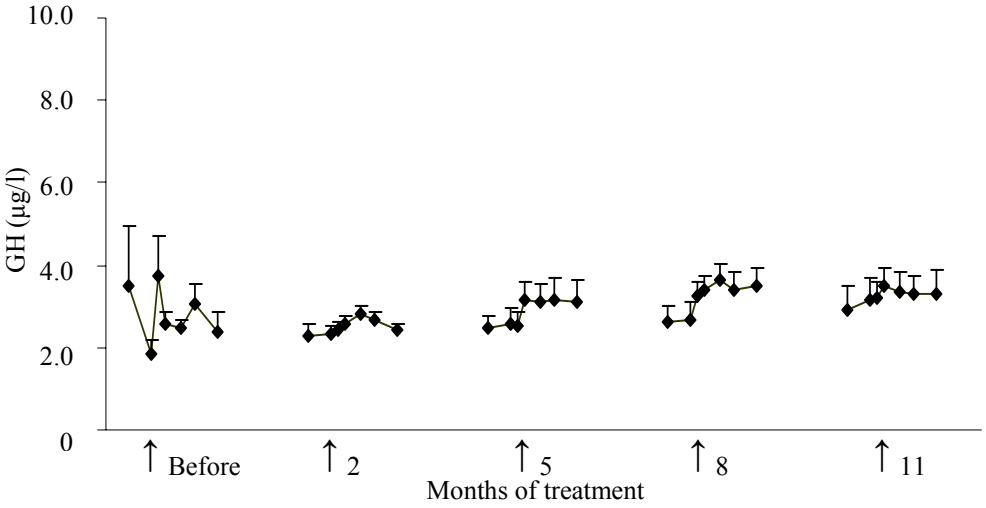

Figure 2. Plasma GH responses (mean ± SEM) in 5 Beagle bitches in a combined anterior pituitary function test (see also legend to Fig. 1).

Differences in basal plasma prolactin concentration before and during treatment with MPA were not significant. In each sampling period, suprapituitary stimulation resulted in a significant rise ($P<0.001$) in plasma prolactin concentration. Differences in the maximal increment and the AUC for prolactin before and during treatment with MPA were not significant (Table 4, Fig. 4).

Basal plasma TSH concentration at 8 months of MPA treatment was significantly higher than before treatment ($P=0.03$) and at 5 months of treatment ($P=0.05$). In each sampling period, suprapituitary stimulation resulted in a significant rise ($P<0.001$) in plasma TSH concentration. Differences in the maximal increment and the AUC for TSH before and during MPA treatment were not significant (Table 4, Fig. 4).

Basal plasma α-MSH concentration was 20.7 ± 4.9 pmol/l before treatment and 20.3 ± 5.3, 21.3 ± 5.3, 25.6 ± 8.4, and 32.1 ± 4.4 pmol/l at the four sampling times during MPA treatment. Differences in the mean plasma α-MSH concentrations were not significant.

Discussion

The tip of one uterine horn and the corresponding ovary were used as control tissues in another study. Nevertheless, all bitches had an oestrous cycle between the surgical procedure and the start of the MPA treatment. Chaffaux and co-workers (1981) demonstrated in dogs that basal plasma gonadotrophin levels and the response of these hormones to an intramuscular injection of a GnRH analogue were unaffected by unilateral ovariectomy. Furthermore, the difference in plasma FSH concentration between intact and unilateral oophorectomized women was not significant (Cooper and Thorp 1999). Thus the dogs used in this study can be regarded as having an intact hypothalamus-pituitary-ovarian axis.

The results of this study demonstrate that treatment with MPA affects the hypothalamic-pituitary-ovarian axis. Oestrus, ovulation, and a subsequent luteal phase did not occur in any of the bitches during treatment with MPA, as judged by the lack of external signs of oestrus and low plasma levels of progesterone. Although the design followed in this study does not allow for a mechanistic examination of the mode of oestrus suppression in the bitch, and thus subtle effects of MPA on the reproductive axis may have been missed, the prevention of oestrus by MPA in the present study cannot be ascribed to a significant reduction in circulating levels of either FSH or LH. On the contrary, during the first months of MPA treatment there was an increase in basal plasma FSH without a concomitant change in basal plasma LH. The progestin-induced change in FSH concentration was not observed by Colon et al. (1993) and its recognition may be explained by the repeated sampling employed in the present study.

	Before treatment	Months after starting treatment with MPA			
		2	5	8	11
ACTH basal (pmol/l)	3.8 ± 0.86	6.0 ± 0.68	4.2 ± 0.55	5.3 ± 0.35	4.9 ± 0.37
ACTH increment (pmol/l)	68 ± 14	59 ± 7.0	47 ± 4.6	64 ± 8.1	42 ± 7.0
ACTH Tmax (min)	5 5 – 10	10 5 - 30	10 5 – 30	10 5 – 10	10 5 – 45
ACTH AUC (pmol/l*45min)	1840 ± 286	2005 ± 212	1498 ± 94	2027 ± 180	1276 ± 231[a]
Cortisol basal (nmol/l	47.6 ± 4.6	60.1 ± 11.4	58.1 ± 18.0	52.5 ± 9.90	58.4 ± 13.7
Cortisol increment (nmol/l)	380 ± 39.1	238 ± 12.1[b]	258 ± 19.1[b]	231 ± 24.3[b]	226 ± 16.2[b]
Cortisol Tmax (min)	30 30 – 45	30 30 - 45	45 30 - 45	30 20 - 45	30 20 – 30
Cortisol AUC (nmol/l*45min)	12199 ± 1121	7825 ± 331[b]	8042 ± 572[b]	7785 ± 707[b]	7585 ± 433[b]

Table 3. Characteristics of ACTH and cortisol secretion in 5 Beagle bitches in a combined anterior pituitary function test (see also legend to Table 1). [a] *significantly different from 2 and 8 months after starting treatment with MPA;* [b] *significantly different from before MPA treatment.*

The results of this study thus indicate that the progestin-induced changes in gonadotrophin release are confined to FSH secretion. One mechanism for the differential control of LH and FSH secretion is gonadal feedback. Both oestradiol and inhibin can specifically suppress FSH synthesis and secretion (Mann et al. 1992; Shupnik 1996). Reduced secretion of these ovarian hormones can explain the elevated circulating FSH concentration during the first months of MPA treatment. On the other hand, the gonadal peptide activin specifically stimulates FSH secretion. Consequently, a temporary progestin-induced change in the secretion of activin might also explain the initial divergent basal levels of LH and FSH. Other important factors in gonadotrophin control are the frequency and amplitude of GnRH pulses, which have been shown to differentially alter LH and FSH gene expression and secretion (Haisenleder et al. 1991; Shupnik 1996; Vizcarra et al. 1997).

The elevated basal plasma FSH level during the first months of MPA treatment may thus be due to a direct inhibitory effect of MPA at the ovarian level, resulting in suppression of the ovarian secretion of oestradiol or inhibin, or stimulation of activin release. In this context, increased plasma gonadotrophin levels and low ovarian oestradiol production have been reported in women treated with progestins (Poindexter et al. 1993; Heikinheimo et al. 1996). Observations in monkeys indicate that the inhibitory effects of progesterone on follicular development persist even in the presence of elevated plasma FSH levels, providing additional

evidence that progestins may have a direct effect at the ovarian level (Goodman and Hodgen 1982). In women, there are also indications for a hypothalamic site of progestin action (Couzinet and Schaison 1993). An initial progestin-induced change in the pattern of hypothalamic secretion of GnRH may therefore be an alternative explanation for the rise in the basal plasma FSH level during the first months of MPA treatment observed in this study.

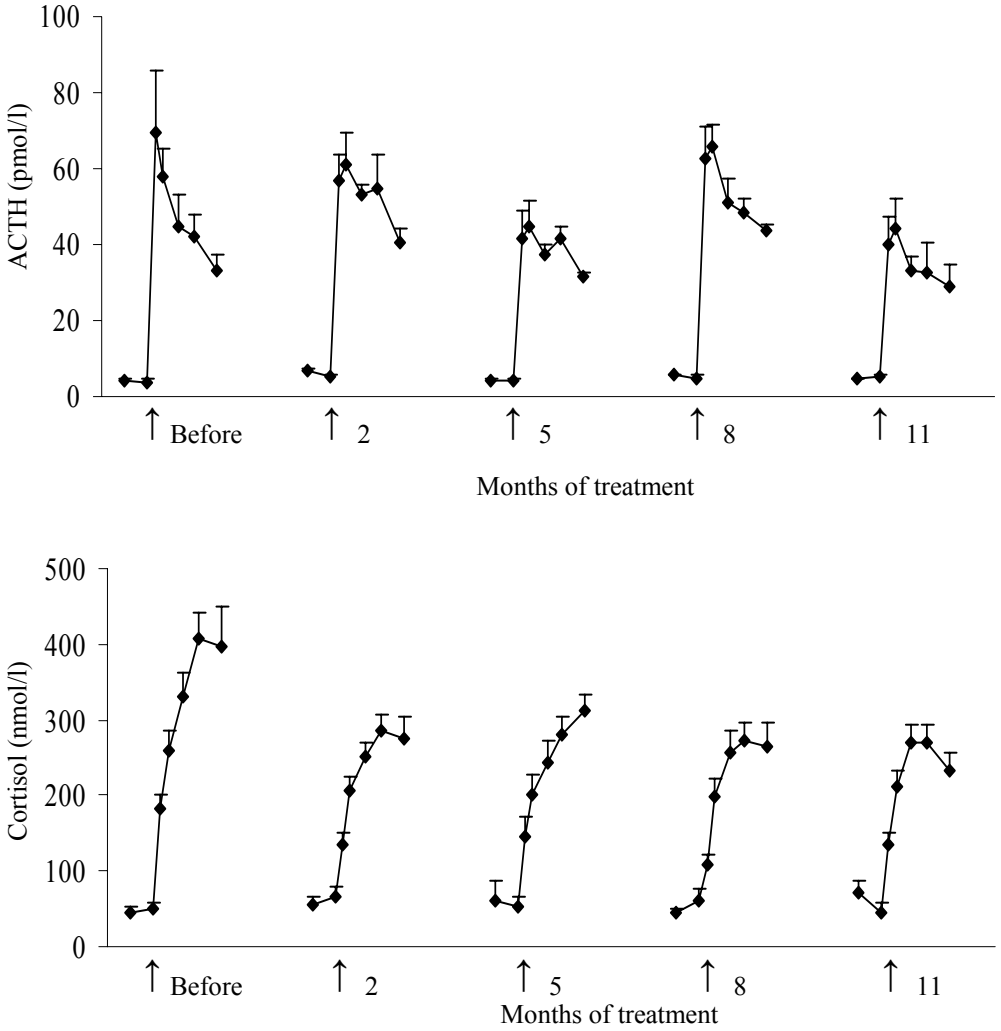

Figure 3. Plasma ACTH and cortisol responses (mean ± SEM) in 5 Beagle bitches in a combined anterior pituitary function test (see also legend to Fig. 1).

	Before treatment	Months after starting treatment with MPA			
		2	5	8	11
TSH Basal (µg/l)	0.09 ± 0.03	0.21 ± 0.06	0.14 ± 0.04	0.22[a] ± 0.08	0.17 ± 0.07
TSH Increment (µg/l)	0.76 ± 0.25	1.01 ± 0.21	0.88 ± 0.17	1.13 ± 0.27	0.91 ± 0.25
TSH Tmax (min)	10 5 – 20	20 10 - 30	30 10 - 30	10 10 - 20	10 10 – 30
TSH AUC (µg/l*45min)	23.4 ± 6.13	34.1 ± 7.95	30.0 ± 5.57	36.2 ± 7.62	28.7 ± 6.09
PRL basal (µg/l)	8.51 ± 2.49	9.07 ± 3.17	11.39 ± 6.74	4.73 ± 0.83	7.95 ± 2.49
PRL increment (µg/l)	53.2 ± 18.0	42.4 ± 14.3	28.5 ± 7.2	66.2 ± 20.1	33.2 ± 6.6
PRL Tmax (min)	10 5 – 20	10 5 - 10	10 5 - 20	10 5 - 10	10 5 – 20
PRL AUC (µg/l*45min)	1086 ± 231	1038 ± 272	698 ± 211	1418 ± 260	806 ± 148

Table 4. Characteristics of TSH and prolactin secretion in 5 Beagle bitches in a combined anterior pituitary function test (see also legend to Table 1). [a] *significantly different from before MPA treatment and at 5 months of MPA treatment.*

With continuing MPA treatment, basal plasma FSH returned to pretreatment levels and the pituitary FSH response to suprapituitary stimulation decreased. These observations may be explained by down-regulation of the pituitary GnRH receptors due to continuous GnRH stimulation (Belchetz et al. 1978). The high GnRH secretion associated with MPA treatment postulated in the previous paragraph may therefore have resulted in desensitization of the response of the gonadotrophes to GnRH. The decline to pretreatment FSH levels and the decrease in responsiveness of pituitary FSH secretion to GnRH are probably part of the oestrus-preventing effects of MPA, because increased FSH secretion is a critical event in the initiation of ovarian folliculogenesis (Kooistra et al. 1999a,b). In other words, MPA treatment for oestrus prevention may prohibit the normal rise in plasma FSH concentration during late anoestrus.

The present results confirm previous findings that progestins alter the GH-IGF-1 axis in the bitch (Eigenmann and Rijnberk 1981). Basal plasma GH concentration tended to increase gradually during the course of the MPA treatment, but the change was not significant. In another study (Concannon et al. 1980), plasma GH concentration did not rise in 27 out of 36 MPA-treated bitches. However, the significant increase in circulating IGF-I concentration during MPA treatment in the present study is consistent with excessive exposure to GH (Selman et al. 1994a). Plasma IGF-1 concentration may thus be a more sensitive indicator than plasma GH concentration for the effect of the progestin treatment on the GH-IGF-1 axis.

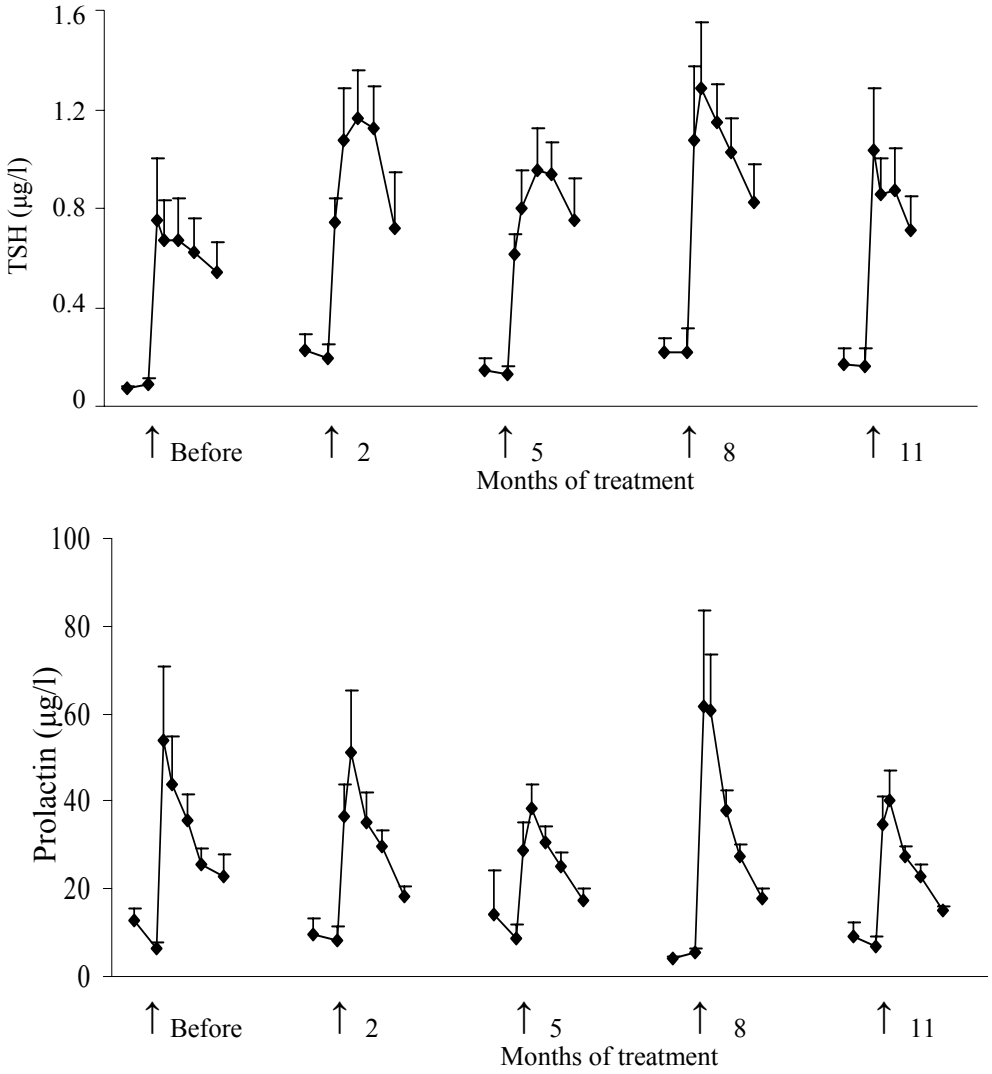

Figure 4. Plasma TSH and prolactin responses (mean ± SEM) in 5 Beagle bitches in a combined anterior pituitary function test (see also legend to Fig. 1).

In contrast to previous observations (Meij et al. 1996a), the GH response to suprapituitary stimulation prior to MPA treatment was not significant. This may be due to ageing (Bhatti et al. 2002), since the dogs in our study were considerably older than those in the study of Meij et al. (1996a). In addition, a relatively high plasma GH level before suprapituitary stimulation in one of the dogs, probably explained by sampling during a GH pulse, had a substantial influence on basal plasma GH concentration. In agreement with previous observations (Selman et al. 1991), there was no pituitary GH response to suprapituitary stimulation during MPA treatment in three of the four tests. This may be ascribed to the negative feedback effect of the nonepisodically secreted mammary GH. It has been demonstrated in humans that GH exerts its negative feedback effect by stimulating hypothalamic somatostatin secretion (Berelowitz et al. 1981). Additionally, the GH-induced elevated circulating IGF-1 levels also inhibit pituitary GH secretion (Hartman et al. 1993).

In addition to interacting with the progesterone receptor, MPA also has a relatively high affinity for the glucocorticoid receptor (Selman et al. 1996). Suppression of the HPA axis was thus expected during MPA treatment, as was reported in both humans (Willemse et al. 1990) and dogs (Selman et al. 1994c, 1996). Indeed, we found the cortisol response to stimulation to be decreased with MPA treatment, although ACTH secretion was only slightly affected, possible because MPA affects the HPA axis for only 2-3 weeks (Selman et al. 1994c). Although this leads to adrenocortical atrophy (Selman et al. 1997), by 3 weeks (Selman et al. 1994c) or 4 weeks (present study) after administration of MPA, the initially suppressed ACTH (and cortisol) concentrations can have returned to normal.

MPA treatment causes significant increases in body weight. The intrinsic glucocorticoid properties of MPA (Selman et al. 1996), leading to increased appetite, may have contributed to this increase in body weight. However, it is difficult to attribute this effect to the glucocorticoid action of MPA alone, since treatment with MPA also affected the GH-IGF-1-axis.

As the luteal phase in the bitch progresses, circulating progesterone concentration decreases and prolactin secretion increases (Kooistra et al. 2002). This association has also been demonstrated in pregnant and pseudopregnant bitches (Steinetz et al. 1990; Okkens et al. 1997). Moreover, administration of a progesterone-receptor antagonist to pregnant bitches causes plasma prolactin levels to rise sharply (Galac et al. 2000). Nevertheless, in agreement with previous studies (Concannon et al. 1980; Rutteman et al. 1987), progestin treatment did not affect prolactin secretion in the present study. This may be explained by the persistently high progestin status.

Mean basal plasma TSH concentration was higher at 8 months after the start of MPA treatment than it was before treatment and at 5 months after the start of treatment. There were no significant alterations in the TSH response to suprapituitary stimulation. Although basal plasma TSH concentrations were elevated at 8 months after the start of the treatment, they were still within the reference range for TSH in our laboratory. Others found that MPA treatment had no effect on mean circulating TSH levels (Frank et al. 1979), but several authors have reported that total T4 and/or T3 values increase during the progesterone-dominated luteal phase

and pregnancy in bitches (Reimers et al. 1984; Dixon 2004; Feldman and Nelson 2004). It remains to be seen whether these changes have clinical significance, but the results of the present study emphasize the importance of considering progestin use when studying the hypothalamic-pituitary-thyroid axis.

In conclusion, MPA successfully prevented the occurrence of oestrus, ovulation, and a subsequent luteal phase. The effects of chronic MPA treatment on adenohypophyseal function included increased FSH secretion, unaffected LH secretion, activation of the mammary GH-induced IGF-I secretion, slightly activated TSH secretion, suppression of the HPA-axis, and unaffected secretion of prolactin and α-MSH.

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CHAPTER 8

Pulsatile plasma profiles of FSH and LH before and during medroxyprogesterone acetate treatment in the bitch

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Abstract

The aim of this study was to investigate the effect of medroxyprogesterone acetate (MPA) on pulsatile secretion of gonadotrophins in the bitch. Five intact Beagle bitches were treated with MPA in a dose of 10 mg/kg body weight subcutaneously at intervals of 4 weeks for a total of 13 injections, starting during anoestrus. The 6-h plasma profiles of luteinizing hormone (LH) and follicle stimulating hormone (FSH) were determined before and 3, 6, 9, and 12 months after the start of MPA treatment.

After 6 months of MPA treatment basal plasma LH concentration was increased significantly. Basal plasma FSH concentration and the area under the curve above the zero level (AUC_0) for FSH were significantly higher after 3 months of MPA treatment than before or after 9 and 12 months of treatment. MPA treatment did not significantly affect pulse frequency, pulse amplitude, or AUC above the baseline for either LH or FSH. During treatment 58 significant LH pulses were identified, and although each LH pulse coincided with an increase in plasma FSH concentration, in 17 cases the amplitude of the increase was too small to be recognized as a significant FSH pulse.

In conclusion, MPA treatment did not suppress basal plasma gonadotrophin levels in the bitches. On the contrary, it caused a temporary rise in the basal concentration of both FSH and LH, which may be due to a direct effect of MPA on the ovary. In addition, several LH pulses were not accompanied by a significant FSH pulse, suggesting that MPA treatment attenuated the pulsatile pituitary release of FSH.

Introduction

Synthetic progestins such as medroxyprogesterone acetate (MPA) are used frequently in dogs to suppress the ovarian cycle. Whether this contraceptive effect of progestins is exerted on the hypothalamus by interfering with the pulsatile secretion of gonadotrophin-releasing hormone (GnRH), on the pituitary by attenuating the synthesis and/or release of luteinizing hormone (LH) and/or follicle stimulating hormone (FSH), or directly on the ovary is still unclear.

Three studies have addressed this issue by determining basal circulating gonadotrophin concentrations and by performing GnRH challenge tests (McCann et al., 1987, Colon et al. 1993, Beijerink et al. 2007). In the first two studies, in which bitches were treated with the progestins MPA or megestrol acetate, respectively, it was concluded that mean basal circulating LH concentration remained unchanged during progestin treatment. In addition, there was no significant change in mean basal circulating FSH concentration during progestin treatment (Colon et al. 1993). GnRH challenge tests, which assess the pituitary sensitivity to GnRH, have yielded conflicting results. GnRH-induced increases in plasma LH concentration did not differ from those in control dogs in one study (Colon et al. 1993), while in another study the increase in plasma LH following GnRH administration was less than that in control dogs (McCann et al. 1987). In the study by Beijerink et al. (2007), MPA treatment caused a temporary rise in basal plasma FSH concentration at two months after the start of the treatment without affecting basal plasma LH concentration. With continuing MPA treatment, basal plasma FSH concentration returned to pre-treatment levels and the pituitary FSH response to suprapituitary stimulation decreased significantly.

Characterization of the pulsatile plasma profiles of gonadotrophins accurately reflects hypothalamic-pituitary dynamics and is an appropriate tool for investigating the mode of action of contraceptive methods in women (Dericks-Tan et al. 1992, Hemrika et al. 1993a,b, Schleussner et al. 2001). However, most studies have been performed in women using combined oral contraceptives and not in those receiving injectable progestins. There is no published information on the effects of MPA on pulsatile gonadotrophin secretion in bitches. In order to unravel the oestrus-preventing mechanism of MPA in the bitch, this study was undertaken to characterize the pulsatile plasma profiles of LH and FSH before and during treatment with MPA.

Materials and methods

Animals, treatment and collection of blood samples

This study was carried out in 5 healthy intact Beagle bitches aged 3 to 9 years and weighing 9.0 to 10.3 kg; they had never been treated with progestins. They were housed in

pairs, fed a commercial diet once daily, and given water *ad libitum*. They were accustomed to the laboratory environment and procedures such as collection of blood samples. Serum progesterone concentration was measured regularly to determine the stage of the oestrous cycle. Throughout the study the general condition of the dogs was monitored by physical examination.

In the dogs used in this study, the tip of the right uterine horn and the corresponding ovary were excised to serve as control tissues for another study. This surgical procedure was performed 245 ± 42 days (mean ± SD) before the start of treatment with MPA. After the surgery, all dogs went through one complete oestrous cycle.

Treatment with the synthetic progestin depot preparation MPA (Depo-Promone®, Pharmacia Animal Health, Puurs, Belgium) was begun during anoestrus in a dose of 10 mg/kg body weight subcutaneously at intervals of 4 weeks for a total of 13 injections. Three days before the start of treatment with MPA, the mean plasma progesterone concentration was 0.9 ± 0.1 nmol/L (mean ± SEM).

The 6-h plasma profiles of LH and FSH were determined before and 3, 6, 9, and 12 months after the start of treatment with MPA. Profile sampling was always performed immediately before the next 4-weekly administration of MPA. Blood samples were collected from the jugular vein at 15-min intervals between 0800 and 1400, placed immediately in chilled EDTA-coated tubes, and centrifuged at 4º C for 10 min at 1500 *g*. Plasma was stored at – 25º C until analysis.

Hormone measurements

Plasma progesterone concentration was measured by a previously validated radioimmunoassay (RIA) (Henry et al., 1987). The intra-assay and interassay coefficients of variation (CV) were 7.1% and 8.8%, respectively. The limit of quantitation was 0.15 nmol/l.

Plasma FSH concentration was measured by a heterologous canine immunoradiometric assay (IRMA) (AHC004, Biocode SA, Liège, Belgium), validated previously (Beijerink et al. 2007). The intra-assay and interassay CV for values above 1.6 µg/l were 3.2% and 15%, respectively. The limit of quantitation was 1.5 µg/l.

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 GD Niswender, Colorado State University, CO, USA), radio-iodinated bovine LH-7981 as prepared for our bovine LH assay (Dieleman and Bevers 1987), and canine pituitary standard LER 1685-1 (a gift of Dr LE Reichert, Albany Medical College, NY, USA) were used in this assay. For values above 0.5 µg/l, the intra-assay CV was 2.3% and the interassay CV was 10.5%. The limit of quantitation was 0.3 µg/l.

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 SD 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 SD, 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 pulse amplitude (maximal increment from basal level), the pulse frequency, the area under the curve above the baseline (AUC_b), and the area under the curve above the zero level (AUC_0).

Subsequently, analyses were performed with SAS version 9.1 for Windows (Insightful Corp., Seattle, WA, USA). Body weight before and after the study was compared by a paired Student's *t*-test. Basal hormone concentration, AUC_b, AUC_0, and peak amplitude for both FSH and LH were compared using a mixed model with dog as random effect and period (before, and 3, 6, 9, 12 months after MPA treatment) as categorical fixed effects factor. The periods were compared pairwise at a comparisonwise significance level using Tukey's technique to adjust for multiple comparisons. Pulse frequency was compared pairwise between the period before treatment and the other periods for both FSH and LH by the Wilcoxon rank sum test stratified for dog at a comparisonwise significance level using Bonferroni's technique to adjust for multiple comparisons. Results are presented as mean ± SEM or median and range. $P<0.05$ was considered significant.

Ethics of experimentation

The study protocol was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium.

Results

No signs of oestrus were observed during the 12 months of MPA treatment. Plasma progesterone concentrations were low after 5 months (0.6 ± 0.3 nmol/l; mean ± SEM) and 12 months (0.6 ± 0.1 nmol/l) of treatment. Body weight on the day of the final injection of MPA (12.4 ± 0.7 kg) was significantly higher than on the day of the first injection (9.5 ± 0.3 kg) ($P<0.02$).

A representative example of LH and FSH pulse patterns before and during MPA treatment in one bitch is shown in Figure 1. Pulsatile gonadotrophin secretion was observed in 4 of the 5 dogs during anoestrus before the start of MPA treatment and in all 20 plasma profiles collected during treatment. Prior to treatment 8 significant LH pulses were identified by the Pulsar programme in the 6-hour profiles and each coincided with a significant FSH pulse. There were no FSH pulses without LH pulses. During MPA treatment, 58 significant LH pulses were identified. Each coincided with an increase in plasma FSH concentration and 41 of these were recognized as significant FSH pulses by the Pulsar programme. In one bitch, at 9 months of MPA treatment, there was one significant FSH pulse without a significant LH pulse.

Basal plasma LH concentration was significantly higher after 6 months of MPA treatment than before treatment. Basal plasma FSH concentration and AUC_0 for FSH were significantly higher after 3 months of treatment than before or after 9 and 12 months of MPA treatment. MPA treatment did not affect pulse frequency, pulse amplitude, or AUC_b for either LH or FSH (Table 1).

Discussion

The tip of one uterine horn and the corresponding ovary had been removed for use as control tissues in another study. Nevertheless, all bitches had an oestrous cycle between the unilateral ovariectomy and the start of the MPA treatment. Chaffaux et al. (1981) demonstrated that basal plasma gonadotrophin levels in dogs and the response of these hormones to an intramuscular injection of a GnRH analogue are not affected by unilateral ovariectomy. Furthermore, the difference in plasma FSH concentration between intact and unilateral oophorectomized women was not significant (Cooper and Thorp 1999). Thus the dogs used in this study can be regarded as having an intact hypothalamic–pituitary–ovarian axis.

The prevention of oestrus in these bitches by MPA cannot be attributed to a reduction in plasma levels of either FSH or LH, as has been proposed by others (Kutzler and Wood 2006). On the contrary, MPA treatment caused a temporary increase in the basal plasma FSH level and the AUC_0 for FSH at 3 months and in the basal plasma LH level at 6 months of treatment. No progestin-induced changes in basal plasma gonadotrophin concentrations were reported in earlier studies (McCann et al. 1987, Colon et al. 1993). However, in the present longitudinal study the same dogs were tested before and several times during MPA treatment by mapping the pulsatile patterns of hormone secretion, which more accurately reflect hypothalamic-pituitary dynamics than do measurements of basal hormone concentration.

The increase in plasma gonadotrophin levels during MPA treatment may be due to a direct action of MPA on the ovary that suppresses ovarian hormone secretion. Ovarian hormones such as oestradiol and inhibin constitute an essential component of the negative feedback loop and a direct inhibitory effect of MPA on the ovary could explain the temporary elevation of circulating gonadotrophin concentrations. In women, progestins have also been reported to increase plasma gonadotrophin levels by inhibiting ovarian secretion of oestradiol

(Poindexter et al. 1993, Heikinheimo et al. 1996, Obruca et al. 2001). The observation in monkeys that the inhibitory effects of progesterone on follicular development persist even in the presence of elevated plasma FSH levels provides further support for the notion that progestins have a direct effect on the ovary (Goodman et al. 1982). In addition, in the male dog there are indications that the effect of progestins is the result of a direct action on the epididymal phase of sperm development, without suppression of plasma LH concentration (England 1997). After the increase at 3 and 6 months of MPA treatment, basal gonadotrophin secretion returned to pre-treatment levels. Thus, during prolonged treatment the contraceptive effect of MPA in dogs may also involve suppression of pituitary release of gonadotrophins.

	Before treatment	After 3 months	After 6 months	After 9 months	After 12 months
LH basal (µg/l)	1.54 ± 0.02	2.09 ± 0.28	2.42 ± 0.38[#]	1.77 ± 0.15	1.74 ± 0.13
FSH basal (µg/l)	6.69 ± 0.97	11.35 ± 1.97[*]	8.25 ± 1.55	6.71 ± 1.09	6.83 ± 0.75
LH peak amplitude (µg/l)	27.08 ± 8.87	15.19 ± 3.10	14.62 ± 1.96	16.83 ± 6.80	12.21 ± 1.65
FSH peak amplitude (µg/l)	9.98 ± 3.94	5.56 ± 0.49	4.69 ± 0.79	10.49 ± 5.26	4.76 ± 0.52
LH pulse frequency (peaks/6 h)	2 (0 – 2)	3 (2 – 4)	3 (2 – 4)	3 (2 – 4)	3 (2 – 4)
FSH pulse frequency (peaks/6 h)	2 (0 – 2)	2 (1 – 3)	2 (2 – 2)	2 (1 – 3)	2 (1 – 3)
AUC_b for LH (µg/l*6 h)	15.99 ± 5.95	16.37 ± 1.91	13.90 ± 2.71	16.60 ± 5.38	11.49 ± 0.57
AUC_b for FSH (µg/l*6 h)	11.17 ± 4.52	7.32 ± 1.34	6.81 ± 1.68	8.13 ± 3.90	5.34 ± 1.33
AUC_0 for LH (µg/l*6 h)	25.21 ± 5.95	28.82 ± 0.87	28.30 ± 3.75	27.21 ± 5.13	21.89 ± 1.17
AUC_0 for FSH (µg/l*6 h)	51.34 ± 1.09	75.38 ± 11.59[*]	56.27 ± 10.31	48.41 ± 8.17	46.38 ± 4.64

Table 1. Pulse characteristics for 6-h plasma profiles of FSH and LH in five healthy Beagle bitches before and 3, 6, 9, and 12 months after the start of treatment with medroxyprogesterone acetate (10 µg/kg, every 4 weeks). The values are expressed as mean ± SEM, except for pulse frequency, which is expressed as median and range. [#] significantly different from before treatment, [*] significantly different from before treatment and after 9 and 12 months of MPA treatment

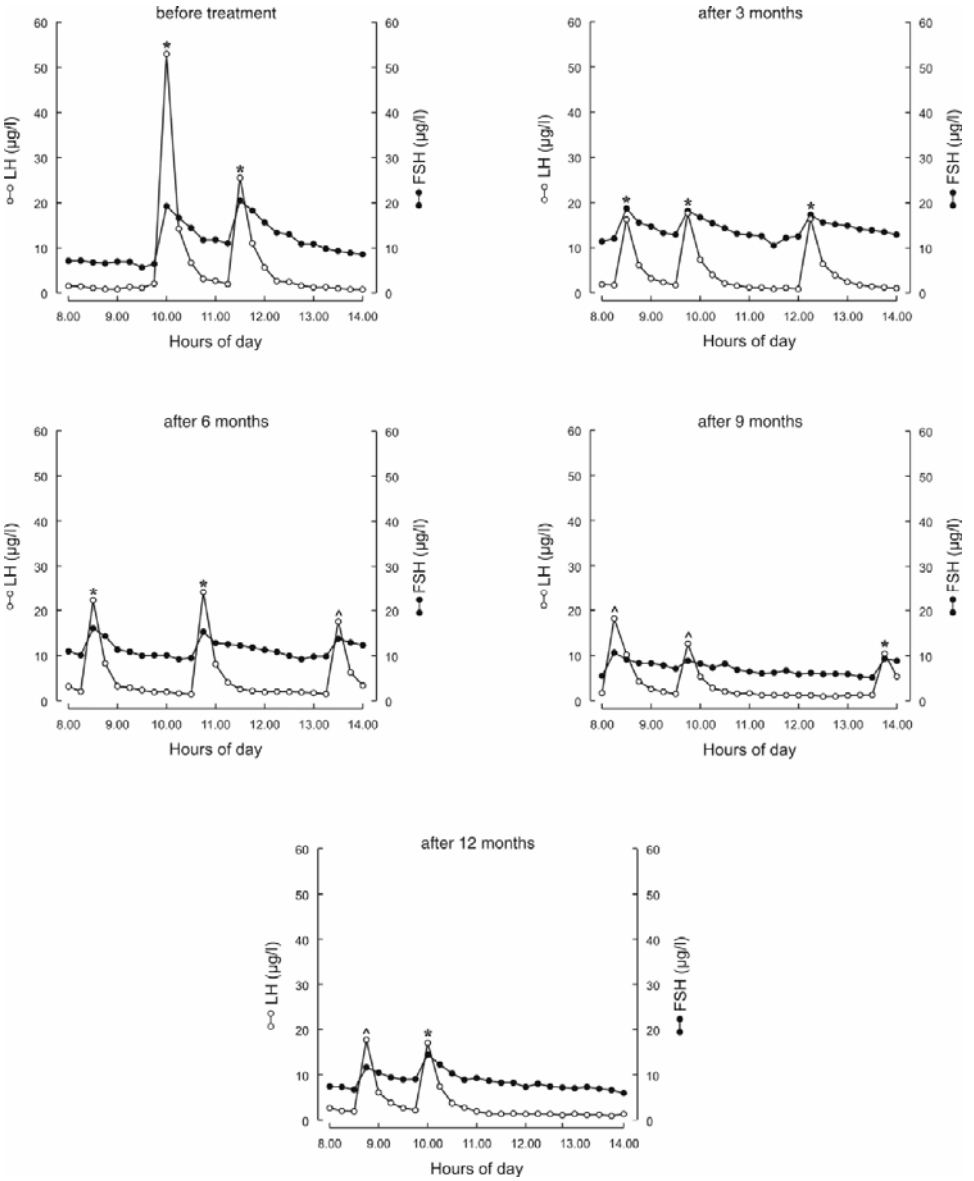

*Figure 1. The 6-h plasma profiles of FSH (black squares) and LH (white triangles) in a 3-year-old Beagle bitch before, and 3, 6, 9, and 12 months after the start of treatment with medroxyprogesterone acetate (10 µg/kg, every 4 weeks). *significant pulses of both FSH and LH identified by the Pulsar programme, ^significant pulse of LH without a concurrent significant pulse of FSH*

From the present study it is evident that pulsatile LH and FSH release is maintained during MPA treatment, but the results also indicate subtle changes in the pulsatile release of the gonadotrophins. A substantial number of significant LH pulses was detected by the Pulsar programme during MPA treatment, and although each coincided with an increase in plasma FSH concentration, several were too small to be recognized as significant FSH pulses. These observations suggest that MPA suppresses pituitary secretion of FSH. This phenomenon has also been observed during the physiological luteal phase (Kooistra et al. 1999). The decrease in pituitary release of FSH during MPA treatment may be caused by decreased pituitary sensitivity to endogenous GnRH pulses, as has been demonstrated by GnRH challenge tests (Beijerink et al. 2007).

In women, MPA is known to prevent ovulation by inhibiting the midcycle surge of FSH and LH, whereas the tonic release of these gonadotrophins is unchanged (Mishell DR Jr 1977, 1996, Jain et al. 2004) or slightly suppressed (Perez-Lopez et al. 1975). It has been hypothesized that in women MPA inhibits the positive feedback effect of oestradiol on the hypothalamic-pituitary axis, which normally stimulates the midcycle release of LH. The pituitary responsiveness of LH and FSH to suprapituitary stimulation does not change, suggesting that also in women the pituitary is not the primary site of inhibition of ovulation (Perez-Lopez et al. 1975, Mishell et al. 1977, Ismael et al. 1987).

It can be hypothesized that the oestrus-preventing effect of MPA is partly due to the intrinsic glucocorticoid activity of MPA. MPA is less specific than progesterone in its receptor binding, having affinity for both the progesterone receptor and the glucocorticoid receptor (Selman et al. 1996). In vivo experiments have shown that MPA has strong glucocorticoid agonistic properties, resulting in suppression of the hypothalamic-pituitary-adrenocortical axis (Selman et al. 1994, Beijerink et al. 2007). Suppression of the hypothalamic-pituitary-ovarian system by increased glucocorticoid activity is seen in the prolongation of anoestrus that is common in bitches with chronic cortisol excess (Rijnberk 1996, Feldman and Nelson 2004).

In conclusion, MPA treatment did not result in suppression of basal plasma gonadotrophin levels in the bitches. On the contrary, it caused a temporary rise in the basal concentration of both basal FSH and LH, attributable to a direct effect of MPA on the ovary. In addition, several LH pulses were not accompanied by a significant FSH pulse, suggesting that MPA treatment attenuated the pulsatile pituitary release of FSH.

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CHAPTER 9

Summarising discussion and conclusions

The oestrous cycle of the domestic bitch is unique with regard to its considerable length compared with that of most other domestic animals. The follicular phase and spontaneous ovulations are followed by a luteal phase having an average duration of about 2 months, irrespective of pregnancy. A non-seasonal anoestrus, with a duration that may last from 2 to 10 months, follows each oestrous cycle. The anoestrus can be prolonged by oestrus-preventing drugs such as progestins.

In the general introduction of this thesis (**Chapter 2**) an overview is given of pre-existing knowledge concerning 1) the hypothalamus-pituitary system, 2) the canine oestrous cycle, with a focus on the anoestrus, and 3) the endocrine effects of progestins used to prolong the duration of anoestrus. The general aim of the studies in this thesis was to get further insight into the endocrinology of the physiological (**Part I**) and the progestin-induced (**Part II**) canine anoestrus.

Studies performed throughout recent years have made clear that during the course of canine anoestrus many endocrinological changes take place at the hypothalamic, pituitary, and ovarian level (Concannon et al. 1986, Jeffcoate 1993, Van Haaften et al. 1994, Tani et al. 1996, 1997, 1999, Kooistra et al. 1999a, Inaba et al. 2002). Apart from these changes in the hypothalamus-pituitary-gonad axis, there are indications of involvement of dopaminergic influences. Administration of dopamine-2 receptor agonists results in lowering of the plasma prolactin concentration and shortening of the anoestrus (Okkens et al. 1985, Onclin et al. 1995). For this reason, it has been suggested that the shortening of the anoestrus by dopamine-agonists is the result of the suppression of prolactin secretion, as prolactin may inhibit gonadotrophin release (Sauder et al. 1984, Park et al. 1993, Yazigi et al., 1997). However, metergoline in a low dosage, which lowers prolactin as a serotonin antagonist, did not shorten the anoestrus (Okkens et al. 1997). The study described in **Chapter 3** is a report on the effect of different doses of the dopamine agonist bromocriptine on plasma prolactin concentration and the interoestrous interval. The results of this study provide forceful evidence that the bromocriptine-induced shortening of the interoestrous interval is not triggered by a decline in plasma prolactin concentration, since in bitches receiving a low dose of the dopamine agonist bromocriptine there was significant shortening of the interoestrous interval without a decrease in plasma prolactin concentration. In line with this, under physiological conditions plasma prolactin concentrations are low during canine anoestrus (Olson et al. 1982, Kooistra and Okkens 2001) and no obvious changes in plasma prolactin concentration have been observed during the transition from anoestrus to the follicular phase in the bitch (Olson et al. 1982).

Consequently, termination of anoestrus is probably due to some other dopamine-agonistic action of bromocriptine. The bromocriptine-induced shortening of the anoestrus may be ascribed to the effects of dopamine agonists on FSH secretion. Shortening of the interoestrous interval by bromocriptine in a dose that also lowers plasma prolactin concentration is associated with a premature increase in basal plasma FSH concentration without a concomitant increase in basal plasma LH concentration (Kooistra et al. 1999b). This is similar to the endocrine events described during late physiological anoestrus (Kooistra et al. 1999a).

Chapter 4 is a report on the effect of treatment with a low dose of the serotonin antagonist metergoline on the interoestrous interval, plasma prolactin concentration, and the 6-hour pulsatile plasma profiles of FSH and LH. In a high dose metergoline also has dopamine agonistic effects, whereas in a low dose metergoline only has serotonin antagonistic effects. The results of this study again show that administration of a low dose of the serotonin antagonist metergoline does not shorten the interoestrous interval, despite a decline in plasma prolactin levels after the start of the treatment. The plasma profiles of LH and FSH were similar to those observed during physiological anoestrus (Kooistra et al. 1999a), but different from those observed during anoestrus shortened by a dopamine agonist (Kooistra et al. 1999b). 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 FSH secretion.

Taken together, it can be concluded that not a decrease in circulating prolactin concentration, but an increase in circulating FSH should be considered a critical event required for ovarian folliculogenesis and consequently the termination of anoestrus in the bitch. In this respect there are similarities with the situation in women, in whom observations during gonadotrophin-induced ovulation have emphasized that plasma FSH concentrations must exceed a certain level before pre-antral 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 the plasma FSH concentration above a certain threshold, resulting in the induction of premature follicular development. Because the serotonin antagonist metergoline does not induce a premature increase in FSH secretion, an earlier than expected oestrus does not occur.

It can be difficult to verify the neuter status of dogs with an unknown reproductive status. In addition, it is occasionally difficult to differentiate ovariectomized (OVX) dogs having remnant ovarian tissue from completely OVX dogs. The study in **Chapter 5** is a report on the effects of GnRH administration on the plasma concentrations of reproductive hormones in intact and OVX bitches. The data presented in this study demonstrate that provocative testing of the pituitary-ovarian axis using GnRH may be helpful in differentiating between bitches in anoestrus and neutered bitches. First, basal plasma LH concentration was significantly higher in OVX bitches than in anoestrous bitches. Nevertheless, taking into account the pulsatile nature of LH release (Concannon 1993, Kooistra et al. 1999a), overlapping of plasma LH concentrations between intact and OVX bitches may be expected. In line with this expectation, the study of Löfstedt et al. (2002) demonstrated that a single high LH value measured by a commercial test kit was not reliable in indicating whether a bitch was intact.

Second, GnRH administration caused a significant rise in plasma oestradiol concentration only if ovarian tissue is present, indicating that determining the plasma concentrations of oestradiol before and after GnRH administration may be used to differentiate between anoestrous bitches with and without ovarian tissue. The results also demonstrated that

measurement of plasma progesterone and testosterone concentrations before and after GnRH administration does not aid in distinguishing between anoestrous and OVX bitches.

The study reported in **Chapter 6** describes the basal and GnRH-induced plasma concentrations of FSH and LH in four anoestrous and four OVX bitches. The GnRH-induced plasma LH concentrations in the anestrous bitches overlapped with the basal plasma LH concentrations in the OVX bitches, again indicating that a single plasma LH concentration cannot reliably differentiate between anoestrous and OVX bitches. However, in this first report on the effect of GnRH-challenge on the plasma FSH concentration in both anoestrous and OVX bitches, indications were found that measurement of the plasma FSH concentration by a newly available FSH IRMA in a single plasma sample may prove reliable for determination of neuter status. Even the maximal plasma FSH level after GnRH administration in the intact bitches did not exceed the lowest plasma FSH level during the entire GnRH-stimulation test in the OVX bitches. Other investigators have also suggested that the pituitary capacity to secrete FSH in the absence of ovarian negative feedback is far greater than that ever observed in the intact bitch (Olson et al. 1992, Concannon 1993). Such a difference could be expected if there is a specific inhibition of FSH as opposed to LH secretion in intact bitches, e.g. due to ovarian derived inhibin (Shupnik 1996). Also in women, the plasma FSH level usually increases more than the plasma LH level when the ovaries are functionally inactive or surgically removed (Bulun and Adashi 2003, Genuth 2004). This indicates that differences in circulating FSH concentration between intact and OVX bitches could be greater than within-group variability. Further studies with larger number of dogs are warranted to investigate the potential of a single plasma FSH concentration.

Taken together, the results of the studies described in **Chapters 5 and 6** provide a basis for the diagnosis of remnant ovarian tissue and verification of neuter status in the bitch.

Part II of this thesis comprises reports on the endocrinology of the progestin-induced canine anoestrus. Progestins, such as medroxyprogesterone acetate (MPA), are commonly used to prevent oestrus in the bitch. In the past decennia, several endocrinological effects and side-effects have been reported. For a summary of these effects in the bitch, the reader is referred to the general introduction of this thesis.

Chapters 7 and 8 are reports on the effect of MPA on canine adenohypophyseal function. In the first study five bitches were treated with MPA and their adenohypophyseal function was assessed in a combined adenohypophyseal function test (Meij et al. 1996) before and 2, 5, 8, and 11 months after the start of MPA treatment. MPA was administered subcutaneously at a dose of 10 mg/kg every four weeks. Blood samples for determination of the plasma concentrations of ACTH, cortisol, GH, IGF-I, LH, FSH, prolactin, α-MSH, and TSH were collected at -15, 0, 5, 10, 20, 30, and 45 min after suprapituitary stimulation. In the second study the 6-hour plasma profiles of FSH and LH were assessed in the same five bitches before and 3, 6, 9, and 12 months after the start of MPA treatment.

The results of both studies demonstrate that treatment with MPA affects the hypothalamic-pituitary-ovarian axis. Oestrus, ovulation, and a subsequent luteal phase did not occur in any of the bitches during treatment with MPA. The prevention of oestrus by MPA

cannot be ascribed to a significant reduction in circulating levels of either FSH or LH, as has been proposed by others (Kutzler and Wood 2006). On the contrary, during the first months of MPA treatment there was an increase in basal plasma FSH and LH. This progestin-induced increase in gonadotrophin concentration was not observed in previous studies (McCann et al. 1987, Colon et al. 1993), and its recognition may be explained by the repeated sampling employed in the present studies. The elevated gonadotrophin levels during the first months of MPA treatment may be due to a direct oestrus-preventing effect of MPA at the ovarian level, resulting in suppression of the ovarian secretion of oestradiol or inhibin (Mann et al. 1992, Shupnik 1996), or stimulation of activin release.

With continuing MPA treatment, basal plasma gonadotrophin concentrations returned to pretreatment levels and the pituitary FSH response to GnRH stimulation decreased (**Chapter 7**), while several LH pulses were not accompanied by a significant FSH pulse (**Chapter 8**), suggesting that MPA treatment attenuated pituitary FSH sensitivity to endogenous GnRH. These effects during continuing MPA treatment are probably part of the oestrus-preventing effects of MPA, because increased FSH secretion is a critical event in the initiation of ovarian folliculogenesis (Kooistra et al. 1999a,b). In other words, chronic MPA treatment for oestrus prevention may prohibit the normal rise in plasma FSH concentration during late anoestrus.

The aim of the study in **Chapter 7** was also to provide an integral picture of the effects of MPA treatment on adenohypophyseal function. The results confirmed previous findings that progestins alter the GH-IGF-I axis in the bitch (Eigenmann et al. 1983, Selman et al. 1994a). Basal plasma GH concentrations tended to increase gradually during the course of the MPA treatment, although this rise was not statistically significant. However, the significant increase in circulating IGF-I concentrations during MPA treatment in our study indicates indirectly excessive exposure to GH (Selman et al. 1994a). Plasma IGF-I concentrations may thus be a more sensitive indicator than plasma GH concentrations for the effect of progestin treatment on the GH-IGF-I axis.

Besides an interaction with the progesterone receptor, MPA also has a relatively high affinity for the glucocorticoid receptor (Selman et al. 1996). Consequently, suppression of the hypothalamic-pituitary-adrenocortical (HPA) axis was expected during MPA treatment, as has been reported before (Selman et al. 1994b, 1996). However, the results of the study reported in **Chapter 7** indicate that the effects on ACTH secretion characteristics were limited. Because the supra-pituitary stimulation test was carried out four weeks after the injection of MPA, ACTH release most likely had returned to pre-treatment values within this timeframe. In contrast, the adrenocortical component of the HPA axis, the maximal cortisol increment and the AUC for cortisol after suprapituitary stimulation, were significantly lower during MPA treatment than prior to treatment, which was comparable to previous observations (Selman et al. 1994b). Apparently, the suppression of the ACTH secretion was severe enough to cause atrophy of the adrenocortical zona fasciculata. It can also be hypothesized that the oestrus-preventing effect of MPA is partly due to the intrinsic glucocorticoid activity of MPA. MPA is less specific than progesterone in its receptor binding, having affinity for both the progesterone receptor and the glucocorticoid receptor (Selman et al. 1996). Suppression of the

hypothalamic-pituitary-ovarian system by increased glucocorticoid activity, i.e., prolonged anoestrus, is also seen in bitches with chronic cortisol excess (Rijnberk 1996).

The basal plasma TSH concentrations were elevated at 8 months after the start of the MPA treatment, although they were still within the reference range for TSH in our laboratory. Our results conflict with those of others, who found no effect of MPA treatment on mean circulating TSH concentrations (Frank et al. 1979). One may speculate that MPA had a direct effect on the thyroid gland as a result of its inherent glucocorticoid properties, leading to a slight rise of the plasma TSH concentration (Kemppainen et al. 1983).

No changes in prolactin or α-melanocyte-stimulating hormone secretion were observed. The absence of an effect of MPA treatment on plasma prolactin concentrations is in agreement with previous studies (Concannon et al. 1980, Rutteman et al. 1987) and may be explained by the absence of a clear-cut decrease in progestational activity, which is known to be a trigger of prolactin release (Galac et al. 2000).

The following conclusions can be drawn:

- Bromocriptine-induced shortening of the interoestrous interval is partly dose dependent and involves a dopamine-agonistic mechanism other than via the lowering of plasma prolactin concentration.
- Serotonin antagonist-induced lowering of plasma prolactin concentration is not associated with shortening of the interoestrous interval. The pulsatile plasma profiles of LH and FSH are 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 GnRH-induced rise in plasma oestradiol concentration can be used to verify whether ovarian tissue is present in a bitch.
- Since the maximum plasma FSH level after GnRH administration in intact bitches did not exceed the lowest plasma FSH level during the entire GnRH-stimulation test in ovariectomized bitches, a single plasma FSH measurement may provide reliable information on the neuter status of a bitch.
- The prevention of oestrus by MPA cannot be ascribed to a significant reduction in circulating levels of either FSH or LH.
- Pulsatile LH and FSH release is maintained during MPA treatment, but with continuing MPA treatment, pituitary FSH sensitivity and/or release to GnRH is attenuated.
- The elevated basal plasma gonadotrophin levels during the first months of MPA treatment may be due to a direct oestrus-preventing effect of MPA at the ovarian level.

- The effects of chronic MPA treatment on adenohypophyseal function also include activation of the mammary GH-induced IGF-I secretion, slightly activated TSH secretion, suppression of the hypothalamic-pituitary-adrenocortical axis, and unaffected secretion of prolactin and α-MSH.

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CHAPTER 10

Samenvatting en conclusies

De oestrische cyclus van de hond is door haar aanzienlijke lengte uniek in vergelijking met die van veel andere dieren. De folliculaire fase en ovulatie worden gevolgd door een luteale fase, die gemiddeld twee maanden duurt en die niet door het optreden van een eventuele dracht beïnvloed wordt. Een niet-seizoensgebonden anoestrus van 2 tot 10 maanden volgt op elke oestrische cyclus. Deze anoestrus kan aanzienlijk verlengd worden door toediening van oestrus-preventieve middelen, zoals progestagenen.

In de algemene introductie van dit proefschrift (**Hoofdstuk 2**) is een overzicht gegeven van reeds bekende inzichten in 1) de werking van het hypothalamus-hypofyse systeem, 2) de oestrische cyclus van de hond, met nadruk op de anoestrus, en 3) de hormonale effecten van progestagenen, die gebruikt worden om de duur van de anoestrus te verlengen. Het doel van de studies die zijn beschreven in dit proefschrift was om meer inzicht te verkrijgen in de endocrinologie van de fysiologische (**Deel I**) en de door progestagenen geïnduceerde (**Deel II**) anoestrus van de hond.

De laatste decennia is duidelijk geworden dat tijdens het verloop van de anoestrus vele hormonale veranderingen plaatsvinden op het niveau van de hypothalamus, de hypofyse, en de ovaria (Concannon et al. 1986, Jeffcoate 1993, Van Haaften et al. 1994, Tani et al. 1996, 1997, 1999, Kooistra et al. 1999a, Inaba et al. 2002). Los van deze veranderingen in de hypothalamus-hypofyse-ovarium-as, zijn er aanwijzingen voor betrokkenheid van het dopaminerge systeem, dat onder andere de prolactine-secretie reguleert. Toediening van dopamine-2 receptor agonisten resulteert namelijk in een verlaging van de plasmaconcentraties van prolactine en een verkorting van de anoestrus (Okkens et al. 1985, Onclin et al. 1995). Dit leidde tot de hypothese dat de verkorting van de anoestrus door dopamine-agonisten het gevolg is van onderdrukking van de prolactinesecretie, omdat prolactine de afgifte van de gonadotrofe hormonen, luteïniserend hormoon (LH) en follikel stimulerend hormoon (FSH), kan remmen (Sauder et al. 1984, Park et al. 1993, Yazigi et al. 1997). Metergoline, in een lage dosering werkzaam als een serotonine-antagonist, onderdrukt echter ook de plasmaconcentraties van prolactine, terwijl de duur van de anoestrus niet wordt verkort (Okkens et al. 1997). **Hoofdstuk 3** beschrijft het effect van verschillende doseringen van de dopamine-agonist bromocriptine op de plasmaconcentraties van prolactine en de duur van het interoestrus interval. De resultaten van deze studie leveren extra bewijs dat de door bromocriptine geïnduceerde verkorting van de anoestrus niet wordt veroorzaakt door een verlaging van de prolactine-afgifte, omdat ook honden die een lage dosering van de dopamine-agonist toegediend kregen een significante verkorting van de anoestrus vertoonden zonder dat er van een verlaging van de plasmaconcentraties van prolactine sprake was. In overeenstemming hiermee zijn plasmaconcentraties van prolactine laag tijdens de fysiologische anoestrus (Olson et al. 1982, Kooistra en Okkens 2001) en zijn er geen duidelijke veranderingen in de plasmaconcentraties van prolactine waargenomen tijdens de overgang van anoestrus naar een nieuwe folliculaire fase (Olson et al. 1982).

De beëindiging van de anoestrus moet daarom veroorzaakt worden door een ander dopamine-agonistisch effect van bromocriptine. De door bromocriptine veroorzaakte verkorting van de anoestrus zou verklaard kunnen worden door effecten op de secretie van

FSH. Verkorting van de anoestrus door bromocriptine gaat namelijk naast een verlaging van de plasmaconcentraties van prolactine ook gepaard met een vroegtijdige toename van de basale plasmaconcentratie van FSH, terwijl de basale plasma LH concentratie niet toeneemt (Kooistra et al. 1999b). Deze toename van de basale plasmaconcentratie van FSH wordt ook waargenomen tijdens de late fysiologische anoestrus (Kooistra et al. 1999a).

Om dit verder te onderzoeken is in **Hoofdstuk 4** het effect onderzocht van een behandeling met een lage dosering van de serotonine-antagonist metergoline op de duur van de anoestrus, de plasmaconcentratie van prolactine, en de pulsatiele afgifte van FSH en LH. In een hoge dosering heeft metergoline tevens dopamine-agonistische effecten, terwijl in een lage dosering metergoline alleen serotonine-antagonistische effecten heeft. De resultaten van deze studie laten wederom zien dat de toediening van een lage dosering metergoline de anoestrus niet verkort, ondanks een afname van de plasmaconcentraties van prolactine. De pulsatiele plasmaprofielen van LH en FSH waren vergelijkbaar met die zoals ze tijdens het voortschrijden van de fysiologische anoestrus werden waargenomen (Kooistra et al. 1999a), maar verschillend van de patronen waargenomen tijdens de anoestrus verkort door dopamine-agonisten (Kooistra et al. 1999b). De vervroegde inductie van de oestrus door dopamine-agonisten kan daarom niet worden veroorzaakt door een daling in de plasmaconcentraties van prolactine, maar is het gevolg van een ander dopamine-agonistisch effect zoals een stimulatie van de FSH-secretie.

Samenvattend kan worden geconcludeerd dat niet een afname van de plasmaconcentratie van prolactine, maar een toename van de FSH-secretie moet worden beschouwd als de oorzaak voor follikelontwikkeling en premature oestrusinductie bij behandeling met dopamine-agonisten. Met betrekking tot dit punt zijn er opmerkelijke overeenkomsten met de situatie bij de vrouw. Bij vrouwen waarbij een ovulatie werd opgewekt met behulp van gonadotrofe hormonen is gebleken dat het plasma-FSH een bepaalde concentratie moet overschrijden voordat preantrale follikels, die het FSH-gevoelige stadium hebben bereikt, zich verder kunnen ontwikkelen (Brown 1978, Schoemaker et al. 1993). De resultaten van dit onderzoek leiden tot de hypothese dat dopamine-agonisten bij de hond een vervroegde toename van de FSH concentratie boven een bepaalde drempelwaarde bewerkstelligen, resulterend in een vervroegde follikelontwikkeling. Omdat de toediening van de serotonine-antagonist metergoline niet een vervroegde toename van de FSH-secretie veroorzaakt, vindt er geen versnelde follikelontwikkeling plaats.

Het kan erg lastig zijn om te achterhalen of een hond met een onbekende geschiedenis qua cyclus is geovariëctomeerd of niet. Daarnaast kan het moeilijk zijn om zekerheid te verkrijgen of resterend ovariëel weefsel is achtergebleven in eerder geovariëctomeerde honden. De studie beschreven in **Hoofdstuk 5** beschrijft de effecten van de toediening van gonadotrofine releasing hormoon (GnRH) op de plasmaconcentraties van verschillende voortplantingshormonen in intacte anoestrische en compleet geovariëctomeerde honden. De resultaten laten zien dat stimulatie van de hypofyse-ovarium-as met behulp van GnRH erg nuttig kan zijn in het onderscheiden van intacte anoestrische en geovariëctomeerde teven. De

basale plasma-LH concentratie was significant hoger in de geovariëctomeerde honden in vergelijking met de intacte anoestrische honden. Omdat LH pulsatiel wordt afgegeven (Concannon 1993, Kooistra et al. 1999a), kan echter een overlap van plasma-LH concentraties tussen intacte en geovariëctomeerde honden worden verwacht. In een andere studie bleek ook dat het meten van een enkele plasma-LH waarde (met een commerciële test kit) niet betrouwbaar was in het bepalen of de hond al dan niet intact was (Löfstedt en Vanleeuwen 2002).

Het toedienen van GnRH veroorzaakte voorts alleen een significante stijging in de plasmaconcentratie van oestradiol als er ovariëel weefsel aanwezig was. Dit geeft aan dat het bepalen van de plasmaconcentratie van oestradiol voor en na GnRH-toediening gebruikt kan worden om dieren met en zonder ovariëel weefsel van elkaar te onderscheiden. Daarnaast bleek dat het meten van de plasmaconcentraties van progesteron en testosteron voor en na GnRH-toediening niet nuttig is om intacte anoestrische en geovariëctomeerde honden te onderscheiden.

In **Hoofdstuk 6** zijn de basale en GnRH-geïnduceerde plasmaconcentraties van FSH en LH in vier anoestrische en vier geovariëctomeerde honden beschreven. De GnRH-geïnduceerde piek in de plasmaconcentratie van LH in de anoestrische honden overlapte de basale plasmaconcentratie van LH in de geovariëctomeerde honden, hiermee opnieuw duidelijk makend dat een enkele plasma-LH bepaling niet betrouwbaar is om onderscheid te maken tussen anoestrische en geovariëctomeerde honden. De basale en GnRH-geïnduceerde plasmaconcentraties van FSH in anoestrische en geovariëctomeerde honden bleken echter geen overlap te vertonen. Mogelijk zal in de toekomst een enkele bepaling van de plasmaconcentratie van FSH, gemeten met de onlangs ontwikkelde FSH IRMA, kunnen aantonen of een hond intact is of niet. De capaciteit van de hypofyse om FSH te secerneren in de afwezigheid van negatieve terugkoppeling van de ovaria blijkt veel groter te zijn dan de capaciteit die is waargenomen in intacte teven (Olson et al. 1992, Concannon 1993). Zo'n verschil kan worden verwacht als er een specifieke onderdrukking van de FSH- ten opzichte van de LH-afgifte in intacte dieren is, zoals bijvoorbeeld als gevolg van het door het door de ovaria geproduceerde hormoon inhibine (Shupnik 1996). Ook bij vrouwen stijgt de plasmaconcentratie van FSH doorgaans meer dan de plasmaconcentratie van LH als de ovaria inactief danwel verwijderd zijn (Bulun en Adashi 2003, Genuth 2004). Dit geeft aan dat de verschillen in circulerende FSH concentraties tussen intacte en geovariëctomeerde dieren groter zouden kunnen zijn dan de variatie binnen de afzonderlijke groepen. Studies met grotere aantallen honden zijn nodig om te bepalen hoe bruikbaar een enkele bepaling van de plasmaconcentratie van FSH voor dit doel is.

Samenvattend, de resultaten van de studies in de **Hoofdstukken 5 en 6** vormen een basis voor de diagnose van rest-ovariëel weefsel en voor de verificatie van het al dan niet geovariëctomeerd zijn van een hond.

In **Deel II** van dit proefschrift zijn studies beschreven betreffende de endocrinologie van de door progestagenen geïnduceerde anoestrus. Progestagenen, zoals medroxyprogesteron

acetaat (MPA), worden regelmatig voor oestruspreventie bij teven gebruikt. In de laatste decennia zijn verschillende endocrinologische effecten en neveneffecten gerapporteerd. Voor een samenvatting van deze effecten bij de hond wordt de lezer verwezen naar de algemene introductie van dit proefschrift.

In **Hoofdstuk 7 en 8** worden de effecten van MPA op de functie van de hypofysevoorkwab bij de hond beschreven. In de eerste studie werden bij vijf teven de effecten van supra-hypofysaire stimulatie onderzocht, waarbij gebruikt gemaakt werd van een gecombineerde functietest voor de hypofysevoorkwab (Meij et al. 1996), uitgevoerd vóór en 2, 5, 8, en 11 maanden na de start van de behandeling met MPA. MPA werd iedere vier weken onderhuids toegediend in een dosering van 10 mg/kg. Bloedmonsters voor de bepaling van de plasma concentraties van het bijnierschorsstimulerend hormoon (ACTH), cortisol, groeihormoon (GH), insuline-achtige groeifactor-I (IGF-I), LH, FSH, prolactine, en schildklierstimulerend hormoon (TSH) werden verzameld op -15, 0, 5, 10, 20, 30, en 45 minuten na supra-hypofysaire stimulatie. De functie van de pars intermedia van de hypofyse werd onderzocht door bepaling van de basale concentratie van plasma α-melanocyt-stimulerend hormoon (α-MSH). In de tweede studie werden de pulsatiele plasmaprofielen van FSH en LH in dezelfde 5 honden vóór, en 3, 6, 9, en 12 maanden na de start van de behandeling met MPA bepaald.

De resultaten van beide studies tonen aan dat de behandeling met MPA effecten heeft op de hypothalamus-hypofyse-ovarium-as. Oestrus, ovulatie, en een daaropvolgende luteale fase werden gedurende de behandeling met MPA niet waargenomen. Oestruspreventie door middel van MPA kon bij onze honden niet worden toegeschreven aan een significante afname van de plasmaconcentraties van FSH of LH, zoals door anderen is gesuggereerd (Kutzler en Wood 2006). Sterker nog, gedurende de eerste maanden werd de MPA behandeling gekenmerkt door een toename van de basale plasmaconcentraties van FSH en LH. Deze door progestagenen geïnduceerde toename in de concentraties van gonadotrofe hormonen werd niet gezien in eerdere studies (McCann et al. 1987, Colon et al. 1993), wat verklaard kan worden door de frequentere en herhaalde bloedafnames die zijn toegepast in de huidige studies. De toegenomen plasmaconcentraties van FSH en LH gedurende de eerste maanden van MPA behandeling zijn mogelijk het gevolg van een direct effect van MPA op het niveau van het ovarium, resulterend in een vermindering van de ovariële oestradiol- en/of inhibinesecretie, of stimulatie van de activine-afgifte (Mann et al. 1992, Shupnik 1996).

Bij voortgaande MPA-behandeling namen de basale plasma FSH- en LH-concentraties weer af tot hun niveau van voor de MPA-behandeling, terwijl de hypofysaire FSH-respons op GnRH-stimulatie afnam (**Hoofdstuk 7**). Ook werd bij verscheidene LH-pulsen geen gelijktijdige significante FSH-puls waargenomen (**Hoofdstuk 8**), suggererend dat MPA-behandeling de gevoeligheid van de hypofysevoorkwab om FSH af te geven als reactie op GnRH vermindert. Deze effecten van MPA-behandeling zijn zeer waarschijnlijk belangrijk voor de oestrus-preventieve werking van MPA, omdat een toename van de FSH-secretie een voorwaarde is voor folliculogenese (Kooistra et al. 1999a,b). In andere woorden, het gedurende

meerdere maanden onder invloed staan van MPA voorkomt mogelijk de normale toename van de plasma-FSH concentratie tijdens de progressie van de anoestrus.

Het doel van de studie in **Hoofdstuk 7** was tevens het verschaffen van een integraal beeld van de effecten van MPA-behandeling op de functie van de hypofysevoorkwab. De resultaten bevestigen eerdere bevindingen dat progestagenen de GH-IGF-I-as beïnvloeden (Eigenmann et al. 1983, Selman et al. 1994a). Basale plasmaconcentraties van GH stegen geleidelijk gedurende de MPA-behandeling, alhoewel de stijging niet statistisch significant was. De significante stijging van circulerend IGF-I gedurende de MPA-behandeling in ons onderzoek impliceert echter indirect de overmatige blootstelling aan GH (Selman et al. 1994a). Plasmaconcentraties van IGF-I zijn dus mogelijk een meer gevoelige indicator dan plasmaconcentraties van GH voor het in kaart brengen van de effecten van progestagenen op de GH-IGF-I-as.

Naast een interactie met de progesteronreceptor heeft MPA ook een relatief hoge affiniteit voor de glucocorticoïdreceptor (Selman et al. 1996). Daarom werd een onderdrukking van de hypothalamus-hypofyse-bijnierschors-as gedurende MPA-behandeling verwacht, zoals dit ook al eerder was aangetoond (Selman et al. 1994b, 1996). De resultaten van de studie beschreven in **Hoofdstuk 7** laten echter zien dat de effecten op de ACTH-secretie gedurende MPA-behandeling beperkt bleven. Mogelijk had de ACTH-secretie zich intussen hersteld, omdat de supra-hypofysaire stimulatie ongeveer 4 weken na injectie van MPA uitgevoerd werd. De onderdrukking van de cortisolsecretie kon wel worden aangetoond en was vergelijkbaar met resultaten in een vorige studie (Selman et al. 1994b). Blijkbaar was de onderdrukking van de ACTH-secretie in de weken direct na MPA toediening sterk genoeg om aanleiding te geven tot atrofie van de zona fasciculata van de bijnierschors.

Een andere hypothese voor het effect van oestrus-preventie door MPA is dat het gedeeltelijk veroorzaakt wordt door de intrinsieke glucocorticoïde activiteit van MPA. MPA is minder specifiek dan progesteron in de receptorbinding en heeft een affiniteit voor zowel de progesteronreceptor als de glucocorticoïdreceptor (Selman et al. 1996). Onderdrukking van het hypothalamus-hypofyse-ovarium-systeem door verhoogde glucocorticoïd activiteit met als gevolg een verlenging van de anoestrus, wordt namelijk ook gezien bij honden met een chronische overmaat aan cortisol door het syndroom van Cushing (Rijnberk 1996).

De basale plasmaconcentraties van TSH waren 8 maanden na MPA-behandeling gestegen, maar ze bleven toch binnen de referentiewaarden van ons laboratorium. Onze resultaten zijn in tegenspraak met die van anderen, die geen effect van MPA-behandeling op de circulerende TSH-concentratie hebben gevonden (Frank et al. 1979). Mogelijk heeft MPA door de intrinsieke glucocorticoïde werking een direct effect op de schildklier, hetgeen via terugkoppeling op de hypofyse een lichte stijging van de plasmaconcentraties van TSH tot gevolg zou kunnen hebben (Kemppainen et al. 1983).

MPA-behandeling veroorzaakte geen veranderingen in de secretie van prolactine of α-melanocyt-stimulerend hormoon. Dat MPA geen effect heeft op de plasmaconcentraties van prolactine is in overeenstemming met bevindingen uit eerdere studies (Concannon et al. 1980, Rutteman et al. 1987) en kan worden verklaard doordat er geen duidelijke afname van

progestagene activiteit was. Van dergelijke abrupte dalingen is namelijk bekend dat ze leiden tot verhoogde afgifte van prolactine (Galac et al. 2000).

De volgende conclusies kunnen worden getrokken:

- Bromocriptine-geïnduceerde verkorting van de anoestrus is gedeeltelijk dosisafhankelijk en behelst een dopamine-agonistisch mechanisme dat niet via een verlaging van de plasmaconcentratie van prolactine werkt.
- Als de plasmaconcentratie van prolactine wordt verlaagd door toediening van de serotonine-antagonist metergoline wordt er geen verkorting van de anoestrus waargenomen. De pulsatiele plasmaprofielen van LH en FSH zijn tijdens deze behandeling vergelijkbaar met de profielen gedurende het verloop van de fysiologische anoestrus, maar verschillend van de profielen tijdens de anoestrus verkort door dopamine-agonisten. Daarom kan de vervroegd optredende oestrus gedurende de behandeling met dopamine-agonisten niet verklaard worden door een daling van de plasmaconcentratie van prolactine, maar moet een gevolg zijn van een ander dopamine-agonistisch effect, zoals een toename van de basale FSH secretie.
- De GnRH-geïnduceerde stijging in de plasmaconcentratie van oestradiol kan gebruikt worden om na te gaan of ovariëel weefsel aanwezig is in een vrouwelijke hond.
- Een enkele bepaling van de plasmaconcentratie van FSH kan mogelijk zeer betrouwbare informatie leveren over het al dan niet geovariëctomeerd zijn van een hond, omdat in onze studie de hoogst gemeten plasmaconcentratie van FSH na GnRH-toediening in intacte teven de laagst gemeten plasmaconcentratie van FSH in geovariëctomeerde teven niet oversteeg.
- De preventie van oestrus door MPA kan niet toegeschreven worden aan een significante daling in de circulerende plasmaconcentraties van FSH of LH.
- De pulsatiele afgifte van LH en FSH wordt gehandhaafd gedurende MPA-behandeling. Met het voortschrijden van de behandeling neemt echter wel de gevoeligheid van de hypofysevoorkwab voor GnRH en/of de afgifte van FSH na een GnRH-puls af.
- De verhoogde basale plasmaconcentraties van de gonadotrofe hormonen gedurende de eerste maanden van MPA-behandeling zouden verklaard kunnen worden door een direct oestrus-preventief effect van MPA op het niveau van de ovaria.
- De effecten van langdurige MPA-behandeling op de functie van de hypofysevoorkwab behelzen ook de activatie van mammair GH-geïnduceerde IGF-I secretie, licht gestegen TSH secretie, onderdrukking van de hypothalamus-hypofyse-bijnierschors-as, en een onveranderde afgifte van prolactine en α-MSH.

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Curriculum Vitae

De schrijver van dit proefschrift werd op 3 maart 1979 geboren in Tubbergen. Na het behalen van het VWO-diploma aan de Rooms-katholieke Scholengemeenschap St.-Canisius te Almelo in 1997, startte de schrijver met de studie Diergeneeskunde aan de Faculteit Diergeneeskunde van de Universiteit Utrecht. Gedurende zijn studie was hij lid van de studentenvereniging Veritas, het Veterinair Dispuut Veritas, en de Veterinaire Studenten Kegelclub 'Duim in 't Gat'. In 2001 nam hij deel aan het 'Excellent tracé programma', waarbij hij gedurende 15 maanden onderzoek heeft verricht naar de regulatie van de anoestrus bij de hond bij de Hoofdafdeling Geneeskunde van Gezelschapsdieren van de Universiteit Utrecht. Deze onderzoeksperiode werd afgerond met het diploma 'Master of Veterinary Research'. De studie Diergeneeskunde werd in 2005 voltooid. Hierna werd de schrijver gedurende 6 maanden aangesteld als AIO bij de Hoofdafdeling Geneeskunde van Gezelschapsdieren. Aansluitend volgde hij aldaar een algemene klinische roulatie (2005-2006) en vanaf september 2006 is de schrijver daar werkzaam als Specialist in Opleiding Interne Geneeskunde van Gezelschapsdieren. De schrijver is in augustus 2006 getrouwd met Maureen Koopmans.

The author of this thesis was born in Tubbergen, The Netherlands, on March 3, 1979. After finishing his secondary school education (Rooms-Katholieke Scholengemeenschap St.-Canisius Almelo) in 1997, he started to study veterinary medicine at the Faculty of Veterinary Medicine of Utrecht University. During his study, he was a member of the students association Veritas, Veterinair Dispuut Veritas, and the Veterinaire Studenten Kegelclub 'Duim in 't Gat'. In 2001 he was invited to participate in the Faculty's 'Excellent track' research programme. At the Department of Clinical Sciences of Companion Animals he investigated the regulation of the anoestrus in the dog for 15 months, and received the MVSc degree. After graduating as a DVM in 2005, he was a PhD-student for six months at the Department of Clinical Sciences of Companion Animals. Thereafter, the author followed an internship in companion animal medicine (2005-2006). From September 2006 onwards a residency in companion animal internal medicine was started. In August 2006 the author married to Maureen Koopmans.

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