Reproductive endocrinology of the dog

Effects of medical and surgical intervention

Jeffrey de Gier

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Reproductive endocrinology of the dog

Effects of medical and surgical intervention

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Effecten van medicamenteus en chirurgisch ingrijpen

(met een samenvatting in het Nederlands)

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Voor mijn ouders

Contents

1

Aims and scope of the thesis

The reproductive cycle of the domestic bitch *(Canis lupus familiaris)*, a mono-oestrous species, is characterized by a follicular phase with spontaneous ovulations, followed by pregnancy lasting 58-65 days or a non-pregnant luteal phase of about 75 days, and a non-seasonal anoestrus of 2-10 months. In the domestic male dog, spermatogenesis is initiated at puberty and is continuous from about 6 months of age onwards. Both the reproductive cycle and spermatogenesis are physiologically controlled by an integrated regulatory network: the hypothalamic-pituitary-gonadal axis (HPG axis). The gonadotrophic cells in the anterior lobe of the pituitary produce and secrete follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in a pulsatile fashion. The granulosa and theca cells in the ovary produce and secrete hormones such as oestradiol, testosterone, progesterone, and inhibin in response to FSH and LH. The types and amounts of reproductive hormones released in females are subject to cyclic changes in the functional status of the ovarian follicles and corpora lutea. Leydig cells and Sertoli cells in the testis secrete oestradiol, testosterone, and inhibin in response to both gonadotrophins. In both males and females, these gonadal hormones provide feedback to the HPG axis.

In the general introduction of this thesis **(Chapter 2)** a summary is given of the nomenclature and functional aspects of the different parts of the pituitary-gonadal axis in female and male dogs, with emphasis on changes during the different phases of the oestrous cycle and current knowledge about medical and surgical interventions.

Information concerning the hormonal changes during the follicular, ovulatory, and early luteal phase in the bitch is generally based on low-frequency sampling schemes. In the study described in **Chapter 3** several unresolved questions concerning hormonal changes around the time of ovulation are addressed. For example, the exact temporal relation between the preovulatory rise in plasma progesterone concentration and the preovulatory LH surge in the bitch is uncertain, which precludes use of an evidence-based choice for the measurement of either hormone in predicting the ovulation period. There is also debate about the role of oestradiol in relation to the initiation of the preovulatory LH surge in the bitch. During the follicular phase, plasma oestradiol-17β concentration increases gradually, peak concentration being reached around the start of the preovulatory LH surge. Oestradiol-17β exerts a negative feedback effect on the secretion of the gonadotrophic hormones during most stages of the oestrous cycle. However, in most mammals there is evidence that the preovulatory LH surge is triggered by the preovulatory oestradiol-17β surge, indicating a temporary positive feedback effect. Furthermore, the possible occurrence and relevance of a preovulatory prolactin surge in bitches, as has been reported in other mammalian species, remains to be elucidated. Finally, information concerning the plasma α-melanocyte-stimulating hormone concentration during the follicular, ovulatory, and early luteal phase is lacking in the bitch. In rats, melanocortins such as α-melanocyte-stimulating hormone mediate the preovulatory surges of LH and PRL. Also in women α-melanocyte-stimulating hormone has been reported to stimulate LH release.

There is little information about the temporal relation between the preovulatory LH and FSH surges in the bitch. Although it is known that each FSH pulse occurs concomitantly with an LH pulse in all stages of the oestrous cycle and in anoestrus, differential regulation of FSH and LH has been reported in dogs. Therefore, the aim of the study described in **Chapter 4**

was to describe in detail the temporal relation between plasma concentrations of LH and FSH around the time of ovulation in the bitch by measuring these plasma gonadotrophins in individual animals.

There are several clinical applications for evaluation of the pituitary-gonadal axis in dogs, such as to confirm the suspected presence of remnant gonadal tissue in dogs in which gonadectomy has been performed and to evaluate disorders of sexual development. The pulsatile pattern of secretion of the gonadotrophins considerably reduces the worth of hormone measurements in a single blood sample. Instead, a provocation test of the pituitary-gonadal axis using GnRH has been advocated, but appropriate studies of this are scarce. Therefore, the aim of the study reported in **Chapter 5** was to obtain more insight into the effects of GnRH administration on plasma concentrations of FSH, LH, oestradiol, and testosterone in anoestrous bitches and male dogs of different breeds, before and after gonadectomy.

Surgical castration of male dogs is a routine procedure in veterinary medicine for a variety of elective and medical indications. Recently, it has become possible to chemically castrate male dogs using a GnRH agonist such as deslorelin. The continuous administration of a GnRH agonist, as opposed to the physiological pulsatile secretion of hypothalamic GnRH, results in down-regulation of the GnRH receptors and desensitization of the pituitary gonadotrophs. Consequently, basal plasma concentrations of LH, FSH, oestradiol, and testosterone decline, followed by arrest of spermatogenesis. In the study described in **Chapter 6** we compared the effects of surgical and chemical castration on the pituitary-testicular axis by means of GnRH-stimulation tests.

In the dog, corpora lutea are the sole source of progesterone, which is essential for maintaining pregnancy. Ovarian progesterone production in the dog is independent of pituitary support during the first half of the luteal phase or gestation. However, prolactin plays an important role in maintenance of the corpora lutea during the second half of the luteal phase. Most methods for terminating unwanted pregnancy in the bitch utilise interference with the pregnancy-maintaining influence of progesterone. This can be done by inducing luteolysis with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) or the dopamine agonist bromocriptine. A more direct way of interfering with the pregnancy-maintaining effects of progesterone is to administer a competitive antagonist of the progesterone receptor, such as aglepristone, which is also effective in terminating unwanted pregnancy during the pituitary-independent part of the luteal phase.

The significant decline in plasma progesterone prior to the onset of spontaneous parturition is considered to be essential for normal whelping. Since the triggering mechanism for parturition remains unclear, it has been difficult to select or develop drugs which are useful for the induction of parturition in the dog. Ideally, such a drug should induce whelping with a high efficiency and within a predictable, short interval after administration. In addition, it should induce a normal parturition without side effects, making it safe for both the bitch and the puppies. Because of their anti-progesterone effect, progesterone-receptor blockers such as aglepristone and mifepristone have been widely investigated for their use as abortifacients. Progesterone-receptor blockers may also be useful for the induction of whelping. The aim of the study reported in **Chapter 7** was to evaluate the efficacy and safety of the progesterone-

receptor blocker aglepristone for inducing parturition in the bitch. Additionally, in the study described in **Chapter 8** the changes in plasma concentrations of 15-ketodihydroprostaglandin-$F_{2\alpha}$ (PGFM), cortisol, ACTH, LH, FSH, prolactin, progesterone, and oestradiol around the time of parturition in spontaneously whelping bitches were compared with those in bitches in which parturition was induced with aglepristone on day 58 of pregnancy.

Inhibition of progesterone synthesis with a competitive inhibitor of the 3β-hydroxysteroid dehydrogenase/isomerase system (3β-HSD) might be an alternative method to terminate unwanted pregnancy. 3β-HSD expression has been demonstrated in canine luteal cells throughout the luteal phase and inhibition of progesterone synthesis by the 3β-HSD inhibitor epostane has been shown to be effective in bitches for termination of pregnancy. Although epostane is not available for use in veterinary medicine, another 3β-HSD inhibitor, trilostane, has been registered for the treatment of hypercortisolism in dogs. However, the safety and potential usefulness of trilostane for induction of abortion and parturition in dogs have not been evaluated. In the study described in **Chapter 9** we assessed the effects of the 3β-HSD inhibitor trilostane on plasma concentrations of progesterone and prolactin during the luteal phase in healthy, non-pregnant bitches.

In **Chapter 10** the results of the studies described in this thesis are summarised and discussed.

2

General introduction

Part of the general introduction has been published:

Physiology of the canine anoestrus and methods for manipulation of its length

J. de Gier, N.J. Beijerink, H.S. Kooistra, A.C. Okkens

Reproduction in Domestic Animals 2008;43, Suppl 2: 157-164

The onset and maintenance of both cyclic oogenesis and continuous spermatogenesis are dependent on endocrine regulation. Endocrine signals from the hypothalamus, pituitary, and gonads, and their interaction form the hypothalamic-pituitary-gonadal axis (Figure 1). Many external factors—such as from the environment (light-dark, temperature) and other endocrine glands such as the adrenals and thyroids—may influence reproductive function.

Nomenclature and functional aspects of the pituitary gland

The hypothalamus and the pituitary (anterior lobe, posterior lobe, pars intermedia) provide a beautiful example of the close interaction between endocrine and neural regulation. Many key elements are neither purely endocrine nor purely neural. Three different mechanisms can be discerned with regard to the regulation of the pituitary by the hypothalamus (Meij et al. 2010):

1) endocrine regulation of the anterior lobe by means of a portal circulation
2) a neurosecretory pathway via the neurohypophysis
3) direct innervation by nerve fibres of the pars intermedia

The portal circulation connects a neuroendocrine to an endocrine system (Figure 1). The neuroendocrine system, located in the anterior and middle portion of the ventral hypothalamus, consists of clusters of peptide- and monoamine-secreting cells. These produce releasing hormones and inhibiting factors which are transported by nerve fibres to terminals in the outer layer of the median eminence. In this part of the hypothalamus they are released into capillaries of the hypothalamic-hypophyseal portal system. In the anterior lobe of the pituitary they regulate hormone production and secretion (Meij et al. 2010).

In the neurosecretory pathway, neurons in the anterior hypothalamus produce hormones which are transported by nerve fibres that pass through the ventral hypothalamus and pituitary stalk to end on fenestrated blood vessels in the neurohypophysis. The hormones are stored in secretory vesicles in the terminal ends of the nerve fibres and secreted into the systemic circulation in response to an appropriate stimulus.

The last regulatory mechanism in the pituitary is direct innervation of the pars intermedia by nerve fibres from the hypothalamus. This direct neural control is largely a tonic (dopaminergic) inhibitory influence. The main hormones secreted by the hypothalamus and pituitary are shown in Figures 1 and 2.

The cells of the adenohypophysis (anterior lobe and pars intermedia) differentiate along three main pathways (Meij et al. 2010):

1) Corticotrophs and melanotrophs both express pro-opiomelanocortin (POMC). Corticotrophs secrete adrenocorticotrophic hormone (ACTH) and melanotrophs secrete α-melanocyte-stimulating hormone (α-MSH).
2) Gonadotrophs secrete follicle-stimulating hormone (FSH) and luteinizing hormone (LH).

3) Somatotrophs, lactotrophs, and thyrotrophs, the Pit1-dependent cell lines, secrete growth hormone (GH), prolactin, and thyroid-stimulating hormone (TSH), respectively

As in other species, ACTH-immunoreactive cells in the foetal adenohypophysis of the dog are the first to differentiate from the pituitary progenitor cells (Sasaki and Nishioka 1998). The distribution of the various secretory cells in the anterior lobe in man is not random but has a topological and numeric organization, and this may also be true for the dog. The human anterior lobe consists of a central wedge containing thyrotrophs (10%) and corticotrophs (15%), and lateral wings containing somatotrophs (50%) and lactotrophs (15%). Gonadotrophs (10%) are distributed diffusely throughout the gland (Yeung et al. 2006).

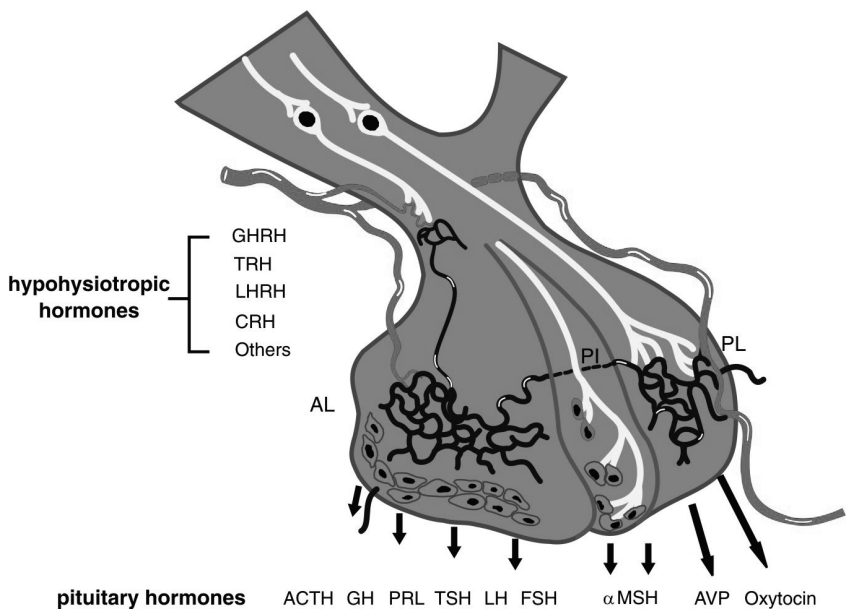

Figure 1. *Schematic representation of the relation between the hypothalamus and the pituitary anterior lobe (AL), pars intermedia (PI), and posterior lobe (PL). GHRH = growth hormone-releasing hormone, TRH = thyrotrophin-releasing hormone, LHRH = luteinizing hormone-releasing hormone, CRH = corticotrophin-releasing hormone, ACTH = adrenocorticotrophic hormone, GH = growth hormone, PRL = prolactin, TSH = thyroid-stimulating hormone, LH = luteinizing hormone, FSH = follicle-stimulating hormone, αMSH = α-melanocyte-stimulating hormone, AVP = arginine-vasopressin (Meij et al. 2010).*

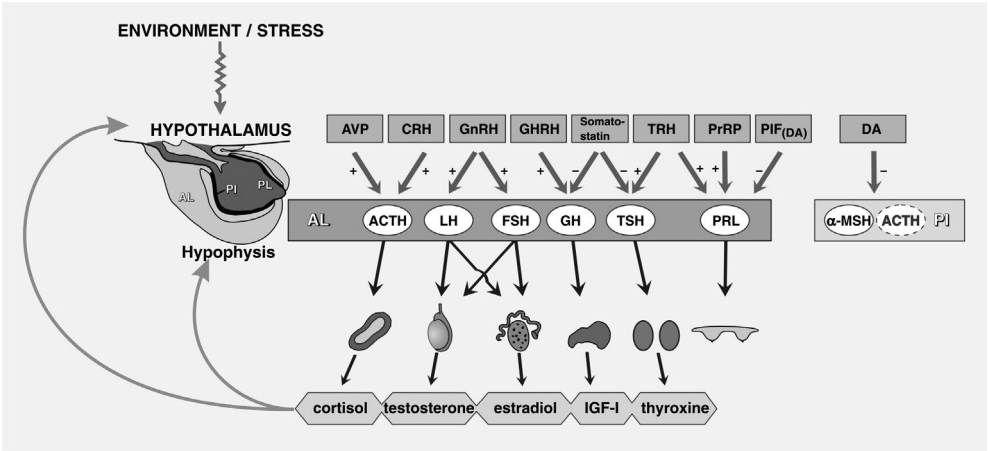

***Figure 2**. Schematic illustration of the hypophysiotrophic regulation of the secretion of hormones by the adenohypophysis. AL = anterior lobe, PI = pars intermedia, PL= posterior lobe, AVP = arginine-vasopressin, CRH = corticotrophin-releasing hormone, GnRH = gonadotrophin-releasing hormone, GHRH = growth hormone-releasing hormone, TRH = thyrotrophin-releasing hormone, PrRP = prolactin-releasing peptide, $PIF_{(DA)}$ = prolactin-inhibiting factor (dopamine), ACTH = adrenocorticotrophic hormone, LH = luteinizing hormone, FSH = follicle-stimulating hormone; GH = growth hormone; TSH = thyroid-stimulating hormone; PRL = prolactin, α-MSH = α-melanocyte-stimulating hormone, IGF-I = insulin-like growth factor-I (Meij et al. 2010).*

Under physiological and most pathological conditions, the basal plasma concentration of each of the six major anterior lobe hormones (ACTH, LH, FSH, TSH, GH, and prolactin) is regulated via a feedback (closed loop) system. Apart from this long-loop feedback, some hormones such as prolactin regulate their own secretion directly by acting on the hypothalamus in a short-loop feedback. Other signals originating within the central nervous system as a result of influences from the environment (temperature, light-dark), stress (pain, fear), and intrinsic rhythmicity can be superimposed on the feedback mechanism (Meij et al. 2010).

Releasing and inhibiting hormones are stored in nerve terminals in the median eminence of the hypothalamus in concentrations 10-100 times higher than elsewhere in the hypothalamus. Their specificity is determined by receptors on individual cells of the anterior lobe. These releasing and inhibiting hormones influence peptide synthesis and/or release in anterior lobe cells by modulating the amount of mRNA, the efficiency of transcription and translation, the processing from preprohormone to hormone, and intracellular degradation of stored hormone. With the exception of dopamine, the hypophysiotrophic hormones are peptides with sequence lengths ranging from 3 to 44 amino acids. Species variation in amino acid sequences can occur with increasing length. Whereas the structures of thyrotrophin-releasing hormone (TRH), gonadotrophin-releasing hormone (GnRH), and somatostatin (3, 10, and 14 amino acids, respectively) are identical in all mammals studied, the structure of growth hormone-releasing hormone (GHRH) varies. Yet corticotrophin-releasing hormone (CRH), with 41 amino acids, is identical in man, dog, horse, and rat (Mol et al. 1994).

Secretion of ACTH

ACTH is synthesized from a well-characterized precursor molecule, pro-opiomelanocortin (POMC), which also gives rise to a number of other peptides that are co-released with ACTH. ACTH secretion by the anterior lobe is regulated by the hypothalamus and central nervous system via neurotransmitters that cause the release of hypophysiotrophic hormones, such as corticotrophin-releasing hormone (CRH) and arginine-vasopressin (VP). The latter are considered to be the predominant stimulating neurohormones for ACTH *in vivo* (Keller-Wood and Dallman 1984). ACTH almost exclusively controls the synthesis and release of glucocorticoids by the two inner zones of the adrenal cortex. In turn, corticosteroids inhibit ACTH release at multiple target sites, of which two have been identified unequivocally: corticotrophic cells in the anterior lobe and neurons in the hypothalamus that produce CRH and VP. ACTH and other POMC peptides also exert a growth-promoting effect on the adrenal cortex (Wulffraat et al. 1987).

The POMC-producing cells of the PI lack glucocorticoid receptors and are thus resistant to glucocorticoid suppression. Hypothalamic dopaminergic influence is held responsible for inhibiting the expression of glucocorticoid receptors (Antakly et al. 1985; Antakly et al. 1987). Despite a high concentration of bioactive ACTH in the canine PI (Halmi et al. 1981), there is no evidence of active secretion of ACTH from the PI in the dog.

Secretion of GH

The hypothalamic neurohormones—GH-releasing hormone (GHRH) and somatostatin—play a central role in anterior lobe secretion of GH (Plotsky and Vale 1985). Episodic GH release can also be elicited by synthetic GH-secretagogues (GHSs) (Momany et al. 1981). These exert their effect on GH release by acting through a specific GHS receptor (McKee et al. 1997) that is not activated by GHRH or somatostatin. About a decade ago, an endogenous ligand (ghrelin) for the GHS receptor was identified (Kojima et al. 1999).

In the dog, excessive production of GH can be induced by endogenous progesterone or by exogenous progestogens used for oestrus prevention (Eigenmann et al. 1983). The progestogen-induced GH production in this species originates from foci of hyperplastic ductular epithelium of the mammary gland (Selman et al. 1994). In normal cyclic bitches the mean basal plasma GH concentration has also been reported to be higher, because less GH is secreted in pulses during the luteal phase than during anoestrus (Selman et al. 1991; Kooistra et al. 2000).

The effects of GH can be divided into rapid metabolic actions and slow or long-lasting hypertrophic actions. The rapid metabolic responses are produced by direct interaction of GH with the target cell and result in enhanced lipolysis and restricted glucose transport across the cell membrane due to anti-insulin effects. In addition, GH has a direct growth-promoting effect by stimulating cell differentiation. These slow anabolic effects are mainly mediated via growth factors synthesized in the liver and known as insulin-like growths factors (IGFs).

Secretion of LH and FSH

The pituitary gonadotrophs secrete both FSH and LH, while each of the other pituitary hormones is secreted by a specific cell type. Both FSH and LH consist of an α and a β subunit, encoded by different genes (Gharib et al. 1990). The pattern of secretion of both FSH and LH reflects

the multifaceted integration of hypothalamic, pituitary, and peripheral signals. The GnRH pulse generator in the hypothalamus exhibits spontaneous, synchronized autorhythmicity in the release of the decapeptide GnRH (Mellon et al. 1990). It translates neural signals into a periodic, oscillatory GnRH signal in a coordinated manner to drive and control the pituitary gonadotrophic components (Knobil 1974; Knobil 1980). The pituitary gonadotrophs release LH and FSH in a pulsatile pattern in response to the GnRH rhythmic signals (Kooistra et al. 1999a). Each pulse of LH and FSH is induced by a pulse of GnRH (Shacham et al. 2001), but in most mammalian species, differential secretion of LH and FSH has been demonstrated. This indicates the presence of regulatory mechanisms that allow independent secretion of LH and FSH. One of these mechanisms is gonadal feedback, because both oestradiol and inhibin can specifically suppress FSH synthesis and secretion (Shupnik 1996). Another mechanism is 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). A change in the frequency of GnRH pulses can modify the ratio of FSH to LH released (Wildt et al. 1981).

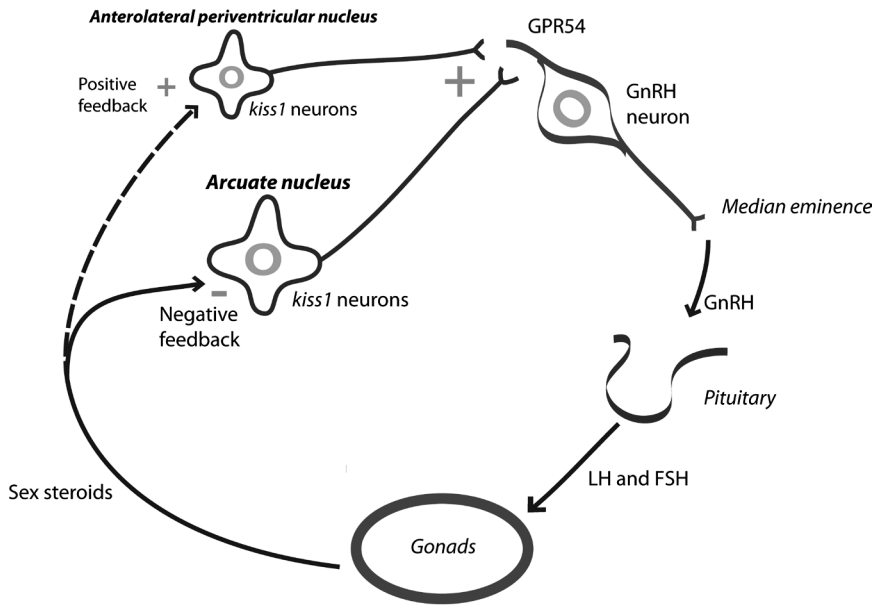

***Figure 3**. Simplified model of kisspeptin-mediated positive and negative feedback regulation of gonadotrophin-releasing hormone (GnRH) secretion. LH = luteinizing hormone, FSH = follicle-stimulating hormone, GPR54 = kisspeptin receptor. The positive feedback loop is mainly present in females.*

Kisspeptin, a recently discovered neuropeptide hormone, is thought to play a crucial role in coordinating negative and positive feedback pathways within the hypothalamic-pituitary-gonadal axis (Figure 3) (Clarkson et al. 2008; Oakley et al. 2009; Tena-Sempere 2010). Most of the current understanding of the role of kisspeptin in regulating reproduction has been obtained from studies in rodents. Kisspeptin is secreted by kiss1 neurons, which express the *Kiss1* gene that encodes kisspeptin. These neurons are located in the hypothalamus, mainly in the arcuate nucleus (ARC)

and the anteroventral periventricular area (AVPV) (Smith et al. 2005a; Smith et al. 2005b). A distinctive feature of kiss1 neuronal populations is sexual dimorphism, adult females having more kiss1 neurons than do males, especially in the AVPV (Kauffman et al. 2007; Clarkson et al. 2009). Kiss1 neurons express receptors for oestradiol and androgens, which are lacking in GnRH neurons (Tena-Sempere 2010). Furthermore, most GnRH-neurons express kisspeptin receptors (GPR54) (Irwig et al. 2004). Disrupting the kisspeptin/GPR54 pathway in GPR54 knockout mice stops the negative feedback regulation of gonadotrophin secretion (Dungan et al. 2007). In addition to the role of kiss1 neurons in negative feedback regulation of GnRH secretion, in female rodents the kiss1 neurons in the AVPV have been implicated in the positive feedback regulation of the preovulatory LH surge induced by the increasing plasma oestradiol concentrations during the follicular phase (Kinoshita et al. 2005; Smith et al. 2005b; Smith et al. 2006; Clarkson et al. 2008). Hence gonadal steroid hormone feedback on hypothalamic GnRH release is now believed to be mediated through kiss1 neurons (Figure 3).

FSH and LH regulate gonadal steroid biosynthesis and initiate and maintain germ cell development. Granulosa, theca, interstitial, and luteal cells in females and Leydig and Sertoli cells in males are capable of secreting hormones in response to LH and FSH. In females, the type and amount of hormones released vary according to the morphological and functional status of the follicle and corpus luteum.

Secretion of α-MSH

Circulating α-MSH, which like ACTH is synthesized from the precursor molecule POMC, mainly originates from the pars intermedia. The predominant melanotrophic A cells are the typical pars intermedia cells, staining strongly for α-MSH and weakly for ACTH. The B cells resemble the corticotrophs of the anterior lobe, staining intensely for ACTH but not for α-MSH (Halmi et al. 1981).

Hormone release from the canine pars intermedia has been reported to be under the influence of a strong and permanent dopaminergic inhibition. Dopamine infusions do not lower the basal plasma concentration of α-MSH (Orth et al. 1988) but the administration of dopamine antagonists such as haloperidol causes a definite increase in plasma α-MSH concentration in dogs (Kemppainen and Sartin 1987).

Secretion of prolactin

The secretion of prolactin by the anterior lobe 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). In addition, several substances are known to have prolactin-releasing activity, such as serotonin, TRH, and nitric oxide (Garthwaite and Hagen 1979; Lafuente et al. 1994; Mol and Rijnberk 1997; Yen and Pan 1999). Also, a specific peptide that promotes prolactin release in the hypothalamus has been identified and characterized (Hinuma et al. 1998).

Neurogenic factors also influence prolactin secretion. Suckling and milking are almost immediately followed by prolactin release. Gonadal steroids are also important modulating factors in the control of prolactin secretion. Oestrogens rapidly induce an enhanced prolactin

response to TRH in dogs (Rutteman et al. 1987). Progesterone has a modulating effect on prolactin secretion in the dog and prolactin secretion increases during the second part of the luteal phase in non-pregnant bitches (Kooistra and Okkens 2001), albeit less than in pregnant and overtly pseudopregnant bitches (De Coster et al. 1983; Overgaauw et al. 1998; Overgaauw et al. 1998). The finding that both administration of a progesterone receptor antagonist to pregnant bitches and ovariectomy during the luteal phase results in significant elevation of plasma prolactin concentration (Galac et al. 2000; Lee et al. 2006) also supports the role of progesterone in prolactin secretion.

The most familiar role of prolactin in mammals is stimulation of mammary gland growth and lactation. Prolactin increases mitosis in mammary gland epithelial cells during development and probably also promotes lobuloalveolar differentiation in the late luteal phase in non-pregnant bitches and during pregnancy and lactation (Brisken et al. 1999). Prolactin also affects gonadal function, playing an important role in maintaining corpora lutea function in the second half of the luteal phase in both pregnant and non-pregnant bitches (Okkens et al. 1990; Kooistra and Okkens 2002).

The gonads

The ovaries

The ovaries lie caudal to the kidneys at the level of the third or fourth lumbar vertebra. The ovaries of the dog are completely enclosed in a peritoneal pouch, the ovarian bursa (Schaefers-Okkens and Kooistra 2010). The surface of the ovaries is covered by the germinal epithelium of the cortex and is free of serosa. Germ cells growing inward from the cortex give rise to follicles, many of which degenerate and become atretic. Tertiary follicles develop during the follicular phase and become visible at the surface of the ovary due to the considerable increase in the amount of follicular fluid they contain.

The testes

The testes lie obliquely within the scrotum, their long axis directed caudodorsally (De Gier and van Sluijs 2010). Tubules with seminiferous epithelium, the site of spermatogenesis, make up approximately 80% of the testis. They are composed of supporting cells and spermatogenic cells. During spermatogenesis spermatogonia develop into spermatozoa (De Gier and van Sluijs 2010). Sertoli cells lining the seminiferous tubules have an important supportive function during spermatogenesis. They express androgen receptors and receptors for FSH. Sertoli cells are thought to regulate development of the germ cells by synthesis and secretion of molecules that act upon them. Androgens and their receptors are essential for maintenance of spermatogenesis, whereas males are still fertile without the influence of FSH (Holdcraft and Braun 2004). Between the seminiferous tubules lie groups of interstitial or Leydig cells, which are the main constituent of the endocrine portion of the testis and produce androgens, driven by LH (Dohle et al. 2003; Holdcraft and Braun 2004).

The synthesis and secretion of gonadal steroid hormones

All steroid hormones are derived from cholesterol by enzymatic conversions (Figure 4). The major rate-limiting step in steroidogenesis is the translocation of (hydrophobic) cholesterol from the outer mitochondrial membrane across the aqueous intermembranous space to reach the inner membrane (Strauss III 2009). This translocation process is enhanced by the *steroidogenic acute regulatory protein* (StAR). Firstly, cholesterol is converted in the mitochondria to pregnenolone. Pregnenolone is then further metabolized outside the mitochondria to several other steroids via various pathways (Figure 4) (Galac et al. 2010b). Cytochrome P-450 enzymes are responsible for most of the enzymatic conversions from cholesterol to steroid hormones. The machinery for steroidogenesis is compartmentalized, in that specific cell types can accomplish several of the sequential steps but rarely can they generate all types of steroid hormones. For example, oestrogen biosynthesis from cholesterol requires at least two tissues or cell types and thus oestrogen production can be modulated independently at different levels. In the ovary, LH acts on the theca cells that produce androgen precursors, whereas FSH stimulates the granulosa cells to aromatize these molecules to oestrogens (Figure 5) (Strauss III 2009).

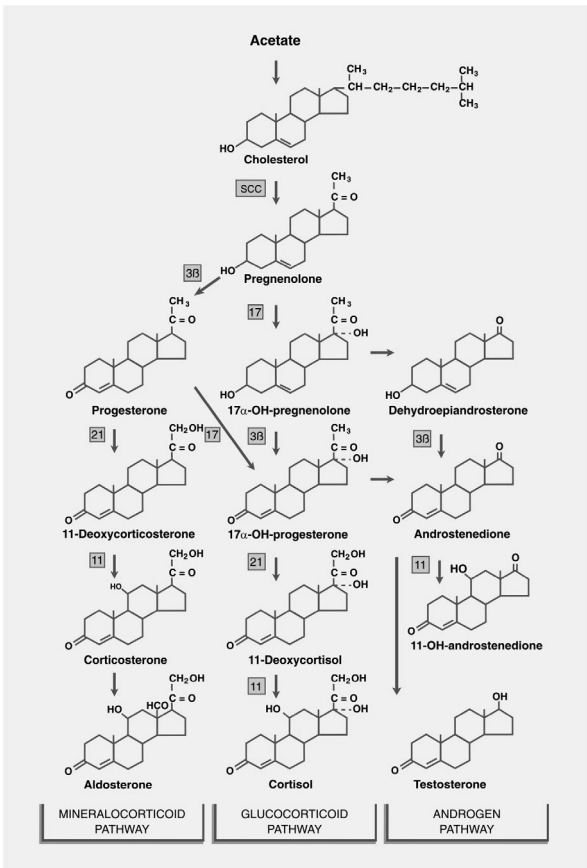

Figure 4*. Major pathways in steroid biosynthesis. SCC = cholesterol side chain cleavage, 3β = 3β-hydroxysteroid dehydrogenase, 11 = 11β-hydroxylase, 17 = 17α-hydroxylase/17, 21 = 21-hydroxylase (Galac et al., 2010).*

Steroidogenic cells cannot store the hormones, which are therefore secreted immediately after biosynthesis, but intratesticular testosterone is mainly bound to androgen binding protein and secreted into the seminiferous tubules (Dohle et al. 2003). This provides the high concentrations of testosterone needed to ensure spermatogenesis (Holdcraft and Braun 2004). For several other effects of testosterone, such as the exocrine prostate function, testosterone must first be converted to dihydrotestosterone (DHT) by the NADPH-dependent enzyme 5α-reductase (Figure 5) (De Gier and van Sluijs 2010). The negative feedback effect of testosterone on LH release is primarily, but not exclusively, via aromatization of testosterone to oestradiol (Figure 5) (Santen 1975; Schnorr et al. 2001; Rochira et al. 2006).

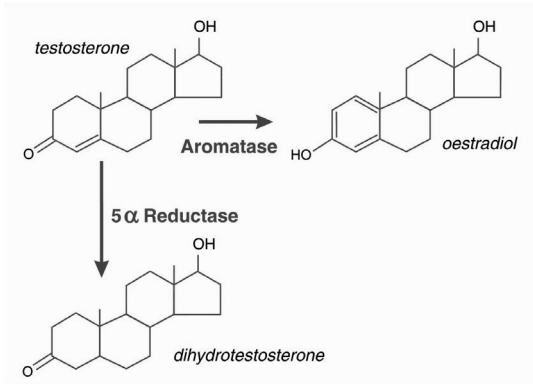

Figure 5*. Schematic illustration of the conversion of testosterone to dihydrotestosterone and oestradiol, catalysed by 5α-reductase and aromatase, respectively (De Gier and van Sluijs, 2010).*

The pituitary-ovarian axis, anoestrus, and the oestrous cycle in bitches

The bitch is a non-seasonal, mono-oestrous animal in which the oestrous cycle can be classified as behaviour oriented or according to ovarian function (Figure 6).

The behaviour-oriented classification distinguishes pro-oestrus, oestrus, and metoestrus (dioestrus). Pro-oestrus is defined as the period from the onset of sanguineous vaginal discharge and vulvar swelling to the first willingness to accept mating. During oestrus the bitch accepts mating and the vulva begins to shrink and soften. Metoestrus (dioestrus) begins when the bitch no longer accepts mating and ends when plasma progesterone concentration declines for the first time to <3 nmol/l (Schaefers-Okkens and Kooistra 2010).

The classification of the oestrous cycle according to ovarian function distinguishes the follicular phase, the phase of preovulatory luteinization and ovulation, and the luteal phase (Schaefers-Okkens and Kooistra 2010). During the follicular phase, tertiary follicles develop and produce oestradiol-17β. Plasma oestradiol-17β concentration increases gradually during the early follicular phase, leading either to a plateau interval or a sharp increase just before the beginning of the preovulatory LH surge, with peak concentrations about 1-2 days before the preovulatory LH surge (Olson et al. 1982; Schaefers-Okkens and Kooistra 2010). The

external signs of pro-oestrus, such as hyperaemia and oedema of the vulva and sanguineous vaginal discharge, are related to high concentration of oestradiol-17β. Vaginoscopy during the early follicular phase reveals that the vaginal mucosal folds are swollen, very pale, and have a smoothly rounded (balloon-like) surface. Plasma oestradiol-17β concentration declines at the end of the follicular phase and plasma progesterone concentration begins to rise as a result of partial luteinization of granulosa cells. Plasma LH concentration is low during the follicular phase, with frequent increases of short duration (Kooistra et al. 1999a). Plasma FSH concentration is relatively high at the beginning of the follicular phase but declines to low levels during the progression of the follicular phase, probably as a result of negative feedback by oestradiol-17β and inhibin.

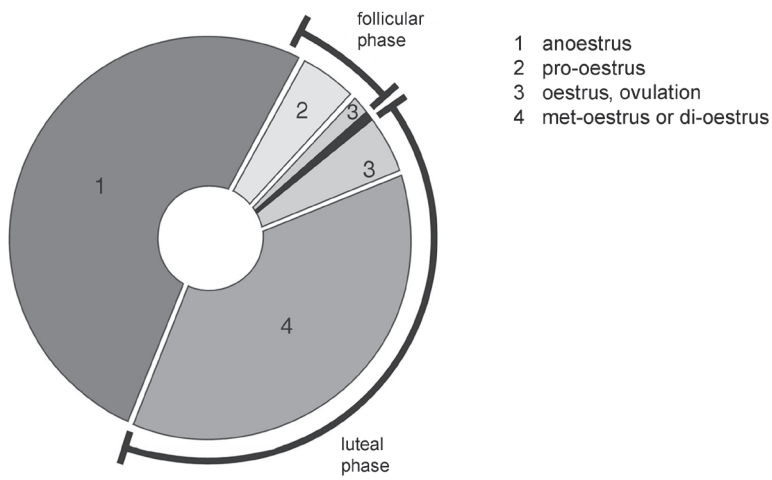

***Figure 6**. Schematic representation of the oestrous cycle and anoestrus in the dog (Schaefers-Okkens and Kooistra 2010).*

The onset of oestrous behaviour normally occurs at the same time as the preovulatory LH surge, but in some bitches it begins days earlier and in others not until days thereafter, or never. Vaginoscopy during this phase reveals shrinkage of the vaginal mucosa and many longitudinal folds. Plasma progesterone concentration is around 6-13 nmol/l at the time of the LH surge and 15-25 nmol/l at the time of ovulation, 1.5-2 days later. The concentration of progesterone, originating from the corpora lutea, increases in the peripheral blood during the remainder of oestrus and the onset of the luteal phase (Schaefers-Okkens and Kooistra 2010). Then a plateau is reached, after which progesterone declines slowly to a basal level of 3 nmol/l for the first time about 75 days after the onset of the luteal phase. The patterns of secretion of LH and FSH during the luteal phase are characterized by a fluctuating baseline with occasional distinct elevations, indicating pulsatile secretion (Kooistra et al. 1999a; Kooistra et al. 1999b).

In the dog, corpora lutea are the sole source of progesterone, which is obligatory for maintaining pregnancy (Sokolowski 1971). Ovarian progesterone production in the dog is

independent of pituitary support during the first half of the luteal phase or gestation (Okkens et al. 1986). During the second half of the luteal phase or pregnancy, prolactin plays an important role in maintaining the corpora lutea (Okkens et al. 1985b; Okkens et al. 1986; Okkens et al. 1989; Okkens et al. 1990; Onclin and Verstegen 1997; Onclin et al. 2000; Kooistra and Okkens 2002; Kowalewski et al. 2011). The transition from the luteal phase to anoestrus is gradual and varies considerably among non-pregnant bitches.

In pregnant bitches, during the last 1–2 days prior to whelping plasma progesterone concentration decreases rapidly (Edqvist et al. 1975; Chakraborty 1987; Van der Weyden et al. 1989), while plasma 15-ketodihydroprostaglandin-$F_{2\alpha}$ (prostaglandin metabolite) concentration begins to increase (Concannon et al. 1988; Meier and Wright 2000). The plasma prostaglandin metabolite level reflects the peripherally active prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) concentration, as $PGF_{2\alpha}$ itself has a short half-life and is rapidly converted to prostaglandin metabolite. As a result of the decrease in progesterone and the rise in $PGF_{2\alpha}$, myometrial activity gradually increases (Van der Weyden et al. 1989), leading to the onset of parturition. The decline in plasma progesterone may be considered essential for normal parturition and inhibiting the decline by implanting medroxyprogesterone acetate (MPA) resulted in its failure (Concannon and Hansel 1977). Although treated bitches were visibly distressed and fluids were discharged from the vagina, no puppies were expelled. The bitches either died with puppies *in utero* or a Caesarean section was required (Flint et al. 1978; Meier and Wright 2000).

It is not clear what triggers the onset of whelping in the dog. In ruminants, elevated levels of foetal cortisol trigger parturition via increased oestrogen production in the placenta at the expense of progesterone production. The elevated level of oestrogen stimulates the production of prostaglandin $F_{2\alpha}$, resulting in increased myometrial activity and softening of the cervix (Whittle et al. 2000). No data on hormone concentrations in canine foetal blood are available and circulating oestrogen concentrations in the bitch appear to decrease rather than increase towards parturition (Edqvist et al. 1975; Van der Weyden et al. 1989; Hoffmann et al. 1994; Onclin et al. 2002).

Progression from early to late anoestrus in the bitch is characterized by increased release of GnRH by the hypothalamus. Especially during late anoestrus, GnRH pulse frequency is significantly increased (Tani et al. 1996). Pituitary sensitivity to GnRH and the indirect response of the ovary to GnRH in bitches in early and advanced anoestrus have been investigated by means of intravenous administration of graded doses of GnRH. The resulting circulating LH and oestradiol concentrations were dose-dependent and significantly higher in advanced anoestrus. In addition, GnRH-induced LH and oestradiol profiles were positively correlated (Van Haaften et al. 1994). These results indicate that during the course of anoestrus there is increased pituitary sensitivity to GnRH and ovarian responsiveness to gonadotrophins (Jeffcoate 1993; van Haaften et al. 1994).

In order to study the role of gonadotrophins during the transition from anoestrus to the follicular phase, 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 an LH pulse. The mean plasma LH concentration of the smoothed baseline and the mean area under the curve (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 were significantly higher during late anoestrus than during mid- and early anoestrus (Kooistra et al. 1999a; Onclin et al. 2001). These observations suggest that an increase in circulating FSH concentration is a key event in ovarian folliculogenesis in the dog and consequently in 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 point 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 enhanced 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).

In addition to increased hypothalamic GnRH release, increased basal plasma FSH, and increased sensitivity of the pituitary and ovaries during progression of anoestrus, several other factors may be involved in the initiation of folliculogenesis and termination of anoestrus. In some bitches increased LH pulsatility has been observed shortly before the start of pro-oestrus (Concannon et al. 1986; Kooistra et al. 1999b; Tani et al. 1999; Beijerink et al. 2004). The exact role of this remains uncertain. 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 increased LH pulsatility at the end of anoestrus provides a stimulus to follicles which are no longer receptive to FSH but have acquired sufficient LH receptors.

In addition, enhanced expression of the genes encoding for the oestrogen receptor (Tani et al. 1997) and P450 aromatase (which catalyses oestrogen biosynthesis in the canine hypothalamus) during anoestrus has been reported in dogs (Inaba et al. 2002). However, although sporadic elevations are observed, plasma oestradiol concentration is usually low during anoestrus and does not begin to rise until approximately one month before the preovulatory LH surge (Jeffcoate 1993). Furthermore, there are no manifestations of oestrogen influences on the reproductive tract or on sexual behaviour during anoestrus, and vaginal endoscopy or cytology reveals no evidence of oestrogenic stimulation until late anoestrus.

There is also some evidence that factors that decrease opioidergic activity promote LH release and termination of anoestrus (Concannon 1993). Treatment with naloxone, an opioid antagonist, stimulated LH release in nearly all stages of the oestrous cycle (Concannon and Temple 1988). Furthermore, no changes in the expression of the FSH receptor have been found during canine anoestrus (McBride et al. 2001). The latter study demonstrated that anoestrus in bitches is due neither to failure of expression of the canine FSH receptor nor to a change in splicing to an inactive form.

Medical and surgical interventions in the pituitary-gonadal axis and their consequences

Dopaminergic influences and induction of a premature oestrus

In addition to previously-mentioned changes in the hypothalamic-pituitary-ovarian axis, there is evidence of involvement of dopaminergic influences in the initiation of a new follicular phase in the bitch. Administration of dopamine agonists, such as bromocriptine and cabergoline, is associated with both 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. 1999b; Verstegen et al. 1999; Gobello et al. 2002; Beijerink et al. 2003). 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. 1985a), but is also due to shortening of the luteal phase (Okkens et al. 1985a; Okkens et al. 1990). The shortening of the luteal phase is probably caused by a decrease in secretion of prolactin, the main luteotrophic factor in the bitch (Okkens et al. 1990; Onclin and Verstegen 1997).

It has been hypothesized that shortening of anoestrus by dopamine agonists is also the result of suppression of prolactin secretion, as prolactin may inhibit gonadotrophin release. Indeed, it has been demonstrated in various mammalian species that high circulating concentrations 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 concentration was observed during physiological hyperprolactinaemia in lactating sows, while the lowering of plasma prolactin concentration by bromocriptine led to a rise in plasma LH concentration in these animals (Bevers et al. 1983). Yet, under physiological conditions plasma prolactin concentration is 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 doses of the serotonin receptor antagonist metergoline (Okkens et al. 1997), even though the plasma prolactin concentration was 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 raised the question whether administration of a dopamine agonist in a dose too low to suppress prolactin secretion could still shorten anoestrus in the bitch. To investigate this, 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 (Beijerink et al. 2003). In the bitches receiving the 5 µg dose, the difference between the average plasma prolactin concentration prior to and during treatment was not significant. In contrast, bitches receiving the 20 or 50 µg dose had significant lowering of plasma prolactin concentration during bromocriptine treatment . The mean ± SEM 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. In all three groups the interval was significantly shorter than 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. This study provided

further evidence for the assumption that premature oestrus is not induced by a decrease in plasma prolactin concentration.

In addition to inhibition of prolactin release, the bromocriptine-induced shortening of anoestrus is also associated with a quick rise in basal plasma FSH without a concomitant increase in basal plasma LH (Kooistra et al. 1999b). These results further support the notion that in the bitch an increase in circulating FSH should be considered a critical event required for ovarian folliculogenesis. Treatment with bromocriptine may cause an increase in plasma FSH concentration to a level that enhances development of follicles. This is similar to the endocrine events during late anoestrus in untreated bitches (Kooistra et al. 1999a).

A role for dopamine in the control of reproduction has been demonstrated in different mammalian species, although the effects of dopamine in the bitch differ from those in other species (Beck et al. 1978; Havern et al. 1994; Besognet et al. 1997). In other species 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.

In contrast to dopamine agonists, low doses of the serotonin receptor antagonist metergoline do not shorten anoestrus (Okkens et al. 1997). It may therefore be expected that the changes in gonadotrophin release associated with dopamine agonist-induced shortening of anoestrus will not be observed during treatment with low doses of metergoline. To investigate the effects of a low dose of a serotonin antagonist on the pulsatile secretion of FSH and LH, 0.1 mg metergoline per kg body weight orally twice daily was administered to bitches starting 100 days after ovulation (Beijerink et al. 2004). The mean interoestrous interval in the eight treated bitches was as expected not shortened, despite decreased plasma prolactin concentration. Moreover, during the first weeks of treatment there were no significant changes in the pulsatile plasma profiles of FSH or LH. These findings indicate that the lowering of plasma prolactin by the serotonin antagonist does not lead to increased secretion of FSH.

In addition to administration of dopamine agonists, a follicular phase may also be induced by administration of GnRH, gonadotrophins, eCG, hCG, and oestrogens (for review see (Kutzler 2007)). Although many protocols exist for oestrus induction in bitches, the fertility results of these methods, with the exception of administration of dopamine agonists, are variable and generally poor. Some methods are also too costly or labour intensive to be suitable for veterinary practice.

Aglepristone, a progesterone-receptor antagonist, and prostaglandin $F_{2\alpha}$ have also been reported to shorten the interoestrous interval if administered during the luteal phase (Romagnoli et al. 1993; Galac et al. 2004).

Suppression of gonadal function in female and male dogs

Surgical intervention

Gonadal function in dogs—the production of germ cells and the production and secretion of hormones—can be controlled medically or surgically. In bitches, ovariectomy has several advantages. A single procedure is effective. It considerably lowers the risk of mammary

cancer if performed before approximately 2.5 years after the first oestrus (Schneider et al. 1969). It also prevents the development of pyometra and progesterone-induced growth hormone excess (Selman et al. 1997). There are, however, also several disadvantages such as the risk of complications during anaesthesia and surgery and the irreversibility of the procedure. There is also the possibility of side-effects such as changes in the hair coat and urinary incontinence. There are indications that the risk of urinary incontinence is greater if the procedure is performed prior to the first oestrus. Furthermore, it has been shown that early-age gonadectomy is associated with an increased rate of cystitis and that age at gonadectomy is negatively correlated with the rate of urinary incontinence (Spain et al. 2004). Urinary incontinence occurs mainly in large breeds, while the Boxer, Dobermann, Bouvier des Flandres, Giant Schnauzer, Irish Setter, Miniature Poodle, Old English Sheepdog, Weimaraner, and Rottweiler appear to be especially at risk (Thrusfield et al. 1998).

Surgical castration of male dogs is a routine procedure in veterinary medicine for a variety of both elective and medical indications. Examples of elective indications for castration are (1) behavioural problems ascribed to the influence of androgens, such as inter-male dominance, urine marking, roaming, and mounting and (2) persistent purulent discharge from the prepuce (Maarschalkerweerd et al. 1997; Giammanco et al. 2005). Castration also prevents the development of testicular tumours and several prostatic disorders, of which benign prostatic hyperplasia is the most important (Johnston et al. 2001; Renggli et al. 2010). However, castration is also associated with disease processes such as prostatic adenocarcinoma and haemangiosarcoma (Teske et al. 2002; Kutzler 2010). Furthermore, the increased risk of obesity and the moderate predictability of the anticipated behavioural changes (Hopkins et al. 1976; Maarschalkerweerd et al. 1997; Neilson et al. 1997) makes elective gonadectomy in male dogs a disputable surgical procedure.

Diagnosis of the presence of gonadal tissue and the pituitary-gonadal axis before and after gonadectomy

During the follicular phase in bitches, demonstrating the presence of ovarian tissue is straightforward. Vaginoscopy and vaginal cytology can be used to recognize the influence of oestrogens (Schutte 1976; Schaefers-Okkens and Kooistra 2010) and cytology is more reliable than a single measurement of plasma oestradiol (Shille and Olson 1989). During the progression to the late follicular phase, ovulation, and the luteal phase, an elevated plasma progesterone concentration provides evidence for the presence of ovarian tissue (Okkens et al. 1981).

Demonstrating the presence of ovarian tissue during anoestrus is challenging. There are no obvious clinical or behavioural differences between the anoestrous bitch and the ovariectomized bitch. Vaginal cytology 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 clinically relevant concentrations of the gonadally-derived hormones oestradiol, progesterone, and testosterone ceases with gonadectomy. However, the ranges of plasma oestradiol concentrations in bitches before and after ovariectomy overlap (Jeffcoate

1993; Frank et al. 2003). In gonadally intact male dogs there is wide variation in basal testosterone concentration (Gunzel-Apel et al. 1994). This might preclude confirmation of the presence of testicular tissue in animals in which the testicles cannot be located. However; reference values based on a large number of dogs of various breeds are lacking. The loss of negative feedback of gonadal steroids causes a rapid increase in the concentration of circulating gonadotrophins (Chaffaux et al. 1981; Olson et al. 1992; Concannon 1993; Jeffcoate 1993; Lofstedt and VanLeeuwen 2002; Reichler et al. 2004), while their secretion pattern remains pulsatile (Concannon 1993). Baseline gonadotrophin levels may provide useful information, but due to the pulsatile secretion pattern, overlap of plasma values between intact and gonadectomized animals may be expected and thus the diagnostic value of a single hormone measurement is questionable (Jeffcoate 1993; Lofstedt and VanLeeuwen 2002).

To differentiate between dogs with and without gonadal 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 concentrations of LH and oestradiol (Van Haaften et al. 1994). Increased plasma concentrations of LH and testosterone after GnRH administration have also been reported in male dogs (Knol et al. 1993). Information about the response to exogenous GnRH after ovariectomy is limited to a few studies. Studies comparing the response to GnRH administration in male dogs and anoestrous bitches before and after gonadectomy are lacking.

Medical prevention of oestrus and suppression of testicular function

Oestrus prevention can be accomplished with several types of drugs, of which progestogens are the most important. Androgens can also be used for this purpose but primarily for short-term prevention. As androgens are not currently registered for this use they are not included in this discussion.

The mechanism of the contraceptive activity of progestogens is still unclear. In many species there is evidence that contraceptive progestogens reduce serum concentrations of gonadotrophins. However, high doses of medroxyprogesterone acetate (MPA) administered to beagle bitches for several months did not reduce the increased circulating concentrations of LH in ovariectomized bitches nor did it lower LH concentrations in intact bitches (McCann et al. 1987). In another study high contraceptive doses of megestrol acetate (MA) did not suppress basal gonadotrophin secretion during anoestrus, nor was the pituitary hypersecretion of LH and FSH that occurs in ovariectomized bitches suppressed (Colon et al. 1993). Beijerink et al. examined 6-h plasma profiles of FSH and LH in five bitches before and at 3, 6, 9, and 12 months after the start of MPA treatment (Beijerink et al. 2007). The results of this study demonstrate that treatment with MPA scarcely 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. However, the prevention of oestrus by MPA could not be ascribed to a significant reduction in circulating levels of either FSH or LH. On the contrary, during the first months of MPA treatment basal plasma FSH and LH concentrations increased. This progestogen-induced increase in gonadotrophin concentration was not observed in the studies of McCann et al. (1987) and Colon et al. (1993), and its recognition may be explained by the repeated

sampling employed in the study by Beijerink et al. (2008). The elevated plasma gonadotrophin concentrations during the first months of MPA treatment may be due to a direct inhibitory effect of MPA at the ovarian level, resulting in suppression of ovarian secretion of oestradiol or inhibin (Mann et al. 1992; Shupnik 1996). With continuing MPA treatment, basal plasma gonadotrophin concentrations returned to pre-treatment levels. In addition, the pituitary FSH response to GnRH stimulation decreased, suggesting that MPA treatment attenuated pituitary FSH sensitivity to endogenous GnRH (Beijerink et al. 2007). Pulsatile FSH and LH release was maintained during MPA treatment, but there were indications that changes occurred in the pulsatile release of the gonadotrophins. In general, LH pulses coincided with FSH pulses, but during MPA treatment several LH pulses were accompanied by increases in FSH so small as to be insignificant (Beijerink et al. 2007).

The progestogens most frequently used for oestrus prevention in the dog are proligestone and MPA. The single subcutaneous dose of proligestone recommended by the manufacturer ranges from 10 mg/kg for a dog of about 60 kg, to 30 mg/kg for one of 3 kg. For MPA, the single subcutaneous dose is 2 mg/kg (maximum 60 mg per animal). These drugs should be administered during anoestrus, approximately 1 month before onset of the expected follicular phase. After the administration of proligestone oestrus is usually suppressed for 9-12 months and after MPA it may be up to 2-3 years. For that reason the manufacturer does not recommend using MPA in animals intended for breeding at a later date. When MPA is administered orally, 5 mg once daily for a maximum of 21 days (10 mg during the first 5 days for large dogs) oestrus is suppressed for 2-9 months.

Use of progestogens for oestrus prevention may lead to the following side-effects:

1) Development of cystic endometrial hyperplasia (CEH)-endometritis (Sokolowski and Zimbelman 1974).
2) Prolonged gestation if conception occurs after a progestogen is administered subcutaneously at the onset of the follicular phase; Caesarean section may be needed.
3) Hypersecretion of growth hormone, which may lead to diabetes mellitus (Selman et al. 1997). The growth hormone excess can be treated successfully with the progesterone receptor blocker aglepristone (Bhatti et al. 2006).
4) Increased risk of neoplastic transformation of mammary tissue, ranging from hyperplasia, adenomatous hyperplasia, and adenomas to malignant tumours. The progestogen-induced transformation begins with proliferation of undifferentiated terminal ductal structures, so-called terminal end buds (Russo and Russo 1991), which increases susceptibility of the mammary tissue to malignant transformation.

With the exception of prolonged pregnancy, these side-effects are largely dependent upon the total progestogen exposure. Using the recommended doses the exposure may be greater with MPA and MA than with proligestone, the latter being a rather weak progestogen.

GnRH agonists or GnRH antagonists may also be used for oestrus prevention and suppression of testicular function. The first-generation GnRH antagonists are unsuitable for clinical use in dogs because of cost, inconvenience for long-term use, and side-effects (Vickery et al. 1989). A single dose of the third-generation GnRH antagonist acyline has recently been shown to be safe in male and female dogs. In male dogs there were short-term and reversible effects such as deterioration of sperm quality with a concurrent decrease in plasma testosterone concentration (Valiente et al. 2007; Garcia Romero et al. 2009). In bitches a single dose of acyline was highly effective in preventing ovulation. A normal oestrous cycle occurred spontaneously in all treated bitches after about 20 days (Valiente et al. 2009c). However, simultaneous administration of acyline and a GnRH agonist implant did not prevent oestrus in nine of twelve bitches, five of which also ovulated (Valiente et al. 2009b).

GnRH agonists administered in high doses over a long period of time prevent oestrus by pituitary down-regulation. However, the early stimulatory effect of GnRH analogues may cause signs of oestrus, if administered during anoestrus and sometimes even if administered during the luteal phase. It is possible to prevent the induction of oestrus by administering a GnRH agonist to pre-pubertal bitches at 4 months of age but not at 7 months, although in the latter dogs oestrus is delayed for a long time (Rubion et al. 2006; Trigg et al. 2006). However, Rubion et al. (2006) reported that GnRH agonist implants administered to bitches before puberty prevented reproductive function for 1 year. Following removal of the implant oestrus occurred naturally in 7 of 10 bitches and was induced in the other three after 1.2-14.3 months. Induction of oestrus by implantation of a GnRH agonist during anoestrus can also be prevented by the prior administration of a progestogen. The efficacy of this appears to depend on the interval between the beginning of progestogen treatment and the implantation, the stage of anoestrus, the dose of progestogen, and the type of GnRH analogue (Wright et al. 2001; Sung et al. 2006). Furthermore, adding progestogens to prevent the initial stimulation of GnRH agonists cancels the advantage of not administering progestogens for oestrus prevention.

The use of GnRH agonists as slow-release implants for suppression of reproductive function in male dogs has been amply studied and implants have been available for clinical use in male dogs in recent years (Vickery et al. 1984; Trigg et al. 2006). This is in contrast to the situation in bitches because of oestrous cycle-related problems. Shortly after administration of a GnRH agonist implant, plasma LH, FSH, oestradiol, and testosterone increase and then decrease, followed by the arrest of spermatogenesis (Vickery et al. 1984; Okada et al. 1994; Goericke-Pesch et al. 2009). The effects of these slow-release formulations of GnRH agonists are fully reversible (Tremblay and Belanger 1984; Dube et al. 1987; Lacoste et al. 1989; Trigg et al. 2001; Junaidi et al. 2003; Goericke-Pesch et al. 2009; Junaidi et al. 2009).

Terminating unwanted pregnancy and induction of parturition

Most methods for termination of unwanted pregnancy in the bitch rely on interference with the pregnancy-maintaining influence of progesterone. Complete luteolysis is very difficult to achieve during the first few weeks after ovulation in the dog (Oettle et al. 1988; Romagnoli et al. 1996). In pregnant bitches $PGF_{2\alpha}$ only induces luteolysis and subsequently abortion when multiple doses are administered beginning 10 to 30 days after fertilization (Oettle et al. 1988; Romagnoli et al. 1996). Administration of the dopamine agonists bromocriptine or cabergoline or the serotonin antagonist metergoline during the pituitary-dependent part of the luteal phase may also induce luteolysis and abortion or premature parturition, by inhibiting secretion of the luteotrophic hormone prolactin (Onclin et al. 1993; Nothling et al. 2003). Combining this with administration of $PGF_{2\alpha}$ gives more reliable induction of luteolysis and thus abortion, with fewer side-effects (Onclin and Verstegen 1999). Administration of the GnRH antagonist acyline also induces luteolysis and termination of midterm pregnancy in bitches (Valiente et al. 2009a). A more direct way of interfering with the pregnancy-maintaining effects of progesterone is the administration of a progesterone receptor antagonist such as aglepristone (Van Look and Bygdeman 1989). This method is also effective in terminating unwanted pregnancy during the pituitary-independent part of the luteal phase (Galac et al. 2000; Fieni et al. 2001b).

Since the triggering mechanism for parturition remains unclear, it has been difficult to select or develop drugs that are useful for the induction of parturition in the dog. Ideally, the drug should reliably induce whelping within a predictable, short interval. In addition, it should be safe for the bitch and the puppies, inducing normal parturition without side-effects. In the bitch, prolactin is an important luteotrophic factor during the second half of gestation. However, dopamine agonists are not useful for the induction of whelping because their effect may be delayed for several days. In addition, the interval between initiation of dopamine agonist treatment and the onset of parturition is quite unpredictable. Furthermore, treatment with inhibitors of prolactin secretion would reduce or even abolish lactation after parturition. Prostaglandin $F_{2\alpha}$ and its synthetic analogues also induce regression of the corpora lutea, resulting in the termination of pregnancy. Like dopamine agonists, prostaglandins must be administered to the bitch for several days before luteolysis occurs (Fieni et al. 1997). In addition, treatment with prostaglandins is often accompanied by side-effects such as tachypnoe, salivation, vomiting, and diarrhoea (Oettle 1982; Feldman et al. 1993; Fieni et al. 1997; Fieni et al. 1997; Moriyoshi et al. 1999), and there may be increased risk of an abnormal parturition (Williams et al. 1999).

In addition to termination of unwanted pregnancy, progesterone-receptor blockers may be useful for induction of whelping. Studies with the progesterone receptor blocker mifepristone have had variable results. Nohr et al. (1993) could only induce the initial stage of parturition that did not proceed beyond dilatation of the cervix. In contrast, Van der Weyden et al. (1989) observed normal parturition in five bitches treated with this drug. Fieni et al. (2001a) induced parturition with a single treatment with the progesterone-receptor blocker aglepristone, followed by a standard additional treatment with alfaprostol (a prostaglandin $F_{2\alpha}$ analogue) or oxytocin. Riesenbeck et al. (1999) described a single case of prolonged pregnancy that was successfully terminated by treatment with aglepristone in combination with prostaglandin $F_{2\alpha}$.

The 3β-hydroxysteroid dehydrogenase/isomerase system (3β-HSD) catalyses the final step in the synthesis of progesterone (Figure 4). Therefore, inhibiting progesterone synthesis with a competitive inhibitor of the 3β-hydroxysteroid dehydrogenase/isomerase system (3β-HSD) might also be used to terminate unwanted pregnancy (Potts et al. 1978). 3β-HSD expression has been demonstrated in canine luteal cells throughout the luteal phase and inhibition of progesterone synthesis by the 3β-HSD inhibitor epostane has been shown to be effective in bitches for termination in early stages of pregnancy (Keister et al. 1989; Kowalewski et al. 2006). Epostane is not available for use in veterinary medicine, but another 3β-HSD inhibitor, trilostane, has been registered for the treatment of hypercortisolism in dogs (Neiger et al. 2002; Ruckstuhl et al. 2002; Galac et al. 2010a), although its effectiveness and safety for induction of abortion or parturition in dogs has not been studied.

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3

Temporal relations between plasma concentrations of luteinizing hormone, follicle-stimulating hormone, oestradiol-17β, progesterone, prolactin, and α-melanocyte-stimulating hormone during the follicular, ovulatory, and early luteal phase in the bitch

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Abstract

Compared with other domestic animals, relatively little is known about the changes in, and temporal relations between, reproductive hormones around the time of ovulation in the domestic bitch. Therefore, plasma concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH), oestradiol-17β, progesterone, prolactin (PRL), and α-melanocyte-stimulating hormone (α-MSH) were determined one to six times daily from the start of the follicular phase until 5 days after the estimated day of ovulation in six Beagle bitches.

In all bitches, the preovulatory LH surge was accompanied by a preovulatory FSH surge. A preovulatory PRL or α-MSH surge was not observed. The preovulatory FSH and LH surges started concomitantly in four bitches, but in two bitches the FSH surge started 12 h earlier than the LH surge. The FSH surge (110 ± 8 h) lasted significantly longer than the LH surge (36 ± 5 h). In contrast with the preovulatory FSH surge, the preovulatory LH surge was bifurcated in four of six bitches. The mean plasma LH concentrations before (1.9 ± 0.4 μg/L) and after (1.9 ± 0.3 μg/L) the LH surge were similar, but the mean plasma FSH concentration before the FSH surge (1.6 ± 0.3 U/L) was significantly lower than that after the FSH surge (3.1 ± 0.2 U/L). In most bitches the highest plasma oestradiol-17β concentration coincided with or followed the start of the preovulatory LH surge. In five of the six bitches the plasma progesterone concentration started to rise just before or concurrently with the start of the LH surge.

In conclusion, the results of this study provide evidence for the differential regulation of the secretion of LH and FSH in the bitch. In addition, the interrelationship of the plasma profiles of oestradiol-17β and LH suggests a positive feedback effect of oestradiol-17β on LH surge release. The start of the preovulatory LH surge is associated with an increase in the plasma progesterone concentration in this species.

Introduction

The oestrous cycle of the domestic dog, a mono-oestrous species, is considerably longer than that of most other domestic species. Spontaneous ovulations are followed by a luteal phase that lasts about 75 days in the non-pregnant bitch, and by a non-seasonal anoestrus of about 2–10 months (Concannon 1993; Schaefers-Okkens 1996). The onset of pro-oestrus is usually characterized by a sanguineous vaginal discharge and it lasts for 3–17 days. Oestrus, which is defined as the period of receptivity to mating, has a duration varying from 3 to 21 days. The follicular phase lasts until ovulation, which usually takes place within 3 days after the start of oestrus behaviour. The occurrence of the preovulatory luteinizing hormone (LH) surge and ovulation cannot be predicted reliably by determining the start of oestrus (Wildt et al. 1978).

Unlike most other species, in dogs the duration of the preovulatory LH surge is relatively long, ranging from 1 to 5 days (Wildt et al. 1978; Concannon 1993; Onclin et al. 2002). For example, in cattle the preovulatory LH surge lasts only 8 h (Dieleman 1984). In dogs, ovulation is assumed to occur approximately 2–3 days after the preovulatory LH surge (Phemister et al. 1973; Concannon et al. 1977; Wildt et al. 1978). It is difficult to assess the exact time of ovulation in the bitch with the use of non-invasive techniques, such as ultrasonography, because it is not easy to differentiate between preovulatory follicles and young cavitated corpora lutea (England and Yeager 1993; Silva et al. 1996). Even more invasive techniques, such as laparoscopy and histological examination of excised ovaries, do not allow exact determination of the time of ovulation (Phemister et al. 1973; Concannon et al. 1977; Wildt et al. 1978; Silva et al. 1996).

In dogs, follicle-stimulating hormone (FSH) pulses occur concomitantly with LH pulses in all stages of the oestrous cycle and in anoestrus (Kooistra et al. 1999a). The preovulatory LH surge is also associated with a surge in FSH secretion (Olson et al. 1982; Concannon 1993). However, there is little detailed information about the temporal relation between the preovulatory surges of LH and FSH in the bitch.

During the follicular phase, the plasma oestradiol-17β concentration increases gradually and peak levels differ considerably between oestrous cycles both within and between individual bitches (Concannon et al. 1975; Olson et al. 1982). According to Wildt et al. (Wildt et al. 1979), the preovulatory oestradiol-17β surge probably triggers the preovulatory LH surge. In contrast, Concannon et al. (Concannon et al. 1979; Concannon 1993) have reported that the start of the preovulatory LH surge was associated with a decrease in the plasma oestradiol-17β concentration. Onclin et al. (Onclin et al. 2002) reported that the plasma oestradiol-17β concentration reached a maximum 24–48 h before the peak of the preovulatory LH surge. The latter findings contrast with observations in other species, such as humans and sheep, in which oestradiol is thought to exert a positive feedback effect on the release of the preovulatory LH surge. In these species, the preovulatory LH surge usually starts when the plasma oestradiol concentration is high but not yet decreasing (Liu and Yen 1983; Evans et al. 1997; Karsch et al. 1997). In healthy cyclic women, administration of increasing amounts of oestradiol in the mid-follicular phase induces an LH surge (Liu and Yen 1983). In sheep, oestradiol is required for LH surge initiation but not for LH surge maintenance (Evans et al. 1997).

In the bitch, the preovulatory LH surge is associated with an increase in the plasma progesterone concentration (Concannon et al. 1975). However, the exact temporal relation between the rise in plasma progesterone concentration relative to the preovulatory LH surge is uncertain. This preovulatory rise in plasma progesterone level is not seen in most other domestic species but has been reported in women (Liu and Yen 1983). In women, the oestrogen-induced LH surge is potentiated by progesterone (Cano and Aliaga 1995). In cattle, progesterone is synthesized by ovarian granulosa cells before ovulation but because it remains in the follicular fluid, there is no preovulatory rise in the plasma progesterone concentration (Sunderland et al. 1994).

In addition to hypothalamic inhibitory and stimulatory signals (Kooistra and Okkens 2002), gonadal steroids modulate the pituitary secretion of prolactin (PRL) in the bitch (Steinetz et al. 1990; Okkens et al. 1997; Galac et al. 2000; Kooistra and Okkens 2002). Conversely, PRL may influence the secretion of gonadotrophins. In humans and sows, lactational anoestrus occurs, which is ascribed to the hyperprolactinaemia-induced inhibition of gonadotrophin pulsatility (Bevers et al. 1983; Quesnel and Prunier 1995; Vekemans 1997). In addition, a preovulatory PRL surge has been reported in other species (Djahanbakhch et al. 1984; Arbogast and Ben-Jonathan 1990; Buys et al. 1990; Gonen and Casper 1990; Bowen and Keyes 1999), but its occurrence and relevance in bitches remain to be elucidated.

Differential enzymatic processing of proopiomelanocortin yields several biologically active melanocortins, including adrenocorticotrophic hormone and α-melanocyte-stimulating hormone (α-MSH). In rats, melanocortins, acting through the melanocortin 4 receptor, mediate the preovulatory surges of LH and PRL (Alde and Celis 1980; Scimonelli and Celis 1990; Watanobe et al. 2001). α-MSH has also been reported to stimulate LH release in women (Limone et al. 1997). Furthermore, α-MSH has been detected in follicular fluid of women and the circulating concentration of α-MSH is highest in the late follicular phase (Facchinetti et al. 1988; Mauri et al. 1990). In the bitch, information concerning the plasma α-MSH concentration during the follicular, ovulatory, and early luteal phase is lacking (Kooistra et al. 1997).

The aim of this study was to learn more about changes in and temporal relations between plasma concentrations of LH, FSH, oestradiol-17β, progesterone, PRL, and α-MSH around the time of ovulation in the bitch.

Animals, materials and methods

Animals

Six healthy Beagle bitches, 4–6 years of age, were used in this study. All had been born and raised at the Department of Clinical Sciences of Companion Animals and were accustomed to the laboratory environment and procedures such as blood collection. They were housed singly or in pairs in indoor–outdoor runs, fed on a standard commercial dog food once daily, and given water ad libitum.

Definition of oestrous cycle stages

The early follicular phase was defined as starting on the first day of pro-oestrus and lasting until vaginoscopy revealed shrinkage of the vaginal mucosa for the first time. The late follicular phase was defined as starting on the first day that shrinkage of the vaginal mucosa was observed and lasting until the estimated day of ovulation. The plasma progesterone concentration was used to estimate the day of ovulation (van Haaften et al. 1989; Silva et al. 1996). The early luteal phase was defined as starting on the estimated day of ovulation and lasting for 5 days.

Clinical examinations

The study protocol was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Utrecht University, The Netherlands.

All dogs were clinically examined three times weekly for the presence of swelling of the vulva and serosanguineous vaginal discharge. The first day serosanguineous vaginal discharge was observed was considered to signify the onset of pro-oestrus. From the onset of pro-oestrus vaginoscopy was performed three times weekly using a pediatric proctoscope (cat. no. 8834.08, R. Wolf, Germany) connected to a light source (cat. no. 4220 LP, R. Wolf, Germany). During vaginoscopy the mucosa of the cranial part of the vagina was inspected for the presence of swelling and/or shrinkage.

Collection of blood samples

Blood samples were collected daily of each individual bitch during the entire experimental period which consisted of the three defined oestrous cycle stages; the early follicular phase, the late follicular phase, and the early luteal phase (Table 1). The number of blood samples collected each day for the determination of plasma concentrations of LH, FSH, oestradiol, progesterone, PRL, and α-MSH differed per hormone and per oestrous cycle phase (Table 2). Retrospectively, blood sampling was started for each individual bitch at a different interval to the peak of the preovulatory LH surge (ranging from 296 to 80 h before the peak of the preovulatory LH surge) (Table 1).

Table 1*. Identification, age and duration of the experimental period in each individual bitch*

Bitch identification	Age (years)	Duration of experimental period (days)				Start of blood sampling in relation to preovulatory LH surge
		Total	I	II	III	Days before T = 0
A	5	20	9	6	5	12
B	6	12	0	7	5	3
C	6	15	7	3	5	6
D	5	18	5	8	5	10
E	4	13	3	5	5	6
F	4	14	3	6	5	5

T = 0 is the moment that the highest plasma LH concentration was measured during the preovulatory LH surge; I, early follicular phase; II, late follicular phase; III, early luteal phase.

***Table 2**. Blood sample frequency per 24 h for each hormone during three stages of the oestrous cycle*

	LH	FSH	Oestradiol-17β	Progesterone	Prolactin	α-MSH
Early follicular phase	3	3	3	1	3	3
Late follicular phase	6	6	6	3	6	3
Early luteal phase	6	6	1	1	6	3

Time of blood sampling; once daily, 8:00; three times daily, 8:00, 16:00, and 24:00; six times daily, 4:00, 8:00, 12:00, 16:00, 20:00, and 24:00. Blood samples were collected from the jugular vein, immediately placed in chilled lithium heparin-coated tubes, and centrifuged at 4 °C for 10 min at 1500×g. Plasma was stored at −25 °C until assayed.

Hormone determinations

Plasma LH concentrations were measured with a heterologous radioimmunoassay (RIA) as described previously (Nett et al. 1975; Kooistra et al. 1999a). The intra-assay and inter-assay coefficients of variation for values above 0.5 µg/L were 2.3 and 10.5%, respectively. The lowest detectable amount of LH was 0.3 µg/L (Kooistra et al. 1999a).

Plasma FSH concentrations were measured with a human immunometric sandwich assay (Amerlite, Amersham, UK) as described previously (Kooistra et al. 1999a). The intra-assay coefficient of variation was <5%. The inter-assay coefficients of variation were 5% at a level of 35 U/L and 9% at a level of 3 U/L. The lowest detectable amount of FSH was 0.5 U/L (Kooistra et al. 1999a).

Plasma oestradiol-17β concentrations were measured with a solid-phase RIA using 125I (Count-A-Count TKE; Diagnostic Products Co., Los Angeles, CA) according to the manufacturer's instructions with slight modifications as described previously and validated for the dog (Dieleman and Bevers 1987; van Haaften et al. 1994). The intra-assay and inter-assay coefficients of variation were 14 and 11.8%, respectively. The lowest detectable amount of oestradiol-17β was 7 pmol/L.

Plasma progesterone concentrations were measured with a previously validated RIA (Dieleman and Schoenmakers 1979; Okkens et al. 1985). The intra-assay and inter-assay coefficients of variation were 11 and 14%, respectively. The lowest detectable amount was 0.13 nmol/L.

Plasma PRL concentrations were measured with a previously validated heterologous RIA (Okkens et al. 1985). The intra-assay and inter-assay coefficients of variation were 3.5 and 11.5%, respectively. The lowest detectable amount of PRL was 0.8 µg/L.

Plasma α-MSH concentrations were measured with a RIA using an antiserum to synthetic human α-MSH, as described previously (Meij et al. 1997). This antiserum reacted equally with α-MSH and desacetyl-α-MSH. The antiserum had <0.1% cross-reactivity with ACTH (1–39) and 4% cross-reactivity with ACTH (1–24). The inter-assay coefficient of variation was 23% and the lowest detectable amount of α-MSH was 1.2 pmol/L.

Data analysis

The peak plasma LH concentration detected during the preovulatory LH surge was taken to indicate time (T) = 0 h. The start of the preovulatory LH and FSH surges and the rise in plasma progesterone concentration were defined as the first measurement that exceeded the mean of the period 72–28 h before T = 0 plus 1 standard deviation (S.D.), and was followed by a measurement which met the same requirements. Similarly, the end of the preovulatory LH and FSH surges were defined as the last measurement that exceeded the mean concentration plus 1 S.D. of the period of 100–144 h after T = 0 and was preceded by a measurement which met the same requirements.

The preovulatory surges were considered bifurcated when the decline between two consecutive plasma concentrations was more than 2 S.D. of the mean plasma LH concentration in the period 72–28 h before T = 0.

The mean plasma PRL and α-MSH concentrations were calculated for the period 72 h before to 160 h after the preovulatory LH surge.

Differences in mean plasma LH and FSH concentrations before and after the preovulatory LH/FSH surge were analyzed by paired Student's t-test (two-tailed). The difference in duration of the mean preovulatory LH and FSH surges was analyzed by independent Student's t-test. Values are expressed as mean ± S.D. $P < 0.05$ was considered statistically significant.

Results

In all bitches a preovulatory LH surge was detectable and lasted 36 ± 5 h. The mean peak plasma LH concentration was 18.7 ± 5.8 µg/L. The mean plasma LH concentrations during the period 72–28 h prior to T = 0 (1.9 ± 0.4 µg/L) did not differ from those during the period 100–144 h after T = 0 (1.9 ± 0.3 µg/L). In four of the six bitches the LH surge was bifurcated (Fig. 1). In two of these bitches the dip in LH levels lasted for at least 4 h, i.e., two consecutive samplings. In the remaining two bitches the decrease comprised one measurement.

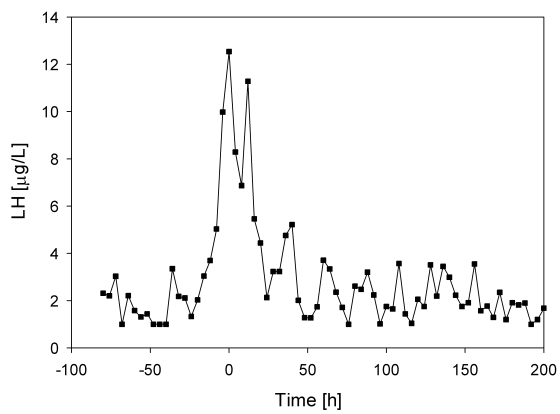

Figure 1*. The plasma concentration of LH (■, µg/L) of Beagle bitch B, 6 years old, during the late follicular and early luteal phases. Note the bifurcated preovulatory LH surge.*

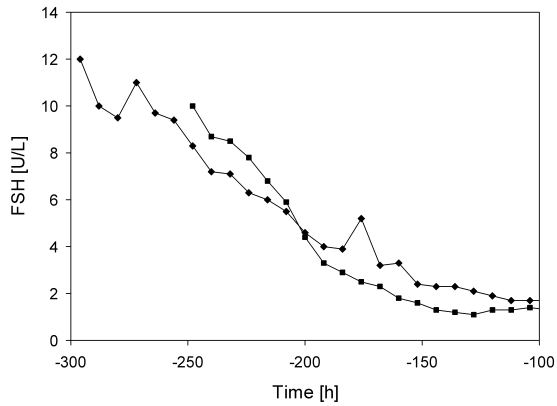

***Figure 2**. Plasma FSH concentrations of Beagle bitch A, 5 years old (♦, U/L) and Beagle bitch D, 5 years old (■, U/L) during the follicular phase. Note the decrease in the plasma FSH concentration in the early follicular phase.*

In all bitches a preovulatory FSH surge was present and lasted 110 ± 8 h which was significantly longer (P < 0.001) than the LH surge (36 ± 5 h). The mean peak plasma FSH concentration was 13.8 ± 2.0 U/L. The mean plasma FSH concentration during the period 72–28 h before T = 0 (1.6 ± 0.3 U/L) was significantly lower (P < 0.001) than that during the period 100–144 h after T = 0 (3.1 ± 0.2 U/L). In two bitches the plasma FSH concentrations at the start of the sampling period, 296 and 248 h before the preovulatory LH surge peak concentration, were relatively high, 12 and 10 U/L, respectively, but declined to basal pre-LH surge values 112 and 184 h before T = 0, respectively (Fig. 2). The preovulatory FSH surge started concomitantly with the preovulatory LH surge in four bitches (Fig. 3). In the other two bitches the LH surge started 12 h later than the FSH surge. In none of the bitches the FSH surge was bifurcated.

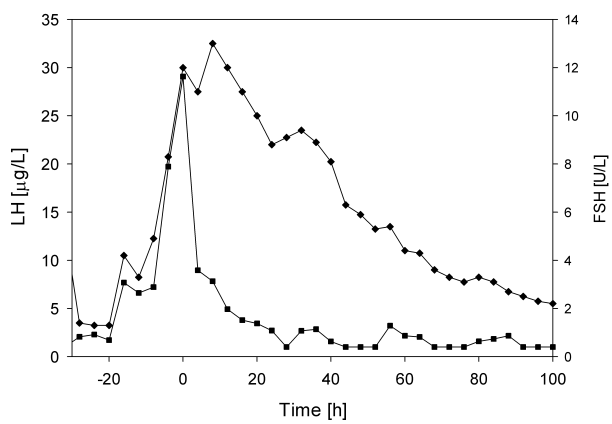

***Figure 3**. Plasma concentrations of LH (■, µg/L) and FSH (♦, U/L) in Beagle bitch D, 5 years old, during the peri-ovulatory period. Note the simultaneous start of the preovulatory FSH and LH surge.*

In all bitches the plasma oestradiol-17β concentration increased gradually during the early follicular phase but increased sharply just before the beginning of the preovulatory LH surge in four bitches (Fig. 4). In the remaining two bitches a sharp rise before the preovulatory LH surge was not observed. The mean preovulatory plasma oestradiol-17β peak concentration was 353 ± 140 pmol/L. In three bitches the highest plasma oestradiol-17β concentration coincided with the start of the LH surge (Fig. 4), and in the other three the highest plasma oestradiol-17β concentration was measured 4 and 16 h before and 4 h after the start of the preovulatory LH surge peak. The mean plasma oestradiol-17β concentration decreased to basal values (35.2 ± 8.8 pmol/L) 80 h after the preovulatory LH surge peak concentration.

The mean plasma progesterone concentration during the period 140–40 h before T = 0 was 2.2 ± 1.0 nmol/L. In three bitches the plasma progesterone concentration started to rise 12–4 h before the start of the LH surge, in two bitches these two events coincided, and in the remaining bitch the plasma progesterone concentration only started to rise 20 h after the start of the LH surge. The rate of increase in plasma progesterone concentration differed between the bitches. After the initial rise in plasma progesterone concentration around the start of the LH surge, the plasma progesterone concentration remained at this level for 72–88 h in four bitches (Fig. 5) and then increased to the high levels normally found during the first half of the luteal phase.

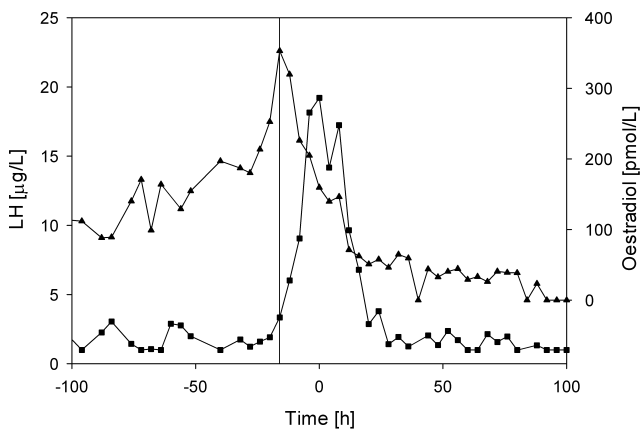

Figure 4. *Plasma concentrations of LH (■, µg/L) and oestradiol-17β (E2, ▲, pmol/L) from 100 h before until 100 h after the preovulatory LH surge in Beagle bitch A, 5 years old, showing a preovulatory surge in plasma oestradiol-17β concentration that coincided with the start of the preovulatory LH surge.*

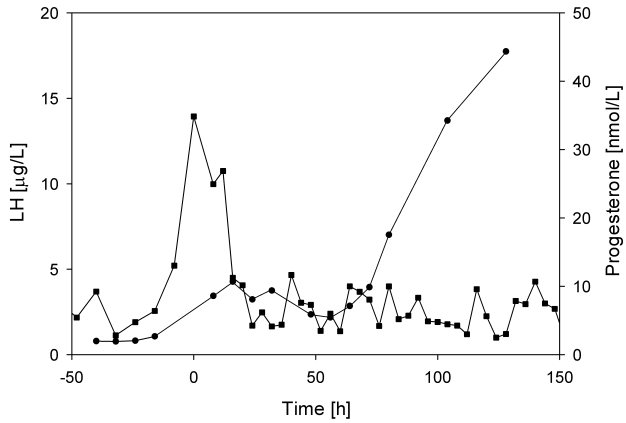

Figure 5. *Plasma concentrations of LH (■, µg/L) and progesterone (P4, ●, nmol/L) during the peri-ovulatory period in Beagle bitch C, 6 years old. After the initial increase, the plasma progesterone concentrations remained stable for 3 days.*

The mean plasma PRL concentration was 3.9 ± 1.1 µg/L and remained constant throughout the oestrous cycle. A preovulatory PRL surge was not detected. One high plasma PRL concentration (66.0 µg/L) was found in one of the six bitches at 84 h before T = 0.

The mean plasma α-MSH concentration was 7.8 ± 2.6 pmol/L. Although there were substantial intra- and interanimal variations, the mean plasma α-MSH concentration did not change significantly in the three stages of the oestrous cycle.

Discussion

The results of this study show the variation in and the temporal relation between the hormones that are considered important with regard to regulation of the oestrous cycle and ovulation in the bitch. In all bitches, ovulation was preceded by an LH surge. The mean duration of the preovulatory LH surge was 36 h, which is similar to findings of Onclin et al. (Onclin et al. 2002) but shorter than that reported by Wildt et al. (Wildt et al. 1978). These differences may be due to different sampling frequencies and different cut-off points. In four of the six bitches the preovulatory LH surge was bifurcated, and in two of these four bitches the dip even lasted at least 4 h. This bifurcated preovulatory LH surge was also described by Wildt et al. (Wildt et al. 1978) in four of twenty-five bitches, using a twice daily sample frequency. Given the relatively short duration of the dip, the bifurcation of the preovulatory LH surge would easily be missed with a low-frequency sampling schedule. This bifurcation has not been described in other species, in which the duration of the LH surge is often much shorter. In sheep, an elevated level of GnRH in the pituitary portal blood is required for the initiation and maintenance of the LH surge (Karsch et al. 1997). Our results suggest that the preovulatory GnRH surge also shows a temporary decline in dogs.

Progression from early to late anoestrus is associated with an increase in basal plasma FSH concentrations without a concomitant rise in basal LH concentrations, which suggests that in the bitch an increase in circulating FSH is critical for the initiation of ovarian folliculogenesis and thus for the termination of anoestrus (Kooistra et al. 1999a). Plasma FSH concentrations were relatively high at the start of the sampling period in two bitches, and the concentrations declined to the relatively low levels present in the other bitches during pro-oestrus. In the latter four bitches, plasma FSH concentrations were not high during the start of the follicular phase, probably because blood sampling was started at a somewhat later stage in the follicular phase, as shown by the shorter interval between the first FSH measurement and detection of the peak of the preovulatory LH-surge in these four bitches. The decline in plasma FSH concentration during the early follicular phase and the difference in FSH concentration before and after the preovulatory FSH surge may be explained by a negative feedback effect of oestradiol and inhibin, secreted by the growing follicles (Olson et al. 1982; McNeilly et al. 2003). In line with the findings of Wildt et al. (Wildt et al. 1978), the mean plasma LH concentration was not significantly different before and after the preovulatory LH surge.

In all bitches, ovulation was preceded by an FSH surge that lasted about three times longer than the preovulatory LH surge. This may be explained, at least in part, by the longer half-life of FSH than LH. As in other mammals, in dogs, the pattern of glycosylation of FSH probably differs from that of LH, and because of this LH may be cleared from the circulation faster than FSH (Chappel et al. 1983; Schwartz 1995). In contrast to the LH surge, the preovulatory FSH surge was not bifurcated. Furthermore, the preovulatory FSH and LH surges started concomitantly in four bitches, but in two bitches the FSH surge started 12 h earlier than the LH surge. These observations suggest differential regulation of FSH and LH secretion in the dog. GnRH is the main regulator of the secretion of FSH and LH by the pituitary gonadotrophic cells (Rijnberk 1996). Differential regulation of FSH and LH secretion may be ascribed to the frequency and amplitude of GnRH pulses (Haisenleder et al. 1991; Mann et al. 1992; Shupnik 1996; Vizcarra et al. 1997). In addition, a specific hypothalamic FSH-releasing factor (Yu et al. 1997) and gonadal feedback may play a role in the differential or non-parallel secretion of FSH and LH (Mann et al. 1992; Shupnik 1996). Even though FSH pulses occur concomitantly with LH pulses in all stages of the oestrous cycle and anoestrus in the dog, there is also evidence of differential regulation of FSH and LH secretion in this species (Kooistra et al. 1999a; Kooistra et al. 1999b).

Plasma oestradiol-17β concentrations increased sharply in the last 24 h before the highest plasma oestradiol-17β concentration was measured in four bitches. The peak in plasma oestradiol-17β concentration coincided with the start of the preovulatory LH surge in three of these bitches and was reached 4 h after the start of the preovulatory LH surge in the fourth bitch. In the remaining bitches the highest plasma oestradiol-17β concentrations were measured 4 and 16 h before the start of the preovulatory LH surge. These results suggest that the increase in plasma oestradiol-17β concentration acts via positive feedback on GnRH release, and hence LH secretion, to cause the preovulatory LH surge. In most mammals, increasing plasma oestradiol concentrations have a positive feedback effect on LH release during the late follicular phase (Liu and Yen 1983; Evans et al. 1997; Karsch et al. 1997).

In contrast, Concannon et al. (Concannon et al. 1979; Concannon 1993) have shown that administration of different amounts of oestradiol-17β to previously ovariectomized Beagle bitches induced an LH peak only after the discontinuation of oestradiol treatment, which led to the hypothesis that oestradiol-17β exerts a negative feedback effect on LH release in the dog. A recent study also showed that administration of increasing doses of oestradiol benzoate did not trigger an LH surge in bitches during anoestrus (Klein et al. 2003). Thus, the time window during which oestrogens exert a positive feedback effect may be very short. There appears to be a clear difference in the possibility of inducing an LH surge by oestradiol-17β between ovariectomized bitches or bitches in anoestrus versus bitches during the follicular phase. An increased sensitivity of the hypothalamus–pituitary–ovarian axis to oestradiol-17β during pro-oestrus would explain the restricted period for a positive feedback effect of oestradiol-17β. This is supported by the increased expression of oestrogen receptor genes in the mediobasal hypothalamus and the pituitary found during the transition from anoestrus to pro-oestrus (Tani et al. 1997; Hatoya et al. 2003).

The plasma progesterone concentration started to rise immediately before or concurrently with the start of the LH surge in five bitches. The preovulatory rise in progesterone is suggested to have an important role in facilitating the preovulatory LH surge in dogs (Concannon et al. 1979; Concannon 1993). However, the preovulatory rise in plasma progesterone concentration is not vital to trigger the LH surge in humans. In women concomitant and reproducible LH and FSH surges can be induced by the administration of oestradiol-17β alone (Liu and Yen 1983).

After the initial rise in the plasma progesterone concentration around the start of the LH surge, the plasma progesterone concentration remained at about the same level for 3–4 days in four of the bitches. A similar phenomenon has been described in women (Laborde et al. 1976; Hoff et al. 1983). In cattle, however, a distinct change in the oestradiol/progesterone ratio in follicular fluid in favour of progesterone is found on the day before ovulation, but this preovulatory rise in progesterone is not reflected by increased plasma progesterone concentrations (Sunderland et al. 1994). The variation in duration of the period in which plasma progesterone concentrations were stable could reflect a variation in the interval between the preovulatory LH surge and ovulation. This would suggest that measurement of the preovulatory LH surge is not very reliable for determining the exact time of ovulation. Indeed, in bitches the period in which ovulation occurs ranges from as early as 24 h until more than 96 h after the preovulatory LH surge (Wildt et al. 1978). After this period of a relatively stable plasma progesterone concentration during the preovulatory LH surge, the plasma progesterone concentration rose sharply again, probably after ovulation, i.e., due to further luteinization of the corpora lutea. The rapid rise in plasma progesterone concentration may, therefore, be a more reliable marker of ovulation than the preovulatory LH surge. Indeed, determination of the optimal mating time by repeated measurement of the plasma progesterone concentration has been reported to be very successful in the bitch (Okkens et al. 1993; Okkens et al. 2001).

Plasma PRL and α-MSH concentrations did not change significantly during the follicular and early luteal phase, as reported earlier (Olson et al. 1982). Although α-MSH plays a role in the regulation of PRL secretion in the rat (Alde and Celis 1980; Scimonelli and Celis 1990; Watanobe et al. 2001), we found no correlation between the plasma profiles of PRL and

α-MSH, which is in accordance with earlier observations in the bitch (Kooistra 2000). A high plasma PRL concentration was measured only once and was probably due to a PRL pulse, which are of low frequency and short duration in the bitch (Kooistra 2000). Alternatively, this high plasma prolactin concentration may have been part of a very short preovulatory PRL surge, which have been reported to occur in several species (Djahanbakhch et al. 1984; Arbogast and Ben-Jonathan 1990; Buys et al. 1990; Gonen and Casper 1990; Bowen and Keyes 1999).

In conclusion, the results of this study demonstrate concurrent preovulatory surges of FSH and LH, but not of PRL or α-MSH surge, in six Beagle bitches. Our results also provide evidence for the differential regulation of the secretion of LH and FSH in the bitch. The interrelationship of the plasma profiles of oestradiol-17β and LH suggests that oestradiol-17β has a positive feedback effect on LH surge release. Lastly, the start of the preovulatory LH surge is associated with an increase in the plasma progesterone concentration in this species.

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4

Differential regulation of the secretion of luteinizing hormone and follicle-stimulating hormone around the time of ovulation in the bitch

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Abstract

Plasma concentrations of luteinizing hormone (LH) and follicle stimulating hormone (FSH) were determined 3–6 times daily in six Beagle bitches from the start of the follicular phase until 5 d after the estimated day of ovulation. The aim of the study was to gain more detailed information regarding the changes in and the temporal relation between these hormones around the time of ovulation. In all bitches, the preovulatory LH surge was accompanied by a preovulatory FSH surge. The mean duration of the preovulatory FSH surge (110 ± 8 h) was significantly longer than that of the preovulatory LH surge (36 ± 5 h). The FSH surge started concomitantly with the preovulatory LH surge in four bitches, and 12 h before the start of the LH surge in the other two bitches. The preovulatory LH surge had a bifurcated pattern in four bitches. The mean plasma LH concentration before (1.9 ± 0.4 µg/L) and after (1.9 ± 0.3 µg/L) the preovulatory LH surge were similar. The mean plasma FSH concentration during the period 72–28 h before the preovulatory LH surge (1.6 ± 0.3 U/L) was lower ($P < 0.001$) than that during the period 100–144 h after the preovulatory LH surge (3.1 ± 0.2 U/L). In conclusion, this study demonstrated concurrent preovulatory surges of FSH and LH and provided more evidence for differential regulation of the secretion of FSH and LH.

Introduction

The domestic dog, a mono-oestrous species, has an oestrous cycle which is considerably longer than that of most other domestic species. Spontaneous ovulations are followed by a luteal phase, which lasts about 75 d in the non-pregnant bitch, and a non-seasonal anoestrus of about 2–10 mo (Concannon 1993). Pro-oestrus and early oestrus, characterized by bloody vaginal discharge, constitute the follicular phase, which varies in length from 6 to 20 d. The follicular phase lasts until ovulation, which usually takes place within 3 d after the start of oestrus behaviour. The duration of oestrus varies from 3 to 21 d. The occurrence of the preovulatory luteinizing hormone (LH) surge and ovulation cannot be predicted reliably by determining the start of oestrus (Wildt et al. 1978).

Gonadotrophins play an essential role in the induction of the follicular phase and ovulation. In dogs, follicle stimulating hormone (FSH) pulses appear to occur concomitantly with LH pulses in all stages of the oestrous cycle and anoestrus (Kooistra et al. 1999). The preovulatory LH-surge is also associated with a surge in FSH secretion (Olson et al. 1982). The reported duration of the canine preovulatory LH surge, ranging from 1 to 5 d, is relatively long (Wildt et al. 1978; Concannon 1993; Onclin et al. 2002), compared to other domestic species. In cattle, for example, the duration of the preovulatory LH surge is only 8 h (Dieleman 1984). In humans, however, the duration of the preovulatory LH surge is about 2 d (Hoff et al. 1983). In dogs, ovulation is assumed to take place approximately 2–3 d after the preovulatory LH surge and is accompanied by a strong increase of plasma progesterone concentration (Phemister et al. 1973; Concannon et al. 1977a; Wildt et al. 1978). Detailed information about the temporal relation between the preovulatory LH and FSH surges in the bitch is limited. The aim of this study was to increase the knowledge of changes in and temporal relations between plasma concentrations of LH and FSH around the time of ovulation in the bitch.

Materials and methods

Six healthy Beagle bitches, 4–6 y of age, were used in this study. All had been born 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. They were housed singly or in pairs in indoor–outdoor runs, fed a standard commercial dog food once daily, and given water ad libitum. All dogs were examined three times weekly for the presence of swelling of the vulva and serosanguineous vaginal discharge, which were used as markers of the onset of pro-oestrus. From the first day of observed pro-oestrus, vaginoscopy was performed once daily until shrinkage of the vaginal mucosa was seen for the first time. To estimate the time of ovulation, plasma concentrations of progesterone were determined three times weekly from the start of pro-oestrus. Ovulation was assumed to occur when the plasma progesterone concentration exceeded 16 nmol/L (Concannon et al. 1977a; Wildt et al. 1979; Okkens et al. 1985). Blood samples were collected from the jugular vein, immediately placed in chilled lithium heparin-coated tubes and centrifuged at 4 °C for 10 min at 1500 × g. Plasma was

stored at −25 °C until assayed. Blood samples were collected at 8-h intervals during the early follicular phase, starting on the first day of pro-oestrus, and lasting until the initial observation of shrinkage of the vaginal mucosa. Blood samples were collected at 4-h intervals during the late follicular phase and the early luteal phase, i.e., from the first day of shrinkage of the vaginal mucosa until 5 d after the estimated day of ovulation.

Plasma LH concentrations were determined by heterologous radioimmunoassay (RIA), as described previously (Nett et al. 1975). Plasma FSH concentrations were determined applying a human immunometric sandwich assay (Amerlite, Amersham, UK) as described previously (Kooistra et al. 1999). Plasma concentrations of progesterone were determined by a previously validated RIA (Dieleman and Schoenmakers 1979).

The highest plasma LH concentration detected during the preovulatory LH-surge was taken as T = 0. The start of the preovulatory LH and FSH surges was defined as the first of two consecutive measurements that exceeded the mean plasma hormone concentration plus one standard deviation of the period 72–28 h before T = 0. Similarly, the end of the preovulatory LH and FSH surges was defined as the last measurement that exceeded the mean plasma hormone concentration plus one standard deviation, during the period 100–144 h after T = 0 and was preceded by a measurement which met the same requirement. The preovulatory surges were considered to have bifurcated patterns when the decline between two consecutive plasma hormone concentrations was more than two standard deviations of the mean plasma hormone concentration in the period 72–28 h before T = 0. Differences in basal plasma LH and FSH concentrations before and after the preovulatory LH/FSH surge were analyzed with a paired Student's t-test (two-tailed). The difference in duration of the mean preovulatory LH and FSH surges was analyzed with an independent Student's t-test. Results are given as mean ± S.D. Differences at $P < 0.05$ were considered significant.

Results

In all bitches, a preovulatory LH surge, with a mean duration of 36 ± 5 h, was detectable. The mean peak plasma LH concentration was 18.7 ± 5.8 µg/L. The mean plasma LH concentrations during the period 72–28 h prior to T = 0 and during the period 100–144 h after T = 0 were 1.9 ± 0.4 and 1.9 ± 0.3 µg/L, respectively. In four of six bitches, the LH surge showed a bifurcated pattern (Fig. 1).

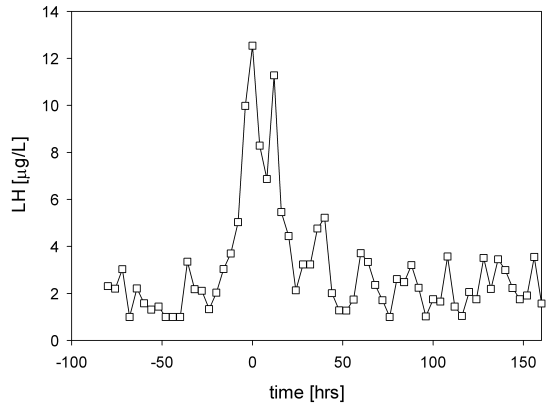

***Figure 1**. The plasma concentration of LH (□) of a 6-y-old beagle bitch during the late follicular and early luteal phase. T = 0 is the highest plasma LH concentration during the preovulatory LH surge. Note the bifurcated preovulatory LH surge*.

In all bitches a preovulatory FSH surge was present. The mean duration of the FSH surge (110 ± 8 h) was longer than that of the LH surge ($P < 0.001$). The mean peak plasma FSH concentration was 13.8 ± 2.0 U/L. The mean plasma FSH concentration during the period 72–28 h before T = 0 (1.6 ± 0.3 U/L) was lower ($P < 0.001$) than that during the period 100–144 h after T = 0 (3.1 ± 0.2 U/L). The preovulatory LH surge started concomitantly with the preovulatory FSH surge in four bitches (Fig. 2), and 12 h after the FSH surge in the other two bitches.

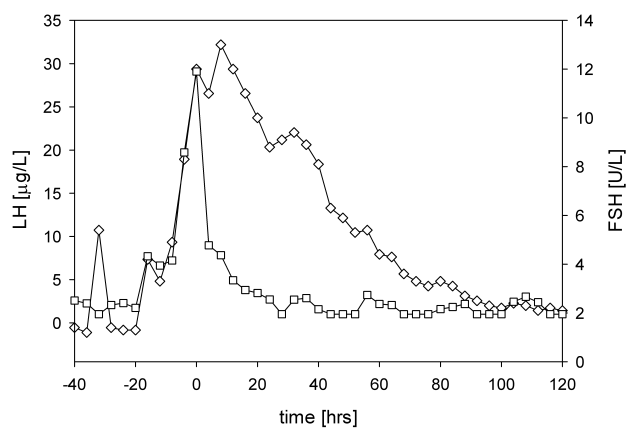

***Figure 2**. Plasma concentrations of LH (□) and FSH (◊) of a 6-y-old beagle bitch during the peri-ovulatory period. T = 0 is the highest plasma LH concentration during the preovulatory LH surge.*

In all bitches, plasma progesterone concentrations increased after the preovulatory LH surge to concentrations measured at the time of ovulation. Five days after the estimated day of ovulation, the mean plasma progesterone concentration in these six bitches was 101 ± 31 nmol/L.

Discussion

The results of this study show the temporal relation between the plasma concentrations of LH and FSH during the peri-ovulatory period. In all bitches, ovulation was determined by measuring plasma progesterone concentration and the start of the luteal phase progressed normally, since high plasma progesterone concentrations were measured within 5 d after the preovulatory LH surge (Phemister et al. 1973; Concannon et al. 1977a; Wildt et al. 1978). (Concannon et al. 1977a; Concannon et al. 1977b)

In all bitches, ovulation was preceded by an LH surge. The mean duration of the preovulatory LH surge was 36 h, which is similar to the findings of Onclin et al. (2002), but shorter than observed in the study of Wildt et al. (1978). Different sample frequencies and different cut-off points may explain these differences. The mean plasma LH concentration before the preovulatory LH surge was not significantly different from that after the preovulatory LH surge, which corresponded to the findings of Wildt et al. (1978).

In four of the six bitches, a bifurcated pattern of the preovulatory LH surge was observed and in two of these four bitches, the dip had a duration of at least 4 h. This bifurcated pattern of the preovulatory LH surge has also been described by Wildt et al. (1978) in 4 out of 25 bitches, using a twice-daily sample frequency. Obviously, taken into account the relatively short duration of the dip, the bifurcated pattern of the preovulatory LH surge can easily be missed when a low-frequency sample schedule is used. In sheep, it has been shown that an elevated GnRH level in the pituitary portal blood is required for initiation and maintenance of the LH surge (Karsch et al. 1997). From the observed significant dip during the preovulatory LH surge in the bitch, it may be hypothesized that the GnRH surge also shows a temporary decline. In other species, in which the duration of the LH surge is often much shorter, a similar bifurcated pattern has not been described.

The mean duration of the peri-ovulatory FSH surge was about three times as long as that of the preovulatory LH surge; this may, at least in part, be ascribed to a longer half-life of FSH than LH. In other mammals, a pattern of glycosylation of FSH that differs from that of LH has been described (Schwartz 1995). It is because of this difference in glycosylation patterns that LH is eliminated from the circulation faster than FSH (Chappel et al. 1983).

The mean plasma FSH concentrations before and after the preovulatory LH surge were significantly different. Furthermore, the preovulatory FSH surge did not show a bifurcated pattern in any of the bitches. Additionally, in two bitches, the FSH surge started 12 h earlier than the LH surge. These observations suggested a differential regulation of FSH and LH secretion in the dog. The main regulator of FSH and LH secretion by the pituitary gonadotrophs is GnRH (Rijnberk 1996). 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; Mann et al. 1992; Shupnik 1996; Vizcarra et al. 1997). In addition, a specific hypothalamic FSH-releasing factor (Yu et al. 1997) and gonadal feedback may play a role in the differential or non-parallel secretion of FSH and LH (Mann et al. 1992; Shupnik 1996).

In conclusion, this study demonstrated concurrent preovulatory surges of FSH and LH and also provided evidence for differential regulation of the secretion of FSH and LH in the bitch.

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5

Effects of gonadotrophin-releasing hormone administration on the pituitary-gonadal axis in male and female dogs before and after gonadectomy

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Abstract

GnRH-stimulation tests were performed in 14 female and 14 male client-owned dogs of several breeds, prior to and 4-5 months after gonadectomy. The aim of the study was to obtain more insight into the pituitary-gonadal axis in intact and neutered dogs and to establish reference values.

Basal plasma LH and FSH concentrations were increased significantly after gonadectomy in both bitches and male dogs. In both males and females ranges of the basal plasma FSH concentrations, before and after gonadectomy, did not overlap as opposed to the overlap in ranges of the basal plasma LH concentrations. Prior to gonadectomy basal plasma LH concentrations were lower and basal plasma FSH concentrations were higher in bitches than in male dogs. After gonadectomy these basal values did not differ significantly. GnRH administration before gonadectomy resulted in an increase in plasma LH and FSH concentrations in both genders. GnRH administration after gonadectomy produced an increase only in plasma LH concentrations in both genders, and a just significant increase in plasma FSH in castrated male dogs.

GnRH administration before gonadectomy resulted in a significant increase in plasma testosterone concentration in both genders. In males ranges of basal and GnRH-stimulated plasma testosterone concentrations before and after gonadectomy did not overlap.

Basal plasma oestradiol concentrations were significantly higher in intact males than in castrated males and their ranges did not overlap. The basal oestradiol concentrations in bitches before and after ovariectomy were not significantly different. At 120 min after GnRH administration, ranges of plasma oestradiol concentration of intact and ovariectomized bitches no longer overlapped.

In conclusion, basal plasma FSH concentration appears to be more reliable than basal plasma LH concentration for verification of neuter status in both male and female dogs. The basal plasma testosterone concentration appears to be reliable for verification of neuter status in male dogs. The plasma oestradiol concentration at 120 min after GnRH administration can be used to discriminate between bitches with and without functional ovarian tissue.

Introduction

The presence or absence of functional gonadal tissue in a male or female domestic dog can be uncertain in certain clinical settings, particularly if the animal's reproductive history is unknown. Furthermore, there may be a functional ovarian remnant after incomplete ovariectomy (OVX), or a disorder such as aneuploidism (e.g., 77, XO), or a disease process such as oophoritis that results in nonfunctional gonadal tissue (Johnston 1989; Nickel et al. 1991). Lastly, long-term treatment with gonadotrophin-releasing hormone (GnRH) agonists may result in downregulation of the hypothalamic-pituitary-gonadal-axis and consequent nonfunctioning of gonadal tissue (Junaidi et al. 2003; Goericke-Pesch et al. 2010).

In female dogs, the question whether or not functional gonadal tissue is present is especially relevant when a bitch that is presumed to be neutered exhibits clinical signs associated with ovarian hormones, such as a vaginal discharge or attractiveness to male dogs. However, determining whether functional ovarian tissue is present is particularly challenging during anoestrus. Vaginal cytology and vaginoscopy have no diagnostic value in this phase, although they are very useful in demonstrating oestradiol influence during the follicular phase (Olson et al. 1982; Shille and Olson 1989; de Gier et al. 2006; Schaefers-Okkens 2010; Schaefers-Okkens and Kooistra 2010). Furthermore, the plasma progesterone concentration does not discriminate between anoestrous and OVX bitches (Buijtels et al. 2006), although it is unequaled in diagnosing functional ovarian tissue during the phase of preovulatory luteinization/ovulation and the luteal phase (Okkens et al. 1981).

In male dogs, the presence of testicular tissue can usually be determined quite easily by palpation, but the absence of palpable testicles can be due to bilateral cryptorchidism (Olson et al. 1992). In addition, palpable testicular tissue is not synonymous with functional gonadal tissue. Chemical castration with GnRH implants can have resulted in complete or nearly complete suppression of steroidogenesis and thus of spermatogenesis. Although suppression of plasma testosterone concentration to basal levels may result in changed sexual dimorphic behavior in male dogs, differentiation between an intact bilateral cryptorchid and a castrated animal cannot rely solely on its behavior (Olson et al. 1992; Neilson et al. 1997; Goericke-Pesch et al. 2010). On the other hand, in healthy intact dogs the prostate size diminishes approximately 50% after reducing the plasma testosterone concentration to baseline levels (Ludwig et al. 2009). Therefore, evaluation of the prostate size during clinical examination might help to discriminate between the presence or absence of testosterone synthesis and secretion by testicular tissue.

The circulating concentrations of the gonadotrophins—follicle-stimulating hormone (FSH) and luteinizing hormone (LH)—often indicate whether a bitch has functional ovarian tissue and is in anoestrus, or has undergone OVX, since plasma LH and FSH are usually higher in the latter case (Olson et al. 1992; Buijtels et al. 2006; Beijerink et al. 2007). Unfortunately, the pulsatile pattern of secretion of these pituitary hormones may cause overlapping ranges of plasma levels between gonadally intact and OVX bitches, especially for plasma LH. Conversely, ranges of plasma FSH concentrations have been reported not to overlap between anoestrous bitches with functional ovarian tissue and OVX bitches (Reichler et al. 2004;

Beijerink et al. 2007). Plasma LH and FSH concentrations are higher in castrated male dogs than in those with functional testicular tissue (DePalatis et al. 1978). As in bitches, overlapping ranges have been reported for plasma LH but not for FSH (Olson et al. 1992).

Better differentiation between dogs with and without functional gonadal tissue can be achieved by testing with GnRH, a hypothalamic decapeptide that stimulates the release of both LH and FSH (Chakraborty and Fletcher 1977; Lacoste et al. 1988; Knol et al. 1993; Van Haaften et al. 1994; Buijtels et al. 2006). The relationship between GnRH and LH is dose-dependent in both male and female dogs (Knol et al. 1993; Van Haaften et al. 1994). In bitches with functional ovarian tissue, GnRH administration during anoestrus causes an increase in the plasma concentrations of LH, FSH, and subsequently oestradiol, the responses being greater in late anoestrus than in the early phase (Van Haaften et al. 1994; Buijtels et al. 2006; Beijerink et al. 2007). In OVX bitches, GnRH administration is reported to increase only plasma LH and not FSH, oestradiol, or progesterone (Chaffaux et al. 1981; Jeffcoate 1993; Buijtels et al. 2006; Beijerink et al. 2007). Similarly, in male dogs with functional gonadal tissue, administration of GnRH causes a rise in plasma LH and testosterone (Falvo et al. 1982; Knol et al. 1993; Purswell and Wilcke 1993), but not FSH (Purswell and Wilcke 1993).

Unfortunately, comparisons of GnRH stimulation tests in gonadectomized dogs and those with functional gonadal tissue have not been paired and have utilized small numbers of mainly beagle dogs. This study was therefore undertaken to evaluate the results of GnRH stimulation tests in larger numbers of male and female dogs of several breeds, both before and after gonadectomy. We intended thereby to establish reference values and to gain more insight into the pituitary-gonadal axis in gonadally intact and neutered dogs.

Animals, materials, and methods

Animals

Fourteen healthy, client-owned bitches were used in this study. According to the owners, all had been in oestrus at least once. All were in anoestrus at the time of inclusion in the study, with a median plasma progesterone concentration of 1.3 nmol/L (range 0.5-2.6 nmol/L), which ruled out the presence of functional corpora lutea. Furthermore, clinical examination revealed that all bitches were not in pro-oestrus or oestrus on the day the first GnRH-stimulation test was performed. They included five mix breed dogs, one American Bulldog, one Australian Kelpie, one Border Collie, one Bouvier des Flanders, one Bull Terrier, one Chow Chow, one Dachshund, one Labrador Retriever, and one Siberian Husky. Their median age at the onset of the study was 18 months (range 10 to 73 months).

Additionally, fourteen healthy, client-owned male dogs were used. All had two scrotal testicles. They included five mix breed dogs, one Chesapeake Bay Retriever, one Golden Retriever, one Maltese, one standard Poodle, two miniature Schnauzers, one Shih Tzu, one St. Bernard dog, and one Weimaraner. Their median age at the onset of the study was 38 months (range 18 to 54 months).

GnRH stimulation test, blood sampling, and hormone assays

A GnRH stimulation test was performed in the week before gonadectomy in all dogs and was repeated at 133 days (median, range 75–221 days) after gonadectomy in the bitches and at 148 days (median, range 110–229 days) after gonadectomy in the males. The difference between males and females in the interval between gonadectomy and the second GnRH stimulation test was not significant.

Blood samples were collected at -40, 0, 10, 60, 90, and 120 minutes, at which times 8, 8, 4, 8, 4, and 4 mL blood, respectively was collected. At T=0, immediately after collecting the second blood sample, a GnRH analogue was administered intravenously via the cephalic vein. Bitches received gonadoreline (Fertagyl®; Intervet/Schering-Plough Animal Health, Boxmeer, The Netherlands) in a dose of 10 µg/kg B.W., and male dogs received buserelin (Receptal®, Intervet/Schering-Plough Animal Health, Boxmeer, The Netherlands) in a dose of 0.4 µg/kg B.W. Blood was collected from the jugular vein in chilled heparin-coated tubes and centrifuged at 1500 X g for 10 min at 4 °C. An aliquot of fresh plasma of each bitch was removed for progesterone assay and then all plasma samples were stored at –25 ºC for other assays. The hormones assayed were progesterone (in bitches at -40 min), LH and FSH (in all dogs at -40, 0, 10, and 60 min), testosterone (in all dogs at 0, 60, and 90 min), and oestradiol (in bitches at -40, 0, 60, and 120 min, and in male dogs at -40, 0, 60, and 90 min).

Plasma progesterone was measured by a ^{125}I-radioimmunoassay (RIA) validated for the dog (Okkens et al. 2001). The intra-assay and interassay coefficients of variation (CVs) were 6% and 10.8%, respectively. The lower limit of quantitation was 0.15 nmol/L.

Plasma LH was measured by a heterologous 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 the 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 CVs 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 FSH was measured by immunoradiometric assay (IRMA) (AHROO4, Biocode SA, Liège, Belgium), according to the manufacturer's instructions and as described previously by Beijerink et al. (2007) (Beijerink et al. 2007). The intra-assay and interassay CVs were 3.0% and 6.0%, respectively. The lower limit of quantitation was 0.5 µg/L.

Plasma testosterone was measured by RIA (Coat-A-Count® Total Testosterone, Diagnostic Product Corporation, Los Angeles, CA) according to the manufacturer's protocol with previously described modifications to increase the sensitivity (Buijtels et al. 2006). The intra-assay and interassay CVs were 5% and 6%, respectively. The lower limit of quantitation was 51 pmol/L.

Plasma oestradiol-17β was measured by RIA (Coat-A-Count TKE; Diagnostic Products Corporation, Los Angeles, CA) according to the manufacturer's instructions with modifications as described previously (Dieleman and Bevers 1987) and validated for the dog (Van Haaften et al. 1994). The intra-assay and interassay CVs were 14% and 11.8%, respectively. The lower limit of quantitation was 7 pmol/L.

Analysis and presentation of data

Statistical analysis was performed using SPSS® for Windows, version 16.0.1 (SPSS Inc., Chicago, IL, USA). If plasma concentrations of LH, FSH, oestradiol, progesterone, or testosterone were below the limit of quantitation, the respective lower limit value was assigned to that sample. Basal LH, FSH, and oestradiol concentrations were calculated as the mean of the values in the samples collected at 40 min and directly before GnRH administration. Nonparametric tests were used because the plasma LH, FSH, oestradiol, and testosterone data were not normally distributed. Differences between both sexes were compared by the Wilcoxon rank sum test for one hormone at a given time. Within-sex differences before and after gonadectomy were compared by the Wilcoxon signed rank test. Within-sex changes in hormone concentration during GnRH-stimulation tests were analyzed by an ANOVA for repeated measures with a simple contrast, the basal plasma concentration as reference category, and a Bonferroni correction. $P<0.05$ was considered significant. Results are presented as median and range.

The term overlap of ranges was used when data ranges before and after gonadectomy were compared and overlap was present. If both data ranges did not share any common values - no overlap was present - and cut-off values with corresponding sensitivity, specificity, and 95% confidence intervals (95% CI) were calculated by receiver operating characteristic (ROC) analysis with gonadectomy as the positive state. Only cut-off values with 100% sensitivity and 100% specificity are given.

Plots for the graphic presentation of data were created with Sigmaplot 11 (Systat Software Inc., Chicago, IL, USA). In the box-and-whisker plots the box represents the interquartile range from the 25th to the 75th percentile. The horizontal bar through the box indicates the median and the whiskers represent the main body of data between the 5th and 95th percentiles.

Ethics of experimentation

This study was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Utrecht University. All owners of the dogs included in this study read and signed an informed consent form in which all procedures concerning this study were explained.

Results

The results of GnRH-stimulation tests before and after gonadectomy and the derived ratios are presented in Tables 1 and 2 and Figures 1-6.

Basal plasma LH concentrations were significantly higher after gonadectomy in both males and females. In bitches the ranges of values before and after OVX did not overlap (Table 1). ROC analysis revealed the plasma LH cut-off value to be 3.4 µg/L. In males the ranges of the values before and after surgical castration did overlap (range 1.0 µg/L – 11.3 µg/L and range 2.5 µg/L – 17.1 µg/L, respectively) (Table 1).

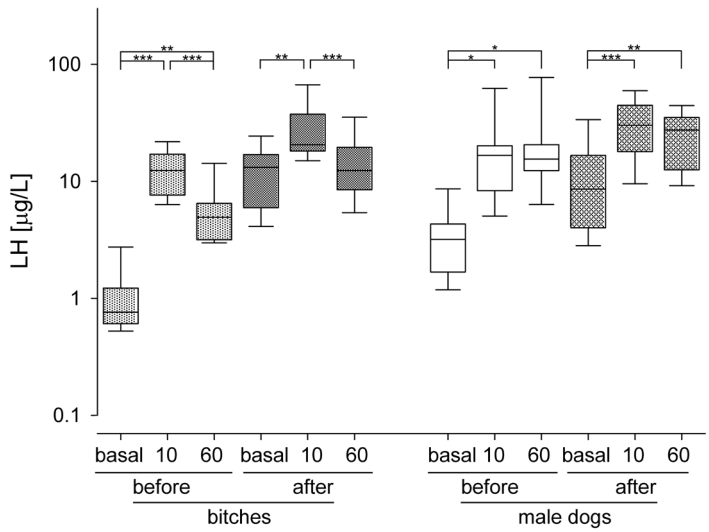

Figure 1. *Plasma LH concentrations before and at 10 and 60 min after GnRH administration in 14 bitches and 14 male dogs, before and after gonadectomy. Asterisks denote a significant difference: *: P <0.05; **: P < 0.01; ***: P < 0.001. Note the logarithmic scale of the y-axis.*

In 7 of 14 bitches and 10 of 14 male dogs the highest GnRH-stimulated plasma LH concentration before gonadectomy was higher than the basal value after gonadectomy. In all dogs, both before and after gonadectomy, plasma LH was increased at 10 min after GnRH administration and in all male dogs and most bitches it was still higher than the basal level at 60 min (Table 1 and Figure 1). In contrast, after gonadectomy it was no longer above the basal value at 60 min in four of the 14 bitches. Both before and after gonadectomy, the plasma LH concentration was significantly lower at 60 min than at 10 min after GnRH in bitches, in contrast to male dogs. In both bitches and male dogs the GnRH-stimulated plasma LH concentrations were significantly higher after gonadectomy, but the ranges of the values overlapped. Before gonadectomy the basal plasma LH concentration was significantly lower in bitches than in male dogs, while after gonadectomy the difference was not significant (Fig. 1).

Table 1 *Hormone concentrations in GnRH stimulation tests before and after gonadectomy in bitches (n=14) and male dogs (n=14).*

	Bitches				**Male dogs**			
	anoestrus		**after gonadectomy**		**with functional testicular tissue**		**after gonadectomy**	
	median	**range**	**median**	**range**	**median**	**range**	**median**	**range**
Basal LH (µg/L)	0.76	0.48 – 3.27	13.16	3.56 – 29.39	3.19	1.01 – 11.25	6.54	2.46 – 17.11
LH 10 min after GnRH (µg/L)	12.38	5.77 – 23.23	20.59	12.92 – 68.07	16.29	3.93 – 92.61	25.52	9.44 – 52.80
LH 60 min after GnRH (µg/L)	4.94	2.95 – 19.27	12.38	4.58 – 44.10	15.41	5.63 – 103.92	24.28	8.59 – 37.57
Basal FSH (µg/L)	4.17	1.18 – 8.08	53.01	13.9 – 82.88	2.17	0.50 – 6.43	26.57	8.88 – 59.66
FSH 10 min after GnRH (µg/L)	12.60	4.75 – 22.26	50.90	16.17 – 86.07	2.56	0.50 – 8.43	30.89	9.76 – 71.27
FSH 60 min after GnRH (µg/L)	12.39	5.19 – 19.54	50.64	16.57 – 89.22	3.15	0.54 – 10.16	31.92	10.94 – 75.95
Basal testosterone (nmol/L)	0.06	0.05 – 0.10	0.05	0.05 – 0.06	17.44	7.46 – 34.84	0.06	0.05 – 0.07
Testosterone 60 min after GnRH (nmol/L)	0.07	0.05 – 0.15	0.05	0.05 – 0.05	25.64	15.93 – 45.28	0.05	0.05 – 0.07
Testosterone 90 min after GnRH (nmol/L)	0.09	0.05 – 0.15	0.05	0.05 – 0.06	25.90	16.97 – 43.90	0.06	0.05 – 0.07
Basal oestradiol (pmol/L)	17.82	10.76 – 29.85	12.91	8.77 – 16.45	35.94	25.11 – 60.05	11.97	9.21 – 17.64
Oestradiol 60 min after GnRH (pmol/L)	27.92	18.25 – 47.65	13.73	7.05 – 19.09	39.80	30.25 – 58.95	10.38	7.92 – 18.25
Oestradiol 90 / 120 min after GnRH (pmol/L)	35.10	23.86 – 54.77	14.34	8.30 – 18.72	44.40	23.94 – 62.81	13.57	9.45 – 20.45

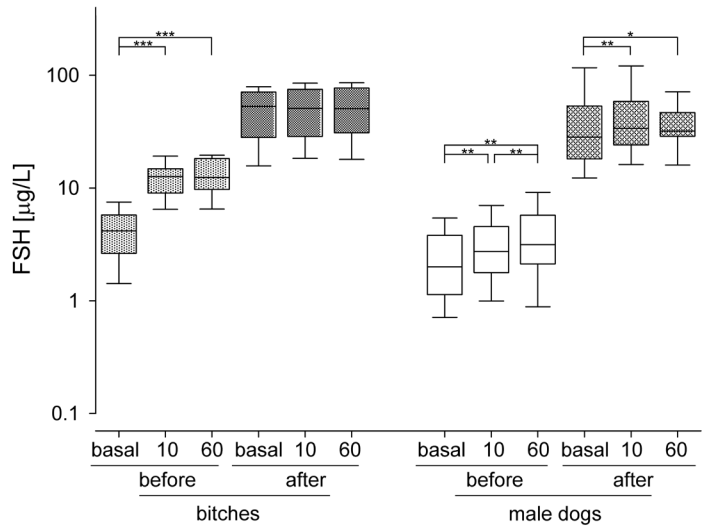

Figure 2. *Plasma FSH concentrations before and at 10 and 60 min after GnRH administration in 14 bitches and 14 male dogs, before and after gonadectomy. Asterisks denote a significant difference: *: P < 0.05; **: P < 0.01; ***: P < 0.001. Note the logarithmic scale of the y-axis.*

Basal plasma FSH concentration was increased after gonadectomy in both sexes (Table 1). ROC analysis revealed cut-off values of 11.0 µg/L for bitches and 7.7 µg/L for male dogs. In all dogs before gonadectomy and in all males after castration, plasma FSH was increased at 10 and 60 min after GnRH administration (Table 1 and Figure 2). After OVX, plasma FSH was increased in only nine bitches at 10 min and in 11 bitches at 60 min after GnRH, and the difference between basal and GnRH-stimulated FSH values was not significant. Moreover, the ranges of the FSH values before and after OVX overlapped at 10 min and 60 min after GnRH (Table 1). There was no overlap in ranges of the corresponding values in male dogs before and after castration. Taken as a group the highest GnRH-stimulated plasma FSH concentration before gonadectomy was higher than the lowest basal FSH concentration after gonadectomy in both sexes. However, in all individuals basal plasma FSH concentration after gonadectomy was higher than the highest GnRH-stimulated value before gonadectomy. In male dogs before gonadectomy plasma FSH concentration increased significantly between 10 and 60 min after GnRH administration. However, none of the differences in plasma FSH between 10 and 60 min after GnRH administration in bitches before or after gonadectomy or in male dogs after gonadectomy were significant (Fig. 2). GnRH-stimulated plasma FSH concentrations were significantly higher after gonadectomy in both males and females. Before gonadectomy, but not after, basal plasma FSH concentrations were significantly higher in bitches than in male dogs.

The LH:FSH ratios in bitches before and after OVX were comparable (Table 2, Fig. 3) and those in castrated male dogs were also similar to those in bitches. However, the ratios in intact male dogs were significantly higher than those in castrated male dogs and bitches, irrespective of the presence or absence of functional ovarian tissue. The ranges of the LH:FSH

ratios before and after castration of the male dogs did not overlap. ROC analysis revealed a cut-off ratio of 0.44. In all dogs the LH:FSH ratio was increased at 10 min after administration of GnRH. It then decreased in 10 of 12 male dogs after gonadectomy of which an LH:FSH ratio was available and in all bitches, both before and after gonadectomy.

Table 2 *LH:FSH ratios in GnRH stimulation tests before and after gonadectomy in bitches (n=14) and male dogs (n=14). T = time after GnRH administration (min).*

	Bitches			
	Anoestrus		**after gonadectomy**	
	median	**range**	**median**	**range**
LH:FSH Basal	0.23	0.07 – 0.60	0.24	0.16 – 0.39
LH:FSH T=10	1.02	0.48 – 1.57	0.58	0.25 – 1.28
LH:FSH T=60	0.42	0.19 – 0.99	0.27	0.15 – 0.54
	Male dogs			
	before gonadectomy		**after gonadectomy**	
	median	**range**	**median**	**range**
LH:FSH Basal	1.59	0.51 – 3.63	0.27	0.14 – 0.38
LH:FSH T=10	5.42	2.60 – 11.06	0.83	0.35 – 1.77
LH:FSH T=60	5.55	2.30 – 10.37	0.56	0.29 – 1.29

The ranges of basal and GnRH-stimulated plasma testosterone concentrations in male dogs before and after gonadectomy, did not overlap. ROC analysis of basal plasma testosterone concentration revealed a cut-off value in male dogs of 3.77 nmol/L. In contrast, the ranges of the basal and GnRH-stimulated plasma testosterone concentrations in bitches before and after OVX overlapped, albeit that both basal and GnRH-stimulated concentrations were significantly higher before OVX. Before gonadectomy in male dogs plasma testosterone increased in all but two animals at 60 min after GnRH administration and in all animals at 90 min. In bitches, before gonadectomy plasma testosterone concentration increased in all but three animals at 60 min after GnRH administration and in all but two at 90 min. Plasma testosterone was significantly higher at 90 min after GnRH administration than at 60 min in bitches but not in males. After gonadectomy, GnRH administration caused no increase in plasma testosterone in either males or females. Basal and GnRH-stimulated plasma testosterone concentrations before gonadectomy were significantly higher ($P < 0.001$) in male dogs than in anoestrous bitches (Fig. 4), and their ranges did not overlap. ROC analysis revealed cut-off values for the basal, T=60 min, and T=90 min values of 3.8 nmol/L, 8.04 nmol/L, and 8.56 nmol/L, respectively.

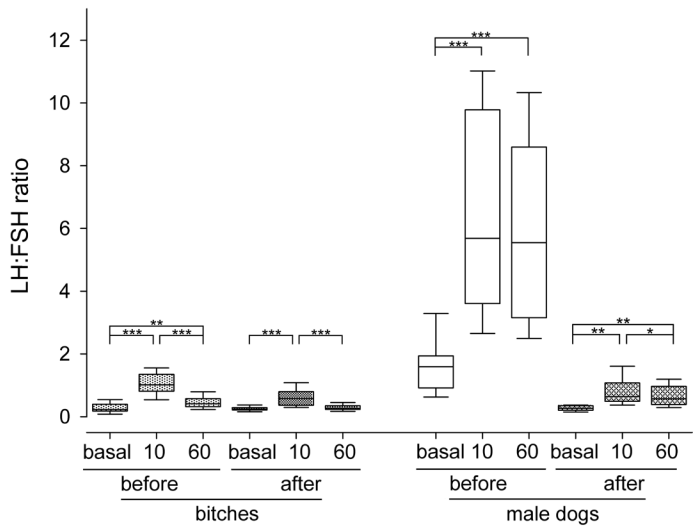

Figure 3. *LH:FSH ratios before and at 10 and 60 min after GnRH administration in 14 bitches and 14 male dogs, before and after gonadectomy. Asterisks denote a significant difference: *: P < 0.05; **: P < 0.01; ***: P < 0.001.*

The basal plasma oestradiol concentration was significantly higher in gonadally intact than in castrated male dogs and both ranges did not overlap. ROC analysis revealed a cut-off value of 21.4 pmol/L. The difference in basal plasma oestradiol concentration between anoestrous and neutered bitches was not significant. In all bitches the administration of GnRH before gonadectomy led to increased plasma oestradiol concentrations at 60 and 120 min, and the values at 120 min were significantly higher than those at 60 min (Fig. 5). Administration of GnRH after OVX did not cause a significant change in plasma oestradiol concentration in 3 bitches at 60 min or in 4 bitches at 120 min. GnRH administration before gonadectomy led to an increase in plasma oestradiol concentration in 12 of the 14 male dogs at 60 and/or 90 min. However, the difference between basal and GnRH-stimulated values was not significant before or after gonadectomy. Plasma oestradiol concentrations at both 60 and 120 min after

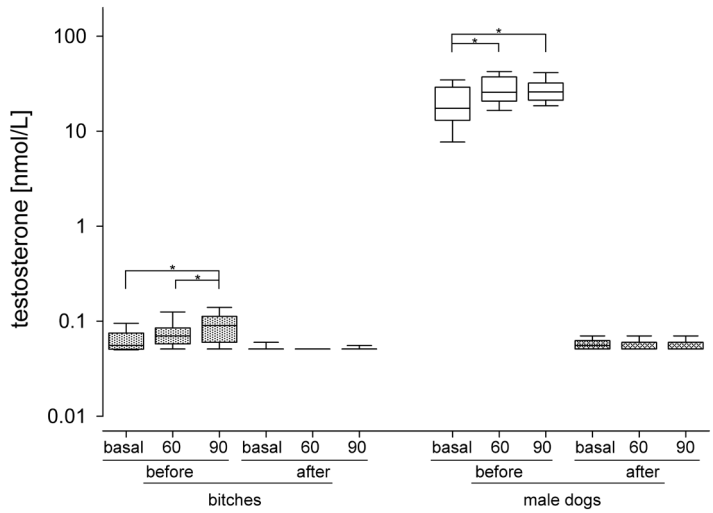

Figure 4. *Plasma testosterone concentrations before and at 60 and 90 min after GnRH administration in 14 bitches and 14 male dogs, before and after gonadectomy. Asterisks denote a significant difference: *: P < 0.05; **: P < 0.01; ***: P < 0.001. Note the logarithmic scale of the y-axis.*

GnRH administration were significantly higher in bitches before OVX. There was, however, a very slight overlap in the ranges of the plasma oestradiol concentrations at 60 min after GnRH administration between bitches sampled before and after OVX (range 18.3 – 47.7 pmol/L and range 7.1 – 19.1 pmol/L, respectively). In one bitch plasma oestradiol at 60 min after GnRH was higher after OVX than before. There was no overlap in the ranges of the plasma oestradiol concentrations at 120 min after GnRH between bitches sampled before and after OVX (Table 1) and ROC analysis revealed a cut-off value of 21.3 pmol/L. Basal plasma oestradiol concentration before gonadectomy was significantly higher in males than in females (P<0.001) but after gonadectomy the difference was not significant.

Basal LH:testosterone ratios after gonadectomy in bitches (median 325, range 89 – 587) and in male dogs (median 197, range 48 – 309) were much higher than before gonadectomy in bitches (median 14.8, range 7.9 – 32.7) or in male dogs (median 0.21, range 0.05 – 0.49) and the ranges did not overlap. ROC analysis revealed a cut-off value of 61 in bitches and 24 in male dogs. Basal LH:testosterone ratios were lower in all intact male dogs than in castrated male dogs and bitches, irrespective of the presence or absence of functional ovarian tissue (Figure 6). The difference in LH:testosterone ratios between bitches and male dogs after gonadectomy was not significant.

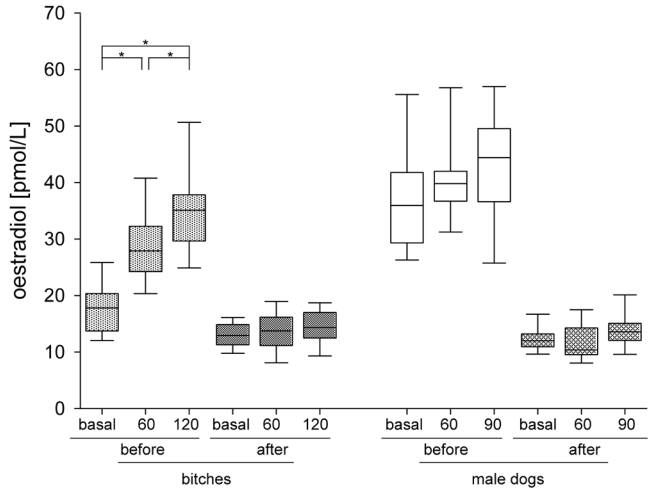

Figure 5. *Plasma oestradiol concentrations before and after gonadectomy: before and at 60 and 120 min after GnRH in 14 bitches and before and at 60 and 90 min after GnRH in 14 male dogs. Asterisks denote a significant difference: *: P < 0.05; **: P < 0.01; ***: P < 0.001.*

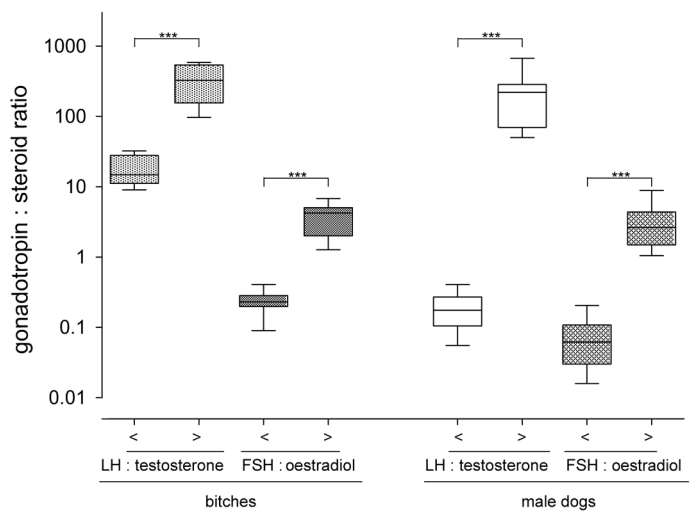

Figure 6. *Plasma LH:testosterone and FSH:oestradiol ratios before (<) and after (>) gonadectomy in bitches and male dogs. Asterisks denote a significant difference: ***: P < 0.001. Note the logarithmic scale of y-axis.*

The basal FSH:oestradiol ratios in bitches (median 4.22, range 0.84 – 7.03) and male dogs (median 2.63, range 0.96 – 9.69) after gonadectomy were higher than those in bitches (median 0.23, range 0.08 – 0.47) and male dogs (median 0.06, range 0.01 – 0.23) before gonadectomy and the ranges did not overlap. ROC analysis revealed a cut-off value of 0.66 in bitches and 0.60 in male dogs. Before, but not after, gonadectomy, basal FSH:oestradiol ratios were significantly higher ($p<0.001$) in bitches than in male dogs (Figure 6).

Discussion

In agreement with previous findings (Chaffaux et al. 1981; Olson et al. 1992; Concannon 1993; Jeffcoate 1993; Lofstedt and VanLeeuwen 2002; Reichler et al. 2004; Buijtels et al. 2006; Beijerink et al. 2007), basal plasma concentrations of both FSH and LH were significantly higher after gonadectomy in both male and female dogs. This is due to the loss of negative feedback of gonadal steroid and protein hormones, which mainly influence the hypothalamus-pituitary-gonadal axis, probably via the KiSS-1/gpr-54 system (Chaffaux et al. 1981; Olson et al. 1992; Concannon 1993; Jeffcoate 1993; Lofstedt and VanLeeuwen 2002; Reichler et al. 2004; Buijtels et al. 2006; Beijerink et al. 2007; Oakley et al. 2009; Roseweir and Millar 2009; Pineda et al. 2010; Tsutsui et al. 2010). However, the basal plasma FSH concentration appears to be more reliable than the basal plasma LH concentration for verification of the neuter status in dogs. Although we observed no overlap in ranges of the basal plasma LH values in bitches before and after OVX, the ranges of the basal LH values in male dogs before and after castration did overlap substantially, in agreement with the results of Olson et al. (1992). In contrast, in both bitches and male dogs there was no overlap in ranges of the basal plasma FSH concentrations before and after gonadectomy. In line with these findings, Beijerink et al. (2007) showed that a single measurement of plasma FSH can be used to verify the neuter status in bitches. Similarly, in women the basal plasma FSH concentration is more informative regarding ovarian reserve than is the basal plasma LH concentration (Toner 1993; Scott and Hofmann 1995; Bulun and Adashi 2003; Genuth 2004).

Reichler et al. (2004) showed that in bitches the elevated basal plasma LH and FSH concentrations after OVX slowly decrease, with a nadir at 10 weeks after OVX. Subsequently, both rise again (Reichler et al. 2004). In the present study, the interval between gonadectomy and the second GnRH stimulation test in the bitches was 75-221 days. In agreement with the observations of Reichler et al. (2004), the lowest basal plasma LH and FSH concentrations were observed in the neutered bitch which had undergone OVX only 75 days before the second GnRH-stimulation test. However, the study of Beijerink et al. (2007) was performed in bitches that had been gonadectomized more than one year prior to the measurement of plasma FSH. These observations suggest that basal plasma FSH concentration may be a more reliable indicator of neuter status if measured more than 6 months after gonadectomy.

Our results suggest that basal plasma LH values in bitches—or FSH values in both bitches and male dogs—that are above their respective cut-off values indicate that the animal has been gonadectomized. However, the cut-off values in bitches were based on measurements during anoestrus. Higher plasma gonadotrophin levels can occur at other times in the cycle, such as during the preovulatory LH and FSH surges. Furthermore, gonadotrophins are secreted in a pulsatile fashion (Concannon 1993; Kooistra et al. 1999) and fortuitous blood sampling during a pulse can result in high LH and FSH values. Indeed, in the present study, GnRH-stimulated LH and FSH values before gonadectomy, which reflect the potential height of a gonadotrophin pulse, were sometimes found to be higher than basal values after gonadectomy. Consequently, basal plasma LH and FSH concentrations should be used with caution to differentiate gonadally intact dogs from gonadectomized dogs. In order to diminish

the influence of pulsatile hormone secretion, we used the mean of two samples collected 40 min apart as the basal plasma concentration.

In agreement with previous findings, GnRH administration resulted in a clear increase in plasma LH in both male and female dogs, both before and after gonadectomy (Chakraborty and Fletcher 1977; Reimers et al. 1978; Jeffcoate 1993; Knol et al. 1993; Purswell and Wilcke 1993; Van Haaften et al. 1994; Buijtels et al. 2006; Beijerink et al. 2007). However, a significant decrease in plasma LH concentration between 10 and 60 min after GnRH administration was only observed in bitches, both before and after OVX. Its absence in male dogs is in contrast with the findings of Knol et al. (1993) and Junaidi et al. (2003). Even if a gonadorelin dose as high as 100 µg/kg B.W. i.v. was administered to male dogs, a sharp increase in plasma LH concentration at 10 min was followed by a decrease at 60 min. The explanation could be that we used buserelin in the male dogs and gonadorelin in the bitches, because gonadorelin was not available at the start of data collection in the male dogs. In several species it has been shown that buserelin is more potent than gonadorelin in releasing LH and FSH: 19 times more potent in rats, 20 to 40 times more potent in humans, and more than 50 times more potent in bovines (Sandow et al. 1973; Kuhl et al. 1976; Dericks-Tan et al. 1977; Chenault et al. 1990). We thus used a dose of buserelin 25-fold lower than that of gonadorelin, to compensate for the difference in potency. On the other hand, buserelin has a longer plasma half-life than does gonadorelin, which could be responsible for the prolonged increase in plasma LH after its administration, rather than a biological difference between male and female dogs. This is supported by findings of Grootenhuis et al. (1990), who also administered buserelin to male dogs and observed no change in plasma LH and FSH concentrations between 10 and 60 min after intravenous administration of 0.5 µg/kg b.w.. In addition, Hoffmann and Schneider (1993) administered buserelin to bitches during early anoestrus in the same dose we used in male dogs and observed the peak plasma LH concentration at 25 to 40 min, which is later than occurs after gonadorelin (Purswell and Wilcke 1993; Van Haaften et al. 1994). Consequently, differences between males and females in the present study are only discussed if a biologically relevant difference was found, independent of the type of GnRH that was used, such as differences in basal plasma hormone concentrations.

GnRH administration caused an increase in plasma FSH concentration in both males and females before gonadectomy, but the increment was less than that in plasma LH. In agreement with findings of Beijerink et al. (2007), GnRH administration did not result in a significant rise in plasma FSH concentration in bitches after OVX, while in male dogs after castration the increase was just significant. Purswell and Wilcke (1993) observed no stimulation of FSH within the first hour after administering gonadorelin to intact male dogs, but the dose was quite low (0.7 µg/kg). The relatively low response to GnRH stimulation of FSH compared to that of LH may be ascribed to differential regulation of pituitary gonadotrophin secretion. The intracellular mechanisms for storage and release are different for LH and FSH. This view is supported by in vitro studies that have shown that, while LH and FSH are produced in the same cell type, they are stored in different granules (Moyle and Campbell 1995; Ascoli and Puett 2009) and the magnitude of FSH secretion in response to secretagogues is smaller than that of LH (Chowdhury and Steinberger 1975; Muyan et al. 1994).

The concentration of GnRH-stimulated plasma FSH declined more slowly than that of LH. This can be ascribed to the longer half-life of FSH, probably due to differences between them in the pattern of glycosylation (Schwartz 1995). Consequently, LH is cleared more rapidly than FSH from the circulation (Flack et al. 1994).

Basal plasma concentrations of LH and FSH before gonadectomy were lower and higher, respectively, in bitches than in male dogs. After gonadectomy the differences in the basal LH and FSH concentration between males and females were not significant. This is reflected in the LH:FSH ratio, which was significantly higher in male dogs before castration than in the other three groups. The fact that the LH:FSH ratio did not differ significantly between gonadectomized female and male dogs suggests a primary role for gonadally produced steroids and proteins in the differential regulation of gonadotrophin secretion. The relatively high LH:FSH ratio in gonadally intact male dogs may therefore be explained by their relatively high plasma oestradiol concentrations compared to those in anoestrous bitches and gonadectomized animals. Via negative feedback both oestradiol and inhibin specifically suppress FSH synthesis and secretion (Shupnik 1996; Roseweir and Millar 2009). The relatively high plasma LH concentration in gonadally intact male dogs is more difficult to explain. Kumar et al. (1980) found that the hypothalamus contained significantly higher concentrations of GnRH in the gonadally intact male dog than in anoestrous bitches, potentially leading to higher basal LH concentrations in intact male dogs. Other important factors in the differential control of gonadotrophin secretion are the frequency and amplitude of GnRH pulses. A higher frequency of pulses results in relatively higher LH gene expression and LH secretion (Vizcarra et al. 1997; Ferris and Shupnik 2006; Ascoli and Puett 2009). It may be hypothesized that the circulating gonadal hormones in male dogs result in a pulsatile secretion pattern of GnRH that preferentially stimulates LH secretion, probably mediated via the KiSS-1/gpr-54 system (Roseweir and Millar 2009).

Basal plasma testosterone concentrations were significantly higher before than after gonadectomy in both male and female dogs, indicating that the gonads are the main source of circulating testosterone in intact dogs. The amount of testosterone produced by the ovaries depends on the phase of the reproductive cycle, and appears to be highest on the day of the preovulatory LH surge (Olson et al. 1984; Concannon and Castracane 1985). In male dogs, the highest plasma testosterone concentration stimulated by GnRH after gonadectomy was much lower than the lowest basal plasma testosterone concentration before gonadectomy. This suggests that a male dog can be considered as having no or insufficient functional testicular tissue when the basal testosterone concentration is below the cut-off value. A single measurement of plasma testosterone thus appears to reliably verify neuter status in males.

GnRH administration resulted in a significant increase in plasma testosterone concentration in both male and female dogs before gonadectomy. This has been reported previously for male dogs (Knol et al. 1993; Purswell and Wilcke 1993; Junaidi et al. 2007), but to the best of our knowledge not for bitches. After gonadectomy basal and GnRH-stimulated plasma testosterone levels were low, but often still detectable, indicating an extragonadal source of testosterone. This is most likely the reticular zone of the adrenal cortex.

The difference in basal plasma oestradiol concentrations between anoestrous and gonadectomized bitches was not significant, indicating that this measurement cannot be used to differentiate between them, as was reported previously (Frank et al. 2003; Buijtels et al. 2006).

In a study by Buijtels et al. (2006), GnRH produced a definite increase in oestradiol concentration in all anoestrous bitches but oestradiol remained undetectable in OVX bitches. This led to the conclusion that in bitches plasma oestradiol concentration can only be increased significantly when functional ovarian tissue is present. GnRH also caused a significant rise in plasma oestradiol in bitches with remnant ovarian tissue, these frequently having higher basal plasma oestradiol concentrations than anoestrous bitches (Buijtels et al. 2010). However, in the present study plasma oestradiol concentration also increased after GnRH administration in several of the ovariectomized bitches, indicating that a GnRH-induced increase in plasma oestradiol concentration is not proof of the presence of functional ovarian tissue. At 120 min after GnRH administration the ranges of plasma oestradiol concentrations in intact and ovariectomized bitches did not overlap, and hence the plasma oestradiol response to GnRH at this time can be used to discriminate between bitches with and without functional ovarian tissue. The detectable plasma oestradiol in the OVX bitches may be explained by conversion of androstenedione, produced in the adrenal cortex, into oestradiol. In addition, oestradiol can also be produced from aromatization in fat cells, hair follicles, and the liver (Nelson and Bulun 2001).

The ranges of the basal plasma oestradiol concentrations in gonadally intact and castrated male dogs did not overlap. GnRH administration did not result in a significant increase in plasma oestradiol concentrations before gonadectomy, although in 12 of the 14 male dogs plasma oestradiol concentration was found to be higher than basal after GnRH administration at 60 and/or 90 min post GnRH. A possible explanation for the lack of a significant increase in plasma oestradiol after GnRH in gonadally intact male dogs could be that oestradiol is aromatized from testosterone. This probably results in a slower increase in oestradiol concentration after GnRH administration than occurs in bitches. Furthermore, the longest interval between GnRH administration and measurement of plasma oestradiol was 90 min (compared with 120 min in bitches) and the maximum plasma oestradiol concentration might not have been reached at that time. There was a significant increase in plasma oestradiol concentration after GnRH when a larger number of males was used (unpublished data), which supports the hypothesis that GnRH administration in gonadally intact male dogs does increase plasma oestradiol.

Dynamic tests are usually chosen to investigate endocrine disorders, such as the ACTH-stimulation test to diagnose Addison's disease and the TSH-stimulation test to diagnose hypothyroidism (Rijnberk and Kooistra 2010). The results of the present study demonstrate that the GnRH-stimulation test can be used to differentiate between dogs with functional gonadal tissue and those that have been gonadectomized. It would be preferable if this could be achieved with a single blood sample, but for this purpose basal plasma LH and FSH concentrations should be used with caution. The alternative might be to measure endogenous hormone pairs in a single blood sample, such as the cortisol:ACTH ratio to diagnose

Addison's disease (Javadi et al. 2006) and the aldosterone:renin ratio to diagnose primary hyperaldosteronism (Javadi et al. 2005). Taking into account the opposite directions in which the circulating gonadotrophin and steroid hormone concentrations move after gonadectomy, the results of the present study demonstrate that the LH:testosterone and FSH:oestradiol ratios permit differentiation between gonadally intact and gonadectomized dogs by use of a single blood sample. Moreover, these ratios had the highest discriminatory power in differentiating gonadally intact from gonadectomized dogs.

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6

The pituitary-testicular axis in dogs before and after surgical castration or chemical castration with the GnRH agonist deslorelin

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Abstract

Chemical castration is achieved by implanting a GnRH agonist to reduce circulating testosterone concentration to castrate levels. It is an increasingly popular alternative to surgical castration in male dogs. Because of the paucity of information about the pituitary-testicular axis following chemical castration, we performed GnRH-stimulation tests in a large number of male dogs prior to and following surgical or chemical castration, to obtain reference values and to compare the effects of these two forms of castration.

In intact male dogs GnRH administration resulted in increased circulating levels of LH, FSH, oestradiol, and testosterone. After surgical castration basal and GnRH-induced plasma FSH and LH concentrations increased markedly, FSH more so than LH. Chemical castration with the GnRH agonist deslorelin resulted in lower plasma levels of LH and FSH and consequently plasma oestradiol and testosterone concentrations were similar to those after surgical castration. GnRH administration to chemically castrated male dogs caused a significant increase in the plasma concentration of LH but not of FSH.

By 4.5 months after implantation of the GnRH agonist, the pituitary gonadotrophs were not completely desensitized in all dogs and the increase in LH but not FSH indicates differential regulation of the release of these gonadotrophins.

Introduction

Castration of male dogs is a routine procedure in veterinary medicine for a variety of elective and medical indications. Examples of the elective indications are (1) behavioural problems ascribed to the influence of androgens such as inter-male dominance, urine marking, roaming, and mounting, and (2) persistent purulent discharge from the prepuce (Maarschalkerweerd et al. 1997; Giammanco et al. 2005). Examples of the medical indications are benign prostate hyperplasia and perianal adenoma, with their sequelae (Johnston et al. 2001; Willard 2003; Renggli et al. 2010).

Surgical castration has traditionally been the method of choice to render male dogs infertile and remove the source of male sex hormones such as testosterone. However, surgical castration is an invasive procedure and irreversible, and behavioural problems attributed to the influence of androgens are not always solved by surgical castration (Hopkins et al. 1976; Maarschalkerweerd et al. 1997; Neilson et al. 1997). Therefore, a reversible trial therapy is desirable. Also, androgen-dependent medical problems often arise in middle-aged and older dogs (Johnson 2003; Willard 2003; Lopate 2010; Renggli et al. 2010), in which the anaesthetic risk can be substantial and surgery thus undesirable. Hence there is a need for a reversible alternative to surgical castration, such as chemical castration. Progestogens such as delmadinone acetate and osaterone acetate can be used to reversibly suppress the effects of androgens with little or no suppression of the pituitary-testicular axis and little or no effect on spermatogenesis (Lange et al. 2001; Tsutsui et al. 2001). The “calming” effect on behaviour, as has also been described in horses, may be effected at the level of the central nervous system (Roberts and Beaver 1987; Perkins 2004). We are aware of no reports of studies in dogs comparing behavioural changes induced by progestogens with those occurring after surgical castration. Treatment with progestogens can result in growth hormone excess and diabetes mellitus (Kooistra et al. 1997; Court et al. 1998). Galactorrhea can occur as a result of increased prolactin secretion after termination of progestogen treatment (Braun et al. 1984; Lee et al. 2006). The potential side-effects and the lack of scientific data concerning the effects on behaviour of progestogens negatively influences their potential use as trial therapy in dogs where castration may be the desired end result.

In recent years it has become possible to achieve chemical castration with GnRH agonists (Trigg et al. 2006). Circulating testosterone concentration is reduced to castrate levels by continuous administration of a GnRH agonist. For this purpose, slow-release formulations have been developed from which a GnRH agonist, such as deslorelin or azagly-nafarelin, is released into the systemic circulation (Vickery et al. 1984; Trigg et al. 2001; Junaidi et al. 2003; Goericke-Pesch et al. 2010). The resulting continuously high circulating concentration of GnRH agonist, in contrast to pulsatile secretion of hypothalamic GnRH, results in down-regulation of the GnRH receptors and desensitization of the pituitary gonadotrophs (Jones et al. 1976; Zilberstein et al. 1983; Hazum and Schvartz 1984; Vickery et al. 1984; Junaidi et al. 2007). Plasma concentrations of LH, FSH, oestradiol, and testosterone thus decrease, followed by arrest of spermatogenesis (Vickery et al. 1984; Okada et al. 1994; Goericke-Pesch et al. 2009). The effects of these slow-release formulations of GnRH agonists have

been shown to be fully reversible (Tremblay and Belanger 1984; Dube et al. 1987; Lacoste et al. 1989b; Trigg et al. 2001; Junaidi et al. 2003; Goericke-Pesch et al. 2009; Junaidi et al. 2009b).

Information about the effects of surgical or chemical castration on the pituitary-testicular axis in male dogs is sketchy. In most studies only basal plasma hormone levels have been measured (DePalatis et al. 1978; Vickery et al. 1982; Lacoste et al. 1988; Günzel-Apel et al. 1990; Paramo et al. 1993; Inaba et al. 1996; Junaidi et al. 2003; Junaidi et al. 2009a; Goericke-Pesch et al. 2010). The function of the pituitary-testicular axis may be evaluated better by performing a GnRH-stimulation test. In most of the reports of GnRH-stimulation tests and hCG-stimulation tests in healthy dogs, only circulating concentrations of LH and testosterone, but not oestradiol and FSH, were measured (Mialot et al. 1988; Knol et al. 1993; Gunzel-Apel et al. 1994; Junaidi et al. 2007). FSH was also measured in two studies (Vickery et al. 1984; Purswell and Wilcke 1993) but there has been no report of GnRH-stimulation tests with measurements of LH, FSH, oestradiol, and testosterone, before and after castration, in a large number of male dogs.

The aim of this study was to examine the effects of surgical and chemical castration on the pituitary-testicular axis in male dogs. GnRH-stimulation tests were performed in 42 privately-owned and clinically healthy male dogs both before and after surgical castration (n=18) or chemical castration (n=24) with implants containing 4.7 mg deslorelin (Suprelorin®, Virbac Nederland BV, Barneveld, The Netherlands). Reference values were obtained for plasma LH, FSH, testosterone, and oestradiol in intact male dogs.

Animals, materials, and methods

Animals

Forty-two client-owned dogs were used in this study. The inclusion criteria were that the dog was healthy, had two scrotal testicles, and was at least 18 months old and thus had a mature hypothalamic-pituitary-testis axis at the start of the study (Mialot et al. 1988; Günzel-Apel et al. 1990). The dogs were assigned to surgical castration (SC) or chemical castration (CC), according to the owners' preference.

Surgical castration was performed in 18 dogs (six mix breed dogs, one Beagle, one Chesapeake Bay Retriever, two Golden Retrievers, one Maltese, one standard Poodle, one Rottweiler, two miniature Schnauzers, one Shih Tzu, one St. Bernard dog, and one Weimaraner). Their mean (± SD) age and body weight at the onset of the study were 33 (± 12.9) months and 22.4 (± 18.7) kg, respectively.

Twenty-four dogs were chemically castrated with implants containing 4.7 mg deslorelin, all from one batch (Suprelorin®, Virbac Nederland BV, Barneveld, The Netherlands). The implants were injected subcutaneously between the shoulder blades. This group consisted of one mix breed dog, one American Cocker Spaniel, one American Staffordshire Terrier, one Belgian Shepherd Dog, one Bichon Frisé, one Bouvier des Flandres, one Canadian White Shepherd, one miniature Dachshund, one Short-haired Dutch Shepherd Dog, one English

Bulldog, three Flatcoated Retrievers, one German Wire-haired Pointing Dog, three Labrador Retrievers, one miniature Pincher, one Rottweiler, two Tibetan Terriers, one Västgötaspets, and one Welsh Springer Spaniel. Their mean age and body weight at the onset of the study were 41 (± 22.6) months and 26.2 (± 14.2) kg, respectively.

Testicular length (a) and width (b) (including scrotal skin) were measured with a caliper. As a measure of testicular size the surface of the oval defined by the length and width of the testicles was calculated as area (cm^2) = 1/2*a* * 1/2*b* * π (Goericke-Pesch et al. 2010).

GnRH-stimulation test, blood sampling, and hormone assays

A GnRH-stimulation test was performed in the week before castration in all dogs and was repeated at 162 ± 8 (mean ± SEM) days after surgical castration and at 133 ± 4 days after chemical castration. All GnRH-stimulation tests were performed after an overnight fast. Blood samples were collected at -40, 0, 10, 60, and 90 minutes, at which times 8, 8, 4, 8, and 4 mL blood, respectively, were collected. At T=0, immediately after collecting the second basal blood sample, the GnRH analogue buserelin (Receptal®, Intervet/Schering-Plough Animal Health, Boxmeer, The Netherlands) was administered intravenously via the cephalic vein in a dose of 0.4 µg/kg B.W. Blood was collected from the jugular vein in chilled, heparin-coated tubes and centrifuged at 1500 X g for 10 min at 4 ºC. The plasma samples were stored at –25 ºC until used for assays of LH and FSH (at -40, 0, 10, and 60 min), testosterone (at 0, 60, and 90 min), and oestradiol (-40, 0, 60, and 90 min).

Plasma LH concentration was measured by a heterologous radio-immunoassay (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 the 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 (CVs) 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 FSH concentration was measured by immunoradiometric assay (IRMA) (AHROO4, Biocode SA, Liège, Belgium), according to the manufacturer's instructions and as described previously (Beijerink et al. 2007). The intra-assay and interassay CVs were 3.0% and 6.0%, respectively. The lower limit of quantitation was 0.5 µg/L.

Plasma oestradiol-17β concentration was measured by RIA (Coat-A-Count TKE; Diagnostic Products Corporation, Los Angeles, CA) according to the manufacturer's instructions with modifications as described previously (Dieleman and Bevers 1987) and validated for the dog (Van Haaften et al. 1994). The intra-assay and interassay CVs were 14% and 11.8%, respectively. The lower limit of quantitation was 7 pmol/L.

Plasma testosterone concentration was measured by RIA (Coat-A-Count® Total Testosterone, Diagnostic Product Corporation, Los Angeles, CA) according to the manufacturer's protocol with previously described modifications to increase the sensitivity (Buijtels et al. 2006). The intra-assay and interassay CVs were 5% and 6%, respectively. The lower limit of quantitation was 51 pmol/L.

Analysis and presentation of data

Statistical analysis was performed using SPSS® for Windows, version 16.0.1 (SPSS Inc., Chicago, IL, USA). The sum of left and right testicular size was used for statistical analysis. Data concerning testicular size, body weight, and time between castration and the second GnRH stimulation test were normally distributed. Results are presented as mean ± SD or mean ± SEM and differences were analysed by the Student's t-test.

If the plasma concentration of LH, FSH, oestradiol, or testosterone was below the limit of quantitation, the lower limit value was assigned to the sample. Basal LH, FSH, and oestradiol concentrations were calculated as the mean of the values at 40 min and immediately before GnRH administration. The pre-treatment plasma hormone concentrations in all dogs before and after GnRH administration were used as reference values for the GnRH-stimulation test in intact male dogs.

Nonparametric analysis was used for comparison of LH, FSH, oestradiol, and testosterone values because the plasma concentrations were not normally distributed. Differences between treatment groups for one hormone at a given time were compared by the Wilcoxon rank sum test. Within-group differences before and after gonadectomy were compared by the Wilcoxon signed rank test. Within-group changes in hormone concentration during the GnRH-stimulation test were analysed by an ANOVA for repeated measures with a simple contrast, the basal plasma concentration as the reference category, and Bonferroni correction. $P<0.05$ was considered significant. Results are presented as median and range.

Cut-off values with corresponding sensitivity, specificity, and 95% confidence intervals (95% CI) were calculated by receiver operating characteristic (ROC) analysis with "after treatment" as the positive state. Only cut-off values with 100% sensitivity and 100% specificity are given.

Plots for the graphic presentation of data were created with Sigmaplot 11 (Systat Software Inc., Chicago, IL, USA). In the box-and-whisker plots the box represents the interquartile range from the 25^{th} to the 75^{th} percentile. The horizontal bar through the box indicates the median and the whiskers represent the main body of data between the 5^{th} and 95^{th} percentiles.

Ethics of experimentation

This study was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Utrecht University. All owners of the dogs included in this study read and signed an informed consent in which all procedures concerning this study were explained

Results

Before treatment the difference between the cumulative mean (± SEM) testicular size in the SC dogs (13.3 ± 1.9 cm^2) and the CC dogs (15.1 ± 1.2 cm^2) was not significant. The mean cumulative testicular size decreased to 8.5 ± 0.9 cm^2 ($P<0.001$) after chemical castration.

In the SC dogs the difference between mean body weight (± SEM) before castration (22.4 ± 4.4 kg) and after castration (22.9 ± 4.4 kg) was not significant. In the CC dogs body weight after chemical castration (27.3 ± 3.0 kg) was higher ($P=0.003$) than before treatment (26.2 ± 2.9 kg).

Table 1 *Plasma concentrations of LH, FSH, oestradiol, and testosterone, before and after GnRH administration in 42 healthy and gonadally intact male dogs of different breeds*.

	median	**range**
Basal LH (µg/L)	2.7	1.0 – 12.2
LH 10 min after GnRH (µg/L)	16.3	3.2 – 116.4
LH 60 min after GnRH (µg/L)	16.3	5.6 – 103.9
Basal FSH (µg/L)	2.4	0.4 – 14.0
FSH 10 min after GnRH (µg/L)	3.2	0.5 – 20.4
FSH 60 min after GnRH (µg/L)	4.3	0.5 – 23.3
Basal oestradiol (pmol/L)	36.9	24.6 – 73.2
Oestradiol 60 min after GnRH (pmol/L)	41.4	29.7 – 73.8
Oestradiol 90 min after GnRH (pmol/L)	43.7	23.9 – 74.9
Basal testosterone (nmol/L)	14.0	1.3 – 46.8
Testosterone 60 min after GnRH (nmol/L)	24.2	12.8 – 84.5
Testosterone 90 min after GnRH (nmol/L)	25.3	10.4 – 67.0

The data from all dogs before surgical or chemical castration (N=42) were used to determine reference values for the GnRH-stimulation test in intact male dogs (Table 1, Figure 1). In the gonadally intact dogs GnRH administration caused an increase in plasma LH and FSH at 10 min and in plasma oestradiol and testosterone at 60 min. Plasma FSH and oestradiol concentrations increased further between 10 and 60 and between 60 and 90 min, respectively. The differences in GnRH-stimulated plasma LH and testosterone concentrations between 10 and 60 min and 60 and 90 min, respectively, were not significant. The ranges of GnRH-stimulated plasma LH, FSH, oestradiol, and testosterone concentrations overlapped with the ranges of the basal values.

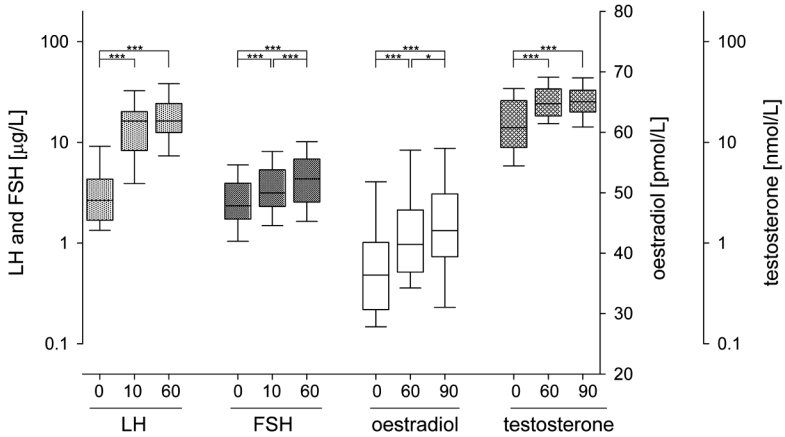

Figure 1. *Plasma LH and FSH concentrations before and at 10 and 60 min after GnRH administration, and plasma oestradiol and testosterone concentrations before and at 60 and 90 min after GnRH administration in 42 gonadally intact male dogs of different breeds. Asterisks denote a significant difference: *: P <0.05; ***: P < 0.001. Note the different scales of the y-axis.*

The basal and GnRH-stimulated plasma LH concentrations after SC were higher than those before castration (P=0.001 and P=0.01, respectively). In contrast, basal and GnRH-stimulated plasma LH concentrations were lower after CC than before CC (P<0.001) (Table 2 and Fig. 2). ROC analysis revealed a cut-off value of 1.1 µg/L for basal plasma LH concentration in CC dogs. In both SC and CC dogs, before and after castration, plasma LH concentrations were significantly higher than the basal value at T=10 and T=60 min after GnRH administration (Fig. 2). In all individual dogs, before and after SC and before CC, plasma LH concentration was increased at T=10 min and T=60 min after GnRH administration. After chemical castration, no increase in plasma LH concentration was observed in one dog at T=10. In this and two other dogs plasma LH concentration at T=60 was lower than the basal value.

Basal and GnRH-stimulated plasma FSH concentrations were higher after than before SC castration (P<0.001). In contrast, basal and GnRH-stimulated plasma FSH concentrations were lower after CC than before (P<0.001) (Table 2 and Fig. 3). ROC analysis revealed a cut-off value of 7.7 µg/L for basal plasma FSH concentration in SC dogs. In all dogs before castration and in the SC dogs after castration, plasma FSH concentration at T=10 and T=60 min after GnRH administration was higher than the basal value (Fig. 3). After CC, plasma FSH concentration at T=10 and T=60 min after GnRH administration was similar to the basal value (Table 2 and Fig. 3). In all individual dogs, before and after SC and before CC, plasma FSH concentration at T=10 min and T=60 min after GnRH administration was higher than the basal value.

The basal LH:FSH ratios in the two groups before castration were similar. In SC dogs the basal LH:FSH ratio decreased after surgical castration without overlapping of the ranges before and after castration (Table 2). ROC analysis revealed a cut-off for the basal LH:FSH ratio of 0.45. In CC dogs the basal LH:FSH ratio increased after chemical castration, with overlapping of the ranges before and after castration. After castration the basal LH:FSH ratio was higher in CC than in SC dogs (P<0.001).

Table 2 *Plasma concentrations of LH, FSH, oestradiol, and testosterone, the LH:FSH ratio, and the testosterone:oestradiol (Te:E2) ratio before and after GnRH administration, prior to and following surgical (n=18) or chemical (n=24) castration.*

	Surgical castration						**Chemical castration**					
	before treatment			**after treatment**			**before treatment**			**after treatment**		
	median	**range**	**n**	**median**	**range**	**n**	**median**	**range**	**n**	**median**	**range**	**n**
Basal LH (µg/L)	3.2	1.0-11.3	14	8.6	2.5-36.0	14	2.6	1.1-12.2	24	0.68	0.4-1.0	23
LH 10 min after GnRH (µg/L)	16.7	3.9-92.6	14	30.2	9.4-65.1	14	15.1	3.2-116.4	24	1.11	0.4-3.8	22
LH 60 min after GnRH (µg/L)	15.5	5.6-103.9	13	27.4	8.6-49.0	13	19.2	6.0-71.4	24	0.80	0.4-2.7	21
Basal FSH (µg/L)	2.0	0.4-6.4	16	28.4	8.9-128.3	15	3.1	0.9-14.0	24	0.5	0.5-3.6	22
FSH 10 min after GnRH (µg/L)	2.7	0.5-8.4	16	32.1	9.8-137.5	15	3.6	1.2-20.4	24	0.5	0.5-3.6	22
FSH 60 min after GnRH (µg/L)	3.2	0.5-11.1	14	32.0	10.9-78.8	14	4.8	1.3-23.3	24	0.5	0.5-3.7	22
Basal LH : FSH	1.59	0.5 – 3.6	14	0.27	0.1 – 0.4	13	1.0	0.2 – 7.2	24	1.3	0.2 – 1.8	22
Basal oestradiol (pmol/L)	36.5	25.1-60.1	17	12.2	9.2-19.1	16	36.3	24.6-73.2	21	14.58	8.6-19.9	21
Oestradiol 60 min after GnRH (pmol/L)	40.0	30.3-59.0	15	11.3	7.9-18.3	14	42.7	29.7-73.8	21	13.52	8.6-28.0	20
Oestradiol 90 min after GnRH (pmol/L)	44.3	23.9-62.8	17	13.1	9.5-20.5	13	43.2	31.4-74.9	20	14.79	10.8-24.2	17
Basal testosterone (nmol/L)	17.4	1.3-34.8	18	0.05	0.05-0.07	18	11.0	3.8-46.8	24	0.05	0.05-0.7	23
Testosterone 60 min after GnRH (nmol/L)	25.5	15.9-45.3	17	0.05	0.05-0.07	17	23.9	12.8-84.5	24	0.06	0.05-8.2	23
Testosterone 90 min after GnRH (nmol/L)	25.7	17.0-43.9	17	0.05	0.05-0.07	15	24.9	10.4-67.0	23	0.09	0.05-7.7	22
Basal Te:E2 x 1000	562.5	211 – 1046	17	4.6	2.7 – 7.1	16	335	122 – 997	21	4.3	2.9 – 33.3	21

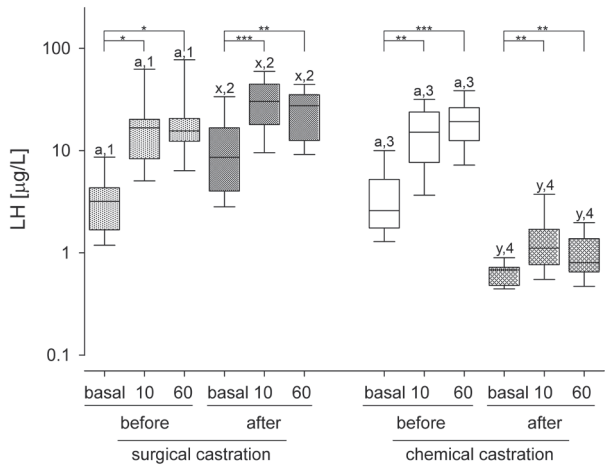

Figure 2. *Plasma LH concentration before and at 10 and 60 min after GnRH administration, before and after surgical (n=18) or chemical (n=24) castration. Asterisks denote a significant difference between basal and post-GnRH values: * P < 0.05, ** P < 0.01, *** P < 0.001. Letters denote a significant difference (P<0.001) between SC and CC dogs for values at the same time in the GnRH-stimulation test. Numbers denote a significant difference (P<0.001, except SC basal: P=0.001; SC T=10 min: P=0.01; SC T=60 min: P=0.01) between pre- and post-castration values within the SC or CC groups. Note the logarithmic scale of the y-axis.*

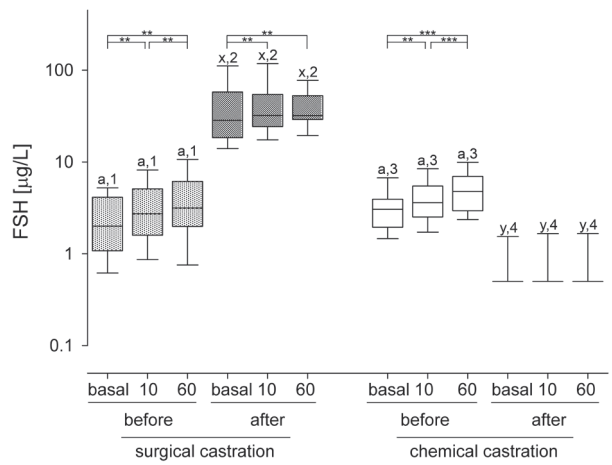

Figure 3. *Plasma FSH concentration before and at 10 and 60 min after GnRH administration, before and after surgical (n=18) or chemical (n=24) castration. Asterisks denote a significant difference between basal and post- GnRH values: ** P < 0.01, *** P < 0.001. Letters denote a significant difference (P<0.001) between SC and CC dogs for values at the same time in the GnRH-stimulation test. Numbers denote a significant difference (P<0.001) between pre- and post-castration values within the SC or CC groups. Note the logarithmic scale of the y-axis.*

Basal and GnRH-stimulated plasma oestradiol concentrations in SC and CC dogs were similar, both before and after castration, and in both groups were lower (P<0.001) than those before castration (Table 2 and Fig. 4). ROC analysis revealed cut-off values of 22.1 and 22.2 pmol/L for basal plasma oestradiol concentration in SC and CC dogs, respectively. Before castration, plasma oestradiol concentration was significantly increased at T=90 min after GnRH administration in both SC (P=0.02) and CC (P<0.001) dogs and in the CC dogs also at T=60 (P<0.001). Before surgical castration, plasma oestradiol was only slightly elevated at T=60 (P=0.06) (Table 2 and Fig. 4). After castration in both SC and CC dogs, plasma oestradiol values at T=60 and T=90 min after GnRH administration were similar to the basal value.

In both the SC and CC dogs basal and GnRH-stimulated plasma testosterone concentration was higher before than after castration (P<0.001) (Table 2 and Fig. 5). After castration GnRH-stimulated plasma testosterone concentration was higher in the CC dogs than in the SC dogs at T=60 min (P=0.02) and T=90 min (P=0.008). ROC analysis revealed cut-off values for basal plasma testosterone of 0.7 nmol/L in SC dogs and 2.2 nmol/L in CC dogs. In both groups plasma testosterone increased significantly by T=60 min after GnRH administration and was unchanged at T=90 min (Figure 5). After surgical or chemical castration, plasma testosterone concentration at both T=60 min and T=90 min after GnRH administration was similar to the basal value.

The basal testosterone:oestradiol ratios in the SC and CC dogs were similar, both before and after castration (Table 2). In both groups there was an approximately one-hundred fold decrease after castration (P<0.001). ROC analysis revealed cut-off ratios of 109 for SC and 78 for CC dogs.

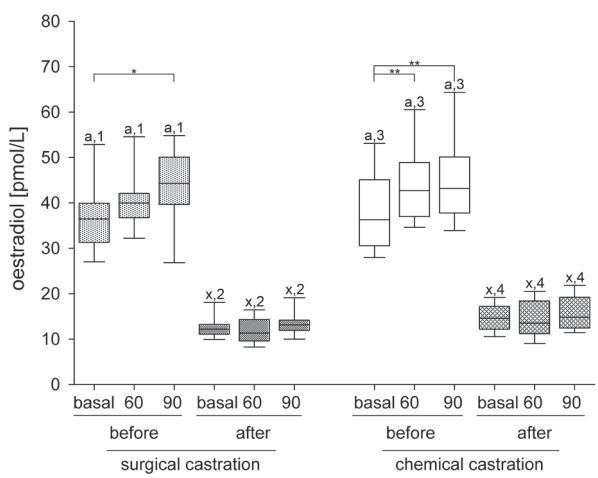

Figure 4. *Plasma oestradiol concentration before and at 60 and 90 min after GnRH administration, before and after surgical (n=18) or chemical (n=24) castration. Asterisks denote a significant difference from the basal value: * P < 0.05, ** P < 0.01. Letters denote a significant difference (P<0.001) between SC and CC dogs at the same time in the GnRH-stimulation test. Numbers denote a significant difference (P<0.001) between pre- and post-castration values within the group*.

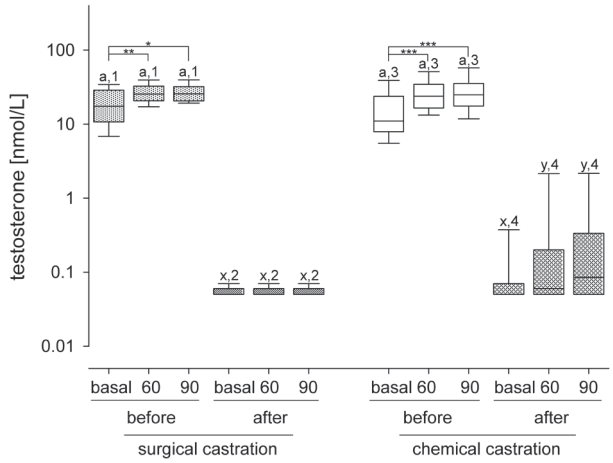

Figure 5. *Plasma testosterone concentration before and at 60 and 90 min after GnRH administration, before and after surgical (n=18) or chemical (n=24) castration. Asterisks denote a significant difference from the basal value: * P < 0.05, ** P < 0.01, *** P < 0.001. Letters denote a significant difference (P<0.001) between SC and CC dogs at the same time in the GnRH-stimulation test. Numbers denote a significant difference (P<0.001) between pre- and post-castration values within the group. Note the logarithmic scale of the y-axis.*

Discussion

Reference values for plasma LH, FSH, oestradiol, and testosterone were determined in 42 male dogs of several breeds before and after administration of the GnRH analogue buserelin. Reference values are needed for evaluation of the pituitary-gonadal axis in dogs in various clinical situations. An example is in determining whether a dog with an unknown history is cryptorchid or castrated. Pulsatile secretion of the gonadotrophins limits the worth of hormone measurements in a single blood sample. A provocation test of the pituitary-gonadal axis using GnRH has been proposed as an alternative, but reference data are scarce and mostly based on small numbers of animals in a few breeds (Freshman et al. 1990; Kawakami et al. 1993; Knol et al. 1993; Purswell and Wilcke 1993; Gunzel-Apel et al. 1994; Junaidi et al. 2007).

GnRH administration caused a significant increase of the plasma concentrations of LH, FSH, oestradiol, and testosterone in the intact male dogs. Results were generally similar to those previously reported but instead of the short-lived increase in plasma LH after GnRH administration observed in most of the reported studies, (Jones et al. 1976; Falvo et al. 1982; Kawakami et al. 1993; Knol et al. 1993; Purswell and Wilcke 1993; Gunzel-Apel et al. 1994; Junaidi et al. 2007), we found plasma LH to be still increased at T=60 min. This is probably because buserelin has a longer half-life than native GnRH (Karten and Rivier 1986; Grootenhuis et al. 1990; Padula 2005). LH is the main regulator of testosterone secretion and the interval between the increase in plasma LH and that of plasma testosterone has been shown to be 30-60 min in dogs (DePalatis et al. 1978; Knol et al. 1993; Gunzel-Apel et al. 1994). This also explains why plasma testosterone concentration did not change between T=60 min and T=90 min (DePalatis et al. 1978)

Chemical castration with deslorelin implants first causes stimulation of gonadotrophin secretion and then secondary hypogonadism (Trigg et al. 2001; Junaidi et al. 2007; Ludwig et al. 2009). Consequently, testis size had decreased significantly in the CC dogs at 4-5 months after implantation This can probably be interpreted as confirmation of the arrest of spermatogenesis at the stage of conversion of spermatogonia to primary spermatocytes, as has been reported (Tremblay and Belanger 1984; Vickery et al. 1984; Cavitte et al. 1988; Paramo et al. 1993; Goericke-Pesch et al. 2009; Junaidi et al. 2009b; Ludwig et al. 2009). However, we did not evaluate spermatogenesis by sperm analysis or by histological examination of the testes.

In agreement with previous findings, basal plasma concentrations of both FSH and LH were significantly higher after surgical castration (DePalatis et al. 1978; Olson et al. 1992) and significantly lower after administration of the deslorelin implant (Cavitte et al. 1988; Junaidi et al. 2007; Goericke-Pesch et al. 2009). After surgical castration the negative feedback of the gonadal steroids and protein hormones on GnRH secretion ceases abruptly. This is probably mediated via the KiSS-1/gpr-54 system, as the Kisspeptin neurons, which induce GnRH secretion by the GnRH neurons, express the sex steroids receptors that the GnRH neurons lack (Oakley et al. 2009; Roseweir and Millar 2009; Tsutsui et al. 2010). The removal of negative feedback is followed by increased secretion of LH and FSH (DePalatis et al. 1978; Winter et al. 1982; Winter et al. 1983). In contrast, plasma LH and FSH concentrations decreased after CC. This is in keeping with the proposed mechanism of action—downregulation of GnRH receptors and desensitization of pituitary gonadotrophs due to continuously elevated plasma GnRH (Jones et al. 1976; Zilberstein et al. 1983; Hazum and Schvartz 1984; Vickery et al. 1984; Junaidi et al. 2007). GnRH secretion is also elevated after gonadectomy but probably still pulsatile (Concannon 1993), as suggested by the increased but pulsatile plasma LH and FSH levels after ovariectomy in bitches (Concannon 1993). Due to pulsatile secretion of GnRH there is no desensitization of the gonadotrophs. Thus it is not an increase in basal plasma GnRH or GnRH agonist but the cessation of pulsatile secretion, as seen after administration of a slow-release GnRH agonist, that desensitises gonadotrophic cells after CC. This emphasizes the importance of the pulsatile nature of GnRH secretion in the regulation of gonadotrophin secretion.

After surgical castration basal and GnRH-stimulated plasma FSH increased more than did LH, as reported previously in both male dogs and bitches (Olson et al. 1992). In contrast to LH, the increase in basal plasma FSH was so great that there was no overlap between pre- and post-castration values. Hence the cut-off value had 100% sensitivity and specificity in distinguishing gonadally intact dogs from dogs after SC. Although LH and FSH are secreted by the same gonadotrophic cells, stimulated by GnRH, they are differentially regulated in the bitch (de Gier et al. 2006). Differential feedback effects of the gonadally secreted steroid and protein hormones, probably via the KiSS-1/gpr-54 system, constitute part of the differentially regulated pituitary gonadotrophin secretion (Shupnik 1996; Roseweir and Millar 2009). For example, the negative feedback by oestradiol and inhibin on the gonadotrophs mainly targets FSH secretion (Shupnik 1996; Hayes et al. 2001; O'Connor and De Kretser 2004; Roseweir and Millar 2009). This suggests that negative feedback by gonadally secreted hormones is more important for FSH secretion than for LH secretion and explains why after SC plasma FSH

increases more than plasma LH. The difference in effect of gonadectomy on basal plasma LH and FSH is also reflected by the decrease in the basal LH:FSH ratio following castration.

The decrease in basal plasma LH concentration following castration by long-term GnRH administration has been shown in several studies in dogs (Vickery et al. 1984; Cavitte et al. 1988; Paramo et al. 1993; Inaba et al. 1996; Junaidi et al. 2003; Goericke-Pesch et al. 2009; Ludwig et al. 2009). However, plasma LH concentration increased significantly after GnRH administration approximately 4.5 months after administration of the deslorelin implant, indicating that the pituitary gonadotrophs were not completely desensitized in all dogs at this time. Junaidi et al. (2007) performed GnRH-stimulation tests at different intervals after administration of an implant containing deslorelin and observed no increase in plasma LH at 100 days after start of treatment (Junaidi et al. 2007). This difference may be explained by (1) their use of native GnRH, whereas we used the more potent buserelin (Padula 2005), (2) their use of smaller dogs (15-22 kg versus 26.2 ±14.2 in our study), resulting in a higher deslorelin dose per kg body weight, and (3) the shorter interval after castration (100 days versus 133 days in our study). It is possible that some dogs in our study were completely downregulated at an earlier time but had started to regain pituitary responsiveness to GnRH. Partial reversal of the effect of deslorelin would be compatible with the suggestion by Junaidi et al. (2009a) that the dose-response relationship for deslorelin is expressed with respect to the maximum duration of suppression and not the degree of suppression.

There are few published data on basal plasma FSH concentrations before and after chronic treatment with GnRH agonists in dogs. Goericke-Pesch et al. (2009) demonstrated a similar and significant effect of the GnRH agonist azagly-nafarelin on basal FSH and the findings of Cavitte et al. (1988) were highly suggestive of such an effect by the GnRH agonist D-Trp6-LH-RH. This is consistent with the expected downregulation of pituitary gonadotrophin secretion. In contrast to plasma LH, plasma FSH did not increase in CC dogs after GnRH administration. The difference in response of FSH and LH to GnRH stimulation after chemical castration may also be ascribed to differential regulation of pituitary gonadotrophin secretion. GnRH induces the secretion of both LH and FSH but preferentially LH (Urban et al. 1988). In vitro studies have shown that LH and FSH are stored in different granules within the same secretory cell (Moyle and Campbell 1995; Ascoli and Puett 2009). The magnitude of the FSH response to secretagogues is smaller than that of LH (Chowdhury and Steinberger 1975; Muyan et al. 1994). LH release is highly responsive to increased GnRH pulse frequency (Marshall et al. 1991), whereas plasma FSH concentration increases in response to *decreased* GnRH pulse frequency (Gross et al. 1987).

Basal and GnRH-stimulated plasma oestradiol concentrations decreased to similar levels after surgical and chemical castration and the approximately 50% decrease in basal plasma oestradiol after castration that we observed is similar to the findings of others (Ludwig et al. 2009; Goericke-Pesch et al. 2010). Plasma oestradiol was still above the detection limit of the assay after SC, pointing to extragonadal production. Although peripheral aromatization of androgens to oestradiol contributes to the total plasma oestradiol concentration, the adrenals are probably the main source of plasma oestradiol after castration (Santen et al. 1980).

Basal and GnRH-stimulated plasma testosterone concentration decreased approximately 200-400 fold after surgical and chemical castration. GnRH administration after chemical castration did not induce a significant increase in plasma testosterone, as was also shown by Junaidi et al. (2007). Despite the absence of a GnRH-induced increase in testosterone with CC, GnRH-stimulated plasma testosterone was higher in CC than in SC dogs. This indicates that the pituitary gonadotrophs were not completely desensitized in all dogs, as was also suggested by the increase in LH after GnRH stimulation in CC dogs. Although the highest measured plasma testosterone concentrations in dogs after CC were 8.2 and 7.7 nmol/L, at T=60 and T=90, respectively, the corresponding median plasma concentrations were 0.06 and 0.09 nmol/L, indicating that most dogs were still completely downregulated.

The basal testosterone:oestradiol ratio was similar in both groups before and after treatment. It decreased approximately 50 fold after treatment, indicating a much more pronounced effect on plasma testosterone than on plasma oestradiol. Mischke et al. (2002), in a study on endocrinological findings in male dogs with neoplastic and degenerative diseases, suggested that the testosterone:oestradiol ratio more accurately predicts the clinical effects of the gonadally secreted steroids in dogs with testicular tumours than do plasma concentrations of testosterone or oestradiol alone (Mischke et al. 2002). Based on this, the similar testosterone:oestradiol ratio after surgical and chemical castration would predict no difference in the effects of the two methods on such effects as prostate function. Indeed, the decrease in prostate size resulting from chronic treatment with GnRH agonists has been reported to be similar to the that after surgical castration (Vickery et al. 1984; Berry et al. 1986; Lacoste et al. 1989a; Kawakami et al. 1995; Junaidi et al. 2009b; Goericke-Pesch et al. 2010). A potential limitation of the use of GnRH agonists alone for treatment of androgen-dependent benign prostate hyperplasia (BPH) is the transient rise in plasma testosterone concentration during the first days of treatment, with the attendant risk of exacerbation of the disease, although there have been few reports on this (Junaidi et al. 2003; Ludwig et al. 2009; Goericke-Pesch et al. 2010; Ström Holst et al. 2010)

In conclusion, administration of GnRH to intact male dogs induced increased secretion of the pituitary gonadotrophins LH and FSH and the sex steroids oestradiol and testosterone. Surgical castration resulted in increased plasma concentrations of LH and FSH. Chemical castration by continuous release of the GnRH agonist decreased plasma LH and FSH and consequently reduced plasma oestradiol and testosterone levels to values similar to those after surgical castration. Administration of the GnRH analogue buserelin to chemically-castrated male dogs induced a significant increase in plasma LH, indicating that the pituitary gonadotrophs were not completely desensitized in all dogs at 4.5 months after administration of the deslorelin implant. However, plasma FSH did not increase in chemically castrated male dogs after administration of the GnRH agonist, which indicates differential regulation of LH and FSH release.

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7

Induction of parturition in the bitch with the progesterone-receptor blocker aglepristone

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Abstract

The triggering mechanism for parturition in the bitch remains unclear. Consequently, the development of drugs to successfully induce parturition in the dog has been difficult. The aim of this study was to evaluate the efficacy of the progesterone-receptor blocker aglepristone for the induction of parturition in beagle bitches. The course of parturition was therefore investigated in six parturitions induced by aglepristone and in six spontaneous parturitions. In addition, data were collected on pup survival and growth rates. Aglepristone was administered twice with a 9 h interval on day 58 of pregnancy. If parturition did not proceed a standard intervention protocol was applied.

Expulsion of the first pup occurred between 32 and 56 h after the first treatment with aglepristone, at which time the plasma progesterone concentration was still elevated. Accordingly, the gestation length of the bitches in the induced group (59.5 ± 0.2 days) was significantly shorter than that of the spontaneously whelping bitches (62.2 ± 0.5 days). The expulsion phase length, the inter-pup interval, the number of puppies born dead, and the number of clinical interventions needed during parturition did not significantly differ between the spontaneously whelping and the induced group. Pup survival and mean birth weights in the two groups did not differ significantly and aglepristone treatment had no significant influence on the growth rates.

The results of this study show that aglepristone is an effective drug which can be used safely for the induction of parturition in the dog.

Introduction

Progesterone is necessary for maintaining pregnancy (Sokolowski 1971). In the dog, the corpora lutea are the sole source of progesterone during gestation (Sokolowski 1971; Tsutsui 1983). Plasma progesterone concentrations decrease significantly prior to the onset of parturition while at the same time myometrial activity increases (van der Weyden et al. 1989). The decline of plasma progesterone concentrations may be considered essential for normal whelping. Inhibition of the decline in progesterone concentration by implanting medroxy-progesterone acetate (MPA) resulted in failed parturitions (Concannon and Hansel 1977). Although the MPA-treated bitches were visibly distressed and fluids were discharged from the vagina, no puppies were expelled. The treated animals either died with puppies in utero or a caesarean section had to be performed.

Parturition in the dog takes place after a pregnancy with an average length of 61.4 days, measured from the single day of mating which was determined on the basis of the plasma progesterone pattern (Okkens et al. 2001). It is not clear what triggers the onset of whelping in the dog. In ruminants, elevated levels of foetal cortisol trigger parturition via increased oestrogen production at the expense of progesterone production in the placenta. The elevated levels of oestrogen stimulate the production of prostaglandin F2α resulting in increased myometrial activity and softening of the cervix (Whittle et al. 2000). In dogs, data on hormone concentrations in foetal blood are not available, and circulating oestrogen concentrations in the bitch seem to decrease instead of increase towards parturition (Edqvist et al. 1975; van der Weyden et al. 1989; Hoffmann et al. 1994; Onclin et al. 2002).

Since the triggering mechanism for parturition remains unclear, it has been difficult to select or develop drugs that are useful for the induction of parturition in the dog. Ideally, this drug should induce whelping with a high efficiency and within a predictable, short time frame after treatment. In addition, treatment should be safe for the bitch and her puppies, i.e. it should induce a normal parturition without side effects.

In the bitch, prolactin is an important luteotropic factor during the second half of gestation. The dopamine agonists cabergoline and bromocriptine induce abortion in this period by suppressing the release of prolactin (Post et al. 1988; Jochle et al. 1989; Onclin et al. 1993; Onclin and Verstegen 1997). However, dopamine agonists are not useful for the induction of whelping since an effect may be expected only after several days. In addition, the time between the start of dopamine agonist treatment and the onset of parturition is quite unpredictable. Furthermore, treatment with prolactin secretion inhibitors would reduce or even abolish lactation after parturition. Prostaglandin F2α and its synthetic analogues also induce regression of the corpora lutea resulting in the termination of pregnancy. Just as dopamine agonists, prostaglandins must be administered for several days before luteolysis takes place in the bitch. In addition, treatment with prostaglandins is often accompanied by side effects such as tachypnoe, salivation, vomiting, and diarrhoea (Oettle 1982; Feldman et al. 1993; Fieni et al. 1997; Moriyoshi et al. 1999), and there may be an increased risk of an abnormal parturition process (Williams et al. 1999).

Progesterone-receptor blockers such as aglepristone (RU 46534) and mifepristone (RU 38486) are competitive antagonists of the progesterone receptor (van Look and Bygdeman 1989; Cadepond et al. 1997). Because of their anti-progesterone effect, these drugs have been widely investigated for their use as abortifacient agents (Concannon et al. 1990; Sankai et al. 1991; Linde-Forsberg et al. 1992; Galac et al. 2000; Fieni et al. 2001b). In addition, progesterone-receptor blockers may be useful for the induction of whelping as well. Studies with the progesterone-receptor blocker mifepristone have shown variable results. Some researchers could only induce an incomplete parturition, which did not proceed beyond the stage of dilatation of the cervix (Nohr et al. 1993). In contrast, van der Weyden et al. (van der Weyden et al. 1989) reported a normal course of parturition in five bitches treated with this drug. Fieni et al. (Fieni et al. 2001a) induced parturition with a single treatment with the progesterone-receptor blocker aglepristone, followed by a standard additional treatment with alfaprostol (a prostaglandin F2α analogue) or oxytocin. Riesenbeck et al. (Riesenbeck et al. 1999) described a single case of prolonged pregnancy that was successfully terminated by treatment with aglepristone in combination with prostaglandin F2α.

The aim of this study was to evaluate the efficacy and safety of the progesterone-receptor blocker aglepristone for the induction of parturition in the bitch. To this extent, the course of parturition, i.e. the length of the expulsion phase, the mean inter-pup interval and the number of clinical interventions, were investigated, and data were collected on pup survival during parturition, postnatal survival and growth rates.

Materials and methods

Animals

Eleven healthy beagle bitches, with ages ranging from 1.5 to 5 years and body weights ranging from 9.8 to 17.3 kg, were used in this study. All dogs had been born and raised at the Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine in Utrecht, The Netherlands, and were accustomed to procedures such as routine clinical examinations and jugular venipuncture. All dogs were examined three times weekly for the presence of swelling of the vulva and a serosanguineous vaginal discharge, which were considered to signify the onset of pro-oestrus. Plasma concentrations of progesterone were determined thrice weekly during (pro)oestrus with a rapid ^{125}I-RIA and the bitches were mated once with one of three male dogs based on the peri-ovulatory rise of progesterone values as described previously by Okkens et al. (Okkens et al. 2001). The day of mating was considered to be day 0 of gestation. Between days 23 and 28 of gestation, pregnancy was confirmed by ultrasonography in all dogs (Aloka S 50-500, 3.5-MHz probe, Biomedic Nederland, Almere, The Netherlands).

Until the sixth week of pregnancy, the bitches were housed in pairs in indoor–outdoor runs, fed a commercially available dry dog food once daily, and given water ad libitum. Starting 3 weeks before the expected parturition, the pregnant bitches were housed individually and were fed three times daily. From day 53 of pregnancy, bitches were housed in a separate kennel equipped with a whelping basket and a heating lamp.

On day 53 of pregnancy, a radiographic diagnosis of pregnancy was made and the number of foetuses was established. In order to create homogenous experimental groups, bitches were assigned to the spontaneously whelping (n = 6) or to the induced group (n = 6) on the basis of the number of foetuses, their age, and their parity. Six bitches were primiparous (three in each group), three others gave birth for the second time (one in the induced group and two in the spontaneously whelping group) and one bitch for the third time (induced group). One of the bitches was used twice, once in the induced group (her first pregnancy) and 7 months later in the spontaneously whelping group. Because of differences in parity, litter size and siring male, this bitch was not treated as her own control, and the results were thus statistically treated as independent data (Nos. 3 and 11). The resulting spontaneously whelping and induced group were similar with respect to litter-size, age and parity. The three siring male dogs were also evenly distributed over the two groups.

Experimental protocol

The study protocol was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Utrecht University, The Netherlands.

Starting on day 54 of pregnancy, a general physical examination of the bitches was performed daily. Rectal temperatures were measured at 01:00, 08:00, 13:00 and 19:00 h.

From day 54 of pregnancy until 4 days after parturition, blood samples were collected by jugular venipuncture 2–4 times a day in EDTA-coated tubes. Within 5 min after collection they were centrifuged at 1500 × g and 4 °C, and plasma was stored at −20 °C until analysis. Blood samples were taken at 08:00 and 19:00 h on days 54, 55 and 56 of pregnancy, at 01:00, 08:00, 13:00 and 19:00 h on day 57 of gestation and on each of the following days up to and including the first complete day after the day on which the last puppy was born. On the second, third and fourth day after the day of expulsion of the last pup, blood samples were taken twice daily at 08:00 and 19:00 h. All samples taken at 08:00 h and those taken at 19:00 h from day 57 until the first day after parturition were selected for determination of the plasma progesterone concentration. An analysis of various hormones was performed in all blood samples, however, not all of the results were used in this study, but in related studies.

The packed cell volume (PCV) was determined every 2–3 days, starting on day 54 of gestation until the last day of sampling. PCV values declined significantly from an average of 34% at 54–55 days of pregnancy to a nadir of 30% between 62 and 64 days ($P < 0.01$). There was no difference in PCV between the spontaneously whelping and the induced group. The volume of blood samples was not adjusted to match this decline in PCV.

On day 58 of pregnancy, bitches assigned to the induced group were weighed, and examined ultrasonographically to assess the presence of any dead foetuses before the start of treatment. Subsequently, at 10:00 and at 19:00 h these bitches were treated with aglepristone (30 mg/ml dissolved in oily solvent; Alizine®, Virbac, Carros, France) at a dose of 15 mg/kg late-pregnancy body weight sc. Each dose was distributed over two injection sites: one high up in the neck and the other more caudally between the shoulder blades. The injection sites were massaged for 1 min following treatment. At 31 h after the first treatment with aglepristone, vaginoscopy was performed to check for cervical dilatation. In addition,

the foetuses were monitored by means of transabdominal ultrasonography to assess the presence of any deceased foetuses.

Management of parturition and puppies

The dogs of both groups were regularly observed for parturient behavior via a camera connected to a monitor in a separate room. Parturition was constantly monitored, but bitches were not disturbed. However, if parturition did not proceed, a standard intervention protocol was applied. When bitches had made straining movements regularly and intensely for 45 min prior to whelping of the first puppy or for 30 min prior to any subsequent puppy without any externally visible progress, digital vaginal exploration was performed. When bitches had shown only non-intense, abdominal straining efforts for 1.5–2 h, digital vaginal exploration was also performed. During vaginal exploration, additional straining was evoked by massaging the dorsal vaginal wall. When a puppy was felt inside the birth canal gentle manual traction was exerted on the pup to aid the birth. When no pup was present in the birth canal, a second digital vaginal exploration took place after 30 min. When, at that time, a puppy had advanced towards or had entered the birth canal, the bitch was left again for 30 min, after which, if no pup had been expelled, the bitch was treated with 2 IU oxytocin sc (Intervet Nederland B.V., Boxmeer, The Netherlands). However, if the pup had not advanced in caudal direction upon the second exploration, bitches were treated with 2 IU oxytocin sc without delay. This procedure was repeated after 45 min if no pup had been expelled. Forty-five minutes thereafter, a course of 2 IU oxytocin treatments was started, given every 1.5–2 h until the birth of the next puppy.

After parturition, the puppies were weighed daily until the age of 31 days. Tube feeding with milk replacement (Denkadog Doggylac, IPP, Apeldoorn, The Netherlands) was started 4–6 times a day when puppies lost weight or had not shown a weight gain of 10% above their birth weight within 48 h after parturition. Puppies were de-wormed at 2 weeks with Vitaminthe® (niclosamide and oxibendazol, Virbac Laboratories, Carros, France), and at 4 and 6 weeks of age with Drontal dog® (praziquantel, pyrantel embonate and febantel, Bayer B.V. Division Animal Health, Mijdrecht, The Netherlands). The puppies were vaccinated for distemper and canine parvovirus at 6 weeks of age (Nobivac® Puppy DP, Mycofarm, De Bilt, The Netherlands). At 7 weeks, the puppies were adopted. Puppies which were born dead or died after parturition were pathologically examined.

Progesterone determinations

Plasma concentrations of progesterone in the samples taken around the time of parturition were measured by a previously validated ^{3}H-RIA using extraction with hexane (Dieleman and Schoenmakers 1979; Okkens et al. 1985). The intra-assay and inter-assay coefficients of variation were 11% and 14%, respectively. The limit of quantitation was 0.13 nmol/l.

Data analysis

Statistical analysis was performed using SPSS® for Windows, version 11.0.1 (SPSS Inc., Chicago, IL, USA) and SAS/STAT® (SAS Institute Inc., Cary, NC).

The mean rectal temperature was calculated from the average temperature value of each bitch during 8 h periods around the time of the expulsion of the puppies. Changes in mean PCV and rectal temperatures were analyzed with Repeated Measures ANOVA. Differences in mean PCVs between the spontaneously whelping and the induced group were compared with Student's t-test with Bonferoni correction.

Litter size, age, and parity of the two groups of bitches were compared using Student's t-test. The number of interventions during parturition, the length of the expulsion phase, i.e. the time from the expulsion of the first until that of the last pup, the mean inter-pup interval, i.e. the duration of the expulsion phase divided by the number of puppies minus 1, and the live birth weight of the puppies were also compared with Student's t-test.

Mean growth of the puppies between days 0 and 10 and between days 0 and 31 was analyzed in a mixed model with treatment, litter size, and birth weight as fixed factors. Litter number was included as a random factor. Pup weights were log-transformed to increase linearity.

In order to compare the mean plasma progesterone concentrations between the two groups, the complete sampling period was divided into five time intervals. Period 1: from day 54 until 10:00 h on day 58 of gestation ('late gestation'); period 2: the 30 h period before expulsion of the first pup ('before parturition'); period 3: from the expulsion of the first pup until the expulsion of the last pup ('during parturition'); period 4: 0–24 h after the expulsion of the last pup ('the day after parturition'); period 5: 24–72 h after the expulsion of the last pup ('the second and third day after parturition'). For each bitch, a mean plasma progesterone concentration was calculated for each of the periods. This mean value was entered into the statistical analysis. It should be noted that the dogs in the induced group received the first dose of aglepristone at the end of period 1.

Because blood samples were not available for each dog during parturition (period 3), an ANOVA for Repeated Measures was performed in each group for the periods 1, 2, 4, and 5 only. To apply this ANOVA to the data in the induced group, the data were made homogeneous by transformation with a natural logarithm (ln). Mean plasma hormone concentrations in the spontaneously whelping group and the induced group were compared with Student's t-test with Bonferoni correction. Data are expressed as mean ± S.E.M. $P \leq 0.05$ was considered significant.

Results

Local side effects from the aglepristone treatments were observed in five out of six treated bitches. In four of these a thickening of the subdermis which lasted for about 2–3 weeks after treatment could be discerned. In one bitch local necrosis of the skin overlying the injection site occurred. All side effects disappeared without any treatment.

On days 58 and 59 of pregnancy, no dead foetuses were observed during transabdominal ultrasonography of bitches in the induced group. Vaginoscopy on day 59, at 31 h after the first aglepristone treatment, revealed foetal membranes in the cranial part of the vagina indicating

cervical dilatation in five of the six treated bitches. In one bitch (No. 10) no cervical dilatation was seen at that time, but when vaginoscopy was repeated 20 h later, the cervix of this bitch had also dilated.

Changes of the mean rectal temperature in the spontaneously whelping and the induced group did not differ significantly (Fig. 1). Mean rectal temperatures started to decline between 40 and 48 h before expulsion of the first pup and reached a nadir of 37.1 ± 0.1 °C at 8–16 h before expulsion of the first pup ($P < 0.05$). After parturition, mean rectal temperatures were significantly higher than the temperatures measured prior to whelping (38.7 ± 0.1 °C, $P = 0.004$).

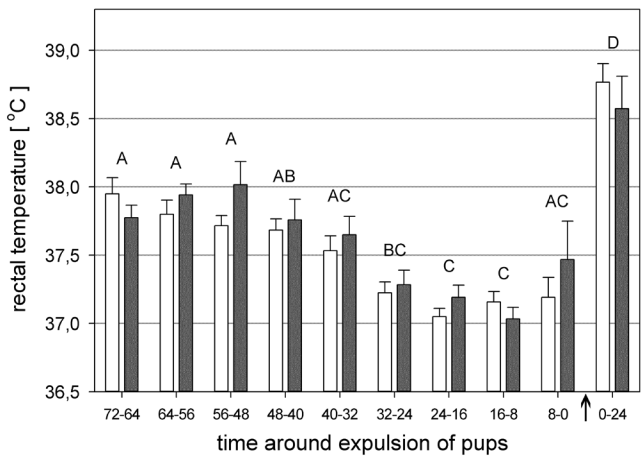

***Figure 1**. Mean (±S.E.M.) rectal temperatures measured every 8 h from 64 to 72 h before the expulsion of the first pup until 0–24 h after the expulsion the last pup in six spontaneously whelping dogs (white bars) and in six dogs in which parturition was induced with aglepristone (black bars). The arrow indicates the time of parturition. The groups did not differ significantly. Different letters A–D denote significant differences between the time intervals.*

The litter size averaged 6.0 ± 1.1 in the spontaneously whelping group, and 7.0 ± 0.4 in the induced group ($P = 0.44$). Expulsion of the first pup occurred between 32 and 56 h after the first treatment with aglepristone with an average of 41.0 ± 3.7 h. As a result, gestation length of the bitches in the induced group (59.5 ± 0.2 days) was significantly shorter ($P = 0.001$) than that of the bitches in the spontaneously whelping group (62.2 ± 0.5 days).

The course of parturition of each bitch is depicted in Fig.2. In the spontaneously whelping group vaginal exploration was performed 18 times. In addition, eight doses of oxytocin were administered, with six of these treatments being administered to a single bitch (No. 1). Because bitch No. 2 showed non-intense straining efforts for 1.5 h without giving birth to a pup, vaginal exploration was done at which time a pup could just be touched. Fifteen minutes later, the first pup was born. In the induced group, 36 vaginal explorations were performed and 16 treatments with oxytocin were given, with eight of these being administered to a single bitch (No. 10). The number of interventions during parturition between the groups did not differ significantly ($P = 0.26$).

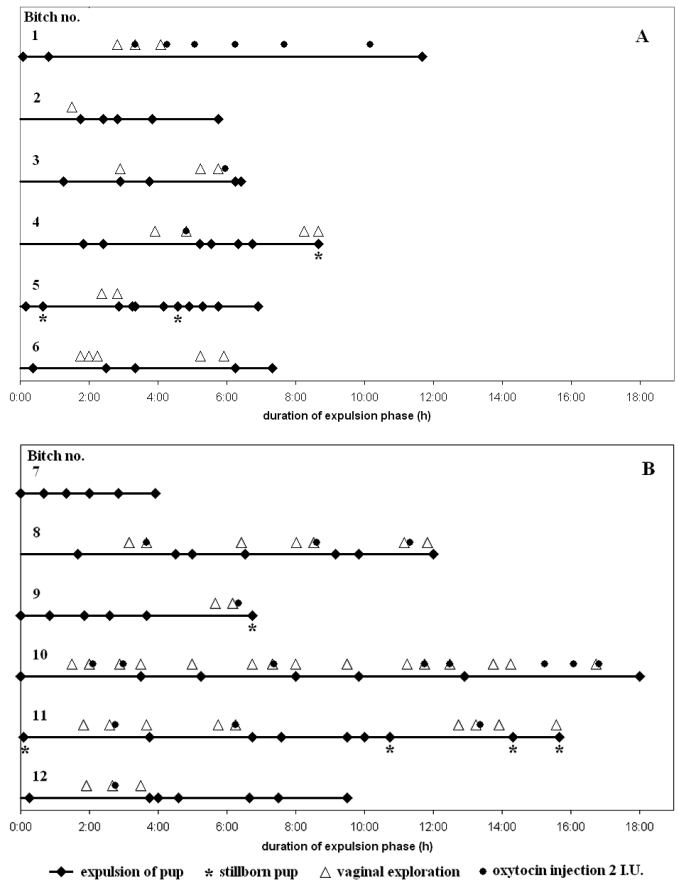

Figure 2. *The course of parturition of six spontaneously whelping bitches (A) and six dogs in which parturition was induced with aglepristone (B), applying a standard protocol for the management of parturition in both groups. The black horizontal lines refer to the length of the expulsion phase, the black diamonds denote the time of expulsion of a pup, the white triangles indicate the times of digital vaginal exploration, and the black dots denote injections of 2 IU oxytocin. Puppies born dead are marked with an asterisk below the diamond. With one exception (bitch No. 2, spontaneously whelping group), no interventions in the course of parturition were necessary before the birth of the first pup in either group.*

No significant difference was observed between the spontaneously whelping and the induced group regarding the length of the expulsion phase (6.7 ± 1.2 and 10.6 ± 2.2 h, respectively, P = 0.15) or the mean inter-pup interval (1.9 ± 0.8 and 1.7 ± 0.3 h, respectively, P = 0.80).

In the spontaneously whelping group, 3 out of 36 puppies were born dead versus 5 out of 42 in the induced group. Post-mortem examination revealed asphyxia as the cause of death.

The mean birth weight of the puppies in the spontaneously whelping group was 293 ± 13 g (n = 33) versus 310 ± 5 g (n = 37) in the induced group (P = 0.23). In both groups, 2 out of 6 litters needed supplementary tube feeding for 1–2 weeks after parturition. Aglepristone treatment did not significantly influence the mean growth rates. The mean growth rate during

the first 10 days was 30 ± 0.8 g/day (n = 35) in the induced group and 40 ± 0.7 g/day (n = 33) in the spontaneously whelping group (P = 0.11). Throughout the entire follow-up period of 31 days the mean growth rates were 48 ± 0.5 g/day (n = 35) and 52 ± 0.4 g/day (n = 31) in the induced and spontaneously whelping group, respectively (P = 0.20).

During the first 7 weeks, 2 puppies out of 33 in the spontaneously whelping group died and 2 out of 37 in the induced group. In the spontaneously whelping group, one pup was euthanized on day 22 after parturition because of severe growth retardation despite supplementary tube feeding. Post-mortem examination revealed no abnormalities that could explain the retarded growth. The second pup in the spontaneously whelping group died from an ileocolic intussusception. In the induced group, one pup died from an intussusception of the jejunum, the other from an infection with coccoid bacteria.

The plasma progesterone profiles of an individual bitch from the spontaneously whelping and one from the induced group are shown in Fig. 3. Between day 54 and day 58 of pregnancy, the mean plasma progesterone concentration in bitches from the spontaneously whelping group was significantly higher (P < 0.01) than in the 30 h period before parturition (Fig. 4). In the days after parturition, mean plasma progesterone concentrations were significantly lower (P < 0.05) than during the two periods before parturition. In the bitches from the induced group, the mean plasma progesterone concentration did not differ significantly between late gestation and the 30 h period before parturition. The day after parturition, the mean plasma progesterone concentration had decreased significantly (P < 0.01). On the second and third day after parturition the mean plasma progesterone concentration was significantly lower than during the previous period (P < 0.05).

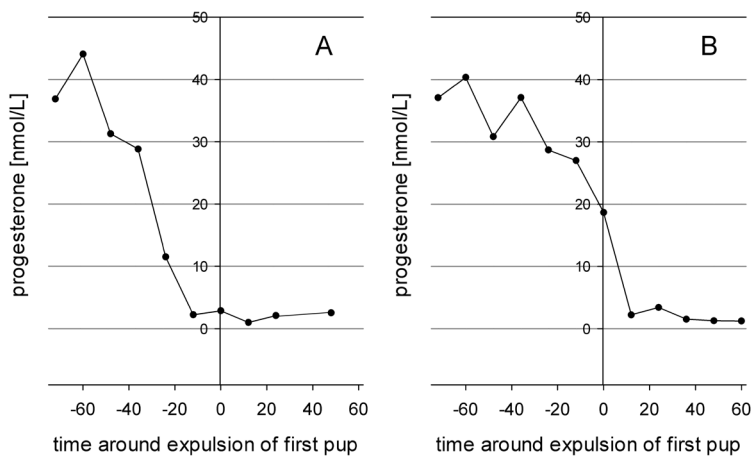

Figure 3. *Plasma progesterone concentrations around the time of expulsion of the first pup (0) of a spontaneously whelping 3-year old beagle bitch (A) and a 1.5-year old beagle bitch in which parturition was induced with aglepristone (B). The black triangles indicate the time of aglepristone treatment.*

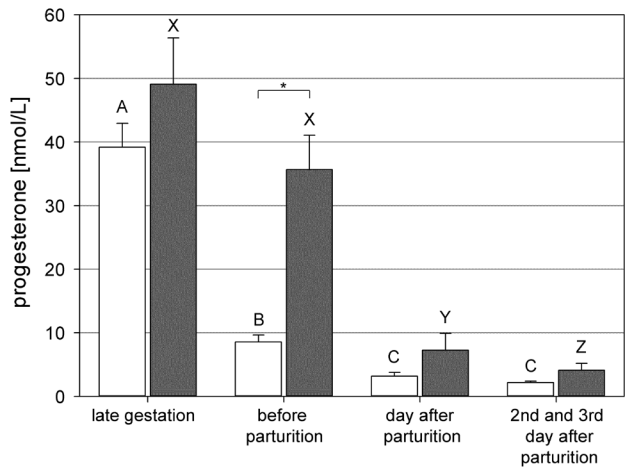

Figure 4. *Plasma progesterone concentrations (mean ± S.E.M.) in six spontaneously whelping bitches (white bars) and in six bitches in which parturition was induced with aglepristone (black bars) in late gestation (day 54 until 10:00 h on day 58 of pregnancy), before parturition (30–0 h before expulsion of the first pup), the day after parturition (0–24 h after expulsion of the last pup), and the second and third day after parturition (24–72 h after the expulsion of the first pup). Different letters A–C and X–Z denote significant differences within the spontaneously whelping and the induced group, respectively. Significant differences between groups are indicated with an asterisk. The first dose of aglepristone was given at the end of the late gestational period.*

In late gestation, before aglepristone treatment, mean plasma progesterone concentrations of the spontaneously whelping and the induced group did not differ significantly. During the last 30 h before parturition, the mean plasma progesterone concentration in the bitches from the induced group was significantly higher ($P < 0.01$) than in the spontaneously whelping group. During the expulsion phase, the mean plasma progesterone concentration in the induced group (22.1 ± 4.9 nmol/l, n = 5) was significantly higher than the concentration in the spontaneously whelping group (5.9 ± 1.2 nmol/l, n = 6, $P = 0.04$). In the post-partum periods, the mean plasma progesterone concentrations did not differ significantly between the two groups.

Discussion

The results of this study demonstrate that the progesterone-receptor blocker aglepristone is an efficient and safe drug for the induction of parturition in the dog. Aglepristone treatment induced parturition with a high efficiency. The bitches in the induced group had a significantly shorter gestation length compared with the spontaneously whelping bitches. In addition, parturition occurred within a relatively short and predictable time frame, on average at 41 h (range 32–56 h) after the first aglepristone treatment. No side effects were observed in the bitches, except a local inflammation reaction at the injection site, which has been reported previously in dogs and cats (Galac 2001; Gorlinger et al. 2002; Galac et al. 2004). No significant difference was

found between the two groups with regard to the length of the expulsion phase and the mean inter-pup intervals. In addition, the number of puppies born dead and the growth rate of living puppies were similar between the groups.

In the induced group the expulsion of puppies occurred despite the presence of high plasma concentrations of progesterone, much higher than the value considered to be necessary for maintaining pregnancy (approximately 6.4 nmol/l) (Concannon et al. 1977). This indicates an effective progesterone-receptor blocking action by aglepristone.

Previous studies were variably successful in inducing parturition with progesterone-receptor blockers. In one study, repeated treatments with mifepristone (6 mg/kg, sc) between days 57 and 59 of gestation in three beagle bitches resulted in an incomplete parturition within 26–40 h after the start of treatment. Only one bitch gave vaginal birth to a single pup, and the three animals had to undergo a caesarean section (Nohr et al. 1993). In another study, mifepristone (7.5 mg/kg bw per day) was administered orally to five beagle bitches from day 57 after mating until the birth of the first pup. Parturition occurred between 26 and 70 h after the first treatment, when plasma progesterone concentrations ranged between 8.6 and 29.6 nmol/l. One bitch needed additional treatment with oxytocin (two doses of 1 IU each) to complete whelping (van der Weyden et al. 1989). Fieni et al. (Fieni et al. 2001a) induced parturition with aglepristone (a single dose of 15 mg/kg bw, sc) on day 58 of gestation, with either 0.08 mg/kg bw of the PGF2α analogue alfaprostol, or 0.15 IU/kg bw oxytocin given 24 h later and every 2 h onwards as a standard treatment until the expulsion of the last pup. On average, parturition in these bitches occurred 32 h after the aglepristone treatment, but alfaprostol or oxytocin had also been administered at that time. In the present study, the two subcutaneous treatments with aglepristone, administered with an interval of 9 h, appeared to induce a whelping process that was highly similar to that of the spontaneously whelping animals. This suggests that aglepristone treatment induces a normal parturition.

In spontaneously whelping bitches increasing plasma prostaglandin concentrations probably induce luteolysis, which results in the onset of labor (Concannon and Hansel 1977; Williams et al. 1999; Meier and Wright 2000). In this study, the expulsion of the first pup in bitches from the induced group occurred in the presence of high plasma progesterone concentrations, indicating that luteolysis had not been completed yet. In the post-partum periods, however, the plasma progesterone concentrations in the induced group had decreased significantly, and were similarly low to those in the spontaneously whelping group. This indicates that in both groups the corpora lutea have a decreased function at this stage. Linde-Forsberg et al. (Linde-Forsberg et al. 1992) induced abortion in mid-pregnancy with a different progesterone-receptor blocker, mifepristone, and found plasma concentrations of prostaglandin F2α-metabolite to increase after treatment, while progesterone concentrations were decreasing. This premature cessation of the luteal phase was also observed after treatment of bitches for mid-gestation abortion with aglepristone (Galac et al. 2000). It is likely that plasma prostaglandin concentrations also increase after aglepristone treatment for parturition induction, possibly due to placental dehiscence. Furthermore, it may be hypothesized that, in the induced group, the occupation of the progesterone receptors by aglepristone had reduced the auto-regulatory positive feedback of progesterone on its own secretion (Rothchild 1981).

The determination of the optimal mating time by measurement of the plasma progesterone concentration after the start of pro-oestrus was 100% successful. All bitches became pregnant after a single mating. The bitches in the induced group were treated with aglepristone at day 58 of pregnancy, at which stage the puppies would be viable for birth, but at which time in the vast majority of cases the normal parturition process would not yet have started (Concannon et al. 1978; Concannon et al. 1988; Okkens et al. 2001). Our findings on postnatal survival of pups demonstrate that with accurately defined gestational age day 58 is a safe time for the induction of whelping in healthy dogs.

A significant drop in rectal temperature was measured on the day before parturition in both the spontaneously whelping and the induced group. In accordance with previous reports (Concannon and Hansel 1977; Concannon et al. 1977; van der Weyden et al. 1989), the pre-partum drop of rectal temperatures in the spontaneously whelping group was temporally related with a decrease in plasma progesterone concentrations. It has been suggested that in dogs progesterone has a thermogenic effect within the thermoregulatory system. Similar observations about the effects of progesterone on the thermoregulatory center have been made in intact and ovariectomized rats in which exogenous progesterone was found to be thermogenic (Freeman et al. 1970). The rapid decline in progesterone concentrations before parturition results in a transient drop in body temperature of dogs until other thermoregulatory factors become readjusted and restore the balance (Concannon and Hansel 1977; Williams et al. 1999; Meier and Wright 2000). In the induced group, the drop in rectal temperature before parturition occurred in the presence of a high plasma progesterone concentration, which may be indicative of a progesterone antagonistic effect of aglepristone on the thermoregulation center.

In contrast to the protocol used by Fieni et al. (Fieni et al. 2001a), who administered alfaprostol or oxytocin as a standard treatment starting 24 h after administration of aglepristone, in the present study no standard treatment with uterotonic drugs was applied after aglepristone administration. This protocol enabled us to study the exclusive effects of aglepristone on the induction of parturition and avoided the possible administration of uterotonic drugs to bitches with a closed cervix. In fact, one bitch in our study did not show cervical dilatation at vaginoscopy 31 h after the first treatment with aglepristone. In all bitches, the aglepristone treatment alone was sufficient to lead to the expulsion of the first pup, within 32–56 h after the start of treatment. This is somewhat longer than the time interval observed by Fieni et al. (Fieni et al. 2001a). However, a comparison between the two studies is not possible since a different protocol was used; furthermore, other factors that differ between the research colonies of beagle dogs, such as genetic make-up and body condition, may very well have an influence on the course of parturition.

A strict protocol for the management of parturition was used in both groups to minimize the risks for the bitches and puppies around parturition. This did not interfere with the aim of the study, i.e. to investigate the efficacy and safety of aglepristone for the induction of parturition. The length of the expulsion phase did not differ significantly between the spontaneously whelping and induced group. There was a large variation between bitches with respect to the number of interventions during parturition, but no significant difference was found between the groups. This suggests that aglepristone treatment does not affect the course of parturition.

Despite the significant difference in gestation length between the groups, mean birth weight of the puppies was not significantly different between the groups. This was an unexpected finding because Evans and Sack (Evans and Sack 1973), Salazar and Yllera (Salazar and Yllera 1991) and Moriyoshi et al. (Moriyoshi et al. 1996) reported a very pronounced growth rate of canine foetuses in late pregnancy in the dog.

After parturition most puppies grew steadily, but two litters in both groups received supplementary tube feedings. This suggests that aglepristone treatment had no effect on either the milk production of the bitches or the weight gain of the puppies. Also, there were no indications that aglepristone treatment affected the pre-weaning mortality rate, since survival to weaning was similar in both groups.

In conclusion, premature parturition was successfully induced in six healthy beagle bitches with the progesterone-receptor blocker aglepristone. The onset of whelping occurred within a short and predictable time frame. There was no need for additional treatment before the start of the expulsion phase, but just as in the group with the spontaneous onset of whelping it was necessary to monitor parturition closely to ensure that labor proceeded in due course. Aglepristone treatment did not affect pup survival and only minor side effects were seen in the bitches. These observations indicate that aglepristone is an effective and safe drug for the induction of parturition in dogs.

A future application of treatment with aglepristone could be the induction of parturition in bitches with a prolonged pregnancy. This would be a practical alternative to a caesarean section, which at present is the sole effective treatment for prolonged gestation. However, more research is needed to determine the etiology behind a prolonged pregnancy and the potential use of a progesterone-receptor blocker for the induction of parturition in such a case.

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8

Hormonal changes in spontaneous and aglepristone-induced parturition in dogs

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Abstract

To increase our understanding of the endocrine changes associated with parturition in dogs, plasma concentrations of progesterone (P4), 15-ketodihydroprostaglandin F2α (PGFM), oestradiol-17-β (E2β), cortisol, ACTH, prolactin (PRL), LH, and FSH were measured in six spontaneously whelping bitches and in six bitches in which parturition was induced with the progesterone-receptor blocker aglepristone on day 58 of pregnancy.

Expulsion of pups in the induced group took place in the presence of P4 concentrations that were still elevated. PGFM concentrations increased before parturition in both groups, but levels were lower in the induced bitches. PGFM levels reached a maximum in both groups during parturition and quickly decreased in the spontaneously whelping group after parturition, but remained elevated in the induced group. In both groups, cortisol concentrations reached similar maximum levels during the last 30 h before the onset of expulsion. During the 3 days postpartum, cortisol concentrations were higher in the induced group. The highly variable ACTH concentrations did not differ significantly throughout the study within or between groups. In both groups, E2β concentrations decreased and PRL concentrations increased between the late gestational period and the 30-h period before parturition. Concentrations of both LH (spontaneously whelping group) and FSH (both groups) decreased between late gestation and the postpartum period. The results of this study illustrate the hormonal changes around parturition in the bitch, and reveal that aglepristone-induced parturition is associated with still incomplete luteolysis, an altered PGFM profile, and elevated postpartum cortisol concentrations as compared with spontaneously whelping dogs.

Introduction

Progesterone (P4) is necessary for maintaining pregnancy in mammals (Heap et al. 1977). In the dog the corpora lutea are the sole source of P4 during gestation (Sokolowski 1971; Tsutsui 1983). Ovarian P4 production is independent of luteotrophic support from the pituitary during the first half of gestation in this species (Concannon 1980; Okkens et al. 1986). Maintenance of the corpora lutea during the second half of the luteal phase or pregnancy is mainly a function of prolactin (PRL) and possibly gonadotrophic hormones (Post et al. 1988; Vickery et al. 1989; Okkens et al. 1990; Onclin and Verstegen 1997; Onclin et al. 2000).

Parturition in the dog takes place after a pregnancy with an average length of 61.4 days, when bitches are mated once on the guidance of the preovulatory increase of the plasma P4 concentration (Okkens et al. 2001). During the last 1–2 days prior to whelping, the plasma P4 concentration decreases rapidly (Edqvist et al. 1975; Chakraborty 1987; Van der Weyden et al. 1989), while the plasma 15-ketodihydroprostaglandin-F2α (PGFM) level begins to increase (Concannon et al. 1988; Meier and Wright 2000). The plasma PGFM level reflects the peripherally active prostaglandin F2α (PGF2α) concentration, as PGF2α itself has a short half-life and is rapidly converted to PGFM. As a result of the decrease in P4 and the rise in PGF2α concentrations, myometrial activity gradually increases (Van der Weyden et al. 1989), which leads to the onset of whelping.

It remains unclear which signal(s) trigger(s) these hormonal changes associated with whelping in the dog. In sheep and goats, a foetal corticoid signal is the trigger for parturition (Flint et al. 1978; Whittle et al. 2001). The subsequently rising oestrogen levels enhance prostaglandin production during gestation, which in turn increases uterine myometrial contraction activity in sheep. In dogs, data on foetal hormone secretion are not available, and circulating oestrogen concentrations in the bitch decrease rather than increase towards parturition (Edqvist et al. 1975; Van der Weyden et al. 1989; Onclin et al. 2002). In addition, the circulating oestrogens seem to be of ovarian rather than placental origin in dogs (Nishiyama et al. 1999; Onclin et al. 2002).

Although it remains to be elucidated which factors result in pre-partum luteolysis in the dog, the sharp decline of the plasma P4 concentration before whelping appears to be essential for a successful parturition (Concannon et al. 1988; Keister et al. 1989; Van der Weyden et al. 1989). Progesterone-receptor blockers such as aglepristone and mifepristone are competitive antagonists of the progesterone receptor, and also have affinity for the glucocorticoid receptor (van Look and Bygdeman 1989; Cadepond et al. 1997). The anti-progesterone effect of these drugs has been used for the induction of abortion or whelping (Van der Weyden et al. 1989; Concannon et al. 1990; Sankai et al. 1991; Galac et al. 2000; Baan et al. 2005).

The aim of this study was to increase our knowledge of the endocrine changes associated with parturition in the dog. In addition, we compared the hormonal changes in spontaneously whelping bitches with those in bitches in which parturition was induced on day 58 of pregnancy with aglepristone.

Materials and methods

Animals

Animal data and maintenance procedures, the methods to determine optimal mating time, and management of parturition were as previously described(Baan et al. 2005). The study protocol was approved by the committee for the use of animals in research and education (DEC) of the Faculty of Veterinary Medicine, Utrecht University, The Netherlands.

Blood samples were collected, from six spontaneously whelping dogs and six dogs in which parturition was induced with the progesterone-receptor blocker aglepristone, from day 54 of pregnancy until 4 days after parturition. Frequency and times of blood sampling are indicated in Table 1. A total of 16 blood samples were taken during the expulsion phase, 6 in the spontaneously whelping group and 10 in the induced group. Thirteen of these samples were collected within 30 min after the birth of a pup (n = 9) or after vaginal exploration (n = 4).

Table 1. *Blood sampling protocol and hormone determinations of bitches in the spontaneously whelping and in the induced group*.

Day	Time	Hormone determinations
Day 54 –56 of pregnancy	08:00	PGFM, Cort, ACTH, LH, FSH, P4, E2β
	19:00	PGFM, Cort, LH, FSH, PRL
Day 57 of pregnancy – 1st day after expulsion of the last pup	01:00	PGFM, Cort, LH, FSH, PRL
	08:00	PGFM, Cort, P4, E2β
	13:00	PGFM, Cort, ACTH, LH, FSH, PRL
	19:00	PGFM, Cort, P4
2nd, 3rd and 4th day after expulsion of the last pup	08:00	PGFM, Cort, P4, E2β
	19:00	PGFM, Cort, ACTH, LH, FSH, PRL

PGFM = prostaglandin F2α metabolite; Cort = cortisol; ACTH = adrenocorticotrophic hormone; LH = luteinizing hormone; FSH = follicle-stimulating hormone; PRL = prolactin; progesterone = P4; E2β = oestradiol-17-beta.

On day 58 of pregnancy, bitches assigned to the induced group were treated at 10:00 and at 19:00 h with a subcutaneous dose of 15 mg aglepristone (Virbac BV, The Netherlands) per kg late-pregnancy body weight.

Hormone determinations

Plasma P4 concentrations were measured by a previously validated 3H-RIA using extraction with hexane (Dieleman and Schoenmakers 1979; Okkens et al. 1985). The intra-assay and interassay coefficients of variation (CVs) were 11% and 14%, respectively. The sensitivity was 0.13 nmol/L. Possible interference with the assay by aglepristone was investigated as follows. Aglepristone was administered subcutaneously, twice with a 9-h interval (t = 0 and t = 9 h) at a dose of 15 mg/kg body weight to five intact female beagle bitches during anoestrus. Blood samples for determination of the plasma P4 concentration were collected prior to aglepristone administration (t = −46 h, −22 h and 0 h), and twice daily thereafter until 60 h after the first aglepristone dose. The mean (±S.E.M.) P4 concentration before aglepristone

administration was 0.8 ± 0.3 nmol/L. The mean P4 concentration 60 h after the first dose of aglepristone administration was 1.0 ± 0.6 nmol/L. The general linear model for repeated measures showed that the P4 concentrations did not change significantly (P = 0.22) after aglepristone administration in any of the bitches.

Plasma PGFM concentrations were measured by RIA, as described previously (Kindahl et al. 1976; Granstrom and Kindahl 1982). The intra-assay CVs were between 3.4% and 7.6% at different ranges of the standard curve. The interassay CV was 14%. The sensitivity was 300 pmol/L.

Plasma E2β concentrations were measured by a solid phase 125I-RIA (Coat-a-Count TKE; DPC, Los Angeles, USA) according to the manufacturer's instructions, with modifications as described previously (Dieleman and Bevers 1987) and validated for the dog (van Haaften et al. 1994). The intra-assay and interassay CVs were 14% and 11.8%, respectively. The sensitivity was 7 pmol/L.

Plasma cortisol concentrations were measured by RIA according to the manufacturer's instructions (Coat-a-Count TKC; DPC, Los Angeles, USA). The intra-assay and interassay CVs were 4.3% and 5.2%, respectively. The sensitivity was 5.5 nmol/L.

Plasma adrenocorticotrophic hormone (ACTH) concentrations were measured by RIA according to the manufacturer's instructions (ACTH 65T Kit Nichols Institute Diagnostics, San Juan Capistrano, USA). The intra-assay and interassay CVs were 3.0% and 7.8%, respectively. The sensitivity was 1.0 ng/L.

Plasma PRL concentrations were determined by a previously validated heterologous RIA (Okkens et al. 1985). The intra-assay and interassay CVs were 3.5% and 11.5%, respectively. The sensitivity was 0.8 µg/L.

Plasma luteinizing hormone (LH) concentrations were determined by a heterologous RIA as described previously (Beijerink et al. 2007). The intra-assay and interassay CVs for values above 0.5 µg/L were 2.3% and 10.5%, respectively. The sensitivity was 0.3 µg/L.

Plasma follicle stimulating hormone (FSH) concentrations were determined by a homologous canine IRMA (AHCOO4; Biocode SA, Liège, Belgium) as described previously (Beijerink et al. 2007). The intra-assay and the interassay CVs for values above 1.60 µg/L were 3.5% and 15.1%, respectively. The sensitivity was set at the value of the lowest standard, 1.5 µg/L.

Data analysis

Statistical analysis was performed using SPSS® for Windows, version 11.0.1 (SPSS Inc., Chicago, USA) and SAS/STAT® (SAS Institute Inc., Cary, USA).

In order to compare mean hormone concentrations between the two groups, the complete sampling period was divided into five time intervals. Period 1: from day 54 until 10:00 h on day 58 of gestation ('late gestation'); period 2: the 30-h period before expulsion of the first pup ('before parturition'); period 3: from the expulsion of the first pup until the expulsion of the last pup ('during parturition'); period 4: 0–24 h after the expulsion of the last pup ('the day after parturition'); period 5: 24–72 h after the expulsion of the last pup ('the 2nd and 3rd day after parturition'). For each bitch, a mean hormone concentration was calculated for each of these

periods. This mean value was entered into the statistical analysis. It should be noted that the dogs in the induced group received the first dose of aglepristone at the end of period 1.

Because blood samples were not available for each dog during parturition (period 3), an ANOVA for Repeated Measures was performed in each group for the periods 1, 2, 4, and 5 only. To apply this ANOVA, data that were found not to be normally distributed were either log-transformed (ln; P4 concentrations in the induced group) or reciprocally transformed (1/x; PGFM concentrations in the induced group). Mean hormone concentrations in both group were compared with Student's t-test, with Bonferroni correction.

The concentrations of P4, cortisol, and PGFM during the expulsion phase (period 3) were calculated by taking the mean of the average concentration measured for each bitch during the expulsion phase (n = 6 in the spontaneously whelping group, and n = 5 in the induced group; one dog was not sampled during the expulsion phase). For period 3, mean concentrations of P4, cortisol and PGFM were compared with Student's t-test.

Data are expressed as mean ± S.E.M. A P-value ≤0.05 was considered significant.

Results

The mean gestation length of the spontaneously whelping bitches (62.2 ± 0.5 days) was significantly longer (P = 0.001) than that of the bitches in the induced group (59.5 ± 0.2 days). Length of expulsion phase, mean inter-pup interval, and mean litter size did not differ between both groups. The aglepristone treatment had no effect on the duration of the interval to the next ovulation. The interoestrous interval, i.e., from the ovulation in the cycle in which the bitch was mated until the ovulation of the following cycle was 211 ± 15 days in the induced group and 213 ± 8 days in the spontaneously whelping group (for further details see (Baan et al. 2005)).

Mean P4 and PGFM concentrations did not differ significantly between both groups in the period between days 54 and 58 of pregnancy, but significant changes were found after day 58. Fig. 1A and B illustrates P4 and PGFM concentrations for a single bitch and mean values for the whole group of spontaneously whelping animals, respectively. P4 concentrations decreased to basal levels before expulsion of the first pup, at which time PGFM concentrations were increasing to reach a peak value just after expulsion of the first pup (Fig. 1A). Mean P4 concentration in the spontaneously whelping group decreased significantly (P < 0.01) in the 30-h period before parturition, whereas the mean PGFM concentration increased significantly (P < 0.01) during that same period (Fig. 1B). On the 1st day after parturition, both mean P4 and PGFM concentrations had decreased significantly again.

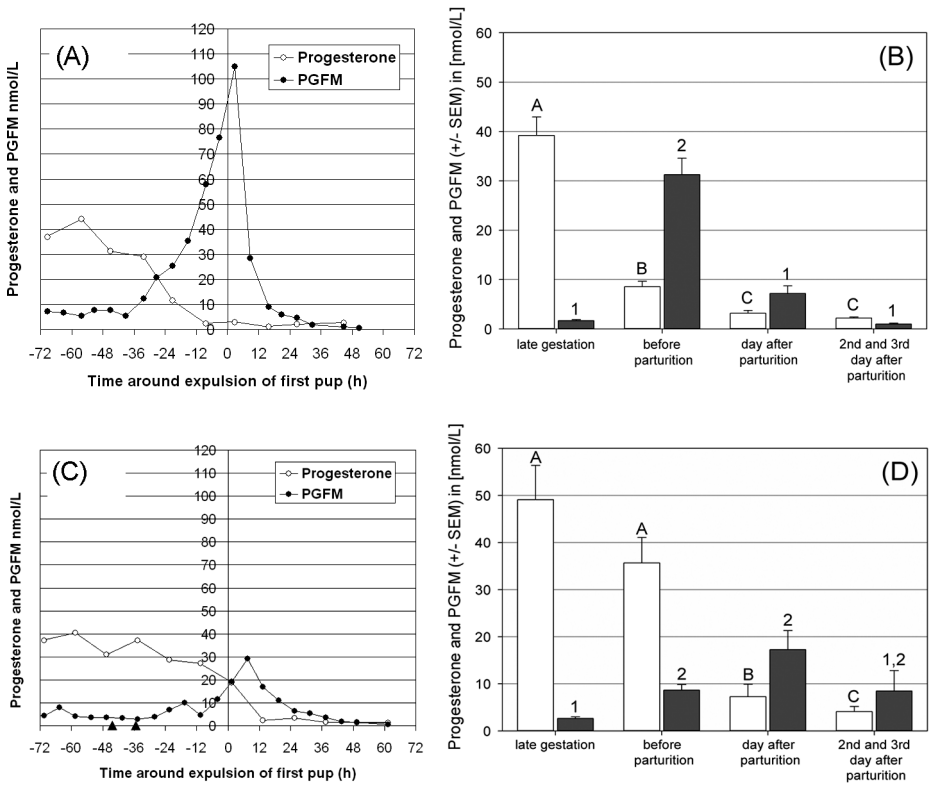

Figure 1. *Plasma concentrations of P4 and prostaglandin F2α metabolite (PGFM) at 12-h (P4) and 6-h (PGFM) intervals around the time of expulsion of the first pup (t = 0) of a spontaneously whelping 3-year-old beagle bitch (A) and a 1.5-year-old beagle bitch in which parturition had been induced (black triangles) with aglepristone (C), and mean (±S.E.M.) plasma P4 (white bars) and PGFM concentrations (black bars) in the bitches from the spontaneously whelping group (B) and the aglepristone-induced group (D). Late gestation: day 54 until 10 a.m. on day 58 of pregnancy; before parturition: 30–0 h before expulsion of the first pup; day after parturition: 0–24 h after expulsion of the last pup; 2nd and 3rd day after parturition: 24–72 h after expulsion of the last pup. The first dose of aglepristone was given to the bitches in the induced group at the end of the late gestational period. Different letters (A–C) and numbers (1, 2) denote significant differences.*

Fig. 1C and D shows P4 and PGFM concentrations for a single bitch and mean values for the whole group of the induced whelping bitches, respectively. The P4 concentration also dropped to basal levels in this group, but this occurred only after – not before – the expulsion of pups. The PGFM concentration started to increase before parturition, but peak concentration remained lower than in the spontaneously whelping bitch (cf. Fig. 1A and C). Overall, in the bitches from the induced group the mean PGFM concentrations increased significantly ($P < 0.05$) between late gestation and the 30-h period before parturition and the day after parturition. PGFM concentrations then dropped during the 2nd and the 3rd day after parturition, but this decline was not significant (Fig. 1D). Despite the increase in the pre-partum period, PGFM

concentrations were significantly lower (P = 0.01) than in the spontaneously whelping group during that period. On the 2nd and 3rd day after parturition, PGFM concentrations in the induced group were significantly higher (P < 0.01) compared with those in the spontaneously whelping group.

In both groups, the mean E2β concentration decreased significantly between the late gestational period and the 30-h period before parturition (P < 0.05 and P < 0.01 for the spontaneously whelping and the induced group, respectively; Fig. 2). No further decrease was observed during the postpartum periods. For each of the four periods, mean E2β concentrations were similar in both groups.

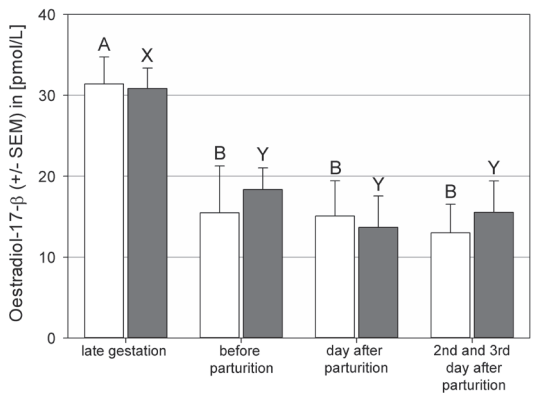

Figure 2. *Mean (±S.E.M.) plasma concentrations of oestradiol-17-β in the bitches from the spontaneously whelping (white bars) and the induced group (black bars) in late gestation, before parturition, the day after parturition, and the 2nd and 3rd day after parturition. Different letters (A, B and X, Y) denote significant differences within the spontaneously whelping and induced group, respectively. The first dose of aglepristone was given to the bitches in the induced group at the end of the late gestational period*.

In both groups, cortisol concentrations increased significantly (P < 0.05) between late gestation and the 30-h period before parturition (Fig. 3A). In the spontaneously whelping group, cortisol concentrations in the postpartum periods were not significantly different from values during late gestation. In the induced group, cortisol concentrations decreased significantly (P < 0.05) on the 1st day after parturition, compared with the 30-h period before parturition, but the levels were still significantly higher (P < 0.05) than those during late gestation. During the 2nd and 3rd day after parturition cortisol concentrations were again similar to those in late gestation. During the two time periods before expulsion of pups, cortisol concentrations did not differ significantly between both groups. During the postpartum periods, cortisol concentrations were significantly higher (P < 0.01) in the bitches from the induced group.

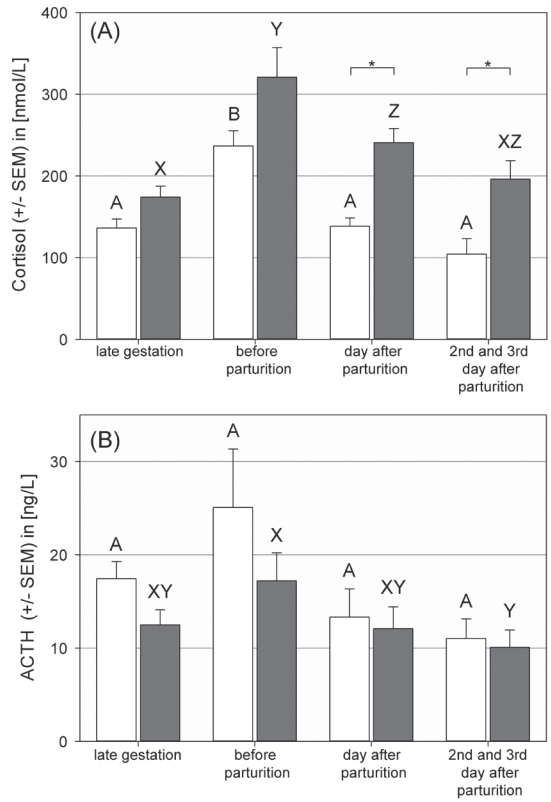

Figure 3. *Mean (±S.E.M.) plasma concentrations of cortisol (A) and adrenocorticotrophic hormone (B) in the bitches from the spontaneously whelping (white bars) and the induced group (black bars) in late gestation, before parturition, the day after parturition, and the 2nd and 3rd day after parturition. Different letters (A, B and X–Z) denote significant differences within the spontaneously whelping and induced group, respectively. Significant differences between groups within one period are indicated with an asterisk. The first dose of aglepristone was given to the bitches in the induced group at the end of the late gestational period*.

There was a large intra-individual variation in ACTH concentrations, especially in the last 30 h before the start of parturition (Fig. 3B). ACTH concentrations did not change significantly throughout the study in bitches from both groups during the first three periods. During the 2nd and 3rd day after parturition, however, ACTH concentrations in the induced group had decreased significantly ($P < 0.05$) compared with the values in the 30-h period before parturition. There was no difference in mean ACTH concentrations between the two groups in any of the periods.

The PRL concentrations in the bitches from the spontaneously whelping group increased significantly ($P < 0.05$) between late gestation and the 30-h period before parturition, while those of the induced group only tended to increase ($P = 0.057$) (Fig. 4). There was no difference in PRL concentrations between the two groups in any of the periods.

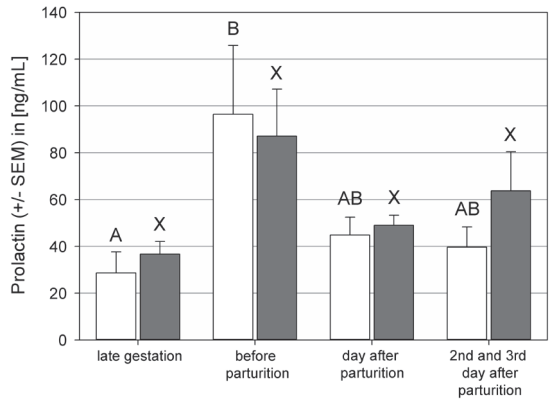

Figure 4. *Mean (±S.E.M.) plasma concentrations of prolactin (PRL) in the bitches from the spontaneously whelping (white bars) and the induced group (black bars) in late gestation, before parturition, the day after parturition, and the 2nd and 3rd day after parturition. Different letters (A, B and X) denote significant differences within the spontaneously whelping and the induced group, respectively. The first dose of aglepristone was given to the bitches in the induced group at the end of the late gestational period*.

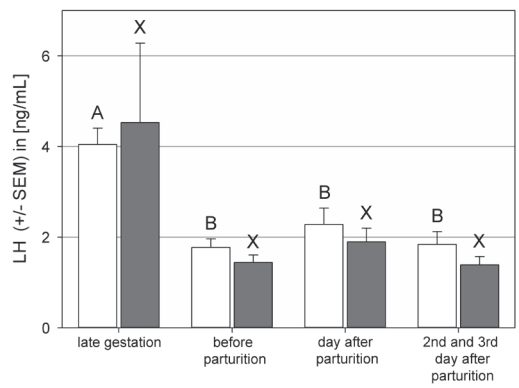

Figure 5. *Mean (±S.E.M.) plasma concentrations of luteinizing hormone (LH) in the bitches from the spontaneously whelping (white bars) and the induced group (black bars) in late gestation, before parturition, the day after parturition, and the 2nd and 3rd day after parturition. Different letters (A, B and X) denote significant differences within the spontaneously whelping and the induced group, respectively. The first dose of aglepristone was given to the bitches in the induced group at the end of the late gestational period*.

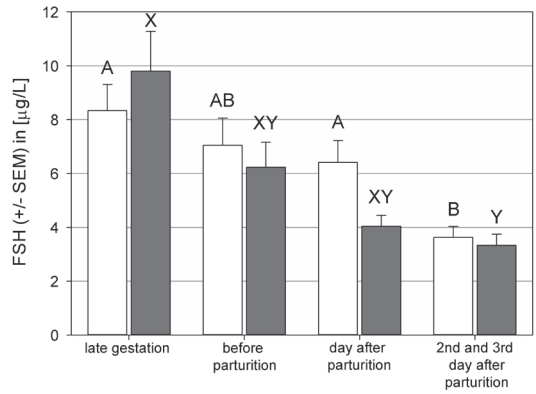

Figure 6. *Mean (±S.E.M.) in the bitches from the spontaneously whelping (white bars) and the induced group (black bars) in late gestation, before parturition, the day after parturition, and the 2nd and 3rd day after parturition. Different letters (A, B and X, Y) denote significant differences within the spontaneously whelping and the induced group, respectively. The first dose of aglepristone was given to the bitches in the induced group between the late gestational period and the period before parturition*.

The LH concentrations in bitches from the spontaneously whelping group decreased significantly ($P < 0.01$) between late gestation and the 30-h period before parturition (Fig. 5). After parturition, LH concentrations remained significantly lower ($P = 0.05$) as compared with the values in the late gestational period. Within the induced group, there was a large variation in LH concentrations between days 54 and 58 of gestation, and there were no significant differences between LH concentrations in any of the periods. LH concentrations did not differ significantly between the two groups in any of the periods. In both groups, FSH concentrations gradually and significantly decreased from late gestation until the 2nd and 3rd day after parturition. During the pre-partum periods, FSH concentrations did not differ between the two groups, but they tended to be higher ($P = 0.026$, with Bonferroni correction $\alpha \leq 0.01$) in the spontaneously whelping group on the day after parturition (Fig. 6).

The P4 concentrations in the samples taken during the expulsion phase were significantly higher in the induced group (n = 5) than in the spontaneously whelping group (n = 6, Table 2). Cortisol and PGFM concentrations did not differ significantly between both groups in this period.

Table 2. *Plasma concentrations (mean ± S.E.M.) of progesterone, cortisol, and prostaglandin F2α metabolite (PGFM) in a single blood sample collected during the expulsion phase*

	Spontaneously whelping group n = 6	Induced whelping group n = 5	P-value
P4 (nmol/L)	5.9 ± 1.2	22.1 ± 4.9	0.036
Cortisol (µg/L)	123 ± 29	141 ± 11	0.575
PGFM (nmol/L)	47 ± 12	23 ± 4	0.121

Discussion

In the spontaneously whelping dogs, the pre-partum decline to basal levels of the P4 concentration coincided with a clear increase of the PGFM concentration, as has been reported before (Concannon et al. 1988). Plasma P4 concentrations in the induced group, however, reached basal levels only after parturition, indicating that luteolysis was not complete at the time of expulsion of the first pup. In line with the important role that PGF2α plays in prepartum luteolysis, a significant, albeit still modest, increase in circulating PGFM concentrations before parturition was noted in the induced whelping dogs, as was also seen in another study (Fieni et al. 2001). The corpora lutea, however, remained functional as reflected by the relatively high P4 concentrations. The level of PGF2α production before parturition in the induced group might have been too low to cause complete luteolysis, or the luteolysis was incomplete because of a lower sensitivity of the corpora lutea to PGF2α at the time of aglepristone administration, i.e., on day 58 of pregnancy. Furthermore, because maximum plasma aglepristone levels are only reached after approximately 2.5 days (unpublished data, provided by the manufacturer), only part of the total number of P4 receptors will be blocked initially and circulating P4 around the time of parturition can still exert its activity at the receptor level, which may have resulted in repression of PGF2α secretion. In addition, P4 may have a stimulating paracrine/autocrine effect on its own production within the luteal cells (Rothchild 1996; Hoffmann et al. 2004).

After parturition, the mean PGFM concentration in the spontaneously whelping group very quickly returned to basal values, contrary to the PGFM concentrations in the induced group. We speculate that the elevated postpartum PGFM concentrations in the induced group are associated with the completion of the luteolytic process after parturition.

In agreement with previous reports (Edqvist et al. 1975; Van der Weyden et al. 1989; Onclin et al. 2002), a significant decrease in oestradiol-17-β concentrations was observed prior to parturition in both groups. Apparently, aglepristone treatment does not affect the plasma profile of this hormone around the time of parturition. The physiological significance of the decreasing E2β concentrations before parturition in the dog is not known. It also remains to be investigated if the ante-partum decrease in gonadotrophic hormone concentrations are associated with the pre-partum oestradiol decrease.

In line with previous studies (Concannon et al. 1978; Hoffmann et al. 1994; Lye 1994; Veronesi et al. 2002), cortisol concentrations increased significantly before parturition (Spitz

et al. 1985)in both groups. After parturition, cortisol concentrations in the induced group were significantly higher than in the spontaneously whelping group, possibly due to partial blocking of glucocorticoid receptors by aglepristone. Blocking of pituitary glucocorticoid receptors results in an increased ACTH release and a subsequently elevated cortisol secretion (Spitz et al. 1985; Wade et al. 1988). The blood sampling frequency used in the present study does not allow strong statements about hormones with highly fluctuating plasma concentrations such as cortisol and ACTH (Kooistra et al. 1997). Elevated postpartum cortisol concentrations in the induced group may also have been caused by a sustained postpartum PGF2α production as reflected in the elevated PGFM concentrations. However, there was no clinical evidence for an elevated stress response in the induced bitches, as postpartum behavior, the number of postnatal losses and the weight-increase of the puppies were similar to those in the spontaneously whelping dogs (Baan et al. 2005).

In the spontaneously whelping group, a significant increase in PRL concentrations was observed before parturition reflecting the modulating effect of P4 on PRL secretion (Steinetz et al. 1990; Okkens et al. 1997; Galac et al. 2000; Kooistra and Okkens 2002). In the induced group, the PRL concentrations before parturition only tended to increase, possibly due to the less abrupt decrease in P4. PRL plays an important role in mammogenesis and lactogenesis (Sinowatz et al. 1980; Brisken et al. 1999). Because in both groups the puppies grew steadily (Baan et al. 2005), it appears that aglepristone does not affect mammary function around the time of parturition, which is important for pup survival and growth after birth.

Both the concentrations of FSH (both groups) and LH (spontaneously whelping group) decreased between late gestation and the postpartum period. This decline in circulating gonadotrophin levels may be due to the increase in PRL secretion (Bevers et al. 1983; Park and Selmanoff 1993; Jedlinska et al. 1995), but an influence of the declining E2β concentration, which has been shown to have an effect on the secretion of gonadotrophic hormones in other periods of the oestrous cycle, may also play a role (de Gier et al. 2006).

In conclusion, the results of this study have further expanded our knowledge on the hormonal changes around parturition in the bitch. After comparison of hormonal patterns between the two groups, it may be concluded that aglepristone-induced parturition is associated with a still incomplete luteolysis, an altered plasma PGM profile, and elevated postpartum cortisol concentrations.

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9

Effects of the 3β-hydroxysteroid dehydrogenase inhibitor trilostane on luteal progesterone production in the dog

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Abstract

Interference with the pregnancy-maintaining influence of progesterone is the basis of most methods for termination unwanted pregnancy in dogs. The currently available methods are based on induction of luteolysis or blocking of the progesterone receptor. Inhibition of progesterone synthesis using a competitive inhibitor of 3β-hydroxysteroid dehydrogenase (3β-HSD) could be another strategy to terminate unwanted pregnancy.

In this study we investigated the effects of the 3β-HSD inhibitor trilostane on corpus luteum function in non-pregnant bitches. Trilostane was administered orally for seven consecutive days in either the pituitary-independent part of the luteal phase (PIP, start of treatment on D11 after ovulation, n=6) or the pituitary-dependent part (PDP, start of treatment on D31 after ovulation, n=6), in an oral dose of about 4.5 mg/kg bw, twice daily. Results were compared with those obtained in control bitches (n=6). ACTH stimulation tests were performed to assess adrenocortical reserve capacity.

Trilostane caused no apparent side effects and ACTH stimulation tests revealed good suppression of cortisol secretion. Trilostane also caused a significant decrease in plasma progesterone concentration. When it was stopped during PIP, progesterone secretion was completely restored and there was no difference in the length of the luteal phase between those dogs and control dogs (99 days, range 70-138 days and 99 days, range 60-112 days respectively). When trilostane was stopped during PDP there was no post-treatment recovery of progesterone secretion and although the luteal phase tended to be shorter (66 days, range 41-101 days) the difference was not significant (p=0.09). Plasma prolactin concentration did not increase after the trilostane-induced decrease in plasma progesterone. The interoestrous interval in dogs treated during PIP (234 days, range 175-269 days) or PDP (198 days, range 120-287 days) was not significantly shorter than the control interval (247 days, range 176-313 days).

In conclusion, trilostane treatment was effective in decreasing plasma progesterone concentration in bitches during the luteal phase, but the dose regimen used in this study produced less clear-cut inhibition of ovarian steroidogenesis than have other strategies to decrease plasma progesterone concentration. Further studies are warranted to determine whether trilostane can be used to terminate unwanted pregnancy in the bitch without inducing adrenocortical insufficiency.

Introduction

The oestrous cycle of the domestic bitch (*Canis lupus familiaris)*, a mono-oestrous species, is characterized by a follicular phase with spontaneous ovulations, followed by a luteal phase of about 75 days and a non-seasonal anoestrus of 2 to 10 months (Schaefers-Okkens and Kooistra 2010). The luteal phase is similar to or somewhat longer than pregnancy in the dog, in contrast to most other domestic species (Okkens et al. 2001).

In the dog, *corpora lutea* are the sole source of progesterone, which is obligatory for maintaining pregnancy (Sokolowski 1971). Ovarian progesterone production in the dog is independent of pituitary support during the first half of the luteal phase or gestation and consequently this part of the cycle may be called the pituitary-independent part of the luteal phase (PIP) (Okkens et al. 1986). During the second half of the luteal phase or pregnancy, prolactin plays an important role in maintenance of the *corpora lutea* and this part of the cycle can therefore be called the pituitary-dependent part of the luteal phase (PDP) (Okkens et al. 1985; Okkens et al. 1986; Okkens et al. 1989; Okkens et al. 1990; Onclin and Verstegen 1997; Onclin et al. 2000; Kooistra and Okkens 2002; Lee et al. 2006)

Most methods for termination of unwanted pregnancy in the bitch rely on interference with the pregnancy-maintaining influence of progesterone. Complete luteolysis is very difficult to achieve in this species during the first weeks after ovulation (Oettle et al. 1988; Romagnoli et al. 1996). In pregnant bitches, $PGF_{2\alpha}$ only induces luteolysis and subsequently abortion when multiple doses are administered from 10 to 30 days after fertilization onwards (Oettle et al. 1988; Romagnoli et al. 1996). Administration of the dopamine agonists bromocriptine or cabergoline or the serotonin antagonist metergoline during the pituitary-dependent part of the luteal phase may also induce luteolysis followed by abortion or premature parturition, by inhibiting secretion of the luteotrophic hormone prolactin (Onclin et al. 1993; Nothling et al. 2003). Combining this with administration of $PGF_{2\alpha}$ gives more reliable induction of luteolysis and thus abortion, with fewer side-effects (Onclin and Verstegen 1999). Administration of the GnRH antagonist acyline also induces luteolysis and termination of mid term pregnancy in bitches(Valiente et al. 2009). A more direct way of interfering with the pregnancy-maintaining effects of progesterone is the administration of a progesterone receptor antagonist such as aglépristone, which is also effective in terminating unwanted pregnancy during the pituitary-independent part of the luteal phase (Galac et al. 2000; Fieni et al. 2001).

Inhibiting progesterone synthesis with a competitive inhibitor of the 3β-hydroxysteroid dehydrogenase/isomerase system (3β-HSD) might also be used to terminate unwanted pregnancy (Potts et al. 1978). 3β-HSD expression has been demonstrated in canine luteal cells throughout the luteal phase and inhibition of progesterone synthesis by the 3β-HSD inhibitor epostane has been shown effective in bitches for termination in early stages of pregnancy (Keister et al. 1989; Kowalewski et al. 2006). Epostane is not available for use in veterinary medicine, but another 3β-HSD inhibitor, trilostane, has been registered for the treatment of hypercortisolism in dogs (Neiger et al. 2002; Ruckstuhl et al. 2002; Galac et al. 2010a).

The aim of this study was to assess the effects of the 3β-HSD inhibitor trilostane on plasma progesterone and prolactin concentrations during the luteal phase in healthy, non-pregnant bitches. ACTH stimulation tests were performed to assess adrenocortical reserve capacity. Trilostane was administered during seven consecutive days during either the pituitary-independent or pituitary-dependent part of the luteal phase and results were compared with those obtained in control bitches.

Animals, materials and methods

Animals, treatment, and collection of blood samples

Sixteen healthy Beagle bitches were used in this study. Two were used twice, first in the control group and then in a treatment group in the luteal phase. The differences in median age at the onset of the study in the pituitary-independent part of the luteal phase (PIP; 6.4 years, range 3.5-7.8 years, n=6), the pituitary-dependent part of the luteal phase (PDP; 4.9 years, range 2.2-8.4 years, n=6), and the control group (4.5 years, range 2.3-7.8 years, n=6) were not significant. All bitches but one had been born and raised at the Department of Clinical Sciences of Companion Animals and were accustomed to the laboratory environment and procedures such as blood collection. They were housed in pairs in indoor-outdoor runs, fed a standard commercial dog food once daily, and given water **ad libitum**.

All dogs were examined thrice weekly for swelling of the vulva and serosanguineous vaginal discharge, which were considered to signify the onset of pro-oestrus. Ovulation (day 1) was estimated by measuring plasma progesterone concentration thrice weekly from the start of pro-oestrus onwards, using a ^{125}I RIA, previously validated for fertility breeding management (Okkens et al. 2001). Plasma progesterone concentrations of 13-16 nmol/L were considered to signify the onset of ovulation. 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 by jugular venepuncture.

Six bitches served as controls and 12 were treated with trilostane (Vetoryl®, Arnolds Veterinary Products, Shropshire, United Kingdom), in a dose of 60 mg at 8.00 a.m. and 8.00 p.m. for seven consecutive days, either during PIP between days 11 and 17 after ovulation or during PDP between days 31 and 37 after ovulation. Bitches receiving trilostane were housed individually to enable observation of possible side effects.

Plasma progesterone and prolactin concentrations were measured daily from three days prior to the start of treatment to 10 days after the last treatment in both treatment groups and daily at 1 and 4 hours after administration of trilostane during the treatment period. Subsequently, blood samples were collected three times weekly until plasma progesterone concentration decreased below 6 nmol/L, which marked the onset of anoestrus. Plasma progesterone and prolactin concentrations in the control bitches were measured daily from day 7 to day 46 after ovulation and subsequently 3 times weekly until plasma progesterone concentration decreased below 6 nmol/L.

ACTH stimulation tests were performed to assess adrenocortical reserve capacity in the treated bitches and to study the effectiveness and safety of the trilostane dosage. Two blood samples were collected 15 minutes apart for measurement of the basal plasma cortisol concentration and then 0.25 mg ACTH (Synacthen, Novartis Pharma, Arnhem, The Netherlands) was administered via the cephalic vein. A sample for measurement of the ACTH-stimulated plasma cortisol concentration was collected 90 min later (Frank et al. 2004; Galac et al. 2010b).

Two ACTH stimulation tests were performed in each bitch, the first at 1 or 2 days before the start of trilostane treatment and the second 2 to 3 hours after the first of the two doses of trilostane on the last day of the treatment period. In the control group an ACTH stimulation tests was performed on days 16 and 36 after ovulation.

To evaluate the effect of trilostane on the pituitary-adrenocortical axis, the cortisol:ACTH ratio (CAR) in plasma was calculated. To evaluate the effect of trilostane on the renin-aldosterone axis, the plasma aldosterone:renin ratio (ARR) was calculated.

Blood samples were collected from the jugular vein, immediately placed in chilled lithium heparin-coated tubes, and centrifuged at 1500 X g for 10 min at 4 °C. Plasma was stored at –25 °C until assayed.

Hormone measurements

Plasma progesterone concentration was measured with a previously validated RIA (Dieleman and Schoenmakers 1979; Okkens et al. 1985). The intra-assay and interassay coefficients of variation were 11% and 14%, respectively. The limit of detection was 0.13 nmol/L.

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

Plasma ACTH concentration was measured by an immunoradiometric assay validated for the dog (Javadi et al. 2006). The interassay coefficient of variation was 7.8% and the limit of detection was 0.2 pmol/L.

Plasma cortisol concentration was measured by a RIA validated for the dog (Javadi et al. 2006). The interassay coefficient of variation was 4 to 6.4% and the limit of detection was 1 nmol/L.

Aldosterone was extracted from 1 ml plasma with dichloromethane. The extract was evaporated and then redissolved in assay buffer for measurement in a RIA as described by Boer et al. (Boer et al. 1983) and validated for the dog (Javadi et al. 2003). The intra- and interassay coefficients of variation were 6% and 14%, respectively. The reference range for plasma aldosterone concentration in our laboratory is 25 to 280 pmol/L (Javadi et al. 2006).

Plasma renin activity (PRA) was measured by incubating 0.5 ml plasma at pH 6.0 for one hour at 37 °C in the presence of inhibitors of angiotensinases and angiotensin I-converting enzyme. After incubation, the samples were deproteinized with 4 mol/L of a 9/1 (v/v) mixture of acetone/ammonia and centrifuged. The supernatants were evaporated and redissolved in assay buffer for measurement of angiotensin I in a RIA as described by Boer et al. (Boer et al. 1983) and validated for the dog (Javadi et al. 2003). The intra- and interassay coefficients of variation were 8% and 15%, respectively. The reference range for plasma renin activity in our laboratory is 65 to 547 fmol/L/s (Javadi et al. 2006).

Calculations and data analysis

The length of the luteal phase was defined as the number of days between ovulation and the decrease in plasma progesterone below 6 nmol/L, which was considered the onset of anoestrus. The interoestrous interval was defined as the number of days between ovulations in two consecutive oestrous cycles. The control interoestrous intervals consisted of those in the control group directly following the studied luteal phase and those in the treated bitches directly preceding the studied luteal phase.

Statistical analysis was performed with SPSS 16.0 for Windows. Differences were calculated using tests for non-parametric data: the Mann-Whitney test for differences between groups and the Wilcoxon signed rank test for differences within groups. A simple linear regression model was used to calculate the correlation between time and plasma prolactin concentration from ovulation onwards.

Results are expressed as median and range and $P < 0.05$ was considered significant. Graphs were created with Sigmaplot 10.0.

Ethics of experimentation

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

Results

Animals

The difference between the median oral dose of trilostane in bitches in PIP (twice daily 4.3 mg/kg bw, range 3.3-4.7 mg/kg bw) and that in bitches in PDP (twice daily 4.5 mg/kg bw, range 4.0-5.3 mg/kg bw) was not significant. Trilostane caused no apparent side effects in any of the bitches in the study.

Effect of trilostane on luteal function

In both treatment groups the median plasma progesterone concentration during the 3 days preceding treatment (PIP: 142 nmol/L, PDP: 109 nmol/L) was higher ($P< 0.05$) than during the first 3 days of treatment (PIP: 63 nmol/L, PDP: 35 nmol/L). In control animals the differences in median plasma progesterone concentration between similar periods of the luteal phase (112 and 114 nmol/L at days 8 to 10 and days 12 to 14, respectively; and 90 and 73 nmol/L at days 28 to 30 and days 32 to 34, respectively) were not significant (Fig. 1). Day-to-day variations in median plasma progesterone and prolactin concentrations in the PIP, PDP, and control groups are shown in Figure 2. While plasma progesterone was decreasing during treatment with trilostane, the change in plasma prolactin concentration was not significant (Fig. 3). In bitches treated during PIP and PDP, as well as in control bitches, there was a positive correlation between time after ovulation and median plasma PRL concentration: $r^2=0.55$, $P < 0.001$; $r^2=0.08$, $P = 0.02$; and $r^2=0.60$, $P < 0.001$, respectively (Fig. 2).

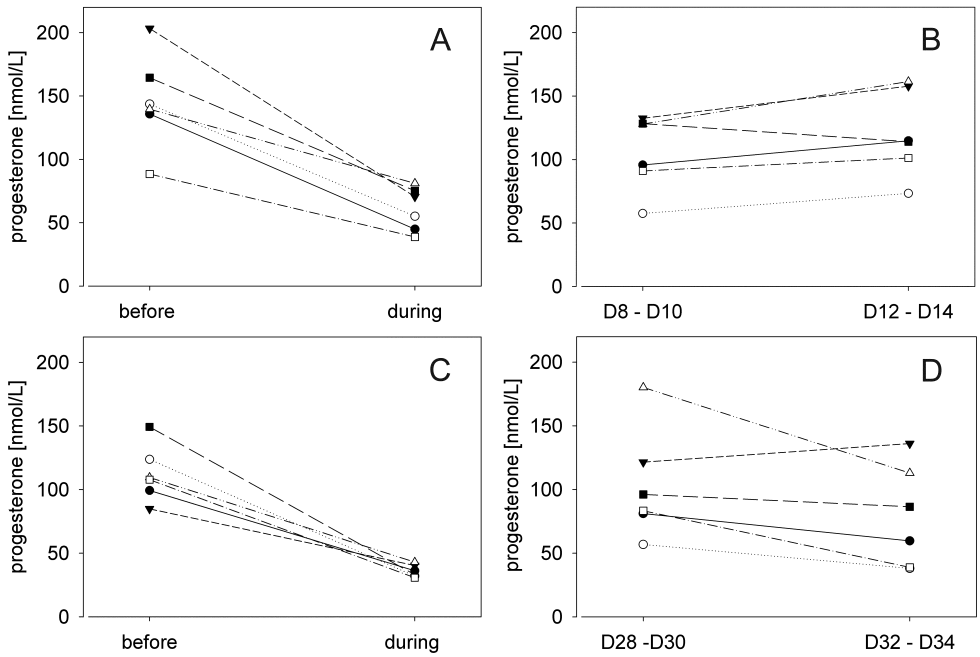

Figure 1. *Plasma progesterone concentrations, median of three days before and three days after start of treatment with the 3β–hydroxysteroid dehydrogenase inhibitor trilostane, in individual Beagle bitches (panels A and C). In the pituitary-independent part of the luteal phase (PIP, panel A) treatment was started on day 11 after ovulation. In the pituitary-dependent part of the luteal phase (PDP, panel C) treatment was started on day 31 after ovulation. In panels B and D median plasma progesterone concentrations in the control group during corresponding periods are depicted.*

The luteal phase was shorter in bitches treated during PDP (66 days, range 41-101 days, n=6) than in those treated during PIP (99 days, range 70-138 days, n=6) ($P < 0.05$), but in both cases the difference in the length of the luteal phase from that in control bitches (99 days, range 60-112 days, n=6) was not significant.

Neither the interoestrous interval following treatment during PIP (234 days, range 175-269 days, n=5) nor that following treatment during PDP (198 days, range 120-287 days, n=5) was significantly shorter than control intervals (247 days, range 176-313 days, n=13).

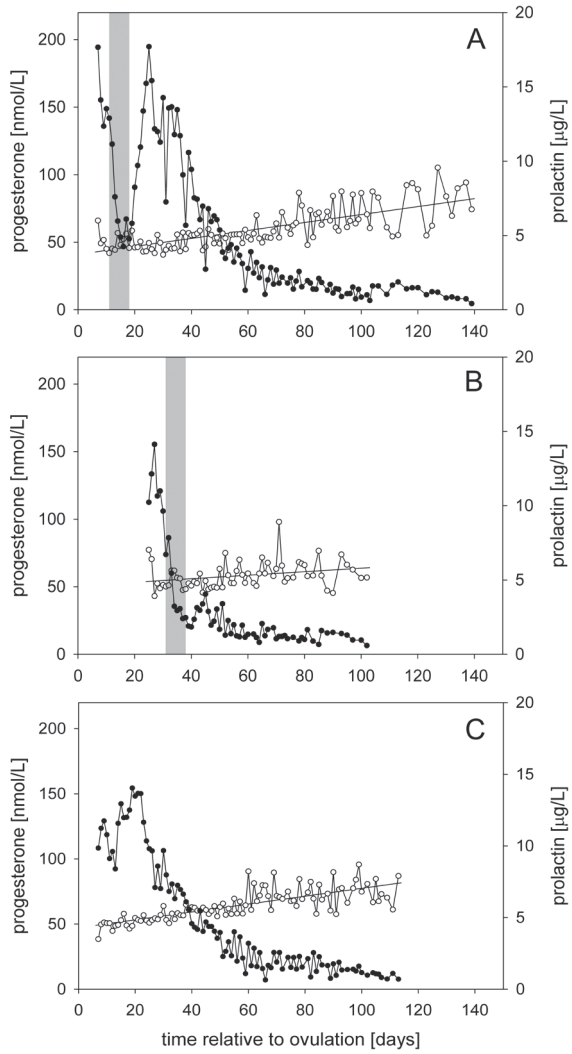

***Figure 2**. Median plasma concentrations of progesterone (solid dots) and prolactin (open dots) in bitches treated during PIP (A), bitches treated during PDP (B), and control bitches (C). Treatment consisted of 60 mg trilostane per bitch twice daily, orally, for seven consecutive days. Day 1 = day of ovulation. Simple linear regression lines for plasma prolactin concentration from Day 1 onwards have been inserted. The treatment period is grey*.

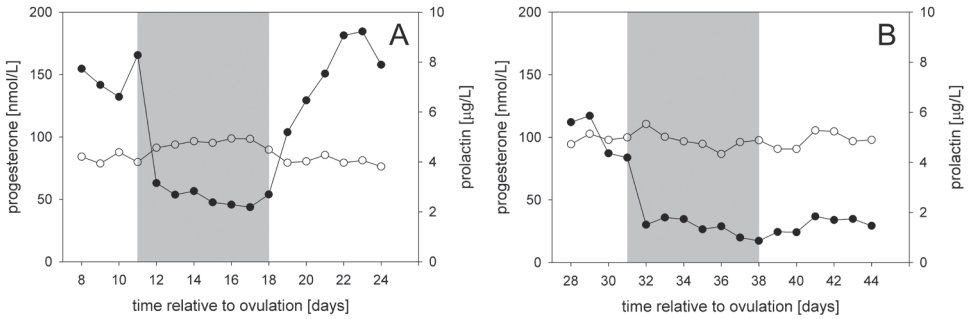

Figure 3. *Changes in median plasma concentrations of progesterone (solid dots) and prolactin (open dots) in beagle bitches treated with the 3β–hydroxysteroid dehydrogenase inhibitor trilostane (60 mg/bitch twice daily, orally) for seven consecutive days. The treatment period is grey. Day 1 = day of ovulation. A: bitches treated during PIP and B: bitches treated during PDP.*

Effect of trilostane treatment on adrenocortical function

ACTH stimulation caused a clear increase in plasma cortisol concentration in all bitches prior to trilostane treatment (Fig. 4). In contrast, the plasma cortisol response to ACTH 2 to 3 hours after trilostane on the last day of treatment was not significant. In both the PIP and PDP treatment groups the post-ACTH plasma cortisol concentration remained below 190 nmol/L, but above 40 nmol/L in 9 of 11 dogs (PIP, range 18-79 nmol/L, n=5; PDP, range 36-178 nmol/L, n=6) (Fig. 4).

In all of the bitches treated during PIP the cortisol:ACTH ratio (CAR) decreased and was lower at the end of treatment (1.87, range 1.44-3.70, n=5) than before treatment (7.60, range 5.10-15.86, n=5) ($P < 0.05$) (Fig. 5). The aldosterone:renin ratio (ARR) was also lower at the end of treatment (0.14, range 0.04-0.34, n=5) than before treatment (0.30, range 0.22-1.06, n=5), but the difference was not significant (P=0.08) (Fig. 5).

In five of the six bitches treated during PDP the CAR was lower at the end of treatment (5.88, range 4.25-10.59, n=6) than before (9.58, range 7.03-17.69, n=6) (P = 0.06) (Fig. 5). ARR was also lower at the end of treatment (0.14, range 0.09-0.43, n=6) than before (0.88, range 0.29-4.08, n=6) ($P < 0.05$) (Fig. 5).

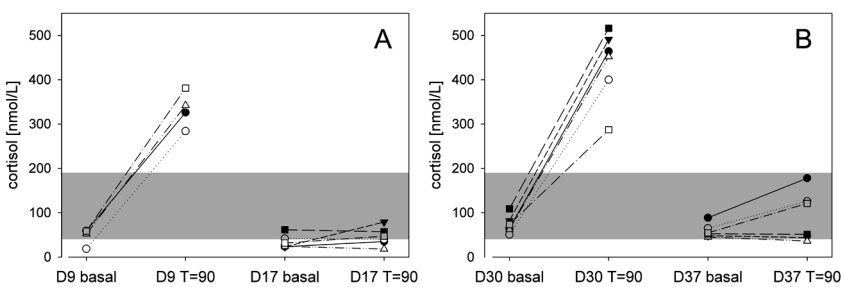

Figure 4*. Plasma cortisol concentrations in individual bitches before and 90 minutes after the intravenous administration of 0.25 mg synthetic ACTH. ACTH stimulation tests were performed before and on the last day of treatment with trilostane (60 mg/bitch twice daily, orally, for seven consecutive days). Treatment started on day 11 (PIP (n=6), panel A) or day 31 (PDP (n=6), panel B). Day 1 = day of ovulation. The preferred post-ACTH plasma cortisol concentration of 40 to 190 nmol/L during trilostane treatment is shown in grey (Galac et al. 2010a).*

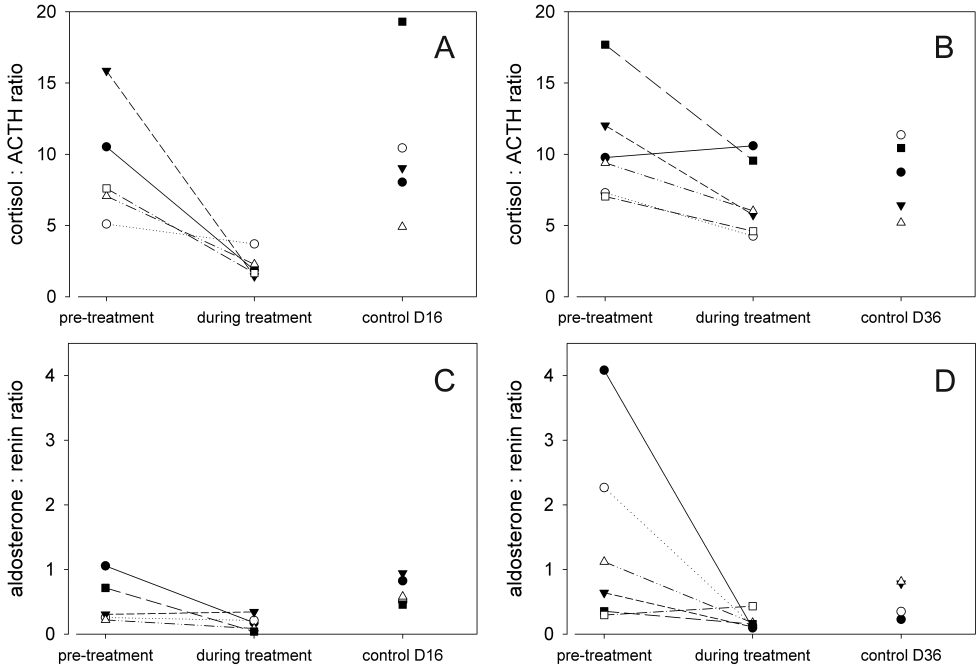

***Figure 5**. Cortisol:ACTH ratio (CAR) and aldosterone:renin ratio (ARR) in individual bitches before and on the last day of treatment with trilostane (60 mg/bitch twice daily, orally, for seven consecutive days). Treatment started on day 11 (PIP; CAR (n=5), panel A; ARR (n=5), panel C) or day 31 (PDP; CAR (n=6), panel B; ARR (n=6), panel D). CAR and ARR in control bitches, measured on days 16 and 36, are also depicted. Day 1 = day of ovulation*.

Discussion

This study shows that gonadal and adrenal steroid synthesis is decreased by trilostane during both the pituitary-dependent and the pituitary-independent parts of the luteal phase in Beagle bitches. The total daily dose of trilostane, 6.6 to 10.6 mg/kg bw, was within the range of 2 to 12 mg/kg bw recommended by the manufacturer. In dogs with pituitary-dependent hypercortisolism, an ACTH stimulation test 2 to 3 hours after the administration of trilostane with food is considered to be the best test to determine the adrenocortical reserve capacity and to establish the optimal dose of trilostane (Neiger et al. 2002; Ruckstuhl et al. 2002; Braddock et al. 2003; Galac et al. 2010a). In accordance with the criteria of Galac et al. (2010a), the post-ACTH plasma cortisol concentration was below 190 nmol/L in all bitches, indicating that the dose used in this study was satisfactory. In addition, this dose had no apparent side effects and the post-ACTH plasma cortisol concentration was above 40 nmol/L in most of the bitches (Galac et al. 2010a). However, that it was below 40 nmol/L in 2 of the 11 bitches during treatment suggests that a higher total daily dose may result in adrenocortical insufficiency.

Trilostane causes a decrease in plasma cortisol concentration (Neiger et al. 2002; Ruckstuhl et al. 2002; Wenger et al. 2004). The resulting loss of negative feedback leads to an increase in plasma ACTH concentration (Witt and Neiger 2004; Sieber-Ruckstuhl et al. 2006; Galac et al. 2010a). These effects of trilostane are nicely reflected in the cortisol:ACTH ratio (CAR) (Javadi et al. 2006). As has been reported in dogs with pituitary-dependent hypercortisolism (Galac et al. 2010a), trilostane treatment caused a decrease in the CAR. Together with the insignificant increase in plasma cortisol concentration in the ACTH stimulation tests, this indicates that the dose of trilostane was sufficient to suppress adrenocortical synthesis of cortisol considerably.

Consistent with its competitive inhibitory effect on 3β-HSD, trilostane not only reduced cortisol secretion but also suppressed aldosterone synthesis. In humans, plasma renin activity (PRA) rather than plasma aldosterone concentration (PAC) is regarded as a guide to decreased mineralocorticoid secretion (Loriaux 2001). Also in dogs with pituitary-dependent hypercortisolism, PRA reflects trilostane-induced changes in aldosterone secretion better than does PAC (Wenger et al. 2004; Galac et al. 2010a). The higher PRA during trilostane treatment can be explained by a trilostane-induced decrease in aldosterone secretion. Subsequently, the decline in PAC results in less circulating volume due to a decrease in aldosterone-mediated renal reabsorption of sodium. The decrease in circulating volume stimulates renin secretion and the activation of the renin-angiotensin system results in a stimulus to aldosterone synthesis and secretion. This sequence of events indicates that conclusions about the effect of trilostane on the renin-aldosterone axis should not rely solely on the basal circulating aldosterone concentration but also on the aldosterone:renin ratio (ARR). The lower ARR observed during trilostane treatment therefore reflects the suppressive effect of trilostane on aldosterone synthesis.

Although trilostane induced a rapid and significant decrease in plasma progesterone concentration in both treatment groups, the resulting concentrations during treatment were well above those considered to be essential for maintaining pregnancy, i.e., 4 to 8 nmol/L (Concannon and Hansel 1977; Onclin et al. 1993). In a study by Keister et al. (1989) in which pregnant bitches were treated with the 3β-HSD inhibitor epostane orally during PIP, starting approximately 15 days after ovulation, plasma progesterone concentration decreased dose-dependently to lower values (4 to 22 nmol/L) than found in the present study (approximately 50 nmol/L) (Keister et al. 1989). Furthermore, the dose range they found to be effective to induce abortion (2.5 to 5 mg/kg BW, SID) (Keister et al. 1989) was lower than the total daily dose used in the present study (6.6 to 10.5 mg/kg BW). This may imply a difference in potency of epostane and trilostane in the bitch. In addition, it has been suggested that epostane preferentially blocks ovarian and placental steroidogenesis in both humans and animals, because in several species epostane induced termination of pregnancy at a dose level that did not have a significant effect on basal plasma cortisol concentration (Potts et al. 1978; Schane et al. 1979; Creange et al. 1981; Birgerson et al. 1986; Birgerson et al. 1987). Another explanation for the difference in effectiveness of epostane and trilostane might be the fact that the bitches in the study of Keister et al. (1989) were mated and presumably pregnant, whereas those in this study were not pregnant.

3β-HSD is expressed almost exclusively in the adrenals and gonads in most species (Simard et al. 1996). In the bitch, 3β-HSD is expressed in the CL and the expression decreases progressively from D15 of the luteal phase onwards (Kowalewski et al. 2006; Kowalewski et al. 2009). 3β-HSD is also expressed in normal adrenal tissue in dogs (Galac et al. 2010c) and thus the effect of 3β-HSD inhibitors on both the ovaries and adrenals should be demonstrable. A difference in inhibition of ovarian 3β-HSD by epostane and trilostane might explain the difference in plasma progesterone levels between bitches treated with trilostane during the luteal phase and mated bitches treated with epostane. This would presumably make trilostane unsuitable for termination of pregnancy in the dose used in this study.

Stopping trilostane during PDP did not cause plasma progesterone to rise to the level observed in the control group over a similar interval after ovulation, whereas full restoration of progesterone secretion did occur following treatment during PIP. This is in agreement with previous studies in dogs in which hypophysectomy was performed or progesterone receptor antagonists were administered during different parts of the luteal phase (Okkens et al. 1986; Concannon et al. 1990; Galac et al. 2000; Galac et al. 2004). Furthermore, the length of the luteal phase in the bitches treated during PDP (66 days) was shorter than that in control bitches (99 days) and bitches treated during PIP (99 days). Therefore, administration of trilostane during PDP may eventually cause abortion or premature parturition at a later stage of pregnancy.

The lack of recovery of progesterone secretion after treatment during PDP may suggest a role for progesterone in the support of it's own secretion during this phase. This has been hypothesized in the dog as well as in other species (Rothchild 1981; Hoffmann et al. 2004; Stocco et al. 2007; Kowalewski et al. 2009). The nuclear progesterone receptor has been shown to be present throughout the luteal phase in dog CL (Hoffmann et al. 2004), suggesting a role for progesterone in corpus luteum function. Studies of the influence of progesterone on corpus luteum function using progesterone receptor antagonists also resulted in shortening of the luteal phase if the antagonists were administered during PDP (Concannon et al. 1990; Galac et al. 2000).

There was no significant shortening of the post-treatment interoestrous interval in bitches that had been treated during either PIP (234 days) or PDP (198 days) compared with control intervals (247 days). This differed from the results of studies in which the progesterone receptor antagonists mifepristone or aglepristone were administered to pregnant bitches around 30 days after ovulation and to non-pregnant bitches around 12 days after ovulation. In those bitches interoestrous intervals were shortened (Galac et al. 2000; Fieni et al. 2001; Galac et al. 2004). However, the interoestrous interval was not shortened in bitches in which parturition was induced with aglepristone at day 58 of pregnancy (Baan et al. 2005).

The results of this study show that there is a positive correlation between the progressive decline in plasma progesterone concentration during the luteal phase and plasma prolactin concentration, which is in agreement with previous studies (Jochle and Andersen 1977; De Coster et al. 1983; Kooistra and Okkens 2002). Other studies have demonstrated a considerable increase in plasma prolactin concentration shortly following the administration of aglepristone during PDP in pregnant bitches (Galac et al. 2000) or after ovariectomy

during PDP (Lee et al. 2006). However, in the present study the treatment-induced decline in plasma progesterone concentration was not followed by a significant increase in plasma prolactin concentration during either PIP or PDP. The lack of a treatment-induced increase in plasma prolactin might be because the intervals between doses were too long for continuous suppression of progesterone synthesis; more frequent administration might have been more effective.

In conclusion, trilostane was effective in decreasing plasma progesterone concentration during the luteal phase, but the treatment regimen used in this study resulted in less clear-cut inhibition of ovarian steroidogenesis than have other reported strategies. Further studies are warranted to determine whether trilostane can be used to terminate unwanted pregnancy in the bitch without inducing adrenocortical insufficiency.

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10

Summarizing discussion and conclusions

The onset of oogenesis and its cyclic continuation, as well as pregnancy, and continuous spermatogenesis, all depend on endocrine regulation. Both the reproductive cycle and spermatogenesis are under the control of an integrated regulatory network, the hypothalamic-pituitary-gonadal axis (HPG axis). The gonadotrophic cells in the anterior lobe of the pituitary produce and secrete follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in a pulsatile fashion. In response to both of the gonadotrophins, the granulosa, theca, and luteal cells in the ovary secrete oestradiol, testosterone, progesterone, and inhibin, and the Leydig and Sertoli cells in the testicle secrete oestradiol, testosterone, and inhibin. These gonadal hormones provide feedback inhibition and stimulation at higher levels of the HPG axis. In the bitch the type and amount of hormones released are subject to cyclic changes of the ovarian follicles and corpora lutea. Environmental and other external factors, as well as internal factors, eg. from the hypothalamic-pituitary-adrenal axis and hypothalamic-pituitary-thyroid axis, may influence reproductive performance.

Chapter 2 provides an overview of the anatomy and function of the different parts of the HPG axis in male and female dogs, with emphasis on changes during the oestrous cycle. This chapter also includes an overview of current surgical and medical interventions applied to the normal HPG axis in dogs.

Current understanding of changes in circulating concentrations of reproductive hormones during the follicular, ovulatory, and early luteal phase is mostly based on low-frequency sampling schemes. Temporal relations among reproductive hormones during this period of the oestrous cycle are even less well understood. This prompted us to study the temporal relations among LH, FSH, oestradiol, progesterone, prolactin, and α-melanocyte-stimulating hormone (α-MSH), by means of high-frequency blood sampling during the follicular, ovulatory, and early luteal phase in the bitch (**Chapter 3**).

Kooistra et al. (1999) have shown that progression from early to late anoestrus is associated with an increase in basal plasma FSH concentration without a concomitant rise in basal plasma LH, which suggests that in the bitch an increase in circulating FSH is critical for initiation of ovarian folliculogenesis and thus for termination of anoestrus. The results of the study reported in **Chapter 3** do indeed show that plasma FSH concentration was relatively high at the beginning of the follicular phase and declined toward the preovulatory LH and FSH surges, which began concomitantly in all bitches studied. The low plasma FSH concentration during most of the follicular phase may be explained by negative feedback effects of oestradiol and inhibin, secreted by the growing follicles (Olson et al. 1982; McNeilly et al. 2003).

Plasma oestradiol concentration rose sharply in close relation to the onset of the preovulatory LH surge in most bitches, suggesting that the increase in plasma oestradiol acts via positive feedback on GnRH release, and hence LH secretion, as has been shown in most mammals during the late follicular phase (Liu and Yen 1983; Evans et al. 1997; Karsch et al. 1997). However, in reported studies in which oestradiol was administered to bitches in anoestrus or after ovariectomy, no LH surge could be triggered (Concannon et al. 1979; Klein et al. 2003). Increased sensitivity of the HPG axis to oestradiol only during pro-oestrus would explain the absence of a positive feedback effect of oestradiol during anoestrus and after ovariectomy. This is supported by increased expression of oestrogen receptor genes in

the mediobasal hypothalamus and the pituitary during the transition from anoestrus to pro-oestrus (Tani et al. 1997; Hatoya et al. 2003). Moreover, in many mammals there is evidence that during this restricted period oestradiol has a positive feedback on kiss1 neurons in the AVPV nucleus, which stimulates GnRH release and subsequently leads to the surges of LH and FSH.

The preovulatory increase in plasma progesterone was closely related to the start of the LH surge in five of the six bitches described in **Chapter 3**. The variable duration of the period in which plasma progesterone concentration was stable—following the initial preovulatory increase and before the second sharp increase, probably after ovulation—could reflect variation in the interval between the preovulatory LH surge and ovulation. This would suggest that measurement of the preovulatory LH surge is not reliable for determining the exact time of ovulation. Wildt et al. (1978) also found that in bitches the period in which ovulation occurs ranges from as little as 24 h to more than 96 h after the preovulatory LH surge. Furthermore, determining the optimal mating time by repeated measurement of plasma progesterone has been reported to be very reliable in the bitch, leading to pregnancy in more than 90% of bitches without fertility problems (Okkens et al. 1993; Okkens et al. 2001). This was also demonstrated in **Chapter 7**. All 12 bitches described in that study became pregnant after one mating based on progesterone measurements, with 6.4 ± 2.2 (mean ± SD) pups per litter. Plasma prolactin and α-MSH concentrations did not change significantly during the follicular and early luteal phases. It is therefore unlikely that during the canine follicular phase prolactin and α-MSH strongly influence the secretion of FSH and LH.

Although it has been reported that each FSH pulse occurs concomitantly with an LH pulse in all stages of the oestrous cycle and in anoestrus (Kooistra et al. 1999), the results of the study presented in **Chapter 4** suggest differential regulation of LH and FSH release in the bitch. Firstly, the periovulatory FSH surge was 3 times as long as the preovulatory LH surge. This can be partly explained by the longer plasma half-life of FSH than of LH (Schwartz 1995). An even more striking difference is that basal plasma FSH concentration was significantly higher after the FSH surge than before the FSH surge. In contrast, basal plasma LH concentration did not change. Furthermore, in none of the bitches did the preovulatory FSH surge have the bifurcated pattern that was present in the LH surge in four of the six bitches. Finally, in two bitches the FSH surge started 12 h earlier than the LH surge. GnRH is the main regulator of the secretion of FSH and LH by pituitary gonadotrophs (Meij et al. 2010). However, the intracellular mechanisms governing the storage and release of FSH and LH are different (Ascoli and Puett 2009) and the magnitude of FSH secretion in response to secretagogues is smaller than that of LH (Muyan et al. 1994). Furthermore, differential regulation of FSH and LH secretion can be explained by the frequency and amplitude of GnRH pulses (Vizcarra et al. 1997). In addition, a specific hypothalamic FSH-releasing factor (Yu et al. 1997; Padmanabhan et al. 2003) and gonadal feedback may play a role in the differential or non-parallel secretion of FSH and LH (Shupnik 1996).

The GnRH-stimulation tests performed in male and anoestrous female dogs of several breeds before and after gonadectomy are reported in **Chapters 5 and 6.** They provide reference values for the diagnosis of clinical conditions that require evaluation of the pituitary-

gonadal axis in dogs, such as the presence of remnant ovarian tissue and disorders of sexual development. In agreement with previous reports, gonadectomy and thus the loss of negative feedback of gonadal hormones on pituitary gonadotrophin release, resulted in significantly higher basal plasma concentrations of both FSH and LH in both male and female dogs (Olson et al. 1992; Reichler et al. 2004; Buijtels et al. 2006). As has been shown in other mammals, the negative feedback of gonadal steroid hormones in dogs is probably mediated via the kiss1/gpr-54 system in the arcuate nucleus in the hypothalamus. The kisspeptin neurons express the sex steroid receptors that the GnRH neurons lack (Oakley et al. 2009; Roseweir and Millar 2009; Tsutsui et al. 2010). As the kiss1/gpr-54 system is thought to be the main regulator of reproduction, future research could be aimed at manipulating its function in order to either stimulate or suppress reproduction in individual animals.

Although the pulsatile secretion of the gonadotrophins reduces the worth of hormone measurements in a single blood sample, basal plasma FSH concentration appeared to be a reliable means of verifying the neuter status in dogs, for we observed no overlap in the ranges of basal plasma FSH before and after gonadectomy in either gender. Basal plasma LH concentration was found less reliable for this purpose because the ranges in male dogs before and after castration did overlap substantially, in agreement with the results of Olson et al. (1992).

When plasma gonadotrophin concentrations in previously gonadectomized animals are used to determine the presence or absence of remnant gonadal tissue, the interval between removal of the gonads and blood sampling is a relevant factor to take into account, as was shown by Reichler et al. (2004). They demonstrated that the elevated basal plasma LH and FSH concentrations after ovariectomy slowly decrease, with a nadir at 10 weeks after ovariectomy. Subsequently, both plasma gonadotrophins rise again (Reichler et al. 2004). In **Chapters 5 and 6**, the interval between gonadectomy and the second GnRH-stimulation test in both males and females was approximately 4.5 months. In agreement with the observations of Reichler et al. (2004), the lowest basal plasma LH and FSH concentrations were observed in a neutered bitch which had undergone ovariectomy only 75 days before the second GnRH-stimulation test. These observations suggest that basal plasma FSH concentration may be a reliable indicator of neuter status if measured more than 6 months after gonadectomy.

Before gonadectomy administration of GnRH caused a greater increase in plasma FSH than in plasma LH in both males and females. After gonadectomy in both males and females, and after chemical castration in males, GnRH caused a significant increase in plasma LH but not in FSH. These findings can be interpreted as further evidence of the differential regulation of LH and FSH secretion by gonadotrophs. FSH is secreted mainly via a constitutive route and thus GnRH plays a smaller overall role in control of its secretion than it does for LH (Ascoli and Puett 2009; Hall 2009).

The basal plasma concentrations of LH and FSH were lower and higher, respectively, in intact anoestrous bitches than in intact male dogs. This may be ascribed to differences in circulating gonadal hormone concentration between anoestrous bitches and males. After gonadectomy, and thus without gonadally secreted hormones, differences in basal LH and FSH concentration between males and females were no longer significant. These findings

suggest a primary role for gonadally produced steroids and proteins in the differential regulation of gonadotrophin secretion. The relatively high LH:FSH ratio in gonadally intact male dogs may be explained by their relatively high plasma oestradiol concentration compared to that in anoestrous bitches and gonadectomized dogs. Via negative feedback, both oestradiol (by its effect on kiss1 neurons) and inhibin specifically suppress FSH synthesis and secretion (Shupnik 1996; Roseweir and Millar 2009). The relatively high plasma LH concentration in gonadally intact male dogs is more difficult to explain. Kumar et al. (1980) found that the hypothalamus contained significantly higher concentrations of GnRH in gonadally intact male dogs than in anoestrous bitches, potentially leading to higher basal plasma LH concentrations in intact male dogs. It may be hypothesized that the circulating gonadal hormones in male dogs result in pulsatile secretion of GnRH, mediated by the hypothalamic kiss1 neurons that preferentially stimulate LH secretion.

The highest plasma testosterone concentration after GnRH administration in gonadectomized male dogs was much lower than the lowest basal plasma testosterone concentration before gonadectomy. This strongly suggests that a single measurement of plasma testosterone reliably verifies neuter status in male dogs. After gonadectomy basal and GnRH-stimulated plasma testosterone concentrations were low, but often still detectable, indicating an extragonadal source of testosterone, most likely the reticular zone of the adrenal cortex.

The difference in basal plasma oestradiol concentration between anoestrous and gonadectomized bitches was not significant. Thus a single measurement of basal plasma oestradiol concentration cannot be used to differentiate between them, as has been reported previously (Frank et al. 2003). Instead, plasma oestradiol concentration measured at 120 min after GnRH administration did discriminate between them. The fact that the plasma oestradiol concentration in the ovariectomized bitches was not undetectable may be explained by conversion from androstenedione produced in the adrenal cortex. Oestradiol can also be produced by aromatization in fat cells, hair follicles, and the liver (Nelson and Bulun 2001).

The half-life of buserelin, the GnRH agonist used in the study reported in **Chapter 6**, is greater than that of GnRH, which may explain the longer increase in plasma LH than reported for GnRH by others (Karten and Rivier 1986; Knol et al. 1993; Padula 2005; Junaidi et al. 2007) and the results in anoestrous bitches presented in **Chapter 5**.

Chemical castration with implants containing 4.7 mg deslorelin resulted in secondary hypogonadism, clearly illustrated by the significant decrease in testis size 4-5 months after implantation (Trigg et al. 2001; Junaidi et al. 2007; Ludwig et al. 2009). The decrease in plasma LH and FSH after chemical castration is consistent with the proposed mechanism of action, which is downregulation of the GnRH receptors and desensitization of the pituitary gonadotrophs via the continuous elevation of plasma GnRH (Jones et al. 1976; Zilberstein et al. 1983; Hazum and Schvartz 1984; Vickery et al. 1984; Junaidi et al. 2007). After gonadectomy, GnRH secretion also increases but the gonadotrophs are not desensitized (Kittok et al. 1984). This may be ascribed to the still pulsatile secretion of GnRH, suggested by the pulsatile secretion of the gonadotrophins (Concannon 1993). Thus it is not an increase in basal plasma GnRH or GnRH agonist but probably the loss of pulsatile secretion after

administration of a slow-release GnRH agonist that desensitises gonadotrophic cells. This emphasizes the importance of the pulsatile nature of GnRH secretion in the regulation of gonadotrophin secretion.

Despite significantly decreased plasma gonadotrophin levels and decreased testis size, both indicating desensitization of the pituitary by the continuously high deslorelin concentration, plasma LH increased significantly after GnRH administration. This indicates that the pituitary gonadotrophs were not completely desensitized in all dogs at 4.5 months after administration of the deslorelin implant. In a study by Junaidi et al. (2007) there was no increase in plasma LH after intravenous injection of gonadorelin 100 days after administration of a deslorelin implant. This may be explained by several differences between their study and the study described in **Chapter 6**. In the latter a more potent GnRH agonist was used, the body weight of the dogs was higher and thus the dose of deslorelin per Kg body weight was lower, and the GnRH-stimulation test was performed at a longer interval after administration of the deslorelin implant than in the study of Junaidi et al. (2007).

Basal plasma oestradiol and testosterone concentrations were decreased to similar levels by surgical and chemical castration. Neither hormone was then increased significantly by administration of GnRH. Despite the absence of a GnRH-induced increase in plasma testosterone in chemically castrated dogs, GnRH-stimulated plasma testosterone levels were higher in chemically castrated than in surgically castrated dogs, which is another indication that, at the time of the GnRH-stimulation test, the pituitary gonadotrophs were not completely desensitized in all dogs. Evaluation of clinical end-points, such as behavioural changes resulting from treatment with deslorelin implants, needs to be assessed to determine the clinical relevance of this finding.

The results of the study reported in **Chapter 7** demonstrate that the progesterone-receptor blocker aglepristone is an efficient and safe drug for the induction of parturition in the dog. In the dog, corpora lutea are the sole source of progesterone, which is obligatory for maintaining pregnancy (Sokolowski 1971). This led us to evaluate a protocol in which only aglepristone, a competitive antagonist of the progesterone receptor, was used to induce parturition. The results were compared with those in spontaneous whelping. The effectiveness of aglepristone to induce parturition was demonstrated by the expulsion of puppies despite high plasma concentrations of progesterone, much higher than that considered to be necessary for maintaining pregnancy (approximately 6.4 nmol/l) (Concannon et al. 1977).

Aglepristone was very efficient in inducing parturition. Gestation was significantly shorter in treated bitches than in those whelping spontaneously. In addition, parturition occurred within a relatively short and predictable interval, averaging 41 h (range 32–56 h) after the first dose of aglepristone. There were no side-effects except local inflammation at the injection site, as reported previously (Galac et al. 2004). The progress of whelping and the survival of pups were not significantly different between the two groups. However, veterinary supervision of induced whelping is warranted, for the number of manipulations (vaginal exploration and oxytocin administration) during parturition was twice as high in the induced group, albeit with a wide range.

Prolonged pregnancy occurs most often when there are only one or two pups, with a risk of foetal death before the onset of parturition due to placental insufficiency. At present, a Caesarean section is the sole effective treatment for prolonged gestation and induction of parturition would be a practical alternative. However, using the protocol reported in **Chapter 7,** several one- and two-pup pregnancies in our clinic progressed only to cervical dilatation, not to complete spontaneous parturition (unpublished observations). The major differences between these clinical cases and the studied population reported in **Chapter 7** are (1) litter size, being one or two pups and six to nine pups, respectively, and (2) the day of pregnancy at the time of aglepristone administration, D58 and D63-65, respectively. In cases of prolonged pregnancy with one or two pups a modification of the protocol described by Fieni et al. (2001) may induce normal parturition, but this is not yet certain. In these cases aglepristone may be administered, preferably not later then day 64 of pregnancy, followed by a low dose of oxytocin 24 h later and then every 1 h until expulsion of the last pup. However, more research is necessary to determine the aetiology of prolonged pregnancy in one- and two-pup pregnancies and the potential use of a progesterone-receptor blocker for the induction of parturition followed by low doses of oxytocin in such cases.

With regard to the hormonal changes in spontaneous and aglepristone-induced parturition in dogs, the study reported in **Chapter 8** demonstrates that parturition in the induced group occurred while luteolysis was still incomplete. The plasma concentration of 15-ketodihydroprostaglandin-$F_{2\alpha}$ (PGFM), a metabolite of $PGF_{2\alpha}$, began to increase before parturition, but the peak concentration was lower than in the spontaneously whelping bitches. Apart from the still high plasma progesterone concentration in the induced group during parturition, the declining plasma PGFM concentration paralleled the postpartum decrease in plasma progesterone, indicating that luteolysis was only completed after parturition. Plasma PGFM concentrations in the induced group were still significantly higher on the 2nd and 3rd days after parturition than in the spontaneously whelping group. Maximum plasma aglepristone levels are only reached after approximately 2.5 days. The result may be that initially the progesterone receptors are only partially blocked and circulating progesterone around the time of parturition can still partially exert its activity at the receptor level, which may have resulted in suppression of $PGF_{2\alpha}$ secretion.

A significant decrease in plasma oestradiol concentration was observed prior to parturition in both groups, as reported previously (Edqvist et al. 1975; Van der Weyden et al. 1989; Onclin et al. 2002). Apparently, aglepristone treatment does not affect the plasma level of this hormone around the time of parturition. The physiological significance of the declining oestradiol concentration before parturition in the dog is not known. It also remains to be determined whether the antepartum decrease in gonadotrophic hormone concentration is associated with the antepartum decrease in oestradiol

Plasma cortisol concentration increased significantly before parturition in both groups, as has been shown previously (Veronesi et al. 2002). After parturition, plasma cortisol concentration was significantly higher in the induced group than in the spontaneously whelping group, possibly due to partial blocking of glucocorticoid receptors by aglepristone. Blocking of pituitary glucocorticoid receptors may have resulted in increased ACTH release

and, subsequently, increased cortisol secretion (Spitz et al. 1985; Wade et al. 1988). However, the blood sampling frequency used in this study does not allow conclusive statements about hormones with highly fluctuating plasma concentrations, such as cortisol and ACTH. Elevated postpartum plasma cortisol concentrations in the induced group may have been caused by the sustained postpartum PGF2α production, as reflected in the elevated plasma PGFM concentrations. However, there was no clinical evidence of excessive stress in the induced bitches, and postpartum behaviour, the number of postnatal deaths, and the increase in body weight of the puppies were similar to those in the spontaneously whelping dogs (**Chapter 7**).

In the spontaneously whelping group, there was a significant increase in plasma prolactin concentration before parturition, reflecting the modulating effect of progesterone on prolactin secretion (Galac et al. 2000; Kooistra and Okkens 2002). In the induced group, plasma prolactin concentration increased only slightly before parturition, possibly because the decrease in plasma progesterone was less abrupt. Prolactin plays an important role in mammogenesis and lactogenesis (Brisken et al. 1999). Since the puppies grew steadily **(Chapter 7)**, aglepristone does not appear to have had a serious effect on mammary function around the time of parturition, which is important for pup survival and growth.

The study reported in **Chapter 9** demonstrates that gonadal and adrenal steroid synthesis is decreased by the 3β-hydroxysteroid dehydrogenase (3β-HSD) inhibitor trilostane during both the pituitary-dependent and the pituitary-independent parts of the luteal phase in Beagle bitches. Although trilostane induced a rapid and significant decrease in plasma progesterone concentration in both treatment groups, the resulting values during treatment were well in excess of those considered to be essential for maintaining pregnancy, i.e., 4 to 8 nmol/L (Concannon and Hansel 1977; Onclin et al. 1993). Increasing the dose of trilostane is probably not a good option, for we found that plasma cortisol concentration after administration of 0.25 µg ACTH was < 40 nmol/L in two of eleven bitches, which is below what is considered to be safe in dogs (Galac et al. 2010).

Epostane, another 3β-HSD inhibitor, prevented pregnancy and decreased plasma progesterone to values (4 to 22 nmol/L) lower than in the study reported in **Chapter 9** (~ 50 nmol/L) (Keister et al. 1989). Furthermore, the dose range found to be effective to induce abortion (2.5 to 5 mg/kg bw sid) was lower than the total daily dose used in the present study (6.6 to 10.5 mg/kg bw). A possible explanation could be that epostane preferentially blocks ovarian and placental steroidogenesis in both humans and animals (Potts et al. 1978; Schane et al. 1979; Creange et al. 1981; Birgerson et al. 1986; Birgerson et al. 1987). These observations presumably make trilostane unsuitable for termination of pregnancy in the dose used in this study.

After the administration of trilostane in the pituitary-dependent part of the luteal phase was stopped, plasma progesterone did not rise to the level observed in the control group at a similar interval after ovulation. However, full restoration of progesterone secretion did occur following treatment during the pituitary-independent part of the luteal phase, in agreement with previous studies using hypophysectomy or progesterone receptor antagonists (Okkens et al. 1986; Galac et al. 2000; Galac et al. 2004). The treatment-induced decline in plasma progesterone concentration was not followed by a significant increase in plasma prolactin

during both phases, as had been expected on the basis of studies of inhibiting progesterone influence (Galac et al. 2000; Lee et al. 2006). Thus more frequent administration of trilostane might have been more effective, as the intervals between doses may have been too great to achieve continuous suppression of progesterone synthesis.

In bitches treated during the pituitary-dependent part of the luteal phase (66 days) the luteal phase was shorter than in control bitches (99 days) and in bitches treated during the pituitary-independent part of the luteal phase (99 days). Hence administration of trilostane during the pituitary-dependent part of the luteal phase may eventually cause abortion or premature parturition at a later stage of pregnancy.

The lack of recovery of progesterone secretion after treatment with trilostane during PDP may suggest a role for progesterone in support of its own secretion during this phase, as has been hypothesized in the dog and other species (Rothchild 1981; Kowalewski et al. 2009).

Conclusions based on this thesis:

- The plasma concentrations of LH and FSH are differentially regulated in dogs, as evidenced by the following:
    - The preovulatory FSH surge does not is not always start simultaneous with the LH surge, but sometimes several hours prior to it.
    - The FSH surge lasts about 110 h, while the LH surge lasts about 36 h and is often bifurcated .
    - After the LH surge, the mean plasma LH concentration returns to the previous level, while after the FSH surge plasma FSH concentration remains higher than before to the surge.
- Oestradiol-17β probably exerts positive feedback on preovulatory LH release in the bitch, as in other species.
- The onset of the preovulatory LH surge is associated with an increase in plasma progesterone.
- Plasma progesterone concentration rises sharply at a variable interval after the LH surge. This sharp rise probably indicates ovulation and thus our findings suggest that measurement of the preovulatory LH surge is an unreliable means of determining the exact time of ovulation.
- No preovulatory surge in plasma prolactin or α-MSH was observed in the bitch.
- Gonadectomy results in increased plasma concentrations of LH and FSH.
- Chemical castration of male dogs by the continuous release of the GnRH agonist deslorelin results in decreased plasma concentrations of LH and FSH and, as a consequence, it lowers plasma oestradiol and testosterone concentrations to values similar to those after surgical castration.

- The ranges of plasma oestradiol concentration in anoestrous and ovariectomized bitches overlap.
- The basal plasma FSH concentration appears to be an appropriate criterion for the presence or absence of functional gonadal tissue in male and female dogs.
- The basal plasma testosterone concentration appears to be an appropriate criterion for the presence or absence of functional gonadal tissue in male dogs.
- GnRH administration in intact male and female dogs induces increased plasma concentrations of LH, FSH, oestradiol, and testosterone.
- The plasma oestradiol concentration 120 min after GnRH administration appears to be an appropriate criterion for the presence or absence of functional gonadal tissue in bitches.
- Administering the GnRH analogue buserelin to chemically castrated male dogs 4.5 months after a deslorelin implant caused a significant increase in plasma LH, indicating that the pituitary gonadotrophs were not completely desensitized in all dogs.
- Aglepristone is an effective and safe drug for the induction of parturition in dogs that are 58 days pregnant and carry 3 or more pups.
- Aglepristone-induced parturition is associated with still incomplete luteolysis, an altered plasma PGFM profile, and elevation of plasma cortisol .
- Trilostane, in an oral dose of ~ 4.5 mg/kg bw bid, decreased plasma progesterone during the luteal phase, but the treatment regimen used in this study resulted in less inhibition of ovarian steroidogenesis than is probably needed for abortion.

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11

Samenvattende discussie en conclusies

De vorming van rijpe eicellen tijdens iedere oestrische cyclus, dracht en spermatogenese, dat in tegenstelling tot de cyclus bij het vrouwelijk dier een continu proces is, zijn afhankelijk van hormonale regulatie. Zowel de oestrische cyclus als spermatogenese staan onder invloed van een geïntegreerd regelsysteem: de hypothalamus-hypofyse-gonade as (HHG-as). Bij een groot aantal diersoorten is aangetoond dat in de hypothalamus het kiss1/gpr-54 systeem de afgifte van “gonadotrophin releasing hormone” (GnRH) stimuleert, dat op zijn beurt zorgt voor de afgifte van follikel-stimulerend hormoon (FSH) en luteïniserend hormoon (LH). Beide worden pulsatiel afgegeven door de gonadotrofe cellen in de voorkwab van de hypofyse. Stimulatie met deze gonadotrofe hormonen van de granulosa, theca en luteale cellen in het ovarium (de eierstok) leidt tot afgifte van oestradiol, testosteron, progesteron en inhibine, terwijl dit in de testis de Leydig- en Sertolicellen stimuleert tot de afgifte van oestradiol, testosteron en inhibine. Deze van de gonaden afkomstige hormonen koppelen op hun beurt terug op de hogere niveau’s van de HHG-as. De door de teef uitgescheiden hormonen worden beïnvloed door cyclische veranderingen in het ovarium waarin zich afhankelijk van de fase diverse ontwikkelingsstadia van de follikels of gele lichaampjes, corpora lutea, bevinden. Vele externe factoren, bijvoorbeeld vanuit het milieu, en interne factoren, bijvoorbeeld de hypothalamus-hypofyse-bijnier of -schildklier as, kunnen de HHG-as beïnvloeden.

Voor een overzicht van de anatomie en de functionele aspecten van de HHG-as bij teven en reuen wordt de lezer verwezen naar **Hoofdstuk 2** van dit proefschrift. In dit hoofdstuk ligt de nadruk op veranderingen tijdens de verschillende fasen van de oestrische cyclus. Daarnaast wordt in **Hoofdstuk 2** een overzicht gegeven van de huidige chirurgische en medicamenteuze interventies in de HHG-as.

De beschikbare informatie over de veranderingen in plasmaconcentraties van de voortplantingshormonen tijdens de folliculaire fase, ovulatie en vroeg luteale fase is vooral gebaseerd op onderzoek waarin met een lage frequentie bloedmonsters werden afgenomen. Informatie over de samenhang van de afgifte van de voortplantingshormonen was nog schaarser. Daarom zijn tijdens de folliculaire, ovulatie en vroeg-luteale fase bij de teef frequent bloedmonsters afgenomen om de onderlinge relatie van LH, FSH, oestradiol, progesteron, prolactine en α-melanocyt-stimulerend hormoon (α-MSH) te bestuderen (**Hoofdstuk 3**).

Kooistra et al. (1999) hebben laten zien dat de basale plasma-FSH-concentraties toenemen bij het voortschrijden van de vroege naar de late anoestrus zonder dat een gelijktijdige stijging van de basale plasma-LH-concentraties werd waargenomen. Dit suggereert dat een toename in circulerend FSH essentieel is voor de start van de ovariële folliculogenese en dus voor het beëindigen van de anoestrus. De resultaten van de studie beschreven in **Hoofdstuk 3** laten inderdaad zien dat de plasma-FSH-concentraties relatief hoog waren aan het begin van de folliculaire fase, waarna er al snel een daling plaatsvond. De plasma-FSH-concentraties bleken laag te zijn in de vorderende folliculaire fase totdat ze gelijktijdig met de plasma-LH-concentraties stegen, leidend tot de pre-ovulatoire LH en FSH pulsen. De lage plasma-FSH-concentratie tijdens het grootste deel van de folliculaire fase kan worden verklaard door negatieve terugkoppeling van oestradiol en inhibine, die worden uitgescheiden door de groeiende follikels (Olson et al. 1982; McNeilly et al. 2003).

Kort voor de start van de pre-ovulatoire LH-puls stegen de plasma-oestradiolconcentraties bij de meeste teven sterk. Dit suggereert dat de toename van de plasma-oestradiolconcentratie een positieve terugkoppeling geeft op de afgifte van GnRH en daarmee ook op de afgifte van LH, zoals werd aangetoond bij de meeste zoogdiersoorten tijdens de laat folliculaire fase (Liu en Yen 1983; Evans et al. 1997; Karsch et al. 1997). Eerdere studies bij de teef toonden echter aan dat toediening van oestradiol tijdens anoestrus of na ovariëctomie geen LH-puls tot gevolg heeft (Concannon et al. 1979; Klein et al. 2003). Het slechts gevoelig zijn voor positieve terugkoppeling van de HHG-as voor oestradiol tijdens de folliculaire fase zou de afwezigheid van een positief terugkoppelingseffect van oestradiol tijdens de anoestrus en na ovariëctomie kunnen verklaren. Deze hypothese wordt ondersteund door de bevinding dat de expressie van de oestrogeen receptor genen in de mediobasale hypothalamus en de hypofyse verhoogd is tijdens de overgang van anoestrus naar de folliculaire fase (Tani et al. 1997; Hatoya et al. 2003). Daarnaast is er bij vele zoogdiersoorten aangetoond dat oestradiol een positief terugkoppelingseffect heeft op de kiss1 neuronen in de AVPV-kern van de hypothalamus, die de afgifte van GnRH bevorderen wat vervolgens leidt tot pre-ovulatoire LH en FSH pulsen tijdens deze periode van de oestrische cyclus.

De pre-ovulatoire stijging van de plasmaprogesteronconcentratie was nauw gerelateerd aan de start van de LH-puls bij 5 van de 6 teven in de studie beschreven in **Hoofdstuk 3**. Na de initiële stijging van de plasmaprogesteronconcentratie bleef deze enige tijd gelijk. Na deze periode van stabilisatie, waarvan de duur nogal varieerde van hond tot hond, steeg de plasmaprogesteronconcentratie scherp door, waarschijnlijk als gevolg van de ovulatie. De variatie in duur van deze periode waarin stabiele plasmaprogesteronconcentraties gezien werden weerspiegelt waarschijnlijk het variabele interval tussen de pre-ovulatoire LH-puls en de ovulatie. Dit suggereert dat bepaling van de pre-ovulatoire LH-puls niet betrouwbaar is voor de exacte bepaling van het optreden van de ovulatie. Wildt et al. (1978) hebben inderdaad aangetoond dat de periode waarin de ovulatie plaatsvindt varieert van 24 tot meer dan 96 uur na de pre-ovulatoire LH-puls. Daarnaast werd aangetoond dat het bepalen van het optimale dektijdstip met behulp van het herhaald meten van de plasmaprogesteronconcentratie bij de teef een betrouwbare methode is met een kans op dracht van meer dan 90% bij teven zonder voorgeschiedenis van fertiliteitproblemen (Okkens et al. 1993; Okkens et al. 2001). De resultaten beschreven in **Hoofdstuk 7** sluiten hierbij aan. Alle 12 teven in die studie werden drachtig na één dekking gebaseerd op het herhaald meten van de plasmaprogesteronconcentratie, waarbij de gemiddelde worpgrootte 6,4 ± 2,2 (gemiddelde ± SD) pups was. De plasmaconcentraties van prolactine en α-MSH vertoonden geen significante veranderingen tijdens de folliculaire en vroeg-luteale fase. Het is daarom onwaarschijnlijk dat prolactine en α-MSH de afgifte van FSH en LH sterk beïnvloeden tijdens de folliculaire fase bij de hond.

Iedere FSH-puls wordt vergezeld van een LH-puls in alle stadia van de oestrische cyclus en anoestrus (Kooistra et al. 1999). Desondanks suggereren de bevindingen beschreven in **Hoofdstuk 4** ook differentiële regulatie van de LH en FSH afgifte. Ten eerste duurde de peri-ovulatoire FSH-puls drie maal zo lang als de pre-ovulatoire LH-puls. Dit kan deels worden verklaard uit de langere plasmahalfwaardetijd van FSH (Schwartz 1995). De gemiddelde plasma-FSH-concentratie na de FSH-puls bleek echter significant hoger te zijn dan voor de

FSH-puls, terwijl de gemiddelde plasma-LH-concentratie voor en na de LH-puls gelijk was. Verder bleek alleen de plasma-LH-concentratie en niet de plasma-FSH-concentratie tijdelijk te dalen bij 4 van de 6 teven (gevorkt patroon). Vervolgens startte de peri-ovulatoire FSH-puls 12 uur eerder dan de pre-ovulatoire LH-puls bij twee van de zes teven. De afgifte van FSH en LH door de gonadotrofe cellen in de hypofyse wordt voornamelijk gereguleerd door GnRH. De intracellulaire mechanismen die de opslag en afgifte van FSH en LH reguleren zijn evenwel verschillend (Ascoli en Puett 2009) en de hoeveelheid afgegeven FSH in reactie op stimulatie met GnRH is kleiner dan die van LH (Muyan et al. 1994). Een aanvullend mechanisme voor de differentiële regulatie van FSH en LH is een verschil in reactie op veranderingen van de frequentie en amplitude van de GnRH pulsen (Vizcarra et al. 1997). Daarnaast spelen terugkoppeling van hormonen vanuit de gonaden en een specifieke FSH-stimulerende factor (Yu et al. 1997; Padmanabhan et al. 2003) waarschijnlijk een rol bij de niet-identieke afgiftepatronen van FSH en LH (Shupnik 1996).

In de **Hoofdstukken 5 en 6** worden de resultaten beschreven van de GnRH-stimulatietesten die uitgevoerd werden voor en na gonadectomie bij reuen en anoestrische teven van verschillende rassen. Deze bevindingen kunnen worden gebruikt als referentiewaarden voor de diagnostiek van aandoeningen waarbij de HHG-as wordt beïnvloed, zoals de aanwezigheid van resterend ovarieel weefsel na ovariëctomie en afwijkingen van de geslachtelijke ontwikkeling. Gonadectomie en daarmee dus het verlies van de negatieve terugkoppeling van de door de gonaden afgegeven geslachtshormonen, resulteerde in een significante stijging van de basale plasmaconcentraties van FSH en LH bij zowel reuen als teven, zoals eerder werd beschreven (Olson et al. 1992; Reichler et al. 2004; Buijtels et al. 2006). De negatieve terugkoppeling door geslachtssteroïdhormonen vindt waarschijnlijk plaats op het niveau van het kiss1/gpr-54 systeem in de hypothalame nucleus arcuatus, zoals werd aangetoond bij andere zoogdierspecies. De kisspeptinneuronen brengen geslachtssteroïdreceptoren tot expressie die ontbreken in de GnRH-neuronen (Oakley et al. 2009; Roseweir en Millar 2009; Tsutsui et al. 2010). Omdat het kiss1/gpr-54 systeem wordt beschouwd als de belangrijkste schakel in de regulatie van de voortplanting zou toekomstig onderzoek naar methoden om de voortplanting te stimuleren of remmen gericht kunnen zijn op het manipuleren van dit systeem.

Het pulsatiele afgiftepatroon van de gonadotrofe hormonen beperkt de waarde van hormoonmetingen in enkelvoudige bloedmonsters. Desondanks bleek de basale plasma-FSH-concentratie toch een betrouwbare methode om de aan- of afwezigheid van ovarieel en testiculair weefsel aan te tonen omdat er bij beide geslachten geen overlap was tussen de plasma-FSH-concentraties voor en na gonadectomie. De basale plasma-LH-concentratie bleek minder betrouwbaar te zijn voor dit doel omdat, overeenkomstig de bevindingen van Olson et al. (1992), bij reuen de plasma-LH-concentraties voor en na castratie wel duidelijk overlap vertoonden.

Het interval tussen gonadectomie en bloedafname blijkt, zoals ook werd aangetoond door Reichler et al. (2004), een relevante factor te zijn als de plasmaconcentraties van de gonadotrofe hormonen worden gebruikt om de aan- of afwezigheid van ovarieel of testiculair weefsel aan te tonen. Reichler et al. (2004) lieten zien dat plasma FSH en LH concentraties, die kort na ovariëctomie stijgen, vervolgens langzaam dalen waarbij de laagste concentratie

op 10 weken na ovariëctomie werd bereikt, waarna de plasmaconcentraties van beide gonadotrofe hormonen weer stijgen. In de **Hoofdstukken 5 en 6** was het interval tussen gonadectomie en de tweede GnRH-stimulatietest bij zowel reuen als teven ongeveer 4,5 maanden. Overeenkomstig de bevindingen van Reichler et al. (2004), werden de laagste basale plasmaconcentraties van LH en FSH gezien bij een teef waarbij de ovaria slechts 75 dagen voor de tweede GnRH stimulatietest waren verwijderd. De basale plasma-FSH-concentratie is daarom waarschijnlijk een uiterst betrouwbare methode om de aan- of afwezigheid van ovarieel of testiculair weefsel aan te tonen wanneer het interval tussen gonadectomie en meting minimaal 6 maanden is.

Voor gonadectomie leidde de toediening van GnRH bij zowel reuen als teven tot een sterkere stijging van de plasma-FSH-concentratie dan van de plasma-LH-concentratie. Na gonadectomie leidde de toediening van GnRH bij zowel reuen als teven en na chemische castratie bij reuen, wel tot een significante stijging van LH, maar niet tot een stijging van FSH. Ook bij andere diersoorten is gevonden dat GnRH een minder grote rol speelt in de afgifte van FSH dan van LH (Ascoli en Puett 2009; Hall 2009). Deze bevindingen kunnen worden geïnterpreteerd als aanvullend bewijs voor de differentiële regulatie van de afgifte van LH en FSH.

De basale plasmaconcentraties van LH en FSH waren respectievelijk lager en hoger in intacte anoestrische teven dan in intacte reuen. Dit kan mogelijk worden toegeschreven aan verschillende plasmaconcentraties van de door de gonaden afgegeven hormonen bij anoestrische teven en reuen. Na gonadectomie, en dus na het wegvallen van de door de gonaden afgegeven hormonen, waren de verschillen tussen reuen en teven niet langer significant. Deze bevindingen duiden op een belangrijke rol van de door de gonaden afgegeven steroïd- en eiwithormonen in de differentiële regulatie van de afgifte van de gonadotrofe hormonen. De relatief hoge LH:FSH ratio bij intacte reuen kan mogelijk worden verklaard door hun relatief hoge plasma-oestradiolconcentratie in vergelijking met anoestrische teven en honden na gonadectomie. Zowel oestradiol (via een effect op de kiss1 neuronen) als inhibine, remmen de FSH-productie en -afgifte via negatieve terugkoppeling (Shupnik 1996; Roseweir en Millar 2009). Het is moeilijker om de relatief hoge plasma-LH-concentratie van de intacte reuen te verklaren. Kumar et al. (1980) toonden aan dat de hypothalamus significant hogere concentraties GnRH bevat bij intacte reuen vergeleken met anoestrische teven, wat mogelijk de oorzaak is van de hogere basale plasma-LH-concentraties bij intacte reuen. Het is waarschijnlijk ook zo dat de door de gonaden afgegeven hormonen via de hypothalame kiss1-neuronen bij reuen een pulsatiel afgiftepatroon van GnRH induceren dat specifiek de afgifte van LH stimuleert.

De hoogst gemeten plasmatestosteronconcentratie na toediening van GnRH bij gecastreerde reuen was veel lager dan de laagst gemeten basale plasmatestosteronconcentratie bij intacte reuen. Derhalve is de bepaling van de plasmatestosteronconcentratie in een enkelvoudig bloedmonster waarschijnlijk een betrouwbare methode om de aan- of afwezigheid van testiculair weefsel aan te tonen. Na gonadectomie waren de basale plasmatestosteronconcentraties en die na toediening van GnRH laag, maar in de meeste gevallen wel meetbaar. Dit wijst op een bron van testosteronproductie buiten de gonaden, waarschijnlijk de zona reticularis van de bijnierschors.

Het verschil in de basale plasma-oestradiolconcentratie tussen anoestrische teven en teven na ovariëctomie was niet significant. Meting van de basale-oestradiol-concentratie in een enkelvoudig bloedmonster kan daarom niet worden gebruikt om te differentiëren tussen anoestrische teven en teven na ovariëctomie, wat overeenkomt met eerdere waarnemingen (Frank et al. 2003). De plasma-oestradiolconcentratie die 120 minuten na de toediening van GnRH gemeten wordt vertoont daarentegen geen overlap tussen beide categoriën teven. Het feit dat de plasma-oestradiolconcentratie bij teven na ovariëctomie niet onmeetbaar laag was kan mogelijk worden verklaard door de omzetting van androsteendion dat wordt geproduceerd in de bijnierschors. Daarnaast kan oestradiol ook worden gevormd door aromatisering in onder andere vetcellen, haarfollikels en de lever (Nelson en Bulun 2001).

In de studie beschreven in **Hoofdstuk 6** werd de GnRH-agonist busereline gebruikt dat een langere plasmahalfwaardetijd heeft dan GnRH. Dit verklaart mogelijk de langduriger toename van de plasma-LH-concentratie in deze studie in vergelijking met de resultaten van anderen (Karten en Rivier 1986; Knol et al. 1993; Padula 2005; Junaidi et al. 2007) en de resultaten bij anoestrische teven beschreven in **Hoofdstuk 5**.

Chemische castratie met implantaten die 4,7 mg deslorelin bevatten induceren secundair hypogonadisme, wat duidelijk wordt geillustreerd door de significante afname van de testisgrootte na toediening van het implantaat (Trigg et al. 2001; Junaidi et al. 2007; Ludwig et al. 2009). De gevonden daling van de plasmaconcentraties van LH en FSH passen bij het vermeende werkingsmechanisme van deslorelin-implantaten. Dit werkingsmechanisme berust op het ongevoelig worden of de desensitisatie van de GnRH-receptoren van de hypofysaire gonadotrofe cellen als gevolg van de continu verhoogde plasma-GnRH-concentratie (Jones et al. 1976; Zilberstein et al. 1983; Hazum en Schvartz 1984; Vickery et al. 1984; Junaidi et al. 2007). De afgifte van GnRH neemt weliswaar ook toe na gonadectomie, maar de gonadotrofe cellen raken dan niet gedesensitiseerd (Kittok et al. 1984). Dat er na gonadectomie geen desensitisatie optreedt zou kunnen worden verklaard doordat het pulsatiele afgiftepatroon van GnRH gehandhaafd blijft, zoals wordt gesuggereerd door de pulsatiele afgifte van de gonadotrofe hormonen (Concannon 1993). De desensitisatie van de gonadotrofe cellen na toepassing van een slow-release GnRH-agonist wordt dus niet veroorzaakt door een toegenomen plasmaconcentratie van GnRH of een GnRH-agonist, maar waarschijnlijk door het verlies van het pulsatiele karakter van de GnRH-afgifte. Dit benadrukt het belang van het pulsatiele karakter van de GnRH-afgifte in de regulatie van de afgifte van de hypofysaire gonadotrofe hormonen.

Ondanks de significante afname van de plasma-gonadotrofe-hormoonconcentraties en de testisgrootte, beide dus waarschijnlijk een gevolg van hypofysaire desensitisatie als gevolg van de continu hoge GnRH-concentratie, steeg de plasma-LH-concentratie significant na GnRH-toediening. Dit toont aan dat de hypofysaire gonadotrofe cellen niet compleet waren gedesensitiseerd bij alle honden op 4-5 maanden na toediening van het deslorelin-implantaat. Junaidi et al. (2007) zagen echter geen toename van de plasma-LH-concentratie na intraveneuze toediening van GnRH op 100 dagen na de toediening van een deslorelin-implantaat. Er zijn meerdere verschillen tussen hun studie en de studie beschreven in **Hoofdstuk 6** die dit zouden kunnen verklaren. In onze studie werd een potenter GnRH-

agonist gebruik, het lichaamsgewicht van de honden was groter met als gevolg een lagere dosis deslorelin per kg lichaamsgewicht (LG) en het interval tussen deslorelin-implantatie en GnRH-stimulatietest was langer dan in de studie van Junaidi et al. (2007).

Na chirurgische en chemische castratie namen de basale plasmaconcentraties van oestradiol en testosteron af tot vergelijkbare lage niveau's. Geen van beide hormonen nam vervolgens significant toe na toediening van GnRH. Ondanks de afwezigheid van een GnRH-geïnduceerde stijging van testosteron in chemisch gecastreerde reuen, waren de GnRH-gestimuleerde plasmatestosteronconcentraties bij deze reuen hoger dan bij chirurgisch gecastreerde reuen, wat een extra aanwijzing is dat, op het moment van de GnRH-stimulatietest, de hypofysaire gonadotrofe cellen niet in alle reuen compleet waren gedesensitiseerd. De klinische effecten van de behandeling met deslorelin implantaten, zoals gedragsveranderingen, moeten worden onderzocht om de klinische relevantie van deze laatste bevinding vast te stellen.

De resultaten van de studie beschreven in **Hoofdstuk 7** tonen dat met de progesteron-receptor-antagonist aglépristone de geboorte bij de hond efficiënt en veilig kan worden geïnduceerd. Bij de hond zijn de corpora lutea, de enige bron van progesteron, noodzakelijk voor het in stand houden van de dracht (Sokolowski 1971). Dit bracht ons ertoe om een protocol te onderzoeken waarmee de geboorte kan worden geïnduceerd door gebruik te maken van de competitieve progesteron-receptor-antagonist aglépristone. De resultaten werden vergeleken met die van spontaan optredende geboortes. De werkzaamheid van aglépristone om de geboorte te induceren werd aangetoond door de uitdrijving van pups ondanks plasmaprogesteronconcentraties die hoger waren dan wat wordt gezien als de grenswaarde waaronder de geboorte op gang kan komen (ongeveer 6,4 nmol/L) (Concannon et al. 1977).

Aglépristone induceerde de geboorte na een drachtduur die significant korter was dan bij de niet behandelde teven waarbij de geboorte spontaan op gang kwam. Daarbij vond de geboorte na toediening van de eerste dosis aglépristone plaats na een kort en voorspelbaar interval van gemiddeld 41 uur (32-56 uur). Er werden geen bijwerkingen gezien, behalve een lokale ontsteking bij enkele teven ter hoogte van de injectieplaats zoals eerder werd beschreven na toediening van aglépristone (Galac et al. 2004). Het verloop van de geboortes en de overleving van de pups waren niet significant verschillend tussen beide groepen. Veterinair toezicht van geïnduceerde geboortes wordt echter geadviseerd omdat het aantal manipulaties tijdens de partus, in de vorm van vaginaal toucher en toediening van oxytocine, twee keer zo hoog was bij de behandelde teven vergeleken met de controle teven. Verlengde dracht wordt het vaakst gezien bij een- en tweelingdrachten, met een risico op foetale sterfte als gevolg van placenta-insufficientie vóór de start van de partus. Op dit moment is de keizersnede de enige effectieve behandeling bij verlengde dracht. Het medicamenteus opwekken van de geboorte zou dus een praktisch alternatief kunnen zijn. De geboortes die geïnduceerd werden bij verschillende één- en tweelingdrachten met het protocol beschreven in **Hoofdstuk 7** stagneerden echter alle tijdens de ontsluitingsfase (niet gepubliceerde observaties). De belangrijkste verschillen tussen deze klinische casus en de populatie beschreven in **Hoofdstuk 7** zijn (1) worpgrootte, één of twee, respectievelijk zes tot negen pups en (2) het moment van toediening van de

aglépristone, dag 58, respectievelijk dag 63-65 na de dekking. In geval van verlengde één- en tweelingdrachten zou een modificatie van het protocol, zoals beschreven door Fieni et al. (2001), mogelijk een normale geboorte kunnen induceren. In deze gevallen wordt aglépristone niet later dan op dag 64 na dekking toegediend, gevolgd door oxytocine in lage doses om het uur startend op 24 uur na de eerste aglépristone toediening en voortgezet tot de geboorte van de laatste pup. Meer onderzoek naar de effecten van dit protocol is echter nodig, als ook onderzoek naar de exacte etiologie van verlengde dracht bij één- en tweelingdrachten bij de teef.

De studie beschreven in **Hoofdstuk 8** toont dat de geboorte bij de geïnduceerde honden plaatsvond terwijl de luteolyse nog niet compleet was. De plasmaconcentratie van 15-ketodihydroprostaglandine-$F_{2\alpha}$ (PGFM), een metaboliet van $PGF_{2\alpha}$, steeg voor de geboorte, maar de hoogste concentratie was lager dan bij de teven waarbij de partus spontaan startte. Naast het optreden van de geboorte bij nog hoge plasmaprogesteronconcentraties bij de geïnduceerde honden, viel de afname van de plasma-PGFM-concentratie samen met de daling van de plasmaprogesteronconcentratie, wat erop duidt dat de luteolyse pas na de geboorte compleet was. De plasma-PGFM-concentraties waren in de geïnduceerde honden op de tweede en derde dag na de geboorte nog steeds significant hoger dan bij de honden waarbij de geboorte spontaan begon. Omdat de maximale plasma-aglépristoneconcentraties pas worden bereikt op ongeveer 2,5 dag na toediening, zijn de progesteronreceptoren waarschijnlijk aanvankelijk partieel geblokkeerd, waardoor progesteron rondom de geboorte nog steeds deels zijn effect heeft op receptorniveau met als gevolg onderdrukking van de afgifte van $PGF_{2\alpha}$.

Voor de geboorte werd bij beide groepen een significante daling van de plasma-oestradiolconcentratie gezien, zoals eerder werd beschreven (Edqvist et al. 1975; Van der Weyden et al. 1989; Onclin et al. 2002). De behandeling met aglépristone beinvloedt kennelijk de plasmaconcentratie van dit hormoon rondom de geboorte niet. De fysiologische functie van de dalende oestradiolconcentratie voor de geboorte is bij de hond niet bekend. Ook de relatie met de daling van de gonadotrofe hormonen voor de geboorte dient nader te worden onderzocht.

Voor de geboorte werd bij beide onderzochte groepen een significante stijging van de plasmacortisolconcentratie gezien, zoals eerder werd beschreven (Veronesi et al. 2002). Na de geboorte was de plasmacortisolconcentratie significant hoger bij de geïnduceerde teven dan die waarbij de partus spontaan begon, mogelijk als gevolg van het partieel blokkeren van de glucocorticoïdreceptoren door aglépristone. Het blokkeren van de hypofysaire glucocorticoïdreceptoren zou tot toegenomen afgifte van ACTH, met als gevolg toegenomen afgifte van cortisol kunnen hebben geleid (Spitz et al. 1985; Wade et al. 1988). De frequentie waarmee bloedmonsters werden verzameld in deze studie laat echter geen definitieve uitspraken toe over hormonen zoals cortisol en ACTH waarvan de plasmaconcentraties sterk fluctueren.

De verhoogde plasmacortisolconcentratie na de geboorte bij de geïnduceerde teven zou kunnen zijn veroorzaakt door de toename van PGF2α, zoals weerspiegeld door de verhoogde PGFM-concentraties. Er waren echter geen aanwijzingen voor excessieve stress

bij de geïnduceerde teven en hun gedrag na de geboorte. De pupsterfte en de groei van de pups waren ook vergelijkbaar met die van de teven waarvan de geboorte spontaan begon (**Hoofdstuk 7**).

Bij de teven waarvan de geboorte spontaan begon werd vóór de geboorte een significante stijging van de plasmaprolactineconcentratie gezien, wat waarschijnlijk een weerspiegeling was van de invloed van progesteron op de afgifte van prolactine (Galac et al. 2000; Kooistra en Okkens 2002). Bij de geïnduceerde teven steeg de plasmaprolactineconcentratie voor de geboorte slechts weinig, mogelijk omdat de daling van de plasmaprogesteronconcentratie minder abrupt was. Prolactine speelt een belangrijke rol bij de mammogenese en lactogenese (Brisken et al. 1999). Omdat de pups goed groeiden **(Hoofdstuk 7)** leek de behandeling met aglépristone geen duidelijk effect te hebben gehad op de functie van de melkklieren rond de geboorte, wat van belang is voor de overlevingskansen van de pups.

De studie beschreven in **Hoofdstuk 9** toont dat de steroïdogenese in de gonaden en bijnieren van Beagles wordt verminderd door de 3β-hydroxysteroid dehydrogenase (3β-HSD)-remmer trilostane tijdens zowel de hypofyse-afhankelijke als de hypofyse-onafhankelijke periode van de luteale fase. Ondanks de snelle en significante daling van de plasmaprogesteronconcentratie, die werd veroorzaakt door trilostane in beide behandelde groepen, waren de laagste waardes tijdens de behandeling duidelijk hoger dan die noodzakelijk zijn om de dracht in stand te houden, nl. 4 - 8 nmol/L (Concannon en Hansel 1977; Onclin et al. 1993). Het verhogen van de dosis trilostane is waarschijnlijk geen goede oplossing, omdat de plasmacortisolconcentratie 1 uur na toediening van 0,25 µg synthetisch ACTH lager was dan 40 nmol/L bij twee van de elf teven, en dus lager dan wat voor honden veilig wordt geacht (Galac et al. 2010).

Een andere 3β-HSD-remmer, epostane, bewerkstelligde dalende plasmaprogesteronconcentraties tot 4 – 22 nmol/L, dus lager dan in de studie beschreven in **Hoofdstuk 9** (~ 50 nmol/L), waardoor de dracht afgebroken werd of abortus geïnduceerd (Keister et al. 1989). Daarnaast waren de doses die effectief waren in het opwekken van abortus (2,5 tot 5 mg/kg LG 1x daags) lager dan de totale dagelijks toegediende dosis in de hier gepresenteerde studie (6,6 tot 10,5 mg/kg LG). Een mogelijke verklaring zou kunnen zijn dat epostane voornamelijk de ovariële en placentaire steroïdogenese remt bij zowel mensen als dieren (Potts et al. 1978; Schane et al. 1979; Creange et al. 1981; Birgerson et al. 1986; Birgerson et al. 1987). Door deze observaties lijkt trilostane ongeschikt om de dracht af te breken met de dosis die werd onderzocht in deze studie.

Na het stoppen van de toediening van trilostane tijdens de hypofyse-afhankelijke periode van de luteale fase, steeg de plasmaprogesteronconcentratie niet tot het niveau dat op het vergelijkbare interval na ovulatie werd gezien in de controlegroep. Na behandeling tijdens de hypofyse-onafhankelijke periode van de luteale fase werd echter wel volledig herstel van de progesteronproductie gezien, wat aansluit bij de bevindingen in eerdere studies waarbij hypofysectomie of de toediening van progesteron-receptor-antagonisten werden onderzocht (Okkens et al. 1986; Galac et al. 2000; Galac et al. 2004). De door de behandeling geïnduceerde afname van de plasmaprogesteronconcentratie werd zowel tijdens de hypofyse-onafhankelijke als hypofyse-afhankelijk periode niet gevolgd door een significante stijging

van de plasmaprolactineconcentratie, wat wel verwacht mocht worden op basis van studies waarin de progesteroninvloed werd geremd of de ovaria werden verwijderd (Galac et al. 2000; Lee et al. 2006). Mogelijk waren de intervallen tussen de toedieningen van trilostane te groot om een continue onderdrukking van de progesteronproductie te bewerkstelligen en zou een meer frequente toediening van trilostane, als het gaat om het stimuleren van de afgifte van prolactine, effectiever geweest zijn.

De luteale fase duurde korter bij teven die werden behandeld tijdens de hypofyse-afhankelijke periode (66 dagen) dan bij de controlehonden (99 dagen) en bij teven die werden behandeld tijdens de hypofyse-onafhankelijke periode (99 dagen). Het toedienen van trilostane tijdens de hypofyse-afhankelijke periode van de luteale fase zou daarom mogelijk abortus of vroeggeboorte kunnen veroorzaken tijdens een later stadium van de dracht.

Het uitblijven van het herstel van de progesteronproductie na behandeling met trilostane tijdens de hypofyse-afhankelijke periode van de luteale fase suggereert tevens dat progesteron een rol speelt bij de instandhouding van zijn eigen productie tijdens deze periode, zoals eerder is gesuggereerd voor de hond en andere diersoorten (Rothchild 1981; Kowalewski et al. 2009).

Conclusies van dit proefschrift:

- De plasmaconcentraties van LH en FSH worden differentieel gereguleerd bij honden, op basis van de volgende bevindingen in dit proefschrift:
  - De peri-ovulatoire FSH-puls start niet altijd gelijktijdig met de LH-puls, maar soms al enkele uren eerder.
  - De FSH-puls duurt ongeveer 110 uur, terwijl de LH-puls slechts ongeveer 36 uur duurt en vaak een gevorkt patroon vertoont.
  - De basale plasma-LH-concentraties vóór en na de LH-puls zijn hetzelfde, maar de basale plasma-FSH-concentratie is hoger na de FSH-puls dan ervoor.
- Evenals bij andere diersoorten heeft oestradiol-17β waarschijnlijk een positief terugkoppelingseffect op de pre-ovulatoire afgifte van LH bij teven.
- De start van de pre-ovulatoire LH-puls valt samen met een stijging van de plasmaprogesteronconcentratie.
- Het interval tussen de pre-ovulatoire LH-puls en de sterke stijging van de plasmaprogesteronconcentratie is variabel. De sterke stijging is waarschijnlijk een indicator voor het optreden van ovulatie en daarom lijkt bepaling van de pre-ovulatoire LH-puls minder betrouwbaar om de optimale dekperiode vast te stellen dan bepaling van de plasmaprogesteronconcentratie.
- Er werd geen pre-ovulatoire piek van prolactine of α-MSH gevonden bij de teef.
- Gonadectomie heeft een stijging van de plasmaconcentraties van LH en FSH tot gevolg.

- Chemische castratie van reuen met behulp van de GnRH-agonist deslorelin leidt tot verlaagde plasmaconcentraties van LH en FSH en dientengevolge tot sterk verlaagde plasmaconcentraties van oestradiol en testosteron, vergelijkbaar met die na chirurgische castratie.
- De basale plasma-oestradiolconcentraties van anoestrische teven en teven na ovariëctomie overlappen.
- De bepaling van de plasma-oestradiolconcentratie op 120 minuten na de toediening van GnRH is een goede diagnostische methode om de aan- of afwezigheid van functioneel ovarieel weefsel aan te tonen.
- De bepaling van de basale plasma-FSH-concentratie is een goede diagnostische methode om de aan- of afwezigheid van functioneel gonadeweefsel aan te tonen bij teven en reuen.
- De bepaling van de basale plasmatestosteronconcentratie is een goede diagnostische methode om de aan- of afwezigheid van testiculair weefsel aan te tonen.
- De toediening van GnRH aan intacte reuen en teven induceert toegenomen plasmaconcentraties van LH, FSH, oestradiol en testosteron.
- Het toedienen van de GnRH-agonist busereline aan chemisch gecastreerde reuen op 4,5 maand na toediening van een deslorelin-implantaat veroorzaakt een significante stijging van de plasma-LH-concentratie. Dit wijst erop dat de hypofysaire gonadotrofe cellen op dat moment niet bij alle honden compleet gedesensitiseerd zijn.
- De geboorte kan bij honden, drachtig van 3 of meer pups, veilig en effectief worden geïnduceerd op dag 58 na de dekking met behulp van de progesteronreceptor-antagonist aglépristone.
- De met behulp van aglépristone geïnduceerde geboorte vindt plaats terwijl de luteolyse nog niet compleet is, de plasmacortisolconcentratie verhoogd is, en het PGFM-profiel anders is in vergelijking met de spontaan optredende geboorte.
- Trilostane, in een orale dosis van ongeveer 4,5 mg/kg LG, 2x daags, verlaagt de plasmaprogesteronconcentratie tijdens de luteale fase, maar het behandelprotocol leidt tot minder remming van de ovariële steroïdogenese dan waarschijnlijk nodig is om abortus op te wekken.

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Karin Albers-Wolthers, beste Karin. Ik mag mij gelukkig prijzen met zo'n enthousiaste nieuwe collega binnen de discipline voortplanting. Omdat jij letterlijk de volgende (of voorgaande?) stap in de regulatie van de voortplanting bestudeert was het gemakkelijk om mijn vragen met betrekking tot kisspeptin aan jou te stellen, met het risico dat ik er een echte Hershey's chocolate kiss aan over hield. Jouw onderzoek is erg spannend en vernieuwend en ik ben blij met mijn betrokkenheid daarbij.

Claudia Vinke, beste Claudia. Het onderzoek naar de deslorelin implantaten is deels endocrinologisch van karakter, zoals beschreven in dit proefschrift, maar er was ook een belangrijk ethologisch deel, waardoor jij aan de wieg staat van deze studie. Mede dankzij jouw morele steun en je geweldige co-begeleiderschap van de studenten die in dit project hebben geparticipeerd is deze intensieve studie haalbaar gebleken. Ik zie uit naar het voortzetten van onze samenwerking als we alle data tot een manuscript gaan verwerken.

Jenny Buijtels, beste Jenny. Jij was de andere promovendus van Hans en Auke die dit jaar promoveerde. Gelukkig was er wat overlap (mooie term toch ☺) tussen onze onderzoeken waardoor we elkaar konden helpen. Als we dan allebei online waren ging dat vaak heel vlot!

Dr. Bruce Belshaw, dear Bruce. As it was our primary goal to get our message across as accurate as possible, I am very grateful that you were willing to edit most of the manuscripts that are part of this thesis. I believe you have improved them a lot.

Bij de verzorging van de kliniekhonden en hun pups zijn meer mensen betrokken dan ik hier kan noemen. Omdat, naast de dagelijkse verzorging, het afnemen van bloedmonsters en het toedienen van farmaca zo van belang zijn voor het welslagen van de onderzoeken wil ik alle betrokkenen bedanken voor hun inzet. Vaste factoren en aanspreekpunten in de jaren dat deze onderzoeken zijn uitgevoerd waren Inge van Duiven en Ries Pel. Ik wil jullie graag, samen met de altijd beschikbare Harry van Engelen, heel erg bedanken voor jullie oplettende medewerking.

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Curriculum Vitae

Born in The Hague, The Netherlands, on May 14th 1973, Jeffrey de Gier graduated from the Groen van Prinsterercollege in The Hague in 1991. He started studying veterinary medicine at Utrecht University in 1991, and graduated in 1999. Subsequently, he worked for two years in a private companion animal practice in Eersel. He started his residency in companion animal reproduction at the Department of Companion Animal Sciences of the Faculty of Veterinary Medicine, Utrecht University in 2001. He passed the board examination of the European College of Animal Reproduction (ECAR) in 2004. From 2007 onwards he is engaged in the PhD project that has led to this thesis, besides teaching animal reproduction to veterinary students and treating patients that are presented to the specialized reproduction unit of the University Clinic for Companion Animals. In 2010 Jeffrey became assistant professor at the Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University.

Jeffrey de Gier is married to Ekelijn Thomas. They have two children, a son, Renzo (2005) and a daughter, Wieke (2007).

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