Reproductive cycles of the domestic bitch☆

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ABSTRACT

Domestic dogs are monoestrous, typically non-seasonal, polytocous, spontaneous ovulators and have a spontaneous luteal phase slightly longer (by approx 5 day) than the 64 ± 1 day luteal phases of a 65 ± 1 day pregnancy, a phase followed by an obligate anestrus before the next 2–3 week "heat" (proestrus-estrus). The resulting inter-estrus intervals of 5–12 months are variable among bitches, commonly 6–7 months, and range from highly variable to regular (to perhaps within ± 5–10 day of sequential 7 month cycle, for instance) within bitches, and across studies and do not vary significantly between pregnant and non-pregnant cycles. Hormone levels reported are those observed in this laboratory using previously reported assays and canine gonadotropin standards unless stated otherwise. Endocrine sequences for dog cycles are not unlike those of many other mammals, including selection of ovulatory follicles by increased LH pulsatility, the occurrence of estrus behavior and LH surge during a decline in the estrogen: progestin ratio, a pronounced preovulatory luteinization as in humans and rodents, and luteotrophic roles for both LH and prolactin. Non-pregnant bitches have a spontaneously prolonged luteal phase, often longer and with a more protracted decline in serum progesterone than in pregnancy as there is no uterine luteolytic mechanism. The obligate anestrus of 8–40 weeks is terminated by poorly understood interactions of environment (e.g. pheromones, possibly photoperiod) and a potential endogenous circannual cycle in sensitivities of hypothalamic dopaminergic, serotonergic and/or opioid pathways.

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1. Introduction

Bitches are monoestrous, typically non-seasonal, polytocous, spontaneous ovulators and have a spontaneous luteal phase similar in length to or a bit longer than the 64 ± 1 day luteal phase of the 65 ± 1 days of pregnancy followed by an obligate anestrus before the next 2–3 week "heat" period (Table 1). Inter-estrus intervals of 5–12 months, typically 6–7 months, range from highly variable to regular within bitches, and averages do not vary significantly or consistently between pregnant and non-pregnant cycles. Pubertal estrus occurs variably at 6–14 months in most breeds, with means positively correlated with breed size. The canine cycle is classically divided into 4 phases (Evans and Cole, 1931)—a 5–20 day proestrus, 5–15 day estrus, 50–80 day metestrus (post-estrus portion of luteal phase), and anestrus typically lasting 80–240 days. These phases reflect, respectively, follicular phase rise in estrogen, the initial luteal phase rise in progesterone and decline in estrogen, the remainder of the luteal phase, and the interval between loss of luteal function and onset of next cycle. Timing within the 160–370 day cycle has been variably reported in days post proestrus onset, estrus onset, metestrus onset, preovulatory LH peak or LH surge. The latter is used as day 0 in this review (Fig. 1) unless otherwise noted. Levels of hormones are primarily those observed in the author's laboratory using previously reported assays and canine gonadotropin standards.

Table 1
Selected features of normal ovarian cycles of domestic dogs.

Parameters and features	Nature, condition or description	Range, duration, or incidence	Average	Comments
Cycle (intervals)	Monoestrus. Polytocous. Non-seasonal	5–12 mo	7 mo	Basenji is seasonal (autumn estrus)
Preovulatory LH surge	Spontaneous	1–3 d	2 d	Onset is "Day 0" of cycle
Onset of surge release of LH	Sufficient to stimulate ovulation	Day 0	1 d	Onset is "Day 0" of cycle
Proestrus signs, behavior	Spontaneous	5–20 d	9 d	Estradiol (E2) increasing
Estrus behavior	Spontaneous	6–11 d	9 d	Declining E2, increasing P4
Estrus behavior onset	Variable	Day −2 to +5	Day 1	Preovulatory luteinization at LH surge
Ovulation	Spontaneous	~60 h post LH surge	–	Nearly synchronous among follicles
Oocyte maturation	Postovulatory, in distal oviduct		Day 4	Estimated at 48–60 h post ovulation
Progesterone first >1 ng/ml	Concomitant with LH surge	Day 0 to +1	Day 0	Used to predict Day 2.5 ovulation
Loss of oocyte viability	Variable among bitches	Day 6–10	Day 7	Estim. in timed-breeding studies
Fertile insemination possible	Including vaginal AI	Day −3 to 10	–	3–7 d survival of uterine sperm
Fertile breeding possible	Natural mating	Day −3 to 8	–	Rarely Day 10
Apparent "cervical closure"	Barrier to natural insemination	Day 7–10	Day 8	Based on AI and contrast studies
Peak fertility	Natural mating	Day 0–5	–	
Peak fertility	Frozen-thawed semen	Day 5–6	–	
Peak vaginal cornification	Vaginal cytology	Day −5 to +1	Day −3	Not clinically useful to time ovulation
Metestrus onset	"Metestrus" vaginal smear	Day 6–10	Day 7 or 8	"Diestrus-1; D1; Metestrus-1; M1" terms used
Metestrus or Diestrus (NP)	Post-estrus luteal phase	45–70 d	60 d	Terms are synonymous in bitch
Luteal phase	Nonpregnant cycles	Day 0 to Day 55–80	to Day 70	Progesterone above 1.5 ng/ml
Luteal phase	Pregnant cycles	Day 0 to Day 62–65	to Day 64 ± 1 d	Parturition Day 65 ± 1 d
LH luteotrophic	Experimental evidence	Day 13–55	–	LH likely luteotrophic throughout
Prolactin Luteotrophic	Experimental evidence	Day 13–55	–	PRL likely luteotrophic throughout
Luteolytic protocols	PGF b.i.d.; PGF-Ag and/or DA-Ag q.d.	Day 25–30 onward	–	Given "to effect" (4–9 d for PGF)
Pregnancy-specific PRL rise	Concomitant with relaxin rise	Day 25–28	Day 27	Significant by Day 30–32
Obligate Anestrus	From P4 < 1 ng/ml until next Proestrus	3–10 mo	5 mo	Avg. longer in some large breeds
Estradiol peak	Late proestrus	Day −3 to 0	Day −1	40–110 pg/ml vs. 5–20 pg/ml in anestrus
Progesterone peak	Early metestrus	Day 20–30	Day 25	15–80 ng/ml vs. 0.5 ng/ml in anestrus
Luteal regression period	Mid and late metestrus; slow, progressive	45–60 d, e.g. Day 25–70	–	Hysterectomy has no effect
Pre-PE LH-pulse rate increase	Circhoral, 60–90 min intervals	Day −35 to −15	8–10 d. pre-proestrus	Duration of 6–10 d, estim.

Notes: LH (luteinizing hormone); d = day(s); Day (day of cycle relative to Day-0 LH surge); NP = nonpregnant; PRL = prolactin; E2 = serum estradiol; P4 = serum progesterone; PGF = prostaglandin F2 alpha; DA = dopamine; Ag = agonist.

This review is based on reports and reviews from this laboratory (Concannon, 1989, 2009; Concannon et al., 1975, 1979, 1980, 1997a,b, 2001a,b, 2006a, 2009) and from, among others, researchers in Osaka (Hatoya et al., 2003); Tsutsui et al. (2007, 2009) in Tokyo; Hoffmann and Kowalewski and colleagues in Giessen and Zurich (Kowalewski et al., 2007); Verstegen, Onclin and colleagues (Verstegen et al., 1999) in Gainesville; England et al. (2009a,b) in Nottingham; Okkens, Kooistra, Rijnberk and colleagues in Utrecht (de Gier et al., 2008); Gobello and colleagues, Wildt and colleagues (Wildt et al., 1981), researchers at Ghent (Van Cruchten et al., 2004), Font-bonne, Reynaud, and colleagues at Alfort (Reynaud et al., 2006), and Nett, Olson and colleagues at Colorado State (Fernandes et al., 1987), as well as reports in ISCFR sym-

posia Proceedings (see Concannon et al., 1989, 1993, 1997a, 2001a,b, 2006b; England et al., 2009a). Important classical texts include Evans and Cole (1931), and Andersen and Simpson (1973).

2. Endocrinology

Endocrine mechanisms of the canine cycle are not unlike those of other mammals as interpreted from hormone profiles and results of experimental manipulations. Steroid assays established for ruminants and humans have been adapted to canine serum and plasma using sample extraction or direct assays with samples from ovariectomized animals controlling for non-specific interference. LH, FSH and prolactin are assayed using either heterol-
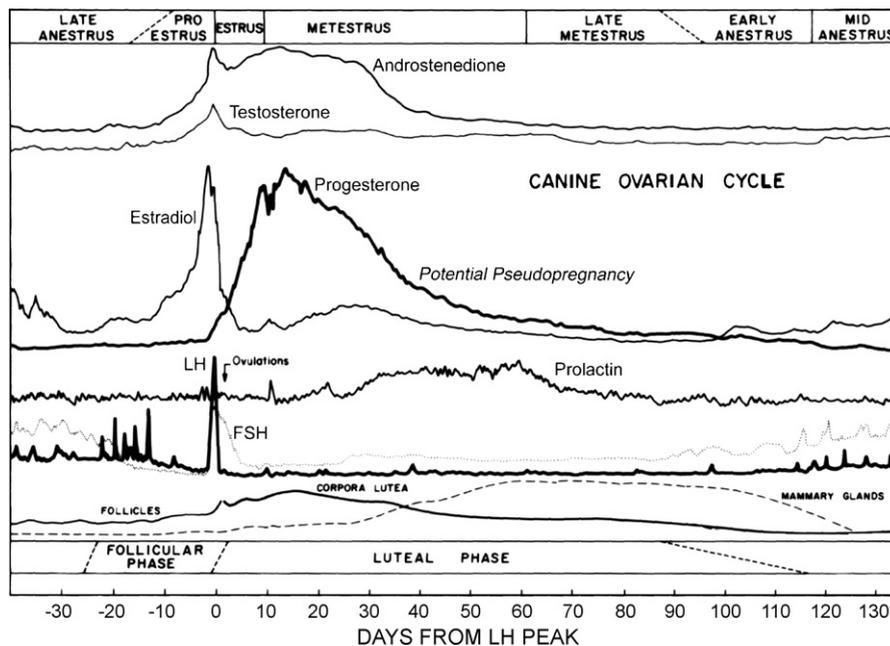
Fig. 1. Schematic of typical changes in concentrations of reproductive hormones in the estrus cycle of the domestic dog, with basal and peaks values being as follows.

Estradiol, 5–10 and 45–120 pg/ml (~20–40 and ~150–450 pmol/l). Pprogesterone, 0.2–0.5 and 15–60 ng/ml (~0.5–1.5 and ~50–200 nmol/l). LH, 0.4–1.5 and 5–40 ng/ml. FSH, 15–40 and 200–400 ng/ml; prolactin, 0.5–2 and 5–30 ng/ml; testosterone, <0.5 and 2–5 ng/ml (<~1 and ~6–16 nmol/l). Androstenedione, 0.5 and 4–11 ng/ml (<~1 and ~12–35 nmol/l).

Salient features include a late proestrus peak in estradiol 1–2 days before the preovulatory LH peak; a preovulatory rise in progesterone reflecting preovulatory luteinization; estrus characterized by falling estradiol concentrations and increasing progesterone concentrations, the latter reaching peak values in early metestrus around day 20–30; a slow decline in progesterone over a 4–8 week period after peak values reflecting slow luteal regression and lack of a uterine luteolytic mechanism; and, a palpable albeit covert increase in mammary size persisting for 3–4 months in every cycle. The transition from the metestrus into anestrus is indicated as imprecise, varying both by definition (e.g. progesterone below 1 or 2 ng/ml, i.e., ~3–6 nmol/l, uterine histological repair) and among bitches due to varying luteal phase durations. Proestrus onset is also indicated as imprecise, varying by criteria and parameter used (i.e., vulval bleeding, estradiol increase, male interest) and among bitches with varying serosanguinous discharge and varying rates of follicle development. Ovulation of immature oocytes at 48–60 h after the LH surge is indicated. The bottom panel shows follicular and luteal phases lasting for near-maximal durations, means reported being about 60–70% of those drawn.

ogous assays based on antisera to ruminant or rodent hormones or homologous assays, with purified canine hormone preparations as standards.

Ovulation occurs in response to an abrupt end-of-proestrus gonadotropin surge resulting in a 1–3 day elevation in LH and a 1–4 day elevation in FSH. Ovulation has been timed to occur about 48–60 h after the LH surge. In bitches, and in contrast to most other mammals, oocyte maturation occurs in the distal uterine tubes ~2 days post ovulation, as in foxes. In humans and many rodents, the preovulatory increase in follicular progesterone production is accompanied by a rise in peripheral progesterone useful for timing ovulation and this is similar in the bitch. Likewise bitches as for many rodents require prolactin in addition to LH for luteotrophin. There is no acute luteolytic mechanism and hysterectomy has no effect on CL function (Olson et al., 1984).

The lengthy luteal phase results in detectable mammary enlargement that in a proportion of bitches can result in a clinical condition, overt pseudo-pregnancy, characterized by gross mammary enlargement accompanied by lactogenesis and lactopoesis. Pregnant cycles are characterized by enhanced progesterone secretion effected by pregnancy-related increases in prolactin after implantation, perhaps in response to increased relaxin; parturition

occurs in response to an abrupt luteolytic rise in systematic PGF immediately prepartum.

2.1. Proestrus

Proestrus occurs when external signs of increased estrogenization are first observed as vulval swelling (edema) usually accompanied by serosanguinous vulval discharge. Proestrus averages 9 days and is characterized by progressive increases in: vulval size and turgor; vaginal epithelial proliferation, cornification, and edema; epithelial cell numbers in vaginal smears; and, vaginal secretion of male-attracting pheromones. Vaginal smear epithelial cell profiles change from being dominated by parabasal cells (accompanied by varying numbers of neutrophils), to being dominated successively by small intermediate squamous cells, large intermediates, and then large cornified cells until finally comprised of entirely (98–100%) cornified cells and virtual absence of neutrophils that no longer traverse the thickened epithelieum. Cornification peaks variably at 1–6 days before the LH surge. The serosanguinous discharge involves serous fluid containing intact and lysed erythrocytes and their hemoglobin originating by diapedesis in the uterus; there are no reliably characteristic changes in their number or appearance. Vaginoscopically,
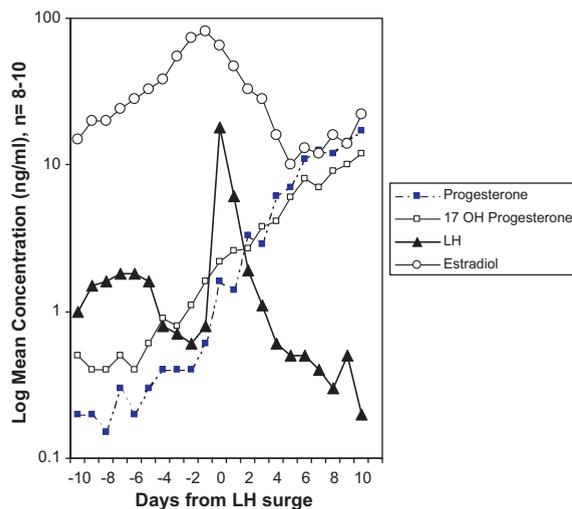
Fig. 2. Mean serum concentrations of LH, estradiol, progesterone, and 17 hydroxyprogesterone in the periovulatory period of beagle bitches (log scale). Salient features include a decreased rate of estradiol increase shortly before the LH surge, abrupt increases in progesterone and 17-OH progesterone at or before the onset of the LH surge, and resulting large pre-LH surge decline in the estrogen: progestin ratio. Mean estrus onset was 0.5, 1.3, 1.8 days after the LH surge in separate beagle studies, times ranging mostly from Day −1 to +3, seldom on Days −2, −3, or +4, and rarely after Day +4.

the mucosa appears edematous, changing progressively from pinkish to white, with serosanguinous fluid on the surface and in deepening vaginal folds that become more prominent in both axes, yielding a smooth cobble-stone appearance (Jeffcoate and Lindsay, 1989).

There is a progressive decline in aggressive response to interested males. Refusals of mounting attempts progressively change from aggressive to ambivalent to playful to passively lying down. Male attraction involves pheromone secretion, and methyl p-hydroxybenzoate was identified as a sex attractant in vaginal secretions of estrous bitches. Small amounts applied to the vulva cause males to become aroused; studies of low ug amounts were discontinued because of hyper-responsiveness and colony disruption (Concannon, unpublished). Serum estradiol increases throughout, from 5 to 15 pg/ml (∼20–55 pmol/l) initially (Fig. 2), to reach peaks of 40–120 (mean 70) pg/ml (∼150–450, mean 255 pmol/l). Proestrus ends with the onset of receptive behavior typically occurring 0.5–3 days after the peak in estradiol and within a day of the preovulatory LH surge. Endocrinologically, physiological proestrus ends with the preovulatory LH surge.

2.2. Estrus

Estrus behavior is characterized by proactive receptivity to mounting by males and increased male-seeking behavior. Estrus lasts a variable length of time, wanes slowly or rapidly after 5–10 days (mean 9 days) but can persist to some extent well beyond the day 8 end of "fertile estrus". Clinically defined, estrus lasts until vaginal anatomy and cytology no longer reflect full or maximal cornification but rather extensive regional desquamation, appearance

of underling non-cornified cells, and epithelial thinning with migration of neutrophils into the lumen, changes that typically occur 6–11 days (average 8 days) after the LH surge. Physiological estrus onset has no distinct cytological correlates, but anatomically is reflected in initial wrinkling and crenulation of the endoscopically viewed vaginal mucosa ± 1 day from the LH surge, as a response to the sharp decline in estrogen: progesterone ratio. Maximal crenulation occurs by day 4–5. In some instances, fertile cycles with normal endocrine profiles can occur with estrus behavior onset as early as 2 days before the LH peak, as late as 6 days after the LH peak, or not at all. Estrus failure has been documented in otherwise fertile cycles by the use of properly timed AI and resulting pregnancies. Some of the variation in onset or occurrence of behavioral estrus within and among bitches involves variation in responses to different males, in behavior being scored in the absence vs. presence of males, and in definitions of estrous onset varying among "receptive reflexes", "standing", "intromissions" and "first ejaculatory copulation or copulatory lock", which in some instances all occur within one day and in others occur completely or incompletely in sequence over several days. Estradiol continues its decline from peak values of late proestrus to intermediate values of 10–20 pg/ml (∼40–90 pmol/l). Serum progesterone rapidly increases above 1–3 ng/ml (3–6 nmol/l) during the preovulatory LH surge, and immediately (or after a 1–3 days pause) rapidly increases further, reaching 10–25 ng/ml (∼30–80 nmol/l) by day 10, at or shortly after the end of estrus. Estrus in the bitch occurs in response to the decline in estradiol that normally begins shortly before the LH surge and continues throughout estrus. Estrus onset is facilitated synergistically by the rapid rise in progesterone resulting from the LH surge. Objectively scored estrus behavior induced by estrogen withdrawal in estrogen-treated ovariectomized bitches has been reported to be more intense, rapid, and synchronous when progesterone was administered at the time of estrogen withdrawal (Concannon et al., 1979).

2.3. Metestrus (diestrus)

Metestrus, the post-estrous portion of the luteal phase, was initially defined behaviorally as starting when estrous-behavior ceases. Using morphological criteria, metestrus begins when a day 6–11 "metestrus" vaginal smear or "metestrus" vaginal mucosa is first detected. Metestrus is considered to last until evidence of the ongoing luteal phase becomes minimal. The end of metestrus, and anestrus onset, are variably defined as when uterine endometrium has undergone histological "repair", when mammary enlargement in response to luteal phase progesterone recedes, and most often in recent decades, when serum progesterone declines to levels persistently below 1 or 2 ng/ml (∼3–6 nmol/l). Serum progesterone increases to peaks of 15–80 ng/ml (∼50–250 nmol/l) between cycle day 20 and 35, and slowly declines thereafter, going below 1 ng/ml (∼3 nmol/l) by day 55–90 (mean 70). Estradiol is variable at intermediate values of 15–30 pg/ml (∼15–110 pmol/l) with profiles to some extent paralleling those of progesterone, higher in mid-luteal phase and then declining. The term "diestrus" is used in some veterinary

texts as a substitute for and synonymous with metestrus in bitches, thereby applying 'diestrus' across species to generally refer to the period of luteal function and avoiding possible misconceptions that 'metestrus' might refer to only a short period after estrus as in descriptions of artiodactyl and rodent cycles. With 'diestrus' used for bitches in that context, unlike in other species, estrus and not diestrus (metestrus) still occupies most of the growth phase of the canine CL, and an intervening anestrus and not diestrus immediately precedes proestrus.

2.4. Anestrus

Canine anestrus (see Okkens and Kooistra, 2006) involves the absence of overt evidence of ovarian activity, is considered to be "obligate" lasting a minimum of 7 weeks after progesterone declines below 1–2 ng/ml, and averages 18–20 weeks. It may initially be important for the normal endometrial repair that is completed around day 120–130. The apoptotic index and percent of degenerated epithelial cells in the endometrium are high during the mid-luteal phase, low in early anestrus and absent by day 120 (Po-yin et al., 2006). Vaginal cytology shows sparse numbers of parabasal cells (and degenerate "squames") and variable but modest numbers of neutrophils. The vaginal mucosa appears thin and red with visible capillaries; the surface is easily traumatized and vaginal cytology difficult to monitor without inducing bleeding with spurious erythrocytes in smears. Serum estradiol is reported to be variable but generally low at 5–10 pg/ml ($\sim$15–35 pmol/l). Basal LH is low (<1–2 ng/ml) between sporadic, variable-height and often-large pulses (3–30 ng/ml) at intervals of 7–18 h or longer. FSH is high (50–400 ng/ml, mean 140) between sporadic pulses slightly above elevated baseline that when detectable are typically concomitant with LH pulses. Serum progesterone remains below 1 ng/ml (under $\sim$4 nmol/l), with a nadir near 400 pg/ml ($\sim$1500 pmol/l) at 30–40 days before proestrus (see Concannon, in Concannon et al., 1993).

2.5. Canine fertility

As confirmed by histological studies and breeding trials, primary oocytes are ovulated 48–60 h post LH surge, undergo maturation at 96–108 h in the distal uterine tube (oviduct), and secondary oocytes remain viable for another 24–48 h (day 5–6) in most bitches, for 72–96 h in some bitches (day 7–8), and 120–144 h (day 9–10) in the extreme. Fertility for single matings is maximal from the LH surge (day 0) until day 5 and wanes rapidly over the next 3 days, in part due to a "cervical closure" at day 7–8. Conception rates following intrauterine AI at day 9–10 after the LH surge average 60% (Tsutsui et al., 2009) vs. rates of less than 10% with vaginal inseminations. Following implantation on day 21, parturition routinely occurs 65 ± 1 days after the LH surge, as early as 55 days and as late as 68 days after mating (Concannon et al., 2001a,b). Canine sperm survive up to 7 days in utero, with mating 5 days before ovulation often being fertile, and super-fecundation is not uncommon. AI with poor quality or frozen-thawed semen is most successful when performed at day 5 or 6 post LH surge and after
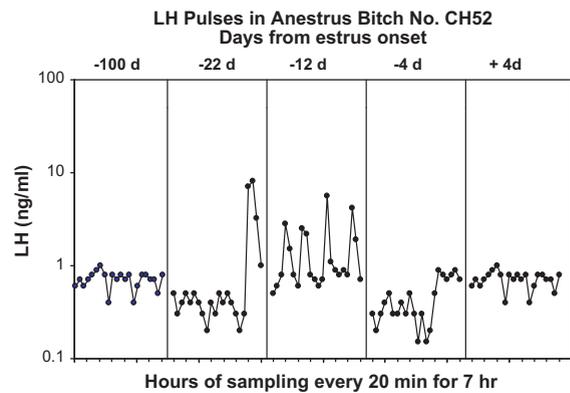

Fig. 3. Concentrations of LH in serum samples collected every 20 min for 7 h in a beagle studied on selected days during anestrus, proestrus, and estrus and shown in relation to the day of preovulatory LH surge. Proestrus onset was at day −8.

oocyte maturation. Canine oocytes appear solid white, are more densely lipid laden than in pigs, and the lipid cannot be centrifuged to the periphery; but, *in vivo* matured oocytes have been used for cloning male and female dogs via somatic cell nuclear transfer (Hong et al., 2009). Normal litter sizes range from 2 to 16, average about 7, breed averages varying from 5 to 8, roughly positively correlated to breed size.

2.5.1. Follicular phase

The follicular phase involves a cohort of 2–8 follicles per ovary detectable as 3 mm large antral follicles visible but not protruding from the surface at 6–10 days before the LH surge, and secreting estradiol sufficient to cause changes in the reproductive tract described for proestrus. It is initiated by a preceding increase in LH pulse frequency (Fig. 3) from intervals averaging > 7 h during anestus to circhorral intervals of less than 2 h (Concannon et al., 1993; Tani et al., 1999). Daily mean LH is thereby elevated for an estimated 6–10 days in the 1–3 weeks before observed proestrus (Fig. 4). The estimated duration is based on daily or more
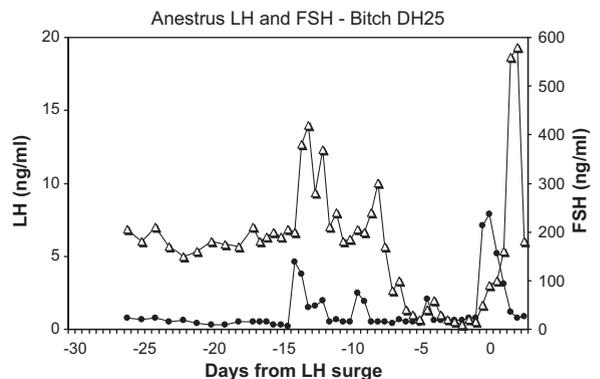

Fig. 4. Serum LH and FSH concentrations at 12–24 h intervals during late anestrus and proestrus in a bitch with 4–8 fold increases in LH, associated with the late anestrus increased frequency of high amplitude LH pulses and increased mean LH levels 300–3000% greater than those earlier in anestrus. In this bitch there was an obvious 100% increase in daily FSH observed, whereas in many instances an FSH increase may not be obvious.

frequent measurements in the period before proestrus, including determination of pulse intervals in 7 h windows of sampling every 15–20 min (Fig. 3), and on the 8–9 day duration of GnRH-agonist administration usually needed to induce fertile proestrus in bitches during anestrus (Concannon, 1993; Concannon et al., 2006a). The increased LH pulsatility is accompanied by FSH pulses, and less prominent increases in mean FSH that may not be significant. The LH increase is the critical factor selecting the ovulatory wave of follicles. Precocious fertile proestrus resulted from injecting purified porcine LH, not FSH (Verstegen et al., 1997). GnRH in pulses every 90 min induced "fertile proestrus", i.e., one resulting in a spontaneous LH surge and fertile ovulation (Concannon et al., 1997a,b). However, pulsatily is not required; persistent elevation of LH for 7–9 days or longer via constant-release GnRH-agonist implants also induces fertile proestrus (Concannon, 1989). Follicle diameters increase to 6–9 mm before the LH surge, and enlarge to 9–12 mm between the LH surge and ovulation; follicle growth and ovulation can be monitored by ultrasound (England et al., 2009a).

2.5.2. Ovulation

Determining the time of ovulation is often critical in breeding management, timing AI, monitoring ovulation-induction, and reproductive experimentation. Where access to rapid LH assays is not available, ovulation is best timed as occurring 2 days after the first abrupt rise in progesterone of > 0.5 ng/ml and reaching ≥ 0.9 ng/ml, an event that occurs concomitantly with the LH surge in over 95% of cycles. When early and frequent measurements are not available, the first day with concentrations ≥ 5 ng/ml is often considered indicative of ovulation in breeding management. A rapid reduction in vulval turgor due to preovulatory declines in the E:P ratio typically indicates ovulation is either eminent or has just occurred. Similarly, intense crenulation of the vaginal mucosa due to declining estradiol is informative, as it becomes maximal 2–3 days after ovulation, and recedes thereafter. Ovarian ultrasound can also determine the time of ovulation with considerable accuracy, based on the transient 1–2 day marked increase in echogenicity of previously anechoic follicles at ovulation, followed by a return of anechoic structures, i.e., day 4 "antral" CL. (see Reynaud et al., 2007). Whether echogenicity at ovulation is due to bleeding, follicle collapse, or change in follicular fluid composition is not known.

The LH-surge to ovulation interval is characterized by a rapid increase in follicle mural cell luteinization, in growth of theca and blood vessels, abrupt increases in serum progesterone and 17-hydroxyprogesterone, and typically further declines in estradiol (Concannon et al., 2009). Increased follicular progesterone is likely to be critically involved in ovulation, as in other species. Progesterone's participation in positive feedback cascades involving PGE and/or oxytocin production and increases in metalloprotease, as in the cow (Fortune, 1994; Fortune et al., 2009), have not been studied in bitches. Details of oocyte maturation (MII at 48–54 h post ovulation), fertilization and early embryo development have been reported and elegantly reviewed (see Reynaud et al., 2006). Interestingly, peripheral cumulus cells undergo mucifi-cation before ovulation but mucification of the compact inner-most cumulus layers and their expansion from the zona are delayed 24–36 h or more after ovulation and appear indicative of the cytoplasmic maturation of the oocyte.

2.5.3. Periovulatory endocrinology and preovulatory LH surge

The proestrus rise in estradiol is accompanied by increases in androstenedione and testosterone reaching peak concentrations averaging ca. 300 and 800 pg/ml (1100–3000 pmol/l), respectively, presumably appearing as excess estrogen precursor (Fig. 1). Whether they cause masculine behaviors (e.g. mounting other bitches) sometimes observed in proestrus is unknown. Follicle secretion of estradiol in late proestrus may be semi-autonomous and self-limiting. Simultaneously with increasing estradiol, LH and FSH are progressively suppressed, often to the lowest concentrations of the cycle except for immediately after the LH surge, reaching ≤ 1 ng/ml and < 50 ng/ml, respectively, due to estradiol and inhibin negative feedback. Inhibin studied using a nonspecific b-INH assay typically paralleled estradiol levels in a preliminary study (Concannon, unpublished). Serum progesterone, reflecting histological detectable sporadic patches of follicle luteinization, increases very slightly during most of proestrus, rising from 0.2–0.4 ng/ml to reach 0.6–0.8 ng/ml 1–2 days before the LH surge, perhaps simply as excess steroid precursor. Preovulatory LH surge release can be defined as the first detectable rise >200% of preceeding mean concentrations and >50% of peak concentrations in frequently collected samples. Day of LH surge onset was either concomitant with the day of the LH peak (70–80%) or one day before (20–30%), varying among studies. The LH surge is likely the more critical parameter, LH being elevated sufficient to cause ovulation independent of further increases. LH peaks in fertile cycles are highly variable, typically ranging from 3 to 40 ng/ml, average 13 ng/ml, and only rarely non-detectable and/or not meeting the aforementioned criteria. LH is typically elevated above 2 ng/ml for 1–3 days, average 2 days, usually peaking in the first 12–18 h. The FSH surge is equally prominent, concentrations rising along with LH from <50 and reaching peaks of 100–600 ng/ml 0–2 days after the LH peak, and declining thereafter less rapidly than LH. LH surge onset typically occurs 0–3 days (average 1 or 2 varying with study) after the peak in estradiol. Thus, estradiol initially declines on average a day before the LH surge, and in some cycles rises to a secondary peak during or after the LH surge. In frequent samples, the LH surge onset cannot be separated temporally from the initial sharp increase in serum progesterone (measured at 6–8 h intervals) or 17-OH progesterone (at 12–24 h intervals). Progesterone increases from 0.5–0.8 ng/ml to 0.9–2.2 ng/ml during, or a few hours before, the onset of LH surge. The concomitant rise in 17-OH progesterone is equally if not more robust (Fig. 2). Increasing 17-OH progesterone may synergize with progesterone in facilitating the LH surge and estrous behavior, as it is also a ligand for the progesterone membrane-receptor reported in the hypothalamus and pituitary of other species.

In the bitch, the proestrus suppression of LH appears sensitive to the rate of increase in estradiol with the LH

surge being initiated by an abrupt decline in the rate of change in the estrogen to progestin ratio. The initial increases in progestins serve to facilitate the rapidity and magnitude of the LH surge (Concannon et al., 1979, 1993, 2009). In ovariectomized bitches with estradiol implants in place, serial injections of estradiol benzoate causing progressive increases in serum estradiol to values 2–4 times normal peak values continued to suppress LH. Preovulatory-like surge release of LH occurred following a decline in estradiol after cessation of injections (or in some bitches after a peak in estradiol occurring despite continued administration due to increased clearance). In similarly treated bitches progesterone administered when estradiol injections were discontinued caused LH surges to occur earlier, more synchronously, and to twice the magnitude obtained without progesterone. The progesterone synergism suggests a facilitative role for increasing progestin immediately before and during the LH surge (Fig. 2) in late proestrus. In bitches as in sheep, increasing estradiol is likely to cause a dose-dependent suppression of hypothalamic GnRH secretion involving a large decrease in pulse amplitude despite an increase in pulse frequency, and resulting in suppression of mean LH (Evans et al., 1994). Direct suppression of gonadotrope LH release may also play a role. The decline in the rate of increase in estradiol in late proestrus that initiates the surge release of LH (Fig. 2) likely results from increased peripheral estradiol clearance as well as from follicles reaching terminal maturation (Concannon et al., 2009).

2.5.4. Luteal phase and progesterone secretion

The canine pattern of luteal growth and slow regression is not dissimilar to that observed in hysterectomized individuals of polyestrous species in which rapid luteolysis depends on uterine PGF production. In both, the luteal phase extends towards or near the duration of normal pregnancy. Hoffmann and colleagues at Giessen have extensively characterized canine luteal biology (see Kowalewski et al., 2007). The growth phase involves up-regulation of PG synthetase, COX2, and PGE production. Increased PGE is presumably mitogenic, angiogenic, anti-apoptotic, vasodilatory, luteo-protective and luteotrophic as in other species. Histological study of periovulatory canine follicles and early CL suggests changes involving a loss of granulosa and rapid in-growth of theca interna cells and blood vessels during and after the LH surge (Concannon, unpublished), and the extent to which granulosa as well as theca contribute to CL formation is not known. Luteal cell diameters are 25–55 μm (average 36) at mid-metestrus, at or shortly after peak progesterone (Dore, 1989). Ultra-structurally, there is apparently only one type of steroidogenic cell, with characteristics not unlike those of large luteal cells of ruminants including abundance of smooth endoplasmic reticulum, high mitochondrial content, and prominent Golgi. Canine luteal cells may be more like those described for rats (Stocco et al., 2007) where smaller and larger luteal cells differ little, if at all, in cellular origin and function and are without the differences seen in ruminants and primates. In rats, as in dogs, the origin of luteal cells as being from granulosa vs. luteal cells is also not clear, and the larger luteal cells of rats are distinguished by

their expression of PRAP (prolactin receptor associated protein) that, interestingly, is only expressed in theca cells of mature follicles. In bitches theca likely contributes significantly to the CL, and the CL are prolactin dependent. LH and prolactin, and potentially progesterone, are luteotrophic. PGF is luteolytic, but is not secreted in amounts that affect CL function in the normal cycle. PGF administered b.i.d. for 4–8 days can effect a transient or permanent functional luteolysis and pregnancy termination. Slow luteal regression over 1–2 months appears to involve constitutive luteal cell functions, and does not involve any decline in LH or prolactin or their receptors (Fernandes et al., 1987). It involves low but evident apoptotic activity with no apparent increase in low-level endogenous PGF production (Luz et al., 2006; Concannon, 2009). Cells of regressed canine CL are multinuclear syncytial giant cells (Dore, 1989).

2.5.5. Luteotropins

LH and prolactin are both luteotrophic by week 2, and likely earlier. Both are required-luteotropins at day 25 and thereafter in both pregnant and non-pregnant cycles (Concannon et al., 1993, 2009). LH suppression by GnRH-antagonist negatively impacts CLs and progesterone secretion as early as day 10 (see Concannon et al., 2009; Valiente et al., 2009). Prolactin lowering doses of dopamine agonist dramatically reduce serum progesterone as early as day 13. After day 27, LH-down-regulating doses of GnRH agonist or prolactin-suppressing doses of dopamine agonist cause transient or permanent cessation of luteal function, keeping serum concentrations of progesterone below 1–2 ng/ml throughout continued treatment and often thereafter. At day 42, highly potent LH-antiserum transiently suppressed serum concentrations of progesterone. Continued expression of StAR and 3BHSD throughout the luteal phase in bitches is documented (Kowalewski et al., 2008) and likely stimulated by LH via adenyl cyclase and protein kinase A pathways as in other species. In vitro, canine mid-luteal phase enzymatically dispersed luteal cells increased their progesterone production with addition of LH or db-cAMP (see Concannon et al., 1993) but not with addition of prolactin or FSH (Concannon and Milvae, unpublished data). Cholesterol ester and homologous plasma were each as stimulatory as LH in vitro with effects additive to those of LH. This suggests that exogenous cholesterol is a chronically rate-limiting factor. Perhaps increased cholesterol uptake is a mechanism whereby prolactin is stimulatory in vivo. Prolactin increases luteal lipoprotein uptake in rodents. Lack of pronounced in vivo effects of prolactin or LH administrations suggests that luteal cells function near maximum at all times, subject to suppression but not stimulation. As in rodents, other luteotrophic effects of prolactin may include increasing LH-R, progesterone membrane receptors (with endogenous progesterone being luteotrophic), expression of steroidogenic enzymes and sterol carrier protein, and decreasing PGF receptors, but these effects remain to be elucidated in canine luteal cells.

Progesterone is likely luteotrophic or antiluteolytic, as in rodents. Progesterone antagonist administered to luteal phase bitches depressed ovarian vascular function, caused inflammatory macrophage invasion of CL, reduced pro-

gesterone secretion, hastened luteal regression and /or reduced cycle length (reviewed in Concannon, 2009),

2.5.6. Progesterone

Progesterone is a uniquely potent steroid in bitches. It can, without estrogen pretreatment, cause endometrial proliferation and mammary hyperplasia during anestrus or in ovariectomized bitches as nearly as extensively as obtained with estrogen pretreatment. Apparently a significant level of constitutive expression of progesterone receptors occurs in canine mammary tissues. Progesterone during the cycle in older bitches can cause acromegalic like symptoms, including hypertrophy of skin and other soft tissues and overgrowth of some bone and cartilage, occurring in response to progesterone-induced hypersecretion of growth hormone by mammary tissue as well as the pituitary, and resulting in increased serum concentrations of IGF-1 (Concannon et al., 1980; Mol et al., 1996; Stovring et al., 1997). Elevations in serum progesterone can also cause diabetic insulin resistance, acting either directly or via increased GH production. Diabetic bitches often need an increase in insulin administration in response to ovarian cycles and pregnancy can cause a diabetic state in predisposed bitches. Synthetic progestins can cause mammary tumors, acromegaly and diabetic states in bitches, in some cases within the recommended dose range as a contraceptive. Excessive doses or long term administration of some progestins have caused mammary adenocarcinoma.

2.5.7. Pregnant cycles

Progesterone is entirely of luteal origin in pregnancy. Mean progesterone in pregnant vs. non-pregnant cycles rarely differ significantly across studies, albeit numerically higher in pregnancy after day 25–30 in most cases. Obvious secondary increases after day 25 occur in most pregnancies but not in non-pregnant cycles. Other observations indicate that progesterone secretion is greatly increased during pregnancy. Mean concentrations after day 25 are numerically higher in pregnant cycles despite an increased plasma volume of distribution sufficient to cause a normo-chromic, normo-cytic anemia comparable to that in humans. Hematocrits begin decreasing between day 25 and 30, reaching nadir near or below 40% at term, and anemia is not resolved until 1–2 months postpartum. Progesterone during pregnancy also remains high despite increased metabolism and hepatic clearance causing fecal progesterone twice that in non-pregnant cycles (Gudermuth et al., 1998). Fecal testosterone and estrogen also increase suggesting heightened luteal secretion of these steroids as well in pregnancy. Increased progesterone secretion is likely to be a result of enhanced prolactin secretion, starting on days 25–28 of pregnancy, mean prolactin being significantly higher by day 32. Prolactin increases simultaneously with the onset of detectable and increasing plasma relaxin concentrations at day 25–27. Relaxin peaks at mid-pregnancy, remains elevated until term, declines at parturition, and appears entirely of placental origin. Various endocrine and non-endocrine changes in pregnant cycles have been reviewed elsewhere including increased insulin resistance (Concannon et al., 1989), increased fibrinogen and c-reactive protein, other alterations in inflammatory and coagulation factors, late pregnancy increases in FSH, prepartum surges in cortisol, PGF and prolactin, and intrapartum oxytocin release (see Concannon et al., 1989, 2006b; Günzel-Apel et al., 2006, 2009).

2.5.8. Reproductive tract, mammary development, and pseudopregnancy

The ovary, fully enclosed in a bursal membrane with a small bursal slit, is typically visible trans-membrane in young animals, but often not in older animals due to bursal fat. The long uterine horns become serpentine during rapid growth in estrus and early luteal phase. The short fundus provides for translocation of ova in estrus. The single cervix is oriented with the os facing nearly ventrad into the anterior vagina, cranial to an often prominent dorsal-median fold (dmf) but visible vaginiscopically, and amenable to transcervical insemination with varying difficulty. The caudal aspect of the dmf may present as a "false cervix", with the occluded vaginal lumen appearing like the cervical os of some species. Oviduct and endometrial responses to estrogen and progesterone appear similar to those described in rodents, and maximal endometrial development requires sequential exposure to estrogen and progesterone, as occurs in normal cycles. Sex steroid receptor changes in reproductive tissues show cyclic changes similar to other species; recent detailed analyses of canine endometrial cells have been extensive (see Van Cruchten et al., 2004; Ververidis et al., 2004). Endometrial ER and PR increase in proestrus, decrease in estrus and metestrus, and are replenished in anestrus. Mitotic indices of endometrial stroma, blood vessels and surface epithelium are maximal under estrogen influence in proestrus and decline thereafter, whereas indices for basal glandular epithelium peak in estrus and metestrus under the early influence of progesterone. Mammary and uterine disease is more common in dogs than in other domestic species, perhaps due to progesterone sensitivity, absence of routine pregnancy, and use of progestins for contraception.

Luteal progesterone, or exogenous progesterone alone, can cause extensive endometrial hyperplasia, and even cystic endometrial hyperplasia (CEH) that can support opportunistic infection and pyometra. Researchers in Osaka found that progesterone suppresses peripheral cellular immunity in metestrus (Sugiura et al., 2004). CEH progressing to pyometra is the most common medical problem of domestic bitches and is usually managed surgically (ovariohysterectomy). Medical management in breeding bitches involves uterotonic and luteolytic effects of PGF injections, using PGF alone, or combined with prolactin-lowering dopamine agonist doses synergistic in inducing luteolysis or with antiprogestin treatment that enhances progesterone withdrawal. The latter causes pre-partum-like changes including opening of the cervix, and thereby facilitates evacuation of uterine contents in response to PGF.

In every normal cycle, sequential exposure to estrogen and progesterone causes significant mammary growth and enlargement detectable by palpation (Fig 1) but is otherwise typically not obvious. A clinical condition, "overt" or "clinical" pseudo-pregnancy, involves a mammary

hyper-response including lactogenesis and lactopoesis and sometimes lactation. There is a mid-late luteal phase increase in prolactin-dependent stimulation of mammary tissues mimicking that of pregnancy. The incidence of some degree of ill-defined overt pseudopregnancy can reach 10–20% or more in some breeds, but is almost negligible in others (e.g. beagles). Precipitated by a premature decline in progesterone that either stimulates prolactin release or increases mammary prolactin-responsiveness seems likely in many cases. Mean prolactin is higher and mean progesterone lower in affected cycles vs. normal cycles (Tsutsui et al., 2007). During the luteal phase ovariectomy often precipitates an overt pseudopregancy, and ovariectomy, PGF induced luteolysis or antiprogestin administration can each increase prolactin (see Concannon, 2009). Clinical pseudopregnancy is often accompanied by, and is sometimes primarily manifested in, behavioral changes mimicking those of the prepartum bitch (e.g. circling, digging, nesting, and defensive behaviors). Clinical management is by prolactin-lowering doses of dopamine agonists. Progestin administration is contraindicated as subsequent progestin withdrawal results in reoccurrence of symptoms. Terminology is confused because the long luteal phase of normal cycles has been referred to as "physiological pseudopregnancy" and analogous to those in rodents.

3. Contraception and induction of estrus

Much of canine reproductive research is directed towards either contraception or induction. Cycles are suppressed by low concentrations of progesterone from s.c. implants; none are commercially available. Oral megestrol acetate is used worldwide, with 2–4 weeks of administrations in early-proestrus or anestrus alternating with 3–4 months untreated, with first and maximum number of treatments varying by jurisdiction. Depo-medroxy-progesterone acetate is approved for canine contraception in some countries, but not others because of complications such as pyometra, acromegaly, diabetes and a significantly increased risk of mammary hyperstimulation, leading to mammary nodules and mammary cancer.

All progestins act to suppress ovarian cyclicity in dogs by preventing increases in LH pulsatility that normally result in proestrus, perhaps acting as anti-estrogens. They do not suppress already low basal LH concentrations but instead allow basal LH to increase above concentrations normally seen in anestrus. Down-regulation of LH and FSH by chronic GnRH-agonist administration provides continuous but reversible estrus-prevention. Commercial agonist formulations (e.g. deslorelin and azagly-nafarelin) have been approved for that use, male contraception or management of selected endocrinopathies, in some countries. The untoward side-effect of acutely inducing fertile estrus upon administration is avoided with treatment of young prepubertal bitches, bitches with high progesterone, or with progestin pre-treatment. Immunologic and cytotoxic modalities for canine contraception are under extensive investigation.

Induction of premature proestrus and estrus has been accomplished with highly variable success rates using estradiol, FSH, FSH plus LH, or eCG, and is typically complicated by follicle luteinization, ovulation failure, short luteal phases or failed implantation. However, human menopausal gonadotrophin, having more LH than FSH potency, may yield better results (Wanke et al., 1997). Prolactin lowering doses of dopamine agonist administered for 7–30 days in anestrous bitches often results in premature but normal proestrus, including normal pre-proestrus increases in LH pulsatility (Concannon, 1993; Spattini et al., 2007). Pulsatile GnRH administration can induce proestrus that progresses into spontaneous fertile estrus. Similarly successful, if given at doses that up-regulate LH to concentrations normally seen prior to proestrus and do not too rapidly down-regulate LH and prevent a spontaneous ovulatory LH surge in response to the estradiol excursion of the induced proestrus, is chronic s.c. GnRH-agonist administration via osmotic pump or proprietary implant for 7–14 days. In one study series, cycle-regulation was effected to the extreme using a GnRH-A with *in vivo* bio-potency 150× native GnRH at doses 0.6–1.8 ug/kg/day; inter-estrus intervals of only 120 days were routinely obtained using 12-day agonist treatments beginning 60 days after PGF induced luteolysis in mid-luteal phase. Cycle intervals of 320 days were routinely obtained by first suppressing estrus with progesterone implants from day 160–252 and then administering GnRH-A at day 305. In comparison control cycle intervals averaged 211 days (Concannon et al., 1989, 2006a). Successful inductions of fertile estrus in bitches by GnRH-A infusion may be related to the fact that moderate to large doses require some 3 weeks to fully down-regulate LH to basal values (see Concannon et al., 2006b, 2009).

3.1. Canine cycle intervals and cycle regulation

The reason why anestrus is so variable in some bitches and not others is not clear. Likewise, the factors leading to late anestrus increases in GnRH and LH pulsatility and initiating proestrus are not well understood. Increased hypothalamic GnRH release activity has been documented in late anestrus (Tani et al., 1996). Factors that may be involved include (1) proximity to other bitches, as pheromones of an estrous bitch can facilitate proestrus onset in other bitches; (2) social interactions, considering effects of dominant females in wolf packs; and (3) subliminal effects of an endogenous circannual cycle. Photo-entrained endogenous circannual cycles seen in seasonally monestrous canids and in autumn-breeding basenji dogs presumably persist as free-running cycles in non-photo-entrained domestic breeds just as they persist in most species of seasonal breeders when deprived of normal photoperiod cues. However, endogenous cycles in dogs are not well documented. Annual cycles of winter nadirs in mean prolactin are reported in male dogs, but study of individual dogs suggests prolactin rhythms may instead be circannual in cycles ranging from 6 to 14 months (Verstegen et al., 2008). Corresponding circannual cycles in sensitivities of hypothalamic dopaminergic, serotonergic and/or opioid pathways are thus suggested to play a role in generating the 7–12 month cycle intervals of the bitch (Concannon, 2009). Changes during the

1–3 months before the increase in LH pulsatility shortly prior to proestrus include (1) serum FSH increasing from high to even higher concentrations (Okkens and Kooistra, 2006); (2) increasing pituitary responsiveness to GnRH; (3) increased LH release in response to opioid antagonist naloxone (Concannon, 1989); (4) increasing numbers of detectable small (2 mm) antral follicles from <2 to >2 per ovary at 30–40 days pre-proestrus (England et al., 2009b); (5) increasing hypothalamic and pituitary ER expression; and (6) increased hypothalamic aromatase expression. Evidence for subliminal waves of dominant follicles during anestrus is modest, and if distinct, they are likely overlapping, yielding a nearly constant pool of responsive dominant follicles. Over 80% of bitches in any stage of anestrus will rapidly enter an induced proestrus in response to physiological doses of GnRH administered circhorrally or of GnRH-A administered constantly, although the latter responses result in fertile estrus more frequently in late vs. early anestrus. Roles of serotonergic, dopaminergic and opioidergic pathways have not been fully documented. The opioid-antagonist naloxone causes acute rises in LH at any time, but especially in late anestrus. Dopamine agonists in doses that lower prolactin can cause a premature proestrus that involves a normal preproestrus-like rise in LH pulsatility, with responses being more rapid in late vs. early anestrus. A response failure in a normally cycling bitch was associated with failure to lower prolactin, suggesting that endogeous changes in sensitivity to dopamine may be important and/or that prolactin may play an inhibitory role. A serotonin antagonist induced precocious proestrus in one study but not in another. Priming by estradiol from a subliminal follicular wave producing elevations in estrogen or androgen could be involved considering the above mentioned changes in ER and aromatase (Hatoya et al., 2003), and a report of a transient rise in estrogen about 40–50 days before proestrus. ER increases during late anestrus, as does hypothalamic aromatase. In bitches previously administered prostaglandin to shorten the luteal phase, estrogen treatment produced a false-proestrus that progressed to a fertile proestrus with estrus being advanced compared to bitches not receiving estrogen (Bouchard et al., 1993). The possibility also exists that there are ovarian or other mechanisms sensitive to changes in availability of progesterone or other ovarian hormones as progesterone declines from the 1 ng/ml in early anestrus to a nadirs of 0.2–0.4 ng/ml around 30 days prior to proestrus (Concannon, 1993). Further study of canine anestrus is needed to explain temporal patterns of canine estrus cycles.

4. Conclusion

The basic endocrinology of the canine estrus cycle appears to be similar to that of many other species, including the role of increased GnRH and LH pulsatility in triggering the 1–2 week follicular phase, the synergistic effect of progesterone in initiating and enhancing preovulatory surge release of LH and estrus behavior following a the follicular phase excursion in serum estradiol, and the spontaneous formation of corpora luteal that develop an absolute requirement for the luteotrophic effects of prolactin as well as LH as in rodents. The relatively long proestrus reflects a 1–2 week follicular phase as occurs in primates. The onset and duration of the relatively lengthy week or so of estrus reflect the stimulatory effect of the associated declining estrogen-progesterone ratio and the apparent absence of a mechanism to abruptly terminate estrus behavior. The spontaneously prolonged luteal phase of approximately two months appears comparable to that of hysterectomized individuals of most polyestrous species and reflects the absence of an acute uterine luteolytic mechanism. While the cellular mechanisms involved are unlikely to be species specific, additional whole-animal and molecular studies are needed to confirm the value of the bitch as a model for basic reproductive phenomena. Poorly understood and in need of study are the role of an endogenous cycle in affecting the duration of anestrus and the 6–12 month inter-estrus intervals, the causal relationships and sequence of the requisite events terminating anestrus, the molecular basis for the delay in oocyte maturation until 2–3 days after ovulation, and the endocrine basis of estrus durations longer and more variable that in most non-candid species.

Conflict of interest statement

The author has no conflict of interest in publishing this review.

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