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Endocrine control of ovarian function in dogs and other carnivores

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Abstract

Ovarian function in dogs is minimally but successfully evolved and adapted for fertility, and represents a basic model for examining the more complex evolution of ovarian activity in other carnivores and mammals in general. Canids are monoestrous, polytocous, spontaneous ovulators with a spontaneous luteal function producing progesterone for the duration of a normal 2-month pregnancy and unaffected by hysterectomy. They have no acute luteolytic mechanism in the absence of pregnancy although PGF is luteolytic and participates in prepartum luteolysis. The cellular mechanisms of luteal and follicular tissues appear unlikely to differ meaningfully from those described in other species, with the spontaneously prolonged luteal function being similar to, and in some instances shorter than, the luteal lifespan of hysterectomized polyestrous species. All or nearly all female caniform carnivore species have photo-entrained annual life-cycles and annual or biennial reproduction. However, the domestic dog, a subspecies of the grey wolf, is an exception and non-seasonal; but, as an exception to the exception, the basenji dog like the dingo, another wolf subspecies, is seasonal, having its cycle in the autumn. The canine obligate anestrus lasts 2-10 months and is terminated by increased GnRH and LH pulsatility. The timing is under multiple regulatory inputs. These include recovery from progesterone effects at variable times after progesterone declines to nadir values; increased dopaminergic and/or decreased opioidergic tones and/or sensitivities, presumably under the influence of an endogenous circannual cycle assumed to persists despite the lack of photo-responsiveness; and, stimulatory pheromonal input from other females (as well as photoperiod in the case of Basenji). The only clear adaptations or unique attributes seen in dogs that are likely beyond what occurred in a more primitive ancestor are two. One, there is a pregnancy specific increase in prolactin that as a potent luteotrophin (as in rodents) acts to enhance progesterone production during pregnancy, which appears likely to be the case in all carnivores. And, two, the bitch has a fertile-mating window as wide as 11 days, and up to 8 days after ovulation. The latter involves the delayed post-ovulatory maturation of oocytes (also seen in foxes), prolonged post-maturation oocyte viability, and a uterine environment hospitable to sperm survival for up to 7 days during estrus. This relative simplicity contrasts to more complicated adaptive strategies like (1) delayed implantation seen in many caniform carnivores (including many mustelids, ursids, and phocid and otarid seals); (2) reflex, induced ovulation (as seen in many feliform carnivores); and (3) prolongation of post-implantation gestation via placental secretion of progesterone (some feliform, some artiodactyls, primates) or gonadotrophin (primates, equids). Also considered in the review are the endocrine mechanisms triggering the LH surge and estrus behavior in dogs, and factors involved in termination of obligate anestrus.

Keywords: anestrus, canine, carnivore, cat, circannual, corpus luteum, delayed implantation, dog, estrogen, estrus, feline, FSH, LH surge, ovary, pregnancy, progesterone, prolactin, pseudopregnancy.

Introduction

This review looks at ovarian function and estrus cycles in dogs from an endocrine perspective and in relation to ovarian activity in other carnivores and mammals in general. The major premise is that, while canine ovarian cycles have unique elements including the timing of oocyte maturation and the slow progression of some events, the underlying mechanisms are likely similar or the same as those demonstrated in more extensively studied species. Further, ovarian function during the canine cycle and pregnancy represents a "basic" form of mammalian ovarian function with few of the many adaptations that have evolved to increase fecundity, accommodate environmental pressures, or longer gestations. The primary resources are the reports on carnivore reproduction that have appeared in past Proceedings of the International Symposium on Canine and Feline Reproduction (Concannon *et al.,* 1989, 1993, 1997a, 2001, 2006, 2009), and other reviews and research reports from our laboratory and others.

The domestic dog and dingo are subspecies of the grey wolf species, *Canis lupus*; the genus Canis consists of three species of wolves (Grey, Red and Ethiopian), three jackal species, and the coyote. The other canid genera include four wild dog species, over 20 species of foxes, and the Asian dhole. Other caniform families in addition to the canids include skunks; badgers, otters, weasels, and other mustelids including mink, polecats and ferrets; seals, walrus and sea lions; raccoons; and bears and pandas. The feliform families in addition to the cats (felidae) include several Malagasy carnivores, mongooses, hyenas, civets, and genets, among others. The felids include the small cats (domestic cats, wild cats, ocelots, cheetahs, lynxes, bobcats, puma and cervals) and the pantherine large-cat

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species (lions, tigers, jaguars, leopards). The importance of the dog as a model species is evidenced by the support and success of the canine genome project financially supported by the National Institutes of Health; furthermore, there are 36 species in the family Canidae of which nine are formally listed as threatened or endangered by extinction (see Songasassen and Wildt, 2002)

Canine ovarian cycle and pregnancy

The ovarian cycle and pregnancy of the domestic dog (C. lupus familiaris) are not only prototypical of those of other canids and most carnivores but also likely exemplify to a large extent the ovarian activity and pregnancies involved in the very early evolution of placental mammals. Domestic dogs are monestrous, non-seasonal spontaneous ovulators with spontaneous luteal function and pronounced progesterone secretion that lasts from 55 to 75 days, average 65 days, and approximately the 2-month duration of pregnancy (Fig. 1 and 2). Thus the CL of the cycle are in most instances sufficient to support a term pregnancy absent support or supplementation by any pregnancy specific mechanisms – a phenomenon critical in a species that has no placental progesterone production or placental gonadotrophin, and a scenario likely present early in mammalian evolution. This physiological pseudo-pregnancy following ovulation is seen in all carnivores studied, either following spontaneous ovulation, or following infertile mating in induced ovulators. It is also comparable to the prolonged luteal phases of hysterectomized individuals in species that have an acute uterine luteolytic mechanism (Fig 3). Although dogs evolved from a photoperiod-entrained seasonal breeder, the primitive wolf, their non-seasonal breeding pattern is potentially similar to the pattern of the progenitor to the original carnivores. Inter-estrus intervals in dogs range from 5 to 13 months, average about 7 months in most breeds, and potentially parallel an endogenous circannual cycle. The pre-ovulatory LH surge and ovulation 2 days later occur after a 1-3 week (average 9 day) period of proestrus. Proestrus is characterized by progressive and eventual full cornification of the vaginal epithelium, serosanguinous discharge of fluid and erythrocytes originating by diapedesis through the uterine capillaries, pheromonal secretion causing increased attraction of males, and progressive and anatomically visible edema and turgor of the vulva and vaginal stroma. All are effects of rising estradiol concentrations. Estrus is concomitant with subsequently declining phase of estradiol concentrations. Estrus begins typically 0-1 days after the LH surge and behaviorally lasts an average of 7 days. Clinical or morphological "estrus" is often defined as ending when the vaginal smears previously characteristic of estrus are no longer predominantly cornified (range days 6-11, average 7.5), with the reminder of the luteal being historically termed metestrus and more recently diestrus. Corroboration is also obtained clinically based on the day that the wrinkling and sacculation of previously smooth edematous and cornified vaginal mucosal folds produced during proestrus ends with folds become visibly less pronounced and appearing more flat and obviously less white (less cornified) with submucosal capillaries again visible as they were prior to the vaginal growth of proestrus - ending typically at 7-9 days after the LH surge.

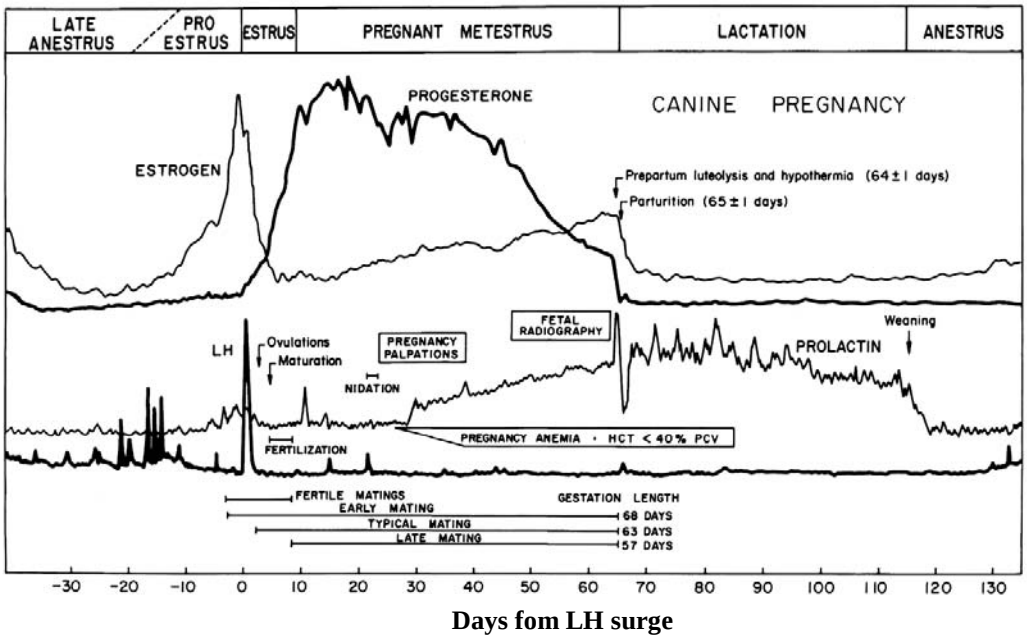


Figure 1. Schematic diagram of changes in circulating estradiol, LH, progesterone and prolactin concentrations during pregnancy in dogs. More recent studies have demonstrated an even more pronounced pregnancy specific rise in prolactin beginning at day 25-30 of pregnancy.

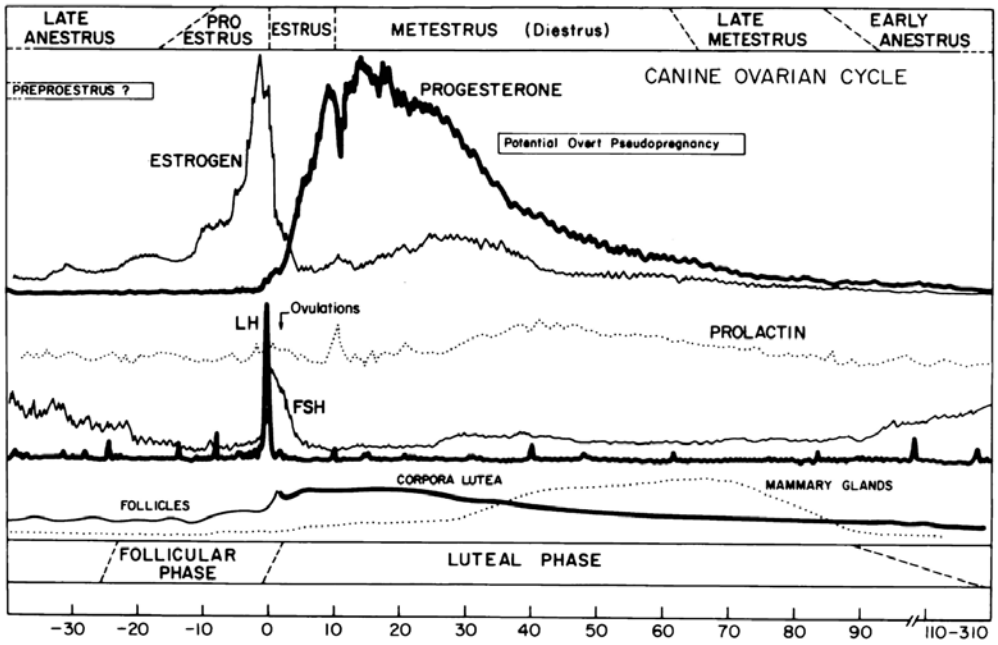


Figure 2. Schematic diagram of changes in circulating estradiol, LH, progesterone and prolactin concentrations during nonpregnant ovarian cycles in dogs.

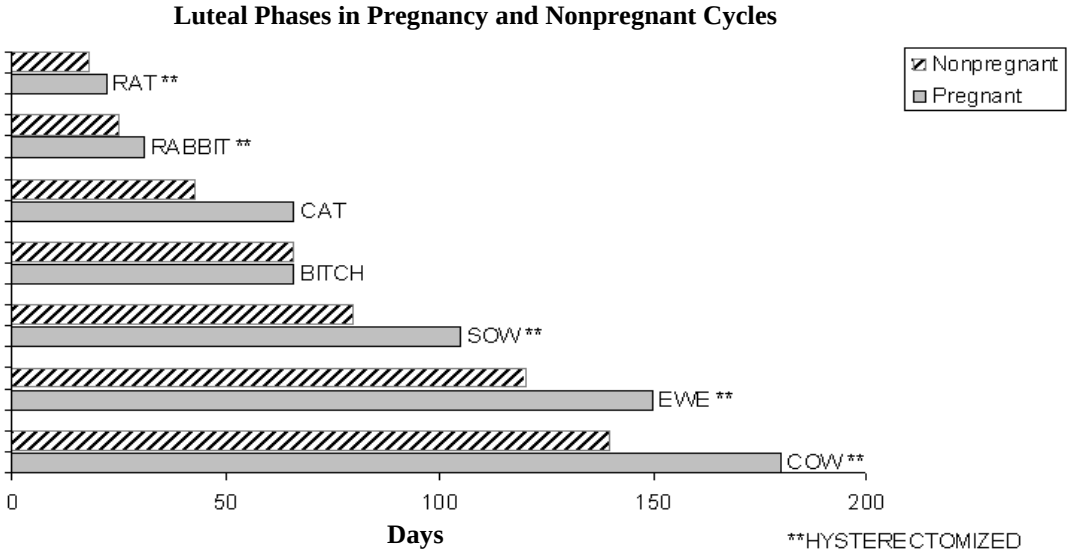


Figure 3. Comparison of the luteal phase duration in the absence of an acute luteolytic mechanism in dogs and in hysterectomized individuals of species that would otherwise have short luteal phases and polyestrous cycles as a result of activation of a uterine luteolytic mechanism in the absence of pregnancy.

Fertility

Dogs are highly fertile, with fertility rates in research and commercial kennels often reaching 95% of mated bitches. Super-fecundation can readily occur when multiple males mate with a bitch before oocyte maturation that is completed in the uterine tubes 2-3 days after ovulation. Mating involves a copulatory lock that lasts one to several minutes (up to 20 min). The high fertility is likely because fertile mating can occur in some instances as early as 5 days before ovulation or as late as 6 days after ovulation and because of prolonged life spans of intrauterine sperm (up to 7 days) and of oviductal oocytes (up to 7-8 days, including up to 5 days after oocyte maturation). Parturition occurs 65 $\pm$ 1 days after the LH surge (day 0), i.e. on average 63 d after ovulation, 64 d after average time of first mating (day 1), and 43 days after

implantation at day 21-22. The same or similar timeframes are seen in most canid species except foxes in which estrus may last only 2-3 days, and pregnancy lasts only 51-53 days, and thus only 35-36 days after implantation at day 16. Plasma progesterone is elevated from the day of the LH surge until 18 h before parturition, peaks around day 20-30 and declines slowly thereafter, is solely of luteal origin, and is dependent on both LH and prolactin secretion. While there is no acute uterine luteolytic mechanism (AULM), exogenous PGF is luteolytic and abortifacient, and endogenous PGF acutely participates in prepartum luteolysis. The circulating levels of progesterone appear to far exceed those required to maintain pregnancy in dogs; in ovariectomized pregnant bitches maintained on exogenous progesterone (Concannon *et al.*, 2001), pregnancy was maintained even though serum concentrations of progesterone were below 2 ng/ml for several days in some animals (Concannon, unpublished).

Follicular phase, estradiol, LH, FSH and progesterone

In dogs, termination of anestrus, onset of proestrus, increased follicle secretion of estradiol, and development of follicles as ovulation-capable follicles all appear due to a pre-proestrus increase in the rate of pulsatile LH release caused by increased GnRH pulsatility (Fig. 4). Whether this event selects a cohort of already-dominant follicles, or whether it first also involves the selection of follicles for dominance, as suggested for other polytocous species (Fortune, 1994), is not known. The possibility remains that during anestrus there are waxing and waning waves of cohorts of large antral follicles that are recruited in response to increases in FSH and from which the more dominate follicles can be selected as ovulatory follicles (Mihm and Evans, 2008), as described for montotocous species. However, there is little or no evidence of waves of follicle development during canine anestrus, and no unique excursions in FSH have been reported, although FSH tends to increase throughout anestrus. Estradiol in canine anestrus has variably been reported as being more variable and on average higher in the month before proestrus (Jeffcoate, 1992) and as becoming lower shortly before proestrus (Olson *et al.,* 1984). In early proestrus, the dominant follicles destined to become the ovulatory follicles are 2-3 mm in diameter and do not protrude above the surface of the ovary but are grossly distinct as being hyperemic and semi-opaque. Proestrus follicles show histological signs of luteinization of the areas of follicle wall many days before the LH surge. During proestrus the follicles become 5-8 mm, and after the LH surge become 9-12 mm by the time of ovulation. One reason to suspect that there are subliminal waves of follicles with increased and then diminished capacity of responding to LH pulses is that, when exogenous GnRH is used to induce proestrus, some bitches fail to respond to elevations in LH comparable to those seen in bitches that do respond (Concannon *et al*., 1989, 1997a; 2006). In anestrus, LH serum concentrations are low $\leq$1 ng/ml, representing only 2-20% of preovulatory peak values, with occasional LH pulses of 2-25 ng/ml typically detected at intervals of 4-24 h. During the week before proestrus pulses maintain their relatively large magnitude but intervals decrease to 60-90 min and mean LH levels are elevated to 3 ng/ml or more (Fig. 5a and b), and distinctly higher than those earlier in anestrus (Olson *et al.*, 1982; Concannon, 1993; Tani *et al*., 1999). During proestrus, LH levels become progressively lower and pulses less detectable or nondectecable due to estrogen negative feedback. LH concentrations increase to peak levels of 4-40 ng/ml (mean 8-15 ng/ml, varying with trials) during the preovulatory surge, with LH peaks typically occurring 1-3 days after the peak in estrogen (Concannon *et al.*, 1975, Nett *et al.*, 1975). In mid and late anestrus, FSH concentrations are elevated, often at or near preovulatory peak concentrations, and average 40% of peak concentrations (Olson *et al.*, 1982; Concannon, 1993). In the week before proestrus, modest concomitant increases in mean FSH that are less pronounced than those of LH (20-50% vs. 300-600% above anestrus levels) and may participate in the onset of proestrus, but appear not to be required as they are minimal in some cycles (Fig. 5b). Basal FSH becomes progressively elevated throughout anestrus without any signs of induced estrogenic activity. It remains unclear in dogs what is the role or bioactivity of the high concentrations of immunoreactive FSH throughout much of anestrus, or the timing of any increased FSH-dependent aromatase activity, or possible changes in FSH-receptor levels. Unclear also are the extent to which pre-proestrus and early proestrus increases in estradiol secretion are the result of increased aromatase synthesis and/or activity or the result of LH-stimulated synthesis of androgen as precursor for already available aromatase. Perhaps in dogs as suggested for pigs there are major autocrine and paracrine changes responsible for development of dominant follicles and their acquisition of LH receptors and expression of IGFBP-4; interestingly the late follicular phase dominant follicles in pigs like the mid-proestrus follicles of dogs likewise show morphological evidence of luteinization (Zhou *et al.*, 1996).

Pulsatile administration of GnRH induces a premature onset of a normal proestrus that progresses to a normal estrus, but so too does non-pulsatile constant administration of GnRH agonist. Therefore, GnRH and LH (FSH) pulsatility are not requirements for normal termination of anestrus. Rather, pulsatiliy likely represents a physiological mechanism whereby mean LH increases but is not subjected to potential down-regulation of the LH response by constantly elevated GnRH. Such downregulation in dogs is a slow,

progressive phenomenon that may be important in estrus induction protocols using higher doses of GnRH-agonist implants. In bitches it takes 3-4 weeks for GnRH down regulation to fully suppress LH levels to basal anestrus-like concentrations (Fig. 6). In contrast, agonists induce proestrus within 3-4 days and estrus and ovulation typically occur within 12-15 days even with low-moderate doses of agonist.

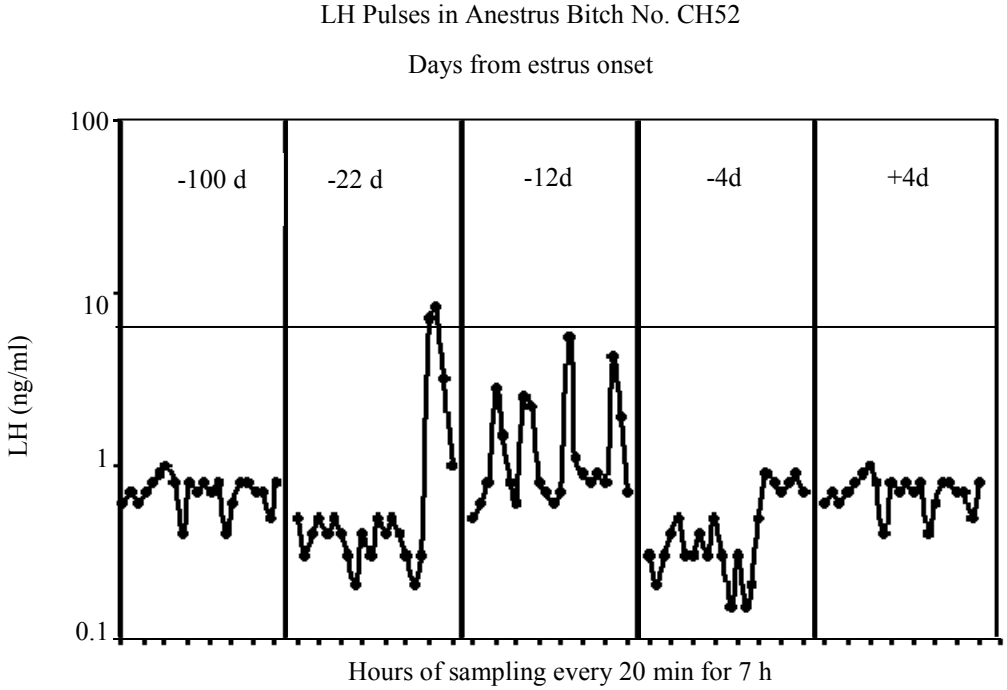


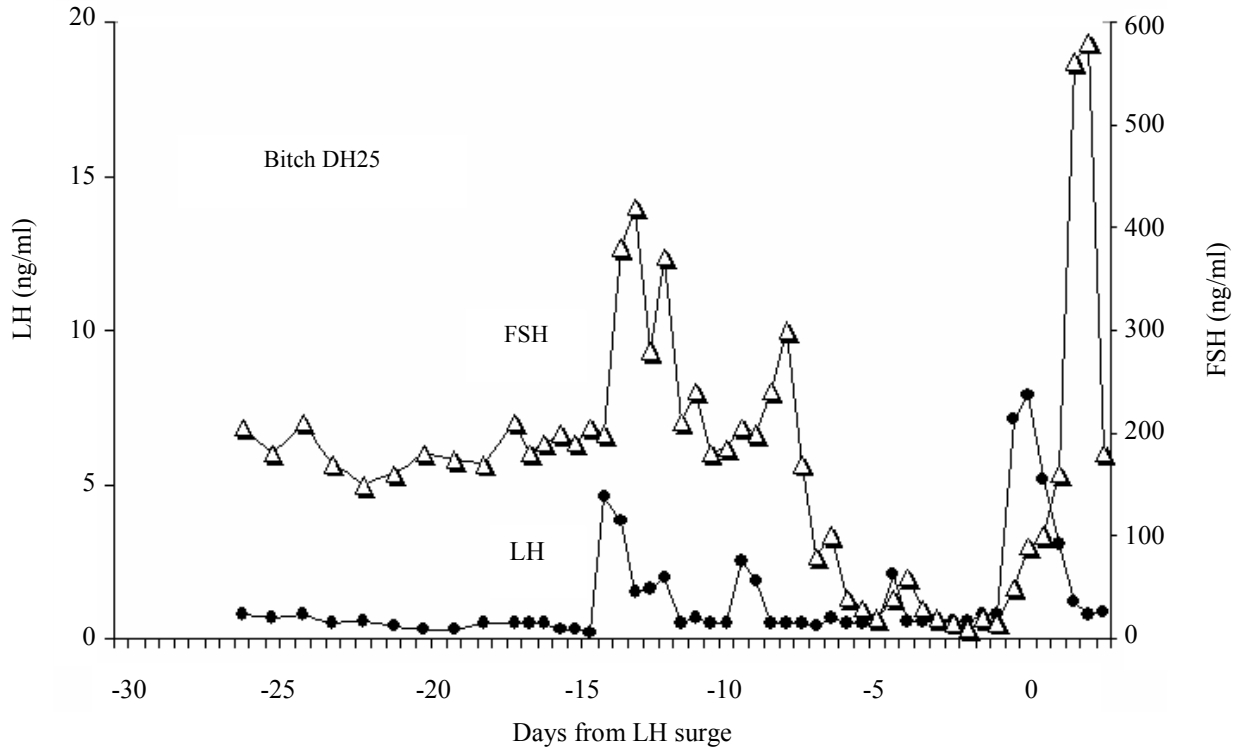
Figure 4. Concentrations of LH in serum samples collected every 20 min for 7 h in a beagle studied on selected days during anestrus, proestrus, and estrus and shown in relation to the day of preovulatory LH surge.

That the increases in LH are more important than those in FSH in the onset of proestrus is suggested by observations that frequent injections of purified porcine LH induced proestrus resulting in fertile estrus in anestrus bitches, while administration of FSH did not (Verstegen *et al.*, 1997).

Estradiol increases throughout proestrus, rising from basal 5-10 pg/ml to reach peaks of 45-120 pg/ml that occur in most instances 1-3 days before the preovulatory LH peak, and 0.5 to 2 days before the onset of the LH surge. The LH surge involves a rise lasting 12-36 h and a subsequent decline of 12 to 24 h, and lasts an average of 1.5 days (ranges 24- 72 h). There are simultaneous increases in FSH and LH during the preovulatory surge, with the elevation in FSH typically resulting in a peak 0.5 –1 day after the peak in LH and a phase of decline to baseline 1-2 days longer than that of LH. The rise in FSH is often only equal to mean concentrations observed in during anestrus, whereas that of LH is typically 10-100 fold higher than means anestrus concentrations.

Serum progesterone rises slowly during proestrus, from basal values of 0.2-0.4 to pre-LH surge levels of 0.6-0.8 ng/ml reflecting partial luteinization of follicles visible histologically as early as 6 days before the LH surge. The rise in progesterone, presumably of follicular origin and representing excess precursor production for estrogen synthesis may begin as early as 1-2 weeks before proestrus (Fig. 7), based on mean progesterone concentrations in early studies on canine anestrus (Concannon, 1993). Progesterone then rises acutely and rapidly concomitant with the onset of the LH surge such that the first acute rise in progesterone cannot be separated from the initial rise LH. The mid and late proestrus follicular phase is potentially autonomous or semi-autonomous, with intrafollicular estradiol being folliculotrophic and with the fate of dominant follicles being predetermined as either atresia or ovulation as the endogenous capacity to further increase estradiol secretion becomes limited. LH and FSH concentrations are both depressed to the lowest levels of the cycle in late proestrus, presumably due to estrogen and inhibin negative feedback. Preliminary study has shown that inhibin concentrations parallel those of estradiol in proestrus and estrus and likely participates in negative feedback during proestrus (Concannon, unpublished). However, GnRH agonist induced proestrus rarely proceeds to full estrus if agonist is discontinued before 8 days, suggesting that a minimum of 7 days of elevated gonadotrophin in generally required to produce autonomous or semi-autonomous ovulatory follicles that can trigger an LH

surge 3-4 days later. Presumably increasing intrafollicular progesterone causes decreases in the ER:PR ratio in follicle cells, as suggested in other species, and participates in the ovulatory process.



Figuire 5a. Changes in LH and FSH during anestrus and proestrus in a bitch with increases in LH 5-8 fold higher than previous mean concentrations, as well as increases in FSH 1-fold higher than in anestrus. This is an example of transiently increased mean LH levels that are typically 300-3000% greater than those earlier in anestrus, occurring as a result of a late anestrus increased frequency of LH pulses of near preovulatory peak magnitude.

Estrus, LH surge, ovulation and sex behavior

Ovulation of immature oocytes occurs 48-60 h after the LH surge. Both the LH surge and overt estrus sex behavior begin either at or, more often, immediately after the peak in estradiol (Concannon *et al.*, 1975; Olson *et al.*, 1982; Concannon, 1993). The precipitating event is the rapid spontaneous decline in the E:P ratio as estradiol attains peak concentrations and/or begins to decline. In some cycles, there may be a secondary rise in estradiol during the LH surge to near peak or peak levels, but at a time when progesterone is rapidly increasing and the E:P ratio declining. The late proestrus spontaneous rise in progesterone and the subsequent initial LH surge-promoted acute rise in progesterone both exacerbate the decline in E:P ratio. Progesterone thereby participates in facilitating both the onset and completion of the LH surge. Increased progesterone is assumed to be among the signals for initiation of oocyte maturation and changes in the pathways leading to the increased intra-follicle PG production required for ovulation as reported in other species. The latter include decreases in cytosolic ER and PR, and binding of progesterone to PMRs (progesterone membrane receptors). It is possible that a gonadotrophin surge regardless of whether it is predominantly or nearly exclusively one of LH or of FSH in activity can elicit ovulation, considering that FSH deficient and LH deficient surges have been identified in a few normal cycles and in some GnRH-agonist induced fertile cycles. The extent to which ovulation has the potential to occur spontaneously at the end of the follicular phase, even though almost always facilitated or precipitated by a ovarian-signaled, properly timed gonadotrophin surge is also not known.

The evidence supporting a critical role for the late proestrus decline in the E:P ratio as the ultimate trigger for LH surge release and onset of estrus behaviors involves the temporal relations among these changes (Fig. 8) as well as studies in ovariectomized bitches administered estradiol with or without subsequent progesterone. The onset of estrus behaviors typically is coincident with the LH surge, but may occur 1-4 days later, and in some instances never occurs despite normal hormone profiles and fertile ovulation demonstrated by AI. The major component of the LH surge, the LH peak, and

estrus onset typically occur 1-3 days after the peak in estradiol, and 0.5 to 1 day after the first detectable acute rise in preovulatory progesterone to ≥0.9 ng/ml. Why estrus-onset is disconnected from LH surge by several days in some cycles is not known but reflects that that different neural pathways with at times different sensitivities to changes in E:P ratio are involved. The rising phase of the LH surge almost always occurs following or during an abrupt decline in estradiol; and, the detection of the onset of the rising phase of the LH surge in samples obtained at 4-8 h intervals cannot be separated from onset of the acute rise in progesterone to ≥ 0.9 ng/ml and typically to 1-2 ng/ml.

In ovariectomized bitches receiving low-dose estradiol-releasing implants and increasing sequential i.m. doses of estradiol benzoate for several days, LH was suppressed to low levels throughout the continuous increases in serum estradiol, even with estradiol concentrations (200-400 pg/ml) well above those of normal proestrus (50-110 pg/ml). When injections stopped, the decline in estradiol was accompanied by preovulatory like surges in LH that were low but within the normal range. In bitches administered progesterone releasing implants at the time that estradiol injections ceased, the progesterone facilitated LH surge release and the surges were significantly higher, more abrupt and synchronized, and in the upper range time of normal preovulatory LH surges. In the few bitches in which a preovulatory like LH surge occurred prior to discontinuation of estradiol injections, subsequent study of the serum estradiol profiles revealed that, in those bitches, serum estradiol declined prematurely while increasing doses of estradiol benzoate were still being administered during the last day of treatment. In those and the other bitches LH surges were initiated only after a decline in estradiol.

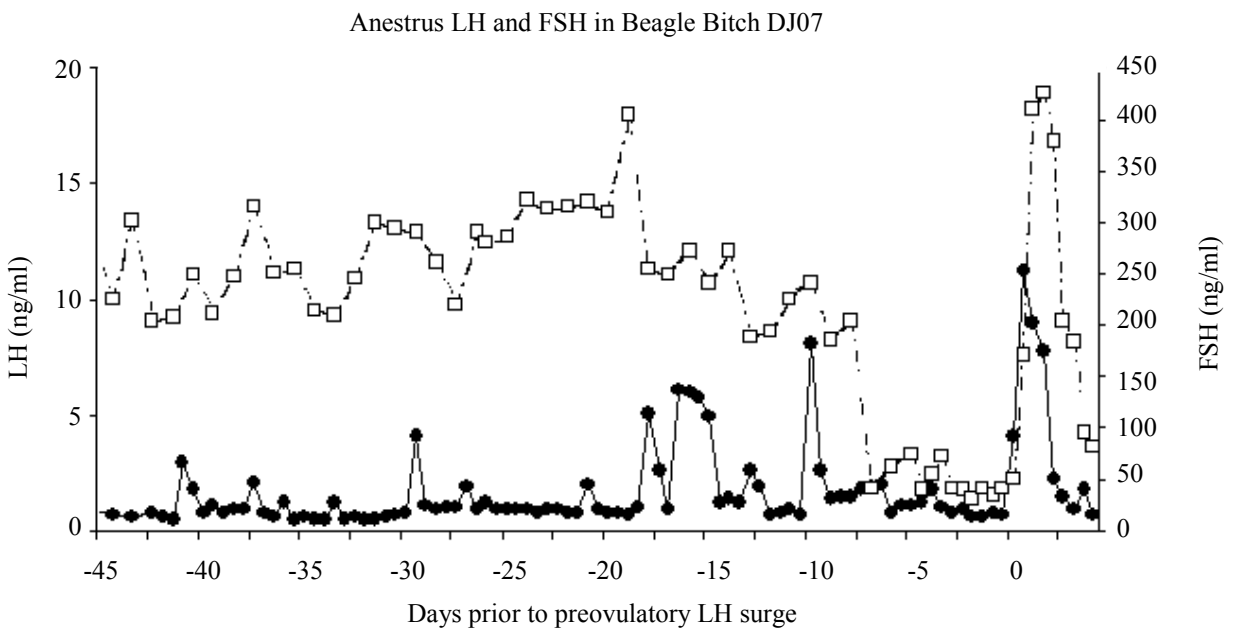


Figure 5b. Changes in serum LH and FSH concentrations in late anestrus and proestrus in a bitch with 4-8 fold increases in LH - and an example of typical cycles in which there is a late anestrus increased frequency of LH pulse of near preovulatory peak magnitude and increased mean LH levels 300-3000% greater than those earlier in anestrus. In this bitch there was no persistent or obvious increase in FSH observed, whereas in some instances FSH increase 50-100% of anestrus values (see Fig. 5a).

In related studies administration of estradiol implants to ovariectomized bitches yielded proestrus behavior, and resulted in modest estrus behavior scores following estrogen withdrawal. When administration of progesterone implants accompanied estrogen withdrawal, estrus behavior scores abruptly increased within 8 h to those typical of normal estrus, increasing not only receptivity (tail deviation, lordosis) but also proceptivity and interest by the males. It is likely, then, that fall in the E:P ratio also has an effect on the amount or composition of vaginal secretion of pheromone(s) responsible for male attraction.

Progesterone metabolite as well as progesterone may play a role in the decline in the E:P ratio effecting the preovulatory LH surge. In sheep, PMRs are expressed in both the pituitary and hypothalamus. PMR binding either progesterone or 17α-OH progesterone causes intracellular calcium mobilization in PMR expressing cells in vitro (Nett, 2007) and in sperm.

We have previously reported that there is a pronounced preovulatory rise in 17α-OH progesterone simultaneous with the rise in progesterone, both slowly throughout proestrus and then acutely at the onset of the

LH surge (Fig. 9). Therefore, in dogs, the late proestrus conditions causing the preovulatory surge release of LH likely include pituitary and hypothalamic effects of these increases in both progesterone and 17α-OH progesterone acting via PMRs, both in triggering and sustaining the LH surge. The continued slow rise in both moieties just before surge onset would participate in facilitating the surge-triggering action of the decline in E:P primarily effected by peaking estradiol. The late proestrus increases in 17α likely supplement the facilitating effect of the simultaneous increases rise in progesterone on the timing and magnitude of the LH surge.

In the above view of the regulation of LH surge release as well as estrus behavior involving a priming or potentiating by rising estradiol, and triggering by a decline in the E:P ratio, the target cells are viewed as being responsive not simply to estrogen concentrations or any particular threshold estrogen concentrations, but to rather the rate of change (first derivative) dE/dt , and the rate of change in E:P ration, dE:P/dt (Fig. 8 and 10a, b).

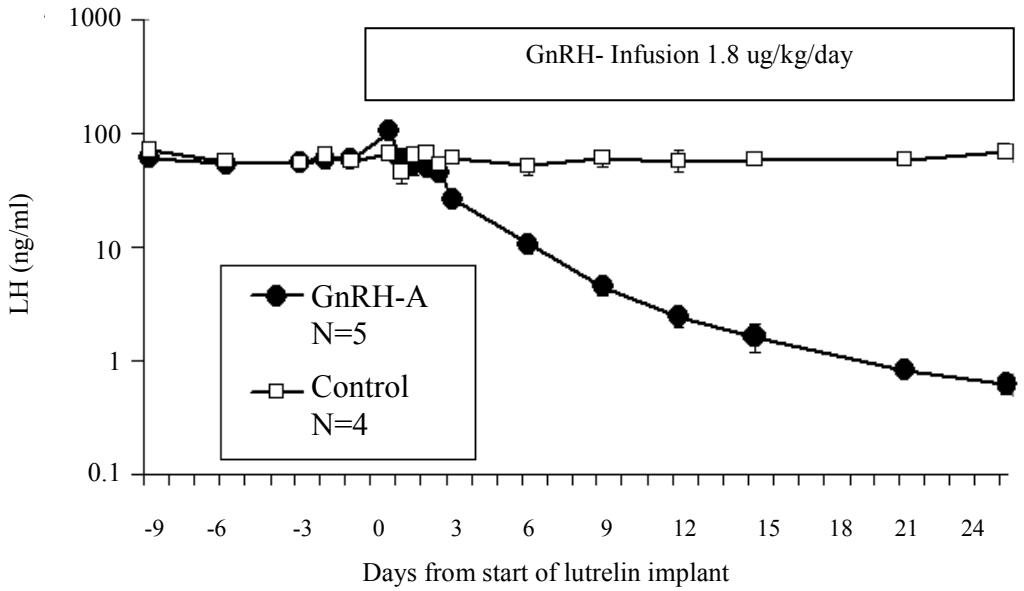


Figure 6. Concentrations of serum LH during down-regulation of LH secretion in ovariectomized beagle bitches by chronic s.c. administration of the GnRH agonist aza-gly-nafarelin by osmotic minipump.

Luteal phase, luteotrophic hormones, and pseudo-pregnancy

The initial rises and then slow declines in progesterone during nonpregnant and pregnant cycles are accompanied by small increases in estradiol after the end of estrus that are presumably of luteal origin and often more evident in pregnant than nonpregnant bitches. The extent to which canine luteal function cell differs significantly if at all from that described in eloquent detail for the rat by Stocco *et al.* (2007) is not known. In rats, small and large luteal cells differ little if at all in cellular origin and function and thus different from the situation described in ruminants and primates, and the origin of rat luteal cells as being from granulosa versus luteal cells is not clear. The large luteal cells of rats are distinguished by their expression of PRAP (PRL-R associated protein) which interestingly is only expressed in theca cells of mature follicles. Follicle luteinization in dogs both during and immediately following the LH surge appears histologically to involve a dramatic loss of granulosa cells morphological integrity and an ingrowths of cells with a morphology the same as the theca interna cells, suggesting theca cells may contribute in large part to luteal formation in this species. Normal luteal function in bitches requires both LH and prolactin as luteotrophic hormones throughout most if not all of the 2 months of elevated progesterone in both pregnant and nonpregnant bitches. CLs are the only source of progesterone in pregnancy and premature luteolysis and abortion occurs with administration of PGF at high doses repeatedly, progesterone antagonist, GnRH antagonist, or prolactin lowering doses of dopamine agonist. The luteal phase is negatively impacted and pregnancy terminated by GnRH antagonist treatments as early as day 12 and any time thereafter, or by continuous LH-down-regulating GnRH-agonist treatment (Valiente *et al.*, 2008a, b). Administration of LH antiserum can cause acute transient reduction in progesterone secretion. (Concannon *et al.*, 1993). LH but not FSH or prolactin acutely stimulated progesterone synthesis by luteal cells in vitro. Prolactin is also clearly luteotrophic by day 12, at which time prolactin lowering doses of dopamine

agonist severely reduce progesterone; PRL quickly becomes a required luteotrophin by day 25, after which time administration of dopamine agonist cannot only reduce but can also permanently or transiently terminate luteal function, shortens the luteal phase, and be abortifacient. After day 25, pregnancy-specific increases in prolactin concentrations greatly increase total progesterone production and extend the functional life of luteal tissue to the end of gestation even in animals in which the luteal phase of the cycle would be otherwise shorter. It is unclear whether, or not, the pregnancy specific rise in prolactin is initiated and maintained by the placental relaxin secretion that begins simultaneously between day 25 and 30 (Fig. 11) and persists throughout pregnancy (Concannon *et al.*, 2001). Relaxin in bitches appears to be entirely of placental origin, and relaxin concentrations observed for varying periods post-partum in some bitches are likely due to retention of placental tissue in endometrial crypts. Reports on both pigs and monkeys suggest a direct PRL-releasing role for relaxin (Bethea *et al.*, 1989).

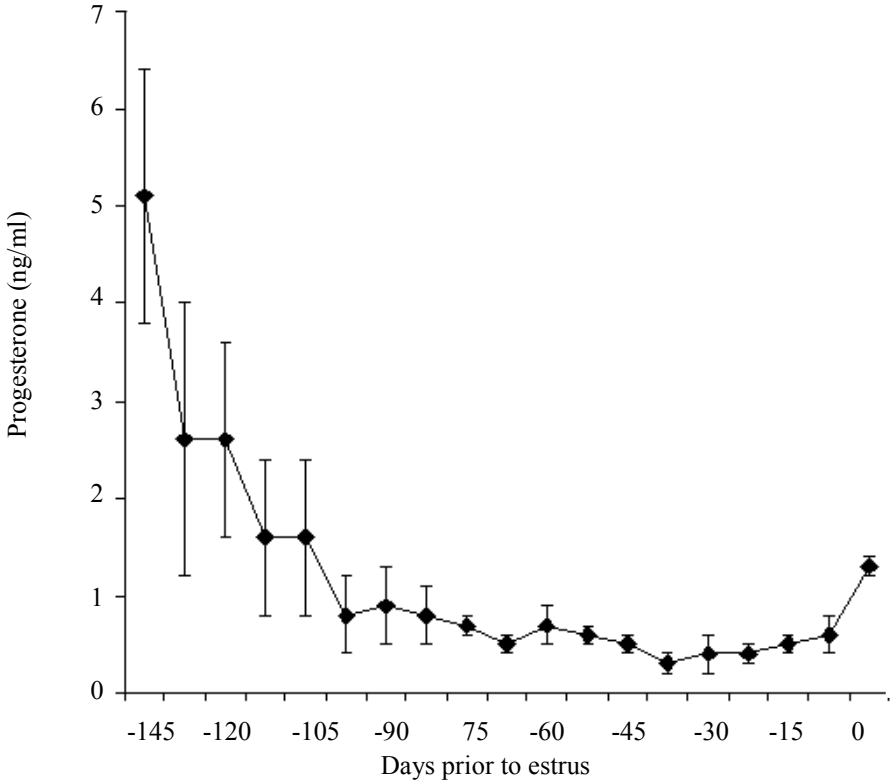


Figure 7. Mean concentrations of plasma progesterone during late metestrus, anestrus and proestrus in bitches, shown in relation to the day of the preovulatory LH surge.

Prolactin-stimulated luteal function and increased progesterone production during pregnancy is likely several-fold that of the nonpregnant cycle, although there is no significant effect on peripheral progesterone concentrations due to dramatically increased progesterone metabolism and dilution by an increased plasma volume of distribution that would otherwise be expected to result in a 25% or greater decrease in circulating hormone concentration. Luteal phase hepato-biliary clearance of progesterone from the circulation is twice as high in pregnant than in nonpregnant bitches based on profiles of fecal progesterone concentrations. Increased uterine mass and mammary gland activity along with the growing placenta likely remove far greater amounts of progesterone from the circulation than occurs in nonpregnant cycles. In preliminary studies, progesterone releasing silastic implants resulted in 3 times greater progesterone concentrations in nonpregnant or ovariectomized bitches than was achieved using the same implants in pregnant bitches in which progesterone was maintained by the exogenous progesterone alone (Concannon *et al.*, 2001; Concannon, unpublished). Fecal estrogen and testosterone profiles suggest that there are also pregnancy specific increases in luteal secretion of these steroids as well (Gudermuth *et al.*, 1998). And, the number of aromatase positive cells in canine CL increase between mid and late gestation (Nishiyama *et al.*, 1999).

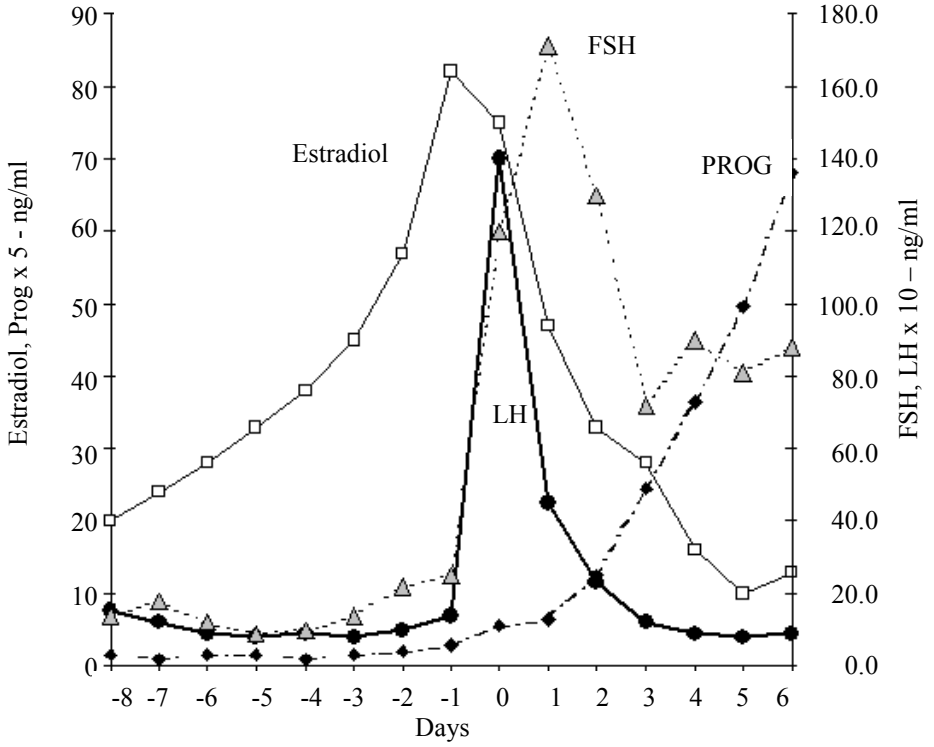


Figure 8. Mean concentrations of serum estradiol, progesterone, LH and FSH in 8 beagle bitches during proestrus and estrus shown aligned to the day of the preovulatory LH surge. Mean time of estrus onset was 0.8 ± 0.5 days after the LH surge.

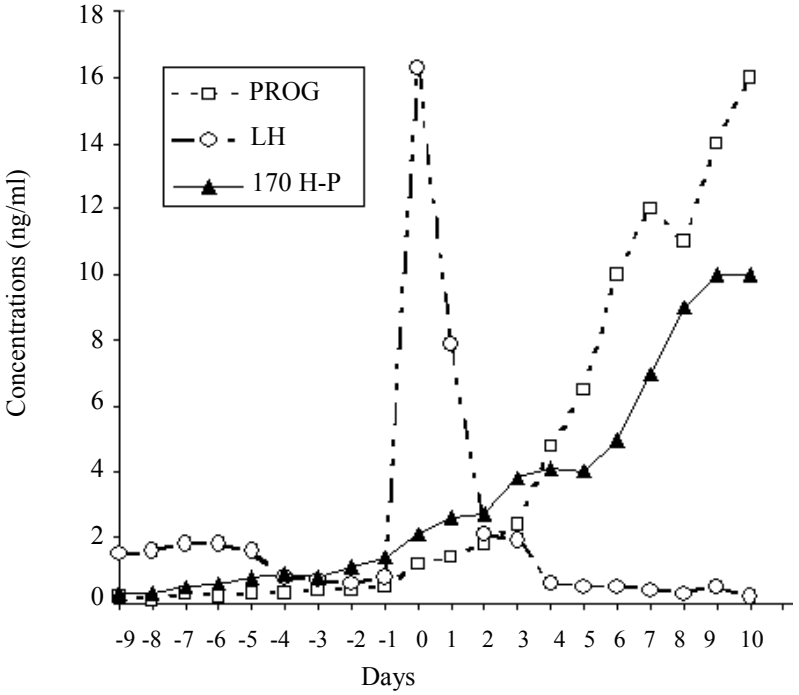


Figure 9. Concentrations of progesterone, 17α-OH progesterone and LH in serum of a beagle bitch during proestrus and estrus.

LH, prolactin and progesterone as luteotrophic hormones

In the dog, LH is assumed as in many other species to act primarily via adenyl cyclase and PKA to increase StAR protein expression, increase expression or activity of various steroidogenic enzymes involved in androgen synthesis, and increasing numbers of gap

junction, but direct studies on canine CL are limited. In vitro, canine luteal cell progesterone production increased acutely with addition of LH, DB-cAMP or phorbol ester, but not prolactin or FSH (Concannon, 1993; Concannon and Milvae, unpublished). StAR and 3bHSD expression occur throughout the luteal phase. The luteotrophic role of prolactin is assumed to be similar to that reported in rodents, and to include that of increased numbers of LH receptors, but could also include as described for rodent models multiple other effects including increasing P450ssc, 3B-HSD, HDL-R, and progesterone membrane receptors (PMR). An effect on PMR could play a significant part in the luteotrophic effects of prolactin since progesterone itself may also be luteotrophic in dogs. The latter is suggested by altered ovarian vascular function, reduced progesterone secretion, hastened luteal regression, and/or reduced cycle length following administrations of progesterone antagonist during the luteal phase or pregnancy (Concannon *et al.*, 1990; Galac *et al.*, 2004; Fieni *et al.*, 2006). Canine CL can synthesize estrogen as well as progesterone, and both could have autocrine effects as the cells also have ER-alpha and PR classic nuclear receptors (Hoffmann *et al.*, 2004). Progesterone has luteotrophic and antiluteolytic properties in other species, and suppresses luteal PGF and PGF-R (see Cai and Stocco, 2005). Progesterone as an autrocrine luteotrophic agent in dogs could be acting via either cytosolic PR or via PMR. Although canine luteal cells, unlike those of the rat, have classical PR, whether, or not, canine luteal cells also possess one or more of the multiple PMR transcripts reported for rat luteal cells that are partially regulated by prolactin (Stocco *et al.*, 2007) has not been reported.

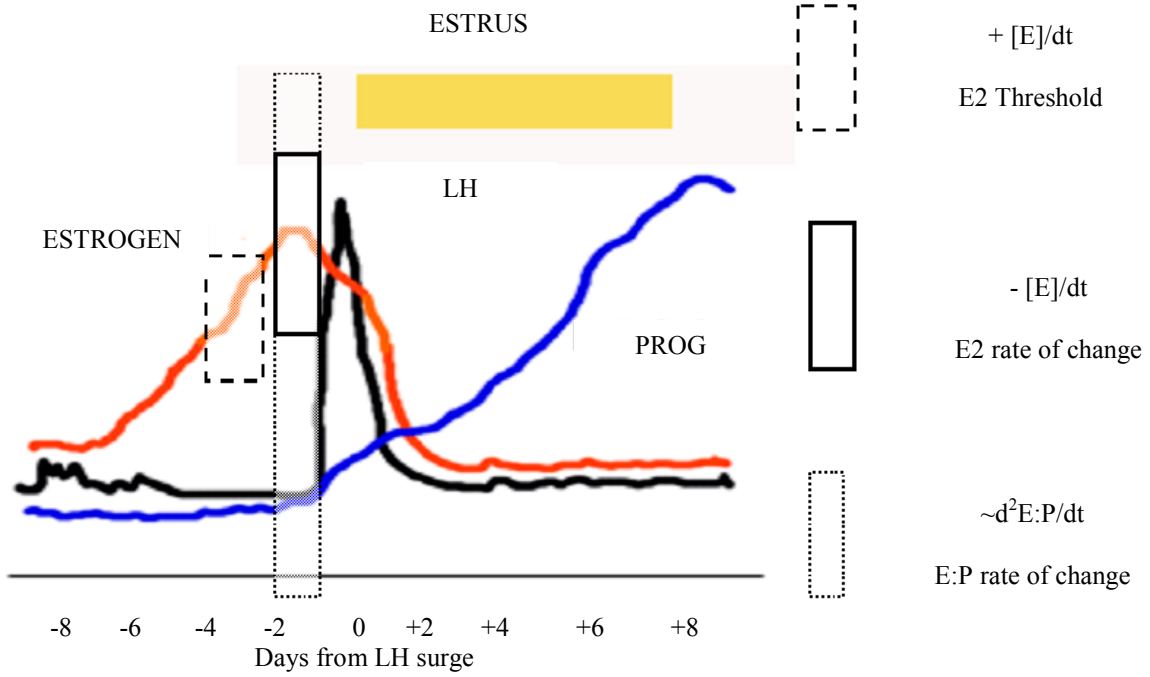


Figure 10a. Schematic representation of changes in serum concentrations of estradiol, progesterone and LH typically observed during proestrus and estrus in dogs, and illustrating the association the preovulatory LH surge with a rapid decline in the E:P ratio. The E2- threshold concept (dashed box) is not compatible with the observations that the LH surge occurs variably following estradiol peaks as low as 30 in some cycles and as high as 110 pg/ml in other cycles. In contrast, the timing of spontaneous LH surges, and results of studies evoking LH surges in estradiol and progesterone treated ovariectomized bitches are compatible with the trigger for the LH surge in dogs being a decline in the rate of change in the E:P ratio (dotted line box). The latter is variably effected by a decline in the rate of increase in estradiol, an estradiol peak, or in most cases an actual post-peak decline in estradiol (solid box), and greatly enhanced by rise in progesterone that is inseparable from the onset of the LH surge.

Luteal regression

In pregnant as well as nonpregnant bitches, luteal function based on plasma progesterone profiles after day 20-30 slowly wanes over a 30 to 50 day period, although a secondary increase beginning between day 25 and 35 occur in some but not all pregnancies. In nonpregnant cycles, the slowly waning luteal phase approximating gestation length is in that sense comparable to those in other species with no AULM and those seen following hysterectomy in artiodactyls and rodent species that have evolved an AULM. It therefore represents the inherent capacity of the corpus luteum as an ephemeral organ that evolved to

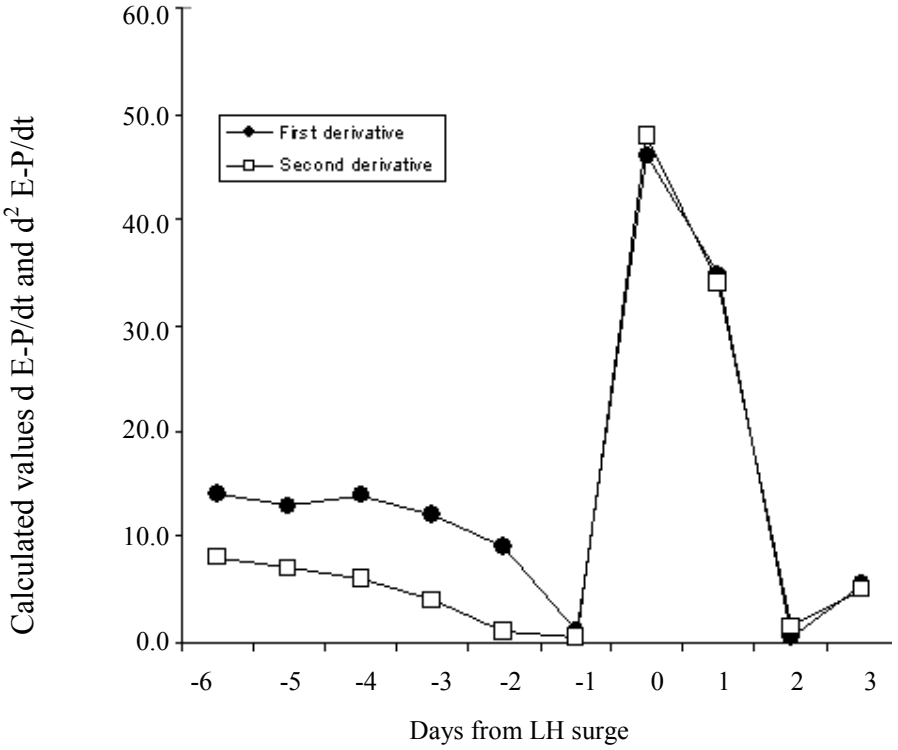
support gestation via progesterone section, with little or no evidence of the changes associated with acute luteolysis in polyestrous species. Hysterectomy does not affect luteal phase length or progesterone concentrations except transiently (Olson *et al*., 1984). Canine luteal regression has been extensively studied by Hoffmann, Kowalewski and colleagues in Giessen (Kowalewski *et al*., 2006; 2008). The slow decline in progesterone is associated with progressively reduced expression of 3ß-HSD after day 15, and little or none of the more pronounced changes observed during rapid luteolysis in polyestrous species or after PFG administration. Endogenous PGF appears not to be involved: luteal COX1 activity is low throughout the luteal phase; COX 2 activity only increases during rapid growth and hypertrophy the first week or two after the LH surge, is reduced by week 3 and low thereafter. Finally, although PGF-R (FP) is constitutively expressed (explaining sensitivity to exogenous or prepartum utero/placental PGF), there was no evidence of luteal expression of PGF synthetase. Although the canine endometrium has low but detectable capacity to produce PGF in vitro (Luz *et al*., 2006), that does not impact the CL *in vivo* since hysterectomy is without substantial effect. The immune system may be involved to some extent; CD8-positive immune cells and MHC II-positive immune cells increased from day 45 to days 60-75 in one report; however, expression of luteal immune cell cytokines including TNF and ILF were not altered throughout the luteal phase. Although apoptotic figures are never very prominent, some low level of apoptosis is involved, with caspase-3 activity detected around day 65, and increases in DNA fragmentation apoptotic index and relative expression of the pro-apoptotic Bax gene in latter stages of the luteal phase. Slow luteal regression is also associated with increased VEFG expression between day 10 and 50 (Mariani *et al*., 2006); but, whether that is a result or cause of decreased luteal function not known, considering the likely luteotrophic effect of progesterone. Luteal cells develop rapidly from progenitor theca and/or granulosa cells that by hypertrophy and along with ingrowing capillaries and stoma eventually fill the follicular/luteal antrum by day 10-15 in most cases. They are preprogrammed to survive the better part of the length of gestation if not longer in dogs and likely in all species that do not have a major placental source of progesterone or placental gonadotrophin. In dogs, LH and prolactin receptors appear to be maintained throughout luteal cell lifespan and there are no declines in either hormone that would explain the slow retrogression (Fernandes *et al*., 1987). Whether or not there are factors protective of the CL from earlier demise other that the presence of receptors for several luteotrophic hormones and absence of an AULM in canids and in hysterectomized polyestrous animals appears to be unstudied.

In pregnant bitches there is a rapid prepartum luteolysis that involves feto-placental PGF release that causes an abrupt decline in progesterone beginning 12-24 h prepartum and reflected in a rise in plasma PGFM before and during the decline in progesterone to below 1 ng/ml. The fall in serum progesterone is not only a prerequisite for parturitions via direct disinhibition of myometrial activity, but it also triggers an acute rise in serum prolactin that may also somehow participate in the cascade of events critical for normal partition. In rats, the administration of a prolactin receptor antagonist can disrupt and/or delay partition independent of its ability to reduce oxytocin secretion (Nephew *et al*., 2007).

Luteal phase pseudopregnancy and acromegaly

The approximately two-month luteal phase in dogs is referred to as a physiological pseudo-pregnancy not only because it occurs spontaneously (as opposed to the comparable pseudopregnancy in rodents induced by mating), but also to distinguish it from the clinical condition(s) that may be associated with it and are referred to as clinical pseudopregnancy or overt pseudopregnancy. The clinical condition typically involves an associated mammary development grossly exaggerated compared to the modest but palpable increase in mammary size that occurs in every normal luteal phase in dogs. The incidence of overt pseudopregnancy is not well documented, but has been suggested as ranging as high as 5-13% in some reviews, and considerably higher in others especially if only modest forms of the condition are included. In two research beagle colonies, the incidence was well below 5%, but beagles are one of the breeds considered less disposed to the condition. The reported changes are sometimes comparable if not indistinguishable from those of late pregnancy or lactation depending on the amount of serous or milk-like fluid production, secretion and let down that occurs. It may also, or instead, involve development of prepartum and postpartum maternal behaviors comparable to those seen in pregnant and/or lactating bitches as the presenting complaint by owner. Susceptibility appears to occur within some individuals more than others, and some breeds are more predisposed than others. While clinically a problem in pet dogs, this biology likely represents the capacity for spontaneous, typically seasonal, communal nursing in some carnivores including mongooses and some wolves, and associated with seasonal increases in prolactin. Overt pseudopregnancy in dogs is prolactin dependent and treated clinically with dopamine agonists. In most cases plasma PRL is elevated above normal and likely involved in the etiology of overt PSP (Tsutsui *et al*., 2007). Apparently a premature and/or more abrupt than normal decline in progesterone occurs and triggers prolactin release and/or increases mammary activity similar to the effects of progesterone withdrawal at parturition (Tsutsui *et al*., 2007).

Another idiosyncrasy of canine luteal function is that the luteal phase rise in progesterone in some older bitches can cause symptoms of acromegaly and insulin resistance due to the hyper-secretion of growth hormone. It is comparable to the elevated GH involved in the acromegaly, insulin resistance and mammary tumor development observed in bitches treated with contraceptive progestins such as medroxy-progesterone acetate (Concannon *et al.*, 1980a; McCann *et al.*, 1987). The hypersecretion of GH is not only from pituitary somatotropes but also, and likely predominantly, from GH-secreting mammary gland cells (Mol *et al.*, 1996); further, mammary expression of GH is not unique in dogs and occurs in humans and rats.



Fgure 10b. Rate of change in a calculated relationship for serum estradiol and progesterone during late-proestrus and early estrus in bitches. The pattern showns a potential pattern of LH secretion as a single function of sensitivity to increasing and then decreasing estradiol negative feedback, with progesterone treated as an estrogen antagonist and thus a facilitator of responses to any reduced rate of increase in estradiol. F1=d[E(pg/ml)-0.1xP(ng/ml)]/dt. F2=d2F1/dt.

Prolactin secretion

In bitches, as in other species, prolactin secretion is primarily under the negative control of dopamine and suppressed by dopamine agonists. The latter are used for treatment of pseudopregnancy, for termination of pregnancy, and for reducing the size of mammary tumors before surgery for tumor or mammary excision. TRH releases prolactin as in most species. In dogs, progesterone also negatively affects prolactin secretion. Prolactin becomes acutely elevated at 1-day prepartum at the time of prepartum luteolysis, and overt clinical pseudopregnancy occurs with moderate frequency in bitches following removal of ovaries during the luteal phase. That progesterone withdrawal is the stimulus for these elevations in prolactin and/or increased prolactin activity is supported by observations that prolactin is often acutely elevated by administration of a progesterone antagonist during the luteal phase, by ovariectomy during the luteal phase, and during the decline of progesterone during PGF-induced luteolysis. Not known is whether, or not, progesterone also modulates prolactin receptors in reproductive tissues in dogs. Interestingly, in some species it has been shown that progesterone suppresses expression of prolactin receptors both in mammary gland and in neurons associated with maternal behavior, and in most species studied progesterone withdrawal at parturition is the stimulus for the onset of lactation. The potential effect of relaxin during pregnancy is discussed elsewhere.

Steroid profiles and steroid metabolism

The aforementioned rise and subsequent decline in serum estradiol in some ovariectomized bitches while continuing to receive increasing doses of estradiol benzoate raises the question as to what are the factors involved in the late-proestrus peak and initial decline in serum estradiol triggering the LH surge. Presumably, estradiol concentrations reaching peak

levels and then beginning to plateau or decline is a function of the development of proestrus follicles to a stage of ovulatory competence, and likely represents a means to signal that stage of development to the H-P axis. However, there must also be a component provided by increased metabolism of the estradiol, both by increased liver microsomes and by increasing mass of estrogen responsive tissues in the reproductive tract. The liver effectively clears the majority of estradiol from the blood during a single pass of the circulation and any significant amount of estrogen-induced increase in steroid catabolism would be expected to impact circulating concentrations of the hormone. The role of increased metabolism in dogs is also born out by the fact that in preliminary studies in ovariectomized steroid treated bitches, the percent increases in serum estradiol were routinely far below those of the increasing estradiol dosages being administered. Likewise, increases in peripheral concentrations of progesterone may only partially reflected the extent of increases in actual progesterone secretion, as mentioned above in regards to canine pregnancy and increased peripheral and/or hepatic clearance.

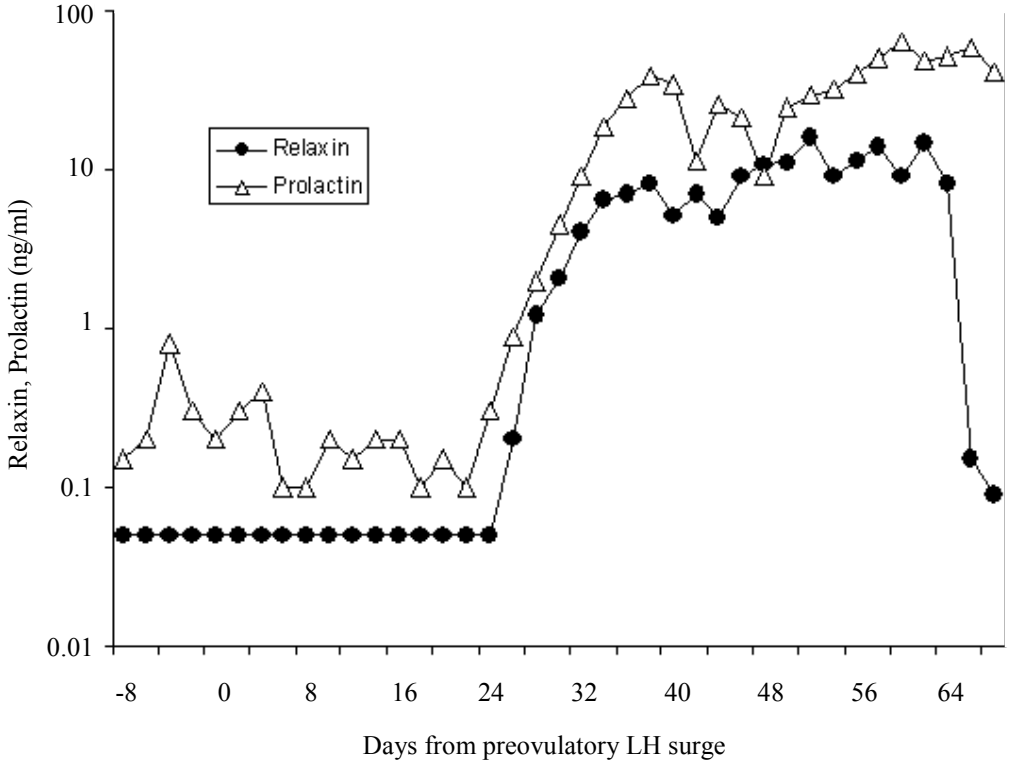


Figure 11. Mean concentrations of relaxin, fibrinogen and prolactin during pregnancy in beagle bitches, shown on a log scale so as to clarify the extent of coincidence in the rise of relaxin and the initial pregnancy specific increase in prolactin. The simultaneous dramatic rise in relaxin at the time that prolactin first appears to be elevated pregnancy-specifically above preceding mean concentrations supports, along with reports of relaxin mediated increases in prolactin secretion in monkeys and pigs, a signaling role for relaxin in pregnancy specific elevated prolactin secretion in dogs.

Anestrus and monoestrus cyclicity

In obligate-anestrus species like the dog, as well as seasonally monoestrus species, a combination of factors that normally function to suppress the H-P axis and LH pulsatility during anestrus become diminished or less effective shortly before the spontaneous onset of proestrus. There is an absolute requirement for cessation of luteal function and/or declines in peripheral progesterone to concentrations no longer suppressive to the H-P-axis. Whether intraovarian concentration of progesterone is important in regulating ovarian follicle responsiveness has not been studied in dogs. Also potentially involved are alterations in hypothalamic opioidergic, serotonergic and/or dopaminergic activity that might increase GnRH pulsatility either directly or as a result of effects on prolactin secretion. Increased GnRH pulsatility might be elicited directly or indirectly or modulated by changes in peripheral and central hormonal milieu that occurs as part of any endogenous circannual cycle that in dogs is not typically photoperiod-entrained. The processes involved may also be modified by exposure to environmental cues including photoperiod, especially in seasonal breeders, as well as a bitch-bitch pheromonal stimulation in domestic dogs. In addition, as anestrus progresses in bitches, gonadotrope sensitivity to GnRH increases, as does the mean concentration of FSH (Okkens and

Koistra, 2006).

Nearly all domestic dog breeds show no evidence of pronounced seasonality, but at least one breed, the basenji, has retained photoperiod-entrainment of the endogenous cycle and breeds in the autumn in both the Northern and Southern hemispheres. Basenji cycles near the equator remain unstudied. The dingo, another subspecies of the grey wolf, is also an autumn breeder in both hemispheres but in equatorial locales may cycle at any time. Most other canid species, like most other carnivores, have retained photoperiod entrainment of circannual cycles to accomplish annual breeding. Arctic and temperate zone carnivores with short 50-65 days gestations breed in early-mid winter; subtropical species typically breeding in autumn or non-seasonally. That most canids have an endogenous circannual cycle entrained by photoperiod argues for the existence for a similar endogenous circannual physiological rhythm that may regulate reproductive hormone secretion in the nonseasonal domestic dog, albeit not entrained to photoperiod. A free-running endogenous circannual cycle of varying length among individuals, similar to those observed experimentally in photoperiodic species in which entraining light cues are either not provided or not processed, might explain a variety of cyclic phenomena in dogs. The latter include apparent spurious timing of hair coat changes (shedding), anecdotal and experimental reports of seasonal or transient decreases in male fertility, and the high variability of estrus cycle intervals among individuals (5-13 months), and similarly variable within some individuals but not others. Some individual dogs have cycles very 6-7 months throughout their reproductive lives, whereas some individuals may have cycles varying over the entire normal range over their lifetime. Anecdotally, we have also observed, in two colonies of experimental beagles, litter-mate bitches that cycled within 1-2 weeks of each other several years in succession. In two instances, a pair of littermates was also synchronized, but one bitch of the pair skipped a cycle and then recycled "on schedule" synchronously with her littermate after an interval of about 1 year. Conversely, many litter-mate bitches become asynchronous before or after their pubertal cycle.

Anestrus involves a variable period following the requisite decline in progesterone below 1 ng/ml; lactation may also impose a specific suppression of LH secretions as LH concentrations are at their lowest during lactation, but pregnancy and lactation does not significantly impact interestrus intervals in most studies. The progesterone profiles during anestrus following parturition have not been detailed. Those for anestrus following nonpregnant cycles show a nadir well below 1 ng/ml reached approximately 3 weeks before the subsequent proestrus. Whether or not the progesterone reflected in concentrations below 1 ng/ml has any central H-P effects and/or intraovarian effects is not known. Circannual or environmentally induced changes in prolactin and/or dopaminergic tone are likely important in that administration of prolactin-lowering doses of dopamine agonist will often shorten anestrus and result in precocious proestrus, with the duration of treatment required becoming shorter (approx. 2 vs. 4 weeks) as anestrus progresses from early (day 120) to last (day 160). Whether, or not, the effect involves dopaminergic effects on hypothalamic GnRH secretion or effects mediated by removal of GnRH release inhibiting effects of prolactin, or both, is not known. Similarly, changes in opioidergic tone could be involved in the termination of anestrus since acute administration of the opioid antagonist naloxone causes acute release of LH, presumably via induction of GnRH release. When during the canine cycle attainment of follicle dominance and the transition to a preponderance of LH-R versus FSH-R occurs, and whether or not one or more inadequate follicular phases accompanied by elevated estradiol secretion typically precede the functional proestrus, are not known. It is possible that there are one or more subliminal waves of gonadotrophin and estradiol secretion that recruit a cohort of follicles to become dominant follicles in mid or later anestrus, with expression of LH-R in amounts that make then sufficiently responsive to a subsequent increase in LH pulsatility as to result in proestrus follicle development. Transient increase in estradiol and FSH in late anestrus are reported. That the mechanisms involved are relatively labile is suggested by anecdotal reports that relocation of a bitch in mid-anestrus close to bitches in proestrus or estrus very often results in occurrence of proestrus a month or more earlier than would otherwise be expected- a presumed pheromone effect.

Comparative aspects of reproductive strategies: seasonality, conservation of reproductive energy, delayed implantation

Monoestrus cycles and spontaneous ovulation appears to be the rule in dogs, wolves, jackals, coyotes and foxes, and gestation lasting to 63 days post-ovulation appears to be the rule in wolves, jackals, coyotes; 53 days in fox species. Among other caniforms and carnivores in general, induced ovulation is common, including most mustelid species, i.e. mink and skunks (although spontaneous ovulation occurs in the striped skunk), and potentially but relatively unstudied in many other caniforms. Among mustelids, a brief season of polyestrous or multiple cycles (follicular phases) as well as return to estrus during delayed implantation occurs. Ovulation is induced by coitus. In black bears, induced ovulation is reported, but in some bear species it occurs spontaneously at a high rate if females are simply housed in proximity to males. In sun bears, spontaneous ovulation followed by an overt lactational pseudopregnancy occurs once annually in non-mated animals. Whether other bears have the potential to be polyestrous or are routinely monoestrus

remains unclear. Pandas, on the other hand, are annually monestrus spontaneous ovulators like the sun bear and most canids. Most felids are induced ovulators but in many species including the domestic cat spontaneous ovulations do occur. Except for the domestic dog, seasonal breeding is the norm among carnivores in temperate and arctic climates; in a few species, distributed in both temperate and subtropical zones, breeding is seasonal in the north or south but non-seasonal towards the equator.

Delayed implantation and luteal function

Another adaptations in many caniform carnivores is embryonic diapause and relatively synchronous delayed implantations occurring in response to season increases in prolactin that ensure seasonally appropriate winter or spring births after post-implantation gestation periods ranging from as little as 40 days (mustelids) or 2 months (bears) to as long as 6-10 months (seals). Delayed implantation allows for synchronous seasonally appropriate implantation and births despite asynchronous breeding dates within species (mid to late winter in mink), or among species. Stripped skunks mate in mid-winter while spotted skunks mate in the late summer or early autumn, yet both give birth in mid-spring after comparable post-implantation gestations, yielding pregnancies of 50-80 days versus 180-220 days respectively. It also allows for occurrence of multiple sires, superfetation and increased litter size in species in which estrus may recur during diapause (mink, badgers). Delayed implantation in carnivores involves a period of suspended development of blastocysts and inadequate luteal function followed by a peri-implantation luteal activation and secretion of markedly higher levels of progesterone caused by increased prolactin secretion. It is reasonable to speculate that the phenomenon also involves embryonic and/or uterine effects of prolactin and/or other seasonally secreted hormone(s) as well as synergistic further increases in prolactin in response to pre- or post-implantation relaxin secretion. In rodents, PRL-R are present in both preimplantation embryo and the endometrium. In mink, implantations and resulting parturitions are advanced by administration of prolactin in late winter and delayed by administration of dopamine agonist (Papke *et al*., 1980). And, study of PRL-R and LH-R RNA expression in untreated and dopamine agonist-treated mink vixens suggest that the seasonal rise in prolactin up-regulates its own receptor and maintains the LH receptor in the CL (Douglas *et al*., 1998), thus causing or facilitating implantation in conjunction with likely uterine and embryonic effects of prolactin. Delayed implantation is primarily a strategy of temperate and polar species with strong environmental pressures either extant or some time in their evolution. The rise in prolactin terminating embryonic diapauses in most or all cases appears to be a function of the endogenous circannual cycle entrained by photoperiod, typically associated with the vernal equinox, or with hibernation (bears, badgers) and/or occurrence of photorefractoriness during hibernation in near-constant darkness.

Pregnancy-associated increased prolactin secretion is assumed to be important for the extended luteal phase of pregnancy in carnivores with post-implantation gestation lengths a month or more longer than in domestic dogs and cats such as the 90-100 day pregnancies in large pantherine cats. However, as seems to occur in domestic cats, late pregnancy in these species may also have the support of effective amounts of placental progesterone production. In contrast to feliform species, caniform species studied including seals and sea lions do not have placental progesterone production, although aromatase may be present. Whether or not pregnancy specific and/or seasonal increases in prolactin are critical for the prolonged post-implantation periods of luteal function of the seals and seal-like caniforms remain unclear. The CL of two species of phocid seals and the stellar sea lion express prolactin receptors and produce progesterone throughout gestation while their placental tissue lacked steroidogenic enzymes. One species of phocid seal, the grey seal, was reported to have placental gonadotrophic hormone activity (Hobson and Boyd, 1984).

Cats

Domestic cats are seasonally or in some cases non-seasonally polyestrous induced ovulators, and manipulation of their breeding seasons by estrus-inducing long-day photoperiods and estrus-suppressing melatonin treatment has been described (Leyva *et al*., 1989). Cats cycle year-round if exposed to equatorial or near equatorial photoperiods. In temperate zones, most cats undergo a seasonal anestrus during the late autumn and/or early winter but are otherwise polyestrous for prolonged periods in the absence of mating or pseudopregnancies. The sequential periods of inter-estrus (2-4 weeks), brief proestrus (0.5-1 day), and estrus (3-10 days) often appear to be associated with periods of low, rising and falling concentrations of estradiol, respectively. Thus, in cats as in the dog, estrus behavior may be primed by a rise in estradiol and subsequently triggered by a peak and/or decline in estradiol. However, some cats will show such subjectively scored estrus behaviors throughout two or more sequential follicular phases and excursions in estradiol. Estrus behavior timing based strictly on receptivity of the male is not readily studied in cats because of the potential for induced ovulation. Feline vaginal and vulval changes occur in response to excursions in estradiol but are not as dramatic as in dogs. Ovulation in response to effective GnRH/LH release occurs more often with two or more copulations in one day, especially if in close succession or allowed

to proceed ad libitum, than following a single copulation. LH release sufficient to cause ovulation is more likely with mating on days 2 or 3 vs. day 1 of estrus, and when estradiol is declining most rapidly. Queens in estrus will initially accept mating 8-24 times within a 24 h with a waning frequency that continues over a 3-5 day period. With ad libitum mating nearly all reflex LH release occurs over the first 12 h after which LH release is limited perhaps due to down regulation, reduced neural or pituitary sensitivity, and/or LH release capacity (Concannon *et al*., 1980b, 1989b). Although cats as a species are classically described as induced ovulators, spontaneous ovulation occurs one or more times a year in 35% of females isolated entirely from males, and over 60 % of females if exposed visually and pherormonally but not physically to males, whether the queens are housed in groups or in individual cages (Lawler, *et al*., 1993; Romagnoli *et al*., 1996; Gudermuth *et al*., 1997). It is not known whether, or not, feline spontaneous ovulations involve vulvo-vaginal-spinal reflexes initiated by self-stimulation and resulting GnRH/LH surges, surge LH release triggered by ovarian steroid changes, or in the absences of any preovulatory gonadotrophin surge. The physiological pseudo-pregnancy of cats following infertile mating, vaginal stimulation or spontaneous ovulation typically only lasts 40-45 days and thus is significantly shorter than in the 65 days of pregnancy. Implantation in cats occurs "early", with attachment occurring at 12 days versus 20 days in dogs, and they thus have a longer post-implantation period (i.e., cat 52 vs. 43 days). In domestic cats, as in dogs, peripheral progesterone in pregnancy is entirely of luteal origin (Verstegen *et al*., 1993), but unlike in dogs there also appears to be sufficient local placental progesterone production to support pregnancy in some queens but not others. Experimentally, 20 to 60% of cats were able to maintain a normal pregnancy following ovariectomy at 40-45 days of gestation (Tsutsui *et al*., 2008). The pregnancy-induced increase in prolactin is more important in cats than in dogs for ensuring persistence of CL function to the end of gestation, especially in queens in which placental progesterone would not be sufficient. Whether or not cat placenta can make progesterone de novo is not known, but it possesses 3ß-HSD and converts pregnenolone to progesterone. In cats, as in dogs, parturition is associated with a preparutm luteolytic mechanism and likewise occurs about 63 days after ovulation.

Wild felids

Many nondomestic felids have reproductive patterns like the domestic cats, with cycle intervals of 2-4 weeks and estrus lasting 3-10 days (Brown *et al*., 2001). Many if not most are also induced obviators. However, based on fecal progesterone profiles some wild felids also show significant incidence of spontaneous ovulation, as in the domestic cat, including the clouded leopard, lion, leopard, margay (Brown *et al*., 2001), but only rarely occurs in cheetah and ocelot. Reproduction is seasonal, with estrus periods concentrated within a 2-4 month breeding season, in some wild felid species and modulated by photoperiod (tiger, snow leopard, Palas cat) whereas others are not seasonal (lions, leopards, pumas, cheetah, margay, ocelot, oncilla) as reviewed by Brown *et al*. (2001). The luteal phase in nonpregnant ovulators typically extends about half the duration of a normal pregnancy, not unlike that in the domestic cat. The lynx species (except bobcats) appear to be seasonally monoestrous induced ovulators, based on birthing dates and hormone profiles, and on mating behaviors like domestic cats, but luteal function, at least in the Iberian lynx is unique. Fecal progesterone studies demonstrate that in both the pregnant and nonpregnant females, luteal function not only persisted the 2 month length of gestation, but after pregnant females give birth, progesterone is again or still elevated and luteal morphology maintained for several months, perhaps acting as a natural contraceptive preventing estrus and breeding out of season (Goeritz *et al*., 2008). A comparable situation of post-partum reactivation of luteal function presumably acting as a natural contraceptive measure occurring after a transient prepartum "luteolysis" has evolved in other mammals including the woodchuck, a sciurid rodent (Concannon *et al*., 1997b). Bobcats do not have this phenomenon, are only weakly seasonal or nonseasonal, and can recycle within the same year and have more than one litter.

Canine ovarian cycle as a basic mammalian ovarian cycle

The canine ovarian cycle can be viewed as relatively unspecialized one. Ovulation and CL formation occur spontaneously. Luteal function is generally adequate to support a gestation involving a modest post implantation period of 43-44 days. The only obvious adaptations to accommodate improved reproductive success appear to be delayed oocyte maturation, pronged lifespan of matured oocytes, and enhancement of luteal progesterone secretion by a pregnancy specific elevation of prolactin concentrations. Using the foregoing as a basis, one can then view additional reproductive adaptations and specializations that have emerged among a variety of carnivore and noncarnivore mammalian species including the following. (1) A conservation of reproductive energy, effort and time occurs by adopting polyestrous cyclicity by means of a uterine luteolytic mechanism (artiodactyls, rodents, and apparently one carnivore – the Hawaiian seal) or nonuterine (primate) acute luteolytic mechanism. (2) Further conservation of energy and time and the development of more rapid polyestrous cyclicity are provided by the strategies of copulation dependent luteal function (many rodents) and/or male dependent or copulation dependent (vaginal reflex) ovulation (many felids and mustelids; seals;

lagomorphs) as well as copulation-dependent formation of functional CL (many rodents). (3) Adoption of seasonal breeding serves as a means to adapt to environmental extremes of temperate and polar latitudes. (4) Adoption of delayed implantation among seasonal breeders with relatively short post-implantation periods serves as a means to greater dissociate the time for optimal mating from the times of implantation and parturition. (5) The prolongation of embryo development and gestation length and the associated prolongation of availability of adequate progesterone is achieved by selection for pregnancy specific anti-luteolytic and/or luteotrophic mechanisms including preimplantation embryonic regulation of uterine prostaglandin pathways, preimplantation and postimplantation secretion of chorionic gonadotrophin, as well as placental progesterone production in addition to feto-placental directed increases in prolactin secretion. A modest lengthening of the post-implantation period of fetal development is an adaptation seen in some carnivores including some with delayed implantations. This apparently occurs by having the fetoplacental unit simply extend the maintenance of luteal function by the mechanism of relaxin-stimulated hyper-secretion of prolactin (phocid seals) and/or by expressing the capacity of the placenta to produce progesterone (some felids).

Canine ovarian research

A relatively large number of laboratories have entered this area because of the large number of canine ovaries available from the surgical neutering of bitches (spays). Canine oocytes are ovulated in prophase of the first meiotic division and undergo maturation in the distal part of the oviduct for 48-72 h. Large oocytes in dogs are more densely lipid laden than those of pigs and centrifugation does not cause significant compaction of the material (Concannon and Wall, unpublished). The lipid laden oocytes require special staining and microscopy for optimal evaluation. Many projects involve improving methodologies for canine oocyte collection, IVM and IVF. One goal is to use the dog (or cat) as a model for endangered canid (or felid) species in which gamete cryopreservation is a goal. For others, work is aimed towards preserving genetic material and/or reproducing small animal models of heritable forms of diseases significant in human medicine. Yet other work is aimed at the potentials for clinical and commercial applications, preservation of traits of individual pets and/or the cloning of individuals using nuclear transfer and ET. Much of such research is hampered by the lack of perfected methods for IVM and IVF and embryo culture, lack of published information on the best methods and timing for ET, availability of synchronized recipients. Harvesting of canine oocytes that will proceed to MII requires collection from antral follicles and the percentage of MII oocytes obtained during IVM is a function of follicle size, independent of stage of the cycle or age, albeit with more large follicles being present on proestrus ovaries. The IVM rate for preselected canine oocytes is very low, but rates of 10-15% have been repeatably achieved (Rodrigues *et al*., 2007). Maturation in TCM199 is negatively affected by fetal calf serum, enhanced by canine estrus serum, enhanced by low levels of FSH but not high levels of FSH or addition of LH, and enhanced by the addition of estradiol combined with human growth hormone. Development of morulae after IVF of IVM matured canine oocytes has been reported by multiple laboratories. Historical and ultrastructural studies of the ovary have been reported. Many laboratories are hampered by lack of the capacity or facilities to produce any significant number of ET recipient females for IVM-IVF produced embryos.

Cloning

To date, two South Korean laboratories have cloned over 50 dogs using somatic cell nuclear transfer into *in vivo* matured oocytes obtained from the oviduct 72 h after ovulation and transferred to recipients at approximately 72 h after ovulation. Cats have also been cloned, including African wild cats cloned using domestic cat mature oocytes and domestic cat recipients (Gomez *et al*., 2004).

Canine ovarian cycle research, contraception, and estrus induction

Canine ovarian cycle research is extensively clinically oriented. One major research avenue involves approaches to contraception of domestic and feral dogs and cats, and captive carnivores. The approaches include anti-GnRH vaccines based on various GnRH-peptide multimers, vaccines based on various zona pellucida protein preparations, vaccines aimed at hormone receptors, delivery of GnRH-linked cyto-toxins to pituitary gonadotropes, and GnRH-agonist administration to down-regulate LH/FSH secretion to prevent cycle occurrence or recurrence. Two commercial GnRH-agonist products for long-term (one-year) cycle suppression are at or near approval for marketing for use in females in several countries – the GnRH-agonist deslorelin in a biodegradable matrix implant (Suprelorin 12, Peptech) that is already approved in some countries for suppression of gonadal function in male dogs, and the GnRH-agonist azagly-nafarelin in a silastic matrix implant (Gonazon, Intervet). Both effectively downregulate LH to basal levels within 3-4 weeks and suppress cycles for 1 year or longer. A major difficulty with the approach is that treatment in anestrus adult dogs typically induces a normal proestrus and estrus that can be fertile and application may be limited to treatment in young prepubertal bitches. Silastic implants releasing low doses of progesterone with first order kinetics are also

under development (Verstegen, 2008, personal communicaton). Some studies are focused on the cost and health benefit ratios of prepubertal versus adult spaying, with focus on side effects of prepubertal spaying include altered long bone growth. Many studies are geared towards determining the efficacy of various regimes for termination of pregnancy in dogs, and involve various protocols using prostaglandins or dopamine agonists singly or in combination, and protocols using the progesterone antagonist alazine, marketed for use in dogs in some countries. The administration of the GnRH antagonist acyline has also been determined to be effective in terminating luteal function and terminating pregnancy, further demonstrating the luteal requirement for LH as a trophic hormone in dogs (Valiente *et al*., 2008). Some ovarian research is focused on the potential role of luteal insufficiency in bitches that have failed to maintain pregnancy and the use of progesterone supplementation. One view is that the problem pregnancies may have insufficient relaxin secretion by the placenta, resulting in inadequate stimulation of prolactin secretion. Research on periovulatory events have focused on the timing of ovulations and progression of oocyte maturation aimed at improved breeding management and application and timing of AI of fresh, chilled or frozen semen, and include use of ultrasound to time and confirm ovulations based on the observed collapse and/or loss of anechoic appearance of the ovulating follicle and subsequent fluid refilling anechoic appearance of the antrum of the early corpus luteum as well as the more common use of progesterone profiles to time ovulation (Reynaud *et al*., 2006). The latter are most accurately used by determining the initial sharp rise in progesterone that is concurrent with the onset of the LH surge. However, in some applications, specific progesterone concentrations typically associated with the time of ovulation (~3-8 ng/ml) or the time of oocyte maturation (8-15 ng/ml) are used, albeit less precisely, especially in instances where earlier samples are not obtained to document the time of the LH surge itself.

Estrus induction research

In cats, protocols using FSH and hCG to induce estrus and ovulation respectively have been reasonably successful and also applied to exotic species. In dogs, in contrast, gonadotrophin based protocols for the most part have induced abnormal follicular development, false estrus or abnormal ovarian function leading to pre- or post-implantation pregnancy failures. In contrast, exogenous GnRH/GnRH agonist protocols have been able to produce normal proestrus, estrus and fertile ovulation resulting in term pregnancies in a high percentage of cases, both in normal anestrus and in animals suspected of prolonged or persistent anestrus. This has been accomplished not only using the physiological approach of pulsatile i.v. GnRH administration every 90 min using programmable infusion pumps, but also using short term (1-3 weeks) constant administration of GnRH super-agonist via s.c. Alzet osmotic mini-pumps (Fig. 12) or administration via biodegradable s.c or submucosal (vulval) implants (Concannon, 1989, 2006; Kutzler, 2007). GnRH agonist induction of fertile estrus will likely have wide application in endangered caniform carnivores, and has been applied successfully in captive wolves using deslorelin implants.

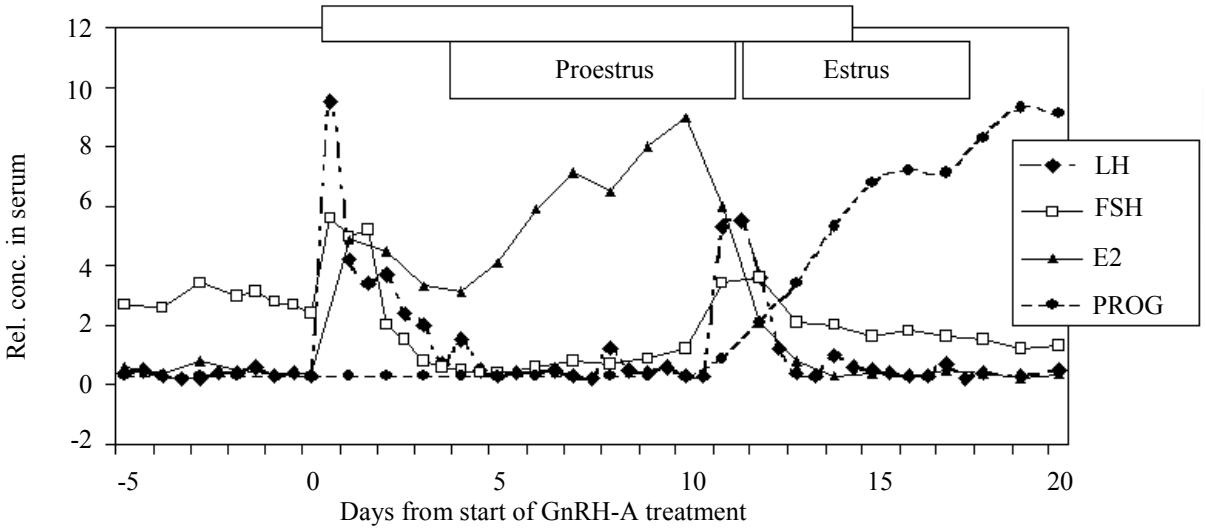


Figure 12. Concentrations of FSH, LH, estradiol and progesterone in a bitch in which administration of the GnRH agonist lutrelin at 1.8 ug/kg/day for 14 days (top bar) induced a proestrus that progressed to a normal estrus, spontaneous LH surge and fertile ovulation. Peak concentration of LH and FSH acutely induced by GnRH-A were 18.4 and 224 ng/ml, respectively; those during the preovulatory gonadotrophin surge were 11.6 and 148 ng/ml, respectively. Maximum concentrations for estradiol and progesterone during study were 85 pg/ml and 19 ng/ml, respectively.

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