Evidence of Heritable Luteal Insufficiency in Portuguese Water Dogs: A Pedigree-Based Investigation

by

Lily R. Lewis, DVM

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
August 9, 2025

Keywords: hypoluteoidism, pregnancy loss, progesterone, corpus luteum, heritable infertility, pedigree analysis

Approved by

Robyn Wilborn, Chair, Professor of Theriogenology, Department of Clinical Sciences
Nancy Merner, Associate Professor, Department of Pathobiology
Emily Graff, Associate Professor, Department of Pathobiology
Candace Lyman, Associate Professor of Theriogenology, Department of Clinical Sciences
Aime Johnson, Associate Professor of Theriogenology, Department of Clinical Sciences

Abstract

Reproductive failure and pregnancy loss is devastating for dog owners both financially and emotionally. While infectious agents are the most common causes of canine pregnancy loss, hormonal aberrations are insidious as there are often no accompanying symptoms. Hypoluteoidism results in insufficient serum progesterone (P_4) concentrations, causing spontaneous abortion as the first symptom. This condition has been documented in dogs, but the underlying pathophysiology is poorly understood. The current literature supports the basis of hypoluteoidism as genetic, although this has not been proven. This is supported by the fact that sporting and working dog groups have lower progesterone concentrations on average throughout gestation compared to other breeds. Devastatingly, there is no genetic test available for early identification of hypoluteoidism. Our clinical Theriogenology team has identified a pedigree of Portuguese Water Dogs (PWDs) in which multi-generational hypoluteoidism has been confirmed. Specifically, two affected individuals (a dam and daughter) have been identified. The two affected individuals were monitored throughout two pregnancies each, in which endogenous serum P_4 fell below baseline prior to Day 63 of gestation. Fortunately, as a result of specialty level care, these pregnancies were successfully managed and maintained using a synthetic progestin and intense monitoring, resulting in four successful litters. Interestingly, two *unaffected* females have also been also identified, which allows us a unique opportunity to investigate the heritability pattern and possible underlying physiology. Using canine whole genome sequencing technologies, and in collaboration with geneticists from the Department of Pathobiology, our team is aiming to identify a causative genetic mutation for hypoluteoidism. Long-term, with a genetic marker identified, we can develop a diagnostic test to screen at-risk individuals, without necessitating a

specialist, and identify high-risk pregnancies to ensure proper care during gestation. Luteal phase deficiency (LPD) is the synonymous disorder in humans, and there is currently no definitive diagnosis in women with this condition**.** Thus, outcomes may be translational and will serve as a springboard to understand of the complex pathophysiology behind premature luteal failure in the canine, and potentially human, species.

Acknowledgements

Funding for this project was provided by the Animal Health and Disease Research Grant within the Office of Research and Graduate Studies at Auburn University College of Veterinary Medicine. My residency program was funded through the American Kennel Club Canine Health Foundation (AKC-CHF) and the Theriogenology Foundation (TF).

I would like to start by thanking all of my committee members who have been a guiding light for me throughout this project and my residency program. To my primary mentor, Dr. Wilborn, a single acknowledgement in this thesis simply cannot convey my immense gratitude for your continuous support, both personally and professionally. You took a chance on me and can only hope to become the type of doctor, mentor, and friend that you are as I proceed throughout my career. To Dr. Lyman - I treasure our connection, and I truly admire how well you treat every clinical case with the individuality it deserves. You have instilled a confidence in me throughout residency that has enabled me to become a more decisive doctor and better critical thinker, and for that I want to thank you. To Dr. Johnson, I sincerely appreciate your responsiveness and dedication to showing up to help when I needed you - both with this project and throughout our times on clinics together. To Dr. Graff – Thank you for your amazing insight and thoughtful contributions you offered to this project. I am especially grateful for all the time you generously gave to mentor and support my fiancé throughout his journey in becoming a clinical pathologist. I've truly valued our time together and will miss you when I leave Auburn. To Dr. Merner – You have been so patient with me in my learning of the processes involved with whole genome sequencing. Thank you for opening your lab to me, and for taking the time to go through things at my pace while I was learning. I particularly appreciated knowing that I could go to you with any problem, or even just

to vent! Your lab was truly a little oasis. Additionally, to Dr. Flaws – though you aren't on my master's committee, it was you that taught me how to be a great scientist and for that I am forever grateful.

I would also like to thank the remainder of the theriogenology team at Auburn University. It takes a village to mold a resident, and every DACT at Auburn were always willing to teach and mentor. I wouldn't be half as capable or confident around farm animals had it not been for Dr. Armstrong, Dr. Klabnik, Dr. Waters and Dr. Rush. All of you were patient and kind while I learned the 'ropes' (pun intended) over in the large animal hospital throughout my time at Auburn. And to Dr. Maxwell and Dr. Hopper, though we only overlapped for a short period, I still carry wisdom that you have shared. I also would not have completed this residency without the support of our small animal theriogenology technician, Maureen Donohue. Maureen – thank you for being a teacher, 'ringmaster', shoulder to cry on, and most importantly – a true friend.

To my resident mates – thank you for being, well, you. Dane, Hannah, and Hayley – you are my Auburn siblings and were my lifeline throughout the highs and lows of residency. I honestly cannot come to terms with the fact that we won't be working together forever. My sadness about leaving Auburn this year is slowly being replaced with joy in knowing that the bond we have built will carry over into the next chapters of our lives. I am so proud of all we have accomplished so far, and I can't wait to witness how we each progress throughout our careers.

Finally, I would like to thank my family. To my fiancé, Paul – I am such a lucky woman to have you by my side. You have provided unwavering support, including moving to two different states as I chased this career, all with a smile on your face. You're my biggest cheerleader and I feel like I can take on the world when you're in my corner. I simply would not and could not be in this position without you, and I cannot way to see what our future holds! To my parents and

siblings- I know that the field of theriogenology is niche, but I sincerely appreciate you taking the time to understand and ask questions – it means more than you know.

Table of Contents

List of Figures

List of Tables

Introduction

The successful birth of live offspring in any mammalian species represents a series of successful physiologic checkpoints that must be achieved – from ovulation, to fertilization, appropriate embryonic development, establishment of a placenta, and successful parturition. Though many aspects of mammalian reproduction have been studied extensively, there are certain areas in which a paucity of data exists. This is a consequence of several factors, the most important of which is likely due to the ethical considerations involved with robust (e.g. *in vivo*) study designs. One area of female reproductive research that seems to lag behind others in the scientific literature is pregnancy loss. Specifically, pregnancy loss in the absence of an identifiable infectious agent– as those have been characterized very well, especially in our domestic animal species. The challenges in studying pregnancy loss are multifactorial, as is often the case with pregnancy loss itself.

Despite this, pregnancy loss is essential to explore as an area of research, especially as our understanding of normal pregnancy and parturition physiology continues to expand. By deepening our understanding of the precise interconnected mechanisms by which pregnancy is maintained and learning how disease states or genetic factors can contribute to aberrations in this normal physiology, we are better equipped to intervene therapeutically. Though often human pregnancy comes to mind when discussing high-risk pregnancy management and interventional medicine, there is a growing field of interest in domestic animal species. Fortunately, mammalian reproduction retains key fundamental similarities across species - allowing veterinarians, medical doctors and other scientists to work translationally.

There is no one classification or definition for the term ‘pregnancy loss’, even within a single species. For instance, though general guidelines exist, there are still debates as to what

constitutes ‘fetal’ vs. ‘embryonic’ loss. In the bitch, these two are usually distinguished based on the presence or absennce of clinical signs, with ‘embryonic’ losses occurring in the absence of passage of a fetus or fetal fluid, a term also known as *resorption.* Fetal loss is most commonly associated with the presence of clinical signs of pre-term labor, including passage of a recognizable fetus and fetal fluids, also known as *abortion*. The cutoff between embryonic and fetal loss is approximately 30-35 days of gestation, with embryonic losses occurring prior to this timeframe, and fetal loss after, though this is slightly debated in the literature (Johnston & Raksil, 1987) (Johnson, 2008).

Pregnancy loss is an unfortunate clinical manifestation of a primary disease state, and not a primary disease process of its own. Thus, the underlying etiology leading to pregnancy loss (e.g. aneuploidy, implantation failure) is used to guide treatment recommendations for future pregnancies. However, in many cases a definitive diagnosis is not obtained – at which point the presumptive diagnosis becomes a broader umbrella term of ‘luteal insufficiency’, ‘luteal failure’, or ‘hypoluteoidism’, implying a lack of steroidogenic support by the corpus luteum to maintain the pregnancy. Though, technically speaking, luteal failure can occur in any species, hypoluteoidism is most described in the bitch and woman.

Hypoluteoidism, as the name depicts, results in insufficient serum progesterone (P_4) concentrations, causing spontaneous abortion. In dogs, abortion is often unfortunately the first symptom. The condition has been documented in canids, but the underlying pathophysiology is poorly understood. In humans, the same condition occurs but is termed ‘luteal phase deficiency’, and the pathophysiology is also multifactorial. Clinically in women, insufficient progesterone secretion causes the normal secretory transformation of the endometrium prior to implantation to be impaired, resulting in miscarriages and pregnancy loss. Although luteal phase deficiency

remains understudied, abnormalities of the luteal phase are reported to occur in up to 35% of women with recurrent pregnancy losses (Arredondo & Noble, 2006).

The current literature supports the basis of hypoluteoidism as genetic, although this has not been proven. In dogs, this is supported by the fact that sporting and working dog groups have lower progesterone concentrations on average throughout gestation compared to other breeds (Hinderer, Ludeke et al. 2021). In women, genetic polymorphisms in the progesterone receptor gene have been identified and have been shown to be associated with recurrent pregnancy loss (N. Khan et al., 2021), also suggestive of a genetic contribution to this condition. However, there is no genetic test available for early identification of hypoluteoidism in any species.

Our clinical Theriogenology team has identified a pedigree of Portuguese Water Dogs (PWDs) in which multi-generational hypoluteoidism has been confirmed. Specifically, both a dam and one of her female offspring were monitored throughout two pregnancies each, in which endogenous serum P_4 fell to baseline (<2.0 ng/mL) prior to Day 63 of gestation. To our knowledge, this represents the first time that a definitive diagnosis of canine hypoluteoidism has been observed and documented in directly related individuals (dam and daughter). Fortunately, as a result of specialty-level care, these pregnancies (n=4) were successfully managed and maintained using a synthetic progestin and intense monitoring, resulting in four successful litters. Interestingly, two *unaffected* females within this pedigree have also been identified, and DNA samples have been archived on all 4 of these related individuals (two affected, two unaffected).

The objectives of this study were to 1) construct a pedigree of affected and unaffected individuals to infer a potential inheritance pattern, 2) assess and characterize the progesterone profiles throughout gestation in affected and unaffected individuals. Our long-term goal will be to perform canine whole genome sequencing on the family of PWDs to determine, similarly to studies

in humans, whether a genetic marker can be identified for hypoluteoidism in the bitch. The clinical implications of identifying a genetic marker will be exceptionally impactful. In the short term, should a genetic link be identified, breeders and veterinarians can make informed decisions regarding the reproductive potential of affected individuals. Affected females can either be removed from breeding pool or bred under careful observation and management as this condition is not life-limiting for the dam. In the long term, identification of a genetic variant enables the development of a screening test, initially in PWDs but with the hopes of expanding to other breeds prior to making breeding or placement decisions. Lastly, given that the synonymous condition in humans is understudied, any knowledge gained on hypoluteoidism in dogs helps to improve our understanding across species.

I. Review of Literature

1. Estrous Cycle in the Bitch

1.1. Cycle Overview

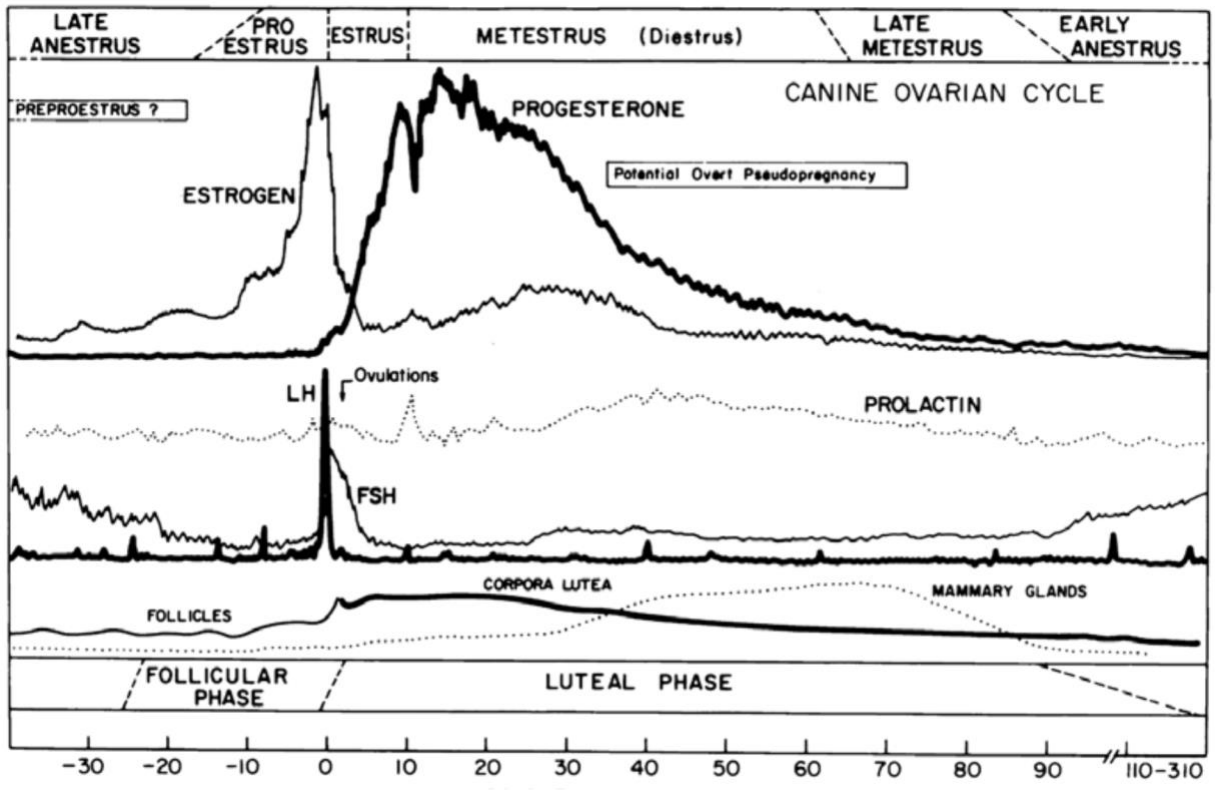


Figure 1 *- Hormonal changes throughout the estrous cycle in the bitch. Reprinted from Concannon et al., 2009.*

1.2. Proestrus

Proestrus in the bitch is a stage of the estrous cycle in which the first signs of reproductive activity are displayed. This phase of the cycle lasts approximately nine days but can range between one to three weeks long (Concannon, 2009). The most obvious outward clinical sign is usually the presence of vulvar discharge, which ranges in consistency and color but most often is light pink and serosanguinous. The vulvar discharge seen in proestrual bitches results from diapedesis of red

blood cells originating from the uterine vasculature. This is in contrast to other mammals, such as primates, that slough superficial layers of the uterine lining during menstruation.

The clinical hallmarks of proestrus include vulvar swelling, vaginal discharge, and attractiveness to males. Early proestrus is characterized by a progressive increase in estradiol (E_2) concentration from approximately 5-10 pg/mL, reaching peaks of 45-120 pg/mL about one to two days prior to the onset of the LH surge (Concannon, 2009). Increased estradiol also acts on the vaginal wall epithelium, causing a transient hyperplasia that will last throughout estrus. This leads to vulvar swelling, characteristic of proestrus. Also correlating to the increase in estradiol, the bitch will secrete pheromones that attracts intact males. It is important to distinguish, however, that most bitches will not stand to be mounted during proestrus.

Vaginal cytology will reveal progressive cornification of the vaginal epithelium, correlating with progressive edema and vaginal wall thickness as proestrus progresses. As such, in early proestrus, there will be a mix of cornified and non-cornified epithelium, erythrocytes and leukocytes (predominantly neutrophils). A proestrual vaginal cytology often appears indistinguishable from one performed in early diestrus. Fortunately, serum progesterone concentrations can be used to differentiate between the two, as baseline progesterone (P_4 <2ng/mL) is expected in proestrus. As proestrus progresses, the percentage of parabasal and small intermediate cells decreases as these cells are replaced by fully-cornified epithelium (Johnston et al., 2001). Bacteria are often present as well, though there is significant variation between bitches as to the bacterial load present.

In early proestrus, follicular stimulating hormone (FSH) concentrations are similar to those seen in anestrus, as this hormone is above baseline at that time (Figure 1). A small increase in this hormone is appreciated (~20% above anestrus) (Concannon, 2009). LH, on the other hand, is

baseline in anestrus, and begins to increase during proestrus. In proestrus, dominant follicles (approximately 2-3mm in diameter) are present in the ovaries. As proestrus progresses, the follicles grow to a diameter of about 5-6mm. Interestingly, follicular dynamics in late proestrus are thought to be 'semi-autonomic', with each follicle having a pre-determined fate (ovulatory or atretic) (Concannon et al., 2009). Follicular growth and the rise in estradiol acts as negative feedback to both LH and FSH, and as proestrus progresses, FSH begins to decline due to negative feedback. LH, however, remains elevated as estradiol modulation to this hormone switches from negative to positive feedback, preceding the LH surge. At the same time, progesterone (P_4) concentrations start to rise at an exponential rate, reflecting pre-ovulatory luteinization occurring within dominant follicles of the canine ovary (Figure 2) (Concannon et al., 2009).

Approximately 24-36 hours following peak estradiol, LH peaks. The LH surge consists of a rise for 12-24 hours, with a subsequent decline of the same duration, with concentrations ranging anywhere between 10-100x to those seen in anestrus. The LH surge is commonly seen as the marker for the start of estrus, however there is some debate in the literature as to the exact onset of estrus in the bitch (Concannon, 2009) (Johnston et al., 2001).

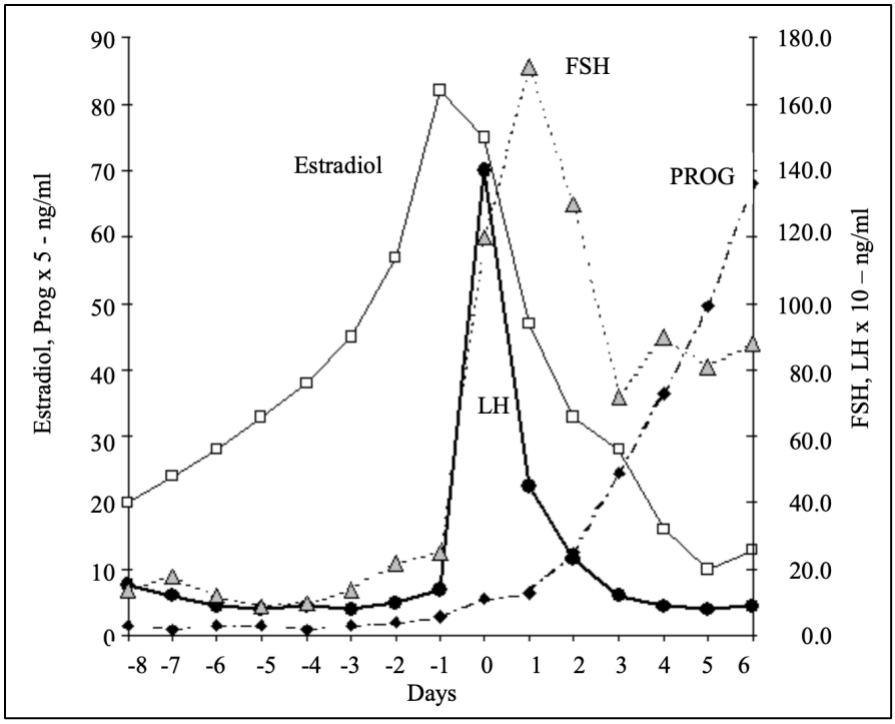


Figure 2 *- Mean concentrations of serum estradiol, progesterone, LH and FSH in 8 beagle bitches during proestrus. Reprinted from Concannon et al., 2009.*

1.3. Estrus

Estrus in the bitch is most universally recognized as the timeframe in which a bitch will stand to be mated, and last approximately 9 days long, though may extend well beyond this when based on behavioral signs alone. Vaginal discharge is still present, though may change to a lighter color and thinner consistency (Johnston et al., 2001).

During late proestrus and into early estrus, there is a decline in the estradiol to progesterone (E:P) ratio (Figure 3). The rise in progesterone is spontaneous and occurs due to preovulatory luteinization of ovarian follicles, a process involving the differentiation of granulosa cells into large luteal cells and theca interna cells into small luteal cells (Chastant-Maillard et al., 2011). The cause for a decline in estradiol is likely multifactorial but thought to be due to increased

metabolism by the liver and sequestration by estrogen-responsive tissues including the vaginal epithelium, uterine endometrium and mammary gland (Concannon et al., 2009). Though much remains to be elucidated as to the molecular impact of this change, it is known that this change is required for the LH surge to occur. In one study, bitches supplemented with estradiol had a suppressed LH surge. Following removal of the exogenous estrogen, the LH surge occurred. Furthermore, a separate group of bitches administered progesterone (P4) implants following removal of estradiol displayed LH surges with serum concentrations approaching the upper range of normal (Concannon et al., 2009). In addition to allowing LH to surge, the decline in serum estradiol also is thought to contribute to the increase in pheromones and mating behavior in the bitch.

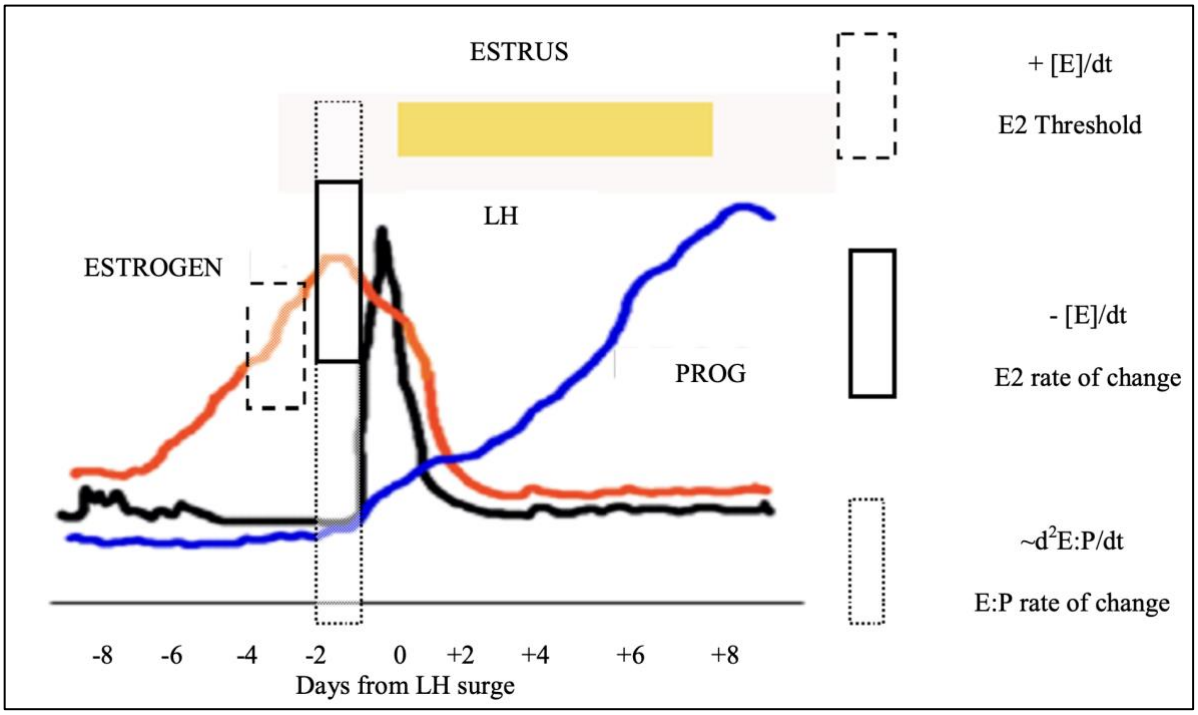


Figure 3 *- Schematic representation of changes in serum concentrations of estradiol, progesterone and LH typically observed during the estrous cycle in bitches. Reprinted from Concannon et al., 2009.*

Follicular changes during estrus include the completion of development of proestrual follicles to a stage of ovulatory competence, with the mean number of ovulatory follicles increasing in correlation to the size of the bitch (Chastant-Maillard et al., 2011). Follicles grow from 0.2-0.6 mm (at the start of proestrus) to upwards of 1 cm at the time of ovulation, though anywhere between 5 and 12 mm at the time of ovulation has been published (Reynaud et al., 2012) (Concannon, 2009) (Concannon et al., 1989). Interestingly, dogs have a higher percentage (upwards of 14%) of follicles containing multiple oocytes (polyoocytic) (Figure 4). For comparison, polyoocytic follicles usually represent <2% of the entire follicular population in mammals (Reynaud et al., 2012). However, due to a high variability in the appearance of cumulus-oocyte-complexes (COCs) observed from polyoocytic follicles, it is currently accepted that most often only one oocyte will be of acceptable quality (Chastant-Maillard et al., 2011).

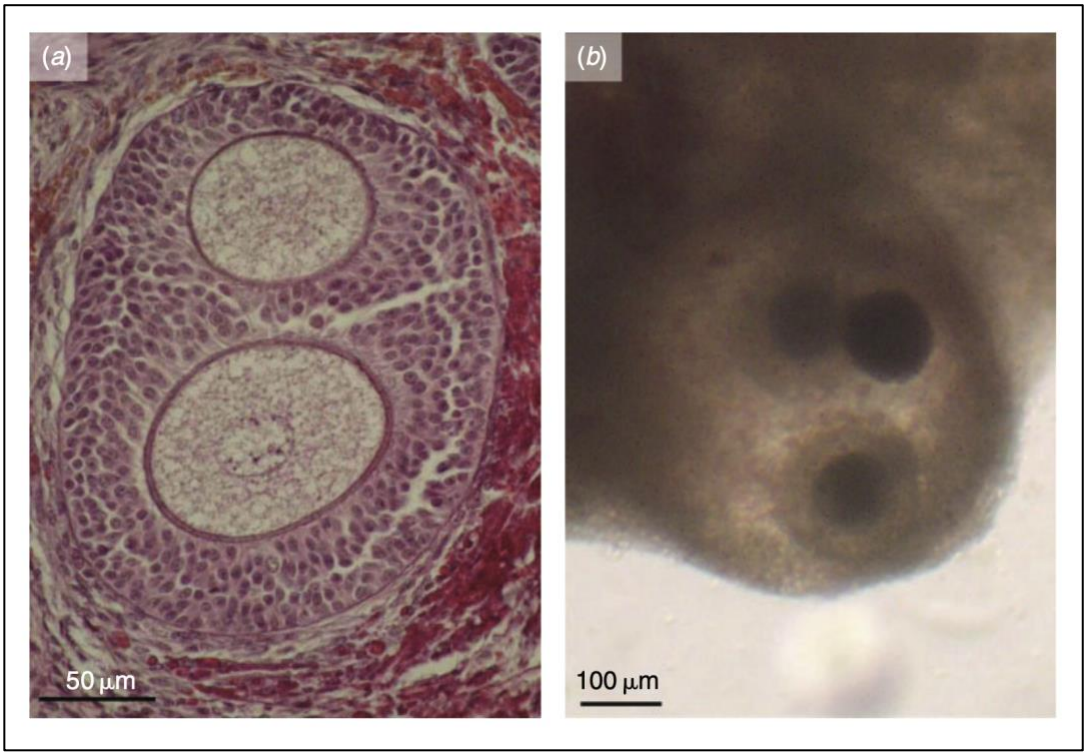


Figure 4 *- Polyoocytic follicles in the bitch presented both histologically (a) with 2 oocytes present and grossly (b) with 3 oocytes present. Reprinted from Chastant-Maillard et al, 2011.*

Luteinization occurs prior to ovulation in the bitch, beginning around the time of the LH surge. This is evident histologically as a loss of follicular granulosa cells and increase of theca interna cells of the follicle, suggesting that the latter play an important rule in luteal function in the bitch (Concannon et al., 2009). This phenomenon is permitted in part by the presence of LH receptors (LHr) earlier in the canine follicle compared to other species – with some reports citing the presence of LHr within the granulosa cells of pre-ovulatory follicles at only 25% of ovulatory size. In contrast, LHr on bovine follicles are visualized when the follicle is closer to 50% of total ovulatory size (Chastant-Maillard et al., 2011). Following the LH surge, mucification (the secretion of mucopolysaccharides such as hyaluronic acid) and cumulus expansion occurs in follicles at least 3mm in diameter. In most mammals, the oocyte resumes meiosis following the LH surge, prior to ovulation. However, the bitch ovulates primary oocytes, arrested in prophase 1 of meiosis, in a germinal vesicle stage (Reynaud et al., 2009) (Reynaud et al., 2012). After ovulation, oocytes remain near the ampulla-isthmus junction for several days, undergoing final maturation prior to fertilization. Resumption of meiosis in the bitch has yet to be fully understood, but granulosa cells are thought to play an important role. In mammals, meiosis is primarily inhibited via Cyclic adenosine monophosphate (cAMP) (Chastant-Maillard et al., 2011) (Sen & Severance, 2018). cAMP is synthesized by the oocyte and cumulus cells surrounding the oocyte, with continuous transport of the molecule occurring via gap junctions within granulosa cell transzonal projections (TZPs). High levels of cAMP cause the activation of protein kinase A, which ultimately phosphorylates and inhibits maturation-promoting factor (MPF). It is the inhibition of maturation-promoting factor that ultimately leads to the continued arrest of oocytes in prophase 1. In the bitch, this arrest is maintained after ovulation (unlike in other mammals), because the granulosa cell projections remain intact for 56- 72 hours post-ovulation, at which point the

projections retract and meiosis is resumed (Chastant-Maillard et al., 2011) (Sen & Severance, 2018) (Reynaud et al., 2009) (Concannon et al., 1989). Oviductal oocytes can live up to 8 days post-ovulation (and 5 days following oocyte maturation) (Concannon et al., 2009).

1.4. Diestrus

The luteal phase in the bitch can be divided into two phases: gonadotropin-*independent* and gonadotropin-*dependent* (Figure 6). As the name suggests, the gonadotropin-*independent* phase defines a time of approximately 2-4 weeks during the first part of diestrus in which the corpus luteum (CL) does not rely on gonadotropins for maintenance. Instead, the CL is maintained by circulating progesterone, estrogens and the maternal immune system (Kowalewski, 2012). The gonadotropin-*dependent* phase occurs after the 4-week mark, at which point the lifespan of the canine CL can only be terminated by hypophysectomy (removal of the hypothalamus) or by medications that mimic this, such as dopamine agonists like cabergoline or bromocriptine. This

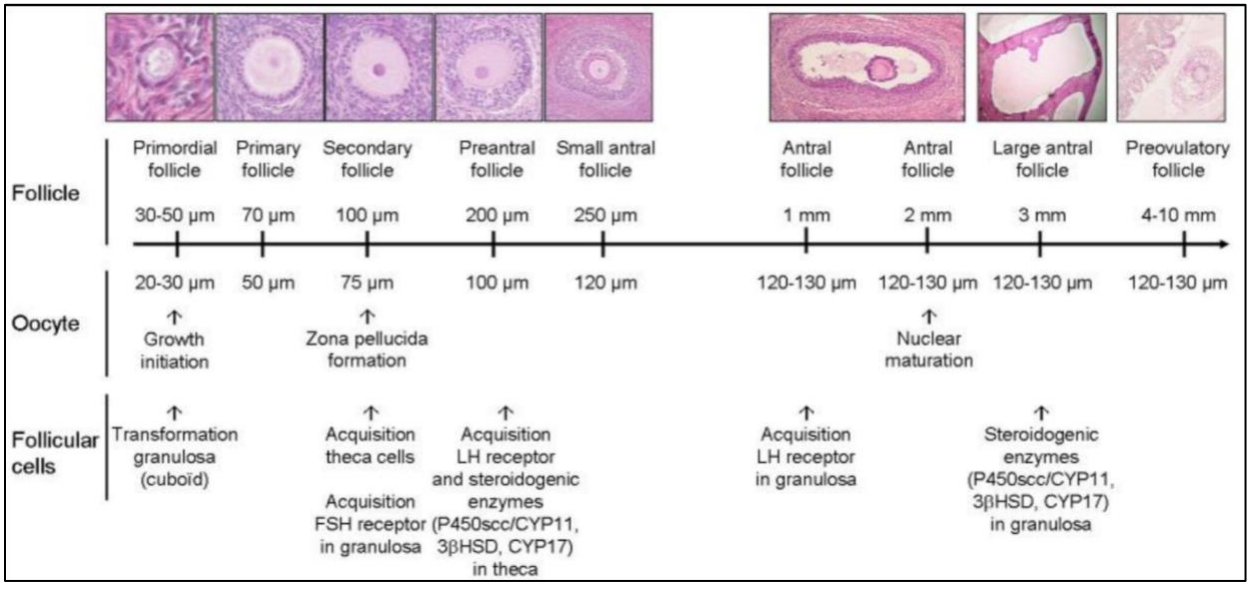


Figure 5 *- Summary of folliculogenesis in the bitch. Reprinted from Reynaud et al., 2020.*

functional withdrawal of prolactin and LH lead to removal of luteotropic support, leading to luteolysis (Kowalewski, 2012) (Concannon, 2009).

In addition to having two distinct phases relating to the reliance of gonadotropin support for CL lifespan, the bitch has a unique estrus cycle in that the hormonal cascade is the same irrespective of pregnancy status. However, there are key differences at certain timepoints within diestrus that are distinct between pregnant and non-pregnant bitches. These differences will be discussed in the section *2.4* and *2.5* describing pregnancy and luteolysis in the bitch.

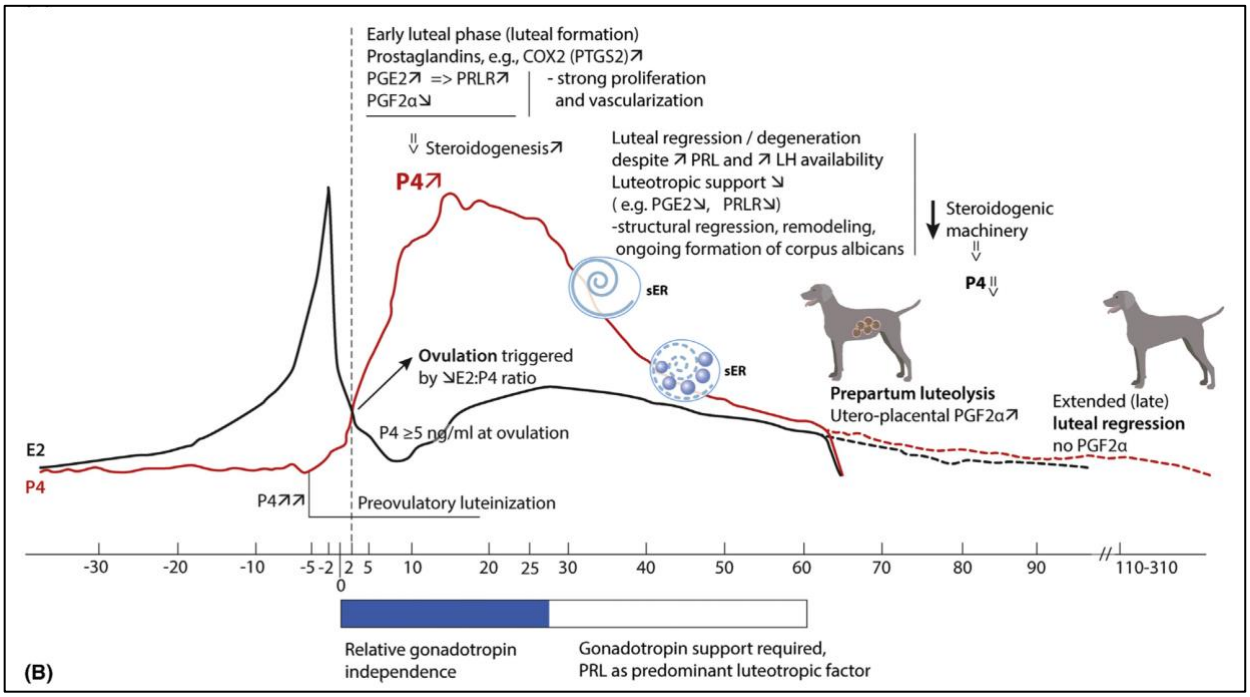


Figure 6 - *Illustration of gonadotropin-dependent and gonadotropin-independent timepoints during diestrus. Luteal dynamics, including differences in luteolysis, are outlined. COX2 (PTGS2), cyclooxygenase 2; PGE2, prostaglandin E2; PGF2a, prostaglandin F2a; PRLR, PRL-receptor; sER, smooth endoplasmic reticulum (first signs of degeneration within sER can be seen at approximately day 35 after ovulation exhibiting "whorl like" structures and proceeds further towards fatty degeneration seen around day 45. Reprinted from the Encyclopedia of Reproduction (Second Edition)- Selected Comparative Aspects of Canine Female Reproduction, M. Kowalewski, 2018.*

1.5. Anestrus

Anestrus occurs following luteolysis in the bitch, with the onset of anestrus correlating with a serum progesterone (P_4) concentration falling below 1 ng/mL (Kowalewski et al., 2020). The interval of anestrus is variable, and can range anywhere between 5-12 months in duration, with 6–7-month intervals being the average (Concannon, 2009). This range and interval can vary between breeds, with some breeds (e.g. German Shepherd dogs) having shorter interestrus intervals (Root Kustritz, 2012). Most of the reproductive hormones are baseline during the majority of anestrus, and the evidence points to follicular waves being quiescent. FSH is one hormone that is elevated and increasing in concentration throughout most of anestrus and decreases just prior to proestrus (Figure 7). The function of elevated FSH in early anestrus is interesting and remains to be elucidated. Estradiol varies throughout anestrus but does reliably increase in the last few weeks, in response to the development of steroidogenically active follicles under the influence of LH and FSH (Concannon et al., 2009). LH increases in both pulse frequency and magnitude in the last week of anestrus, just prior to proestrus. The exact mechanism by which anestrus is terminated in the bitch remains unknown. However, recruitment of a small cohort of follicles with the presence of functional LHr in mid to late anestrus is one postulated mechanism by which proestrus is initiated in the bitch. Other mechanisms include changes to dopaminergic or opioidergic tone. Alterations in the length of anestrus (shortened) were observed in bitches administered dopamine agonists. Similar effects were seen in bitches administered the opioid antagonist, naloxone, suggesting that endogenous opioidergic pathways suppress hypothalamic GnRH secretion in the bitch, as seen in other species (Concannon et al., 2009) (Uenoyama et al., 2022).

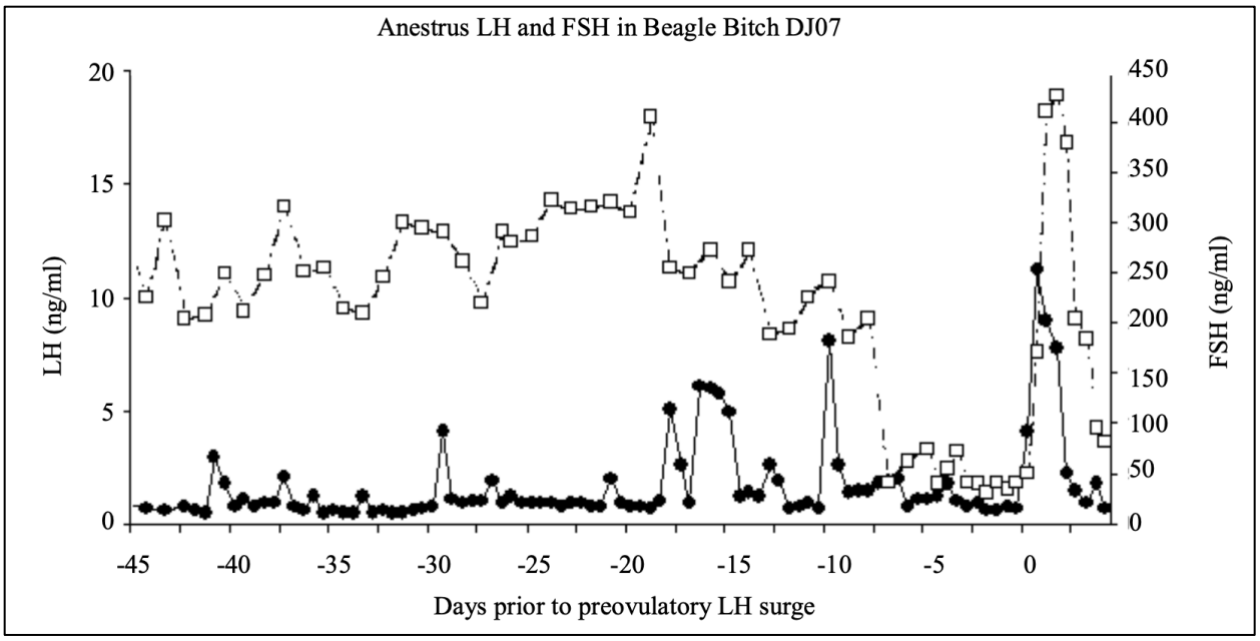


***Figure** 7 - Serum concentrations of LH and FSH during late anestrus and proestrus. Reprinted from Concannon et al., 2009.*

2. Pregnancy in the Bitch

2.1. Fertilization

Mammalian fertilization involves fusion of a spermatozoon and oocyte to form a single-cell zygote, and this process is considered successful once the fusion, or syngamy, of the male and female gametes is complete. Once deposited inside of the female, canine sperm cells migrate rapidly through the cervix, uterine body and uterine horns. In contrast to most domestic species, canine sperm cells primarily reside in the isthmus of the oviduct, as opposed to the uterotubal junction (see Figure 8). The oviductal isthmus, just adjacent to the uterotubal junction, serves as the functional sperm reservoir in bitches (Reynaud et al., 2015) (Chastant-Maillard et al., 2011). Canine sperm cells have a robust lifespan - living up to 10 days in the female reproductive tract - which allows time for oocyte maturation to occur following ovulation (Goericke-Pesch et al., 2012). Sperm cells have been shown to penetrate immature oocytes, but this is the exception rather

than the rule. Even when penetration of the zona pellucida does successfully occur, the fusion of male and female pronuclei is not able to occur prior to oocyte maturation. (Reynaud et al., 2006) (Concannon et al., 1989).

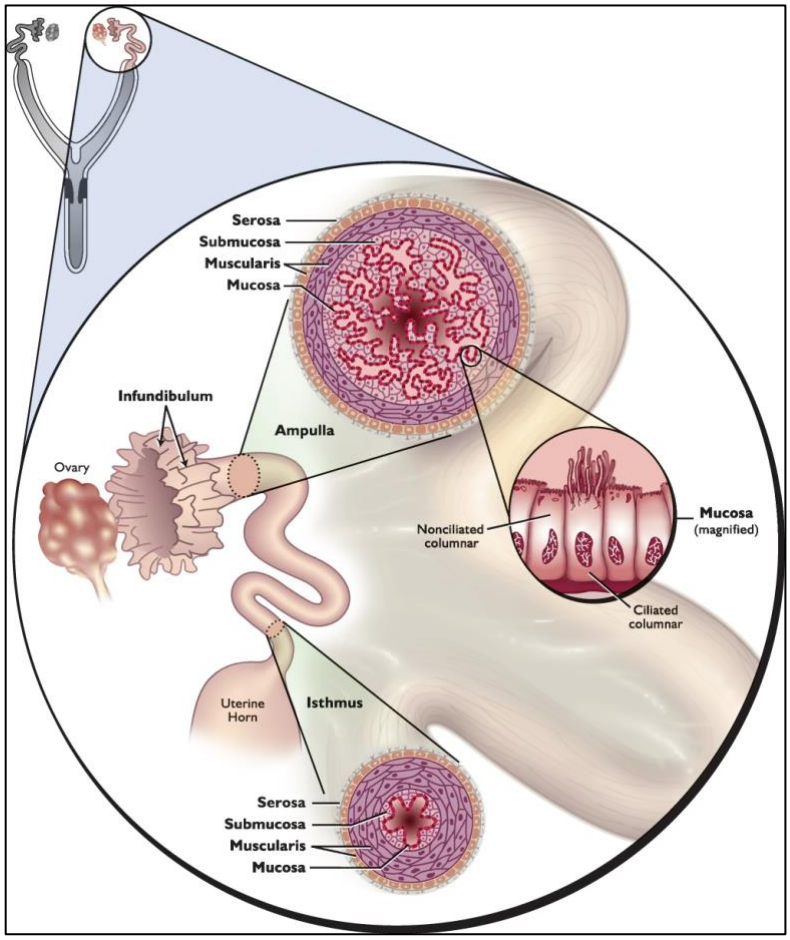


Figure 8 *- Anatomy of the oviduct. Reprinted from Pathways to Pregnancy and Parturition, Senger 2012.*

2.2. Early Embryogenesis and Implantation

Following syngamy in the ampulla of the oviduct, embryo development begins. For a table depicting the stages of embryogenesis and timeline, see Figure 9. Briefly, the two pronuclei are present approximately 100 hours post-ovulation. A morula is present at approximately 230 hours (8-10 days) post-ovulation, at which point the embryos pass into the uterus from the oviduct

(Reynaud et al., 2012). Once within the uterus, the embryos migrate throughout, absorbing histotrophe (uterine secretions provided to sustain embryos prior to attachment). Remarkably, a mechanism for maternal recognition in the bitch has not been identified, likely in part due to the similar hormonal cascade between gravid and non-gravid bitches. Histological changes to the uterus in early pregnancy (as early as days 10-14 post-ovulation) have been identified, however, and are primarily inflammatory in nature, and not specific to the presence of an embryo. Attachment of the blastocyst to the maternal endometrium occurs at 16-18 days post-ovulation, with a more intimate attachment (implantation) occurring by fetal trophoblast cells just days after (Verstegen-Onclin & Verstegen, 2008). Unlike other domestic animal species that use the term 'attachment' in reference to the connection of the embryo to maternal endometrium, implantation is an appropriate term for the bitch as there are regions of decidualization present along the placenta, resulting in the normal loss of maternal tissue at birth.

Table 1

Authors	No. of bitches	Breed	Reference point	Corpora lutea/two ovaries (Mean ± S.E.M.)	Collection rate (%)	No. oocytes/ embryos observed	Observation/ staining method	Period of examination (after ovulation)	Observation periods of oocytes and embryonic stages [h (d) post-ovulation]									
									GV	MII	2 PN	2 cells	3–4-cells	4–6 cells	8 cells	16 cells	Morula	Blastocyst
Holst and Phemister (1971) [7]	36	Beagle	Mating	–	–	53 (→16 cell.) + blastocysts	Photonic microscopy + sections	1–20 d after mating (? d after ovulation)		72 h? (3 d)					120–288 h? (5–12 d)	120–288 h? (5–12 d)		192–480 h? (8–20 d)
Tsutsui (1975) [34]	19	Beagle and mixed breeds	Follicle aspect at laparoscopy	6.7 ± 0.5	88	109	Photonic microscopy	24–216 h (1–9 d)	24 h	48–120 h (2–3 d)	72–96 h (3–4 d)	96–144 h (4–6 d)	120–144 h (5–6 d)		144–168 h (6–7 d)	192 h (8 d)	204–216 h (8.5–9 d)	–
Archbald et al. (1980) [53]	8 (superovulate)	Mixed breeds	Mating (Day 2 of œstrus)	–	–	78	Photonic microscopy	8–12 d after mating (? d after ovulation)				x	x	x	x			–
Renton et al. (1991) [10]	10	–	Progesterone ± LH peak	7.6 ± 0.6	77	47	Photonic microscopy	120–312 h (5–13 d)			120 h (5 d)			144 h (6 d)	120–192 h (5–8 d)		240 h (10 d)	288–312 h (12–13 d)
Concannon et al. (2001) (review) [52]	–	Beagle and mixed breeds	LH peak	–	–	–	–	–			96 h (4 d)	120 h (5 d)	144 h (6 d)	168 h (7 d)	168–192 h (8 d)	216 h (9 d)	240 h (10 d)	264 h (11 d)
Bysted et al. (2001) [25]	9	Beagle	LH peak	7.3 ± 0.6	98	65	Photonic microscopy semi-thin sections	6–12 d after LH (4–10 d after ovulation?)			120 h (5 d)	~144–168 h (6–7 d)			168–192 h (7–8 d)	192 h (8 d)	~216–240 h (9–10 d)	–
Reynaud et al. [35]	50	Beagle and mixed breeds	Ovarian ultrasono-graphy	7.7 ± 0.6	72	195	Confocal microscopy	17–138 h (0.7–5.5 d)	17–44 h	54–83 h	92–124 h	112–138 h	112 h		112–138 h	–	–	–

x: Embryos are present, but stage is not precisely determined.

Figure 9 *- Key events of embryogenesis in the bitch. Reprinted from Reynaud et al., 2006.*

2.3. Placentation

The bitch has an endotheliochorial, zonary, modified deciduate placenta (Figure 10). The zonary placenta includes a unique region called a para-placental zone (also known as a 'pigmented zone' or 'marginal hematoma'). In this region, location alongside the edge of the zonary bands of the chorionic villi, maternal endometrial tissue has been disrupted, resulting in blood clot formation. Functionally, these marginal hematomas metabolize hemoglobin into uteroverdin, ultimately allowing the embryo to obtain iron metabolites from maternal blood (Verstegen-Onclin & Verstegen, 2008). The uteroverdin present within this structure is also responsible for the characteristic hunter-green color seen with placental separation at birth.

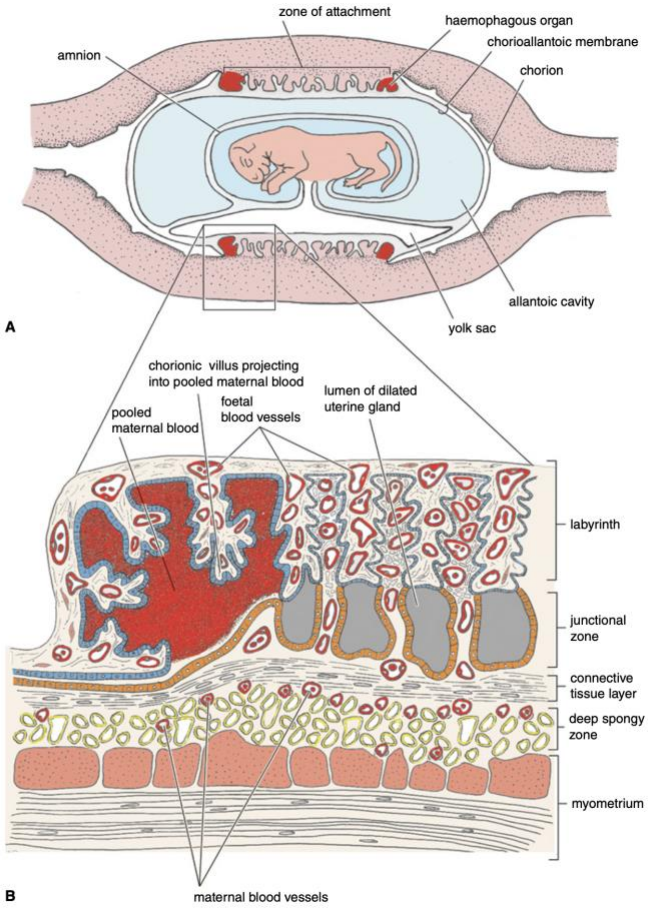


Figure 10 *- Arrangement of canine fetal membranes within the uterus, highlighting the zonary attachment of the chorioallantoic attachment to the endometrium (a), and marginal hematomas (b). Reprinted from Veterinary Embryology, 2nd edition, McGeady et al., 2017.*

On a molecular level, there are some key distinctions to the canine placenta. First, the bitch is the only known domestic mammal that lacks placental steroid production throughout the entirety of gestation. Thus, ovarian support is paramount to a successful, term pregnancy (to be detailed further in subsequent sections). Additionally, deciduate cells are present within the canine placenta and similar in nature to those found in human and rodent placentas, exhibiting steroid receptor expression and involvement with fetal-maternal signaling. However, in contrast to human and rodent deciduate cells, decidualization in the bitch is more localized. Specifically, decidual cells

are concentrated around areas of trophoblastic invasion, rather than ubiquitous throughout the structure (Kowalewski, 2018) (Kowalewski et al., 2020). Deciduate cells are the only population of cells within the canine placenta that express progesterone receptors (P4-r). and oxytocin receptors (OXTR) (Figure 11). Relaxin receptors (RLN-r) are also present on the deciduate cells, but not exclusive to this location (Kowalewski et al., 2020) (Kowalewski, 2023). Though much remains unknown regarding the exact function of these receptors, they are thought to participate in the signaling cascade during pre-partum luteolysis (Figure 11).

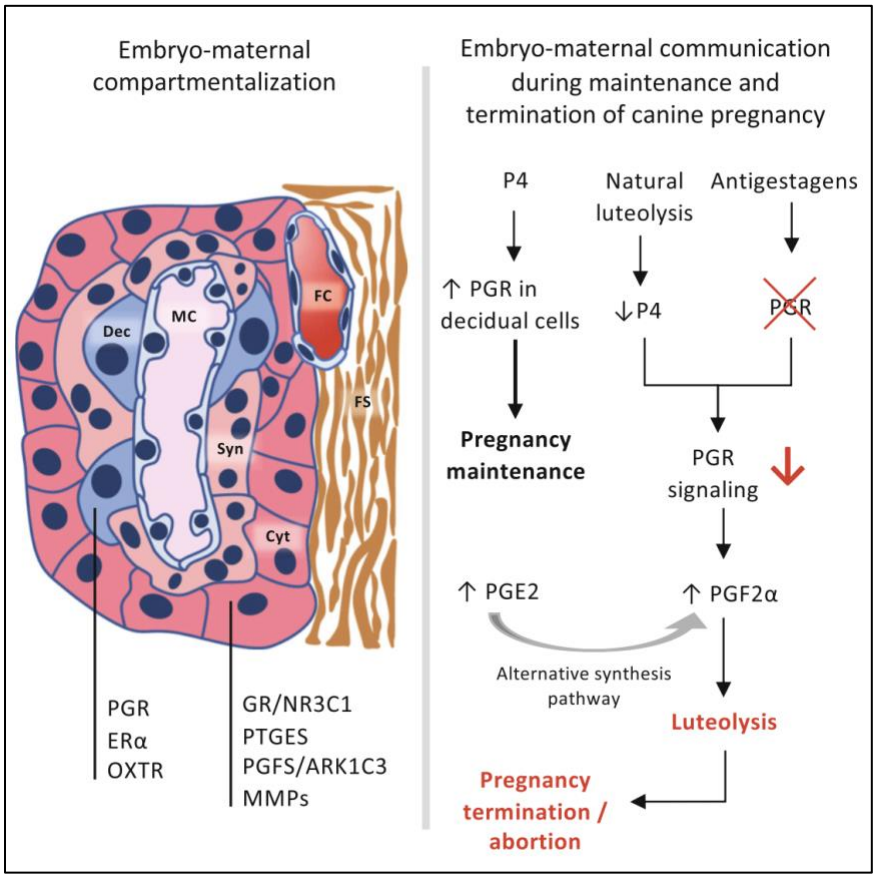


Figure 11 *- Schematic representation of feto-maternal interface within the placenta. Decidual cells are the only cells that express progesterone receptors (PGR) and oxytocin receptors (OXTR). Relaxin receptors are not depicted. Reprinted from Placentation in Mammals (Geisert et al., 2021.) - Chapter: Canine Endotheliochorial Placenta: Morpho-Functional Aspects.*

2.4. Maintenance of Pregnancy

2.4.I Progesterone

Progesterone is a steroid hormone secreted by the corpus luteum in mammals and has many roles that contribute to the maintenance of pregnancy. In canids, progesterone remains elevated for approximately nine weeks regardless of whether a pregnancy has been established. Progesterone will normally increase and peak 20-30 days following the LH surge before declining to basal levels at approximately 60-70 days post LH surge.

Progesterone receptors are present in several tissues in the pregnant bitch including the uterus (endometrium, myometrium), placenta, corpus luteum and cervix. Circulating progesterone binds to P4-r, regulated via paracrine and autocrine mechanisms, to exert effects in order to maintain pregnancy (Papa and Kowalewski 2020). In fact, the development of maternal decidual cells and expression of P4-r is one of the first events that support maintenance of pregnancy in early gestation. When in an environment of elevated steroid (P_4) levels, deciduate cells communicate with fetal trophoblastic cells to ensure the pregnancy is maintained (Kowalewski et al., 2020). Within the uterus, progesterone works to maintain a quiescent and supportive uterine environment for a pregnancy. Specifically, progesterone contributes to the differentiation and integrity of the maternal endometrium, regulates glandular secretions, and suppresses the uterine contractions that are mediated by circulating estrogen (Verstegen-Onclin and Verstegen 2008). Progesterone also increases cervical tone and dampens the maternal immune response, both of which are essential physiological changes to ensure pregnancy maintenance.

In several mammalian species, including the bitch, progesterone stimulates its own production through the influence of important steroidogenic enzymes (notably 3β-Hydroxysteroid

dehydrogenase and P450 side-chain-cleavage enzyme). *In vitro* studies have demonstrated a stimulatory effect of progesterone on these enzymes in murine species (Papa & Hoffmann, 2011). This is particularly important in early diestrus and correlates with the serum progesterone increase seen following ovulation. This autocrine regulation still occurs in late diestrus or pregnancy, but the limiting factor for progesterone production at that point relates to the availability of cholesterol as a substrate for production, rather than the enzymes involved in the steroidogenic pathway (Kowalewski et al., 2011).

Given the importance of progesterone in the maintenance of pregnancy, the metabolism of this hormone has been heavily investigated. In pregnancy, there is an increased amount of progesterone synthesized by the corpus luteum compared to a non-pregnant diestrus (Verstegen-Onclin & Verstegen, 2008). Comparing fecal metabolites of progesterone between pregnant and non-pregnant bitches has corroborated the theory that an increased production of P_4 by the corpus luteum occurs during pregnancy (Gudermuth, 1998). However, this discrepancy is not often captured by routine diagnostic testing, and this phenomenon can be attributed somewhat to an increased P_4 metabolism by the liver. Hemodilution (secondary to an increased plasma volume in the pregnant bitch) and functional removal of P4 (secondary to the increase in uterine and mammary mass and development of the placenta) also contribute. More recent studies have characterized the hepatic clearance to be approximately twice that in pregnant versus non-pregnant bitches, and the plasma dilution effect to cause a ~25% decrease in circulating P4 concentration (Gudermuth, 1998) (Concannon, 2009).

2.4.II Prolactin

In addition to progesterone, canine pregnancy is maintained and regulated by the luteotrophic hormones relaxin and prolactin. Prolactin is a polypeptide hormone that is secreted in

a pulsatile manner by the pituitary gland, from specialized cells called lactotrophs. Prolactin has also been shown to be secreted by the hypothalamus, uterus, and mammary gland (primarily reported in rats) (Freeman et al., 2000). Interestingly, in both humans and rodents, molecules nearly identical to pituitary prolactin have been shown to be produced by deciduate cells of the placenta; Additionally, certain immune cells such as lymphocytes and macrophages have been shown to be a source of prolactin in human and rodent species. If similar mechanisms are confirmed in the bitch, this could suggest an important interaction between the immune system, prolactin, and pregnancy maintenance in the canine species (Freeman et al., 2000).

Prolactin secretion is regulated primarily by the hormone dopamine, with dopamine acting in an inhibitory manner. There is no doubt a complex relationship between these two hormones - both act on receptors throughout a variety of tissues in the body. At the most fundamental level, however, dopaminergic secretion will lead to an inhibition of prolactin synthesis and secretion. Interestingly, *in vitro* studies have also demonstrated dopamine to be stimulatory to prolactin secretion (Freeman et al., 2000). Regulation of prolactin is not from dopamine alone, as there are several other hormones that play a role, albeit to a much lesser extent. These include vasoactive intestinal peptide, serotonin, opioid peptides, oytocin, thyrotropin-releasing hormone, nitric oxide, and estradiol (Freeman et al., 2000) (Kooistra & Okkens, 2001).

During gestation, elevated progesterone supports dopaminergic tone, which is inhibitory to the secretion of prolactin. Despite this, prolactin concentrations increase during the second half of gestation in the bitch, with a gradual rise starting around day 25 post-ovulation. Prolactin secretion persists at this stage due to placental factors stimulating its release, such as relaxin and possibly estradiol, along with increased pituitary sensitivity (Freeman et al., 2000). An acute, sharp increase

in the overall concentration is appreciated 1-day prepartum, correlating with the time of luteolysis, and progesterone falling to baseline levels, prior to parturition (Concannon, 2009).

Prolactin is arguably one of the most important luteotrophic hormones, and it is likely that this hormone acts through several mechanisms to maintain pregnancy. First, it is important to understand the target organs of prolactin. Prolactin acts on prolactin receptors (PRL-r), which are present on a variety of tissues in the body. In the bitch, PRL-r have been shown to be present within the corpus luteum, placenta, endometrium, mammary gland, and pituitary gland (Michel et al., 2012) (Kowalewski et al., 2011). Though not proven in the bitch, extrapolation from other species suggests that PRL-r may also be present in other areas of the brain (e.g. the hypothalamus), on immune cells, and potentially within bone, cartilage, or cardiovascular tissues (Freeman et al., 2000). Of all the locations, receptor expression is highest in corpus luteum but does vary within pregnancy and between pregnant and non-pregnant bitches. In pregnancy, expression was highest both pre- and post-implantation (between days 8-12 and 18-25 post-ovulation, respectively), with a significant decrease appreciated at the time of pre-partum luteolysis (~1 day pre-partum). In non-pregnant dogs, PRL-r expression is high early in diestrus (around day 15 post-ovulation), with a gradual decrease appreciated until the time of luteolysis. Additionally, receptor expression was evaluated within uterine and placental tissues in pregnant bitches, and found to be time-dependent, nearly opposite to expression in luteal cells. Specifically, lower expression was appreciated during the pre-implantation time period and increased within placental tissues mid-gestation. Though a decrease in PRL-r within the utero-placental was seen at the time of luteolysis, the finding was not significant. Characterizing the PRL-r within canine reproductive tissues during gestation has allowed a deeper understanding for why this hormone is so essential in the maintenance of pregnancy.

There is undoubtedly a tight regulation of PRL-r in relation to luteal function and placental development throughout pregnancy. Regarding luteal function, the decrease in PRL-r correlates with the overall trend in progesterone concentrations (high during early diestrus, diminishing until a sharp decline at luteolysis), suggesting that PRL may be an upstream regulator of progesterone secretion. Specifically, the synthesis of progesterone relies on the substrate cholesterol, as previously discussed, but also important enzymes such as steroidogenic acute regulatory protein (StAR) and 3β-Hydroxysteroid Dehydrogenase (3β-HSD). After binding to the PRL-r, PRL acts through a JAK/STAT pathway, which act upstream to StAR and 3β-HSD. To further support that PRL indirectly regulates the production of progesterone, it has been shown that expression of StAR and 3β-HSD decline in parallel with PRL-r expression (Kowalewski et al., 2011). PRL-r are also found on fetal trophoblast cells and uterine glandular epithelium (Kowalewski et al., 2020). Suggested roles of PRL on these tissues include the modulation of trophoblast and placental growth, and potentially nutrient transport and/or placental angiogenesis. Regulation of histotroph ("uterine milk"), though less characterized in canid species, has also been postulated (Kowalewski et al., 2011).

Though often considered pregnancy-specific, an increase in prolactin concentrations is also seen in non-pregnant bitches and serves the same role of maintaining the corpus luteum. This unique aspect of canine reproduction emphasizes the species' reliance on prolactin as an essential luteotropin, regardless of pregnancy status. Specifically, in the non-pregnant bitch, prolactin concentrations remain relatively low throughout the majority of diestrus, with an acute increase around day 50 post-ovulation, correlating with the clinical finding of pseudopregnancy (Kowalewski et al., 2011). Pseudocyesis, or pseudopregnancy, is a normal occurrence in intact female canids, though the clinical signs are variable. Typically, this condition encompasses a

variety of clinical signs including mammary development, galactorrhea, maternal behavior such as nesting, and even abdominal distention. Though this condition is thought to relate more directly to a loss in progesterone following luteolysis (and not secondary to the increase in prolactin), prolactin does play a role. Specifically, prolactin causes milk production, leading to lactation in some bitches with pseudopregnancy (Johnston et al., 2001).

In conclusion, PRL acts to maintain pregnancy through several mechanisms. First, PRL supports the conversion of cholesterol to progesterone by the upregulation and maintenance of StAR and other steroidogenic enzymes. Additionally, based on receptor expression at various timepoints during canine gestation, PRL has been postulated to act via autocrine, paracrine, and/or endocrine manners to maintain pregnancy in a variety of mechanism including placental growth, immune regulation, or direct conceptus support via modulation of uterine glandular secretions. Finally, PRL is a potent luteotropic hormone, maintaining the lifespan of the CL in both pregnant and non-pregnant bitches.

2.4.III Relaxin

Relaxin (RLN) is a peptide hormone that originates primarily from the placenta in the bitch (via fetal cytotrophoblastic cells). However, the CL has also been shown to produce RLN, albeit at a more local level, with minimal impact to blood concentrations of the hormone. RLN can be used clinically as a diagnostic test for pregnancy. In the bitch, RLN is detected in serum around day 20-30 of pregnancy, peaks a few weeks later, and remains elevated through term (Nowak et al., 2018). Similarly to prolactin, relaxin acts on receptors (RXFP1 and RFXP2) that are present on luteal steroidogenic cells, macrophages, and within the anterior pituitary. Lactotrophs, as seen with PRL-r, also express RXFP1, whereas vascular endothelial cells (particularly located around

CL), express RFXP2. Finally, though RLN is produced by fetal placental cells, the receptor RFXP2 is expressed on maternal (decidual) cells (Nowak et al., 2018).

RLN has a variety of roles during canine pregnancy, though much of the precise mechanisms are extrapolated by other species. However, based on the evidence of RNL production and relaxin receptors, there are several proposed mechanisms. In humans, acting primarily through the RXFP1 receptor, RLN promotes the production of nitric oxide (NO), causing vasodilation, and ultimately acts on the smooth muscles of the myometrium and uterine vasculature to reduce uterine contractility and ensure adequate uterine perfusion (Conrad, 2011). In the bitch, RXFP1 receptors have been localized in endothelial cells, suggesting a similar mechanism (Nowak et al., 2018).

Additionally, RLN promotes activation of cAMP on certain target tissues, including the endometrium, and this activation is associated with markers for decidualization, such as prolactin. In rats, RLN has been shown to stimulate production of prolactin from lactotrophs in the anterior pituitary, likely via a cAMP-mediated mechanism (Freeman et al., 2000). Similar mechanisms have also been identified in primates (monkeys) and pigs. Though the direct production of PRL by RLN has yet to be proven in dogs, the identification of relaxin and prolactin in canine lactotrophs is strongly suggestive that relaxin may stimulate prolactin secretion in dogs. Given the important luteotrophic effects of prolactin – secondary stimulation of PRL by RLN would be considered as important mechanism by which this hormone helps to maintain pregnancy in the bitch (Valkovic et al., 2019).

Immunomodulatory effects of RLN have also been demonstrated. In humans, RLN has been shown to modulate the immune system via secretion of pro- and anti-inflammatory cytokine secretion by the uterus throughout pregnancy, ensuring maternal immune tolerance to the fetus, and facilitating implantation (Goldsmith & Weiss, 2009). Though this exact mechanism remains

unproven in the bitch, the discovery of the relaxin receptor, RXFP2, in luteal macrophages suggests there may be a similar immunomodulatory nature to RLN in the support and maintenance of canine pregnancy.

In addition to the growing research characterizing the luteotrophic hormones relaxin and prolactin and their impacts on pregnancy maintenance, previous research studies have demonstrated that a progesterone receptor antagonist, aglepristone (Alizin^{Ò}), not only causes a functional progesterone withdrawal, leading to pregnancy termination, but also decreases circulating RLN and PRL levels in the bitch (Nowak et al., 2018) (Kowalewski et al., 2011). As this medication increases in popularity and availability in the United States, more will likely be discovered about the relationship.

2.5. Luteolysis

Luteolysis is a process by which the corpus luteum (CL) breaks down following the end of the luteal phase of the estrous cycle, prior to normal parturition, or preceding pregnancy loss. The mechanism by which luteolysis occurs varies between species and is often differentiated as structural or functional. Structural luteolysis, as the name implies, involves a structural change to the luteal tissue that makes up the corpus luteum. Functional luteolysis, however, occurs when there is a decline in steroid production by luteal tissue (e.g. decline in serum progesterone concentration). The differentiation between functional and structural luteolysis is often not clinically relevant, but collection or imaging ovarian tissue at the time of suspected luteolysis can differentiate.

There are variations to mechanisms regulating luteolysis between species. For instance, in ruminants, luteolysis is initiated by the secretion of prostaglandin F2a (PGF2a) by the uterus,

leading to the return to estrus (in the event that no conceptus is recognized between day 16-18 of the cycle), or parturition (in pregnant animals) (Youngquist & Threlfall, 2007). In the bitch, regardless of pregnancy status, PGF2a secretion by the endometrium is not the driver for luteolysis. However, there are notable differences between luteolysis in pregnant and non-pregnant bitches, adding to the complexity of luteolysis in this species.

In non-pregnant bitches, luteolysis is most often considered a passive process consisting of regression of luteal tissue and PGF2a secretion does not appear to play a role. This has been demonstrated in early studies performed on non-pregnant dogs investigating luteolytic signals in the bitch. Hysterectomy does not appear to significantly impact the duration of diestrus, which corroborates more recent research findings that uterine prostaglandins are not a driving force behind the luteolytic mechanism. Additionally, neither serum PGF2a, or metabolites, are identified systemically in serum during luteal regression in non-pregnant bitches (Zatta et al., 2017). Although diestrus is approximately the same duration irrespective of pregnancy, functional luteolysis in non-pregnant bitches occurs as early as day 30 post-ovulation (Kowalewski, 2014). Interestingly, this correlates with the timeframe in which the CL requires gonadotropin support (gonadotropin-dependent phase). Despite an increase in systemic prolactin concentrations, progesterone concentrations start to decline. This is thought to mark the onset of functional luteolysis, as despite potent luteotropic support by PRL, the steroidogenic capacity of the CL is diminished. Additionally, there is decreased expression of the key steroidogenic enzymes required for the production of progesterone by the CL (Kowalewski, 2014; Zatta et al., 2017). Structural luteolysis follows functional luteolysis and is most evident around 45-60 days post-ovulation in the non-pregnant bitch (Hoffmann et al., 2004). Much remains unknown regarding the precise mechanism leading to the onset of structural luteolysis. Current literature supports an intrinsic

mechanism that leads to metabolic senescence of luteal cells (Zatta et al., 2017). The immune system also plays a smaller role, with evidence showing that immune cells are present within the CL to play a role in clearance and remodeling of luteal tissue and surrounding vasculature (Hoffmann et al., 2004).

In the pregnant bitch, luteolysis differs in that CL regression occurs sharply and purposefully and is not secondary to degeneration or a loss of steroidogenic capacity. Unlike in other species where prostaglandin is produced by the maternal endometrium, in canids PGF2a is produced by fetal trophoblastic cells within the placenta. This production correlates with fetal maturation, and an acute rise in production of PGF2a occurs around day 60-63 of gestation in normal pregnancies (Kowalewski et al., 2011) (Nowak et al., 2018). It's important to note that fetal death can also trigger production of prostaglandins, leading to luteolysis and parturition. The immune system is also involved in luteolysis in the pregnant bitch and is thought to play a much larger role by causing rapid destruction of luteal tissue. Following PGF2a production by the placenta, immune cells such as macrophages, neutrophils and lymphocytes influx into luteal tissue, accelerating luteal breakdown by the production of pro-inflammatory cytokines (Nowak et al., 2018; Zatta et al., 2017). This immune and inflammatory response precedes the suppression of steroidal enzymes, ultimately leading to a sharp progesterone decline and impending parturition.

Luteolysis in the bitch, regardless of pregnancy status, leads to the removal of progesterone production by the corpus luteum. However, there are significant differences between the mechanisms that drive luteal regression in the pregnant and non-pregnant bitch. In the absence of pregnancy, luteolysis is a slow, autonomous process, driven by an internally programmed timeline. Contrast this with luteolysis in the pregnant bitch, a mechanism that is driven by fetal maturation,

robust immune system involvement, and rapid structural and functional degeneration of the CL, leading to parturition.

3. Pregnancy Loss in the Bitch

3.1. Diagnosing Pregnancy Loss

A definitive diagnosis for the cause of pregnancy loss in the bitch is often difficult to obtain. Regardless of the etiology, the outcome is devastating for the owner and clinician alike. The term pregnancy loss is inclusive of all causes of embryonic death, fetal resorption, abortion at any stage of pregnancy, and stillbirth. Early embryonic death may often go unnoticed, as the timepoint this encompasses (between days 1-20 after fertilization) is before routine pregnancy diagnosis in the bitch (e.g. abdominal ultrasonography, relaxin) (Verstegen-Onclin & Verstegen, 2008). Fetal resorption may be more easily identified, especially in larger litters, based on comparisons between the inner chorionic cavities (size discrepancies, changes to echogenicity, absence of viable fetus). Abortion accounts for only a small component and is defined as the delivery of one or more fetuses that cannot survive outside of the uterus. Finally, it's important to note that both early embryonic death and fetal resorption can be presented in a clinical history as infertility, due to the absence of overt clinical signs in these cases.

3.2. Infectious Causes of Pregnancy Loss in the Bitch

There are several causes of pregnancy loss in the bitch. Broadly speaking, infectious disease is thought to be the most common etiology of pregnancy loss in the bitch (Johnston et al., 2001) (Verstegen-Onclin & Verstegen, 2008). Though almost any infectious agent could lead to

maternal compromise such that pregnancy loss and abortion results, there are a few key infectious agents that are associated with pregnancy loss and abortion in the dog.

3.2.I Brucellosis

Brucella canis is a gram-negative bacterium that causes canine brucellosis. Canine brucellosis is found worldwide, however seroprevalence has been difficult to determine. Brucella species are zoonotic, and canine brucellosis specifically is moderately transmissible to humans (as compared to the primary *Brucella spp*. seen in other species, such as small ruminants, which are highly zoonotic). The most common clinical signs for this condition in dogs include lethargy, lymphadenopathy, diskospondylitis, uveitis, orchitis, epididymitis and acute prostatitis. Regarding the pregnant bitch, the most common clinical presentation is abortion, usually after day 45 of pregnancy. Fetal pathology includes autolysis, edema, hemorrhage, and congested tissues. Following abortion, the bitch may have foul smelling vaginal discharge accompanied by post-partum metritis. Less commonly, neonates may present as a litter that is failing to thrive. Additionally, the bitch may have a history of previous infertility or abortion (Sebzda & Kauffman, 2023).

3.2.II Herpesvirus

Canine herpesvirus, caused by the viral agent *canid alphaherpesvirus 1*, is also very prevalent worldwide and has a much higher incidence than Brucellosis (up to 60-80%, depending on the country) (Verstegen et al., 2008). Clinical signs of healthy adult dogs are generally inapparent in latent infection, and lead to carrier status of infected individuals, with recrudescence possible. Reproductive losses can present at any timepoint in gestation, with late term abortion and the birth of weak neonates slightly more common (likely due to the accuracy of diagnosis of that

that stage). A characteristic “turkey-egg kidney”, describing diffuse renal petechiation seen on necropsy, is considered pathognomonic. Abortion and neonatal death is often limited to the first litter following infection in the bitch, as maternal antibodies are protective in subsequent litters. Treatment is possible, though not without significant commitment and expense, as long-term antiviral therapy is indicated for these cases.

3.3. Non-infectious Causes of Pregnancy Loss in the Bitch

There are a plethora of non-infectious etiologies that can result in pregnancy loss. In many cases, non-infectious conditions result in decreased *fertility* (via altered cyclicity or structural changes to the uterus or uterine environment). However, loss of *pregnancy* following fertilization may instead be the primary clinical symptom. A differentiation as to whether fertility or pregnancy is directly impacted may assist the practitioner in identification of the underlying cause. Considering the extensive range of non-infectious causes of pregnancy loss, this review will be limited to the most well-studied and significant.

Underlying systemic disease and co-morbidities are an important cause for pregnancy loss in the bitch. The most common culprits include diabetes mellitus, hypothyroidism, hyperadrenocorticism and hypoadrenocorticism (Verstegen et al., 2008). Many of these conditions impair the normal hypothalamic-pituitary-gonadal axis by causing alterations to up or down-stream hormones (e.g. excess cortisol in hyperadrenocorticism leading to ovulation failure). Obtaining an accurate diagnosis in these cases is essential as treating the symptoms (rather than the underlying cause) is often unrewarding.

Uterine pathology (e.g., CEH-pyometra complex, acquired scar tissue, developmental disorders) is also a known cause of pregnancy failure in many mammalian species, including the

bitch. Abnormal uterine structure or function will result in the inability for normal conceptus attachment and/or abnormal placental formation.

Medication and toxicants are also an important cause of non-infectious pregnancy loss. In theory, exposure of almost any pharmaceutical or nutraceutical can be toxic to pregnancy at the wrong dose. Numerous medications are contra-indicated during pregnancy due to teratogenic effects including androgens, anabolic and estrogenic steroids, glucocorticoids, antimicrobials, antifungals, antiparasitic agents, antineoplastic agents, analgesics and anti-inflammatories (Verstegen et al., 2008) (Johnston et al., 2001).

Hormonal and immunologic aberrations are also reported causes of pregnancy loss in the bitch. Hypoluteoidism falls into this category (discussed in more detail below). Our specific study is investigating hypoluteoidism in a pedigree of Portuguese Water Dogs, and the potential heritability of this condition.

4. Hypoluteoidism

4.1. Intro to hypoluteoidism

4.1.I Definition

Hypoluteoidism is defined as a premature decline of serum progesterone during gestation and is most commonly observed between days 20 and 35 of pregnancy in the bitch (Becher et al., 2010). Left undiagnosed or untreated, this condition will result in pregnancy loss if progesterone concentrations fall to <2 ng/mL. Although there are limited documented cases in the literature, hypoluteoidism is certainly seen in the clinical setting. Case reports have been documented in German Shepherd Dogs, a Bernese Mountain Dog, an Istrian Shorthaired Hound dog, and an Old English Sheepdog. (Zedda et al., 2017) (Günzel-Apel et al., 2006) (Görlinger et al., 2005)

(Dockweiler et al., 2017). Our present study seeks to determine an underlying genetic cause for hypoluteoidism in the bitch, with the long-term goal of developing a screening test to prevent the financial and emotional burden that pregnancy loss has on owners and clinicians, alike.

4.1.II Significance

Canine pregnancy loss is a devastating emotional and financial loss to breeders. Hypoluteoidism is a condition that, left untreated, will result in spontaneous abortion due to a premature decline in serum P_4 concentrations during gestation. The economic investment involved to ensure a successful fertilization, healthy pregnancy, and minimized risk of labor and delivery of neonates can be substantial. Unfortunately, despite the best efforts of the clinician and owner, a drop in serum progesterone may be missed, as testing for this hormone is not indicated mid-gestation - even in cases with an unknown history (or in cases of primiparous bitches). When diagnosed early, treatment options are available and can be both practical and successful. Thus, accurate and early detection of this condition is essential as therapeutic intervention, in the vast majority of cases, will lead to positive clinical outcomes.

4.2. Concurrent Medical Conditions and Hypoluteoidism

4.2.I Shortened Luteal Phase

A 2006 study by Günzel-Apel et. al. investigated progesterone levels in German Shepherd Dogs to determine a link between cycle length and progesterone levels. In this study, twenty-two non-pregnant and nineteen pregnant German Shepherd dogs were evaluated and assigned to a control or short-cycling group, based on the duration of their interestrus interval, with <5 months considered short-cycling. The study reported that there was a significant association between progesterone levels and a shortened luteal phase (Günzel-Apel et al., 2006). One postulated

mechanism behind the shortened luteal phase is due to the absence of a true anestrus secondary to an inadequate amount (both amplitude and duration) of progesterone. Without this sufficient 'threshold' of progesterone priming on the hypothalamus, anestrus does not occur, and bitches return to heat following diestrus (pregnant or non-pregnant). By that definition, the luteal phase itself is not shortened, but rather the anestrual period of the estrous cycle is shortened. Clinically, 'split heats' – the absence of ovulation and subsequent insufficient rise of progesterone during estrus – may look similar to a shortened luteal phase, as bitches will appear to come back into heat in a few weeks to months. Careful serum progesterone monitoring and clinical correlation (e.g. vaginal cytology to determine stage of cycle) is used to diagnose a split heat from a shortened luteal phase (Meyers-Wallen, 2007).

4.2.II Hypothyroidism

Although a definitive link between hypoluteoidism and hypothyroidism in the bitch has not been proven, hypothyroidism is an endocrinopathy that is often implicated in cases of reproductive failure in the bitch. Specifically, abnormal thyroid hormones (low T_4) have been identified in bitches with pregnancy loss and infertility (Verstegen et al., 2008). However, there are conflicting results on whether fertility is truly impacted in bitches with hypothyroidism. Nevertheless, breeders often scrutinize thyroid levels in their breeding bitches to mitigate any change that fertility could be impacted due to a hypothyroid state. Negative effects on neonatal outcomes have also been demonstrated in experimentally induced hypothyroidism in the bitch. A study by Panciera et. al. showed decreased birth weight and increased periparturient mortality, which the author noted is similar to pregnant women with untreated hypothyroidism (Panciera et al., 2012).

The exact mechanism for impaired fertility and poor neonatal outcomes has yet to be elucidated and is likely multifactorial. In humans, hypothyroidism has been associated with decreased secretion of gonadotropins (LH and FSH), from reduced gonadotropin-hormone (GnRH) pulsatility (Hughes, 2012). Furthermore, humans with hypothyroidism and concurrent polycystic ovarian disease have aberrations to steroidogenesis, including abnormal metabolic clearance of androstenedione and estrone, resulting in more androgenic activity (Arduc et al., 2015). The long-term effects of upregulated androgenic activity (in the female) can include poor follicular and luteal development, resulting in a decline in progesterone production by the corpus luteum. If this also occurs in bitches with hypothyroidism, infertility and/or concurrent hypoluteoidism as a clinical manifestation is certainly plausible. Though much remains unknown about whether or not hypothyroidism is directly linked to hypoluteoidism, further research in this area could yield important insights.

4.3. Etiology of Hypoluteoidism

4.3.I Proposed Mechanism(s)

The exact mechanism behind luteal insufficiency is still unknown. Several mechanisms have been explored, including primary and secondary dysregulation of the corpus luteum, underlying autoimmune disorders, and genetic or familial predisposition.

Arguably the most widely accepted mechanism leading to the characteristic decline in serum progesterone in bitches with hypoluteoidism is a primary, intrinsic failure of the corpus luteum to maintain production of this steroid. In other words, *functional* corpus luteal failure, rather than *structural* luteal failure, is the proposed inciting underlying etiology resulting in low serum progesterone and subsequent pregnancy loss. Support for functional corpus luteal failure is

provided by evidence of elevated circulating prolactin concentrations observed in bitches with a diagnosis of hypoluteoidism, suggesting a downstream effect, rather than a failure from disruption to this luteotrophic hormone (Kowalewski et al., 2011) (Kowalewski et al., 2009). Possibilities for downstream dysregulation include a disruption to the PRL-r signal or expression, defects in the production of steroidogenic enzymes (e.g. StAR, 3β-HSD), or dysregulation of the luteotrophic hormone LH, or it's receptor (LH-r) (Kowalewski et al., 2006) (Johnston et al., 2001). Receptor alterations have also been described in reproductive tissues outside of the corpus luteum. In one case report, an Old English Sheepdog was presented for infertility. In the diagnostic workup, a uterine biopsy was obtained and immunohistochemistry for progesterone receptors was performed. Weak intranuclear immunoreactivity was identified, supporting a problem at the level of the endometrium (rather than the corpus luteum, as previously described). While the clinical relevance of this finding (pertaining to hypoluteoidism specifically) is unknown, the knowledge gained from the study does help to fill the knowledge gaps of pregnancy loss in the bitch (Dockweiler et al., 2017).

Another proposed mechanism that has been explored as a cause for hypoluteoidism is immune system dysregulation. In a study performed by Karachudel et. al., bitches experiencing shortened interestrous intervals, infertility, or pregnancy loss were examined against control dogs. Specifically, serum progesterone, and the presence of anti-progesterone antibodies (IgE, IgG and IgM) were evaluated. Serum progesterone concentrations were significantly lower in bitches with hypoluteoidism, whereas serum prolactin (in German Shepherd Dogs) was significantly higher than controls. Furthermore, increased levels of IgE antibodies against progesterone were found in 6 bitches (including one control bitch), suggesting a possible correlation with hypoluteoidism. Though studies in the bitch on hypoluteoidism have been primarily observational, prospective

studies have been performed in other species. For instance, experimental activation of the innate immune system was performed in a murine model in a study by Erlebacher et. al. Activation of the immune system resulted in a decrease in serum progesterone levels and paradoxical alterations luteinizing hormone receptor (downregulated) and 20α-HSD (upregulated). Furthermore, serum prolactin levels were normal to mildly elevated, consistent with findings of hypoluteoid bitches in the literature (Erlebacher et al., 2004). Additionally (though also observational) in humans, autoimmune conditions such as systemic lupus erythematosus (SLE) have been linked to luteal phase deficiency (Hughes, 2012; Kowalewski et al., 2011).

There is strong support for the driving mechanism of hypoluteoidism to be genetic in origin. Genetic aberrations could explain the current theory of intrinsic luteal failure as an underlying mechanism of hypoluteoidism, and support the immunologic links described in the literature. Furthermore, genetic variants as a leading cause of hypoluteoidism in the bitch is consistent with anecdotal evidence and published case reports investigating this condition in certain breeds and lines of dogs. Unfortunately, the scope of genetic investigation relating to pregnancy loss in the bitch is quite limited. However, evidence from other mammalian species demonstrates evidence for both Mendelian and non-Mendelian inherited disorders resulting in pregnancy loss.

Though there are a plethora of genes involved with maintenance of pregnancy, genetic variants to *CYP11A1*, in both wild-type and experimental models, closely resemble hypoluteoidism in the bitch. *CYP11A1* is a gene that codes for a key enzyme in the steroidogenic pathway (cholesterol side-chain cleavage enzyme, also known as P450scc). P450scc is responsible for converting cholesterol into pregnenolone (P_5) within the inner mitochondrial membrane of luteal cells (Fortune, 2018). In humans, disorders related to mutations of the *CYP11A1* generally follow

an autosomal recessive inheritance pattern, causing impaired steroidogenesis to varying degrees, depending on the type of mutation. Both homozygous and compound mutations are reported, with homozygous mutations resulting in congenital adrenal insufficiency and disorders of sexual development (e.g. gonadal dysgenesis, sex-reversal) (Tee et al., 2013). In compounded mutations of this gene, activity of P450scc is only partially diminished, resulting in conditions that are not limiting reproductive capacity, *per se*, but may lead to failure of an established pregnancy, due to a lack of steroidogenic capacity by luteal and/or placental tissues (Tee et al., 2013) (Rayat et al., 2023). In addition to autosomal recessive, loss-of-function mutations seen clinically in humans, overexpression of the *CYP11A1* has been studied in laboratory settings using transgenic mice, to mimic disease states such as polycystic ovarian syndrome (PCOS). Mice with overexpression of *CYP11A1* had normal copulatory behavior and evidence of normal fertilization, however the transgenic females failed to sustain pregnancy to term. Additionally, aberrations to progesterone secretions were observed, which the authors concluded was a contributing factor to pregnancy loss. Regarding heritability, despite the iatrogenic introduction of a transgene, germline transmission was confirmed by PCR genotyping of the offspring (Chien et al., 2013).

Another autosomal recessive pattern of inheritance is seen in pathogenic mutations involving the gene STAR-Domain containing protein 1 (STARD1). STARD1 codes for an important protein that is also involved in steroidogenesis, Steroidogenic Acute Regulatory protein (StAR). StAR acts one step upstream of CYP11A1 and is responsible for transferring cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane. Complete null mutations in StAR, as documented in humans, cause complete cessation of steroidogenesis. This condition, known as lipoid congenital adrenal hyperplasia, is life-threatening in neonatal life. Without proper diagnosis and treatment, death is imminent secondary to adrenal insufficiency.

With treatment, patients can live, but clinical outcomes include sex-reversal and often infertility. Partial mutations in STARD1 have also been characterized, in both humans and mice. In both species, puberty appears to proceed as expected, due to a threshold amount of preserved steroidogenesis. In mice, with notably shorter lifespans than humans, a progressive buildup of cholesterol within ovarian (and other steroid-producing tissues) occurs, eventually leading to anovulatory cycles and infertility due to impaired progesterone synthesis (Selvaraj et al., 2018). In humans, very similar clinical outcomes are seen, with most of the literature focused on alterations to the menstrual cycle, including an inhibition of progesterone synthesis late in the cycle due to lipid accumulation (Miller, 2017).

In addition to the Mendelian inheritance patterns discussed, non-Mendelian inheritance mechanisms have also been identified as important contributors to the genetic susceptibility for pregnancy loss in women. For instance, an inherited gene variant, PROGINS has been identified in the progesterone receptor gene (PGR). Specifically, the PROGINS complex is a repetitive element belonging to the *ALU* family characterized by the insertion of an approximately 306 base-pairs, accompanied by linked single nucleotide polymorphisms (V660L and H770H) (Tiwari et al., 2015). The *ALU* family is a class of repetitive DNA that is dispersed throughout the human body, makes up a significant proportion of the genome (~10%), and has been implicated in genetic diseases. Though the insertions are typically non-coding, when inserted into functional genomic regions, the ALU insertions can disrupt gene regulation and expression, causing clinical manifestations of disease. (Batzer & Deininger, 2002). In humans, the PROGINS polymorphism is believed to alter progesterone receptor function, reducing the efficacy of receptor signaling and normal endometrial preparation necessary to maintain pregnancy. Clinically, this manifests as

recurrent pregnancy loss and increased risk of moderately preterm delivery (defined as birth between 32-37 weeks of gestation) (Tiwari et al., 2015) (Nebela Khan et al., 2021).

In summary, multiple pathways can disrupt progesterone production and signaling during the luteal phase and pregnancy, including corpus luteum disfunction, immune-mediated luteal failure, and genetic variants altering steroidogenesis or receptor function. Though the precise mechanism causing hypoluteoidism in the bitch remains unknown, these models help to provide an understanding for biological processes that may contribute to pregnancy loss.

4.4. Treatment for hypoluteoidism

4.4.I Indications for Treatment

Treatment for hypoluteoidism is achievable provided the diagnosis has been made prior to compromise of the pregnancy. The current consensus is to reserve treatment for bitches with viable fetuses and a serum progesterone measurement of <5ng/mL (15.7 nmol/L) before 58-60 of pregnancy or following a rapid decline in serum progesterone (total decline of approximately 10-15 ng/mL) between days 20-35 of gestation (Becher et al., 2010). Caution should be used if treating a bitch prior to day 45 of gestation, due to the high likelihood of reported teratogenic side effects on the fetus (below).

4.4.II Progesterone Supplementation and Side Effects

Fortunately, there are treatment options available for dogs diagnosed with hypoluteoidism while pregnant. However, the available treatments are not without risk. Treatment consists of supplementation with either natural progesterone or synthetic progestin. Examples of natural progesterone supplementation include Prometrium (Solvay Pharmaceuticals, Marietta, GA, USA),

and synthetic options include Altrenogest and Megestrol Acetate (MGA)(Johnson, 2008) (Günzel-Apel et al., 2012).

As mentioned, treatment for hypoluteoidism should be carefully considered due to potential teratogenic effects, including masculinization of female puppies, hypospadias, increased incidence of cryptorchidism in male puppies, and limb, facial, or cardiac deformities in either sex (Becher et al., 2010) (Verstegen et al., 2008). Adverse effects of progestogens reported in the treated bitch include increased incidence of cystic endometrial hyperplasia and pyometra (Noakes et al., 2019). There was also a case report of mammary fibroadenoma that had developed under progestin treatment (Zedda, Bogliolo et al. 2017). Anecdotally, in our present study of Portuguese Water Dogs, one of the bitches receiving altrenogest had a male puppy diagnosed with hemilamia (underdeveloped limb(s)) and ectrodactyly (missing digit(s)) at birth. Due to the risk of internal deformities and overall poor prognosis, the puppy was euthanized shortly after birth. The remainder of the litter was healthy but did require supplemental feeding due to reduced milk production from the dam.

4.4.III Ancillary medications

Uterine tocolytics are another class of medications that can be used in conjunction with progestins to help maintain pregnancy. Terbutaline, a β2-adrenergic agonist, can be used off-label for this purpose at a dose of 0.01 mg/kg subcutaneously, up to every 6 hours (titrated to effect) (Wiebe & Howard, 2009). This should be reserved for bitches in late gestation that have clinical signs associated with premature labor, such as nesting behavior, transient decrease in body temperature, or vaginal discharge (Johnson, 2008). Additionally, use of uterine tocolytics *without* progesterone supplementation will not prevent premature labor in cases of hypoluteoidism (and

often in other causes of pregnancy loss), as the inciting cause for parturition is a drop in progesterone secondary to luteal failure.

II. Materials and Methods

1. Animals

This study was conducted at the Small Animal Teaching Hospital at Auburn University in Auburn, AL. Animals involved were client-owned, seeking clinical services from the Small Animal Theriogenology service related to breeding and pregnancy management. All animals were healthy, up to date on vaccinations, and tested negative for *Brucella canis* at the time of breeding. A diagnosis of hypoluteoidism was made if a female had a progesterone concentration of <2ng/mL prior to 60 days of gestation (60 days post-ovulation) in one or more pregnancies. One the first female was positively diagnosed with hypoluteoidism, related individuals were considered to high-risk for pregnancy loss.

2. Sample Collection

Residual canine serum samples were obtained for this study and used with owner consent. Blood collection was performed at the time of clinical services and used for hormonal analysis of serum progesterone, either for ovulation timing or pregnancy monitoring. Following collection, whole blood samples were centrifuged to separate serum. One aliquot was used immediately for hormone analysis, and the remaining serum samples stored at either -20°C or -80°C for future use.

3. Hormone Analysis

Serum hormone concentrations for progesterone were obtained using two platforms, based on lab availability and hospital hours. For after-hours or weekend samples, an IDEXX Catalyst DxÒ analyzer (IDEXX Laboratiries, Westbrook, ME) was performed according to manufacturer instructions. For weekday samples, the serum was submitted to Auburn University's Endocrine

Diagnostic Service, and a validated chemiluminescent immunoassay was performed. Both methods ensured same-day results.

4. Pedigree Construction

A multi-generational pedigree was constructed using breeding records and accessible data from the Orthopedic Foundation for Animals (OFA) and the Portuguese Water Dog Foundation (PWD-F). Breeding records were corroborated by medical records to ensure accurate identification of individuals, and lineage was traced with OFA and the PWD-F. Relationships were verified across multiple generations. Pedigree structure was used to assess potential inheritance patterns for hypoluteoidism.

5. Progesterone Data Analysis

Serum progesterone values were obtained for identified individuals across multiple pregnancies, if applicable. Using medical records from Auburn University's Small Animal Theriogenology Department, the day of ovulation for each individual, for each pregnancy, was determined and this was deemed 'day 0' of gestation. The remaining progesterone values were obtained from medical records and analyzed throughout the entirety of gestation, in mid gestation (defined as days 23-45 post-ovulation) and in late gestation (defined as days 50-60 post-ovulation).

Serum progesterone concentrations were compared between affected and unaffected bitches during mid-gestation (day 23-45 post-ovulation) using an unpaired two-tailed t-test. Subsequent pregnancies, if applicable, were treated as independent individuals. Normality was assumed based on sample distribution, and significance was defined as $p < 0.05$.

I. Results

1. Pedigree

Our preliminary findings support a genetic basis for hypoluteoidism. While once thought to be a rare occurrence, our team has documented this condition in directly related individuals within a pedigree of PWDs. To best investigate the heritability of this condition, we have created a depiction of this pedigree (Figure 12) to highlight the relationship between the affected and unaffected individuals. We have confirmed that one affected female with hypoluteoidism has produced both affected and unaffected female offspring (with the assistance of medical intervention throughout pregnancy).

In the pedigree, females are depicted in circles, and males are depicted in squares. Affected females (defined as females in which serum P_4 dropped below 2 ng/mL prior to 63 days of gestation) are depicted in black (Dam 1, and her daughter, Dam 2). Unaffected females are depicted with a patterned circle (Dam 3 - daughter of Dam 1, and Dam 4, daughter of Dam 2). Lastly, females that are colored in gray are suspected to have hypoluteoidism due to lower-than-expected P_4 values during gestation (serum P_4 of <2 ng/mL not definitively documented – Dam 5 and Dam 6). Interestingly, one of the suspected females (Dam 6) has a common ancestral sire with Dam 1.

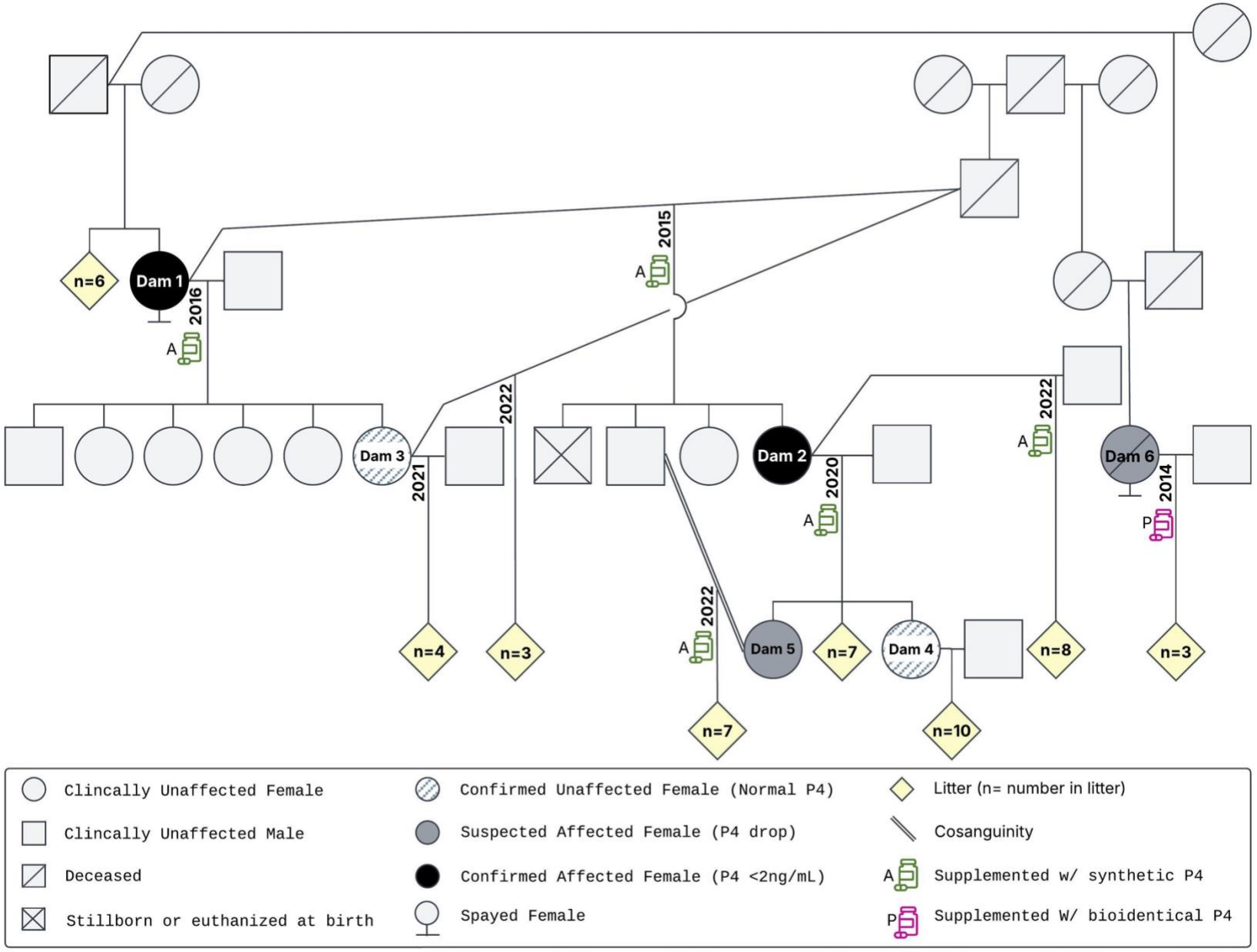


Figure 12 *- Pedigree illustrating affected and unaffected individuals in a cohort of Portuguese Water Dogs evaluated for suspected hypoluteoidism. Generational structure and familial relationships are depicted to assess potential heritable patterns.*

2. Individual Progesterone Concentrations

2.1. Dam 1

Dam 1 was presented to Auburn University's theriogenology service in 2015 for a workup of infertility. Breeding was attempted on three prior heat cycles in this dam, without a successful pregnancy. Serum P_4 concentrations for the 2015 pregnancy were monitored very closely and declined throughout gestation, reaching a nadir of 1.4 ng/mL at day 53 post-ovulation. A similar pattern was observed during her second gestation the following year (2016). Remarkably, once hypoluteoidism was confirmed, medical intervention and close monitoring resulted in successful outcomes for both pregnancies. P_4 concentrations and details of both pregnancies for Dam 1 are illustrated in Figure 14.

2.2. Dam 2

Dam 2, a daughter from Dam 1's first litter, was later presented for breeding and pregnancy management in 2020. Due to the family history of hypoluteoidism, P_4 was closely monitored throughout gestation (Figure 14) Her P_4 declined, reaching a nadir of 1.6 ng/mL on day 56. Treatment with altrenogest was initiated, and the pregnancy was successfully carried to term. A subsequent litter was successfully managed in Dam 2 the following year (2022) with medical therapy initiated on day 47, and a healthy litter of eight puppies was delivered via cesarean section on day 63.

2.3. Dam 3

A third female, out of the second litter of Dam 1, was also bred (Figure 12)**.** As a precaution, serum P_4 was monitored. Although she is a first-degree relative of Dam 1, this female (Dam 3)

does not have a diagnosis of hypoluteoidism. Serum P_4 concentrations remained adequate for maintenance of pregnancy, without the need for medical intervention. She delivered a healthy litter of four puppies on her due date. This same trend was observed in her second litter the following year, resulting in a healthy litter of three puppies. Peak serum P_4 concentration was 32.6 ng/mL at 3 days post-ovulation.

2.4. Dam 4

Dam 4 was bred in fall of 2023. She is the daughter of Dam 2 (affected) and half-sister to Dam 5 (suspect affected, see 'other individuals', below). Though her serum P_4 started to decrease after day 47 of gestation, she did not reach concerning levels (of <2 ng/mL prior to day 60 of gestation). Fortunately, she did not require any progesterone supplementation. Serum P_4 concentrations continued to decline, reaching a baseline value of 0.6 ng/mL on day 62. An elective cesarean section was performed, and she delivered a healthy litter of 10 puppies.

2.5. Other Individuals

There are two other females on the pedigree of interest. Dam 5 is the offspring of Dam 2 (affected), and second generation to Dam 1 (affected). Her P_4 did decline during gestation to 3.4 ng/mL on day 52 post-ovulation, and supplementation with altrenogest was implemented. A P_4 value of <2 ng/mL was not documented prior to day 63 of gestation. She was carried to term and delivered a litter of 7 puppies on day 63 post-ovulation.

Dam 6 also has relations to Dam 1 and was bred in 2013. Her serum P_4 levels also steadily declined throughout pregnancy, reaching a nadir of 3.6 ng/mL on day 49. However,

hypoluteoidism could not be confirmed as she was supplemented with Prometrium®, a bioidentical P_4.

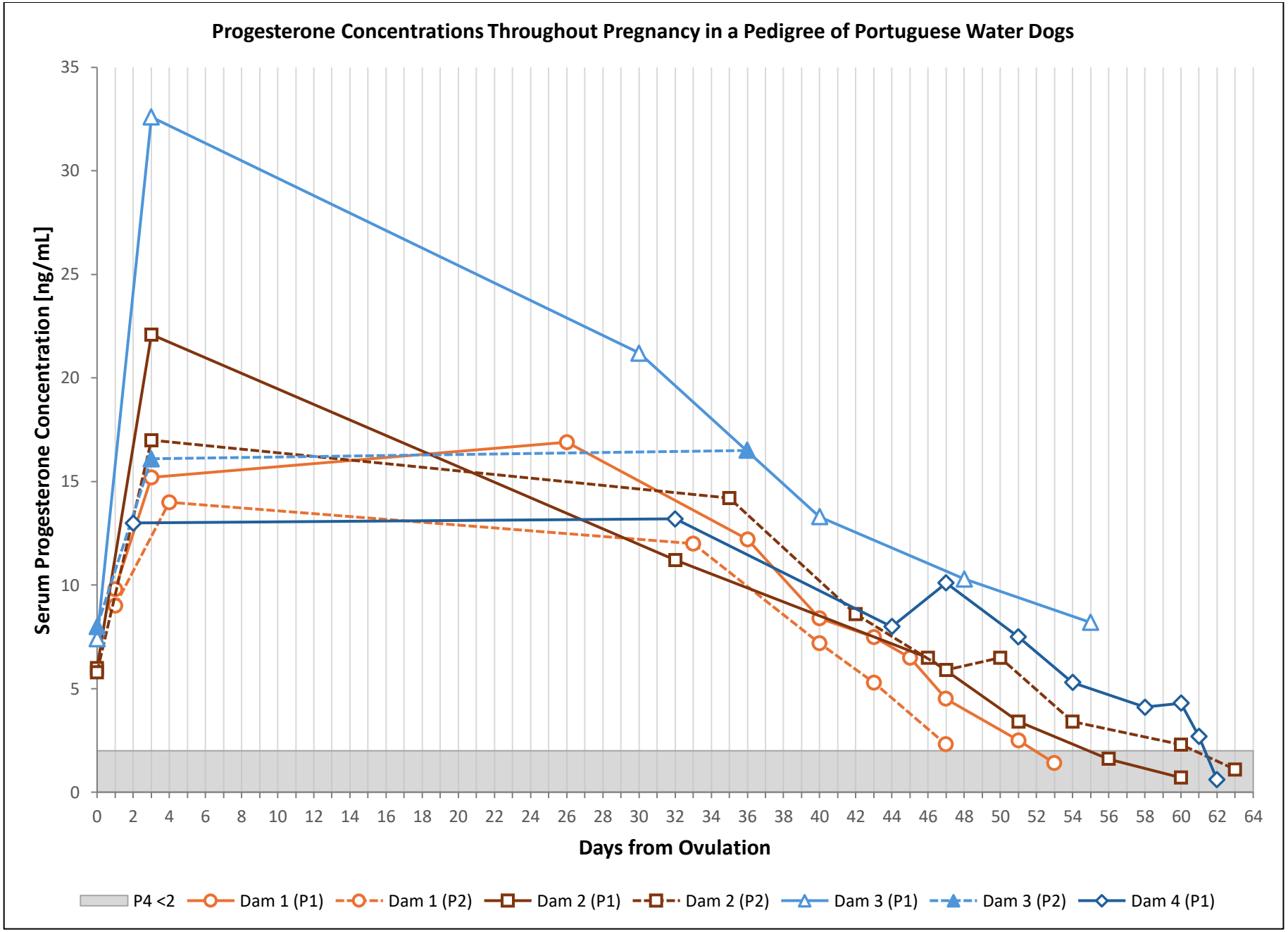
Days from Ovulation	Dam1 (P1)	Dam1 (P2)	Dam2 (P1)	Dam2 (P2)	Dam3 (P1)	Dam3 (P2)	Dam4 (P1)	Dam5 (P1)	Dam6 (P1)
-6			0.8						
-5									
-4	0.6				0.5				
-3		1.8	2.4	2.3				0.5	
-2					2.3	2.3			
-1	2.1	4		3.4			3		
0			6	5.8	7.4	8		4.1	4.5
1	9.8	9							7.1
2							13		
3	15.2		22.1	17	32.6	16.1			
4		14							

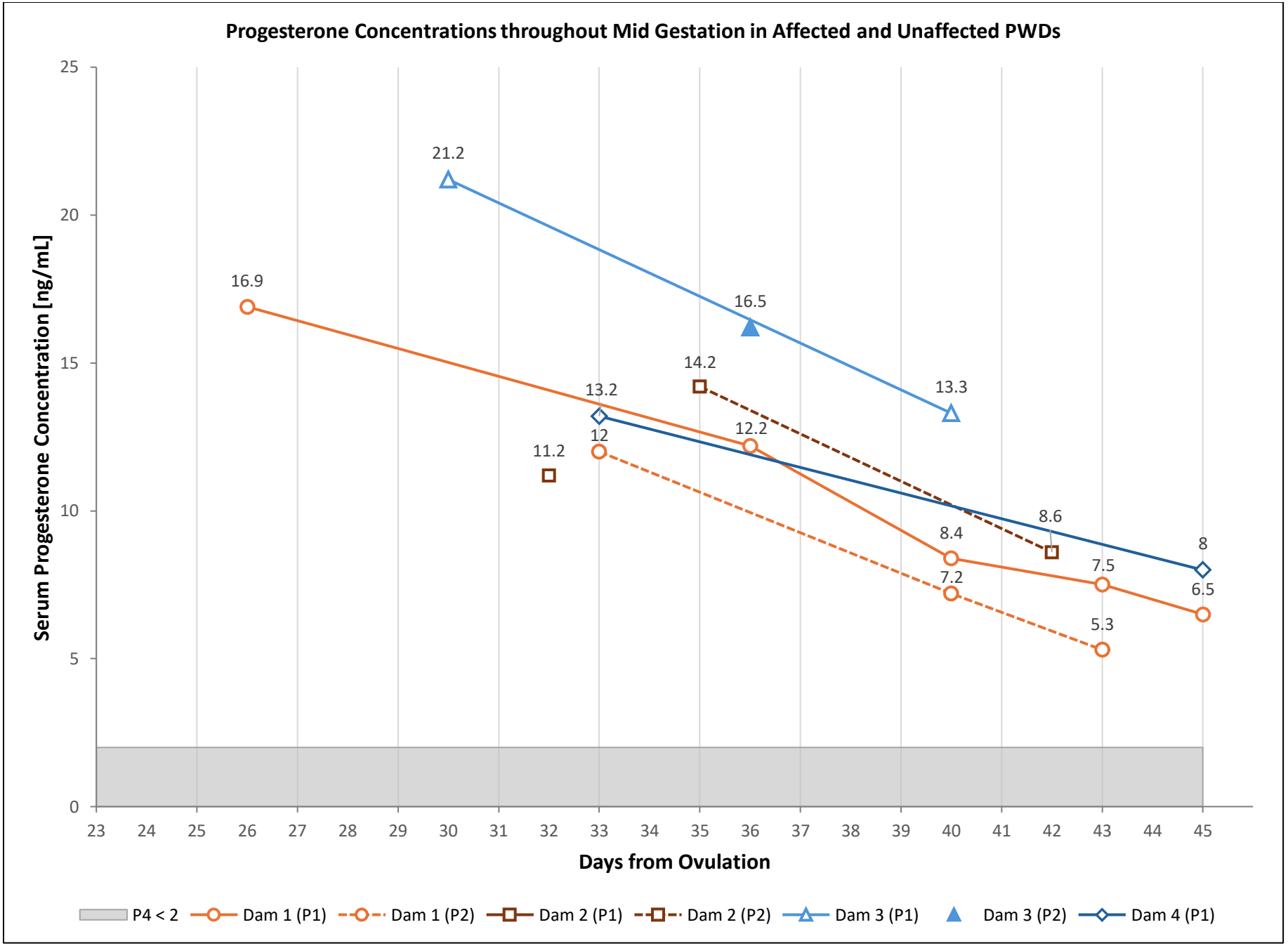
Table 1 *- Progesterone concentrations in all PWDs in relation to days from ovulation.*

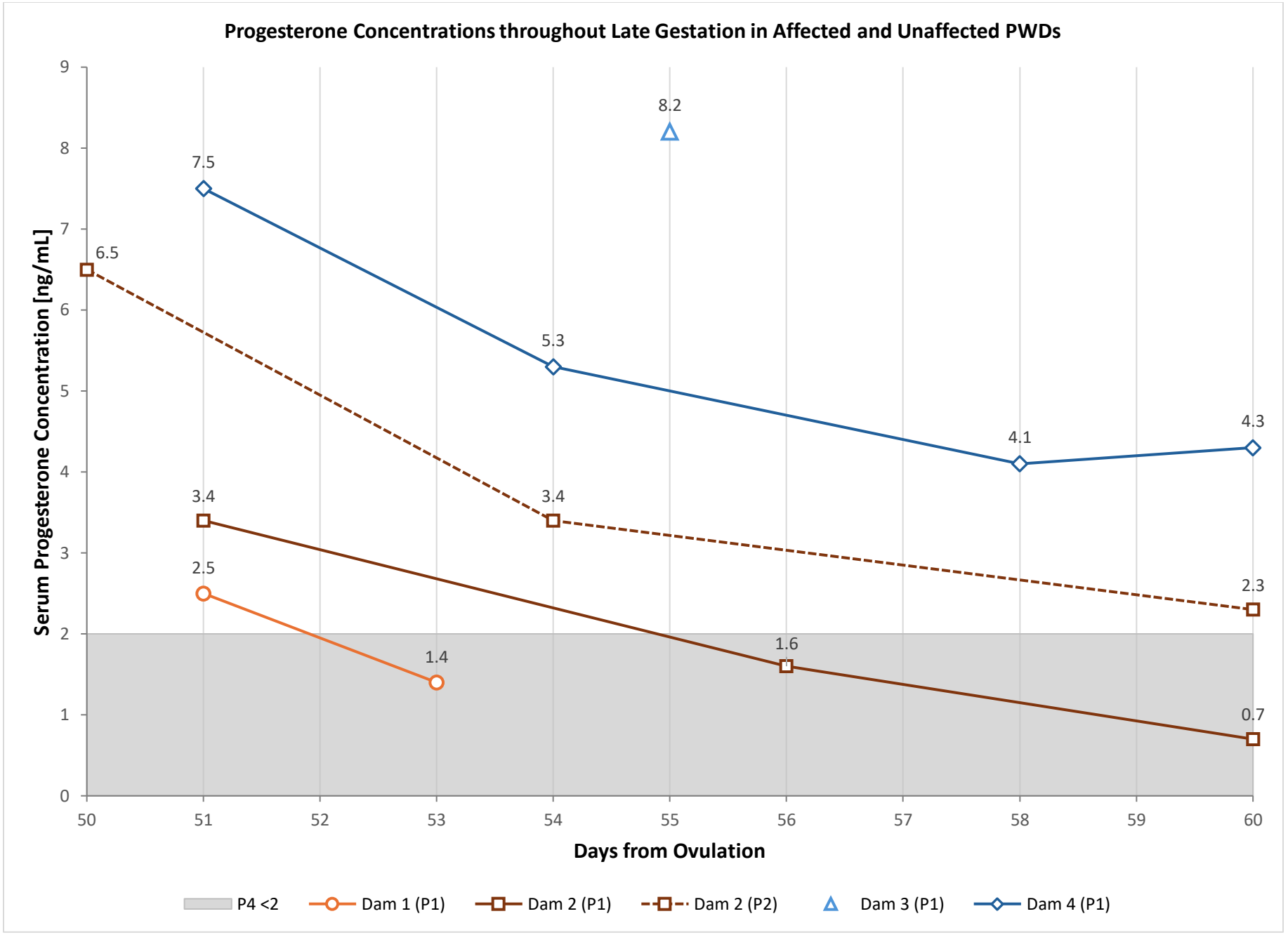
2.6. Progesterone Data Tables

Days from Ovulation	Dam (P1) 1	Dam (P2) 1	Dam (P1) 2	Dam (P2) 2	Dam (P1) 3	Dam (P2) 3	Dam (P1) 4	Dam (P1) 5	Dam (P1) 6
23									
24									
25									
26	16.9								
27									
28									
29									
30					21.2				
31									
32			11.2				13.2		
33		12							
34									
35				14.2					
36	12.2					16.5			11.1
37									
38									
39									
40	8.4	7.2			13.3			5.8	
41									
42				8.6				5.5	
43	7.5	5.3							7.1
44							8	5.9	
45	6.5								6.8
46			6.5						
47	4.5	2.3		5.9			10.1	5.3	6.9
48					10.3				
49								3.6	4.3
50				6.5					
51	2.5		3.4				7.5		
52									3.4
53	1.4								
54				3.4			5.3		
55					8.2			8.9	
56			1.6						
57									
58							4.1		
59									2.8
60			0.7	2.3			4.3		
61							2.7		
62							0.6		
63				1.1				10.8	

Table 2 *- Progesterone concentrations in relation to days from ovulation in all individuals. Red outlined boxes depict progesterone concentrations that met the diagnostic criteria for hypoluteoidism.*


Progesterone Concentrations Throughout Pregnancy in a Pedigree of Portuguese Water Dogs
Serum Progesterone Concentration [ng/mL]
0
5
10
15
20
25
30
35
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64
Days from Ovulation
P4 <2
Dam 1 (P1)
Dam 1 (P2)
Dam 2 (P1)
Dam 2 (P2)
Dam 3 (P1)
Dam 3 (P2)
Dam 4 (P1)


Progesterone Concentrations throughout Mid Gestation in Affected and Unaffected PWDs
Serum Progesterone Concentration [ng/mL]
Days from Ovulation
25
20
15
10
5
0
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
21.2
16.9
16.5
14.2
13.2
13.3
12
11.2
12.2
8.6
8.4
8
7.5
7.2
6.5
5.3
P4 < 2
Dam 1 (P1)
Dam 1 (P2)
Dam 2 (P1)
Dam 2 (P2)
Dam 3 (P1)
Dam 3 (P2)
Dam 4 (P1)


Progesterone Concentrations throughout Late Gestation in Affected and Unaffected PWDs
Serum Progesterone Concentration [ng/mL]
Days from Ovulation
0
1
2
3
4
5
6
7
8
9
50
51
52
53
54
55
56
57
58
59
60
8.2
7.5
6.5
5.3
4.1
4.3
3.4
3.4
2.5
2.3
1.4
1.6
0.7
P4 <2
Dam 1 (P1)
Dam 2 (P1)
Dam 2 (P2)
Dam 3 (P1)
Dam 4 (P1)

3. Comparing Progesterone Concentrations

3.1. Mid Gestation

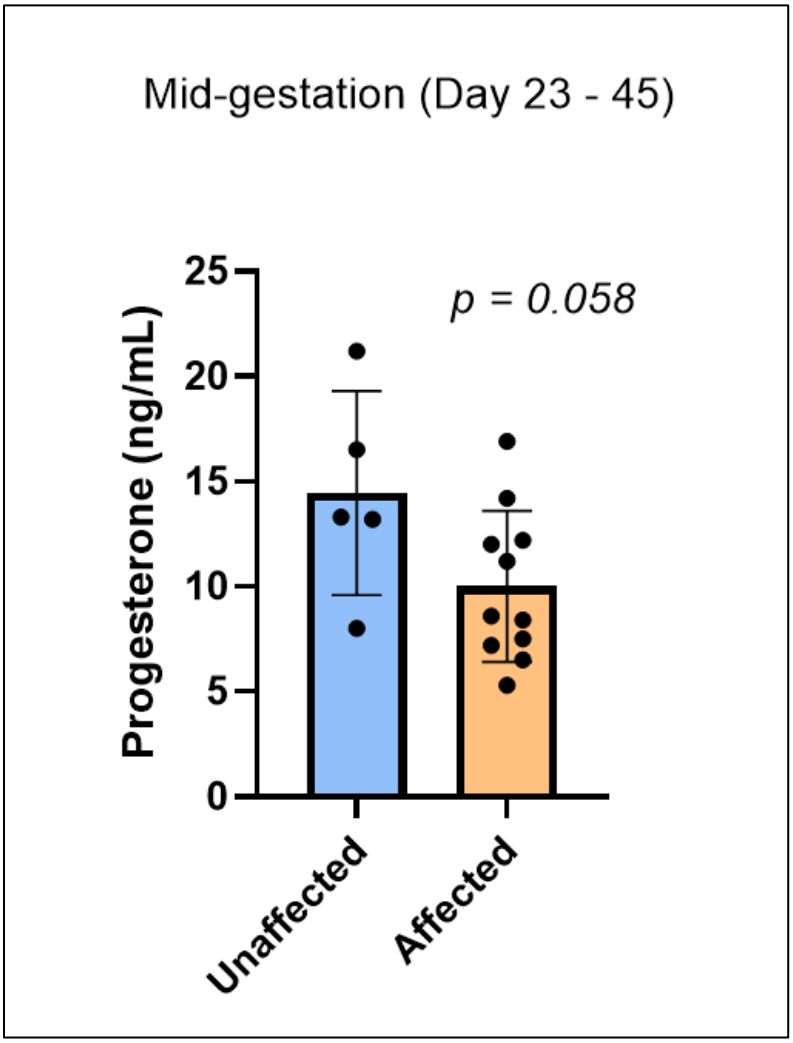


Figure 13 *- Bar graph with scatter plot depicting progesterone concentrations throughout mid-gestation in affected vs. unaffected PWDs.*

Mid-gestation progesterone concentrations (Day 23-45 of gestation) were evaluated to assess for significant differences between affected and unaffected PWDs (Figure 13). Mean progesterone concentrations during mid-gestation were lower in affected pregnancies (mean ± SD: 10.3 ± 1.5 ng/mL vs. 14.8 ± 3.7 ng/mL, respectively). Although affected pregnancies exhibited

numerically lower progesterone levels, this difference did not reach statistical significance, (p= 0.058).

3.2. Late Gestation

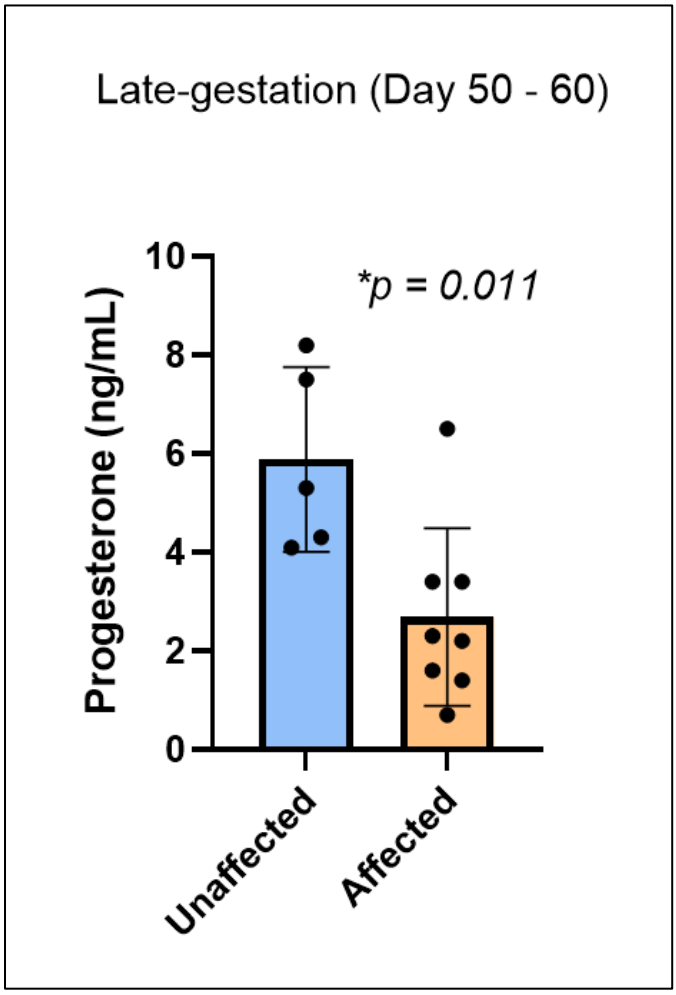


***Figure 14** - Bar graph with scatter plot depicting progesterone concentrations throughout late gestation in affected vs. unaffected PWDs.*

Late-gestation progesterone concentrations (Day 23-45 of gestation) were evaluated to assess for significant differences between affected and unaffected PWDs (Figure 14). Mean

progesterone concentrations during were significantly lower in affected pregnancies (2.6 ± 1.2 ng/mL) compared to unaffected pregnancies (6.8 ± 2.1 ng/mL) (p= 0.011).

II. Discussion

The clinical condition of hypoluteoidism in bitches is one that is highly debated, both in clinical settings and throughout the literature. The cause of this skepticism is understandable, as differences in opinion exist regarding the diagnostic criteria (with most scientists agreeing upon a P_4 of <2ng/mL prior to 60 days of gestations as inclusive) and because of the high potential for concurrent or alternative medical conditions that, similarly to hypoluteoidism, can result in late-term pregnancy loss. However, increasing awareness of this condition among breeders and veterinary professionals has enabled the scientific community to take a closer look at this understudied condition. This has led to publications including several case reports and observational studies on hypoluteoidism in the bitch. However, to the author's knowledge, this study is the first to describe a pedigree of related individuals with hypoluteoidism documented throughout multiple gestations. Notably, unaffected individuals were also identified. The integration of clinical data and pedigree analysis in this study allows for the development of a working hypothesis regarding the potential mechanisms underlying hypoluteoidism in the bitch, with the potential for extrapolation into other species.

In the present study, affected and unaffected individuals were identified based on the inclusion criteria of having serum progesterone concentrations measured below 2 ng/mL prior to 60 days of gestation. This cutoff (of less than 60 days) is important to distinguish, as normal litters can be born as early as 61 days of gestation (assuming accurate ovulation timing is used to calculate gestational age). Our affected females, Dam 1 and Dam 2 (daughter of Dam 1), both met this criterion. The unaffected females, Dam 3 and Dam 4, did not meet this criterion. Comparison of progesterone concentrations between affected and unaffected individuals was significant in late gestation (between days 50-60) and trended towards significant in mid-gestation (days 23-45).

There is potential for selection bias when interpreting progesterone differences between group, since progesterone concentrations are part of the diagnostic criteria for defining affected pregnancies. However, these data were taken as longitudinal measurements across gestation, highlighting a progressive decline in affected individuals. This suggests that luteal dysfunction is the true underlying feature of this disease state, especially considering that the bitch has no placental steroid production during gestation that could contribute to serum progesterone concentrations.

A complete pedigree analysis of the affected lineage of Portuguese Water Dogs was also performed and corroborated using medical records and AKC registration documents. Several potential modes of inheritance were considered, including autosomal recessive and x linked. Autosomal recessive inheritance is supported by the pedigree, with affected females shown to skip generations. One limitation to categorizing the inheritance pattern is absence of male phenotype data. Autosomal recessive inheritance patterns typically appear with equal frequency, regardless of sex. Though this is possible in our pedigree, it is an unknown. This also limits the evaluation of x-linked inheritance, though indirectly supports autosomal recessive inheritance due to the overt *lack* of male disease phenotype.

Several potential genetic mechanisms could explain the phenotype observed in the affected lineage. Mutations in genes critical to steroidogenesis, particularly CYP11A1 and STAR, have been implicated in similar reproductive phenotypes in other species. Specifically, genetic abnormalities with these genes have been associated with complete or partial steroidogenesis at various timepoints in gestation. In humans and murine species, the most vulnerable timepoint for this phenotype to manifest is during the luteal-placental shift in early gestation (~7-12 weeks). However, if pregnancy can be maintained until placental takeover the risk for late-term pregnancy

loss is low, and mutations of CYP11A1 or STAR typically manifest as recurrent early pregnancy loss or infertility. Due to species-specific differences in pregnancy maintenance physiology among mammals, the phenotypic presentation of mutations to genes controlling steroidogenesis is likely to vary. Thus, the possibility for hypoluteoidism to be a mutation to either CYP11A1, STAR, or another regulator of steroidogenesis is highly possible, despite different clinical manifestations of hypoluteoidism.

Mutations, or polymorphisms, affecting the progesterone receptor (PGR) gene represents another potential mechanism underlying hypoluteoidism. PROGINS, an Alu element insertion, of the progesterone receptor (PGR) gene in humans has been associated with recurrent pregnancy loss, caused by impaired endometrial response to progesterone. Similarly to the aforementioned genetic mutations in genes controlling steroidogenesis, PROGINS insertions cause pregnancy loss in humans at the critical timepoint of luteal-placental transition. While these findings are intriguing, the results of the present study contrast slightly with how this mutation manifests in people. Though a direct comparison is difficult to make between the endometrium of the bitch and human during early gestation, maintenance of pregnancy in humans is undoubtedly more reliant on the endometrium at this timepoint. However, mutations or polymorphisms affecting other elements of the progesterone signaling pathway, particularly within autocrine positive feedback mechanisms within the corpus luteum itself, could contribute to the pathogenesis of hypoluteoidism in bitches. Specific mechanisms impacted include progesterone-mediated upregulation of steroidogenic enzymes, intra-luteal prostaglandin signaling, and prolactin-receptors. Due to the strong evidence for a heritable (e.g. autosomal recessive) mutation, further studies are needed to determine if non-Mendelian receptor mutation, similar to PROGINS, is implicated as a cause for hypoluteoidism in the bitch.

While a genetic basis for hypoluteoidism is strongly supported by the pedigree analysis, other mechanisms may contribute or be the direct cause of luteal insufficiency in canines. The potential for an immune-mediated mechanism for hypoluteoidism in the bitch is intriguing, since the immune system is often implicated in cases of pregnancy loss secondary to infectious agents. However, the immune system itself may contribute directly to luteal dysfunction through non-infectious, immunologic mechanisms in the absence of infectious disease. Although immune-mediated pregnancy loss is inherently difficult to definitively diagnose, there is support for mechanism in the literature and our findings. Experimental murine models have showed that activation of the maternal immune system resulted in pregnancy loss due to functional luteal insufficiency, rather than the direct fetal or placental destruction often seen secondary to infectious causes (Erlebacher et al., 2004). Additionally, more specific to canines, one observational study in bitches measures IgE antibodies directly against progesterone in bitches with luteal failure (Krachudel et al., 2013). These data demonstrate a clear link between immune activation in the absence of infectious disease, and in bitches with hypoluteoidism. The data from our study supports these data, as no systemic signs of illness were observed in affected individuals, and all breeding bitches were screened for common infectious abortive diseases (*e.g. Brucella canis*) at the time of breeding. Thus, aberrations to the immune system could certainly be an underlying mechanism leading to hypoluteoidism in the bitch.

Though this study had strong clinical data paired with a robust pedigree, limitations include a small number of individuals evaluated and a lack of data in some cases (e.g. progesterone drop not documented in second pregnancies in individual with an existing diagnosis). Additionally, the lack of phenotypic male data makes confirming the mode of inheritance challenging. Finally,

although environmental factors can theoretically influence pregnancy outcomes, their impact in the present study is likely minimal, but not obsolete.

Despite these limitations, the findings of the present study provide a strong basis for future investigation into the genetic and/or molecular mechanisms leading to hypoluteoidism in the bitch. Future studies can be approached by advanced imaging and/or molecular analyses to evaluate hormone signaling and genetics regulation. Molecular analyses of reproductive tissues are of interest as this could characterize alterations to the receptors important luteotrophic hormones in bitches with hypoluteoidism (e.g. prolacin receptors). Comparing receptor localization in affected versus unaffected bitches could reveal whether impaired receptor signaling or expression contributes to luteal insufficiency. Furthermore, gaining information as to luteotrophic hormone *receptors* rather than circulating hormone concentrations is of interest, as serum prolactin has already been shown to be elevated in hypoluteoid bitches, supporting a mechanism at the level of the receptor. Advanced imaging studies, pending cost in a clinical setting, may be a feasible alternative to tissue analysis. Color doppler ultrasound has been used in other species (e.g. equine) as a marker of luteal health. Extrapolation of this to canine species is reasonable, and comparison between bitches with a previous diagnosis of hypoluteoidism to those unaffected could provide interesting insights, with the potential for use as a diagnostic screening tool if significant changes are observed. Finally, given the familial clustering of hypoluteoidism observed in the present study, and throughout the literature, canine whole genome sequencing (see Appendix) represents a critical next step towards identification of the underlying mechanism of hypoluteoidism in the bitch.

In conclusion, hypoluteoidism remains a poorly understood but clinically significant cause of pregnancy loss. The present study describes, for the first time, a pedigree of Portuguese Water Dogs with hypoluteoidism. Several mechanisms that have been postulated to result in

hypoluteoidism in the bitch, though all remain unproven. Primary and intrinsic failure of the corpus luteum is the most widely accepted mechanism, with immune dysregulation also implicated. In humans and murine models, there are genetic variants that describe mechanisms closely resembling hypoluteoidism, with both Mendelian and non-Mendelian inheritance identified. The findings in this study support a Mendelian (autosomal recessive) inheritance mechanism. Future studies integrating canine whole genome sequencing, tissue analysis, and advanced imaging studies of the corpus luteum will be critical obtaining a complete understanding of the genetic and physiological basis of this condition in the bitch.

III. Appendix

1. Whole genome sequencing (WGS) of selected PWDs

Next generation sequencing has revolutionized gene discovery efforts and has overcome sample size limitations in a multitude of species (Huskey et al., 2020) (Huskey et al., 2021) (Ma et al., 2022). In the present study, PacBio HiFi sequencing will be carried out on four total bitches from the PWD pedigree **(Figure 12),** including the two confirmed affected females (Dam 1 and Dam 2), and two unaffected females (Dam 3 and Dam 4). PacBio sequencing technology was chosen because of its comprehensive and accurate dataset of long reads (up to 25 kb) that have a low degree of bias, an accuracy of >99.9% (Q30), the ability to decipher repeats and GC-rich regions, all of which provide the highest precision for calling all variant types: single-nucleotide variants (SNVs), small insertions and deletions (indels), and structural variants (SVs). Searching for the familial causal variant in the PWD pedigree, all variant types will be investigated. Genomic DNA will be sequenced on a PacBio platform at HudsonAlpha Institute for Biotechnology's Genome Sequencing Laboratory. FASTQ files will be obtained from HudsonAlpha, and the quality of the raw FASTQ files will be determined using FASTQC. Subsequently, the HiFi reads will be trimmed using the program Trimmomatic to remove terminal sequences that do not meet the quality threshold (Bolger et al., 2014). The tool minimap2 will be used for the canine genome alignment, which is the recommended alignment tool by PacBio and is available in SMRT_tools. With several versions of the canine genome available, different alignments (e.g. CanFam3.1, CanFam4, CanFam6) will be carried out to be thorough. Regarding variant calling, PacBio recommends using the program Deepvariant for short variants (https://github.com/google/deepvariant). Subsequently, ANNOVAR (Wang et al., 2010) will be used to annotate the short variant VCF files using gene prediction from Ensembl build version 75.

Regarding SVs, pbsv will be used to call large insertions, deletions, duplications, inversions, and translocations. These variants are initially stored as a svsig file and subsequently processed to VCF using pbsv call. SVs will be annotated using the svpack program (https://github.com/PacificBiosciences/svpack). VCFs will be merged for filtering using Genome Analysis Tool Kit best practices.

1.1. Control samples

Orthopedic Foundation for Animals - Canine Health Information Center (CHIC): The Merner lab has a long-standing relationship with the Orthopedic Foundation for Animals - Canine Health Information Center (CHIC), which has a DNA Bank. In fact, we have already attained 706 blood or buccal-swab derived DNA samples of various purebred dogs for genetic studies. This includes 85 purebred dogs diagnosed with a canine mammary tumor (CMT) representing 32 different American Kennel Club (AKC) recognized breeds - 14 of those dogs were previously whole genome sequenced (Huskey et al., 2020). It also includes 621 purebred dogs of 14 different breeds, which were randomly selected as controls for genotyping purposes. Despite that these already-attained samples do not represent the PWD, assuming the causal variant is rare and specific to the PWD, they can be used for genotyping variants identified in this study. Additionally, the CHIC DNA Bank has 197 DNA samples of PWDs (121 females and 76 males). We will request access to those 197 samples, and, since offspring information is available, we will assume females with offspring are unaffected, which can be additional controls for the study.

1.2. Variant filtering and analysis

We will carry out the filtering/analysis under the assumption that the two confirmed affected females (Dams 1 and 2) are affected; and that the two confirmed unaffected female (Dams 3 and 4) are unaffected.

We will filter the merged and annotated gVCF file based on different potential modes of inheritance (i.e. autosomal dominant, recessive, x-linked). For instance, regarding autosomal dominant, we will search for heterozygous variants identified only in the affected samples. We will filter against the WGS data of the one unaffected family member, as well as the 14 CMT-affected dogs (that we will assume are unaffected). Regarding autosomal recessive, we will search for variants homozygous in only the affected samples and are either heterozygous or not present in controls. We will first focus on coding variants, searching for low-hanging fruit. Variants of interest will be searched for in the EVA, iDOG and Dog10k databases. We will also genotype using CHIC DNA Bank controls.

IV. References

Arduc, A., Dogan, A. B., Bilmez, S., Nasiroglu, I. N., Tuna, M. M., Isik, S.,…Guler, S. (2015). High prevalence of Hashimoto's thyroiditis in patients with polycystic ovary syndrome: does the imbalance between estradiol and progesterone play a role? *Endocrine Research*, *40*(4), 204-210. https://doi.org/https://doi.org/10.3109/07435800.2015.1015730

Arredondo, F., & Noble, S. L. (2006). Endocrinology of Recurrent Pregnancy Loss. *Seminars in Reproductive Medicine*, *24*(01), 033-039. https://doi.org/10.1055/s-2006-931799

Batzer, A. M., & Deininger, L. P. (2002). Alu repeats and human genomic diversity. *Nature Reviews Genetics*, *3*(5), 370-379. https://doi.org/10.1038/nrg798

Becher, A., Wehrend, A., & Goericke-Pesch, S. (2010). [Luteal insufficicency in the bitch - symptoms, diagnosis, consequences and therapy. A review of the literature]. *Tierarztl Prax Ausg K Kleintiere Heimtiere*, *38*(6), 389-396. (Luteale Insuffizienz bei der Hündin - Symptome, Diagnose, Folgen und Therapie. Eine Übersicht der Literatur.)

Bolger, M. A., Lohse, M., & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*(15), 2114-2120. https://doi.org/https://doi.org/10.1093/bioinformatics/btu170

Chastant-Maillard, S., Viaris de Lesegno, C., Chebrout, M., Thoumire, S., Meylheuc, T., Fontbonne, A.,…Reynaud, K. (2011). The canine oocyte: uncommon features of in vivo and in vitro maturation. *Reproduction, Fertility and Development*, *23*(3), 391-402. https://doi.org/https://doi.org/10.1071/RD10064

Chien, Y., Cheng, W.-C., Wu, M.-R., Jiang, S.-T., Shen, J. C.-K., & Chung, B.-C. (2013). Misregulated Progesterone Secretion and Impaired Pregnancy in Cyp11a1 Transgenic Mice1. *Biology of Reproduction*, *89*(4), 91-91. https://doi.org/10.1095/biolreprod.113.110833

Concannon, P. (2009). Endocrinologic Control of Normal Canine Ovarian Function [Article]. *Reproduction in Domestic Animals*, *44*(s2), 3-15. https://doi.org/10.1111/j.1439-0531.2009.01414.x

Concannon, P., Castracane, D., Temple, M., & Montanez, A. (2009). Endocrine control of ovarian function in dogs and other carnivores. *Anim Reprod*, *6*.

Concannon, P., McCann, J., & Temple, M. (1989). Biology and endocrinology of ovulation, pregnancy and parturition in the dog. *Journal of reproduction and fertility. Supplement*, *39*, 3-25.

Conrad, P. K. (2011). Maternal vasodilation in pregnancy: the emerging role of relaxin. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *301*(2), R267-R275. https://doi.org/doi:10.1152/ajpregu.00156.2011

Dockweiler, J. C., Cossic, B., Donnelly, C. G., Gilbert, R. O., Buckles, E., & Cheong, S. H. (2017). Infertility associated with the absence of endometrial progesterone receptors in a bitch. *Reprod Domest Anim*, *52*(1), 174-178. https://doi.org/10.1111/rda.12874

Erlebacher, A., Zhang, D., Parlow, F. A., & Glimcher, H. L. (2004). Ovarian insufficiency and early pregnancy loss induced by activation of the innate immune system. *114*(1), 39-48. https://doi.org/https://doi.org/10.1172/JCI20645

Fortune, E. J. (2018). Ovarian Production of Estradiol: The Two-Cell, Two-Gonadotropin Model. In *Encyclopedia of Reproduction* (pp. 165-171).

Freeman, M. E., Kanyicska, B., Lerant, A., & Nagy, G. (2000). Prolactin: Structure, Function, and Regulation of Secretion. *Physiological Reviews*, *80*(4), 1523-1631. https://doi.org/10.1152/physrev.2000.80.4.1523

Goericke-Pesch, S., Klaus, D., Failing, K., & Wehrend, A. (2012). Longevity of chilled canine semen comparing different extenders. *Anim Reprod Sci*, *135*(1-4), 97-105. https://doi.org/10.1016/j.anireprosci.2012.08.032

Goldsmith, T. L., & Weiss, G. (2009). Relaxin in Human Pregnancy. *Annals of the New York Academy of Sciences*, *1160*(1), 130-135. https://doi.org/https://doi.org/10.1111/j.1749-6632.2008.03800.x

Gudermuth, D. F. (1998). Pregnancy-Specific Elevations in Fecal Concentrations of Estradiol, Testosterone and Progesterone in the Domestic Dog (Canis familiaris). *Theriogenology*, *50*, 237-248.

Görlinger, S., Galac, S., Kooistra, H. S., & Okkens, A. C. (2005). Hypoluteoidism in a bitch. *Theriogenology*, *64*(1), 213-219. https://doi.org/https://doi.org/10.1016/j.theriogenology.2004.12.011

Günzel-Apel, A., Urhausen, C., Wolf, K., Einspanier, A., Oei, C., & Piechotta, M. (2012). Serum Progesterone in Pregnant Bitches Supplemented with Progestin Because of Expected or Suspected Luteal Insufficiency. *Reproduction in Domestic Animals*, *47*(s6), 55-60. https://doi.org/https://doi.org/10.1111/rda.12029

Günzel-Apel, A. R., Zabel, S., Bunck, C. F., Dieleman, S. J., Einspanier, A., & Hoppen, H. O. (2006). Concentrations of progesterone, prolactin and relaxin in the luteal phase and pregnancy in normal and short-cycling German Shepherd dogs. *Theriogenology*, *66*(6), 1431-1435. https://doi.org/https://doi.org/10.1016/j.theriogenology.2006.01.030

Hoffmann, B., Büsges, F., Engel, E., Kowalewski, M., & Papa, P. (2004). Regulation of Corpus Luteum-function in the Bitch. *Reproduction in Domestic Animals*, *39*(4), 232-240. https://doi.org/https://doi.org/10.1111/j.1439-0531.2004.00508.x

Hughes, C. G. (2012). Progesterone and autoimmune disease. *Autoimmunity Reviews*, *11*(6-7), A502-A514. https://doi.org/doi:10.1016/j.autrev.2011.12.003

Huskey, W. L. A., Goebel, K., Lloveras-Fuentes, C., Mcneely, I., & Merner, D. N. (2020). Whole genome sequencing for the investigation of canine mammary tumor inheritance - an initial assessment of high-risk breast cancer genes reveal BRCA2 and STK11 variants potentially associated with risk in purebred dogs. *Canine Medicine and Genetics*, *7*(1). https://doi.org/https://doi.org/10.1186/s40575-020-00084-w

Huskey, W. L. A., Mcneely, I., & Merner, D. N. (2021). CEACAM Gene Family Mutations Associated With Inherited Breast Cancer Risk – A Comparative Oncology Approach to Discovery. *Frontiers in Genetics*, *12*. https://doi.org/10.3389/fgene.2021.702889

Johnson, C. A. (2008). High-risk pregnancy and hypoluteoidism in the bitch. *Theriogenology*, *70*(9), 1424-1430. https://doi.org/10.1016/j.theriogenology.2008.09.010

Johnston, D. S., & Raksil, S. (1987). Fetal Loss in the Dog and Cat. *Veterinary Clinics of North America: Small Animal Practice*, *17*(3), 535-554. https://doi.org/10.1016/s0195-5616(87)50052-3

Johnston, S. D., Root Kustritz, M. V., & Olson, P. S. (2001). *Canine and feline theriogenology* (1st ed.). Saunders.

Khan, N., Zargar, H. M., Ahmed, R., Godha, M., Ahmad, A., Afroze, D., & Masoodi, R. S. (2021). Effect of steroid hormone receptor gene variants PROGINS (Alu insertion) and

PGR C/T (rs1042839) as a risk factor for recurrent pregnancy loss in Kashmiri population (North India). *Journal of Obstetrics and Gynaecology Research*, *47*(12), 4329-4339. https://doi.org/doi:10.1111/jog.15054

Khan, N., Zargar, M. H., Ahmed, R., Godha, M., Ahmad, A., Afroze, D., & Masoodi, S. R. (2021). Effect of steroid hormone receptor gene variants PROGINS (Alu insertion) and PGR C/T (rs1042839) as a risk factor for recurrent pregnancy loss in Kashmiri population (North India). *J Obstet Gynaecol Res*, *47*(12), 4329-4339. https://doi.org/10.1111/jog.15054

Kooistra, H., & Okkens, A. (2001). Secretion of Prolactin and Growth Hormone in Relation to Ovarian Activity in the Dog. *Reproduction in Domestic Animals*, *36*(3-4), 115-119. https://doi.org/https://doi.org/10.1046/j.1439-0531.2001.00311.x

Kowalewski, M. (2012). Endocrine and Molecular Control of Luteal and Placental Function in Dogs: A Review. *Reproduction in Domestic Animals*, *47*(s6), 19-24. https://doi.org/https://doi.org/10.1111/rda.12036

Kowalewski, M. P. (2014). Luteal regression vs. prepartum luteolysis: regulatory mechanisms governing canine corpus luteum function. *Reproductive Biology*, *14*(2), 89-102.

Kowalewski, M. P. (2023). Advances in understanding canine pregnancy: Endocrine and morpho-functional regulation. *Reproduction in Domestic Animals*, *58*(S2), 163-175. https://doi.org/https://doi.org/10.1111/rda.14443

Kowalewski, M. P., Tavares Pereira, M., & Kazemian, A. (2020). Canine conceptus-maternal communication during maintenance and termination of pregnancy, including the role of species-specific decidualization. *Theriogenology*, *150*, 329-338. https://doi.org/https://doi.org/10.1016/j.theriogenology.2020.01.082

Kowalewski, P. M. (2018). Selected Comparative Aspects of Canine Female Reproductive Physiology. In *Encyclopedia of Reproduction* (pp. 682-691).

Kowalewski, P. M., Beceriklisoy, B. H., Aslan, S., Agaoglu, R. A., & Hoffmann, B. (2009). Time related changes in luteal prostaglandin synthesis and steroidogenic capacity during pregnancy, normal and antiprogestin induced luteolysis in the bitch. *Animal Reproduction Science*, *116*(1-2), 129-138. https://doi.org/10.1016/j.anireprosci.2008.12.011

Kowalewski, P. M., Michel, E., Gram, A., Boos, A., Guscetti, F., Hoffmann, B.,…Reichler, I. (2011). Luteal and placental function in the bitch: spatio-temporal changes in prolactin receptor (PRLr) expression at dioestrus, pregnancy and normal and induced parturition. *Reproductive Biology and Endocrinology*, *9*(1), 109. https://doi.org/10.1186/1477-7827-9-109

Kowalewski, P. M., Schuler, G., Taubert, A., Engel, E., & Hoffmann, B. (2006). Expression of cyclooxygenase 1 and 2 in the canine corpus luteum during diestrus. *Theriogenology*, *66*(6-7), 1423-1430. https://doi.org/https://doi.org/10.1016/j.theriogenology.2006.01.039

Krachudel, J., Bondzio, A., Einspanier, R., Einspanier, A., Gottschalk, J., Kuechenmeister, U., & Muennich, A. (2013). Luteal insufficiency in bitches as a consequence of an autoimmune response against progesterone? *Theriogenology*, *79*(9), 1278-1283. https://doi.org/https://doi.org/10.1016/j.theriogenology.2013.02.025

Ma, X., Brinker, E., Graff, C. E., Cao, W., Gross, L. A., Johnson, K. A.,…Wang, X. (2022). Whole-Genome Shotgun Metagenomic Sequencing Reveals Distinct Gut Microbiome Signatures of Obese Cats. *Microbiology Spectrum*, *10*(3). https://doi.org/10.1128/spectrum.00837-22

Meyers-Wallen, V. N. (2007). Unusual and abnormal canine estrous cycles. *Theriogenology*, *68*(9), 1205-1210. https://doi.org/10.1016/j.theriogenology.2007.08.019

Michel, E., Feldmann, K. S., Kowalewski, P. M., Bley, C., Boos, A., Guscetti, F., & Reichler, M. I. (2012). Expression of prolactin receptors in normal canine mammary tissue, canine mammary adenomas and mammary adenocarcinomas. *BMC Veterinary Research*, *8*(1), 72. https://doi.org/10.1186/1746-6148-8-72

Miller, L. W. (2017). Disorders in the initial steps of steroid hormone synthesis. *The Journal of Steroid Biochemistry and Molecular Biology*, *165*, 18-37. https://doi.org/http://dx.doi.org/10.1016/j.jsbmb.2016.03.009

Noakes, D. E., Parkinson, T. J., & England, G. C. W. (2019). *Veterinary reproduction and obstetrics* (Tenth edition. ed.) https://doi.org/https://doi.org/10.1016/C2014-0-04782-X

Nowak, M., Boos, A., & Kowalewski, P. M. (2018). Luteal and hypophyseal expression of the canine relaxin (RLN) system during pregnancy: Implications for luteotropic function. *PLOS ONE*, *13*(1), e0191374. https://doi.org/https://doi.org/10.1371/journal.pone.0191374

Panciera, D. L., Purswell, B. J., Kolster, K. A., Werre, S. R., & Trout, S. W. (2012). Reproductive Effects of Prolonged Experimentally Induced Hypothyroidism in Bitches. *Journal of Veterinary Internal Medicine*, *26*(2), 326-333. https://doi.org/https://doi.org/10.1111/j.1939-1676.2011.00872.x

Papa, P., & Hoffmann, B. (2011). The Corpus Luteum of the Dog: Source and Target of Steroid Hormones? *Reproduction in Domestic Animals*, *46*(4), 750-756. https://doi.org/https://doi.org/10.1111/j.1439-0531.2010.01749.x

Rayat, K. A., Thapar, P., Kaur, M., Singh, S., & Kaur, A. (2023). Association of CYP11A1 Polymorphisms with Recurrent Pregnancy Loss in the Female Population of Punjab. *Journal of Human Reproductive Sciences*, *16*(3), 242-245. https://doi.org/10.4103/jhrs.jhrs_24_23

Reynaud, K., de Lesegno, C. V., Chebrout, M., Thoumire, S., & Chastant-Maillard, S. (2009). Follicle population, cumulus mucification, and oocyte chromatin configuration during the periovulatory period in the female dog. *Theriogenology*, *72*(8), 1120-1131. https://doi.org/10.1016/j.theriogenology.2009.07.006

Reynaud, K., Fontbonne, A., Marseloo, N., Viaris de Lesegno, C., Saint-Dizier, M., & Chastant-Maillard, S. (2006). In vivo canine oocyte maturation, fertilization and early embryogenesis: A review. *Theriogenology*, *66*(6), 1685-1693. https://doi.org/https://doi.org/10.1016/j.theriogenology.2006.01.049

Reynaud, K., Fontbonne, A., Saint-Dizier, M., Thoumire, S., Marnier, C., Tahir, M.,…Chastant-Maillard, S. (2012). Folliculogenesis, Ovulation and Endocrine Control of Oocytes and Embryos in the Dog. *Reproduction in Domestic Animals*, *47*(s6), 66-69. https://doi.org/https://doi.org/10.1111/rda.12055

Reynaud, K., Saint-Dizier, M., Tahir, M. Z., Havard, T., Harichaux, G., Labas, V.,…Chastant-Maillard, S. (2015). Progesterone plays a critical role in canine oocyte maturation and fertilization. *Biol Reprod*, *93*(4), 87. https://doi.org/10.1095/biolreprod.115.130955

Root Kustritz, M. V. (2012). Managing the reproductive cycle in the bitch. *Vet Clin North Am Small Anim Pract*, *42*(3), 423-437, v. https://doi.org/10.1016/j.cvsm.2012.01.012

Sebzda, K. M., & Kauffman, K. L. (2023). Update on Brucella canis. *Veterinary Clinics of North America: Small Animal Practice*, *53*(5), 1047-1062. https://doi.org/https://doi.org/10.1016/j.cvsm.2023.05.002

Selvaraj, V., Stocco, M. D., & Clark, J. B. (2018). Current knowledge on the acute regulation of steroidogenesis. *Biology of Reproduction*, *99*(1), 13-26. https://doi.org/doi:10.1093/biolre/ioy102

Sen, A., & Severance, A. L. (2018). Oogenesis & Ovulation: Oocyte Maturation and Ovulation, Comparative. In M. K. Skinner (Ed.), *Encyclopedia of Reproduction (Second Edition)* (pp. 234-238). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-809633-8.20563-3

Tee, K. M., Abramsohn, M., Loewenthal, N., Harris, M., Siwach, S., Kaplinsky, A.,…Miller, L. W. (2013). Varied Clinical Presentations of Seven Patients With Mutations inCYP11A1Encoding the Cholesterol Side-Chain Cleavage Enzyme, P450scc. *The Journal of Clinical Endocrinology & Metabolism*, *98*(2), 713-720. https://doi.org/10.1210/jc.2012-2828

Tiwari, D., Bose, D. P., Das, S., Das, R. C., Datta, R., & Bose, S. (2015). MTHFR (C677T) polymorphism and PR (PROGINS) mutation as genetic factors for preterm delivery, fetal death and low birth weight: A Northeast Indian population based study. *Meta Gene*, *3*, 31-42. https://doi.org/http://dx.doi.org/10.1016/j.mgene.2014.12.002

Uenoyama, Y., Tsuchida, H., Nagae, M., Inoue, N., & Tsukamura, H. (2022). Opioidergic pathways and kisspeptin in the regulation of female reproduction in mammals. *Frontiers in Neuroscience*, *16*. https://doi.org/https://doi.org/10.3389/fnins.2022.958377

Valkovic, L. A., Bathgate, A. R., Samuel, S. C., & Kocan, M. (2019). Understanding relaxin signalling at the cellular level. *Molecular and Cellular Endocrinology*, *487*, 24-33. https://doi.org/https://doi.org/10.1016/j.mce.2018.12.017

Verstegen, J., Dhaliwal, G., & Verstegen-Onclin, K. (2008). Canine and feline pregnancy loss due to viral and non-infectious causes: a review. *Theriogenology*, *70*(3), 304-319. https://doi.org/10.1016/j.theriogenology.2008.05.035

Verstegen-Onclin, K., & Verstegen, J. (2008). Endocrinology of pregnancy in the dog: A review. *Theriogenology*, *70*(3), 291-299. https://doi.org/https://doi.org/10.1016/j.theriogenology.2008.04.038

Wang, K., Li, M., & Hakonarson, H. (2010). ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. *Nucleic Acids Res*, *38*(16), e164. https://doi.org/10.1093/nar/gkq603

Wiebe, J. V., & Howard, P. J. (2009). Pharmacologic Advances in Canine and Feline Reproduction. *Topics in Companion Animal Medicine*, *24*(2), 71-99. https://doi.org/https://doi.org/10.1053/j.tcam.2008.12.004

Youngquist, R. S., & Threlfall, W. R. (2007). *Current therapy in large animal theriogenology* (2nd ed.). Saunders Elsevier.

Zatta, S., Rehrauer, H., Gram, A., Boos, A., & Kowalewski, P. M. (2017). Transcriptome analysis reveals differences in mechanisms regulating cessation of luteal function in pregnant and non-pregnant dogs. *BMC Genomics*, *18*(1). https://doi.org/https://doi.org/10.1186/s12864-017-4084-9

Zedda, M. T., Bogliolo, L., Antuofermo, E., Falchi, L., Ariu, F., Burrai, G. P., & Pau, S. (2017). Hypoluteoidism in a dog associated with recurrent mammary fibroadenoma stimulated by progestin therapy. *Acta Vet Scand*, *59*(1), 55. https://doi.org/10.1186/s13028-017-0324-x