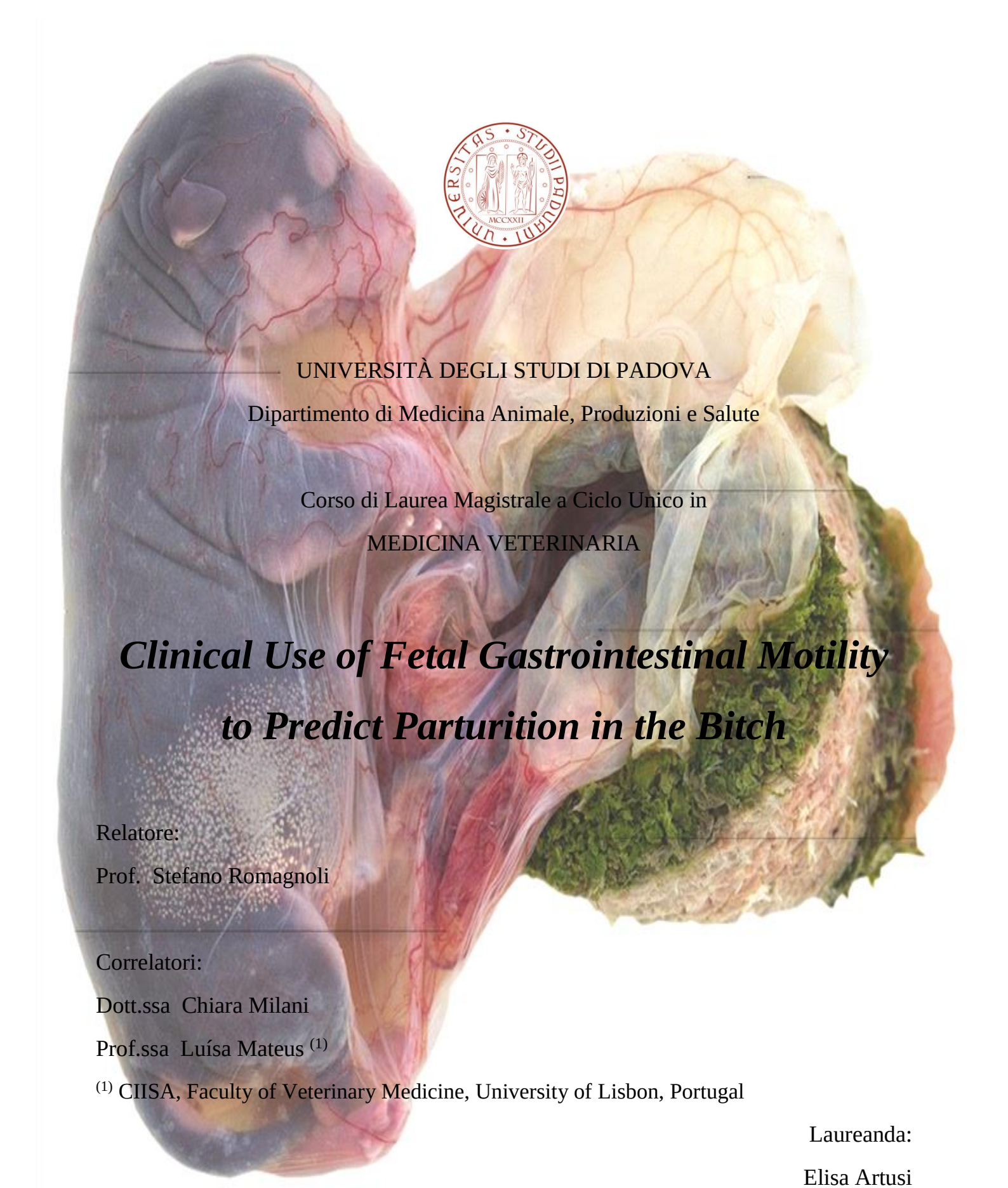

UNIVERSITÀ DEGLI STUDI DI PADOVA

Dipartimento di Medicina Animale, Produzioni e Salute

Corso di Laurea Magistrale a Ciclo Unico in

MEDICINA VETERINARIA

Clinical Use of Fetal Gastrointestinal Motility to Predict Parturition in the Bitch

Relatore:

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“ Non è cane e non è lupo…sa soltanto quello che non è ”.

Ai miei (mezzi) lupi.

INDEX

1. RIASSUNTO

INTRODUZIONE: Una stima adeguata della data del parto nella cagna è un'informazione molto utile, soprattutto per aiutare i clinici a gestire sia parti normali ché problematici. Allo stesso modo è anche utile per pianificare un parto cesareo.
La peristalsi intestinale fetale è un reperto importante, utile in quanto fornisce la prova del completamento dell'organogenesi nel feto canino (Yeager et al., 1992; Kim et al., 2007). La prima visualizzazione della peristalsi intestinale si verifica approssimativamente dal giorno 48 al 54 di gestazione, sebbene non sia costante e possa differire tra i segmenti intestinali (Gil et al., 2015).
OBIETTIVO: Utilizzando un ecografo B-Mode collegato ad una sonda di 4-9 MHz, ricercare la motilità gastrointestinale fetale negli ultimi 10 giorni di gestazione come criterio utile a predire la data del parto nella cagna e correlarla con altri parametri significativi di parto imminente.
MATERIALI E METODI: 20 cagne gravide sono state seguite dal giorno 54 di gravidanza in poi, col fine di valutare: (a) temperatura vaginale e (b) rettale, (c) dosaggio del progesterone (P4) e del (d) cortisolo sierico, (e) battiti cardiaci fetali utilizzando un ecografo B-Mode presentante anche la modalità doppler e (f) motilità gastrointestinale fetale utilizzando lo stesso ecografo.
La correlazione Rho di Spearman è stata utilizzata al fine di comprendere quali relazioni statistiche vi siano tra tutti questi parametri.
RISULTATI E DISCUSSIONE: Questo studio ha osservato una totale assenza di peristalsi fetale in tutti i feti esaminati al gg 54-55. Il primo giorno di gravidanza in cui si riscontra è il 56°. L'esame ecografico dal gg 59 al gg 63 consente una facile ed immediata osservazione della peristalsi intestinale in modo costante in più del 60% di feti esaminati, sebbene in quest'ultimo periodo ci siano stati casi in cui nessun feto esaminato presentasse un evidente movimento intestinale.
La motilità gastrointestinale fetale è correlata negativamente ($p<0.05$) con la temperatura vaginale e rettale.
In accordo con la letteratura, è stata osservata una decrescita della temperatura vaginale e rettale a partire da 24 ore prima del parto, ed una concomitante rapida caduta del livello di P4 sierico e un innalzamento dei valori di cortisolo. Una decrescita nei battiti cardiaci fetali è stata osservata dal gg 54-57 al gg 58-63 di gestazione.
CONCLUSIONE: La motilità gastrointestinale fetale può essere considerata come un indice di maturazione fetale e, se riscontrata in almeno 60% dei feti esaminati, può essere utile nell'indicare che la cagna partorirà in 5 gg o anche meno. Tuttavia, un suo non ritrovamento non può escludere che la cagna possa partorire di lì a pochi giorni, se non ore. Pertanto, questo parametro può essere definito specifico ma non sensibile.
Per affinare la predizione della data del parto in presenza di attività peristaltica fetale, la variazione media di 0.73°C di temperatura vaginale e 0.67°C di temperatura rettale, può aiutare il veterinario a prevedere l'iniziodel parto nelle successive 24 ore.

1. ABSTRACT

INTRODUCTION**:** An accurate estimate of parturition time in the bitch is a very useful information to help clinicians manage normal and abnormal parturitions as well as to plan an induced labor or a cesarean delivery.
Intestinal peristalsis is an important finding that provides evidence of completion of canine fetal organogenesis (Yeager et al., 1992; Kim et al., 2007). The first visualization of intestinal peristalsis occurs at approximately 48 to 54 days of gestation, although peristalsis is not constant and may differ between bowel segments (Gil et al., 2015).
OBJECTIVE**:** To investigate fetal gastrointestinal motility using B-Mode ultrasound connected to a 4-9 MHz probe, in the last 10 days of pregnancy as a criteria to predict the day of parturition in bitches and correlate it with other parameters of impending parturition.
MATERIALS AND METHODS**:** 20 pregnant bitches were followed from day 54 of pregnancy onwards, in order to evaluate: (a) vaginal and (b) rectal temperature, (c) serum progesterone concentration, (d) serum cortisol concentration, (e) fetal heartbeats by B-mode and doppler ultrasound (US) and (f) fetal intestinal peristalsis using US.
Spearman's Rho correlation was used in order to evaluate the statistical relation among all these parameters.
RESULTS AND DISCUSSION: This study observed no fetal peristaltic movements in all the examined fetuses on D54-D55 of pregnancy. The first day in which fetal bowel movement was recorded is D56. The US examination on D59 to D63 revealed an easy and immediate observation of fetal gastrointestinal motility in more than 60% of the fetuses examined, although in this period there have been cases in which none of the fetuses evaluated showed a clear bowel motion.
Fetal gastrointestinal motility is negatively correlated with vaginal and rectal temperature ($p<0.05$). In agreement with previous literature, a decrease in vaginal and rectal temperature 24 hours prior to parturition was recorded, as well as a rapid drop in serum P4 concentration and a rise in serum cortisol level. A decrease in fetal heartbeats was observed from D54-D57 to D58-D63.
CONCLUSION: Fetal gastrointestinal motility might be considered as a criteria to evaluate fetal maturity and, if recorded in at least 60% of the examined fetuses, can be a useful criteria to estimate that the bitch will whelp in 5 days or even less. However, its absence in all fetuses does not rule out, it can't be excluded that the bitch may deliver in a few days or even hours. Fetal gastrointestinal motility can be considered a specific but not a sensitive parameter.
In order to improve the prediction of parturition time when fetal bowel movements are observed, a mean variation of 0.73°C in vaginal temperature and of 0.67°C in rectal temperature, might help the veterinarian in estimate that parturition will occur during the following 24 hours.

This research project has been conducted at the veterinary teaching hospital of the University of Padova. Professor Luísa Mateus has collaborated to the study during her period at University of Padova as Visiting Scientist in 2016, and has provided supervision for the writing of the thesis during my stay at the University of Lisbon within the Erasmus Plus program in September-October of 2016.

Part of this data has been presented at the 8th International Symposium on Canine and Feline Reproduction in Paris on 22-25 June 2016.

2. INTRODUCTION

As veterinary care has progressed, so has the demand for reproductive technologies: semen preservation, estrus induction, ovulation timing and parturition management are only a few improvements that this discipline can offer (Smith, 2007). Nowadays, because of the increase in canine breeding and the high market demand of companion and breeding dogs, veterinary reproductive assistance is much more required than in the past. In breeding kennels as well as in private households matings, pregnancies are generally followed with apprehension by owners who expect to know the exact day of parturition in order to provide an adequate and hygienic environment for the puppies.

An accurate estimation of parturition date is very useful if an elective cesarean section has to be planned or following an unwanted pregnancy, when the estimation of fetal age may be necessary in order for the veterinarian to decide the correct abortion method.

Estimating parturition date is recommended in breeds with high incidence of dystocia, like English and French Bulldogs, Shih Tzu, Boston Terriers, Yorkshire Terriers, Boxers and Pugs. An elective C-section can be planned for bitches which present any of the following criteria: nulliparous bitches with more than 6 years of age; small litter size, such as two fetuses or less; big litter size with eight pups or more; brachycephalic breeds; bitches with a previous history of dystocia (Smith, 2007).

Furthermore, knowing the whelping date might be useful in order to minimize all the issues connected to a premature or late birth. Exceeding 72 days after mating might be due to uterine inertia, which may have a familiar background (Smith, 2007).

The prediction of the parturition date requires reliable methods to be investigated in order to prevent, diagnose and treat an abnormal parturition.

2.1 Physiology of canine pregnancy

Preovulatory events

Fertile estrus is the period that begins with the decline in estrogen concentration and the rise in progesterone (P4) concentration in association with the LH surge, and ends with the sudden reduction in vaginal cornification (Concannon et al., 1989).

Ovulation takes place approximately 2 days after the LH peak. Therefore, the LH surge produces the enlargement and the luteinization of mature follicles which leads to ovulation. It transforms 3-4 mm estrogen-secreting follicles into 8-9 mm progesterone-secreting corpora lutea (Concannon et al., 1977). Oocytes are ovulated as primary oocytes and become fertile only 2 to 3 days after ovulation. The fertile life of mature oocytes lasts another 2-3 days or more, which allows that matings 7 or 8 days after the LH peak can often be fertile (Concannon et al., 1989).

P4 concentration remains low during proestrus and before the preovulatory LH surge, but on average it increase slowly and fluctuate between 0.3 and 1 ng/mL, due to the luteinization effect of the LH (Concannon et al., 1977). P4 value rapidly increases to 1.1-1.9 ng/mL shortly before the preovulatory LH peak and reaches a value between 4 and 10 ng/mL on the ovulation day (Concannon et al., 1983). A range of serum P4 values in different stages of the canine oestrous cycle is displayed in Table n° 1.

The summary of preovulatory events are displayed in Figure n° 1 below.

Stage of the cycle	**Serum P4 values in ng/mL**
Anestrus or Early Proestrus	0-1 ng/mL
LH surge	2-2.9 ng/mL
Ovulation	4-10 ng/mL
Oocytes mature and available for fertilization	10-34 ng/mL

Table n° 1. Serum P4 concentration in different stages of canine reproductive cycle. Values are in ng/mL. Adapted and modified from Feldman and Nelson, Canine and Feline Endocrinology and Reproduction, p. 535, 1996.

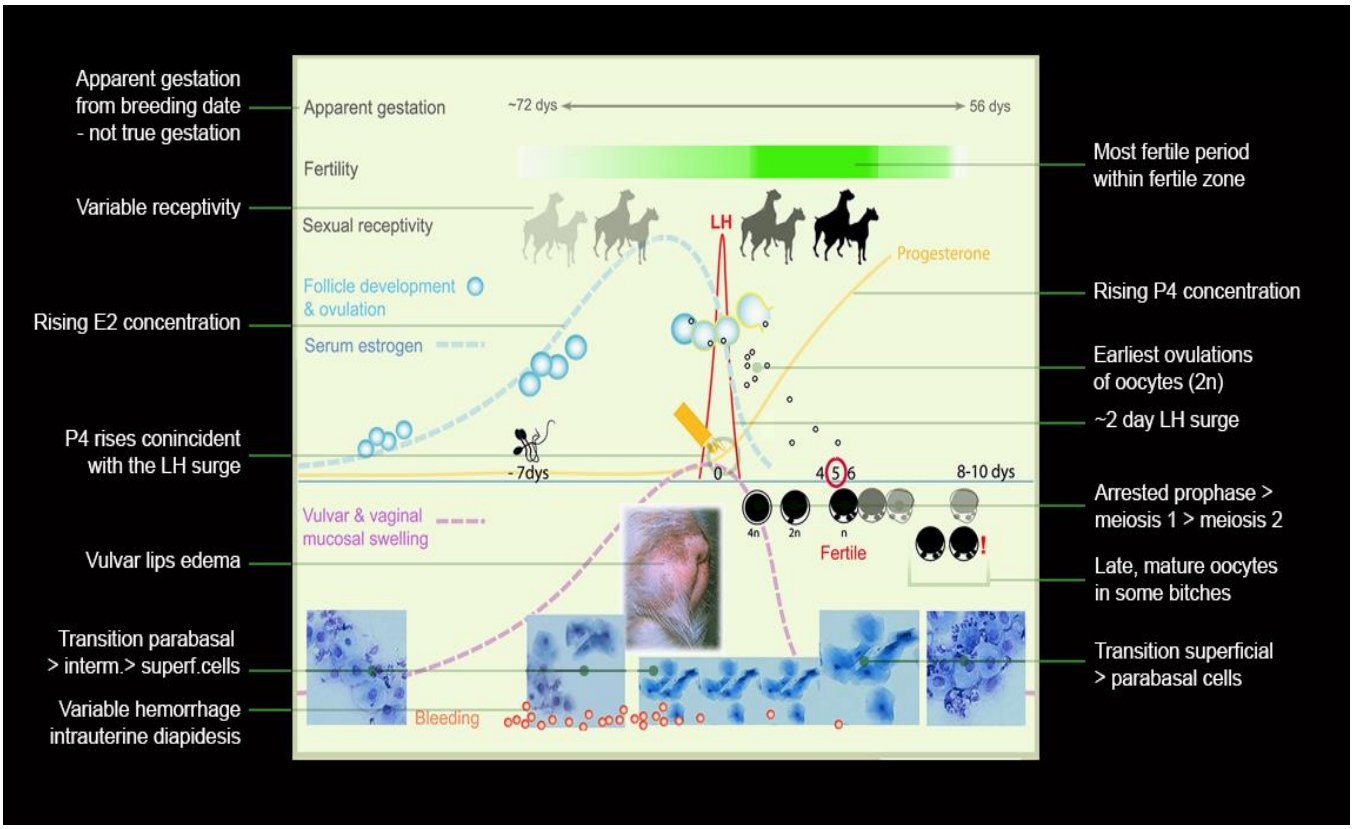


Figure n° 1. Schematic representation of periovulatory endocrine events, behavioral, vulvar and vaginal smears changes during the estrous cycle of the bitch. Adapted from Dr Rob Lofstedt, Library of Reproduction Illustration (LORI), http://loricaninefeline.blogspot.it/search?q=pregnancy.

Implantation events

Canine embryos enter the uterus around day 8-9 after ovulation as zygotes or morulae. In the first 1-2 days migration between horns can occur. Implantation takes place around day 16 to 18 after the LH peak (Johnston et al., 2001c). An indirect indicator of implantation is likely to be the rise of acute phase proteins in the blood of healthy bitches.

Post-implantations events: placentation and maternal changes

In Beagles, uterine swellings at implantation sites are about 1 cm diameter on day 20 after ovulation (Concannon et al., 1989). At this time, localized uterus edema, growth of the embryonic membranes and early placental development take place.

The dog placenta is zonary and endotheliochorial (Figure n° 2 and Figure n° 3). Because of this type of placentation, only 5-10% of immunoglobulins are transferred to the fetuses across the placenta. Therefore, the majority of passive maternal immunity is acquired through colostrum (Johnston et al., 2001c; Romagnoli, 2015).

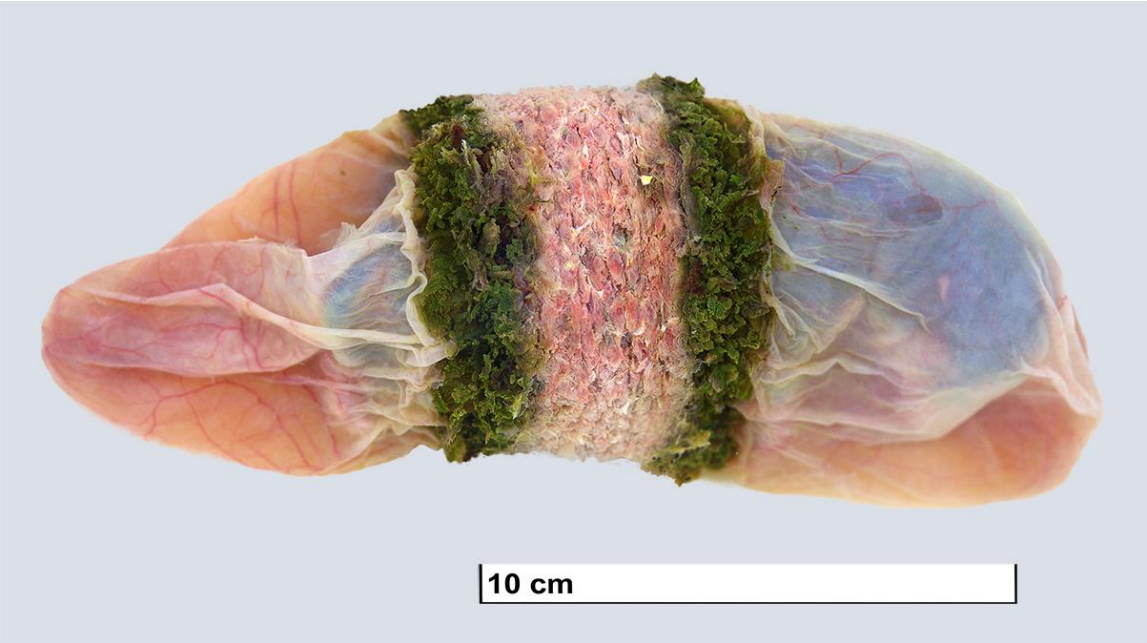


Figure n° 2. Canine fetal-placental unit at approximately 58 days of gestation. Zonary placentation is a wide ring-like structure surrounding the chorion. Adapted from Dr Rob Lofstedt, Library of Reproduction Illustration (LORI), http://loricaninefeline.blogspot.it/search?q=canine+placenta.

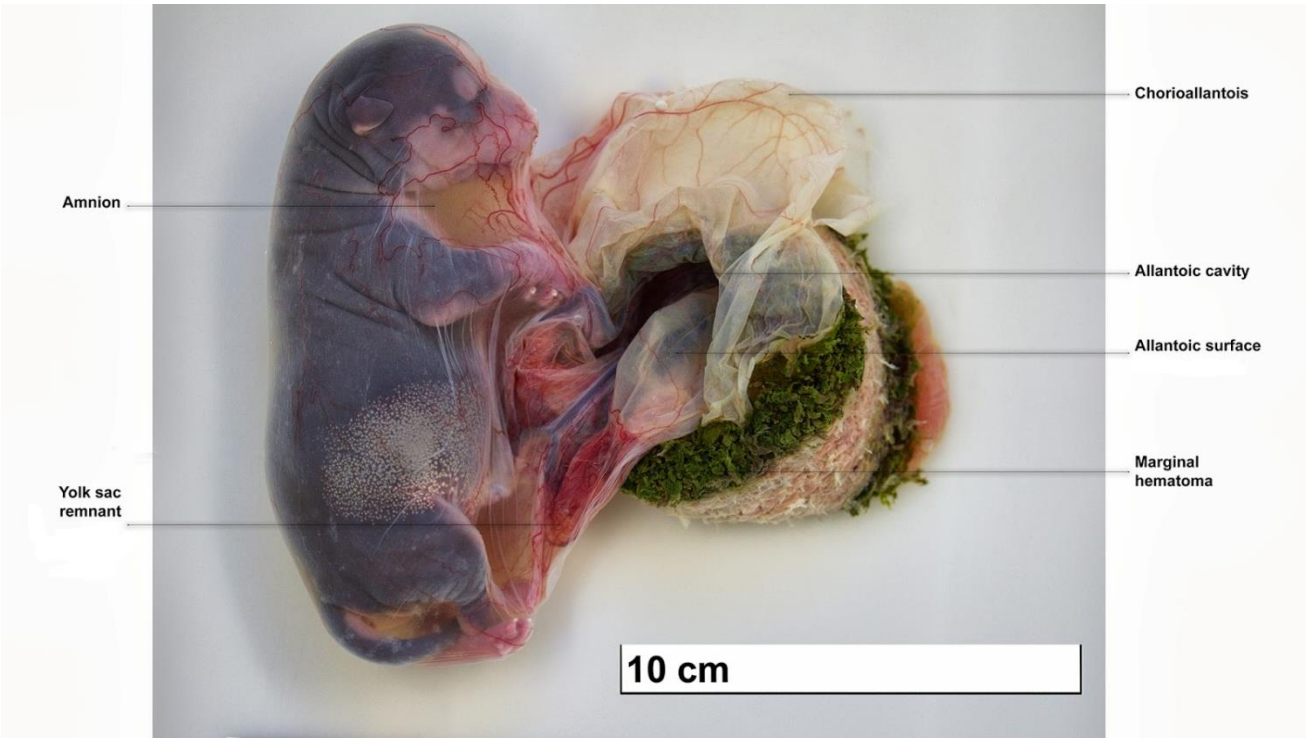


Figure n° 3. Canine fetal-placental unit at approximately 58 days of gestation. In the image above, the fetus has been extracted from the chorion, even if he still lies in the amnion. Different placental parts are named on the sides. Adapted from Dr Rob Lofstedt, Library of Reproduction Illustration (LORI), http://loricaninefeline.blogspot.it/search?q=canine+placenta.

During pregnancy, important maternal blood and body weight changes take place, such as:.

- The acute phase protein fibrinogen concentration increases between day 21 and 30 after the LH surge, reaching values of >280 mg/dl between day 29 and 50, remarkably higher than that of non-pregnant dogs (188±8 mg/dl). Values after day 50 are similar between pregnant and non-pregnant dogs. This result has a clinical relevance, suggesting that fibrinogen assay may be useful for an indirect pregnancy diagnosis in dogs (Concannon et al., 1996). Recently, fibrinogen has been further investigated and values of 250-300 mg/dl around day 25-28 post ovulation were reported. Values of 300 mg/dl at day 28 are considered 100% accurate in diagnosing pregnancy in the bitch provided that no other inflammatory condition is present anywhere else in her organism. Another acute phase protein, haptoglobin, is normally present at 35-50 mg/dl, and in pregnant bitches was founded at 75-100 mg/dl (Romagnoli et al., 2005), suggesting that it might be used as an indirect pregnancy diagnosis method as well.

- Maternal haematocrit, which is around 45-55% in healthy non-pregnant dogs, decreases slowly after implantations, reaching usually levels below 40% at day 35 and below 35% at the end of pregnancy (Concannon et al., 1989). Furthermore, in the last week of pregnancy and in the first days of lactation, reference values for hematocrit, total leucocytes counts, thrombocyte counts, hemoglobin content, mature neutrophil counts, lymphocytes counts, total protein concentration and albumin concentration are a little bit different from the ones reported in non-pregnant bitches and this unawareness may lead to incorrect conclusions of maternal health. A recent study suggested that a different range of reference values for these parameters has to be used when testing bitches in the last week of pregnancy or in early lactation (Table n° 2),
  (Orfanou et al., 2016).

Parameter	Proposed range of reference values
Haematocrit	25.5-33.5%
Total leucocytes	15500-26000 cells μL^{-1}
Thrombocytes	568500-823000 cells μL^{-1}
Haemoglobin	8-10 g dL^{-1}
Mature neutrophils	10500-19500 cells μL^{-1}
Lymphocytes	2700-6000 cells μL^{-1}
Total proteins	4.5-6 g dL^{-1}
Albumins	1.8-2.4 g dL^{-1}
C-reactive proteins	36-152 mg L^{-1}
Total calcium	8.8-10 mg dL^{-1}

Table n° 2. Different blood reference values in the last week of pregnancy and in early lactation in the bitch. Adapted and modified from Orfanou et al., 2016. Proceedings 8th International Symposium on Canine and Feline Reproduction in Paris, France, p. 140.

- Serum glucose concentration is lower in the pre-partum period but higher thereafter, possibly associated with the stress of parturition leading to the release of cortisol, which causes hyperglycemia (Lúcio et al., 2009).

- Another important post implantation event regards the increase in total body weight, which is in average 36% at the end of gestation (Concannon et al., 1989). Almost all the increase in body weight occurs in the second half of pregnancy, thus it is important not overfeed the bitch in the first 4 weeks. Only during the 6th week the amount of food has to be increased of about 30%, because fetal size increases rapidly in the last 3 weeks of pregnancy (Romagnoli, 2015). Hence food appetite, and so food intake, increases proportionally to body weight during late pregnancy (Concannon et al., 1989).

Pregnancy diagnosis

Pregnancy diagnosis in the bitch may be achieved using 3 different methods which may be performed alone or together with the others, in order to maximize the accuracy of the result. Besides, it is important to estimatethe day of gestation in order to choose the best pregnancy diagnosis method.

- Pregnancy diagnosis might be done through palpation at day 20 to 25 of pregnancy, a time in which embryonic vesicles are often detectable, unless the abdomen is too tense or the bitch is fat. It is best performed putting the hand as a sort of "gate", moving it in a cranio-caudal sense: embryonic vesicles are felt like small balls (Figure n° 4). From day 35 onwards these swellings are more than 3 cm in diameter, elongated, and may be difficult to palpate and to distinguish from each other (Concannon et al., 1989; Romagnoli, 2015). However, an ultrasound examination is recommended afterward in order to confirm the positive pregnancy status.

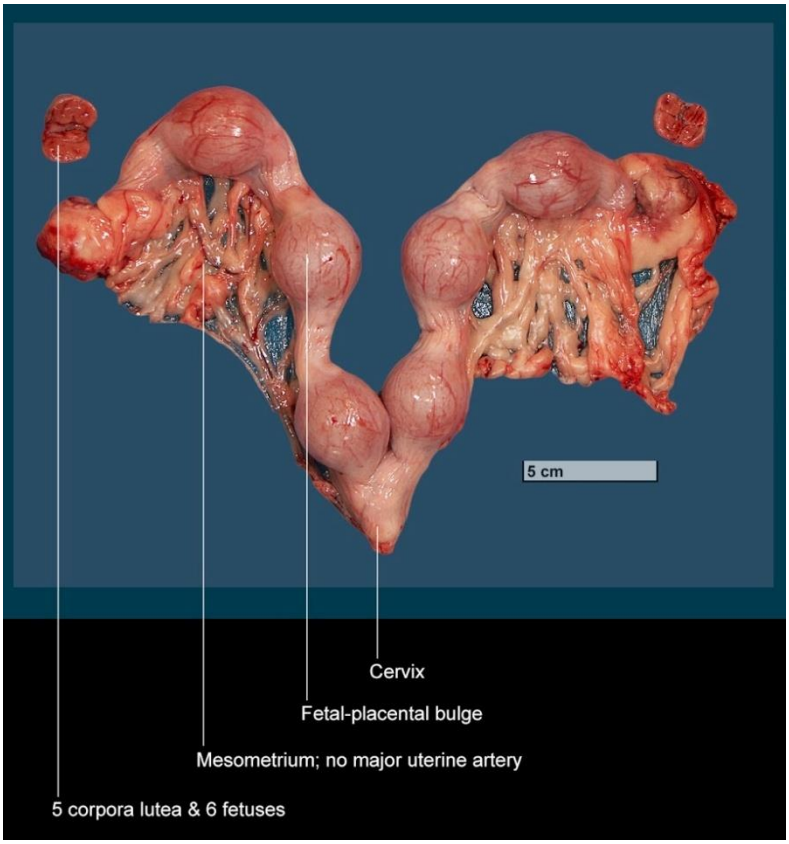


Figure n° 4. Canine pregnant uterus at approximately 25 to 27 days of gestation. In Figure above, each horn contains 3 embryonic vescicles, which can be easily palpate at this gestational time. Adapted from Dr Rob Lofstedt, Library of Reproduction Illustration (LORI), http://loricaninefeline.blogspot.it/search?q=pregnancy.

- Ultrasound examination is the most common and reliable method used for assessment of pregnancy status. Its only disadvantage is the lack of accuracy in counting the number of fetuses. Canine gestational vesicles are detectable on day 18 after ovulation and embryonic heartbeats might be detected around day 23 (Concannon et al., 1989). An example of anechoic embryonic vesicles is displayed in Figure n° 5.

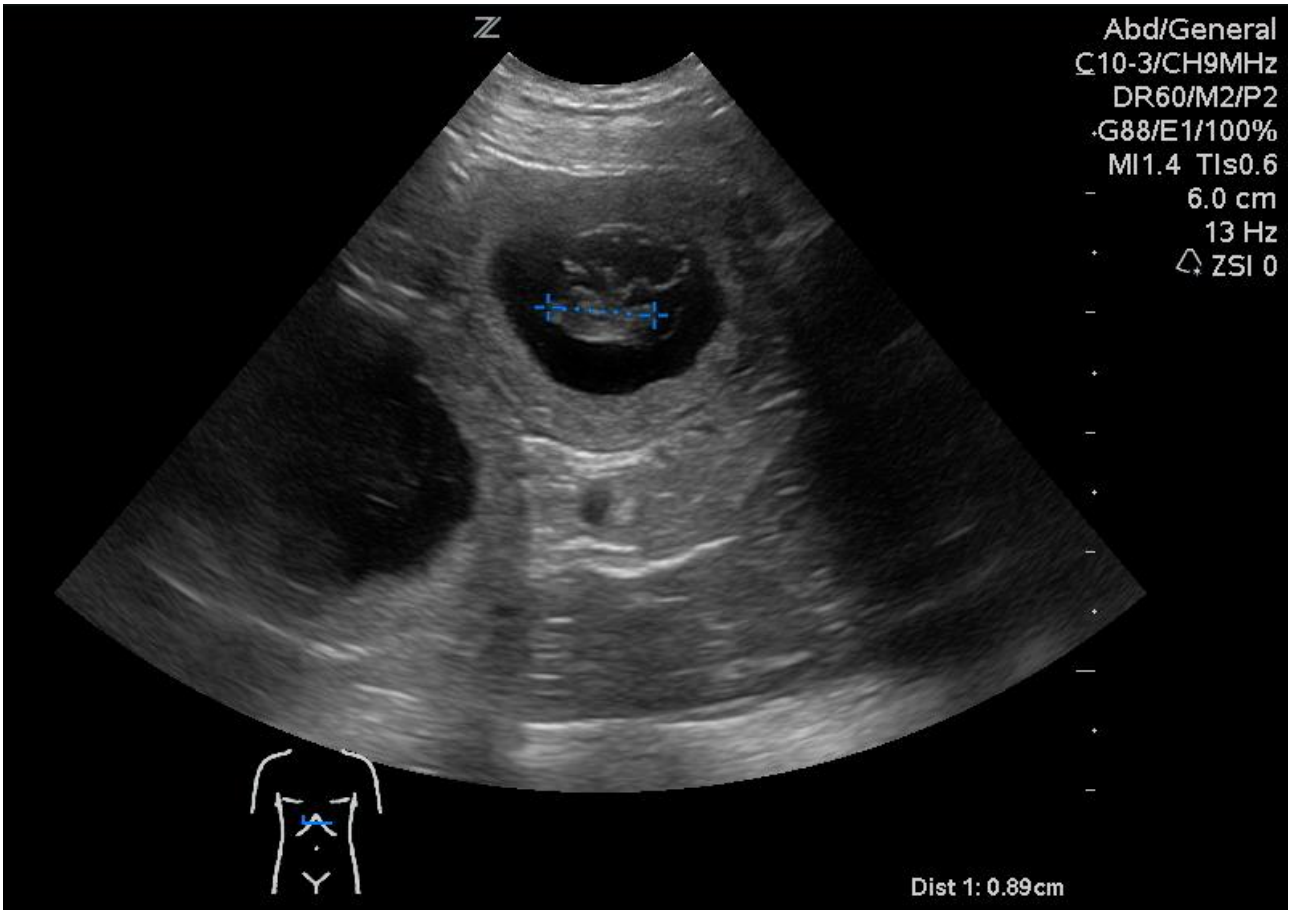


Figure n° 5. Ultrasound image displaying two embryonic vesicles at 28 days from ovulation in a 3-year old Basset Hound bitch. This finding confirms pregnancy diagnosis. The blue dotted line measures the length of the embryo, which is 0.89 cm.

- Radiography can be used for pregnancy confirmation and for fetal development evaluation, but only during the last 2 weeks of gestation (Concannon and Rendano 1983). Fetal skeletons become mineralized and visible on radiography only after day 46 of gestation. Fetal skeleton radiography finds its main application in the evaluation of the litter size. For this reason, usually the litter size x-ray is performed on day 55 of pregnancy or later (Figure n° 6), a time in which all the fetuses will be certainly mineralized.

Figure n° 6. Example of a litter size lateral x-ray performed in a 3 years old Jack Russel bitch on day 59 after ovulation.

Canine gestation length

Canine gestation length can range from 57 to 72 days, estimated as the interval from the day of the first mating to the day of parturition (Concannon et al., 1983), as Figure n° 7 shows. A difference of almost ten days may create practical problems in the management of pregnant bitches. A higher accuracy of estimation of the whelping day may be achieved knowing the day in which LH surge occurred. In fact, the interval between LH peak and parturition is less variable and ranges from 64 to 66 days with an average of 65.1±0.1 days (Concannon et al., 1983). This explains why gestations which last ≤ 61 days result from mating ≥ 3 days after the LH peak and gestations which last ≥ 68 days all occur from mating ≥ 2 days before the LH peak (Concannon et al., 1983).

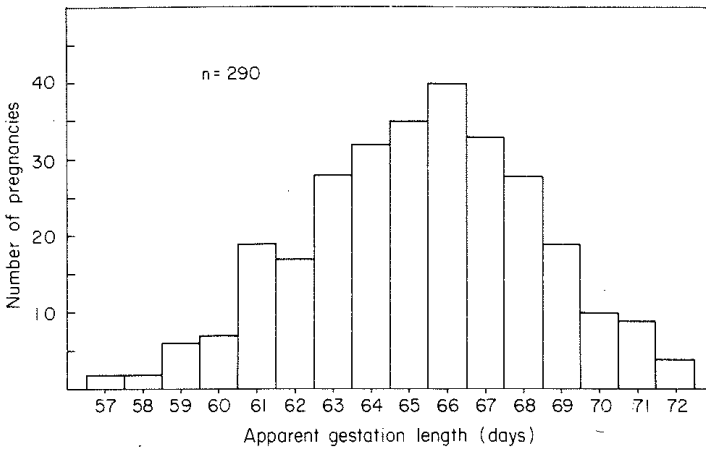


Figure n° 7. Gestation length from the day of the first mating to parturition observed in 290 Beagle bitches. Adapted from Concannon et al., American Journal of Veterinary Research, 44(10):1819-1821, 1983.

Litter size and breed seem to have an influence on the duration of gestation in dogs. Okkens et al. (2001) stated that the mean duration of pregnancy in West Highland White Terriers was 62.8±1.2 days compared to the 61.4±1.7 days duration of German shepherds. Thus, it seems that West Highland White Terriers have longer gestation length. Furthermore, it was observed that pregnancy length was negatively correlated with litter size, such as bitches whelping four or a fewer pups were more likely to have a longer gestation duration than those whelping five or more pups (Okkens et al., 2001; Eilts et al., 2004; Gavrilovic et al., 2008). However, this result was not confirmed in a recent study, in which no significant correlation between gestation length and litter size was observed in Beagle bitches (Seki et al., 2010). In the same study, pregnancies in nulliparous bitches tended to be approximately 1 day shorter than in multiparous ones when calculating from the day of the first mating to the day of parturition (Seki et al., 2010).

2.2 How to predict parturition in bitches

The prediction of parturition date can help veterinarians to manage normal and difficult parturition, being one of the most frequent questions asked by owners.

Parameters useful to predict parturition day and signs of forthcoming parturition are listed in Table n° 3.

Predictor of Parturition date		**Interpretation**
Breeding Dates		Parturition may occur 57-72 d from a single breeding
Hormonal and cytological findings :	Serum luteinizing hormone (LH)	Parturition occurs 64-66 d after serum LH surge
	Serum progesterone (P4)	Parturition occurs 64-66 d after serum P4 concentration of 2-2.9 ng/mL; Parturition occurs 62-64 d after serum P4 concentration of 4-10 ng/mL; Parturition occurs 12-24 h after a drop in circulating P4 concentration to levels <1-2 ng/mL
	Diestrus vaginal smear	Parturition occurs approximately 57 d after the onset of cytological diestrus
Radiographic appearance of fetuses		Mineralized fetal spine, skull and ribs first seen 20-22 d prepartum; caudal vertebrae, fibula, calcaneus and paws observed 2-9 d prepartum; teeth observed 3-8 d prepartum
Ultrasonographic appearance of fetuses		Observation of fetal organs and measurement of inner corionic

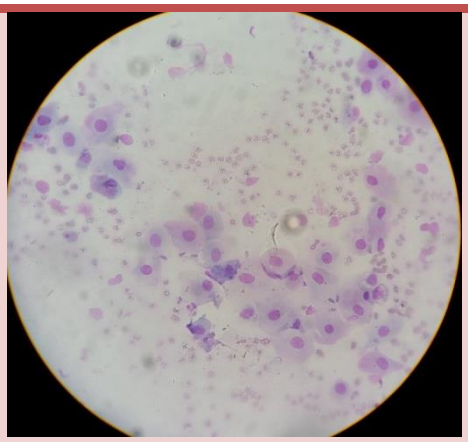
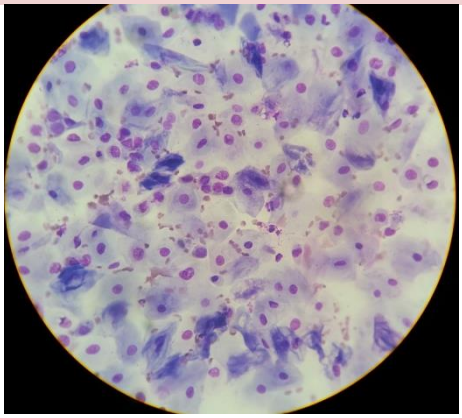
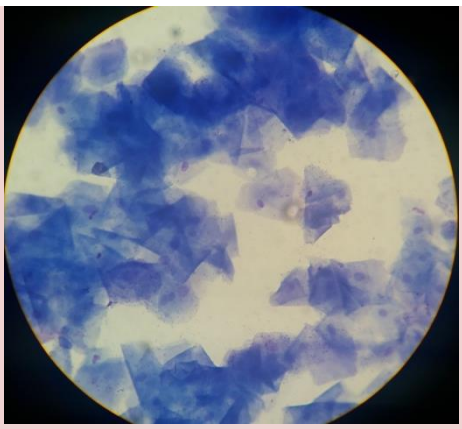
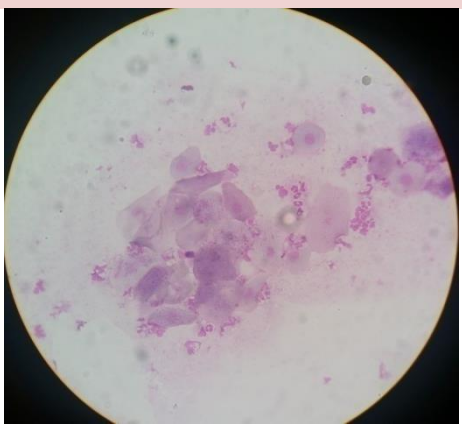
	cavity and biparietal diameter
Rectal Temperature	A drop in rectal temperature of about 0.8°C occurs between 48 and 24 hours prior to parturition
Signs of forthcoming parturition	**Interpretation**
Onset of Lactation	Onset of secretion of milk varies from 2 weeks before, until several days after parturition. Colostrum may appear yellow-tinged and more opaque than milk
Nesting Behavior	Onset ranges from 5 to 7 d prior to onset of parturition
Cervical, Vagino-vestibular and Vulvar relaxation	Increase in serum relaxin concentration near parturition cause increase cervical relaxation. The vagino-vestibular junction and vulva also relax
Lochia	Greenish-black discharge passes from the vulva following placental separation; whelping should occur 30-40 minutes from its presence

Table n° 3. Predictors of onset of parturition in the bitch. Adapted and modified from Johnston et al., Canine and Feline Theriogenology, p. 106, 2001d.

2.2.1 Day of ovulation

Apart from being useful to identify the optimal time of breeding, ovulation timing is the most commonly used tool to predict parturition day. Optimal fertility is achieved when a bitch is bred 2 to 4 days after ovulation. Ovulation day is important when performing artificial insemination (AI) with fresh, refrigerated or frozen semen, as well as to maximize fertility in breeding bitches with an history of standing behavior or conception failure (Johnston et al., 2001b). Many methods to time ovulation have been reported, but the most common one is the combined use of vaginal cytology and serum P4 assay. The use of vaginal endoscopy or ovarian ultrasound is not so frequent, even if both techniques are becoming increasingly available (Romagnoli, 2015).

- Vaginal cytology is considered the simplest way to assess the stage of the oestrous cycle and to confirm estrus. It is a very useful and practical method which can be performed inserting a cotton swab through the dorsal commissure of the vulva and gently pushing it until the middle vagina. Then the swab is rolled on a glass slide and stained with a dye. The dye in excess is removed with water and the glass slide is dried in the air (Romagnoli, 2015). Table n° 4 below shows the different cytological pattern on each stage of the canine estrous cycle.

Stage of the cycle	Vaginal Smear[1]	Description[2]
First half of Proestrus		Parabasal and small intermediate cells are predominant. Percentage of keratinized cells is 30-50%. Erythrocytes are present. Neutrophils may be present.
Second half of Proestrus		By mid to late proestrus, parabasal and small intermediate cells decrease in number and superficial (keratinized) cells increase. Percentage of keratinized and cells is 50-70%. Erythrocytes may be present.
Estrus		Superficial cells are predominant: more than 90% in most bitches. A 100% of keratinized cells are seen in the ovulation day. Erythrocytes may be present. Neutrophils are absent.
Early Diestrus		A drop from 100% keratinized cells to a 30% in 24 hours, indicates that the bitch is in the first day of cytological diestrus. Neutrophils may be present.

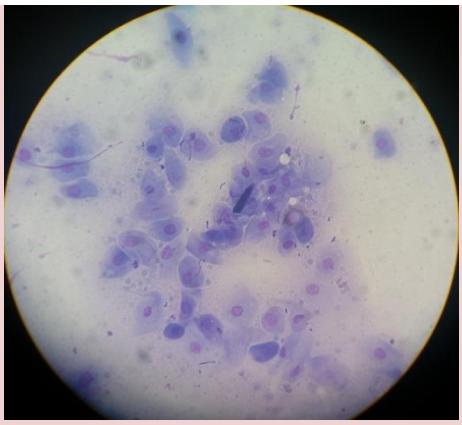
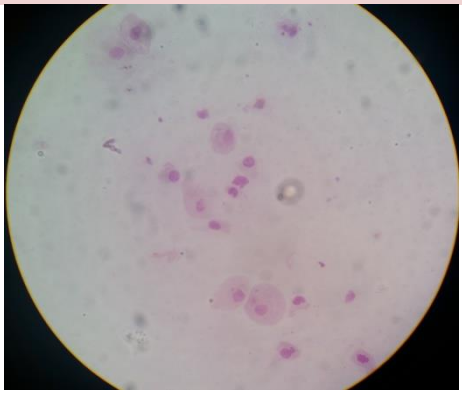
Late Diestrus		Proceeding through the diestrus period, cells keratinization will arrive at 0%, together with an increase in parabasal, small intermediate cells and nucleus. without cytoplasm. Neutrophils may be present.
Anestrus		Poor in cells. The only cells that may be seen are basal and small intermediate ones. Lots of nucleus without cytoplasm can be seen.

Table n° 4. Cytological patterns in different stages of canine estrus cycle. Magnification of 400X. [1]Images by courtesy of Professor Luísa Mateus, CIISA, Faculty of Veterinary Medicine, University of Lisbon, Portugal; [2]Descriptions adapted and modified from Johnston et al., Canine and Feline Theriogenology, p. 36-40, 2001a-b.

- Evaluating thanks to the close relationship between LH and P4, the concentration of serum P4 in serum samples allows the timing of ovulation. In the bitch, an increase in serum P4 concentration 2 to 3 days before ovulation (Johnston et al., 2001b) can be observed. The LH peak corresponds to a P4 value of 2-2.9 ng/mL. Almost 2 days after the LH peak ovulation takes place with a P4 value of 4-10 ng/mL (Concannon et al., 1989). Evaluation of serum P4 concentration with chemiluminescence (CLIA) assay gives an accuracy of nearly 100%. (Romagnoli, 2015).

- Vaginal mucosa undergoes dramatic changes in appearance during the canine estrus cycle, which may be detected using vaginoscopy or endoscopy (Johnston et al., 2001b). Thus, vaginal endoscopy examination can be a useful tool to assess whether or not the ovulation took place. Vaginal mucosa can be seen as edematous, swollen and with some blood during proestrus and early estrus due to estradiol stimulation (Figure n° 8A). As soon as follicular estrogen production stops in association with the increase in P4, vaginal folds become wrinkled. This condition is also called “crenulation” (Figure n° 8B) and it might be considered as an indirect ovulation indicator (Linsday, 1983; Moxon et al., 2012; Romagnoli, 2015).

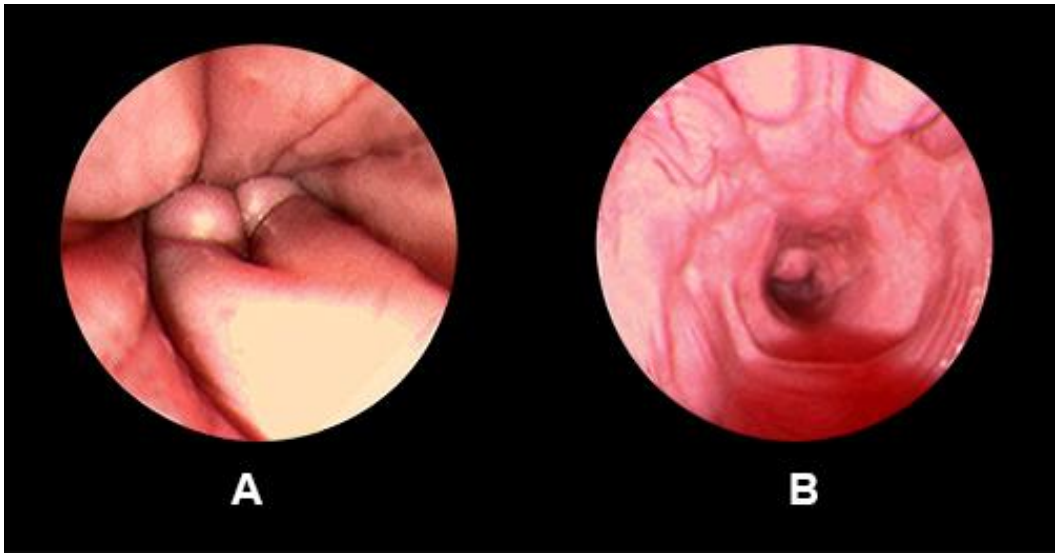


Figure n° 8 A. Endoscopic examination shows an edematous and swollen vaginal folds typical of proestrus and early estrus. Ovulation hasn't taken place already.
Figure n° 8 B. Endoscopy displayed that vaginal folds are become wrinkled. Ovulation occurred.
Adapted from Dr Rob Lofstedt, Library of Reproduction Illustration (LORI),
http://loricaninefeline.blogspot.it/search?q=crenulation

- Ultrasonography is a useful tool to evaluate follicular development, ovulation and corpora lutea growth (Hayer et al., 1993; Davidson and Baker, 2009), but its application is still limited.

  A 3-time daily evaluation of the ovaries can be used to recognize when the ovulation has just occurred (Davidson and Baker, 2009). Follicles have distinct walls and anechoic fluid centers with a distal enhancement and they do not collapse either in the bitch or in the queen. These structures make the ovary surface quite irregular. At the time of ovulation, the anechoic fluid filled follicles gradually become isoechoic to hyperechoic corpora hemorragica. In several days, these will become corpora lutea but they remain anechogenic just like the follicles (Davidson and Baker, 2009). Furthermore, the employment of US in ovulation timing is still marginal also due to the difficulty to observe the ovaries in small sized bitches (Romagnoli, 2015). Figures n° 9-10-11 below display ovary variations that commonly take place before and after ovulation.

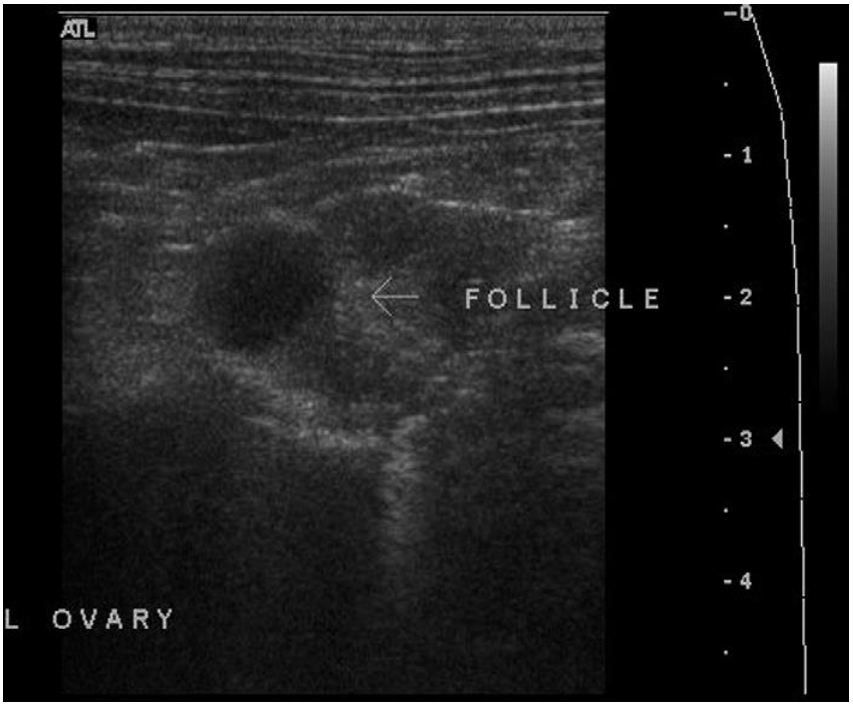


Figure n° 9. Follicles on a canine ovary. Adapted from Davidson and Baker, Topics in Companion Animal Medicine, 24(2):55-63, 2009.

Figure n° 10. Corpora hemorragica are isoechoic compared to the previous follicles. Adapted from Davidson and Baker, Topics in Companion Animal Medicine, 24(2):55-63, 2009.

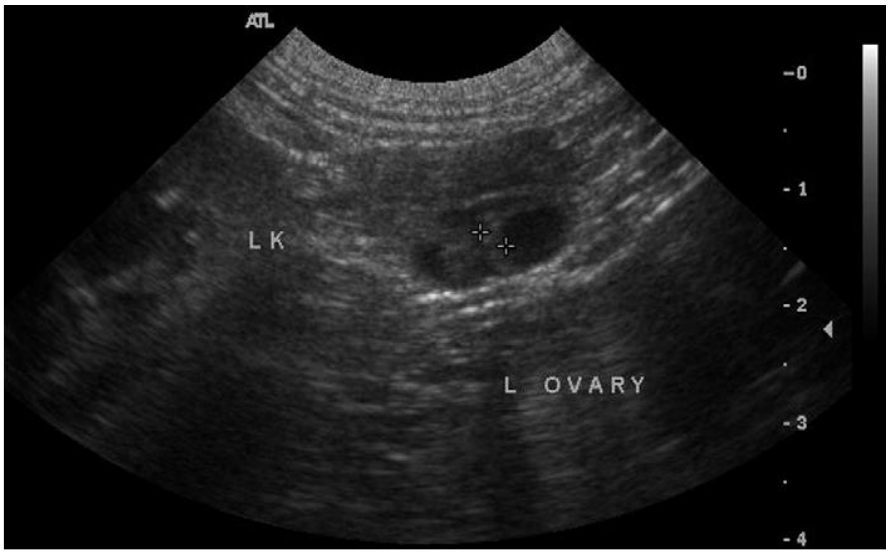


Figure n° 11. Postovulatory left ovary. Corpus luteum has a thin wall and shows presence of fluid within. Adapted from Davidson and Baker, Topics in Companion Animal Medicine, 24(2):55-63, 2009.

2.2.2 Cytological diestrus

Finding the first day of cytological diestrus (D1) means visualizing a drop from a 100% to ≤30% keratinized cells (Figure n° 12A-12B). The interval between D1 and parturition is approximately 57±2 days (Johnston et al., 2001c; Romagnoli, 2015).

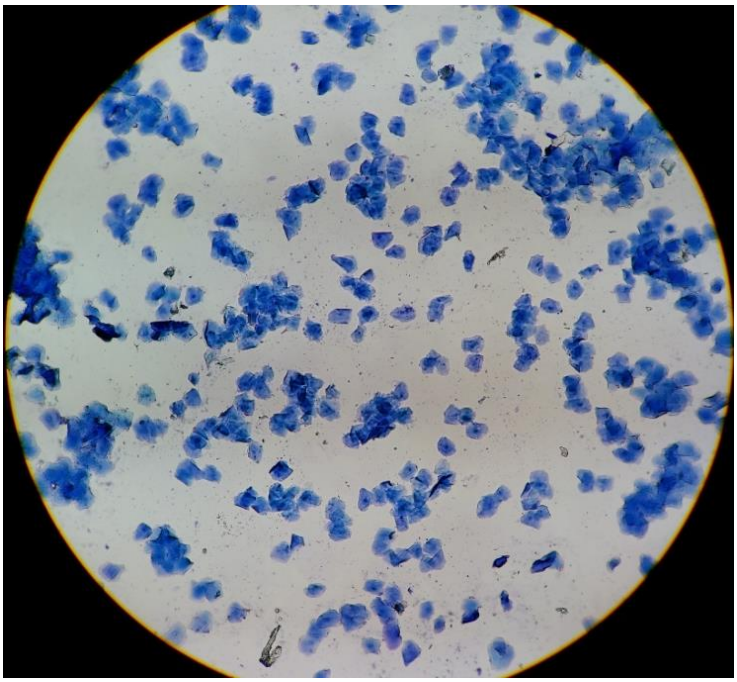


Figure n° 12 A. 100% keratinized vaginal smear (on the left) compared to the first day of cytological diestrus (on the bottom left). Magnification of 100X.
Images by courtesy of Professor Luísa Mateus, CIISA, Faculty of Veterinary Medicine, University of Lisbon, Portugal.

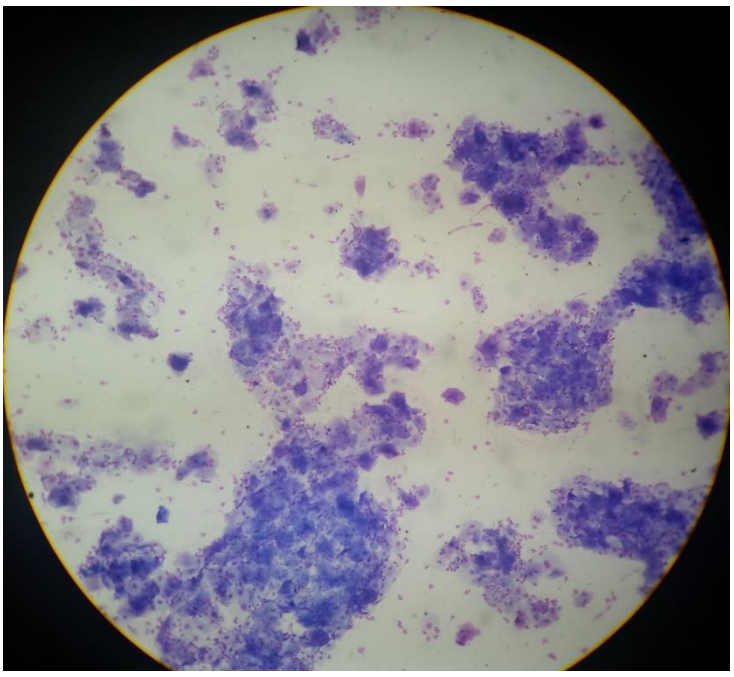


Figure n° 12 B. First day of cytological diestrus. Magnification of 100X.
Images by courtesy of Professor Luísa Mateus, CIISA, Faculty of Veterinary Medicine, University of Lisbon, Portugal.

2.2.3 Radiographic examination

The use of radiography to monitor fetal readiness to parturition is based on the detection of fetal structures within a precise range of days, which is due to the variable degrees of fetal mineralization depending on the day of gestation (Table n° 5).

Feature	Detection (d) after LH surge	
	Mean	Range
Spherical uterine swellings	35	31–38
Ovoid uterine swellings	41	38–44
First evidence of mineralization of the fetal skull	45	43–46
Scapula, humerus, and femur	48	46–51
Radius, ulna, and tibia	52	50–53
Pelvis and all ribs	54	53–59
Coccygeal vertebrae, fibula, calcaneus, and distal extremities	61	55–64
Teeth	61	58–63

Table n° 5. Fetal structures detection using radiography. Adapted from Lopate, Theriogenology, 70:397-402, 2008.

Several factors affect the radiographic quality, starting from the choice of proper X-ray technique (lateral or ventro-dorsal) and the adequate patient restraint. Another important issue is that large litter size may not allow an accurate visualization, making the evaluation of all skeletal components difficult. Also, food or gas in mother's colon, may obscure the correct visualization of the fetuses. It is important to underline that even if the fetus appears completely mineralized at day 58 after the LH surge, they are not fully mature, thus on this stage they wouldn't be able to survive extra uterus (Lopate, 2008).

X-rays can help in providing a rough estimate of fetal age, but this is not considered an accurate method to assess fetal maturation and estimate the correct whelping day (Lopate, 2008).

2.2.4 Rectal and Vaginal temperature

Rectal temperature

Monitoring rectal temperature in the last days of pregnancy, may help to predict parturition time (Concannon et al., 1977; Tsutsui and Murata, 1982; Verstegen-Onclin and Verstegen, 2008; Zonturlu et al., 2008). Transient prepartum hypothermia is observed during the parturition week.
The fall in rectal temperature starts 24 hours prior to parturition, with the nadir occurring at 12 hours and averaging 0.8±0.1°C (Figure n° 14). Rectal temperature then increases at whelping and is maintained for at least 4 days at levels above those recorded prior the prepartum hypothermia (Concannon et al., 1977). Hypothermia follows the decrease in P4 by approximately 12 hours (Figure n° 13), suggesting that the prepartum decrease in rectal temperature is not a physiological factor in the initiation of parturition, but rather a response to the rapid changes in circulating hormone levels. As a matter of fact, P4 has a thermogenic action and its declines leads to the decrease in rectal temperature. Rectal temperature will start rising as the bitch enter in the first stage of labor.

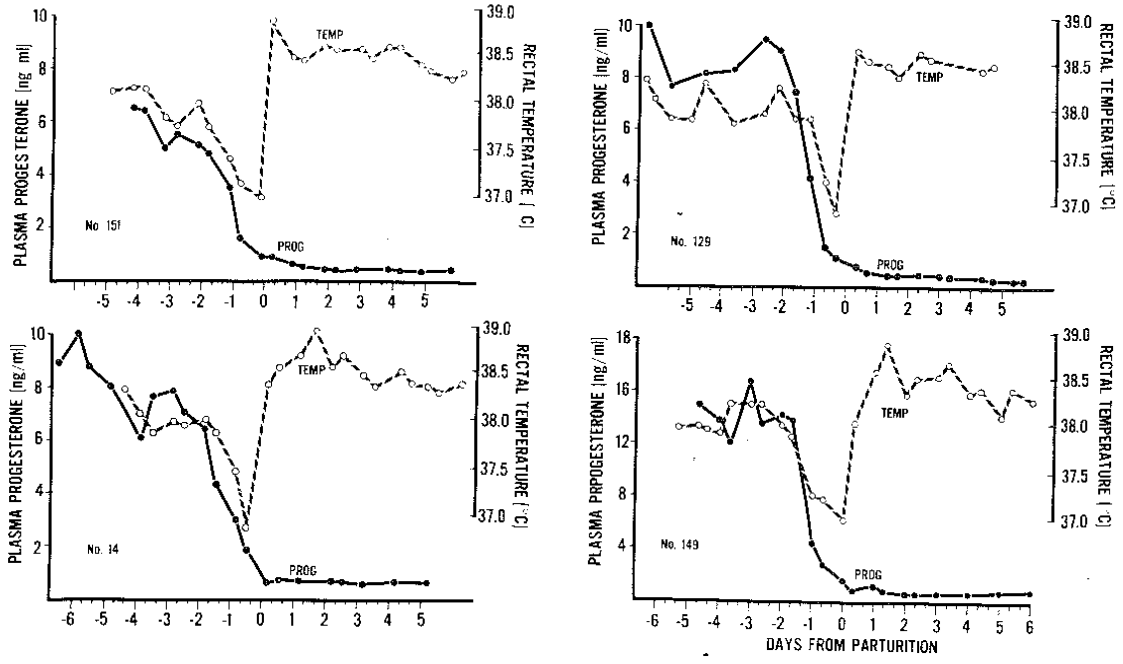


Figure n° 13. Plasma P4 concentration and rectal temperature through the whelping week of 4 Beagle bitches. Adapted from Concannon et al., Biology of reproduction, 4:517-526, 1977.

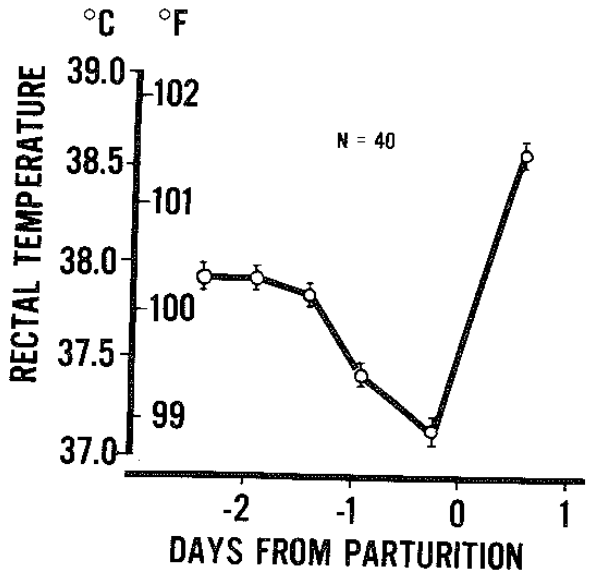


Figure n° 14. Mean rectal temperature around the time of parturition in 40 Beagle bitches. Adapted from Concannon et al., Biology of reproduction, 4:517-526, 1977.

Tsutsui and Murata in 1982 concluded that a decrease in body temperature to 37.5°C or less in the late stage of pregnancy could be a valid indicator to predict impending parturition. In their study, the average body temperature before the observed decline was 38.0°C, and it decreased to 37.0°C, 10 hours before parturition. After that, rectal temperatured gradually rised again until 37.4°C just before parturition (Figure n° 15).

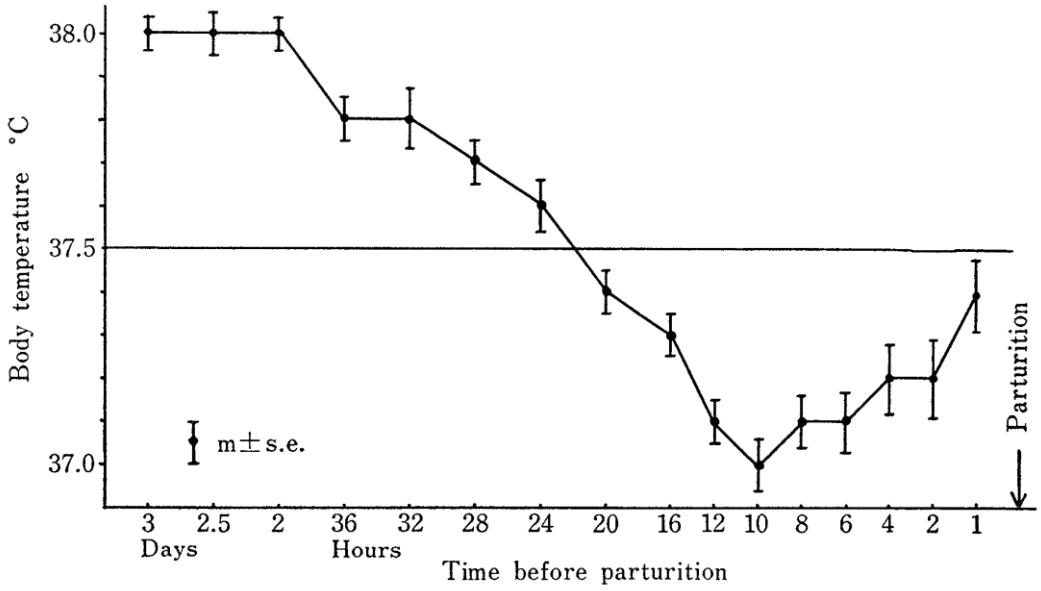


Figure n° 15. Variation in body temperature from 3 days to 1 hour before parturition in 45 pregnant bitches. Adapted from Tsutsui and Murata, Japanese Journal of Veterinary Science, 44(4):571, 1982.

On the other hand, Veronesi et al. (2002) stated that no statistically significant difference in rectal temperature was recorded 48 hours prior to parturition, suggesting that monitoring body

temperature variation could not be a valid method to predict parturition onset. This observation is in disagreement with previous studies (Concannon et al., 1977). In their study, a statistically significant increase in body temperature beginning 12 hours after the onset of parturition was reported, as Figure n° 16 below shows (Veronesi et al., 2002).

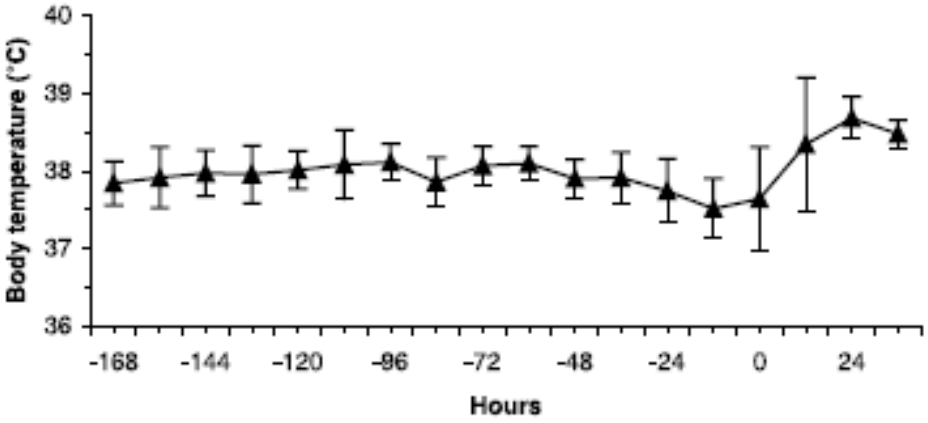


Figure n° 16. Mean body temperature of 7 bitches in peripartum period. Adapted from Veronesi et al., Journal of veterinary medicine. Series A, 49(5):264-268, 2002.

Owners should be instructed to monitor rectal temperature from day 54 to 55 of gestation onwards, in order to understand when parturition is approaching. Rectal temperature should be measured at least three times daily using the same thermometer, which should be inserted at least two centimeters towards the dorsal wall of the rectum, in order to avoid false measurements due to the feces. However, considerable variation in temperature values may occur among bitches and occasionally, there are bitches which do not show any drop in rectal temperature (Veronesi et al., 2002). Therefore, owners must be warned that parturition can occur even if they do not detect a drop in rectal temperature.

Vaginal temperature

Vaginal temperature can be measured using a normal thermometer. Recently, innovative and non-invasive methods to collect continuously body temperature were investigated. Vaginal temperature measurement was compared to the rectal one through the adoption of temperature loggers which were installed in 26 bitches' vagina (Maeder et al., 2012). Their results stated that there is a correlation between vaginal and rectal temperature with a mean difference of 0.0±0.2°C. Thus, vaginal temperature decreases approaching the day of parturition as observed in rectal temperature (Figure n° 17). Even if this method gives the chance to monitor continuously body temperature, an increase in neutrophil was found in vaginal cytology after the removal of the logger, suggesting that

a small inflammation spot might follow this practice. In another study, vaginal temperature loggers loss occurred once, and in two other bitches reinsertion was necessary (Geiser et al., 2014).

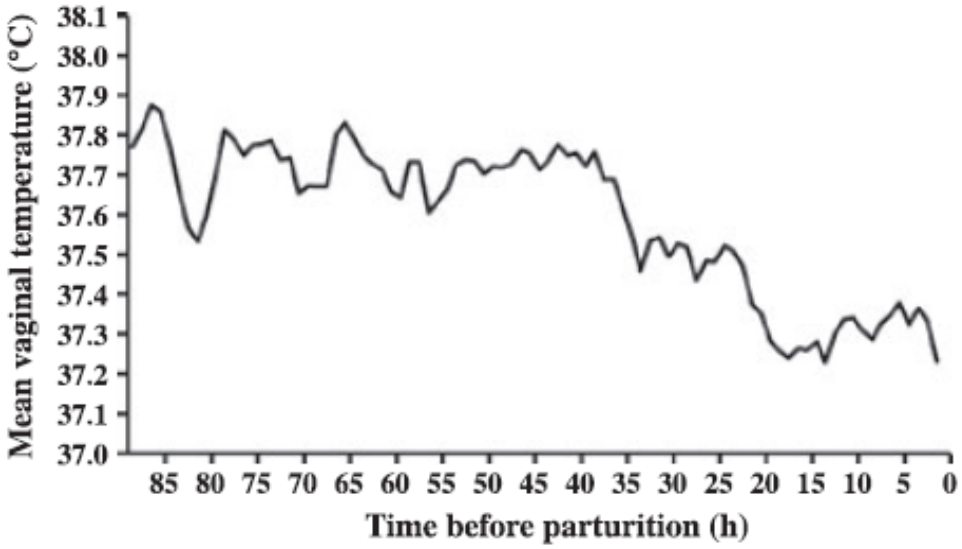


Figure n° 17. Mean vaginal temperature of 16 bitches in the last 90 hours before parturition, recorded with vaginal temperature loggers. Adapted from Geiser et al., Reproduction in domestic animals, 49(1):109-114, 2014

2.2.5 Serum Progesterone and Cortisol concentration

Serum Progesterone concentration

At the beginning of diestrus serum P4 concentration is generally fairly high, reaching values usually above 25 ng/mL at day 10 to 15. On these days, in some bitches, serum P4 concentrations might achieve levels from 50 to greater than 90 ng/mL (Feldman and Nelson, 1996). In a study of Concannon at al. (1975), serum P4 increased rapidly throughout estrus, reached 19.1±2.5 ng/mL on day 10 from LH peak, until a maximum of 22.9±2.7 ng/mL on day 25. It remained elevated until day 30 at 19.9±2.7 ng/mL. Its concentration declined gradually after day 30 in both pregnant and non-pregnant bitches. Although higher values can be reached, it is impossible to differentiate pregnancy serum P4 assay from a non-pregnancy one, as Figure n° 18 below shows.

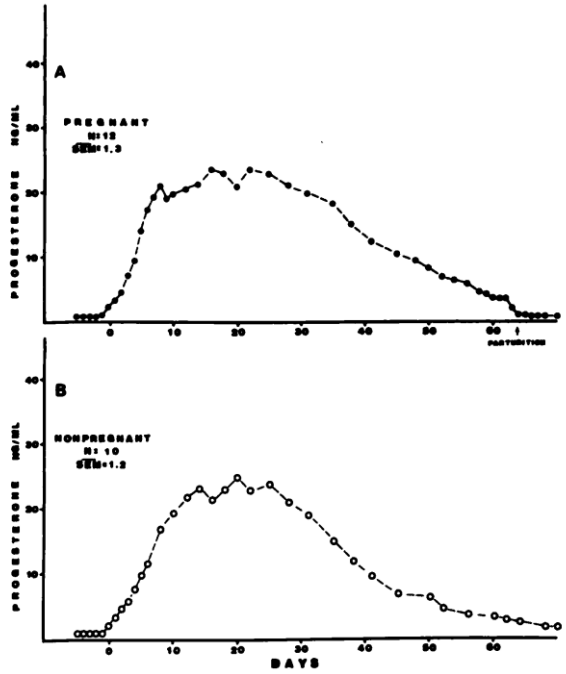


Figure n° 18. Mean plasma P4 levels in pregnant (A) and in non-pregnant bitches (B). Values are aligned to a common day 64 of parturition. Adapted from Concannon et al., Biology of reproduction, 13(1):112, 1975.

Before parturition can occur, a prepartum decrease in serum P4 to less than 1 to 2 ng/mL is necessary (Concannon et al., 1977). A decrease below 1.5 ng/mL between 12 and 40 hours prior the birth of the first pup was observed by Concannon et al. (1978). For this reason, serum P4 assay is a useful test to assess the parturition trigger or to plan an elective C-section.

As a matter of fact, the administration of exogenous P4 during the last days of pregnancy stops the onset of parturition. Such late administration leads to a prolonged gestation, fetal death and maternal compromise (Concannon et al., 1977).

A recent study assessed the diagnostic efficacy of a single P4 determination using chemiluminescent immunoassay (CLIA) to establish if parturition is going to occur the following

day (Rota et al., 2015). P4 concentration lower than 3.4 ng/mL correctly identified bitches that are going to whelp the following day. However, due to individual variations, a single serum P4 measurement has a low diagnostic efficacy, suggesting that more than one measurement or the evaluation of other parameters of impending parturition are needed
(Rota et al., 2015).

Serum Cortisol concentration

The increase in plasma cortisol concentration occurs during late pregnancy in the bitch (Concannon et al., 1977). As literature reports, fetal and maternal factors trigger the beginning of normal parturition. In particular, the rise of fetal corticotrophin-releasing hormone as parturition approaches stimulates the release of fetal adrenocorticotropic hormone (ACTH) and the following release of fetal cortisol. So, maternal and fetal corticosteroids are both important in initiating parturition. This enhancement in cortisol level leads to a prepartum rise in plasma of 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$, also called PGFM, the major metabolite of prostaglandin $F_{2\alpha}$ (PGF). They can stimulate uterine contractions directly or indirectly, due to the stimulation of pituitary gland and the subsequent release of oxytocin (Johnston et al., 2001d).
Serum cortisol levels 2 to 4 days prepartum ranges from 11-43 ng/mL with peak levels of 42-87 ng/mL occurring 8 to 24 hours prior to parturition (Concannon et al., 1978), (Figure n° 19). Furthermore, it has been demonstrated that elevated cortisol level on the day prior to parturition when bitches are usually quiet and lethargic indicates that prepartum elevation in plasma corticoids is part of the physiological mechanism of parturition, and not a demonstration of stress (Concannon et al., 1977).

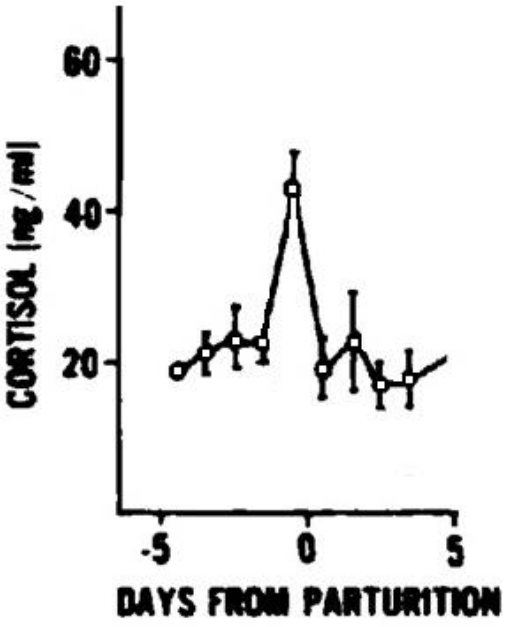


Figure n° 19. Mean serum cortisol 5 days prior to parturition to 5 days after parturition in 7 Beagle bitches.
Adapted and modified from Concannon et al., Biology of reproduction, 19(5):1113, 1978.

In a recent study of Veronesi et al. (2002), basal value of serum cortisol concentration was found to be 45±13nmol/l, while from 24 hours prior to parturition to 12 hours after parturition, levels of 90.4±41.4 nmol/l can be reached (Figure n° 20). This study partially disagreed with Concannon et al. (1978), who stated that serum cortisol level is high prior to parturition but decreases at the beginning of the delivery.

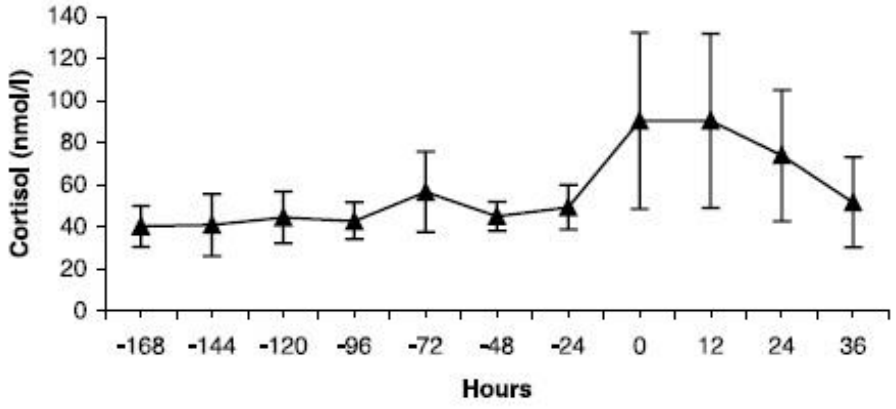


Figure n° 20. Mean serum cortisol concentration of 7 bitches in peripartum period. Adapted from Veronesi et al., Journal of veterinary medicine. Series A, 49(5):264-268, 2002.

Cortisol may play a very important diagnostic role when measured in the amniotic fluid at birth (Bolis et al., 2016). The high cortisol amniotic fluid at birth seems to be associated with short-term survival, suggesting that it could be a useful tool to recognize puppies that need special assistance immediately after birth.

2.2.6 Ultrasound examination

New methods used to increase the accuracy in the prediction of parturition time include the use of ultrasonography (US). The first detection of specific embryonic and fetal structures has been described by several studies as a useful way to assess gestational age (Yeager et al., 1992; Lopate, 2008; Aissi et Slimani, 2008; Beccaglia, 2015). Table n° 6 shows the day of the initial detection of fetal structures.

Fetal structures	**Days after ovulation**
Gestational sac	18
Heartbeat	23
Limb bud	27-31
Diencephalo-telencephalic vescicle	29-33
Stomach	29-33
Urinary bladder	31-35
Skeleton	29-33
Fetal movements	32-34
Lung	34-36
Liver	34-36
Kidneys	41-43
Bowel	57-63

Table n° 6. Days after ovulation in which fetal structures appear for the first time during canine gestation length using ultrasonoghraphy. Adapted and modified from Beccaglia, Proceedings 18th EVSSAR Congress - European Veterinary Society for Small Animal Reproduction, Hannover (Germany), p. 20-25, 2015.

2.2.6.1 ICC and BP measurement

The measurement of inner corionic cavity (ICC) and biparietal diameter (BP) are reliable features to predict gestation date and indirectly parturition time according to many authors.

In the first half of gestation, such as from day 19 to 37 after the LH peak, the measurement of ICC can easily be obtainedand recognized as the gestational sac is seen by US as spherical anechoic structure with well-defined margins (Figure n° 21). While in the second half of pregnancy, such as after day 37 from the LH peak, fetal measurements of the BP can be acquired (Figure n° 22). Furthermore, from day 35 to 58, fetal diencephalon-telecenphalic vesicle (DPTV) can be seen as a symmetric anechoic area with clearly defined margins and it has been suggested to be a marker of

gestational age (Beccaglia and Luvoni, 2004). However, compared to ICC and BP, DPTV cannot be considered accurate enough in predicting parturition time.

Figure n° 21. Ultrasonographic image of inner corionic cavity (ICC) diameter 41 days before parturition in a Tibetan Mastiff bitch. Adapted from Socha et al., Theriogenology, 84(5):779-783, 2015.

Figure n° 22. Ultrasonographic image of biparietal diameter (BP) 17 days before parturition in a Leonberger bitch. Adapted from Socha et al., Theriogenology, 84(5):779-783, 2015.

Specific formulae for ICC and BP have been reported for small (up to 10 kg) and medium (11-25 kg) size dogs (Luvoni and Grioni, 2000). Some authors proposed the application of the equations

described for medium size dogs also in large breeds ones (26-40 kg) with a correction factor in order to increase accuracy (Kutzler et al., 2003). However other authors stated that formulae for medium size dogs might be used also in giant ones (>40 kg) with good accuracy (Socha et al., 2015). A resume of ICC and BP formulae for different bitches' size in order to obtain days before parturition (DBP) is displayed in Table n° 7.

	ICC	**BP**
Small size dog	DBP= (mm-68.68)/1.53	DBP= (mm-25.11)/0.61
Medium size dog	DBP= (mm-82.13)/1.8	DBP= (mm-29.18)/0.7
Large size dog	DBP= (mm-105.1)/2.5	DBP= (mm-30)/0.8
Giant size dog	DBP= (mm-88.1)/1.9	DBP= (mm-23.39)/0.47

Table n° 7. ICC and BP formulae, obtained respectively 19-37 days and 37 days after the LH peak, for small (up to 10 kg), medium (11-25 kg), large (26-40 kg) and giant (>40 kg) size dogs. Adapted from Beccaglia, Proceedings 18th EVSSAR Congress - European Veterinary Society for Small Animal Reproduction, Hannover (Germany), p. 20-25, 2015.

Although ICC and BP are both highly accurate to predict the whelping day, it has been demonstrated that their accuracy might be affected by some factors:

- *gestational period in which the examination is performed*. It has been proved that the estimation of gestational age in dogs is more precise during early pregnancy, such as on day 30 after the LH surge, than at a subsequent time (England et al., 1990; Kutzler et al., 2003). This finding suggests that the accuracy in predicting the parturition date decreases as gestation progress. For instance, BP measurement maintains a high accuracy up to week 6 of pregnancy;
- *breed*. The decline in accuracy approaching the late gestation period might be due to the large variability in fetal growth rate between breeds at this time. Kutzler et al. results, supported the hypothesis that fetuses of small size bitches grow at a slower rate, and fetuses of giant size bitches grow at a faster rate than those of medium and large size bitches (Kutzler et al., 2003). Besides, it seems that equations designed for a single breed are likely to be more accurate to avoid morphological variables present in dogs of the same size. Son et al. (2001), described a prediction table of parturition day based on the measurements of gestational structures in Maltese and Yorkshire Terrier and Groppetti et al. (2015) obtained higher accuracy when using a specific equation adapted only for German Shepherd dogs;
- *litter size*. Apart from the mother's size, it seems that even the litter size might affect the accuracy of these formulae, but in particular it is likely to influence BP: higher accuracy was

obtained in normal litter size when compared to small and large litters (Beccaglia and Luvoni, 2006).

Nowadays, ultrasonographic measurement of fetal structures is considered one of the most common and accurate method for prediction of whelping date during pregnancy and it might be used also when ovulation and the time of mating are unknown. However their accuracy can be affected by many factors. Thus, in order to achieve the most precise results, a combination of different methods is recommended.

2.2.6.2 Fetal Heartbeats

Ultrasonography is the most efficient technique used to monitor fetal development and fetal viability. Normal fetal heart rate should be 2-3 times that of the bitch, and thus greater than 220 beats per minute (bpm); values between 180 and 220 bpm indicate moderate fetal stress whereas values less than 180 bpm indicate severe fetal stress (Zone and Wanke, 2001). During the last 10 days of pregnancy, evaluation of fetal heart rate is a parameter commonly used to monitor and assess fetal wellbeing. Fetal stress resulting primarily from hypoxia, can occur during dystocia and it might be suspected when a decrease in fetal heart rate is detected (Kutzler et al., 2003). Other authors stated that the ratio between fetal heart rate and maternal heart rate compared to the former alone, might be even better to assessed fetal health (Alonge et al., 2016). Together with the observation of fetal heartbeats, also monitoring blood flow changes in umbilical artery might be a useful way to diagnose fetal distress (Giannico et al., 2015).

Furthermore, fetal heart rate has been investigated in many studies in order to understand if it might be a useful criteria to predict parturition in the bitch. According to literature, the first fetal cardiac frequency is detected at day 23 after the LH peak, even if the measurement becomes easier on day 32 (Verstegen et al., 1993). From the initial average values of 214±13.3 bpm, an increase in fetal heart rate value is observed until day 40 from the LH peak (238.2±16.1 bpm). From day 60 to parturition a reduction until 218±6.7 bpm was noted approaching the whelping day (Figure n° 23), (Verstegen et al., 1993).

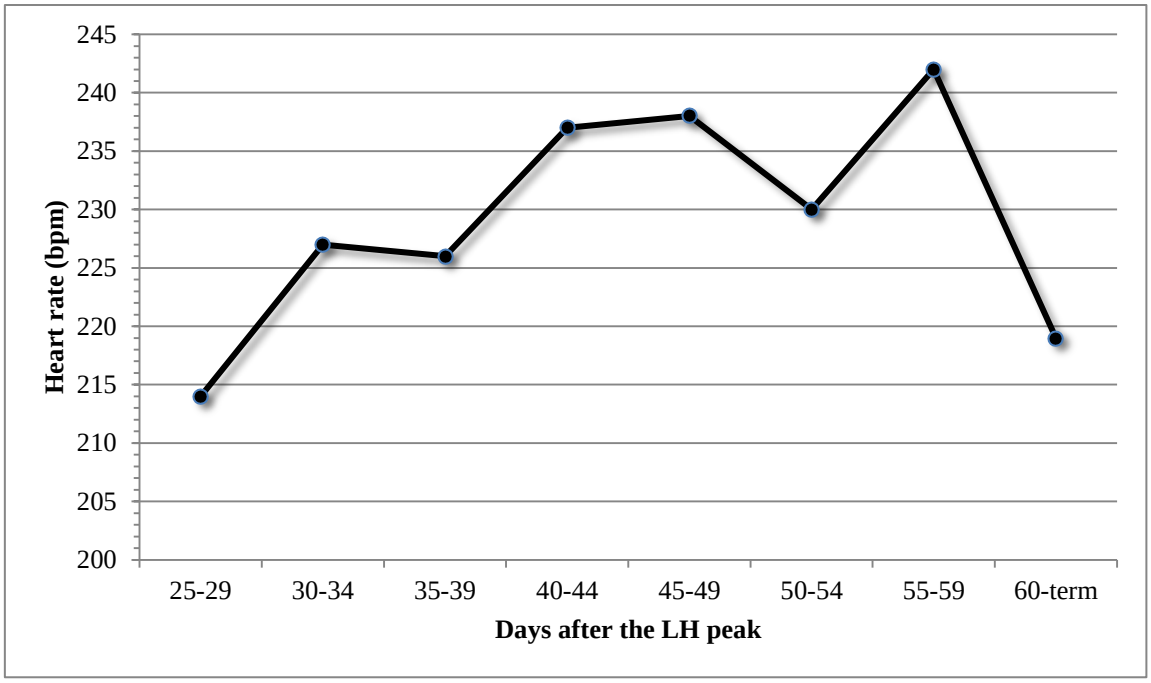


Figure n° 23. Mean fetal heart rate in 15 Beagle bitches. Adapted and modified from Verstegen et al., Journal of reproduction and fertility. Supplement, 47:175-180, 1993.

Fetal heart rate was further investigated and found to have physiological accelerations and decelerations from 72 hours prepartum onwards. In the beginning, heart rate variations occurs only in few fetuses, but approaching the day of parturition all the fetuses displayed accelerations and decelerations (Gil et al., 2014), suggesting this as a useful way to predict impending parturition. In order to better understand the meaning of this phenomenon, when a low fetal heart rate is detected, the fetus should be monitored for a few minutes to establish if an acceleration follows the deceleration observed. If the deceleration period was transient, the fetus is not in distress (Gil et al., 2014).

2.2.6.3. Fetal Gastrointestinal Motility

Fetal bowel is the last organ that can be visualized in an ultrasonographic exam and it indicates indirectly that the gestation is almost due. The time of its first detection is discordant among the authors. Earlier, authors agreed in stating that its first visualization, together with the beginning of intestinal peristalsis, is around 57 to 63 days of gestation (Yeager et al., 1992; Kim et al., 2007). However, a recent study made by Gil et al. (2015), revealed that the first ultrasonographic appearence of fetal intestinal bowel is on day 39 to 44 of gestation and the first detection of peristalsis is around day 48 to 54. This new finding is probably due to the high resolution images that modern ultrasound can provide. Until 2015, there are no previous studies describing the ultrasonographic assessment of fetal canine bowel in literature.

Generally, the term "peristalsis" indicates the intestinal motility. The fetal bowel movements observed by ultrasound, were investigated also in human fetuses (McLain, 1963). It is known that the first material espulsed by the pup, the meconium, can be visualized also in human fetuses. Its presence along the intestine causes the distension of the gut and peristalsis is useful to move the luminal content (Becker et al., 1940). For instance, it seems that the meconium content of the intestine and its gradual displacement to the colon, is responsible for a progressively more efficent peristalsis (Zilianti et Fernández, 1983). The gastrointestinal tract of human infants may play a role also in the regulation of the amniotic fluid. As a matter of fact, yellow and green amniotic fluids were observed in some infants who had an increase in fetal gastrointestinal motility, suggesting that this staining might be secondary to anal sphincter relaxation and so to the release of meconium, as a manifestation of fetal distress (McLain, 1963). Also veterinary literature reports a relationship between fetal intestinal peristalsis and fetal distress. A study performed by Zone and Wanke (2001), reported that bowel movements seem to be observed in all of the puppies that have severe fetal distress together with an heart rate per minute less than 180, whereas peristalsis were observed in only 40% of the fetuses with slight fetal distress together with an heart rate of 180-200 beats per minute. This finding, suggested that the detection of bowel movements assessed by ultrasonography, might be a reliable method to assess fetal distress or at least of physiological distress due to the normal mechanism of parturition (Zone et Walke, 2001).

On the other hand, a recent study did not look at fetal intestinal peristalsis as a fetal distress manifestation, but rather as a useful criteria to predict parturition in bitches (Gil et al.,2015). This author stated that intestinal peristalsis only is observed 5 to 9 days after the visualization of fetal intestine and not at the same time. These fetal intestinal movements are not constant and they may differ along the intestinal tract. This fact implicates that, from day 48 to 54 of pregnancy, only a continuous observation of several minutes with the ultrasound will allow to evaluate this feature

(Gil et al., 2015). This finding changes as the day of parturition approach. From day 57 onwards, instestinal peristalsis is visible immediately due to evident intraluminal fluid. Thus, a superficial interpretation of peristaltic motion as a criteria to evaluate rediness to parturition might result in a misleading judgment of fetal age. In fact, as the first visualization of fetal peristalsis can be as soon as 9 to 15 days before the full term, an inappropriate surgical intervention can lead to the death of the fetus (Gil et al., 2015). Furthermore, the evaluation of the survival probabilty of the fetuses using the detection of their peristaltic movements has been performed. Literature reports that once intestinal peristalsis is found consistently, such as from day 57 onwards (Gil et al. 2015), fetuses would have different probability to survive. This probability is different between natural parturition and C-section and increases approaching day 63. For example, fetuses born on day 57 have 10% chance of survival if delivered naturally and a 20% chance if delivered by C-section, while fetuses born on day 62-63 have 100% chance of survival with both methods (Figure n° 24).

Figure n° 24. Probability of survival for fetuses born from day 57 onwards. Adapted from Gil et al., Theriogenology, 84(5):681-686, 2015.

Figure above suggestes that fetuses were not probably enough mature to survive extra uterus if delivered before day 62. This finding denotes that ultrasound detection of fetal intestinal peristalsis on day 57 onwards is still insufficient to indicate fetal maturity and so readiness to parturition. In order to improve this prediction, the additional evaluation of other parameters is recommended.

2.3 Aim of the study

This study aims to evaluate the prediction of parturition time in bitches. It has the purpose of identify another reliable method to predict the whelping date by investigating ultrasonographically the gastrointestinal motility of canine fetuses in the last ten days of pregnancy. As literature reports, there are other parameters that can be used to predict the whelping day, each of them with a proper accuracy. Thus, the aims of this study were:

1. Investigate fetal gastrointestinal motility in the last ten days of pregnancy;
2. Compare and integrate it with other parameters of impending parturition:
    - Vaginal temperature
    - Rectal temperature
    - Serum progesterone concentration
    - Serum cortisol concentration
    - Fetal heartbeats.

3. MATERIALS AND METHODS

3.1 Animal Data

This study was performed on a population of 20 pregnant bitches of different breeds and sizes presented to the Veterinary Teaching Hospital of the University of Padova between February 2015 and June 2016. Eighteen bitches were presented for ovulation staging and were therefore monitored through vaginal cytology and serum P4 assay to identify the optimal time of mating, while two other bitches were presented for pregnancy diagnosis.

Pregnancy diagnosis by ultrasound were carried out from day 21 to day 30 after the estimated day of ovulation using a commercial ultrasound unit (Zonare, Zonare Medical Systems Inc®, Mountain View, California, USA) with a 4-9 MHz probe.

All bitches were examined 1-5 times from day 54 to 63 of pregnancy depending on owner's availability. Gestational age was assessed during the study and corrected by counting backward from the delivery day (day 63 post-ovulation).

Eleven bitches were followed and assisted directly during whelping. The remaining nine bitches were generally followed by the owners themselves.

Fifteen bitches had normal delivery, while 4 delivered by Cesarean section and one presented dystocia during the expulsion of the first pup. The litter size was between 3 and 12 pups, (Table n° 8).

Bitch number	Name	Breed	Age	Type of delivery	Litter size	Stillbirth - Neonatal death	AI - Natural Mating
1	Pepper	Flat Coated Retriever	4 years	Normal	12 pups	2 - 2	Natural
2	Vicky	Flat Coated Retriever	2 years	Normal	11 pups	1 - 0	Natural
3	Lisa	Whippet	3 years	C-section	8 pups	0 - 0	Natural
4	Ithaca	Basset Hound	3 years	Normal and C-section	11 pups	1 - 0	Natural
5	Bibì	Jack Russel Terrier	3 years	Normal	5 pups	1 - 0	Natural
6	Haka	Jack Russel Terrier	2 years	Normal	5 pups	0 - 0	AI
7	Quincy	Scottish Shepherd	2 years	Normal	8 pups	0 - 0	AI
8	Kate	Australian Shepherd	5 years	Normal	10 pups	0 - 0	Natural
9	April	Pumi	2 years	Normal	7 pups	0 - 0	Natural
10	Milly	Norfolk Terrier	7 years	C-section	5 pups	0 - 0	AI
11	Tilda	Flat Coated Retriever	2 years	C-section	7 pups	0 - 0	Natural
12	Pepper	Flat Coated Retriever	5 years	Natural	12 pups	0 - 0	Natural
13	Fanny	Boxer	4 years	Normal	3 pups	0 - 1	Natural
14	Gaia	Boxer	2 years	Normal	8 pups	0 - 5	Natural
15	Mika	Jack Russel Terrier	2 years	Normal	7 pups	0 - 0	Natural
16	Tikka	Flat Coated Retriever	2 years	Normal	10 pups	0 - 10	Natural
17	Neve	Samoyed	2 years	Normal	7 pups	0 - 0	Natural
18	Uffa	Golden Retriever	3 years	Normal	8 pups	1 - 0	AI
19	Ruby	Flat Coated Retriever	6 years	Normal	7 pups	0 - 0	AI
20	Dana	Bouvier des Flanders	4 years	Normal	8 pups	0 - 0	Natural

Table n° 8. Signalment, type of delivery, litter size, stillbirth - neonatal mortality and artificial insemination - natural matings for 20 bitches followed up during pregnancy to establish fetal maturation through gastrointestinal motility.

3.2 Experimental Protocol

On the first day in which a bitch was enrolled into the research protocol, a complete blood count and serum biochemistry analysis were assessed to determine the health status of the mother. A complete history and physical examination were performed, including evaluation of conjunctival, oral and vaginal mucosas, skin and subcutaneous tissues, retropharyngeal, prescapular and popliteal lymphnodes, abdominal palpation, lung auscultation with assessment of respiration and heart rate. All bitches were in healthy status with all clinical parameters within physiological range (Annex n° 21). Furthermore, from day 52 to day 61 of pregnancy, an X-ray in lateral or ventro-dorsal projection of the bitches' abdomen was performed in order to get more information about the litter size. X-rays obtained are showed in the Annex n° 1-20.

In addition, in each visit the following parameters were recorded:

a) Vaginal temperature;
b) Rectal temperature;
c) Serum progesterone concentration;
d) Serum cortisol concentration;
e) Fetal heartbeats;
f) Fetal gastrointestinal motility.

a) Vaginal temperature

Vaginal temperature was measured at the beginning of each visit, in order to avoid the influence of the stress of the bitch during the manipulations, which could result in a rise in the temperature. Before performing the measurement, the thermometer was disinfected with alcohol and then with betadine.

The measurement was performed using a digital thermometer (Omron® Flex Temp Smart) inserted for 10 seconds into the vestibulum (Figure n° 25). The same thermometer was used in all vaginal measurements.

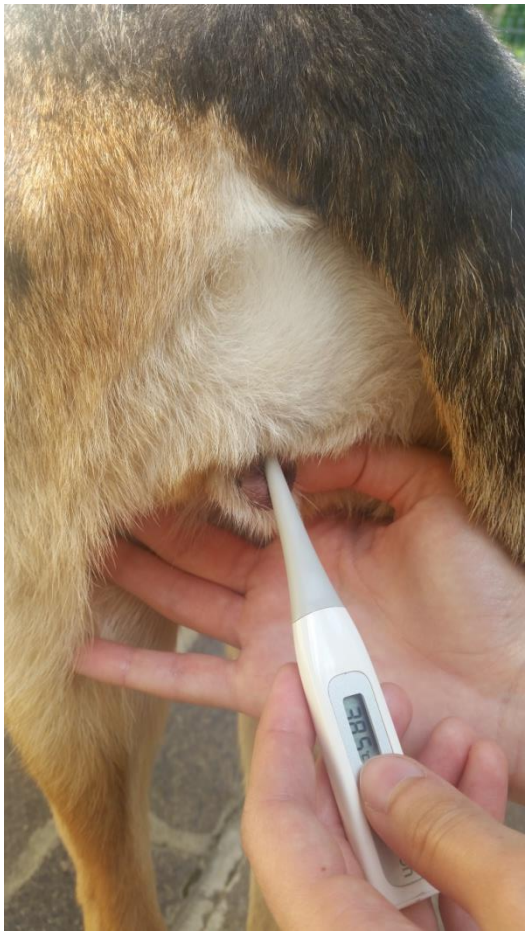


Figure n° 25. Measurement of vaginal temperature. A digital thermometer is inserted into the vestibulum with the left hand, while the vulvar lips are gently opened with the right fingers.

b) Rectal temperature

Rectal temperature was measured at the beginning of each visit with the same type of thermometer used previously. The thermometer was inserted two centimeters into the rectum (Figure n° 26) and kept there for 10 seconds.

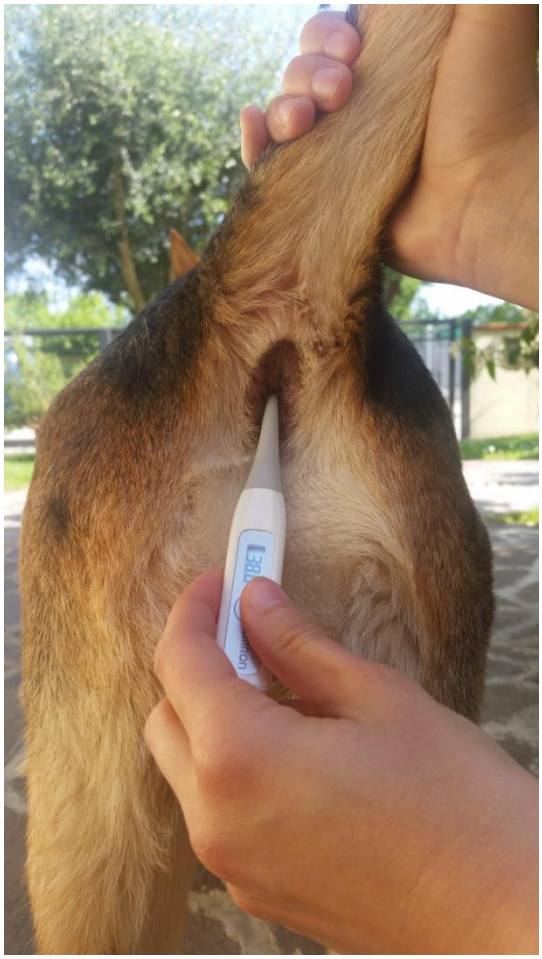


Figure n° 26. Measurement of rectal temperature. A digital thermometer is inserted into the rectum and kept there for at least 10 seconds.

c) Serum Progesterone concentration

Blood collection was performed from the cephalic vein using a 22 gauge needle connected to a 2.5 mL syringe (PIC® Solution), (Figure n° 27). In one bitch which was particularly stressed, the collection was done from the saphenous vein. A period of 10 minutes was allowed for blood clotting, the samples were centrifuged for 5 minutes at 1700 g. Then, the serum was gently transferred in one or more Eppendorf vials using a plastic disposable pipette, labelled and stored at -20°C in the freezer of the hospital laboratory (Figure n° 28).

Figure n° 27. Blood collection from the cephalic vein using a 23 gauge needle and a 2.5mL syringe. The area in which the needle was inserted was clipped when necessary, after which disinfection with alcohol was performed.

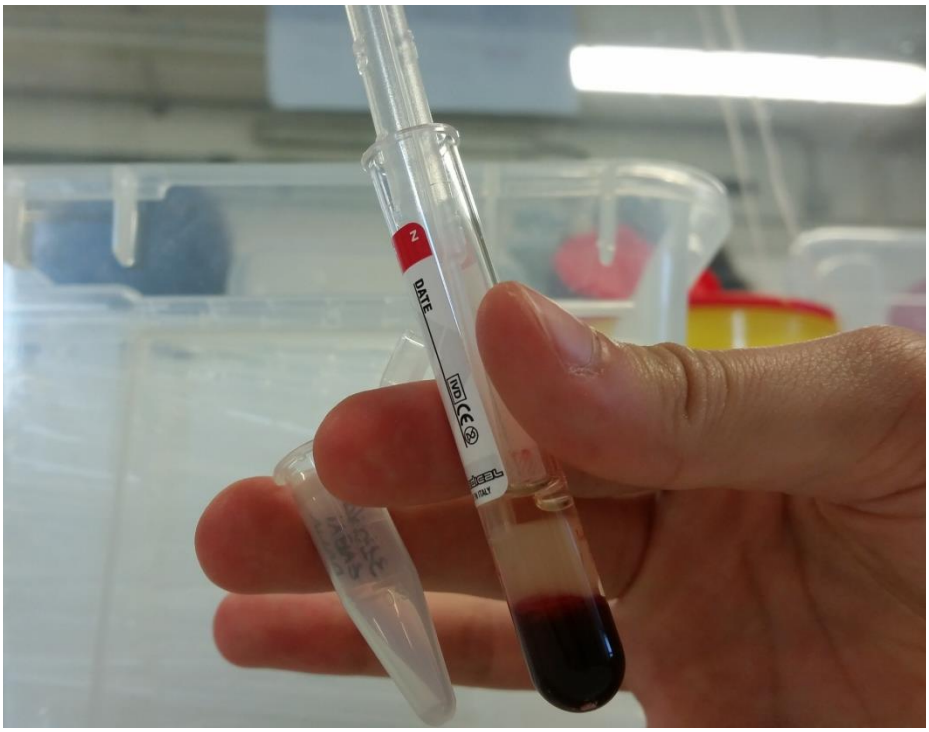


Figure n° 28. Following centrifugation and the complete separation of serum, the latter was gently transferred in one or more eppendorfs using a plastic micropipette.

Measurements of P4 and cortisol concentrations in all serum samples were performed as described below.

P4 was measured by chemiluminescence (IMMULITE 1000) using a commercial kit (IMMULITE®/IMMULITE 1000 Progesterone; Siemens Healthcare Diagnostics Products, USA). As reported, the IMMULITE® Progesterone is a solid phase, competitive immunoassay using chemiluminescent technology (CLIA) which is reported to have an inter-assay coefficient of 5.5% to 12.5%.

The solid phase (also called "bead") is coated with polyclonal rabbit anti-P4 antibody. The liquid phase is made of an alkaline phosphatase, that is bovine calf intestine, conjugated to P4. The sample and the reagent are incubated together with the solid coated phase for 30 minutes. During this time, P4 in the sample competes with the enzyme-conjugated P4 of the reagent for a limited number of antibody binding sites on the bead. After a centrifugal washes, unbound samples and enzyme-conjugated are removed. Finally, chemiluminescent substrate is added to the test. The signal generated is in proportion to the bound enzyme. The time to achieve the first result is 42 minutes. Hormonal assays were performed altogether in one assay at the end of the study. Values are reported in ng/mL.

d) Serum Cortisol concentration

The same serum aliquot used to measure P4 was used also to assay cortisol level. Measurements of all serum samples were performed in only one assay. The inter-assay coefficient is reported to be 6.7% to 8.8%. The procedure employed (IMMULITE®/IMMULITE 1000 Cortisol; Siemens Healthcare Diagnostics Products, USA) was a solid-phase, competitive chemiluminescent enzyme. Cortisol test units contains one bead coated with polyclonal rabbit anti-cortisol antibody, that is highly specific for cortisol.

At the beginning of each consultation, during the history collection, some questions about the past and recent medical history of the bitch were asked to the owner, including if she was taking drugs containing prednisone or prednisolone, in order to rule out falsely elevated cortisol level.

Results of serum cortisol assay are reported in µg/dL.

e) Fetal Heartbeats

Fetal heartbeat evaluation was performed with a 4-9 MHz transducer connected to a commercial ultrasound unit (Zonare, Zonare Medical Systems Inc®, Mountain View, California, USA). The bitch was placed in dorsal or lateral recumbency (Figure n° 29). A specific ultrasound gel (GIMA®, Blue Ultrasound Gel) was applied on the abdomen. A number of fetuses between 1 and 4 was evaluated in each bitch. At least 3 heartbeat measurements for each fetus were recorded.

Figure n° 29. In order to perform the ultrasound exam, bitches were positioned in dorsal or lateral recumbency. The abdomen was gently clipped at the first visit.

f) Fetal Gastrointestinal Motility

Fetal gastrointestinal motility exam was investigated with the same ultrasound equipment and gel used to record fetal heartbeats. The bitch was placed in dorso-ventral or lateral recumbency. One to 4 fetuses for each bitch were examined.

Fetal intestinal peristalsis was evaluated by observing bowel movements across time. Peristaltic waves can be recognized just by following a sinuous movements of bowel loops. At least 3 observations of gastrointestinal motility for each fetus were made, with images and 3 seconds videos recorded (Figure n° 30). Fetal intestinal peristalsis was scored 0 (no bowel movement was present) or 1 (intestine peristalsis was present).

Figure n° 30. An example of bowel visualization in a 58-day old Jack Russel fetus. Trying to keep this view, intestinal peristalsis was investigated and recorded in 3 seconds videos.

The following form was used in each visit to write down all the data mentioned above.

DATE:_____ DAY OF PREGNANCY:_____

Bitch name:
Owner name:
Breed:
Age:
Past and recent medical history:

Day of Ovulation:

CLINICAL PARAMETERS

Mucosa (conjunctival, oral and vaginal):
Lymphnodes (retropharyngeal, prescapular and popliteal):
Abdominal palpation:
Lung auscultation and respiration rate:
Heartbeats per minute:
Vaginal Temperature:
Rectal Temperature:

BLOOD SAMPLE

Haematology and biochemistry *:
Serum Progesterone assay:
Serum Cortisolassay:

X-RAY**

Litter Size:

ULTRASOUND ON DAY:

Fetal Gastrointestinal Motility	1st fetus:
	2nd fetus:
	3rd fetus:
Fetal Heartbeats per minute	1st fetus:
	2nd fetus:
	3rd fetus:

DELIVERY ON DAY:

Normal/C-section:

Litter Size:

Live and/or Stillborn pups:

*= only one time at the first visit; **=only one time from day 55 onwards

3.3 Statistical Analysis

The criteria that accomunate all the 20 bitches is that they have to be in good health. For this reason clinical parameters, a complete blood count and serum biochemistry were performed.

Spearman's Rho correlation was used to better understand the statistical realation between the parameters evaluated.

Statistical significance in vaginal and rectal temperature was investigated using a T-test.

4. RESULTS

Part of these data were presented at the 8th International Symposium on Canine and Feline Reproduction in Paris on 22-25 June 2016.

No physiological problem was reported during clinical examination at the beginning of each visit. All the bitches presented normal conjuntival, oral and vaginal mucosa; retropharyngeal, prescapular and popliteal lymphnodes were normal in shape and size; nothing to report at abdominal palpation and lung auscultation; respiratory rate and heartbeats per minute were within physiological range Results of the clinical examination of every bitch included in the study are presented in the Annex n°21. Blood count and serum biochemistry were performed in all the bitches with the exception of two of them, because the owners did not give their consent. These exams did not reveal any worrisome conditions, only a few parameters were not in physiological range.

As stated in Materials and Methods, parameters evaluated on each visits were:

a) Vaginal temperature
b) Rectal temperature
c) Serum progesterone concentration
d) Serum cortisol concentration
e) Fetal heartbeats
f) Fetal gastrointestinal motility.

a) Vaginal temperature

Vaginal temperature measurements were performed in all the 20 bitches at the beginning of each visit. Individual values are listed in the Annex n° 22.

Mean, Standard Deviation, Standard Error, Minimum and Maximum values are depicted in Table n° 9.

	D54	**D55**	**D56**	**D57**	**D58**	**D59**	**D60**	**D61**	**D62**	**D63**
Samples	N=2	N=2	N=2	N=8	N=7	N=11	N=6	N=8	N=5	N=2
Mean	38.25	38.05	38.15	37.94	38.11	37.72	37.83	37.73	37.26	36.9
Standard Deviation	0.07	0.07	0.21	0.34	0.37	0.29	0.39	0.50	0.42	0.57
Standard Error	0.05	0.05	0.15	0.12	0.14	0.09	0.16	0.18	0.19	0.4
Minimum	38.2	38	38	37.5	37.5	37.3	37.1	36.9	36.6	36.5
Maximum	38.3	38.1	38.3	38.6	38.7	38.1	38.2	38.3	37.7	37.3

Table n° 9. Vaginal temperatures data: mean, standard deviation, standard error, minimum and maximum values are assessed in Celsius degrees (°C). The day of gestation was corrected by counting backward from the day of whelping (D63).

A decrease in vaginal temperature from D54 to D63 was appreciable with a drop from D61 to D62 that averages 0.5°C. Vaginal temperature was strongly and positively correlated with rectal temperature and fetal heartbeats ($p<0.01$), while only a weak positive and negative correlations were found with serum P4 concentration ($p<0.05$) and with serum cortisol concentration ($p<0.05$), respectively (Table n° 17).

The decreasing trend of vaginal temperatures with Standard Error bars is displayed in Figure n° 31.

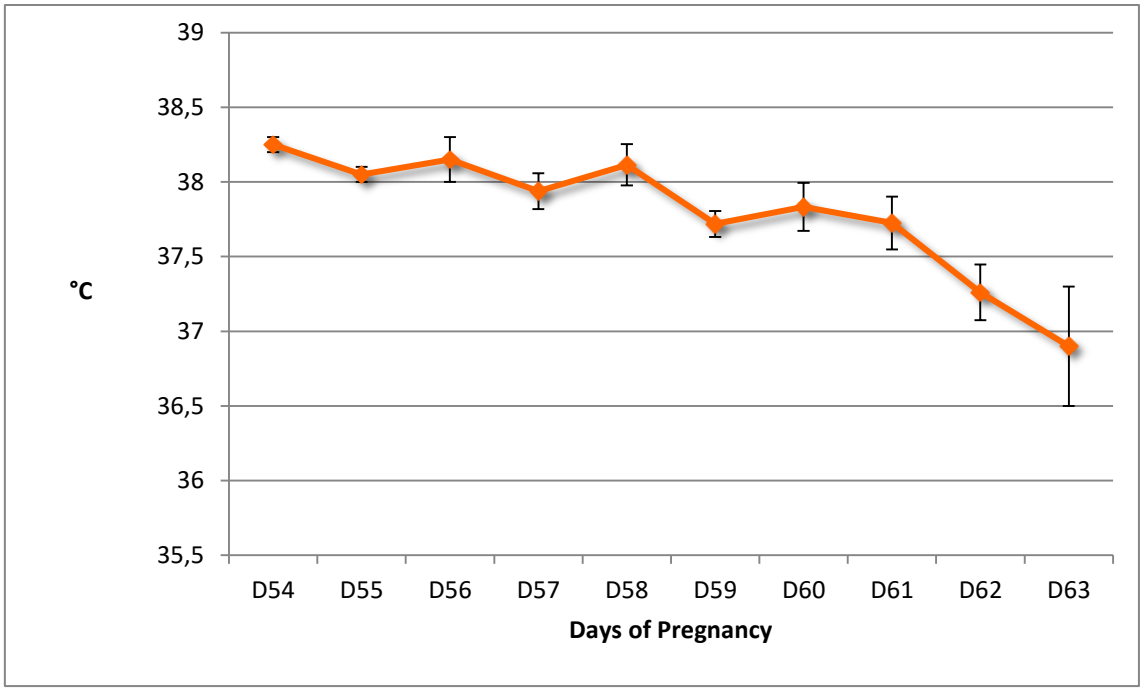


Figure n° 31. Vaginal temperature trend in 20 healthy bitches in the last 10 days of pregnancy. Results are in Celsius degrees (°C) ± SEM.

b) Rectal temperature

Rectal temperature measurements were performed in all the 20 bitches at the beginning of each consultation. Individual values are listed in the Annex n° 23.

Mean, Standard Deviation, Standard Error, Minimum and Maximum values are depicted in the Table n° 10.

	D54	**D55**	**D56**	**D57**	**D58**	**D59**	**D60**	**D61**	**D62**	**D63**
Samples	N=2	N=2	N=2	N=8	N=7	N=11	N=6	N=8	N=5	N=2
Mean	38.5	38.2	38.2	38.09	38.21	38.02	37.77	37.99	37.4	37.35
Standard Deviation	0	0	0	0.33	0.27	0.25	0.45	0.27	0.39	0.5
Standard Error	0	0	0	0.12	0.10	0.08	0.18	0.1	0.18	0.35
Minimum	38.5	38.2	38.2	37.7	38	37.7	37.2	37.6	36.8	37
Maximum	38.5	38.2	38.2	38.7	38.8	38.5	38.5	38.3	37.7	37.7

Table n° 10. Rectal temperatures data: mean, standard deviation, standard error, minimum and maximum values are assessed in Celsius degrees (°C). The day of gestation was corrected by counting backward from the day of whelping (D63).

A decline in rectal temperature was remarkable from D54 to D63 with a drop between D61 to D62 which averages 0.6°C. Rectal temperature was strongly and positively correlated with vaginal temperature and with fetal heartbeats ($p<0.01$), while no correlation was found with serum P4 and serum cortisol concentration (Table n° 17).

The decrease trend of rectal temperatures with Standard Error bars is displayed in Figure n° 32.

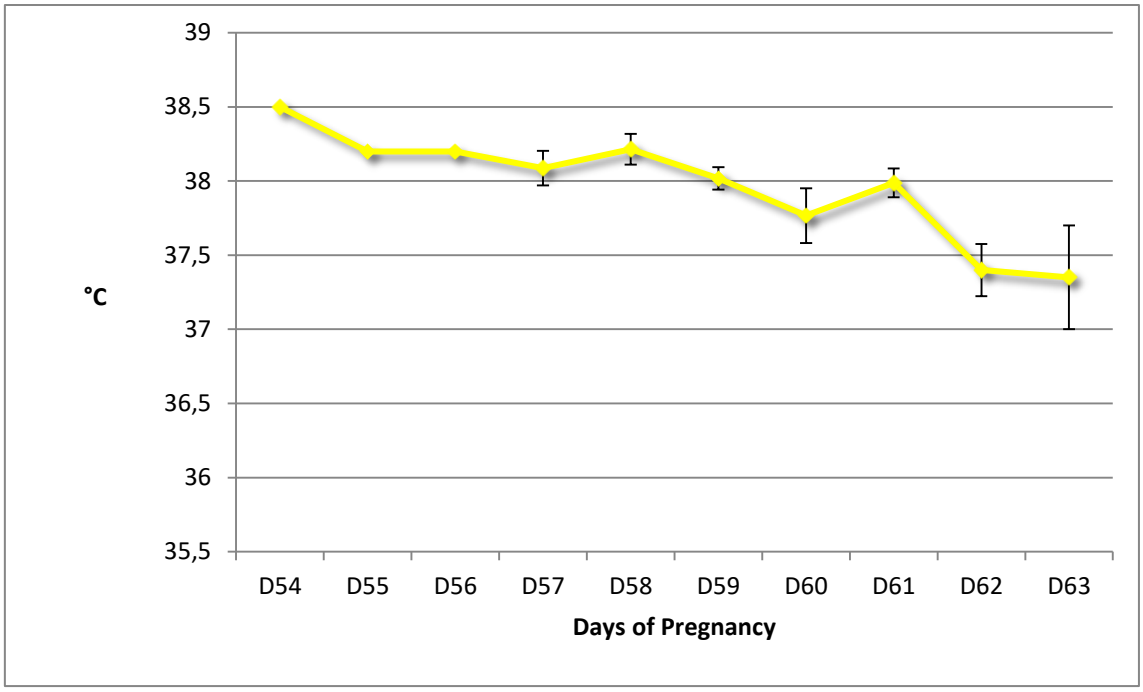


Figure n° 32. Rectal temperature trend in 20 healthy bitches in the last 10 days of pregnancy. Results are in Celsius degrees (°C) ± SEM.

Comparing rectal and vaginal temperature, the former presents higher values than the latter through the last ten days of pregnancy. Only on D60 the mean vaginal temperature is higher than the mean rectal one. As Figure n° 33 shows, both parameters decrease suddenly by day 61 to the parturition day. These two parameters were strongly and positively correlated ($p<0.01$), (Table n° 17).

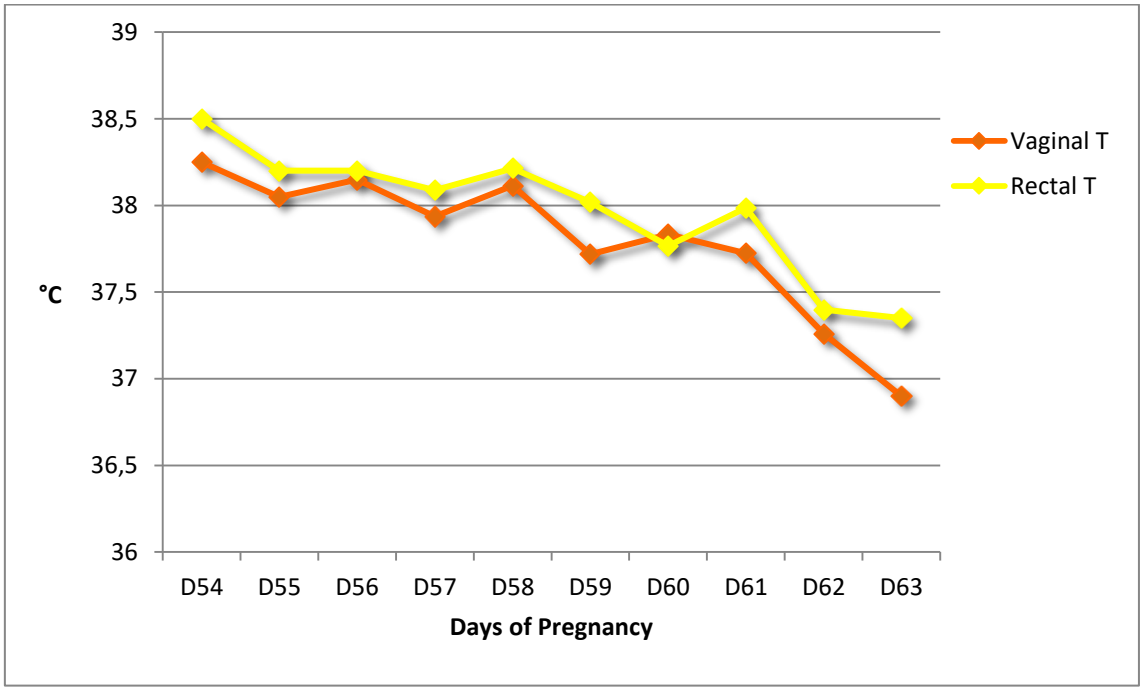


Figure n° 33. Comparison between rectal and vaginal temperature in the last 10 days of pregnancy performed in 20 healthy bitches. The two temperatures decline in parallel. Results are stated in Celsius degrees (°C).

The relevant difference in vaginal and rectal temperature in the last 24 hours prior to parturition was investigated through a T-test statistical analysis. The comparison between mean, standard deviation and standard error values between D54-D61 and D62-D63 for both temperatures are displayed in Table n° 11 and n° 12 below.

	Vaginal temperature		
Days	D54-D61	D62-D63	P <0.001
Samples	N=46	N=7	
Mean	37.89	37.16	
Mean Difference Value	0.73		
Standard Deviation	0.38	0.45	
Standard Error	0.06	0.17	

Table n° 11. Mean of vaginal temperatures in Celsius degrees (°C), mean difference, standard deviation and standard error of the mean between D54-D61 and D62-D63. The day of gestation was corrected by counting backward from the day of whelping (D63).

Table n° 12. Mean of rectal temperatures in Celsius degrees (°C), mean difference, standard deviation and standard error of the mean between D54-D61 and D62-D63. The day of gestation was corrected by counting backward from the day of whelping (D63).

	Rectal temperature		
Days	D54-D61	D62-D63	P <0.001
Samples	N=46	N=7	
Mean	38.06	37.39	
Mean Difference Value	0.67		
Standard Deviation	0.32	0.38	
Standard Error	0.05	0.14	

The T-test shows a statistically significant difference of the vaginal temperature between D54-D61 and D62-D63 (Mean: 0.73°C; $p<0.001$), as well as a statistically significant difference of the rectal temperature between D54-D61 and D62-D63 (Mean: 0.67°C; $p<0.001$).

c) Serum Progesterone concentration

Blood samples were collected in 18 bitches. Bitch n° 11 was supplemented from D27 to D60 of pregnancy with natural P4 (Prontogest®) due to hypoluteodism suspicions. For this reason, this bitch was excluded from serum P4 statistical analysis, in order to avoid false results. Individual values are listed in the Annex n° 24. Blood collection was not performed in Bitch n° 9 because the owner did not give his permission.

Mean, Standard Deviation, Standard Error, Minimum and Maximum values are depicted in Table n° 13.

	D54	**D55**	**D56**	**D57**	**D58**	**D59**	**D60**	**D61**	**D62**	**D63**
Samples	N=1	N=1	N=2	N=7	N=6	N=9	N=5	N=6	N=4	N=2
Mean	3.7	4.5	6.25	5.46	5.83	4.78	4.02	4.17	2.51	0.98
Standard Deviation	0	0	2.33	1.28	1.02	1.27	1.08	1.2	2.45	0.03
Standard Error	0	0	1.65	0.48	0.42	0.42	0.49	0.82	1.22	0.02
Minimum	3.7	4.5	4.6	3.5	4.1	3.4	3.2	2.4	0.95	0.96
Maximum	3.7	4.5	7.9	7.2	6.8	7.4	5.9	6.6	6.1	1

Table n° 12. Serum P4 data: mean, standard deviation, standard error, minimum and maximum values are assessed in ng/mL. The day of gestation was corrected by counting backward from the day of whelping (D63).

A decrease in serum P4 concentration is noticed at D63. The main fall in P4 values is from D61 to D62: a drop which averages 1.66 ng/mL in 24 hours was recorded. However, the high standard deviation recorded on D62 of pregnancy confuse this result. Another appreciable decrease of P4 was detected to be 1.53 ng/mL from D62 to D63.

Serum P4 concentration presents only a weak positive correlation with vaginal temperature ($p<0.05$), (Table n° 17).

Serum P4 assay decreasing trend with Standard Error bars is displayed in Figure n° 34.

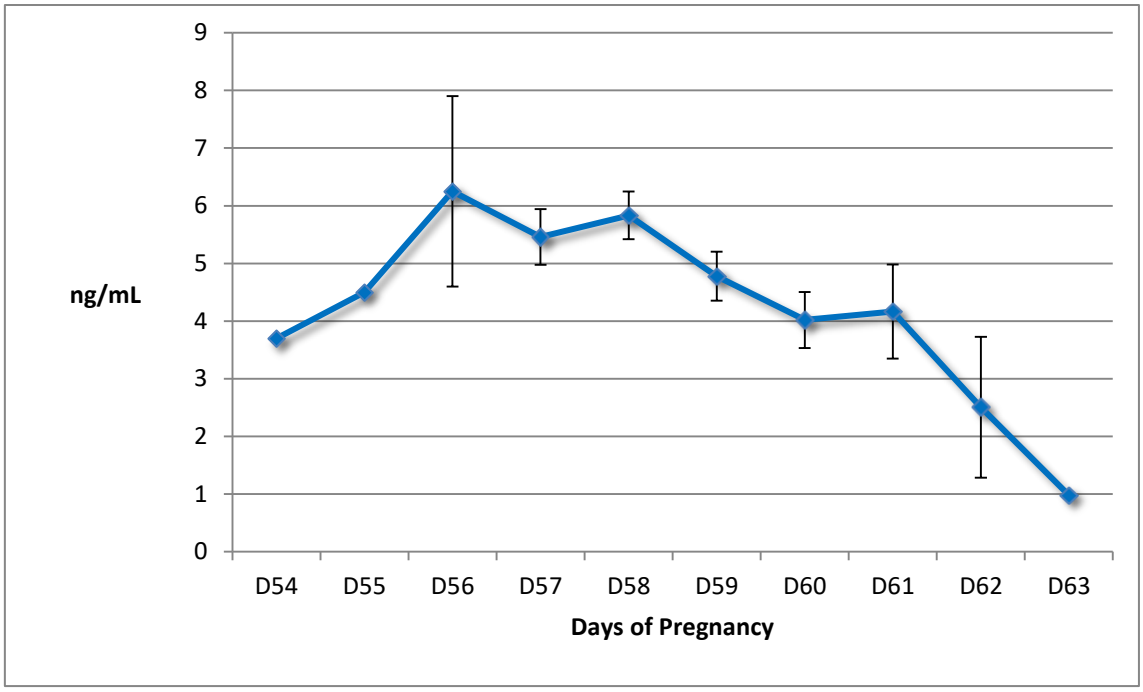


Figure n° 34. Serum P4 assay trend in 18 healthy bitches in the last 10 days of pregnancy. Results are in ng/mL ± SEM.

d) Serum Cortisol concentration

Blood samples were collected in 19 bitches. From the same serum samples used to evaluate serum P4 concentration, also maternal cortisol level was assessed. Individual values are listed in the Annex n° 25.

Mean, Standard Deviation, Standard Error, Minimum and Maximum values are depicted in Table n° 14.

	D54	**D55**	**D56**	**D57**	**D58**	**D59**	**D60**	**D61**	**D62**	**D63**
Samples	N=2	N=1	N=2	N=8	N=7	N=9	N=5	N=7	N=5	N=2
Mean	1.2	3.8	3	2.51	3.63	3.26	3.06	2.89	5.64	4.1
Standard Deviation	0.42	0	1.7	0.81	1.87	1.30	1.53	1.15	1.68	0
Standard Error	0.3	0	1.2	0.29	0.71	0.43	0.68	0.44	0.75	0
Minimum	0.9	3.8	1.8	1.5	0.9	2.3	1.5	1.7	3.5	4.1
Maximum	1.5	3.8	4.2	3.7	5.5	5.9	4.7	4.5	8.1	4.1

Table n° 13. Serum cortisol assay data: mean, standard deviation, standard error, minimum and maximum values are assessed in µg/dL. The day of gestation was corrected by counting backward from the day of whelping (D63).

A rise in serum cortisol level was observed from D54 to D63. A rapid increase in serum cortisol concentration could be observed from D61 to D62, being in average 2.75 µg/dL. In Bitch n° 15 cortisol concentration on D62 was of 8.1 µg/dL. However in the same day, Bitch n° 10 for example, had a serum cortisol concentration of 3.5 µg/dL.

Serum cortisol concentration had a weak negative correlation with vaginal temperature ($p<0.05$).

No correlation was observed with serum P4 (Table n° 17).

Serum cortisol assay trend with Standard Error bars is displayed in Figure n° 35.

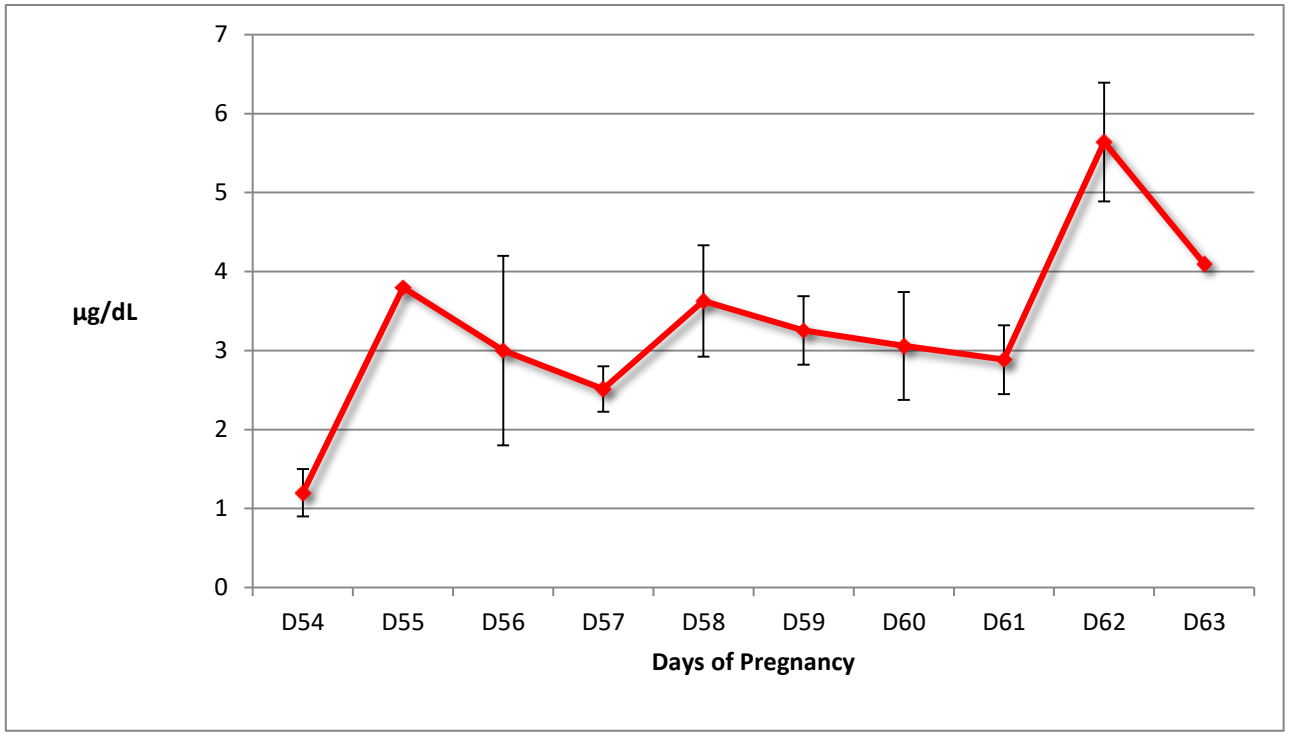


Figure n° 35. Serum cortisol assay trend in 19 healthy bitches in the last 10 days of pregnancy. Results are stated in µg/dL ± SEM.

e) Fetal Heartbeats

Fetal heartbeats were measured in at least 1 fetuses for a maximum of 3 fetuses for each bitch.
At least 3 or more measurements for each fetus were recorded using doppler ultrasound.
Individual values are listed in the Annex n° 26.
Mean, Standard Deviation, Standard Error, Minimum and Maximum values are depicted in Table n° 15.

	D54	**D55**	**D56**	**D57**	**D58**	**D59**	**D60**	**D61**	**D62**	**D63**
Samples	N=6	N=6	N=6	N=22	N=19	N=27	N=13	N=21	N=13	N=8
Mean	226.5	214.5	230	224.75	219.57	219.73	216.83	212.63	204.8	216.33
Standard Deviation	19.09	17.68	9.9	11.89	21.26	11.79	13.64	14.17	15.17	3.79
Standard Error	13.5	12.5	7	4.20	8.04	3.55	5.57	5.01	6.79	2.19
Minimum	213	202	223	207	193	202	200	190	183	212
Maximum	240	227	237	238	256	241	233	240	221	219

Table n° 14. Fetal heartbeats mean, standard deviation, standard error, minimum and maximum values are assessed in beats per minute (bpm). The day of gestation was corrected by counting backward from the day of whelping (D63). Samples indicated with "N" regard the number of fetuses that have been examined on that day.

A slight decrease in fetal heartbeat was noticed from D54 to D63 of pregnancy, (Figure n° 36). As a matter of fact, fetal heartbeats average 223.9 bpm from D54 to D57 and average 215 bpm from D58 to D63. This decrease varies among bitches and presents large standard deviation intervals. For example in the Bitch n° 17 on D59 the average fetal heartbeats recorded was 211 bpm, and this value remained almost constant on D61 (216 bpm) and on D63, being 219 bpm (Figure n° 37).
This parameter was positively correlated with vaginal and rectal temperature ($p<0.01$),
(Table n° 17).

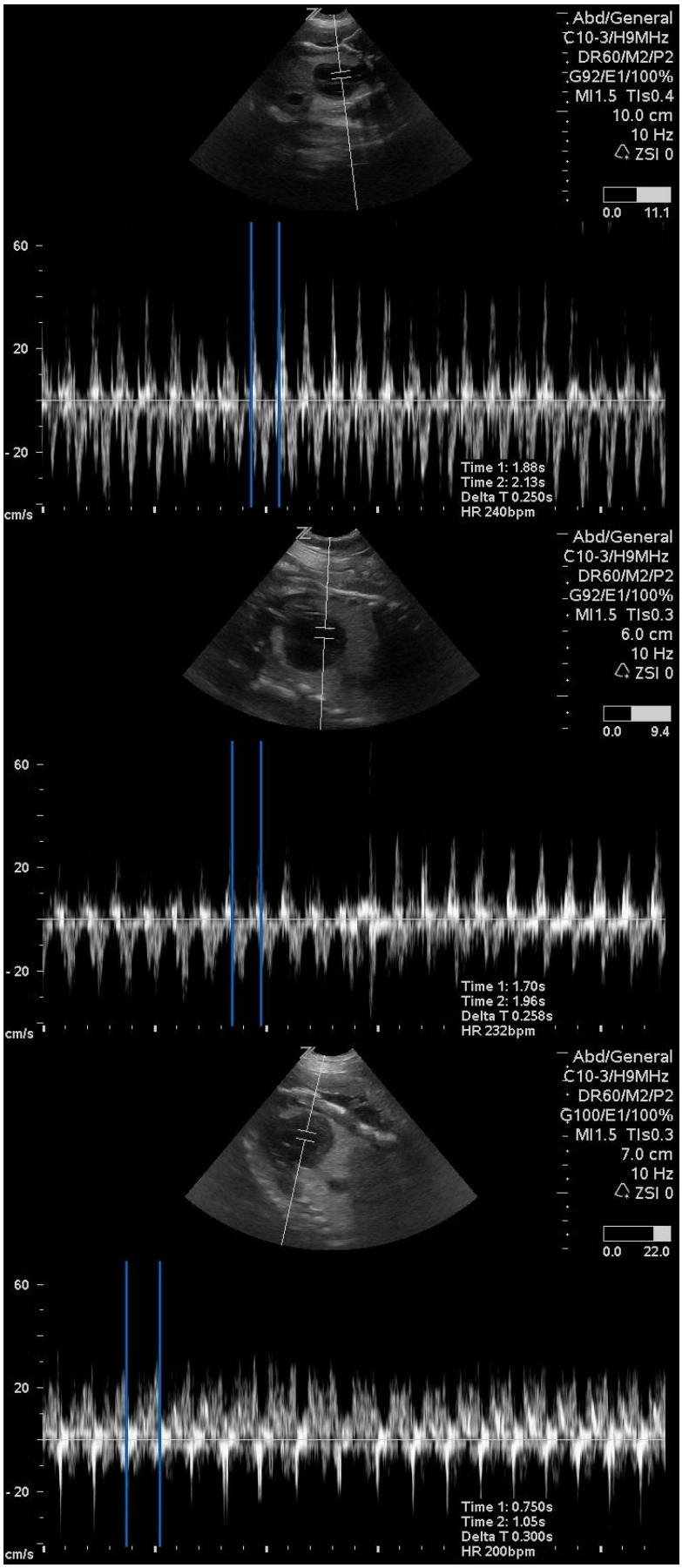


Figure n° 36. Doppler ultrasound image reporting the appreciable decrease in fetal heartbeat (HR, bottom right) in a 57(A), 58(B) and 60(C)-day old fetus of a 3-year old Basset Hound bitch.

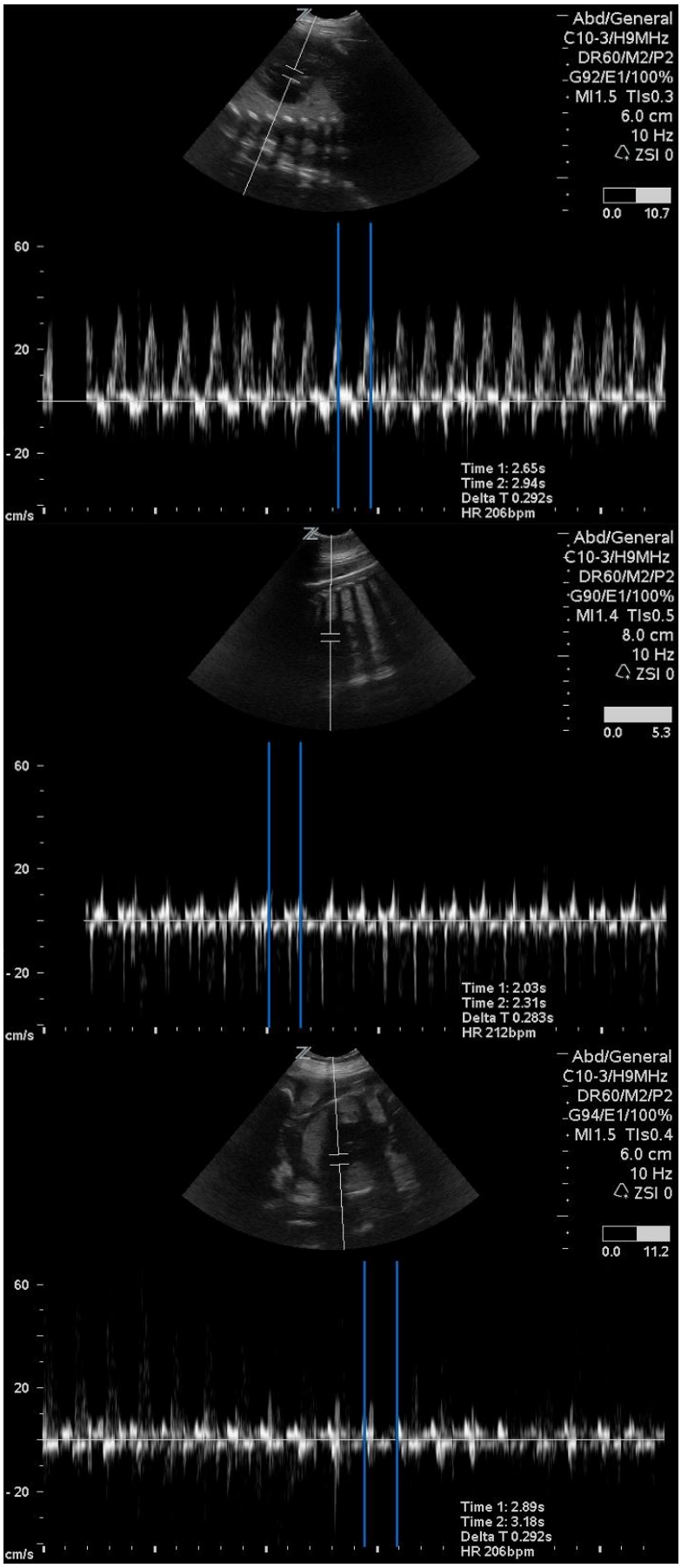


Figure n° 37. Doppler ultrasound image reporting the slight decrease in fetal heartbeat (HR, bottom right) in a 59(A), 61(B) and 63(C)-day old fetus of a 2-year old Samoyed bitch.

Fetal heartbeats trend is displayed in Figure n° 38. The graphic reveals a slight decrease approaching the parturition day.

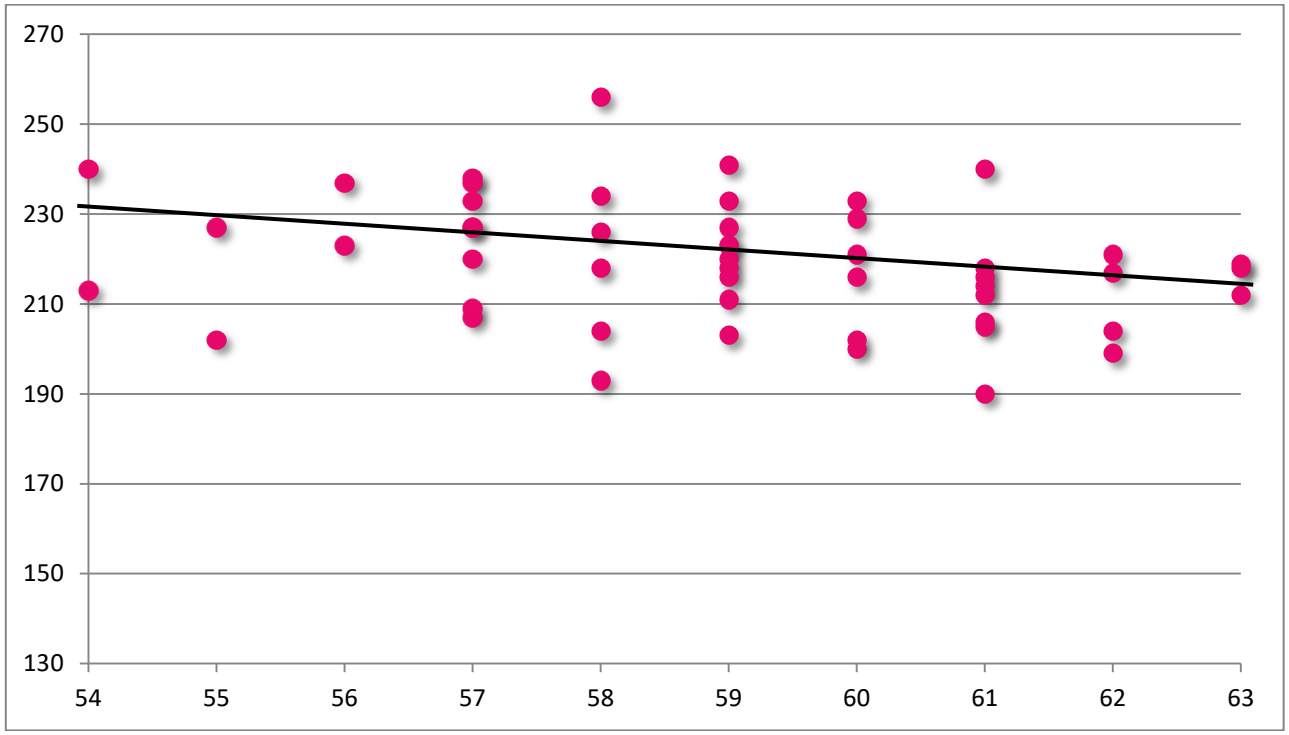


Figure n° 38. Fetal heartbeats mean among fetuses examined in each bitch on each consultation day. The evaluation comprehends 141 fetuses belonging to 20 healthy bitches in the last 10 days of pregnancy. Results are displayed in beats per minute (bpm).

f) Fetal Gastrointestinal Motility

Fetal gastrointestinal motility was measured in at least 1 fetuses for a maximum of 4 fetuses in each bitch on each consultation day. At least 3 or more evaluation of intestinal bowel movement for each fetus were performed. Three seconds videos were recorded in order to catch fetal intestinal peristalsis. Individual values are listed in the Annex n° 27.

Mean, Standard Deviation, Standard Error, Minimum and Maximum values are stated in the Table n° 16 below.

	D54	**D55**	**D56**	**D57**	**D58**	**D59**	**D60**	**D61**	**D62**	**D63**
Samples	N=6	N=6	N=6	N=23	N=21	N=28	N=17	N=22	N=12	N=6
Mean	0	0	16.67	18.75	27.38	66.67	66.66	64.58	63.33	66.67
Standard Deviation	0	0	23.57	20.77	22.93	32.49	36.51	40.28	41.5	47.14
Standard Error	0	0	16.67	7.34	8.67	9.8	14.91	14.24	18.56	33.34
Minimum	0	0	0	0	0	0	0	0	0	33.3
Maximum	0	0	33.3	50.0	66.7	100	100	100	100	100

Table n° 15. Fetal intestinal peristalsis data: mean, standard deviation, standard error, minimum and maximum measurements are reported above in percentage. The day of gestation was corrected by counting backward from the day of whelping (D63). Samples indicated with "N" regard the number of fetuses that have been examined on that day.

In days D54 and D55, no peristaltic movements could be recorded in none of fetuses examined, while from D56 to D58 less than 27.38% of the fetuses showed intestinal motility. In the last 5 days of pregnancy (D59 to D63), more than 63.33% of the fetuses on each day of visit showed gastrointestinal motility reaching values of 66.67%. Bitch n° 13 did not show any peristaltic movements on D59, so did Bitch n° 8 on D60 and D62 and Bitch n° 16 on D61. Fetal gastrointestinal motility statistical analysis shows a negative and significant correlation with vaginal and rectal temperature ($p<0.05$, Table n°17). However, nocorrelation was observed with fetal heartbeats, serum P4 and cortisol concentration, (Table n° 17).

Figure n° 39 shows an example of ultrasound video recorded in our study, showing fetal peristaltic movement.

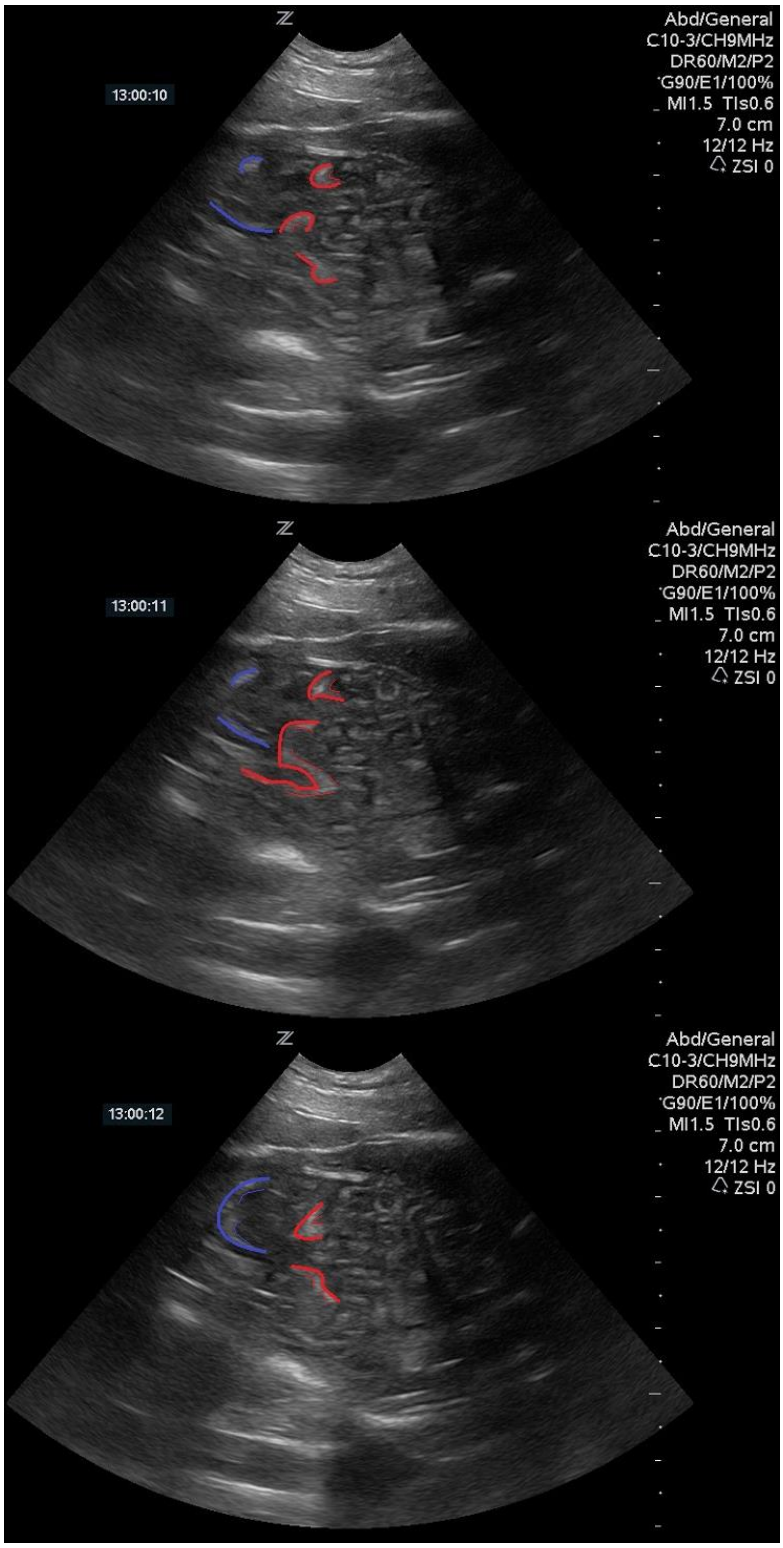


Figure n° 39. Ultrasound image displaying fetal bowel movements in a 60-day old fetus of a 3-year old Basset Hound bitch. Peristalsis in the same bowel loop is highlighted in red in the images above, taken at 1 second interval from each other. Adapted from Artusi et al., Proceedings 8th International Symposium on Canine and Feline Reproduction in Paris, p. 11, 2016

Inserting the data obtained in a histogram graphic, the trend observed was the following (Figure n° 40).

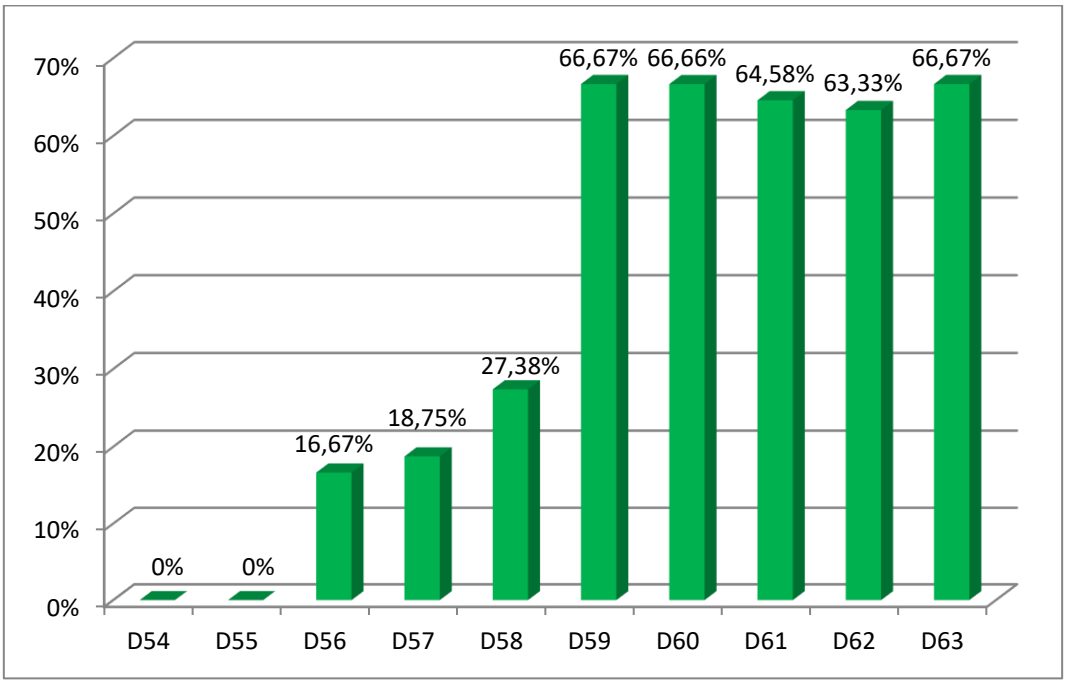


Figure n° 40. Fetal gastrointestinal motility observed in 147 fetuses of 20 healthy bitches in the last 10 days of pregnancy on each consultation day.

However, taking in consideration the number of bitches with the detection of at least 1 fetus with motile intestine on each consultation day during the last 10 days of pregnancy, Figure n° 41 could be obtained.

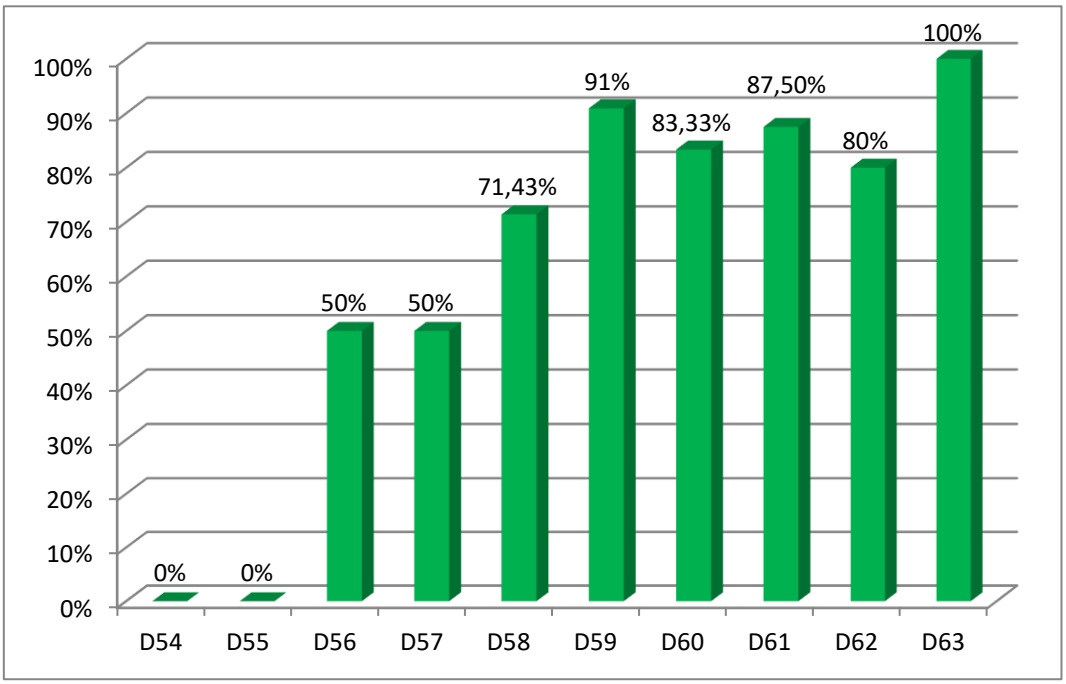


Figure n° 41. Fetal gastrointestinal motility recorded in at least 1 fetus in 20 healthy bitches in the last 10 days of pregnancy.

4.1 Statistical Analysis

In order to better understand how all these parameters can be used to help clinicians in predicting the parturition day, the relation among them was investigated through a Spearman's Rho correlation, as Table n° 17 below shows.

Fetal statistical results, such as fetal gastrointestinal motility and fetal heartbeats, were obtained by the examination of different fetuses on each consultation day, due to the fact that they move inside the uterus resulting in a difficulty in analyzing the same fetuses. This is important for the statistical meaning of this study, due to the casuality among the fetuses examined.

		Fetal bpm	Fetal GIM %	Vaginal T	Rectal T	P4	Cortisol
Fetal Heartbeat	Correlation coefficient	1.000	-.121	.428**	.427**	.220	-.313*
	Significance		.390	.001	.001	.156	.030
	N	54	53	53	53	43	48
Fetal GIM %	Correlation coefficient	-.121	1.000	-.294*	-.271*	-.243	.234
	Significance	.390		.032	.050	.116	.109
	N	53	53	53	53	43	48
Vaginal T	Correlation coefficient	.428**	-.294*	1.000	.773**	.352*	-.337*
	Significance	.001	.032		.000	.021	.019
	N	53	53	53	53	43	48
Rectal T	Correlation coefficient	.427**	-.271*	.773**	1.000	.241	-.213
	Significance	.001	.050	.000		.119	.147
	N	53	53	53	53	43	48
P4	Correlation coefficient	.220	-.243	.352*	.241	1.000	-.042
	Significance	.156	.116	.021	.119		.789
	N	43	43	43	43	43	43
Cortisol	Correlation coefficient	-.313*	.234	-.337*	-.213	-.042	1.000
	Significance	.030	.109	.019	.147	.789	
	N	48	48	48	48	43	48

Table n° 16. Results obtained using a Spearman's Rho correlation. Fetal heartbeats (bpm), fetal gastrointestinal motility (GIM), vaginal temperature (T), rectal temperature, serum progesterone (P4) concentration and serum cortisol concentration were evaluated. Positive, negative or no correlation were found between them.
**Significance level $p<0.01$. *Significance level $p<0.05$.

5. DISCUSSION

5.1 Limits of the study

Before starting the discussion of the results obtained, it is important to underline what kind of limits this study had to deal with.

1. The owners were not so confident in bringing for several times their bitch in the last week of pregnancy, because they were worried about the stressful events in which their dogs would have to be submitted. One of the owners did not allow to perform blood collection in all the consultations and another one asked us to avoid blood collection once. Blood collection was the most stressful event according to the owners;
2. Several owners asked us to perform the ultrasonography rapidly because they were concerned about stressing their pregnant bitch. For these reasons, the observation of fetal intestinal peristalsis, which requires time to catch the better videos, was sometimes hindered, and so shortened;
3. Most of the owners did not agree in bringing their bitch on D62 or D63 of pregnancy and this leads to a low number of data in these two days;
4. Recording fetal gastrointestinal motility was difficult in some cases. In fact, in order to identify peristaltic movements, the bitch has to lay in dorsal or lateral recumbency completely still for a few minutes. As parturition approaches, the bitches were more stressed and manipulations were more difficult than in the first days. For example, in Spring and Summer seasons it was very difficult to perform ultrasound, because of the bitches' panting;
5. Investigating ultrasonographically fetal gastrointestinal motility was also difficult due to the movements of the fetuses themselves, which interfered with the correct visualization of the fetal bowel.

5.2 Parameters of impending parturition

Rectal and vaginal temperature

The results of our study show a significant decrease in the last 24 hours of vaginal temperature, as well as of the rectal temperature. The mean difference measured was more than 0.5 °C for both type of measurement, being the vaginal drop of temperature more sharp than the rectal one, (0.73°C and 0.67°C, respectively). A strong and positive correlation between vaginal and rectal temperature was found in our study.

Even if rectal temperature does not show any correlation with serum P4 concentration, as already Veronesi et al. (2002) affirmed, vaginal temperature shows a weak positive correlation with serum P4 concentration and this might be explained by the physiological thermogenic effect of serum P4. This suggests that the decrease in vaginal temperature is not connected with the initiation of parturition, but rather with a decline in serum P4 concentration.
Rectal temperature measurement can be a reliable way to indicate that parturition is approaching. Our results disagree with Veronesi et al. (2002) who did not record any significant decrease in temperature in the last 48 hours of pregnancy. The mean rectal temperature measured in our study shows a gradual decline during the last 10 days of pregnancy, especially 48 to 24 hours prior to parturition.

According to literature, the evaluation of vaginal temperature reveals that its decline parallels the one of the rectal temperature (Maeder et al., 2012). Maeder et al. (2012) performed this measurement using vaginal loggers. Inflammation spots and recurrent loss of the logger were observed. Our study was performed with a simple digital thermomether and obtained the same results of vaginal loggers. Thus, no significant difference in term of accuracy can be found when measuring vaginal temperature with a digital thermometer, which seems to be as reliable as vaginal loggers.

Even if rectal temperature is widely recognized as a useful method to predict parturition onset, vaginal temperature seems to be a very interesting parameter to investigate better in the future, because it might be more useful than the rectal temperature to predict the parturition time.

Serum progesterone concentration

A gradual decrease in serum progesterone concentration during the last 10 days of pregnancy can be observed, together with a noticeable fall in its value 48 hours prior to parturition. As a matter of fact, serum P4 concentration drops from average values of 4.17±1.2 ng/mL on D61 to 2.51±2.45 ng/mL on D62 to 0.98±0.03 ng/mL on the day of parturition, being in agreement with previous literature which stated that the P4 concentration from 40 to 12 hours prior to the expulsion of the first pup is <1.5 ng/mL (Concannon et al., 1977). The large standard deviation on D62 is due to Bitch n° 10 which still presented a very high level of serum P4 on D62 (6.1 ng/mL). However her serum P4 concentration dropped roughly on D63 to 1 ng/mL.

On D54 and D55 a low P4 value was recorded comparing with other days. However these results were achieved through a single serum sample on each day, so they cannot be considered as reliable as the others, which were all obtained by 2 or more samples.

Serum cortisol concentration

A remarkable increase in serum cortisol level was observed approaching the parturition day. Serum cortisol values increase from 2.89±1.15 µg/dL to 5.64±1.68 µg/dL in the last 48 hours prepartum. Despite the paucity of our data on D63, our study agrees with previous results reported in literature (Concannon et al., 1978; Veronesi et al., 2002).

In Bitch n° 15 this values achieved a concentration of 8.1µg/dL 24 hour prior to parturition, but this increase is not similar among bitches. As a matter of fact, 24 hour prior to parturition, Bitch n° 10 presented a serum cortisol concentration of 3.5 µg/dL, quite similar to the values reported in other bitches in previous days. This suggest that measuring serum cortisol concentration cannot be as reliable as serum progesterone in predicting the parturition day due to the individual difference among the bitches.

As mentioned before, it is very important to know patients who has been treated with prednisone or prednisolone in order to avoid falsely elevated cortisol level. In fact, although some steroids show cross-reactivity, their normal physiological concentrations are low compared to cortisol, so they wouldn't interfere significantly with the IMMULITE® Cortisol procedure. However, it was observed a cross-reactivity of nearly 49% with prednisolone: that's why a false increase in cortisol must be taken in consideration, because it might be present in bitches treated with these drugs.

Fetal heartbeats

Literature reports that from D58 post ovulation until term, a reduction in fetal heartbeat is noticed, going from values of 238.2±16.1 bpm to 218±6.7 bpm (Verstegen et al., 1993). Our study reveals the same results. However in the Bitch n°17 the average fetal heartbeat recorded on D59 remained almost constant to D63. This suggests that the decrease in fetal heartbeats approaching parturition is individual and thus not appreciable in all the bitches.

There was a strong, positive correlation between fetal heartbeats and vaginal and rectal temperature. Such a correlation has not been described before and it is not easy to explain; the two events could just happen at the same time and be unrelated to each other.

5.3 Practical use of fetal gastrointestinal motility

Fetal intestine appearance during serial fetal ultrasonography indicates the end of fetal organogenesis. Gil et al. (2015) stated that fetal bowel can be seen for the first time around D39 to D44 of pregnancy and that the first peristalsis is detectable from D48 to D54. Our results are in disagreement with this study, because on D54-D55 of pregnancy no peristaltic movements were recorded in none of the examined fetuses. Only on D56 the first fetal bowel movements were detected. Furthermore, the same authors stated that from D57 to D62 fetal intestinal peristalsis is visible immediately, suggesting an intestinal movement increasing nearby the day of whelping. This study supports only in part this affirmation. Ultrasound examination of fetal bowel from D56 to D58 of pregnancy showed slow peristalsis movements, so that it made it difficult to record them: in these days, only a continuous observation for several minutes can be able to catch fetal bowel movements. From D59 to D63 the peristaltic waves can be easily observed immediately or after an observation of a few seconds in most of the fetuses. This easier visualization might be due to the increase in fetal bowel maturation approaching the day of parturition.

However, it has to be underlined that this parameter is hindered by the limits of the study described above. Not all the fetuses showed peristaltic movements from D59 onwards and in 4 bitches none of the fetuses presented fetal intestinal movements. This can be explained by the fact that fetal gastrointestinal motility is not constant and may differ among the intestinal tract as stated by Gil et al. (2015). Also on D62 when the fetus is at term and fully mature, some part of the intestine may not show any peristaltic movements, as happened in Bitch n° 8. Furthermore, the visualization of fetal bowel movements requires that the bitch remains completely still, and sometimes it is quite

difficult. Client compliance is another weak point of our research due to owner's apprehension and/or because of the first stage of labour starting. For instance this caused paucity of data on D63 (only two bitches were analyzed on this day) which made our statistical analysis weaker on this time frame. This also can explain the large standard deviation interval reported.

From our results, we might say that 0% of the bitches presents at least 1 fetus with motile intestine on D54-D55, while 50% of them presented this finding on D56-D57. The 100% was reached only on D63, the day of whelping, but the paucity of our data in this day make this last finding not so reliable.

On the other hand, it might be stated that when fetal intestinal peristalsis is seen in at least 1 fetus, the bitch is between D56 to D63, so in the last week of pregnancy. Analyzing the frequency with which the 147 fetuses examined displayed peristaltic movements, more than 60% of them showed gastrointestinal motility from D59 of pregnancy onwards, so the bitch is supposed to be in the last 5 days of pregnancy. In the same way, when less than 30% of the fetuses displayed fetal bowel movements, the bitch might be on D56 to D58 of her pregnancy. When no peristaltic movement is recorded, it cannot be affirmed surely that she is not going to whelp prior to 7 days, because our study recorded cases in which on D59, D60, D61 and even on D62 none of the fetuses examined in different bitches presented a clear gastrointestinal motility. For this large presence of false negative results, fetal gastrointestinal motility seems to be a parameter with high specificity and low sensitivity.

The percentage of fetuses diplaying intestinal peristalsis, remains almost the same from D59 to D63 (from a minimum of 63% to a maximum of 67%). If the veterinarian relies only on this parameter to estimate the fetal age, he might be misled, thinking that the bitch is ready to whelp, when she is not. Gil et al. (2015) stated that the survivor percentage is 100% only when the bitch delivers naturally or by C-section on D62 and D63. Minor survivor percentage is recorded in the previous days. For this reason, the observation of 60% of fetuses with peristaltic movements may not be enough to predict that the fetus is fully mature and ready to be delivered. Thus, it would be important to associate fetal gastrointestinal motility to other parameters of impending parturition.

Unfortunately, fetal gastrointestinal motility statistical analysis reports that it had poor correlations with other parameter of impending parturiton. As a matter of fact, it presents no correlations at all with fetal heartbeats, serum P4 and cortisol concentration. However a weak negative correlation with vaginal and rectal temperature could be found, suggesting that they are both influenced by the approaching of delivery date. Since fetal gastrointestinal motility cannot discriminate the day of

pregnancy from D59 onwards, vaginal and rectal temperature might be helpful due to the fact that they both decrease 24 hours prior to parturition. Thus, finding fetal bowel movements in at least 60% of the fetuses and appreciate at the same time that vaginal and rectal temperature are decreasing with a mean value of 0.73°C and 0.67°C might help the clinicians in improving his prediction of whelping date, stating that in 24 hours or even less the bitch will deliver. Even if measuring serum P4 concentration is still considered the most common method to predict the whelping day, it requires a blood sample and our study faced with worrisome opinions from the owners about this. Vaginal and rectal temperature are commonly monitorated by the owner himself and he must be aware that measuring rectal or vaginal temperature three times per day starting at least one week before parturition, is a good method to display a descendent trend useful to predict the whelping day.

6. CONCLUSION

Fetal gastrointestinal motility can be easily identified after a few seconds of observation on D59 onwards in at least 63% of fetuses. This may allow the small animal clinician to forecast that the bitch should whelp in 5 days or even less. On the other hand, due to the presence of high false negative results, if the fetuses do not display any peristaltic movement between D59 and D63, it cannot be excluded that the bitch will whelp in a few days or even hours. Fetal gastrointestinal motility can be considered as a specific but not sensitive parameter.

Although a larger data set of bitches during the last 5 days of pregnancy would be necessary to reach statistical significance, the assessment of fetal gastrointestinal motility can be considered an interesting tool to evaluate canine fetal maturity. However, the decision on when to plan for a C-section should no be based solely on the observation of fetal gastrointestinal motility. This prediction might be improved by the evaluation of vaginal and/or rectal temperature during the last week of pregnancy. When a mean temperature variation respectively of 0.73°C and 0.67°C is observed and at least 60% of fetuses are displaying fetal gastrointestinal peristalsis, the probability that the bitch will whelp within 24 hours is much greater.

7. ANNEX

Animal Data

Starting from Bitch number 1 proceeding to Bitch number 20, a complete past and recent anamnesis together with all the information regarding their pregnancy and the litter size x-ray, are reported in the Annex n° 1-20 below.

Number	1
Name	Pepper
Breed	Flat Coated Retriever
Age	4 years
Past anamnesis	Nothing to report, she has always been in good health
Recent anamnesis	Nothing to report, she has always been in good health
Estrous monitoring	Yes
Type of mating	Natural
Pregnancy management	Moderate physical activity. She lives outdoors and indoors with other dogs.
Type of delivery	Normal
X-ray	
Litter Size	12 pups. 2 of them were stillbirths and another 2 died a few days later.
Further notes	-

Annex n° 1.

Number	2
Name	Vicky
Breed	Flat Coated Retriever
Age	2 years
Past anamnesis	Surgical removal of a subcutaneous cyst in the sternal region a few months ago. She has always been in good health.
Recent anamnesis	Nothing to report.
Estrous monitoring	Yes
Type of mating	Natural
Pregnancy management	Moderate physical activity. She lives outdoors and indoors among other dogs.
Type of delivery	Normal
X-ray	
Litter Size	11 pups. 1 stillborn.
Further notes	-

Annex n° 2.

Number	3
Name	Lisa
Breed	Whippet
Age	4 years
Past anamnesis	Cardiac problem diagnosed almost 2 years ago. Treated medically and now only a cardiac murmur is detectable.
Recent anamnesis	Foreign body in the stomach, maybe a little coin, is visible on the x-ray.
Estrous monitoring	No
Type of mating	Natural
Pregnancy management	Moderate physical activity. She lives indoors with another dog.
Type of delivery	C-section
X-ray	
Litter Size	8 pups, all alive.
Further notes	Unwanted pregnancy discovered only when pregnancy was at term.

Annex n° 3.

Number	4
Name	Ithaca
Breed	Basset Hound
Age	3 years
Past anamnesis	Nothing to report, she has always been in good health
Recent anamnesis	Episodes of flatulence without diarrhea in the last week. Normal appetite.
Estrous monitoring	Yes
Type of mating	Natural
Pregnancy management	Moderate physical activity. She lives indoors with other dogs.
Type of delivery	Normal and C-section (after the 5th puppy)
X-ray	
Litter Size	11 pups, one stillborn.
Further notes	The first 5 pups were expelled with minor effort in physiological range of time (less than 2 hours between one pup and another). Then, more than 2 hours passed between the 5th fetus and the 6th one. Feathering vagina was performed several times by the author without success. An emergency C-section was done.

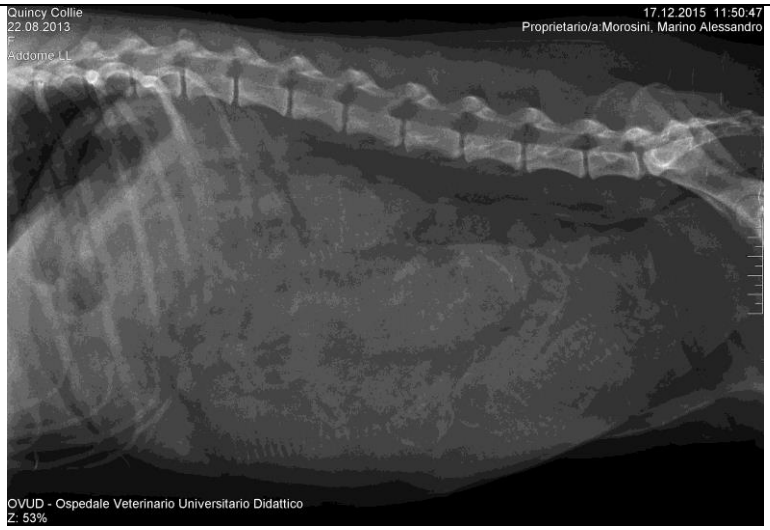
Annex n° 4.

Number	5
Name	Bibì
Breed	Jack Russel Terrier
Age	3 years
Past anamnesis	Nothing to report. She has always been in good health.
Recent anamnesis	Nothing to report. She has always been in good health.
Estrous monitoring	Yes
Type of mating	Natural
Pregnancy management	Moderate physical activity. She lives outdoors and indoors among other dogs.
Type of delivery	Normal
X-ray	
Litter Size	5 pups. 1 stillborn.
Further notes	-

Annex n° 5.

Number	6
Name	Haka
Breed	Jack Russel Terrier
Age	2 years
Past anamnesis	Nothing to report. She has always been in good health.
Recent anamnesis	Nothing to report. She has always been in good health.
Estrous monitoring	Yes.
Type of mating	Artificial Insemination.
Pregnancy management	Moderate physical activity. She lives outdoors and indoors with other dogs.
Type of delivery	Normal
X-ray	
Litter Size	5 pups, all alive.
Further notes	-

Annex n° 6.

Number	7
Name	Quincy
Breed	Scottish Shepherd
Age	2 years
Past anamnesis	Nothing to report. She has always been in good health.
Recent anamnesis	Transparent vaginal discharge was seen 2 weeks before deliver, together with an intensive licking of the vulva, which was slightly reddened. The owner reported also a decrease in appetite. Bacteriological exam of the vulva discharge was done and Staphylococcus coagulase positive was isolated. She was treated with success with amoxicillin-clavulanic acid.
Estrous monitoring	Yes
Type of mating	Artificial Insemination.
Pregnancy management	Moderate physical activity. She lives indoors among other dogs.
Type of delivery	Normal
X-ray	
Litter Size	8 pups, all alive.
Further notes	-

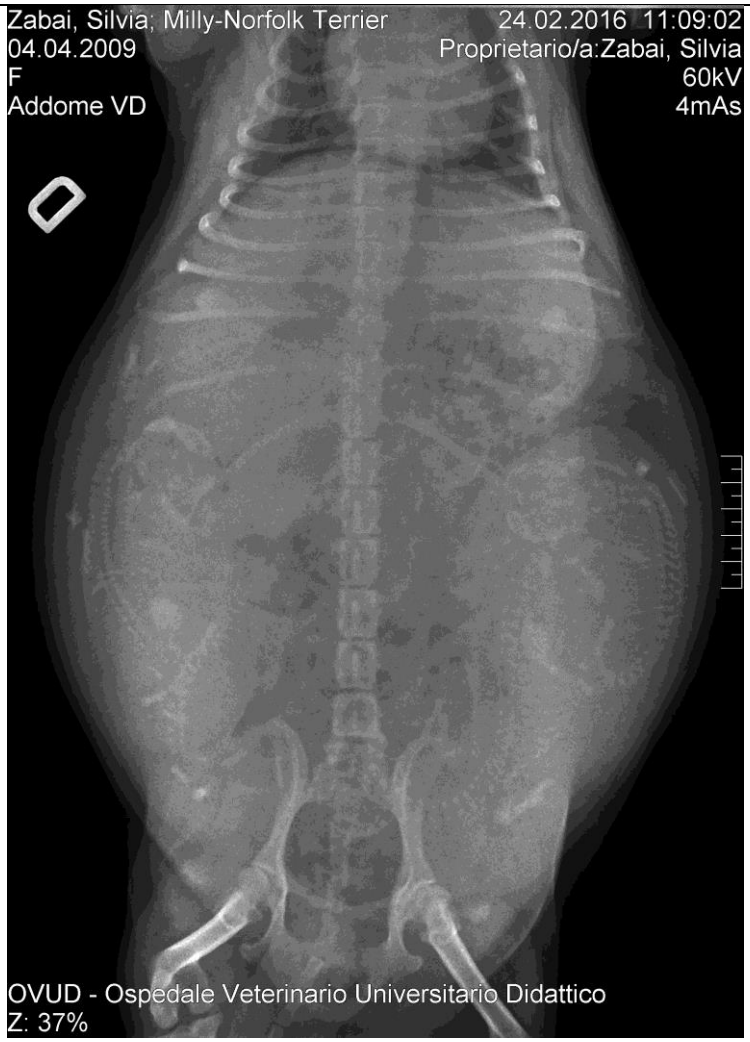
Annex n° 7.

Number	8
Name	Kate
Breed	Australian Shepherd
Age	5 years
Past anamnesis	Nothing to report. She has always been in good health.
Recent anamnesis	Nothing to report. She has always been in good health.
Estrous monitoring	Yes
Type of mating	Natural
Pregnancy management	Moderate physical activity. She lives outdoors and indoors among other dogs.
Type of delivery	Normal
X-ray	
Litter Size	10 pups, all alive.
Further notes	-

Annex n° 8.

Number	9
Name	April
Breed	Pumi
Age	2 years
Past anamnesis	Nothing to report. She has always been in good health. Surgical removal of vaginal rein.
Recent anamnesis	Nothing to report. She has always been in good health.
Estrous monitoring	Yes
Type of mating	Natural
Pregnancy management	Moderate physical activity. She lives outdoors and indoors among other dogs.
Type of delivery	Normal
X-ray	The owner did not allow to perform the x-ray.
Litter Size	7 pups, all alive.
Further notes	-

Annex n° 9.

Number	10
Name	Milly
Breed	Norfolk Terrier
Age	7 years
Past anamnesis	Nothing to report. She has always been in good health.
Recent anamnesis	Nothing to report. She has always been in good health.
Estrous monitoring	Yes
Type of mating	Artificial insemination.
Pregnancy management	Moderate physical activity. She lives outdoors among other dogs.
Type of delivery	Elective C-section.
X-ray	
Litter Size	5 pups, all alive.
Further notes	-

Annex n° 10.

Number	11
Name	Tilda
Breed	Flat Coated Retriever
Age	2 years
Past anamnesis	Nothing to report. She has always been in good health.
Recent anamnesis	She was treated with Prontogest® from day 27 to 60 of pregnancy because hipoluteodism was suspected.
Estrous monitoring	Yes
Type of mating	Natural
Pregnancy management	Moderate physical activity. She lives outdoors and indoors with other dogs.
Type of delivery	C-section
X-ray	
Litter Size	7 pups, all alive.
Further notes	Normal parturition mechanism did not trigger, maybe because of P4 supplementation. So, C-section was performed.

Annex n° 11.

Number	12
Name	Pepper
Breed	Flat Coated Retriever
Age	5 years
Past anamnesis	Nothing to report. She has always been in good health.
Recent anamnesis	Nothing to report. She has always been in good health.
Estrous monitoring	Yes
Type of mating	Natural
Pregnancy management	Moderate physical activity. She lives outdoors and indoors among other dogs.
Type of delivery	Normal
X-ray	
Litter Size	12 pups, all alive.
Further notes	-

Annex n° 12.

Number	13
Name	Fanny
Breed	Boxer
Age	4 years
Past anamnesis	Nothing to report. She has always been in good health.
Recent anamnesis	A few episodes of diarrhoea in the last 2 days. Normal appetite.
Estrous monitoring	Yes
Type of mating	Natural
Pregnancy management	Moderate physical activity. She lives outdoors, with other dogs.
Type of delivery	Normal
X-ray	
Litter Size	3 pups. One of them died the day after without an identifiable reason (fading neonate).
Further notes	-

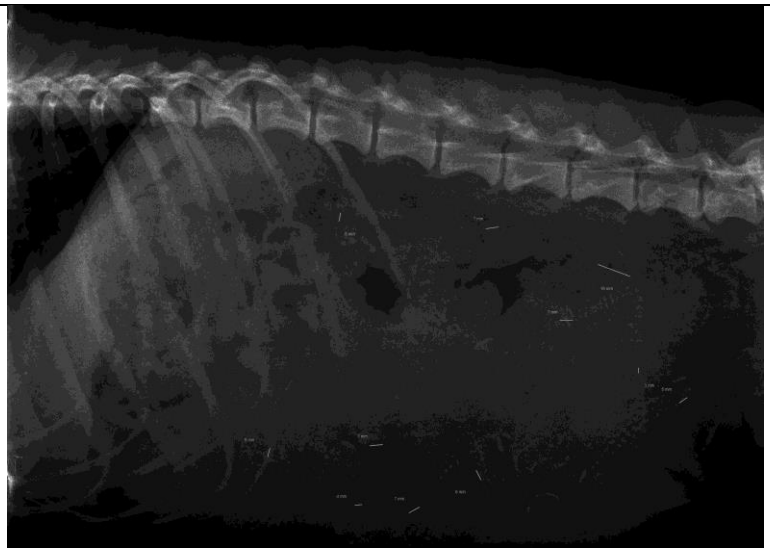
Annex n° 13.

Number	14
Name	Gaia
Breed	Boxer
Age	2 years
Past anamnesis	Nothing to report. She has always been in good health.
Recent anamnesis	In the last week of pregnacy, the bitch had less appetite and the owner observed a few episodes of vomit and diarrhea.
Estrous monitoring	Yes
Type of mating	Natural
Pregnancy management	Moderate physical activity. She lives outdoors, with other dogs.
Type of delivery	Normal.
X-ray	
Litter Size	8 pups. 5 of them presented cleft palate and died a few weeks later.
Further notes	-

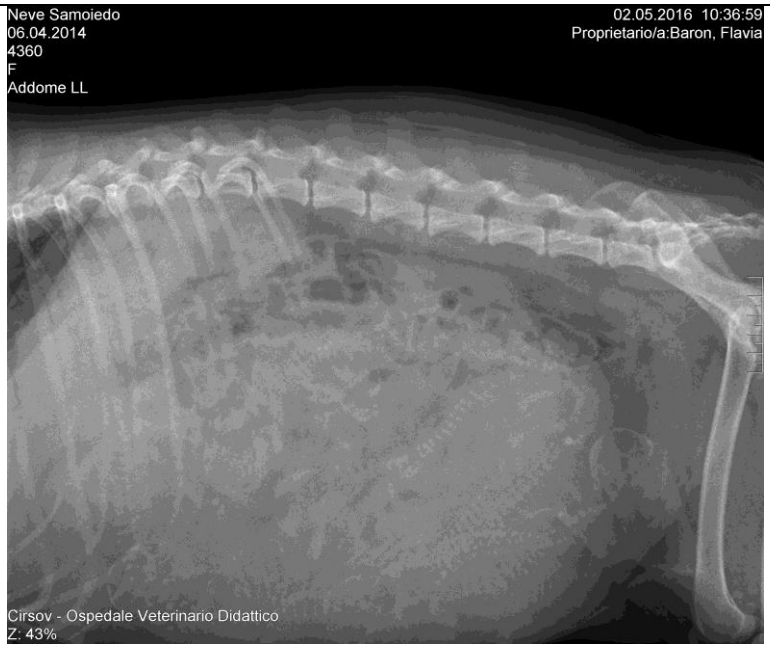
Annex n° 14.

Number	15
Name	Mika
Breed	Jack Russel Terrier
Age	2 years
Past anamnesis	Nothing to report. She has always been in good health.
Recent anamnesis	Nothing to report. She has always been in good health.
Estrous monitoring	Yes
Type of mating	Natural
Pregnancy management	Moderate physical activity. She lives indoors with another dog and three cats.
Type of delivery	Normal
X-ray	
Litter Size	7 pups, all alive.
Further notes	Difficulty during the expulsion of the last pup. The author performed feathering vagina for several times and the pup was reanimated with success.

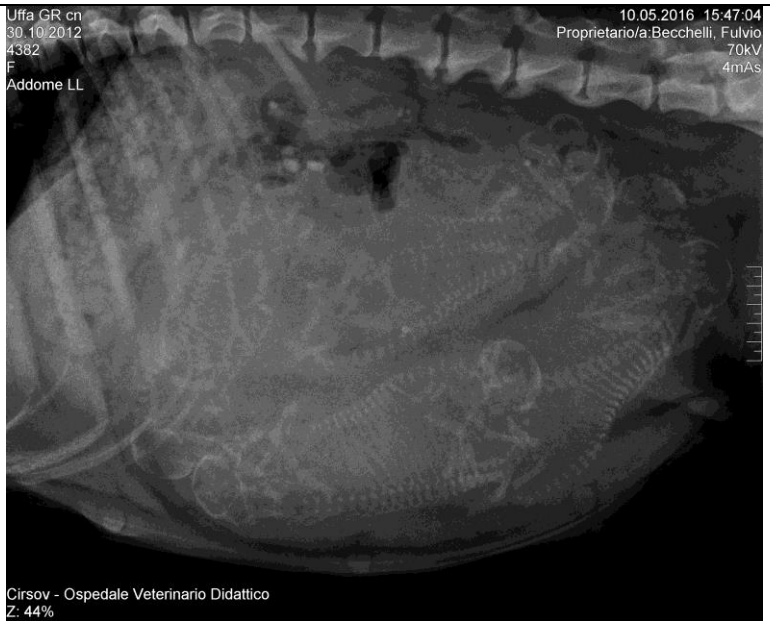
Annex n° 15.

Number	16
Name	Tikka
Breed	Flat Coated Retriever
Age	2 years
Past anamnesis	Nothing to report. She has always been in good health.
Recent anamnesis	Nothing to report. She has always been in good health.
Estrous monitoring	No
Type of mating	Natural
Pregnancy management	Moderate physical activity. The owner did not know that she was pregnant until day 50 of gestation. She lives outdoors and indoors with other dogs.
Type of delivery	Normal
X-ray	
Litter Size	10 pups. All died in a month due to unknown reasons.
Further notes	Unwanted pregnancy.

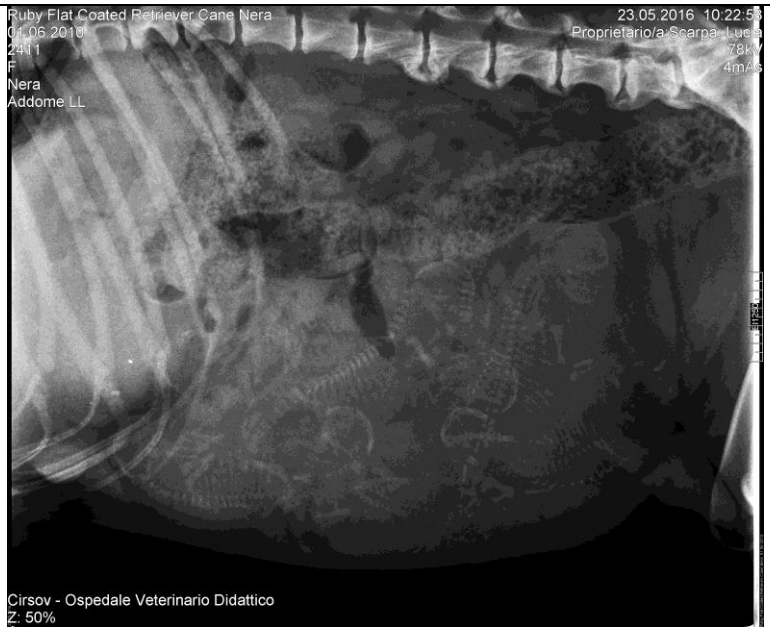
Annex n° 16.

Number	17
Name	Neve
Breed	Samoyed
Age	2 years
Past anamnesis	Nothing to report. She has always been in good health.
Recent anamnesis	In the last 24 hours, a decrease in appetite was observed by the owner, together with a vomit episode. She was treated with success with a gastric protector.
Estrous monitoring	Yes
Type of mating	Natural
Pregnancy management	Moderate physical activity. She lives indoors. No other dogs are present.
Type of delivery	Normal
X-ray	
Litter Size	7 pups, all alive.
Further notes	-

Annex n° 17.

Number	18
Name	Uffa
Breed	Golden Retriever
Age	3 years
Past anamnesis	Nothing to report. She has always been in good health.
Recent anamnesis	Nothing to report. She has always been in good health.
Estrous monitoring	Yes
Type of mating	Artificial insemination.
Pregnancy management	Moderate physical activity. She lives indoors with another dog and a cat.
Type of delivery	Normal
X-ray	
Litter Size	8 pups. 1 stillborn.
Further notes	-

Annex n° 18.

Number	19
Name	Ruby
Breed	Flat Coated Retriever
Age	6 years
Past anamnesis	Nothing to report. She has always been in good health.
Recent anamnesis	Nothing to report. She has always been in good health.
Estrous monitoring	Yes
Type of mating	Artificial insemination.
Pregnancy management	Moderate physical activity. She lives outdoors and indoors with other dogs.
Type of delivery	Normal
X-ray	
Litter Size	7 pups, all alive.
Further notes	-

Annex n° 19.

Number	20
Name	Dana
Breed	Bouvier de Flandres
Age	4 years
Past anamnesis	Nothing to report. She has always been in good health.
Recent anamnesis	Nothing to report. She has always been in good health.
Estrous monitoring	Yes
Type of mating	Natural
Pregnancy management	Moderate physical activity. She lives outdoors with other dogs.
Type of delivery	Normal
X-ray	
Litter Size	8 pups, all alive.
Further notes	Difficult expulsion of the first pup. Obstetrical manipulations were done by the author in order to lubricate the birth canal. A syringe full of ultrasound gel was connected with a small catheter. It was inserted in the vagina and the gel gently pushed into the birth canal. The pup was then extracted and gently reanimated.

Annex n° 20.

Bitches health status

Clinical examination was performed in all 20 bitches. At the beginning of each visit, conjunctival, oral and vaginal mucosa together with retropharyngeal, prescapular and popliteal lymphnodes, abdomen palpation, lung auscultation, respiratory rate and heartbeats per minute (bpm) were evaluated. Also a complete blood count and serum biochemistry were assessed. Annex n° 21 below reports the resume of all the consultations.

Subject number	**Mucosa (conjunctival, oral and vaginal)**	**Lymphnodes (retropharyngeal, prescapular and popliteal)**	**Abdominal Palpation**	**Lung auscultation and respiratory rate**	**Mean Heartbeats per minute**	**Blood count**	**Serum biochemistry**
1	Normal in all consultations	Normal in all consultations	Normal in all consultations	Normal in all consultations	119 bpm	Eosinophils: 10.9 % (1.4-6.2 %)	Alkaline phosphatase: 58UI/L (72-269UI/L); ALT: 22 U/l (33-86U/l); Glycemia: 52mg/dl (90-114mg/dl)
2	Normal in all consultations	Normal in all consultations	Normal in all consultations	Normal in all consultations	119 bpm	Hgb: 12.5g/dl (13.2-17.3 g/dl)	ALT: 22U/l (33-86U/l)
3	Normal in all consultations	Normal in all consultations	Normal in all consultations	Normal in all consultations	135 bpm	-	-

4	Normal in all consultations	Normal in all consultations	Normal in all consultations	Normal in all consultations	128 bpm	WBC: 18.23× 10^3/µl (8.23-15.74× 10^3/µl); Eosinophils: 10.1% (1.4-6.2%)	AST: 17U/l (25-45U/l); ALT: 14U/l (33-86U/l)
5	Normal in all consultations	Normal in all consultations	Normal in all consultations	Normal in all consultations	125 bpm	PLT: 644×10^3/µl (226-401×10^3/µl)	Albumin: 33.7g/L (25.34-31.83g/L); Phosphorus: 3mg/dL (3.7-5.7mg/dL)
6	Normal in all consultations	Normal in all consultations	Normal in all consultations	Normal in all consultations	120 bpm	Segmented neutrophils: 81.2% (61.6-77.3%)	AST: 22U/l (25-45U/l); Cholesterol: 295mg/dL (177-285mg/dL); Total Proteins: 74.6g/L (58-69g/L)
7	Normal in all consultations	Normal in all consultations	Normal in all consultations	Normal in all consultations	128 bpm	Hgb: 12.1 g/dl (13.2-17.3g/dl)	Alkaline phosphatase: 45U/l (72-269U/l)
8	Normal in all consultations	Normal in all consultations	Normal in all consultations	Normal in all consultations	126 bpm	-	ALT: 22.1U/l (33-86U/l); CK: 69U/l (107-280U/l); Albumin: 35.6g/L (25.34-31.83g/L); Cholesterol:

							292mg/dL (177-285mg/dL); Tryglicerides: 104mg/dL (45-96mg/dL)
9	Normal in all consultations	Normal in all consultations	Normal in all consultations	Normal in all consultations	160 bpm	-	-
10	Normal in all consultations	Normal in all consultations	Normal in all consultations	Normal in all consultations	160 bpm	Hgb: 11.1g/dl (13.2-17.3g/dl); PLT: 588×10^3/µl ($226\text{-}401\times10^3$/µl)	GGT: 9.96 U/l (4.54-8.75U/l); Bilirubin: 0.55mg/dL (0.15-0.34mg/dL); ALT: 170.8U/l (33-86U/l); Tryglicerides: 104mg/dL (45-96mg/dL)
11	Normal in all consultations	Normal in all consultations	Normal in all consultations	Normal in all consultations	106 bpm	Lymphocytes: 27.7% (12.5-24.8%)	AST: 20U/l (25-45U/l); ALT: 21U/l (33-86U/l); CK: 67U/l (107-280U/l)
12	Normal in all consultations	Normal in all consultations	Normal in all consultations	Normal in all consultations	116 bpm	Hgb: 11.6g/dl (13.2-17.3g/dl); PLT: 621×10^3/µl ($226\text{-}401\times10^3$/µl);	AST: 16U/l (25-45U/l); ALT: 10.6U/l (33-86U/l)

						Eosinophils: 14.4% (1.4-6.2%)	
13	Normal in all consultations	Normal in all consultations	Normal in all consultations	Normal in all consultations	122 bpm	WBC: 19.37×10^3/µl ($8.23-15.74\times10^3$/µl); Eosinophils:5 17% (1.4-6.2%)	GGT: 0 U/l (4.54-8.75U/l); ALT: 27U/l (33-86U/l); Alkaline phosphatase: 50U/l (72-269U/l); Calcium: 8.6mg/dL (9-10.1mg/dL)
14	Normal in all consultations	Normal in all consultations	Normal in all consultations	Normal in all consultations	120 bpm	WBC: 16.63×10^3/µl ($8.23-15.74\times10^3$/µl); Monocytes: 7.9% (3.7-6%)	Total Proteins: 75.29g/L (58-69g/L); Globulins: 48g/L (30-38.6g/L)
15	Normal in all consultations	Normal in all consultations	Normal in all consultations	Normal in all consultations	107 bpm	Hgb: 12.6g/dl (13.2-17.3g/dl); Lymphocytes: 30.4% (12.5-24.8%)	ALT: 18.2U/l (33-86U/l); Calcium: 7.9mg/dL (9-10.1mg/dL)
16	Normal in all consultations	Normal in all consultations	Normal in all consultations	Normal in all consultations	93 bpm	WBC: 19.11×10^3/µl (8.23-	AST: 21U/l (25-45U/l); ALT: 20U/l (33-86U/l);

						15.74×10^3/µl); Hgb: 11.2g/dl (13.2-17.3g/dl); Monocytes: 21.4% (3.7-6%)	Azotemia: 23mg/dl (27-53mg/dl); Albumin: 23.28g/L (25.34-31.83g/L)
17	Normal in all consultations	Normal in all consultations	Normal in all consultations	Normal in all consultations	108 bpm	Hgb: 12.1g/dl (13.2-17.3g/dl)	AST: 20U/l (25-45U/l); ALT: 18U/l (33-86U/l); Cholesterol: 323mg/dl (177-285mg/dl)
18	Normal in all consultations	Normal in all consultations	Normal in all consultations	Normal in all consultations	86 bpm	Hgb: 12.2 g/dl (13.2-17.3g/dl); Monocytes: 2.1% (3.7-6%); Eosinophils: 12.2% (1.4-6.2%)	ALT: 25U/l (33-86U/l); Cholesterol: 294mg/dl (177-285mg/dl); CK: 595U/l (107-280U/l)
19	Normal in all consultations	Normal in all consultations	Normal in all consultations	Normal in all consultations	102 bpm	WBC: 17.53×10^3/µl (8.23-15.74×10^3/µl); Hgb: 11.3g/dl (13.2-17.3g/dl);	ALT: 22U/l (33-86U/l); Albumin: 21.8g/L (25.34-31.83g/L); Globulins: 44g/L (30-38.6g/L)

						PLT: 837×10^3/µl ($226\text{-}401\times10^3$/µl); Eosinophils: 19.9% (1.4-6.2%)	
20	Normal in all consultations	Normal in all consultations	Normal in all consultations	Normal in all consultations	103 bpm	Hgb: 12.2g/dl (13.2-17.3g/dl)	AST: 20U/l (25-45U/l); ALT: 20U/l (33-86U/l); Albumin: 23.73g/L (25.34-31.83g/L); Globulins: 45g/L (30-38.6g/L)

Annex n° 21. Clinical examination performed in all 20 bitches. At the beginning of each visit, conjunctival, oral and vaginal mucosa together with retropharyngeal, prescapular and popliteal lymphnodes, abdomen palpation, lung auscultation, respiratory rate and heartbeats per minute (bpm) were evaluated. Table above reports the resume of all the consultations. A complete blood count and serum biochemistry was also performed in order to assess maternal health status. Even if some parameters were not within physiological range, these results did not indicate any worrisome condition.

Results

a) Vaginal temperature individual values assessed on each consultation day are reported in Annex n° 22 below.

Vaginal temperature in °C										
Subject number	**D54**	**D55**	**D56**	**D57**	**D58**	**D59**	**D60**	**D61**	**D62**	**D63**
1				38.6	38.7	37.9		38		
2				38.1		37.3				
3			38.3							
4				38	38.2		37.1			
5						37.8	38	37.8		
6					38.1					
7		38.1			37.9					
8				38			37.8	36.9	37.3	
9						37.6				
10								38	37.5	36.5
11	38.2			37.9	38.1			38.3	37.7	
12			38			38	38.1			
13	38.3					38		37.9		
14				37.5		37.4				
15					37.5	37.3			36.6	
16					38.3		37.8	37		
17						37.8		37.9		37.3
18				37.8		37.7				
19		38		37.6					37.2	
20						38.1	38.2			

Annex n° 22. Vaginal temperature measurements were performed in all the 20 bitches at the beginning of each visit in Celsius degrees (°C). The day of gestation was corrected by counting backward from the day of whelping.

b) Rectal temperature individual values assessed on each consultation day are reported in Annex n° 23 below.

Rectal temperature in °C										
Subject number	**D54**	**D55**	**D56**	**D57**	**D58**	**D59**	**D60**	**D61**	**D62**	**D63**
1				38.7	38.8	38.2		38.3		
2				38.2		37.9				
3			38.2							
4				38.3	38		37.2			
5						38.3	37.6	37.6		
6					38.2					
7		38.2			38.1					
8				38			37.5	37.7	37.2	
9						38				
10								38.1	37.7	37
11	38.5			38.2	38.2			38.3	37.7	
12			38.2			37.9	37.8			
13	38.5					38.1		38.2		
14				37.7		37.7				
15					38	38.1			37.6	
16					38.2		38	37.8		
17						37.7		37.9		37.7
18				37.8		37.8				
19		38.2		37.8					36.8	
20						38.5	38.5			

Annex n° 23: Rectal temperatures assessed, after vaginal ones, in all the 20 bitches at the beginning of each visit, in each visit in Celsius degrees (°C). The day of gestation was corrected by counting backward from the day of whelping.

c) Serum P4 assay individual values assessed on each consultation day are reported in Annex n° 24 below.

Serum P4 assay in ng/mL										
Subject number	**D54**	**D55**	**D56**	**D57**	**D58**	**D59**	**D60**	**D61**	**D62**	**D63**
1				5.5	6.6			4.2		
2				3.5		3.7				
3			7.9							
4				7.2	6.3		5.9			
5						3.4		2.8		
6					5.2					
7					6.8					
8				5.2			3.4		0.97	
9										
10								6.6	6.1	1
11										
12			4.6			5.8	3.9			
13	3.7					4.2		2.4		
14				5.3		5.7				
15					6	4.4			2	
16					4.1		3.2	2.4		
17						7.4		6.6		0.96
18				6.9		4.3				1
19		4.5		4.6					0.95	
20						4.1	3.7			

Annex n° 24. Serum P4 assay was evaluated in 18 bitches on each consultation day. Results are stated in ng/mL. The day of gestation was corrected by counting backward from the day of whelping. One of the bitches examined, number 11, was assuming Prontogest® , a natural P4 supplementation therapy, from day 27 to day 60 of her pregnancy because luteal insufficiency was diagnosed. In order to avoid false P4 results in the statistical analysis, she was not inserted in the results.

d) Serum cortisol assay individual values assessed on each consultation day are reported in Annex n° 25 below.

Serum Cortisol assay in µg/dL										
Subject number	**D54**	**D55**	**D56**	**D57**	**D58**	**D59**	**D60**	**D61**	**D62**	**D63**
1				2.2	4.6			2.4		
2				2.9		3.1				
3			4.2							
4				3.7	5.5		4.7			
5						5.9		4.1		
6					2.7					
7					4.9					
8				1.6			1.6		6.1	
9										
10								3.6	3.5	4.1
11	1.5			2.8	1.6			4.5	5	
12			1.8			3.1	1.5			
13	0.9					2.4		1.7		
14				2		2.8				
15					5.2	5			8.1	
16					0.9		3	1.9		
17						2.4		2		4.1
18				1.5		2.3				
19		3.8		3.4					5.5	
20						2.3	4.5			

Annex n° 25. Serum cortisol assay was evaluated in 19 bitches on each consultation day. Results are stated in µg/dL. The day of gestation was corrected by counting backward from the day of whelping.

e) Fetal heartbeats individual measurements evaluated on each consultation day are reported in Annex n° 26 below.

Fetal heartbeats in bpm											
Subject number		**D54**	**D55**	**D56**	**D57**	**D58**	**D59**	**D60**	**D61**	**D62**	**D63**
1	1st fetus				224	222	224		216		
	2nd fetus				231	238	241		200		
	3rd fetus					194	216		198		
2	1st fetus				233		234				
	2nd fetus				217		221				
	3rd fetus				232		243				
3	1st fetus			219							
	2nd fetus			251							
	3rd fetus			241							
4	1st fetus				246	240		199			
	2nd fetus				239	216		195			
	3rd fetus				225	223		206			
5	1st fetus						218		201		
	2nd fetus							185	180		
	3rd fetus							248			
6	1st fetus					185					
	2nd fetus					200					
	3rd fetus										
7	1st fetus		176			190					
	2nd fetus		212			207					
	3rd fetus		218			216					
8	1st fetus				229			202	202	217	
	2nd fetus				238				220		
	3rd fetus								233		
9	1st fetus						241				
	2nd fetus										
	3rd fetus										
10	1st fetus								260	221	208
	2nd fetus								220	217	216
	3rd fetus									225	
11	1st fetus	235			236	257			223	211	228
	2nd fetus	226			230	255				198	198
	3rd fetus	258			248				201	189	228
12	1st fetus			214			220	222			
	2nd fetus			225			207	236			
	3rd fetus			230			233				
13	1st fetus	199					218		229		
	2nd fetus	216					230		209		
	3rd fetus	225					222		204		
14	1st fetus				210		195				

	2nd fetus				190		218				
	3rd fetus				222		195				
15	1st fetus					217	198			163	
	2nd fetus					202	201			180	
	3rd fetus					200	206			205	
16	1st fetus					222		218	209		
	2nd fetus					235		218	202		
	3rd fetus					246		227	206		
17	1st fetus						205		201		217
	2nd fetus						218		228		214
	3rd fetus								218		225
18	1st fetus				210		238				
	2nd fetus				220		236				
	3rd fetus				229		196				
19	1st fetus		224		221					232	
	2nd fetus		216		197					203	
	3rd fetus		240		210					177	
20	1st fetus						220	223			
	2nd fetus										
	3rd fetus						212	243			

Annex n° 26. Fetal heartbeats were evaluated in all the 20 bitches on each day of visit. Results are reported in beats per minute (bpm). The day of gestation was corrected by counting backward from the day of whelping.

f) Fetal gastrointestinal motility individual measurements evaluated on each consultation day are displayed in Annex n° 27 below.

Fetal gastrointestinal motility											
Subject number		**D54**	**D55**	**D56**	**D57**	**D58**	**D59**	**D60**	**D61**	**D62**	**D63**
1	1st fetus				0	0	1		1		
	2nd fetus				0	0	1		0		
	3rd fetus				0		1				
2	1st fetus				1		1				
	2nd fetus				0						
3	1st fetus			0							
	2nd fetus			0							
	3rd fetus			1							
4	1st fetus				0	0		1			
	2nd fetus				0	0		1			
	3rd fetus				0	0		1			
	4th fetus					1					
5	1st fetus						1	1	1		
	2nd fetus						1	1	1		
	3rd fetus							1	1		
6	1st fetus					0					
	2nd fetus					0					
	3rd fetus					0					
7	1st fetus		0			1					
	2nd fetus		0			0					
	3rd fetus		0			0					
8	1st fetus				0			0	1	0	
	2nd fetus				0			0	1		
	3rd fetus				0						
9	1st fetus						1				
	2nd fetus						0				
10	1st fetus								0	1	1
	2nd fetus								0	1	1
	3rd fetus								1	1	1
11	1st fetus	0			1	1			1	1	
	2nd fetus	0			0	0			1	1	
	3rd fetus	0			0	0			1	1	
12	1st fetus			0			1	0			
	2nd fetus			0			1	1			
	3rd fetus			0			0	1			
13	1st fetus	0					0		1		
	2nd fetus	0					0		1		
	3rd fetus	0					0		1		
14	1st fetus				0		0				

	2nd fetus				1		1				
	3rd fetus				0		1				
15	1st fetus					0	0			1	
	2nd fetus					0	1			0	
	3rd fetus					1	0			1	
16	1st fetus					1		1	0		
	2nd fetus					1		1	0		
	3rd fetus					0		0	0		
17	1st fetus						1		0		1
	2nd fetus						0		1		0
	3rd fetus								0		0
18	1st fetus				0		1				
	2nd fetus				0		0				
	3rd fetus				1		1				
19	1st fetus		0		0					0	
	2nd fetus		0		0					1	
	3rd fetus		0		0						
20	1st fetus						1	1			
	2nd fetus						1	1			
	3rd fetus						1	0			

Annex n° 28. Fetal gastrointestinal motility recorded in each fetus on each day of visit of 20 healthy bitches. Number 0 stands for no peristaltic movement recorded, and number 1 stands for the positive visualization of peristaltic motion.

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