UNIVERSIDADE FEDERAL DO PARANÁ

ELAINE MAYUMI UENO GIL

CURRENT ADVANCES IN ULTRASONOGRAPHIC ASSESSMENT OF PREGNANT BITCHES

CURITIBA

2020

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Tese apresentada ao curso de Pós-Graduação em Ciências Veterinárias, Setor de Ciências Agrárias, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Ciências Veterinárias, na área de concentração em Diagnóstico por Imagem.

Orientadora: Profª. Drª. Tilde Rodrigues Froes

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RESUMO

A presente pesquisa buscou contribuir com informações sobre a avaliação ultrassonográfica pré-natal durante a gestação em cadelas, particularmente às características fetais que devem ser avaliadas rotineiramente pelo médico veterinário imaginologista e a padronização de um método diagnóstico invasivo guiado por ultrassom que possibilite o diagnóstico intrauterino de diversas doenças fetais congênitas ou adquiridas. Para tanto, o trabalho foi subdividido em três capítulos independentes. No primeiro capítulo o enfoque da pesquisa foi descrever o desenvolvimento ultrassonográfico do rim fetal canino correlacionando com a idade gestacional. Este trabalho demonstrou que existe uma forte correlação entre a idade gestacional e o comprimento dos rins, que nos permitiu gerar uma equação para estimar a data do parto com alta sensibilidade entre 48 a 52 dias (ou entre a sexta e a sétima semana) de gestação. No segundo capítulo descrevemos sobre a visibilização do timo fetal. O timo é um órgão linfo-epitelial responsável pelo desenvolvimento do sistema imunológico sendo que, o grande interesse na avaliação desse órgão está relacionado a previsão do status imunológico do feto antes do parto. Discorremos neste capítulo sobre a avaliação do timo fetal canino descrevendo sua aparência ultrassonográfica normal e os seus pontos de referencia anatômicos no feto canino, indicando a melhor idade gestacional para sua identificação e definindo a acurácia do exame ultrassonográfico para esse fim e, tentando explicar as possíveis causas da não identificação desse órgão em alguns fetos de cadelas gestantes. O terceiro capítulo refere-se à descrição da técnica de amniocentese guiada por ultrassonografia, acreditamos que esse método diagnóstico invasivo é seguro e pode ser recomendada para vários portes de cadelas gestantes em diferentes idades gestacionais permitindo o diagnóstico precoce de doenças congênitas e assim, possibilitando o planejamento pré-natal do manejo materno-fetal. Portanto, apresento três estudos inéditos cujos achados e conclusões são de grande importância para a reprodução e o diagnóstico por imagem veterinário.

Palavras-chave: Cachorro 1. Gestação 2. Ultrassonografia 3. Desenvolvimento renal 4. Timo fetal 5. Amniocentese 6.

ABSTRACT

The present research sought to contribute with information about prenatal ultrasound assessment during pregnancy in bitches. Describes the fetal characteristics that should be routinely evaluated by the veterinarian imaginologist, and the standardization of an invasive diagnostic method guided by ultrasound that allows the intrauterine diagnosis of several congenital or acquired fetal diseases. Therefore, the research was divided into three independent chapters. In the first chapter, the research focus was to describe the ultrasonographic development of canine fetal kidney correlating with gestational age. This work demonstrated that there is a strong correlation between gestational age and kidney length, which allowed us to generate an equation to estimate the date of delivery with high sensitivity between 48 to 52 days (or between the sixth and seventh week) of gestation. In the second chapter, we report about the ultrasonography aspect of the thymus in intrauterine fetal dog. The thymus is a lymphoepithelial organ responsible for the development of the immune system, and the high interest in evaluation of this organ is related to the prediction of immunological status of fetus before delivery. It was the reason that in the second chapter, we discuss the evaluation of canine fetal thymus; reporting normal thymic ultrasonographic appearance and the anatomic reference point to locate the canine intrauterine fetus. Furthermore, indicating the best gestational age for its identification and defining the accuracy of the ultrasonographic examination for this purpose besides trying to explain the possible causes of non-identification of this organ in some pregnant bitches. The third chapter refers to the description of the technique of amniocentesis guided by ultrasonography. We believe that this invasive diagnostic method is safe and can be recommended for several sizes of pregnant bitches at different gestational ages allowing early diagnosis of congenital diseases and thus enabling the prenatal planning of maternal-fetal management. Therefore, I present three unpublished studies whose findings and conclusions are of great importance for breeding and veterinary diagnostic image.

Keywords: Dog 1. Gestation 2. Ultrasonographic 3. Fetal kidney development 4. Fetal thymus 5. Amniocentesis 6.

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Early results on canine fetal kidney development: ultrasonographic evaluation and value in prediction of delivery time

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Abstract

To date there have been no studies that describe the ultrasonographic evaluation of kidney development in canine fetuses. The aim of this prospective and longitudinal study was to monitor fetal kidney development with ultrasound and use fetal kidney measurements as a complementary biometric index for estimation of gestational age. Ultrasonographic examinations were performed on 15 clinically healthy pregnant bitches every four days from 30th day of pregnancy, until visualization of the fetal renal pelvis was no longer possible. Four distinct periods of ultrasonographic canine fetal kidney development were defined. Kidney length and renal pelvis distention were measured on longitudinal plane images. The fetal kidney ranged from 0.40 cm to 2.30 cm in length, and diameter of the pelvis ranged from 0.06 cm to 0.17 cm, however by the end of gestation the renal pelvis was no longer dilated and so its diameter could not be measured. Statistical analysis confirmed a relationship between gestational age and fetal kidney growth. Ultrasonographic evaluation of fetal kidney development is simple to perform. There is a strong correlation between gestational age and kidney length, which allowed generation of an equation to estimate delivery date with high sensitivity between 48 and 52 days of pregnancy. Fetal organ development can be considered complete when the renal pelvis is no longer dilated, this finding can assist the ultrasonographer in staging the gestation by prompting examination for fetal intestinal motility, which begins at the same gestational age. Measurement of fetal kidney length can be used in conjunction with other methods to estimate gestational age and predict delivery time.

Keywords: dog, pregnancy, ultrasound monitoring, delivery date prediction, gestational age

1.1 INTRODUCTION

Accurate determination of gestational age allows estimation of the time of delivery, such that breeders and veterinarians can plan for an assisted delivery, if required, thus helping to reduce peripartum losses (Kutzler et al, 2003; Luvoni & Beccaglia, 2006). If hormonal assays and determination of ovulation time are not possible, estimation of gestational age can be based on the time of first ultrasonographic appearance of specific embryonic and fetal structures (Shille & Gontarek, 1985; England et al, 1990; Yeager et al, 1992; Lopate, 2008). However, this method is only accurate to within one or two days. Therefore, new methods for more accurate determination of the gestational age in dogs would help to reduce the risk of death as consequence of premature C-section.

Several ultrasonographic examinations, including identification of: fetal liver, stomach, bladder, and heart have been reported to accurately estimate gestational age (Yeager et al, 1992; Topie et al, 2015). Recently, high-definition ultrasound visualization of the renal pelvis in feline fetuses was reported (Topie et al, 2015), and this technique allows the final stage of gestation to be identified more accurately.

Mammalian kidney formation (nephrogenesis) begins in the intermediate mesoderm with early development of the kidney: pronephros, mesonephros, and metanephros. Pronephros and mesonephros are transient excretory systems and disappear without contributing to a permanent renal system and metanephros involves formation of a permanent kidney (Salazar & Yllera, 1991). Depending on the species, nephrogenesis ceases shortly after delivery but, in the dog, the kidney continues to develop in the first week’s post-partum (Sinowatz, 2010). Therefore, it has been hypothesized that due to the different embryological stages of renal development the kidneys might appear ultrasonographically different during distinct stages of pregnancy.

The kidneys are first visible ultrasonographically in the canine fetal abdomen between days 40 to 46 of pregnancy (England et al, 1990) and limited information about their development has been reported. Initially they appear hypoechoic with a dilated, anechoic renal pelvis (Yeager et al, 1992), but as gestation progresses, the renal cortex can be differentiated from the medulla and the less dilated pelvis (Mattoon & Nyland, 2015). However, high-resolution ultrasound imaging provides a better assessment of fetal development, allowing the identification of detailed changes in the appearance of the fetal kidney over shorter periods of time relating to gestational age. In man ultrasound, scanning has been used to monitor the normal development of fetal anatomy and to detect nephropathies based on abnormalities relating to parenchyma and/or size of kidney and renal pelvis (Devriendt et al, 2013; Dias et al, 2014).

There are no previous studies describing the ultrasonographic evaluation of kidney development in canine fetuses. Our hypothesis is that it is possible to monitor the development of fetal kidneys using ultrasonography and to use fetal kidney measurements as a complementary biometric index for estimation of gestational age. The purpose of this study was fourfold: (1) to describe the ultrasonographic development of the canine fetal kidney and correlate this with gestational age; (2) to assess the degree of correlation between gestational age and kidney length; (3) to evaluate the degree of correlation between gestational age and the diameter of renal pelvis; and (4) to determine a regression equation for estimation of gestational age from kidney length.

1.2 MATERIALS AND METHODS

1.2.1 PATIENT SELECTION

Fifteen clinically healthy pregnant bitches were recruited in a prospective and longitudinal study. Breeds represented were: English Bulldog (4), American Staffordshire Terrier (1), Miniature Schnauzer (4), Pug (2), Pekingese (1), French Bulldog (1), Yorkshire Terrier (1) and Chinese Crested (1). Ages ranged from one to six years and weight between 3 and 26 kg. The number of fetuses identified in each pregnancy ranged from three to nine fetuses. Bitches that were unavailable for serial examinations, ie due to late pregnancy (more than 35 days of pregnancy) at the time of the first presentation, date of delivery not reported by owners, and bitches presented with concomitant disease or receiving diuretic drugs were excluded from this study. All fetuses from a pregnancy in which any structural abnormalities were detected by ultrasonography were also excluded. All procedures were conducted in accordance with the institutional Animal Use Committee guidelines.

1.2.2 EQUIPMENT AND ULTRASONOGRAPHIC IMAGINING

Two-dimensional ultrasonographic evaluations were performed using a MyLab™30VET Gold (Esaote, Genova, Italy) with a 7.5 to 12 MHz high-resolution linear multifrequency transducer (LA523 reference – Esaote, Genova, Italy). Bitches were positioned in dorsal recumbency using a sponge trough. Abdominal hair was clipped to optimize ultrasonographic image acquisition and acoustic gel was applied to the transducer. The protocol as described by Gil et al. (2014) was used to evaluate as many fetuses as possible in each bitch, and ultrasonographic images were acquired in a clockwise circle. The gain, focus and depth penetration was adjusted for each fetus during examination to optimize the image quality.

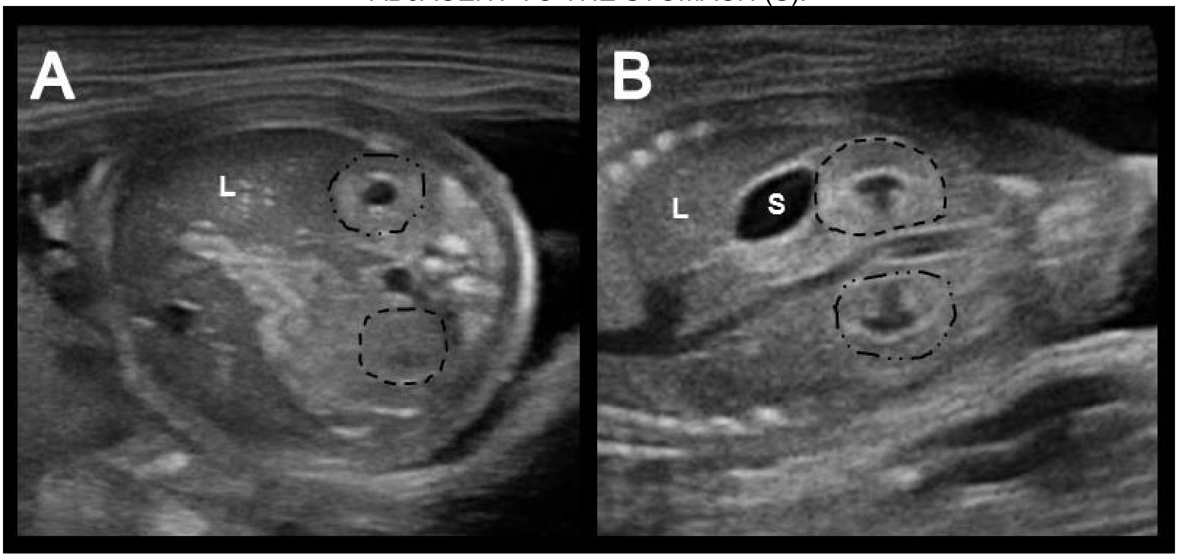
Ultrasonographic examinations were performed from 30th day after first mating or insemination for pregnancy diagnosis. Examinations were repeated every four days until it was no longer possible to visualize the renal pelvis. From that examination onward, daily assessments were made until delivery.

Gestational age was estimated in days of pregnancy, using the descriptions of Yeager et al. (1992) combined with assessment of the development of fetal bowel as reported by Gil et al. (2015). Confirmation of gestational age was made after delivery, in days from delivery, by counting backward (delivery as Day 0); normal gestational duration was considered to be 57 to 63 days due to the variability of proestrus and estrus periods in dogs (Concannon et al, 1983).

Evaluations were performed on as many fetuses as possible on each occasion, but they were not performed if fetal positioning was sub-optimal for ultrasonographic measurements of fetal kidneys. Intrauterine fetal abdomens were assessed in the transverse and dorsal planes of each fetus (FIGURE 1). In all patients, a complete survey of fetal anatomy was performed first; giving priority to the examination of the kidney. Images of fetal kidneys were recorded in longitudinal and dorsal sections (FIGURE 1B). Two experienced ultrasonographers (one of whom is a member of Brazilian College of Veterinary Radiology) were responsible for image acquisition throughout the study (Daniela

Garcia; D.G.; and Elaine Gil; E.G.); evaluations were not blinded since an interobserver study was not the objective of this research.

FIGURE 1. ULTRASONOGRAPHIC IMAGING OF CANINE FETUS DEMONSTRATE: (A) FETAL ABDOMEN IN TRANSVERSE PLANE SHOWS RIGHT KIDNEY (DOTTED TRACES) ADJACENT TO THE LIVER (L) AND LEFT KIDNEY (TRACES); (B) FETAL ABDOMEN IN THE DORSAL PLANE PRESENTS RIGHT KIDNEY (DOTTED TRACES) AND LEFT KIDNEY (TRACES) ADJACENT TO THE STOMACH (S).



SUBTITLE: L, liver; S, stomach.

1.2.3 ULTRASONOGRAPHIC MEASUREMENTS

After identification of the fetus, the fetal abdomen was scanned in a dorsal plane until the kidneys were detected on either side of vertebral column. The right kidney was located immediately caudal to right hepatic lobe and the left kidney just below the stomach, next to the spleen (FIGURE 1B). Kidney length was measured in only one kidney from outer to outer border, at maximal length, in a longitudinal plane, passing through the renal pelvis (FIGURE 2). Care was taken to ensure that the kidney was evaluated at its longest length and the ultrasound calipers were used to measure from the upper to lower border of the kidney, ensuring that the adrenal gland was excluded from this measurement. When the renal pelvis was visible, measurement was made between the pole and the ureteric outlet where possible, avoiding the ureteral exit (FIGURE 3A and 3B). The maximum length of renal pelvis was measured from inner edge to inner edge of the renal tissue.

FIGURE 2. ULTRASONOGRAPHIC IMAGING OF CANINE FETAL KIDNEY IN DORSAL PLANE DEMONSTRATING THE MEASUREMENT OF CANINE KIDNEY LENGTH (RED ARROW) MADE FROM POLE TO POLE.

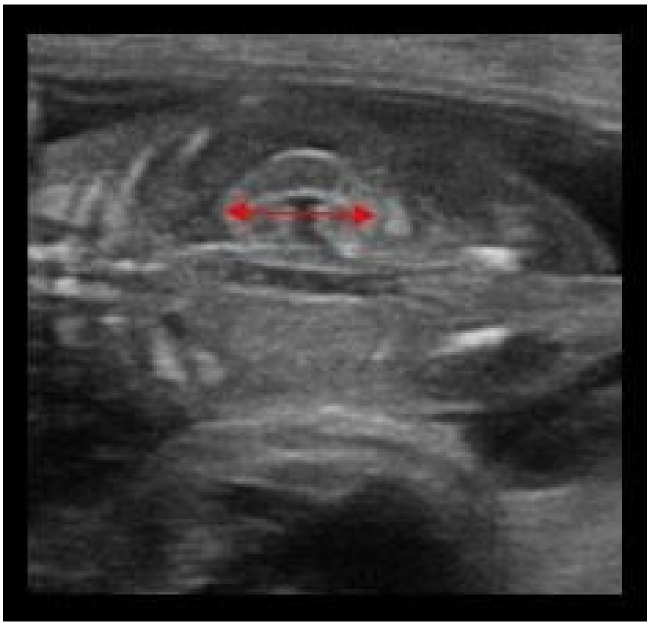
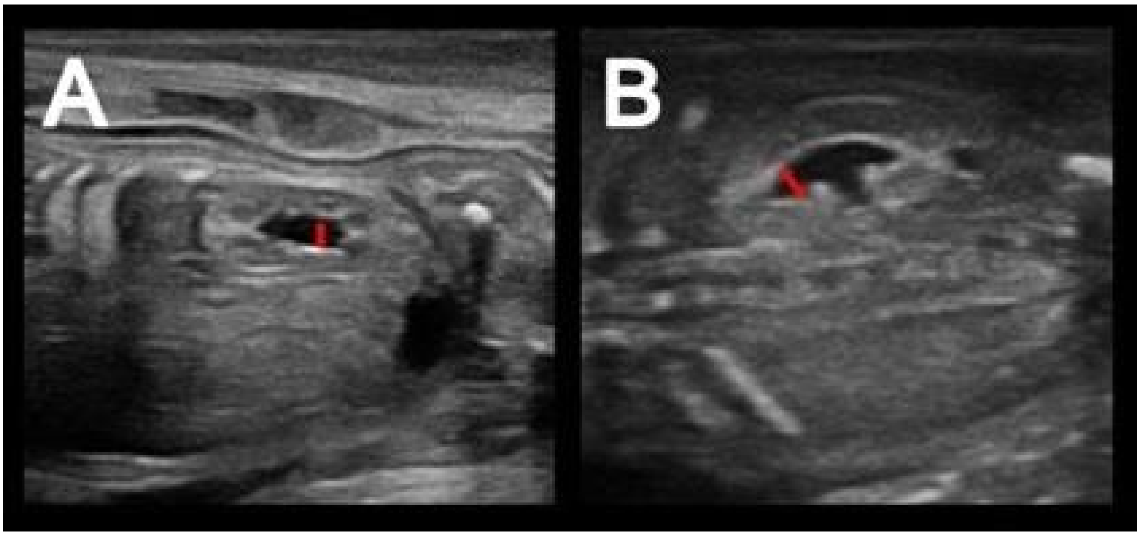


FIGURE 3. ULTRASONOGRAPHIC IMAGING OF CANINE FETAL KIDNEYS IN DORSAL PLANE DEMONSTRATING THE LOCATION OF MEASUREMENT (RED ARROWS) OF THE CANINE FETAL RENAL PELVIS.



Fetal kidney measurements were performed at the end of each examination using ultrasound images recorded, in DICOM format, in IMAGELAB software. Measurements were made in as many fetuses as possible, and at least three fetuses from each bitch were examined at each time point. A single operator (Elaine Gil; E.G.), performed all the measurements in duplicate and the mean values were calculated to reduce experimental variation.

1.2.4 STATISTICAL ANALYSES

Data were collected by one author (Elaine Gil; E.G.) using Microsoft Office Excel (Microsoft Office 2007 for Windows, Redmond, WA, USA). All statistical tests were selected by one statistical professional (Eliane Coimbra; E.C.) and analysis was made using R for Windows (version 3.1.1 Ri386, R Foundation for Statistical Computing, Vienna, Austria, 2014).

Statistical analysis was used to confirm a relationship between increasing gestational age and fetal kidney growth. Shapiro-Wilk test was used to ascertain if data on fetal kidney length and fetal renal pelvis diameter were normally distributed. A descriptive analysis with minimum, maximum, mean, median and standard deviation of the size of fetal kidney and fetal renal pelvis was correlated with gestational age. Pearson's correlation test was applied to verify the degree of correlation between gestational age and kidney length and between gestational age and diameter of the renal pelvis. Multivariate linear regression analysis was employed to create a predictive equation to estimate gestational age by using fetal kidney length or renal pelvis diameter. Finally, receiver operator curve (ROC) characteristics evaluated the power prediction of the equation in estimating gestational age using fetal kidney length; subsequently, sensitivity and specificity of the equation were determined. In all analyses, a P value of $\alpha = 0.05$ was considered significant and significance was analyzed relative to the value set.

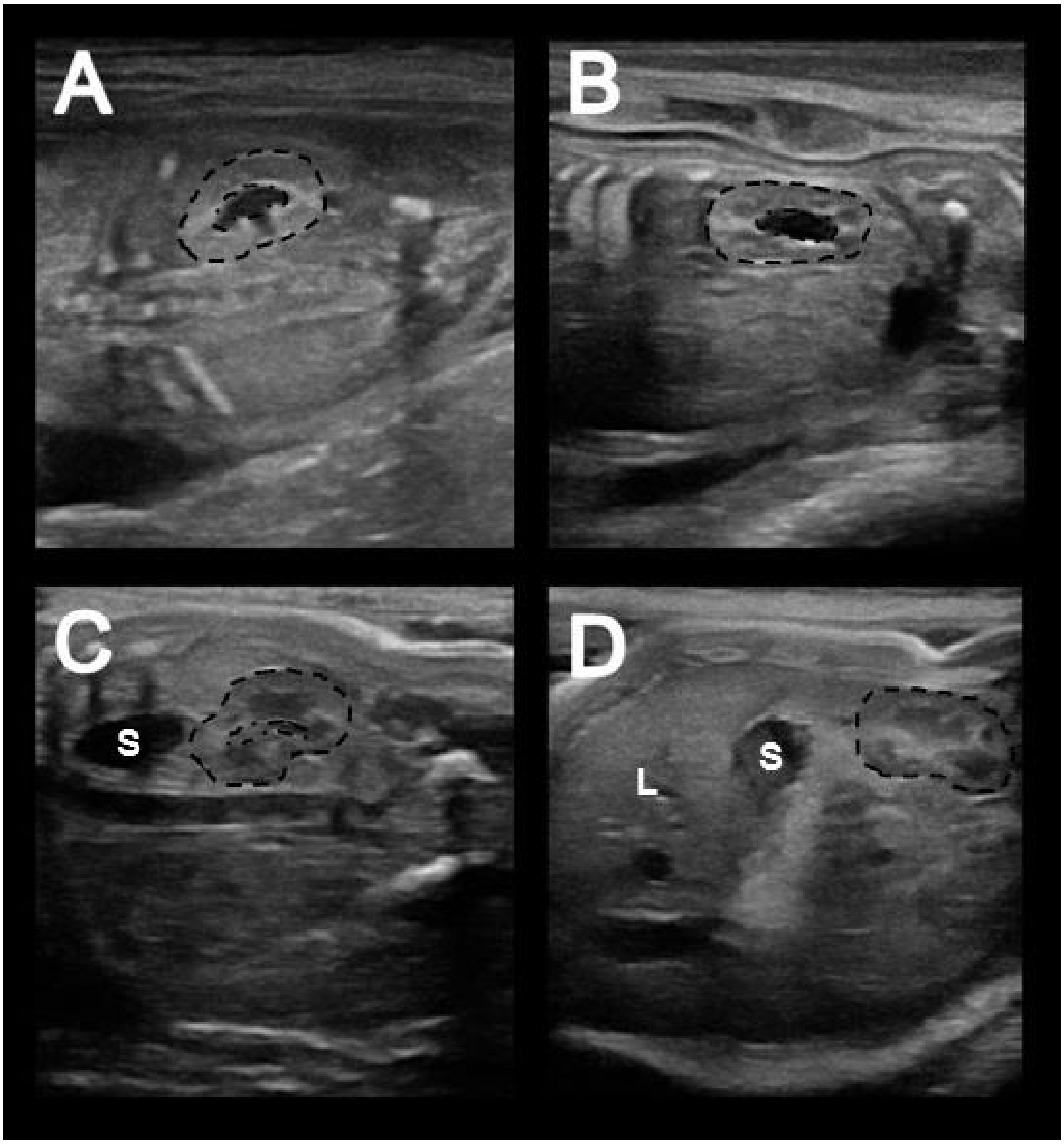
1.3 RESULTS

Fifteen pregnant bitches were included in this study. Measurements from at least three fetuses were analyzed at each time point and all were born healthy. All bitches included in this investigation had at least three fetuses. Thus, at least three measurements of each parameter (kidney length and renal pelvis diameter) were collected per pregnant bitch, amounting to a minimum of 45 measurements per parameter.

During monitoring of fetal organogenesis by ultrasonography, an assessment of the development of fetal kidneys was performed in all pregnant bitches. All fetuses had the same ultrasonographic characteristics of kidney development; so four developmental periods were defined according to the gestational ages as described below:

- Period 1: fetal kidney has a thickened and hyperechoic cortex, with no corticomedullary definition, renal pelvis is dilated with a "mushroom" shape and is filled with anechoic contents (FIGURE 4A);
- Period 2: fetal kidney has a thin hypoechoic cortex (if compared to previous period), part of hypoechoic medulla is identified, some corticomedullary definition visible and renal pelvis is dilated and has a tubular construction filled with anechoic contents (FIGURE 4B);
- Period 3: fetal kidney has thin, hypoechoic cortex (when compared to period 1). There is corticomedullary definition and renal pelvis is only slightly dilated and better visualized in the form of a canaliculus filled with anechoic content (FIGURE 4C);
- Period 4: in this stage, the fetal kidney has a similar appearance to an adult organ (Konde, 1985; Wood & McCarthy, 1990) with a thin cortex and hypo/isoechoic parenchyma as compared to liver, with corticomedullary definition and with no renal pelvic dilatation. However, the renal pelvis has hyperechoic features (similar to its postnatal form) that are not detectable in earlier periods (FIGURE 4D).

FIGURE 4. ULTRASONOGRAPHIC IMAGING OF FETAL KIDNEY IN DORSAL PLANES PRESENTING (A) PERIOD 1: FETAL KIDNEY (TRACE) WITH THICKENED AND HYPERECHOIC CORTICAL, NO CORTICOMEDULLAR DEFINITION, RENAL PELVIS (DOTTED TRACE) WITH "MUSHROOM" SHAPE FILLED WITH ANECHOIC AND DILATED CONTENTS; (B) PERIOD 2: FETAL KIDNEY (TRACE) WITH THIN CORTICAL TENDING TO HYPOECHOIC, PART OF MEDULLA - HYPOECHOIC, PRESENCE OF THE BEGINNING OF FORMATION OF CORTICOMEDULLAR DEFINITION AND RENAL PÉLVIS (DOTTED TRACE) IN TUBULAR FORMAT FILLED WITH ANECHOIC AND DILATED CONTENTS; (C) PERIOD 3: FETAL KIDNEY (TRACE) WITH THIN CORTICAL AND HYPOECHOIC, PRESENCE OF CORTICOMEDULLAR DEFINITION AND RENAL PELVIS (DOTTED TRACE) IN THE SHAPE OF A CANALICULUS FILLED WITH ANECHOIC CONTENT AND SLIGHTLY DILATED; (D) PERIOD 4: FETAL KIDNEY (TRACE) PRESENTS AN IMAGE SIMILAR TO THE ADULT ORGAN (KONDE, 1985; WOOD & MCCARTHY 1990): THIN CORTICAL AND HYPO/ISOECHOIC, WITH CORTICOMEDULLAR DEFINITION AND WITHOUT DILATION OF THE RENAL PELVIS.



SUBTITLE: S, stomach; L, liver.

TABLE 1 shows gestational ages (in days until delivery and estimated days of pregnancy), average, standard deviation, and median from four periods of development of canine fetal kidney.

TABLE 1. DESCRIPTIVE STATISTICS OF THE FOUR PERIODS OF ULTRASONOGRAPHIC EVALUATION OF CANINE FETAL KIDNEY DEVELOPMENT, CORRELATED TO FETAL AGE DETERMINED BY COUNTING BACKWARD CONSIDERING BIRTH AS DAY ZERO OF 15 BITCHES AND 66 FETUSES ANALYZED.

Periods of ultrasonographic development of fetal kidney	Gestational age (days from delivery)	Mean	Median	Standard Deviation	Estimated Gestational Age (days of pregnancy)
Period 1	20 - 24	23	23	2	39 – 43
Period 2	16 - 20	18	18	2	43 – 47
Period 3	11 - 15	13	14	1	48 – 52
Period 4	1 - 5	3	3	1	57 – 62

SUBTITLE: Period 1: fetal kidney with thickened and hyperechoic cortical, no corticomedullar definition, renal pelvis with "mushroom" shape filled with anechoic and dilated contents; Period 2: fetal kidney with thin cortical tending to hypoechoic, part of medulla - hypoechoic, presence of the beginning of formation of corticomedullar definition and renal pelvis in tubular format filled with anechoic and dilated contents; Period 3: fetal kidney with thin cortical and hypoechoic, presence of corticomedullar definition and renal pelvis in the shape of a canaliculus filled with anechoic content and slightly dilated; Period 4: fetal kidney presents an image similar to the adult organ (Konde, 1985; Wood & McCarthy, 1990): thin cortical and hypo/isoechoic, with corticomedullar definition and without dilation of the renal pelvis.

Statistical analysis revealed a normal distribution only for the diameter of fetal renal pelvis (*p-value* = 0.0594). Descriptive statistics (minimum and maximum, average, median and standard deviation) of fetal renal length and fetal renal pelvis diameter during four ultrasonographic periods of development of the canine fetal kidney are reported in TABLE 2.

Pearson's correlation coefficient between gestational age (in days until delivery) and fetal kidney length was r = 0.8926, indicating that the relationship between these variables is strong and positive. Confirming that as parturition approaches kidney length increases. However, coefficient between gestational age (in days until delivery) and fetal renal pelvis diameter was r = - 0.3558, indicating that this ratio is moderately weak and negative, in other words, reduction of the diameter of the fetal renal pelvis has little relationship with the proximity of delivery.

TABLE 2. DESCRIPTIVE STATISTICS OF KIDNEY LENGTH AND RENAL PELVIS DIAMETER OF THE CANINE FETUS, IN CENTIMETERS, ACCORDING TO FOUR ULTRASONOGRAPHIC PERIODS OF CANINE FETAL KIDNEY DEVELOPMENT.

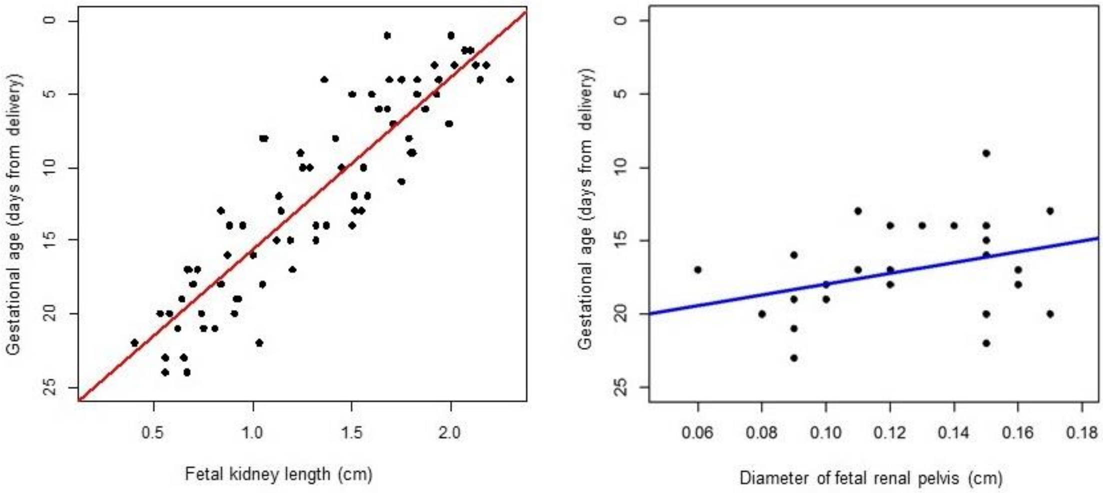
Gestational age (days from delivery) and measured structure	Min-Max	Mean	Median	Standard Deviation
Period 1: 20 to 24 days				
Kidney length	0.40-1.03	0.68	0.65	0.17
Diameter of the pelvis	0.08-0.17	0.12	0.09	0.04
Period 2: 16 to 20 days				
Kidney length	0.53-1.20	0.80	0.75	0.17
Diameter of the pelvis	0.06-0.17	0.11	0.10	0.03
Period 3: 11 to 15 days				
Kidney length	0.84-1.75	1.29	1.32	0.27
Diameter of the pelvis	0.11-0.17	0.14	0.14	0.02
Period 4: 1 to 5 days				
Kidney length	1.36-2.30	1.89	1.93	0.25
Diameter of the pelvis	-	-	-	-

SUBTITLE: Period 1: fetal kidney with thickened and hyperechoic cortical, no corticomedullar definition, renal pelvis with "mushroom" shape filled with anechoic and dilated contents; Period 2: fetal kidney with thin cortical tending to hypoechoic, part of medulla - hypoechoic, presence of the beginning of formation of corticomedullar definition and renal pelvis in tubular format filled with anechoic and dilated contents; Period 3: fetal kidney with thin cortical and hypoechoic, presence of corticomedullar definition and renal pelvis in the shape of a canaliculus filled with anechoic content and slightly dilated; Period 4: fetal kidney presents an image similar to the adult organ (Konde, 1985; Wood & McCarthy, 1990): thin cortical and hypo/isoechoic, with corticomedullar definition and without dilation of the renal pelvis.

FIGURE 5 shows a scatterplot between gestational age in days until delivery by fetal kidney length and gestational age in days until delivery against diameter of the fetal renal pelvis.

From FIGURE 5 it can be concluded that when gestational age, as estimated by canine fetal kidney length, is plotted against days until delivery the points are uniformly close to the line but that on the same plot for gestational age estimated by diameter of the fetal renal pelvis, the points are scattered in relation to the line. These observations indicate that there is a strong correlation between increased size of the fetal kidney and time to delivery, and a weak correlation between time to delivery and reduction in diameter of the fetal renal pelvis.

FIGURE 5. SCATTERPLOT INDICATING THE DEGREE OF CORRELATION BETWEEN GESTATIONAL AGE AND CANINE FETAL KIDNEY LENGTH AND THE DEGREE OF CORRELATION BETWEEN GESTATIONAL AGE AND CANINE FETAL RENAL PELVIS DIAMETER.



Considering the strong correlation ($r = 0.8925$) between gestational age and canine fetal kidney length, a simple linear regression model was adjusted with a slope of -11.77 ± 0.691 ($p < 2.10^{-16}$) and an intercept (point that line intersects y-axis) of 27.41 ± 0.976 ($p < 2.10^{-16}$), the following equation was obtained:

Gestational age (days from delivery) = 27.414 – 11.771 x Kidney length (cm)

This predictive equation has a high accuracy of 80%, sensitivity of 90% and specificity of 70%, demonstrating that this is a reliable model for estimating gestational age. However, TABLE 3 shows the accuracy, sensitivity, and specificity of the equation in the four periods of ultrasonographic development of canine fetal kidney demonstrating in which period the adjusted model best estimates gestational age.

According to TABLE 3, the highest accuracy, sensitivity, and specificity for the equation occurs in period 3. This will be the best time at which to estimate gestational age.

TABLE 3. ACCURACY, SENSITIVITY AND SPECIFICITY OF THE EQUATION FOR ESTIMATING GESTATIONAL AGE (IN DAYS FOR DELIVERY) IN FOUR PERIODS OF ULTRASONOGRAPHIC DEVELOPMENT OF THE CANINE FETAL KIDNEY.

Periods of ultrasonographic development of fetal kidney	Accuracy (%)	Sensitivity (%)	Specificity (%)
Period 1	50.00	50.00	66.67
Period 2	79.41	99.90	70.59
Period 3	99.98	99.95	99.92
Period 4	38.24	50.00	58.82

SUBTITLE: Period 1: fetal kidney with thickened and hyperechoic cortical, no corticomedullar definition, renal pelvis with "mushroom" shape filled with anechoic and dilated contents; Period 2: fetal kidney with thin cortical tending to hypoechoic, part of medulla - hypoechoic, presence of the beginning of formation of corticomedullar definition and renal pelvis in tubular format filled with anechoic and dilated contents; Period 3: fetal kidney with thin cortical and hypoechoic, presence of corticomedullar definition and renal pelvis in the shape of a canaliculus filled with anechoic content and slightly dilated; Period 4: fetal kidney presents an image similar to the adult organ (Konde, 1985; Wood & McCarthy, 1990): thin cortical and hypo/isoechoic, with corticomedullar definition and without dilation of the renal pelvis.

1.4 DISCUSSION

Developments in ultrasonographic technology have increased the number of publications detailing the specific prenatal anatomy of each organ in dogs and cats (Topie et al, 2015; Gil et al, 2015; Gil et al, 2015). Studies detailing specific prenatal renal anatomy in man have been published since 1981 (Lawson et al, 1981). Knowledge of prenatal renal development in people allowed the use of renal biometry as an additional method for estimation of fetal age and to identify congenital kidney diseases (Devriendt et al, 2013; Dias et al, 2014). Based on these references, our objective was to precisely determine ultrasonographic patterns of canine kidney development.

This research has shown that the ultrasonographic appearance of canine fetal kidneys can be divided into four distinct periods (TABLE 1 and FIGURE 4). The description of these periods established the relation between renal size and fetal age, and that the period when this association is most accurate is period 3 (corresponding to 48 to 52 days of pregnancy). In period 1 (39 to 43 days of pregnancy - FIGURE 4A) it is easy to identify that the renal pelvis is dilated and "mushroom" shaped. However, there is no good statistical measure correlating reduction of renal pelvis diameter and gestational age.

In this study canine fetal kidneys were first visualized with ultrasound between 39 and 43 days of pregnancy (TABLE 1, FIGURE 4A), one day before the date previously reported in the literature (England et al, 1990). At this early stage, it is not possible to identify the medullary region, only the dilated renal pelvis in a "mushroom" shape that probably corresponds to an undifferentiated stage of metanephros; a stage in which no calyx is formed and the papillary duct drains fluid direct to renal pelvis (Sinowatz, 2010).

With the development of nephrons and collecting ducts, the kidney becomes divided into cortical and medullary regions, with the cortex mainly composed of renal corpuscles and contorted tubules, whilst the medulla consists of Henle loops and collecting ducts (McGeady et al, 2017). Thus, the identification of these characteristics could be made ultrasonographically in periods 2, 3 and 4 (FIGURE 4B, 4C, and 4D) of canine fetal kidney development from day 43 of pregnancy (TABLE 1). Variations in the renal morphology visualized with ultrasound are probably due to differences in ureteric bud branching and the nephron arrangements associated with these branches (Sinowatz, 2010).

The ability to differentiate between periods 2, 3 and 4 is closely related to the degree of renal pelvis dilatation and, as pregnancy progresses it is possible to monitor the gradual reduction in dilatation until it is no longer visible (FIGURE 4). The start of corticomedullary differentiation associated with dilatation of the renal pelvis into "tubular" shapes marks the beginning of period 2, which occurs between 43 to 47 days of pregnancy (TABLE 1, FIGURE 4B).

Care should be taken when the fetal kidney is evaluated in period 3, as there is only a small difference in degree of fetal renal pelvic dilation compared to period 4 (TABLE 2, FIGURE 4C and 4D), but this differentiation is easier to identify if it is correlated with ultrasonographic development of fetal intestine (Phase 3 and 4) ie the intensity of intestinal motility (Gil et al, 2015). It is helpful to use both parameters as the gestational age of the fetal kidney in development period 3 (48 to 52 days of pregnancy) is similar to the gestational age of intestinal development in phase 3 (48 to 55 days of pregnancy) (Gil et al, 2015).

It is noteworthy that period 3 is longer than the other periods (TABLE 1). This prolongation may lead the ultrasonographer to make errors when estimating the gestational age if ultrasonographic gestational monitoring is not done with care. Thus, the equation should be used at this time, as its accuracy for estimating gestational age is 99% and the standard deviation is ± 0.7 days.

Period 4 represents gestational age from 57 to 62 days of pregnancy (TABLE 1) and during this phase the fetal kidney has a similar ultrasonographic appearance (FIGURE 4D) to that in the healthy adult dog (Konde, 1985; Wood & McCarthy, 1990): the cortex is thin and hypo/isoechoic compared to the liver, there is a well-defined corticomedullary junction and there is no dilatation of the renal pelvis. It is worth remembering that kidneys were examined in the longitudinal plane and perhaps this may have made it harder to visualize discrete dilatations, therefore it is recommended that pelvic dilation in the fetus should be evaluated in the longitudinal plane and not in transverse orientation as for adult dogs (D’Anjou & Penninck, 2015; Nyland et al, 2015).

The authors believe that the fetal kidney in period 4 (57 to 62 days of pregnancy) appears similar to a postnatal kidney, but the ultrasonographic image of fetal kidneys does not allow us to infer anything about functionality. In fact, induction of new nephrons continues until the eighth day of a puppy's post-partum life, and the anatomical appearance of the developing post-partum kidney differs from that of the adult dog kidney (Eisenbrandt & Phemister, 1979).

This study provides verification that the echogenicity of normal fetal kidneys varies during several stages of pregnancy and that in human fetuses this differentiation also occurs at various ages (Hershkovitz et al, 2011). In human fetuses, the clinical significance of differences in renal cortical echogenicity is variable and can occur in both normal kidneys as well as pathological conditions (Devriendt et al, 2013; Hershkovitz et al, 2011).

Canine fetal kidneys can be easily identified in transverse and dorsal planes and subtle movements of laterality must be performed so that organs can be visualized simultaneously. It is always possible to evaluate both kidneys in all canine fetuses, however, only the kidney that was located most ventrally or in the center of the screen kidney was measured at each time point, and always in a longitudinal plane (FIGURE 2 and 3). To optimize visualization for measurement it is essential to view the maximal length of the kidney in a longitudinal plane.

Fetal kidneys (right and left), as in healthy adult dogs, appear to be similar in size to one another (Barella et al, 2012). It is reported that in human fetuses there may be unequal growth of contralateral kidneys (Duval et al, 1985). The size of both kidneys was not compared in this study, however, further investigation is warranted in future research.

It was possible to determine the development of normal fetal kidney length and renal pelvis diameter during four periods of ultrasonographic development of canine fetal kidneys (TABLE 2). Fetal kidney length in human fetuses can be used to diagnose congenital or non-congenital nephropathies associated with fetal developmental abnormalities (Devriendt et al, 2013; Dias et al, 2014). In dogs, there are no reports on the diagnosis of nephropathies in fetuses, however, standardization of fetal kidney size as performed in this study may eventually allow the identification of nephropathies in future cases.

As expected, increase in canine fetal kidney size was highly correlated ($r = 0.8926$) with advancing gestational age, showing that as gestation progresses fetal kidneys grows proportionally. This event allowed the creation of a linear regression model that accurately estimates gestational age from the 5th week of gestation. This model has high accuracy and high sensitivity and a standard error of approximately 0.7 days thus providing another tool that can be used with confidence to estimate gestational age.

The accuracy of this model was tested at each fetal kidney development period to show the best timing for its use. The test had highest accuracy and sensitivity between the 6th and 7th weeks of pregnancy (period 3), and it is at this age that the ultrasonographer often loses the ability to estimate gestational age using organogenesis, as all organs are developed by then. Therefore, the most reliable way to estimate the date of delivery at this age may be by using this model in conjunction with biparietal diameter equations for days until delivery (Beccaglia & Luvoni, 2012).

Several studies have investigated the use of measurements of biparietal diameter to estimate gestational age or time until delivery. Beccaglia and Luvoni (2012) and Beccaglia et al. (2016) describe that the accuracy of these formulae decreases from 95.4% in the 5th week of gestation to 85.3% in the 8th week, so it is proposed that the kidney measurement formula is used simultaneously, since in period 3 between 6th and 7th week this formula has an accuracy and sensitivity of

99% (TABLE 3). However, the accuracy of the kidney model has to be tested in routine daily practice.

The absence of fetal renal pelvic dilation on ultrasonographic examination confirms the end of fetal organ development (organogenesis), but the presence of intestinal motility is an important parameter that can further assist the ultrasonographer in ageing fetuses in this time period. To detect the time of delivery and/or to plan a caesarean section, daily ultrasonographic monitoring from detection of the 4^{th} developmental period of kidney and 4^{th} developmental phase of bowel intestine (Gil et al, 2015) is advised, because from that age fetal heart rate oscillations (Gil et al, 2014) associated with umbilical cord resistivity index (Giannico et al, 2015; Giannico et al, 2016) should be monitored.

There are a number of limitations to this research, the experimental sample was small and the measurement of fetal kidney and pelvis diameter in the transverse plane was not performed. This information, coupled with measurements in the longitudinal plane would allow standardization of canine fetal kidney volume at different gestational ages. The bitches in this study represented a variety of different breeds and weights, so when using the equation to estimate gestational age there were differences between estimated ages in small and medium size breeds as occurs with the formulae for biparietal diameter (Beccaglia & Luvoni, 2012; Beccaglia et al, 2016).

1.5 CONCLUSIONS

Ultrasonographic evaluation of fetal kidney development is simple to perform. This study had shown that there is a strong correlation between gestational age and kidney length, allowing generation of an equation to estimate the date of delivery, with a high sensitivity between 48 to 52 days (or between 6^{th} and 7^{th} week) of pregnancy. The model using the fetal kidney length measurement has to be used as a complementary method to estimate gestational age and predict delivery time with high accuracy.

2. CHAPTER II. TWO-DIMENSIONAL ULTRASONOGRAPHIC ASSESSMENT OF THE CANINE FETAL THYMUS: CHARACTERIZATION AND LOCALIZATION

Two-dimensional ultrasonographic assessment of the canine fetal thymus: Characterization and Localization

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Abstract

To date there have been no studies that describe the ultrasonographic appearance and location of the thymus in canine fetuses. The aims of this prospective and observational study were to describe the normal ultrasonographic appearance and location of the canine fetal thymus correlated with gestational age, to determine the accuracy of ultrasonographic examination of the thymus and to try to explain why it was not possible to identify a fetal thymus in any fetuses in some pregnant bitches. Ultrasonographic examinations were performed on 26 clinically healthy bitches every three days from the 40th day of pregnancy until delivery. The heart was used as an anatomical guide for identification of the thymus, thus, fetuses needed to be positioned for a cardiac examination. The thymus was first visible ultrasonographically around 48-52 days of gestational age or 8-13 days before delivery and continues to be visible, with no changes in morphology, until delivery. The fetal thymus appeared as a heterogeneous structure, with hypo and hyperechoic spindle-shaped lines interspersed in the parenchyma and was delimited by hyperechoic interfaces. It had a triangular shape in the dorsal plane and a rectangular shape in the transverse plane and was always located in the cranial mediastinum. However, the thymus could not be identified in any fetuses in English Bulldog and English Bull Terrier bitches with a body condition score of 4 or 5 weighing > 22 kg. The statistical method applied in this study proved that the main reason for not identifying the canine intrauterine fetal thymus is a high body condition score of the bitch.

Keywords: dog, pregnancy, ultrasonography, gestational age, expected development, fetal thymus

2.1 INTRODUCTION

The thymus is a lymphoepithelial organ that plays an important role in the maturation of the immune system. Evaluation of the thymus is important in human medicine for predicting the immunologic status before and after birth. Furthermore, thymic hypoplasia in human fetuses has been reported in a number of syndromes, due to exposure to toxic substances, and in immunodeficiency disorders (Linch et al, 1984; Ewald & Frost, 1987; Driscoll et al, 1991; Akar et al, 2002).

The mammalian thymus is located in the pericardial mediastinum, anterior to the major vessels of the heart, and ventral to the base of the heart and aortic arch (Haley, 2003). In man, the thymus is described as sonographically hypoechoic to the thyroid and isoechoic to the liver. The echotexture is homogeneous or finely heterogeneous. There are multiple linear focal echogenic structures scattered throughout the thymic parenchyma, which correspond to the septa and blood vessels (Felker et al, 1989; Zalel et al, 2002; Cho et al, 2007).

The canine thymus has not previously been described in an antenatal ultrasonographic examination. Our hypothesis is that the fetal thymus can be assessed by ultrasonographic examination in dogs. Thus, the aims of this paper were threefold: (1) to show that it is possible to evaluate the canine fetal thymus describing its normal ultrasonographic appearance and correlate this with gestational age; (2) to determine the accuracy of the ultrasonographic examination in the detection and evaluation of the canine fetal thymus; (3) to try explain the inability to identify the fetal thymus ultrasonographically in some pregnant bitches.

2.2 MATERIALS AND METHODS

2.2.1 ANIMAL POPULATION

The study was approved by the Institutional Animal Use Committee guidelines and performed between February 2016 and March 2017. Using a prospective observational study design, fetuses in clinically healthy bitches were evaluated to determine the ultrasonographic appearance of the thymus. Inclusion criteria were: bitches available for serial evaluation, bitches with initial gestational age < 45 days, and bitches in which the owner was able to predict the date of delivery. The fetuses also needed to be healthy with no morphological changes in their development. The characteristics of pregnant bitches (breed, weight and body condition score) are shown in TABLE 1. Body condition score (BSC) in bitches was classified from 1 to 5, where score 1 is very thin, 3 is the ideal score and score 5 is obese.

TABLE 1. DESCRIPTION OF CHARACTERISTICS (BREED, WEIGHT AND BODY CONDITION SCORE) OF PREGNANT BITCHES.

Bitch	Breed	Weight (Kg)	Body Condition Score	Bitch	Breed	Weight (Kg)	Body Condition Score
1	Maltês	3	2	**14**	Miniature Schnauzer	7	4
2	Chinese Crest	4	3	**15**	Pekingese	5	3
3	Chinese Crest	5	3	**16**	English Bulldog	24	4
4	Miniature Schnauzer	6.5	3	**17**	English Bulldog	24	4
5	Siberian Husky	15	4	**18**	English Bulldog	22	4
6	Miniature Schnauzer	7	3	**19**	English Bulldog	26	5
7	French Bulldog	6	3	**20**	English Bulldog	26	5
8	Boxer	15	1	**21**	English Bull Terrier	23	4
9	Pug	6	3	**22**	English Bull Terrier	24	4
10	Pug	8	5	**23**	English Bull Terrier	25	4
11	Yorkshire Terrier	3	2	**24**	English Bull Terrier	22	4
12	Chihuahua	3	2	**25**	English Bull Terrier	25	5
13	Miniature Schnauzer	5.5	3	**26**	English Bull Terrier	27	5

2.2.2 B-MODE ULTRASONOGRAPHIC EQUIPMENT AND TECHNIQUES

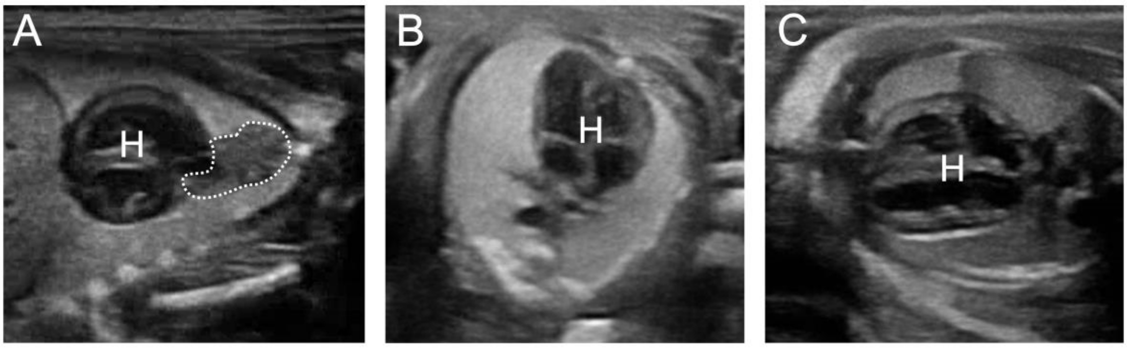
Two-dimensional ultrasonographic evaluations were carried out using two ultrasonography machines: MyLab™30Gold VET (Esaote, Genova, Italy) and Logiq 3 (Wipro GE Healthcare Provate LTD, Bangalore, India); both equipped with high resolution multifrequency linear transducers of 7.5 to 12 MHz and 7 to 12 MHz respectively. The bitches were restrained in dorsal recumbency using a sponge trough, abdominal hair was clipped, and acoustic gel was applied to the transducer to optimize the ultrasonographic image. The gain, focus, and other adjustments were made during the examination to optimize the image for each fetus. The same examination protocol was used in all bitches and the ultrasonographic images were acquired in a clockwise circle as described by Gil et al. (2014).

2.2.3 ESTABLISHMENT OF GESTATIONAL AGE AND ULTRASONOGRAPHIC EXAMINATIONS

Ultrasonographic evaluations in pregnant bitches occurred from the 40th day after the first mating or insemination (as reported by the owners) every three days. On each visit fetal organogenesis was assessed and gestational age estimated (Yeager et al, 1992; Gil et al, 2015; Gil et al, 2018). From the first day of visualization of the fetal thymus, ultrasonographic assessments continued to be done every three days to identify possible changes in the organ's appearance until delivery. Gestational age was confirmed after delivery, in days to delivery, by counting backward (delivery as day zero). The normal gestational length was considered to be 57 to 63 days, due to the variable pro-estrus and estrus periods in bitches (Concannon, 1983). Measurement of hormonal concentrations were not made.

One experienced ultrasonographer (a specialist from the Brazilian College of Veterinary Radiology and Federal Council of Veterinary Medicine) and an experienced cardiologist (a member of the Brazilian Society of Cardiology) performed the ultrasonographic examinations and were responsible for image acquisition throughout the study. Recordings of ultrasonographic studies, of the fetal thorax, were obtained using images in which the fetus was positioned in dorsal (FIGURE 1A) and transverse (FIGURE 1B and 1C) recumbency.

FIGURE 1. ULTRASONOGRAPHIC IMAGES OF FETAL THORAX SHOWING THE CARDIAC STRUCTURES IN TRANSVERSE PLANE: A - SHORT-AXIS AT SITE OF PAPILLARY MUSCLE, INITIAL DETECTION OF THYMUS – DOT DELIMITED; IN LONGITUDINAL PLANE: B - FOUR-CHAMBER VIEW AND C - LONG-AXIS VIEW.



SUBTITLE: H, HEART.

2.2.4 IDENTIFICATION OF THE CANINE FETAL THYMUS

Ultrasonographic identifications of the thymus were made in as many fetuses as possible in each bitch, and in at least four fetuses from each pregnant bitch. When the fetus was identified by ultrasonographic examination and was in the ideal position for evaluation, the fetal thorax was assessed in the dorsal (FIGURE 1A) and transverse (FIGURE 1B and 1C) planes. All fetuses needed to be positioned for a cardiac examination because the heart was used as an anatomical guide for the identification of the thymus.

When the fetus was positioned correctly, the fetal cardiac window was created in transverse (short-axis at the site of the papillary muscle view – FIGURE 1A) and longitudinal (four-chamber view and long-axis view – FIGURE 1B and 1C) views in real-time, as described by Giannico et al., (2015). After the identification of the fetal heart, dedicated "fan” movements were made with the transducer to identify the thymus. When the thymus imaging view appears, the heart is no longer visible. The fetal thymus was then examined.

2.2.5 STATISTICAL ANALYSIS

Statistical analysis was used to describe and analyze the data from the thymus view in fetuses from 26 bitches. A descriptive analysis of the response variable (dependent) "thymus view" ("yes" and "no") was described in relation to the independent variables. The independent variables analyzed were: breed, age (years), weight (kg) and BCS. A regression dichotomous logistics was employed to verify the relationship between those variables. Logistic regression models were adjusted considering the dichotomous response "thymic viewing fetuses" as 1 (yes) and 0 (no), considering as explanatory variables, breed, age, weight and BCS of bitches. R software [free distribution i386 version 3.1.2 which can be found in www.r-project.org in the AOD (Overdispersed Analysis of Data) packet] was used for analysis.

2.3 RESULTS

The thymus in all fetuses became visible at a gestational age of 48-52 days, or 8-13 days before delivery and continued to be visible with no change to its morphological characteristics until the time of delivery. The structure was identified in bitches 1 to 15, but not in bitches 16 to 26. The bitches in which fetal thymuses could not be identified were all English Bulldog and English Bull Terrier breeds, with BCS 4 or 5 and weighed > 22 kg.

The fetal thymus appeared as a structure with heterogeneous echotexture with spindle-shaped hypo and hyperechoic lines interspersed in the parenchyma and was delimited by hyperechoic interfaces that allowed its differentiation from the adjacent lungs. The lung and the fetal cardiac silhouette were the anatomical referral points for identification of the thymus. Thus, it was easily found in dorsal views (FIGURE 1A), but less easy to identify in both transverse views (FIGURE 1B and 1C).

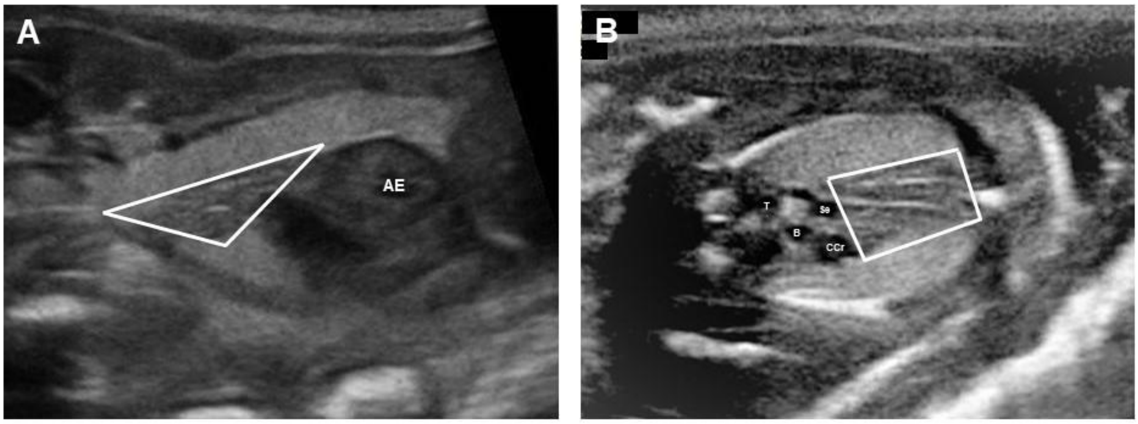
To view the entire organ in the dorsal plane, slight lateral slips with the transducer were performed to the left side of the fetus, to isolate the fetal thymus by removing part of the heart from the ultrasonography screen and avoiding the places where the bones of fetal ribs, sternum and/ or vertebrae create posterior acoustic shadowing. The fetal thymus has a triangular shape and was located in the cranial mediastinum extending to the base of heart, supported by the pericardium of the left cardiac chambers and surrounded by the left lung lobes. However, the pericardium and the lung lobe fissures are not clearly defined on ultrasonographic images. (FIGURE 2A).

To identify the fetal thymus in the transverse plane the four-chamber and long-axis heart views were made (FIGURE 1B and 1C). The ultrasonographer then moved the transducer in a cranial direction along the fetus until the heart was no longer seen and the thymus appears. The places where fetal rib bones create a posterior acoustic shadowing artifact should be avoided. In this section, the parenchyma has a rectangular shape and is located in the cranial mediastinum, between the sternum and three thoracic vessels: cranial vena cava, brachycephalic trunk and left subclavian artery; laterally surrounded by both lungs (FIGURE 2B).

TABLE 2 shows the estimates of the parameters of the logistic regression model adjusted to BCS data and visualization, or not, of the thymus. Note that the explanatory variable "BCS" is significant at a level of 0.05 and 0.01 of probability. The probability of visualization of fetal thymus is increased when the BCS is lower

(FIGURE 2). The probabilities have been adjusted according to BCS, and the model deviation was D(y:μ) = 16.38 (with 24 degrees of freedom), indicating an appropriate adjustment.

FIGURE 2. ULTRASONOGRAPHIC IMAGES OF FETAL THORAX DEMONSTRATING: A - FETAL THYMUS IN DORSAL PLANE WITH A TRIANGULAR SHAPE AND LOCATED ADJACENT TO, AND SUPPORTED BY, THE PERICARDIUM OF LEFT CARDIAC CHAMBERS AND SURROUNDED BY THE LUNGS; AND B - FETAL THYMUS IN TRANSVERSE PLANE SHOWING A RECTANGULAR SHAPE AND LOCATED BETWEEN GREAT VESSELS AND STERNUM AND SURROUNDED LATERALLY BY THE LUNGS. THE THYMUS HAS A HETEROGENEOUS ECHOTEXTURE WITH SPINDLE-SHAPED HYPO AND HYPERECHOIC LINES INTERSPERSED IN THE PARENCHYMA AND IS DELIMITED BY HYPERECHOIC INTERFACES.



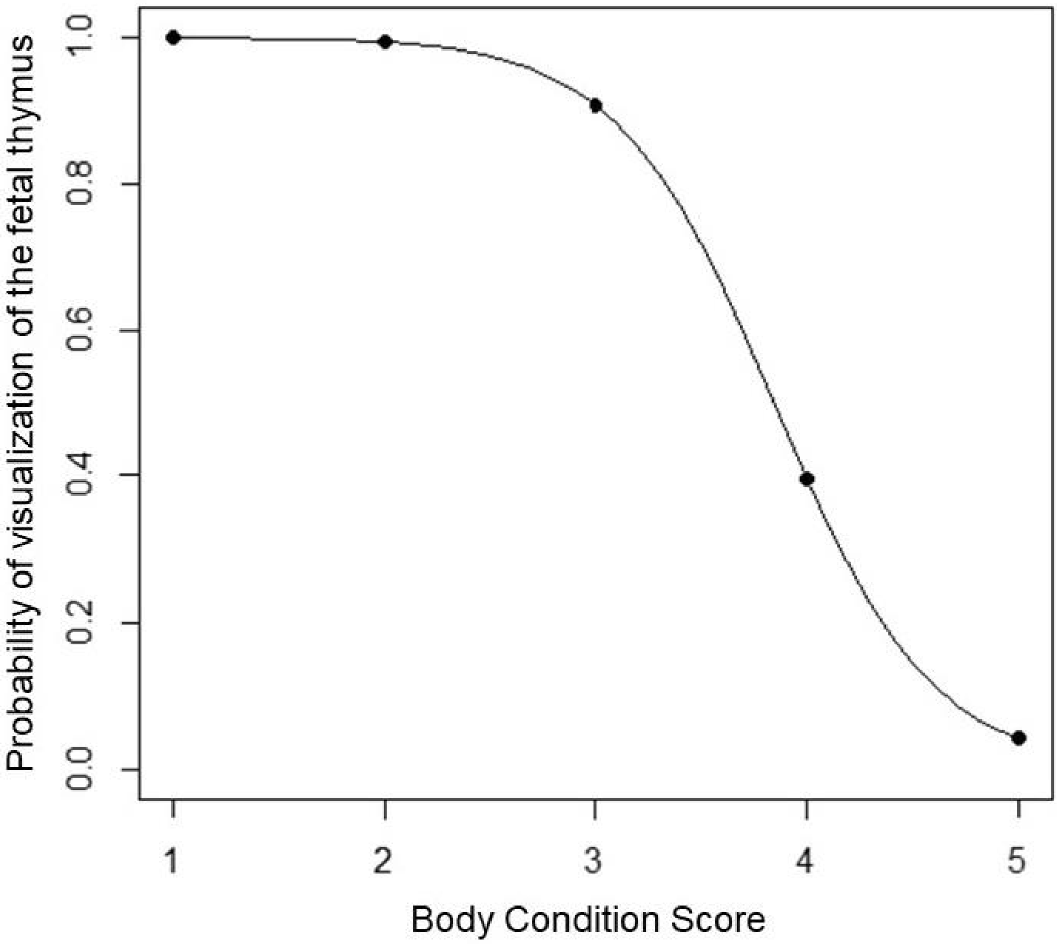
SUBTITLE: AE, left atrium; CCr, cranial vena cava; B, brachycephalic trunk; Se, left subclavian artery; T, trachea.

The probability of viewing the thymus is very high for bitches with scores 1 (99.95%) and 2 (99.31%), while when the BCS of the bitch is 3, the probability decreases to 90.67% but is still high. At BCS 4 the probability of thymic visualization decreases to 39.52%, and finally, when the score is 5, viewing probability is only 21.4% (FIGURE 3). Except for the BCS, the other variables were not significant, so they are not required to explain the thymus view in fetuses and were not included in the model.

TABLE 2. ESTIMATES OF THE LOGISTIC MODEL FITTED TO THE DATA ON THE FETAL THYMUS VISUALIZATION.

Effect	***Parameter***	***Estimate***	***Standard error***	***Z***	***P value***
Intercept	β_0	10.370	4.130	2.511	0.0120
Body Condition score	β_1	-2.699	1.068	-2.528	0.0115

FIGURE 3. GRAPH SHOWING THE PROBABILITY OF VISUALIZING CANINE FETAL THYMUS IN RELATION TO THE BCS OF THE BITCH.



2.4 DISCUSSION

High-resolution ultrasonography devices have enabled the capture of detailed images of the development of canine fetal organs such as bowel, lungs, kidneys (Gil et al, 2015; Banzato et al, 2017; Gil et al, 2018) and, in cats, the description of the specific prenatal anatomy of several organs (Topie, 2015). We believe that the high resolution of ultrasonography equipment now allows sufficient refinement of the image to provide details and analysis of canine fetal structures not previously described. In this study we show that it is possible to identify the canine intrauterine fetal thymus using ultrasonography.

In human medicine, pioneering research on the ultrasonographic appearance and location of the fetal thymus was described 30 years ago (Felker et al, 1989). However, it was not until the 2000's that there was a significant interest in studying the fetal thymus. This was primarily due to the development of ultrasonography devices that allowed operators to evaluate, describe and measure this organ more precisely (Zalel et al, 2002; Machlitt et al, 2002) but also due to an increasing recognition of the relationship of thymus to development of fetal immune system (Sciaky-Tamir et al, 2015).

The identification of the canine fetal thymus was possible in late pregnancy (48-52 days of pregnancy or 8-13 days before delivery) and the appearance was unchanged until the time of delivery. Normal thymopoiesis occurs at 45 days of pregnancy and at this time the distribution of subsets of thymocytes is virtually identical to those of the postnatal thymus. At this stage of development the cellularity of the fetal thymus changes which may explain why it is more easily seen on ultrasonography (Felsburg, 2002).

Visualization of the fetal thymus defines the beginning of eighth week of pregnancy, at this stage the fetus is bigger, thoracic structures are better defined, lungs are brighter ultrasonographically making the identification of the thymus easier. All of these factors contribute to, and facilitate, the identification of the thymus on ultrasonographic examination as described in the human fetus (Cho et al., 2007).

For accurate identification of the fetal thymus, correct device's settings are essential. In particular the focus and gain must be optimized to allow differentiation of the contrast between adjacent structures. In the canine fetus the hyperechoic lung was the structure adjacent to the thymus that allowed the delimitation of the thymus due to an evident difference in echogenicity, similar to the findings reported in human fetuses (Felker et al, 1989).

It is considered important to analyze the thymus in human fetuses because some authors believe that the reduction in the size of fetal thymus, identified by ultrasonographic examination can be a marker for the detection of maternal-fetal diseases such as: intrauterine growth restriction (Olearo et al, 2012), genetic diseases such as microdeletion 22q (Bataeva et al, 2013) and trisomy 21, 18 and 13 (Karl et al, 2012), congenital heart defects (Li et al, 2011), histological chorioamnionitis (Aksakal et al, 2014) and pre-eclampsia (Mohamed et al, 2011). Based on these references, we chose to describe the appearance, shape and location of the canine fetal intrauterine thymus according to the findings of these reports. It was hoped that it may be possible in future to analyze the relationship of the thymus shape at routine prenatal ultrasonographic examination on fetal development in dogs. Changes in thymic shape may be correlated with congenital diseases, immune development of the canine fetus and gestational age.

The canine fetal thymus has a heterogeneous echotexture with spindle-shaped hypo and hyperechoic lines interspersed in the parenchyma and it is delimited by hyperechoic interfaces that facilitate its differentiation from adjacent lungs (FIGURE 2). The appearance of the canine fetal thymus remains unchanged until the end of gestation, which is in contrast to what has been reported in human fetuses in which the fetal thymus tends to change its echogenicity as pregnancy progresses, probably due to changes related to development (Felker et al, 1989).

The location of canine fetal thymus reported here was similar to that described in human fetuses: cranial mediastinum, adjacent to thoracic vessels and trachea, supported at the base of heart, and between lungs (Zalel et al, 2002). It was found in two fetal planes (dorsal and transverse - FIGURE 2A and 2B), but with different shapes (triangular and rectangular shapes - FIGURE 2A and 2B) which is similar to the human fetal thymus that has a triangular shape and a rounded quadrilateral in the two planes (Felker et al, 1989). The location in the two planes has been described in similar ways for the canine and human fetal thymus. However, the transverse plane is the most commonly described for identifying the human fetal thymus where it is located between the sternum and the great vessels of the heart (‘the three vessels view’ – pulmonary artery, aorta and superior vena cava, with the trachea to the right of the vessels) (Zalel et al, 2002). This differs from what was reported in this study in which canine intrauterine fetal thymus was viewed more cranially in the mediastinum and found between the cranial vena cava and aortic branches (FIGURE 2B).

In our study, the probability of ultrasonographic identification of the canine fetal thymus in bitches with a BCS > 4 significantly reduces to 39.52% making it highly likely the organ will not be found in these fetuses. The fetal thymus was not found in Bitches 16 to 26 (TABLE 1) and similar reports for failure to identify the thymus in human fetuses are described (Felker et al, 1989; Zalel et al, 2002). It is speculated that, in females with a high BCS which have a thick ventral layer of fatty tissue deposits and possibly enlarged mammary glands, the distance between the fetus and the abdominal wall increases. The subcutaneous fatty tissue deposit in the ventral wall hinders the return of high-frequency ultrasonic waves and reduces the definition of the image, making it almost impossible to make detailed analysis of some intrauterine fetal structures. Also, in these particular pregnant bitches the capacity for use of a lower transducer is more limited, which may reduce the ability to see the thymus, a delicate and small intrathoracic structure. Statistical analysis confirmed the high BCS of pregnant females as the main cause of failure to identify the canine fetal thymus (TABLE 2), even to the population included bitches with a high BCS but weighing < 20 kg (Bitch 5, 10 and 14 - TABLE 1) in which it was possible to identify the canine fetal thymus.

Other explanations have been put forward in several studies with human fetuses and were related to image quality and maternal body habitus (Cho et al, 2007) or maternal obesity (Zalel et al, 2002), as shown in this research. In human fetuses, the explanation for failure to visualize the thymus was related to fetal position and acoustic shadow caused by the proximal clavicle and ribs (Felker et al, 1989), which was not the case in this study because the canine fetal thymus was identified regardless of the fetal positioning, and maneuvers with the transducer were performed so that the shadows of the bones did not obstruct the visualization.

This research had two major limitations which are: absence of large breed bitches in the sample and the small sample size of pregnant bitches with a high percentage of females in which the fetal thymus was not identified. No measurements of the size of the intrauterine fetal thymus were made which would have been useful to help in the identification of possible reduction or increase in size in routine gestational ultrasonographic examinations. Despite this, this study standardizes the ultrasonographic views for the normal canine intrauterine fetal thymus, and describes localization of this organ in the fetal thorax during routine gestational ultrasonographic examination.

In summary, the canine fetal thymus has a similar appearance to that described in studies of the thymus in human fetuses (Cho et al, 2007). It is important not to misidentify this structure in canine intrauterine fetus as it could be confused with a congenital mass or with an abnormally echotextured lung area. The shape of the canine intrauterine fetal thymus makes it difficult to measure the organ, suggesting that additional research is needed to develop methods of measuring this structure. It is harder to identify the thymus in the fetuses of pregnant bitches which have increased ventral body wall fat deposits. Further studies are required to provide normal size measurements and to investigate the usefulness of routine ultrasonographic examination in the evaluation of the canine intrauterine fetal thymus for the diagnosis of congenital or acquired diseases.

2.5 CONCLUSION

The identification of the canine fetal thymus by ultrasonography is feasible in bitches with a BCS < 3 and from 48 to 52 days of pregnancy.

3 CHAPTER III. THE TECHNIQUE OF PERCUTANEOUS ULTRASONOGRAPHIC-GUIDED FINE-NEEDLE ASPIRATION BIOPSY FOR AMNIOTIC FLUID COLLECTION IN PREGNANT BITCHES

The technique of percutaneous ultrasonographic-guided fine-needle aspiration biopsy for amniotic fluid collection in pregnant bitches

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Abstract

Amniocentesis is an invasive diagnostic technique that allows the aspiration of amniotic fluid from the gestational vesicle and can be performed under continuous ultrasonopgraphic guidance. The purpose of this prospective and longitudinal study is to describe the ultrasonographic-guided amniocentesis technique by discussing the indications, limitations, and complications in pregnant bitches. Twenty-one pregnant dogs were included and punctured at least at two gestational ages in a total of 118 punctures. The transducer was placed perpendicular to the bitch's skin and sterile material was used when the pool of amniotic fluid in the gestational vesicle was located. Punctures were performed every five days in a different vesicle at each gestational age, selecting the point closest to the abdominal wall. The freehand technique was used and the needle was introduced laterally to the transducer, so that the transducer and the needle form an angle of 45º with each other. The site needle punction was selected according to the location of the fetus in abdominal cavity. The needle was insert into cranial or caudal region of the gestational vesicle, adjacent to the head or posterior region of the fetus and avoiding the zonary placenta. Amniocentesis was considered successful when the collection of 1 mL of amniotic fluid was aspirated, with a success rate of 49.15%. Learning curves were constructed according to the size of pregnant females and the puncture attempts in each gestational period, regardless of the size, the success rate tends to fall as the pregnancy progresses, being more evident at the end of pregnancy. Relationship between the puncture attempts and the size of the females and the puncture attempts and the gestational age were not statistically significant. Only two bitches (French Bulldog and Schnauzer) presented complications after the second amniocentesis, approximately on the 50^{th} day of pregnancy. The first bitch aborted all the fetuses and the second presented fetal death of the entire litter. Amniocentesis ultrasonographic-guided technique was described, standardized and considered safe with 90.48% of pregnant bitches free from complications.

Keywords: Pregnancy, ultrasonography, invasive diagnostic method, amniocentesis,

3.1 INTRODUCTION

The real-time image formation in B-mode performed by ultrasonography allows the detection of movement associated with the identification of any changes in the organs. The ability to guide a biopsy procedure is an advance in the ultrasonography scanning technique that provides a quick and safe method of obtaining a cytological sample of tissue or abdominal organs, with success and safety being strictly related to direct visualization of the needle during a guided biopsy (Hager et al, 1985; Barr, 1995). The guidance of biopsy needles by ultrasonography has been widely used in canine patients to obtain organ samples for cytological and histopathological evaluation (Crystal et al, 1993; Wood et al, 1998; Nyland et al, 2002; Wang et al, 2004; Britt et al, 2007; da Costa et al, 2008; Watson et al, 2011; Feeney et al, 2013; Pierini et al, 2017; Sumner et al, 2018; Kim et al, 2019; Thiemeyer et al 2019; Fleming et al, 2019).

The invasive diagnostic method that allows the withdrawal of amniotic fluid from the gestational vesicle through a needle transabdominally and under the continuous direction of ultrasonography, in order to obtain a sample is called amniocentesis (Gui et al, 2016). In women, the indications for this procedure vary according to the gestational age since in the first trimester this procedure is suggested for the diagnosis of chromosomal abnormalities or disorders in a single gene, and in the final trimester the recommendations are related to the assessment of lung maturity fetal, microbial invasion of the amniotic cavity and intra-amniotic inflammation (Gordon et al, 2002; Cruz-Lemini et al, 2014).

In veterinary medicine, ultrasonographic-guided amniocentesis has been used in cattle (Garcia & Salaheddine, 1997; Kamimura et al, 1997), equine (Schmidt et al, 1991; Smith et al, 1997; Ripley et al, 2019), goats (Lovell et al, 1995), rodents (Serriere et al, 2006) and canines (Bonte et al, 2017) with several recommendations: to analyze the physiology and biochemical behavior of the amniotic fluid, validation of the technique in several species, in the detection of fetal infection in utero, determination of sex and fetal genotyping, and evaluation of fetal lung maturation. Through another approach, in canines and sheep this technique was commonly described and performed during exploratory laparotomy and after exposure of the uterus (Prestes et al, 2001; Groppetti et al, 2015;

Dall'Ara et al, 2015; Bolis et al, 2017; Veronesi et al, 2018) and not by ultrasonographic-guided transabdominal technique.

To our knowledge, the technique of percutaneous ultrasound-guided amniotic fluid fine-needle aspiration biopsy has not been reported in dogs. Only a recent study reports the collection of ultrasonographic-guided amniotic fluid at the end of pregnancy in female dogs (Bonte et al, 2017), however the detailed description of the technique was not carried out. We hypothesize that amniocentesis is a safe technique to be performed under the aid of ultrasonography and can be recommended at different gestational ages in pregnant bitches. The purpose of this article is to describe the technique, discuss the indications, limitations, and complications of percutaneous ultrasonographically guided biopsy of the canine amniocentesis.

3.2 MATERIALS AND METHODS

3.2.1 ANIMAL SELECTION

A prospective and longitudinal study, approved by the Ethical Committee of the School of Veterinary Medicine of Federal University of Parana with protocol nº. 035/ 2016, was carried out from April/ 2016 to August/ 2017. Twenty-one clinically healthy pregnant bitches of various races, ages, weights, and primiparous or pluriparous from private owners were recruited with the specific objective of validating and standardizing the technique of amniotic fluid biopsy with a fine-needle guided by ultrasonography.

The characteristics of pregnant bitches (breed, age, size, litter size) are shown in TABLE 1. The bitches were classified according to the body weight of each breed according to the Federation Cynologique Internationale, considering as small size bitches with body weight less than 10kg, medium size bitches with body weight between 10-20kg and large size bitches with body weight above 20kg.

TABLE 1. DESCRIPTION OF CHARACTERISTICS (SIZE, BREED, AGE AND LITTLER SIZE) OF THE PREGNANT BITCHES SELECTED TO PERFORM THE GUIDED AMNIOCENTESIS.

Size	Breed	Age	Litter Size
Small	Miniature Schnauzer	03 years	04 puppies
	Miniature Schnauzer	04 years	04 puppies
	Miniature Schnauzer	02 years	05 puppies
	Miniature Schnauzer	02 years	04 puppies
	Miniature Schnauzer	03 years	02 puppies
	Miniature Schnauzer	01 year	03 puppies
	Miniature Schnauzer	02 years	02 puppies
	Miniature Schnauzer	01 year	04 puppies
	Mixed breed	02 years	08 puppies
	French Bulldog	02 years	03 puppies
	French Bulldog	03 years	07 puppies
	French Bulldog	02 years	08 puppies
	French Bulldog	01 year	07 puppies
	French Bulldog	03 years	02 puppies
	French Bulldog	02 years	01 puppy
	French Bulldog	03 years	06 puppies
Medium	Pittbull	03 years	06 puppies
	Australian Cattle	02 years	06 puppies
	Chow-Chow	01 year	05 puppies
Large	Siberian Husky	05 years	08 puppies
	Great Dane	06 years	18 puppies

The exclusion criteria used were: non-pregnant bitches, pregnant bitches unavailable for serial examinations, very early pregnancy (more than 40 days) at the first presentation, and date of delivery not informed by the owners, bitches with abdominal skin disorders and bitches that have an associated systemic infection. Any pregnant bitch that does not fit these exclusion items was considered able to participate in the study.

3.2.2 EXAMINATION METHOD AND ULTRASONOGRAPHIC EQUIPMENT

The bitches were prepared for ultrasonographic examinations: positioned in the supine position under a foam trough, trichotomy of the abdominal region was performed, and ultrasound gel was applied to the abdomen and transducer to improve image acquisition. The examination protocol described by Gil et al, (2014) was used to evaluate the most significant number possible of fetuses in each bitch and the images were formed following a clockwise order in the abdomen of the pregnant bitch.

Two devices were used to perform two-dimensional ultrasonographic examinations and for punctures: MyLab™30Gold VET (Esaote, Genova, Italy) and Philips Affiniti 50G (Philips, Amsterdã, Netherlands), both coupled to high-resolution 7.5 to 12 MHz and 5 to 12 MHz multi-frequency linear transducers respectively. In addition, to optimize the image quality for each individual fetus, adjustments in gain, focus and depth were made when necessary.

3.2.3 DEFINITION OF GESTATIONAL AGE

One experienced operator (10 years) performed all ultrasonographic examinations to consistently ascertain the proper development of conceptuses and their gestational age. For pregnancy diagnosis, ultrasonographic examinations were performed from the 30th day after first mating or insemination (reported by the owners). After the gestational diagnosis of the bitches, ultrasonographic examinations were scheduled on the 40th day of pregnancy to perform the procedures, and the return visits every five days. From the 58th day of pregnancy, daily assessments were made until delivery when clinically necessary.

Gestational age was estimated in all ultrasonographic examinations following the descriptions performed by Yeager et al, (1992) added to identification of fetal intestinal and renal development as reported by Gil et al, (2015) and Gil et al, (2018) respectively; associated with the use of gestational formulas by measuring some fetal parameters (Lopate, 2018). To confirm the gestational age, counting backward was performed using the date of delivery as day zero, in days for delivery, and the standart duration of pregnancy considered was 58 to 63 days of pregnancy due to the particularities of bitch's estrous cycle (Concannon et al, 1983). Thus, the dosage of hormones was not performed.

3.2.4 PREPARATION AND MATERIAL FOR THE BIOPSY PROCEDURE WITH ULTRASONOGRAPHIC-GUIDED FINE-NEEDLE

All procedures were authorized by the owners who were aware of the possible risks and signed a consent form. Chemical containment was not necessary, and the pregnant bitches were contained only physically.

The ultrasonographic evaluation before the procedure was based on a scan of the uterine cavity, in serial transversal and longitudinal views of the bitch's abdomen, to define the location of the placentas, amniotic fluid pool, fetal position and fetal movements. Transplacental needle insertion was avoided.

The sonographer washed his hands with chlorhexidine and used sterile gloves. The dog's abdominal region was cleaned, and asepsis was performed with sterile gaze soaked with chlorhexidine. The transducer was covered with a sterile glove, with gel inside to improve ultrasonic transmission (FIGURE 1).

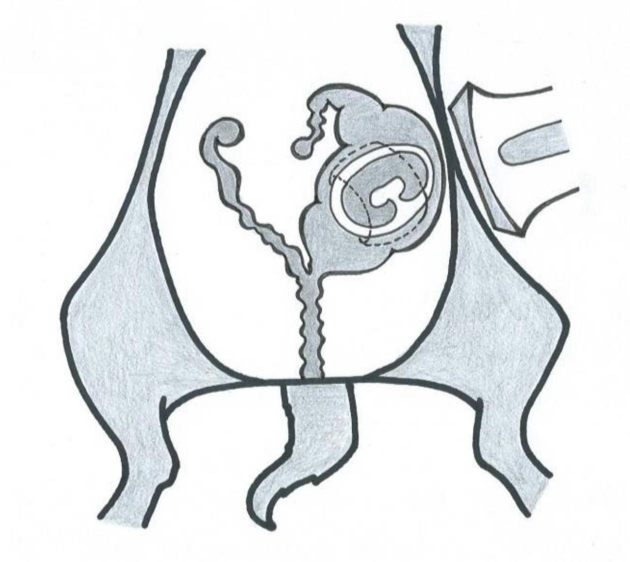
The complete procedure was performed by the same ultrasonographer with the aid of an ultrasonographic machine that allowed continuous visualization of the needle tip. The transducer was placed perpendicular to the bitche's skin to obtain a transverse or longitudinal plane of the gestational vesicle. For the puncture, a 22G needle (30x0.7 mm) coupled to a 10 mL syringe, both sterile were used (FIGURE 1), therefore, the most appropriate location in the gestational vesicle and in the abdomen of the bitch was selected and the procedure of fine-needle puncture guided by ultrasound was performed and subsequently described in detail (FIGURE 2).

The punctures were performed every five days from the 40th day of gestation and considered successful when the collection of 1 mL of amniotic fluid was aspirated. For this study, one gestational vesicle by gestational age was chosen to perform the ultrasonographic-guided puncture, always selecting the one that was positioned closest to the abdominal wall (FIGURE 3) regardless of its location in the bitch's abdomen.

FIGURE 1. MATERIAL USED FOR THE AMNIOCENTESIS PROCEDURE IN PREGNANT BICHES: STERILE GAZE, TRANSDUCER COVERED BY STERILE GLOVE, CHLORHEXIDINE, 22G STERILE NEEDLE, 10ML STERILE SYRINGE, AND STERILE GLOVES.

FIGURE 2. PHOTOGRAPHIC IMAGE PRESENTING THE MATERIAL USED (STERILE GLOVES ON THE OPERATOR AND TRANSDUCER, SYRINGE AND NEEDLE) DEMONSTRATING THE NEEDLE POSITION IN RELATION TO THE TRANSDUCER IN THE PREGNANT BITCH ABDOMEN.

FIGURE 3. SCHEMATIC DRAWING OF THE ABDOMEN OF THE PREGNANT BITCH DEMONSTRATING HOW TO CHOOSE THE GESTATIONAL VESICLE THAT WAS POSITIONED NEXT TO THE ABDOMINAL WALL.



Puncture attempts were unlimited until it was considered successful, preferably, a different gestational vesicle at each gestational age was punctured. In addition, at the end of each procedure, a thorough ultrasonographic assessment of the fetus, the respective punctured gestational vesicle and of the pregnant bitch was performed for at least 15 minutes for active bleeding, presence of fetal movement, evaluation of fetal heart rate and the research for free liquid adjacent to the gestational vesicle, ruling out the possibility of uterine rupture.

3.2.5 STATISTICAL ANALYSIS

Puncture attempts at each gestational age and different sizes of pregnant bitches were noted and tabulated by an author (E.G.) using Microsoft Office Excel (Microsoft Office 2016 for Windows, Redmond, WA, USA). The statistical tests were selected and applied by a professional statistic (E.C.) using the “vcd”, “stats” and “tigerstats” packages in the R for Windows software (version 3.5.1 Ri386, R Foundation for Statistical Computing, Vienna, Austria, 2016). In all analyses, a P-value of $\alpha = 0.05$ was considered significant, and significance was analyzed relative to the value set.

First, a descriptive analysis of the distribution, minimum, maximum, mean, median and standard deviation of the number of attempts to perform the puncture according to the size of the pregnant females (small, medium and large) was performed. Subsequently, learning curves were constructed to assess the degree of difficulty of the puncture technique according to the size of the pregnant bitches at each gestational age. Finally, Pearson's correlation test was applied to assess whether the number of puncture attempts was related to the litter size or the gestational age. In order to verify the association between the number of puncture attempts and the bitches' size, a contingency table was constructed, and Pearson's contingency test was applied because the variable "size" is categorical. Afterwards, the Chi-square test was used to assess the degree of association between these variables, considering that the value close to one indicates a strong relationship.

3.3 RESULTS

Twenty-one clinically healthy pregnant bitches were included in this study. All of them were punctured at least in two gestational ages resulting in 118 punctures, of which 58 (49.15%) were considered successful. Two bitches presented complications after the procedure: a French bulldog and a Schnauzer. Both of them two days after the second amniocentesis at approximately the 50th days of pregnancy. The French bulldog has aborted all the fetuses and the Schnauzer presented fetal death of the entire litter. All the others pregnant bitches followed the pregnancy until the end and gave birth to healthy puppies. TABLE 2 shows the distribution, minimum, maximum, mean, median, and standard deviation of the number of performed ultrasonographic-guided amniocentesis attempts according to the females size (small, medium and large).

3.3.1 INSERTION OF THE NEEDLE

The complete procedure was performed by a single person (E.G.) using the freehand technique under the guidance of ultrasonography, which enabled the continuous visualization of the needle. The ultrasonographic transducer was placed perpendicular to the female's skin, taking care that it does not slip (FIGURE 2). The needle was tilted up to 45º, in relation to the longitudinal plane of the abdomen, at the intended location of entry, adjacent to the transducer that remains in the same transverse plane.

TABLE 2. DISTRIBUTION OF DESCRIPTIVE STATISTICS PRESENTING MINIMUM, MAXIMUM, MEAN, MEDIAN AND STANDARD DEVIATION OF THE NUMBER OF PERFORMED ULTRASONOGRAPHIC-GUIDED AMNIOCENTESIS ACCORDING TO THE SIZE OF PREGNANT BITCHES AT LEAST IN TWO GESTATIONAL AGES.

Size	Attempts	Min.	Máx.	Mean	Median	Standard Deviation
Small	78	1	5	1.902	2	1.031
Medium	14	1	4	2	2	1.069
Large	26	1	7	2.6	2	1.624

The site of entry was selected according to the location of the fetus, preferably in the cranial or caudal region of the amnion, adjacent to the head or the posterior region of the fetus and avoiding the zonary placenta (FIGURES 4 and 5). The insertion of the needle has four stages: abdominal skin puncture, uterine puncture, entry into the amniotic cavity and advance of the needle.

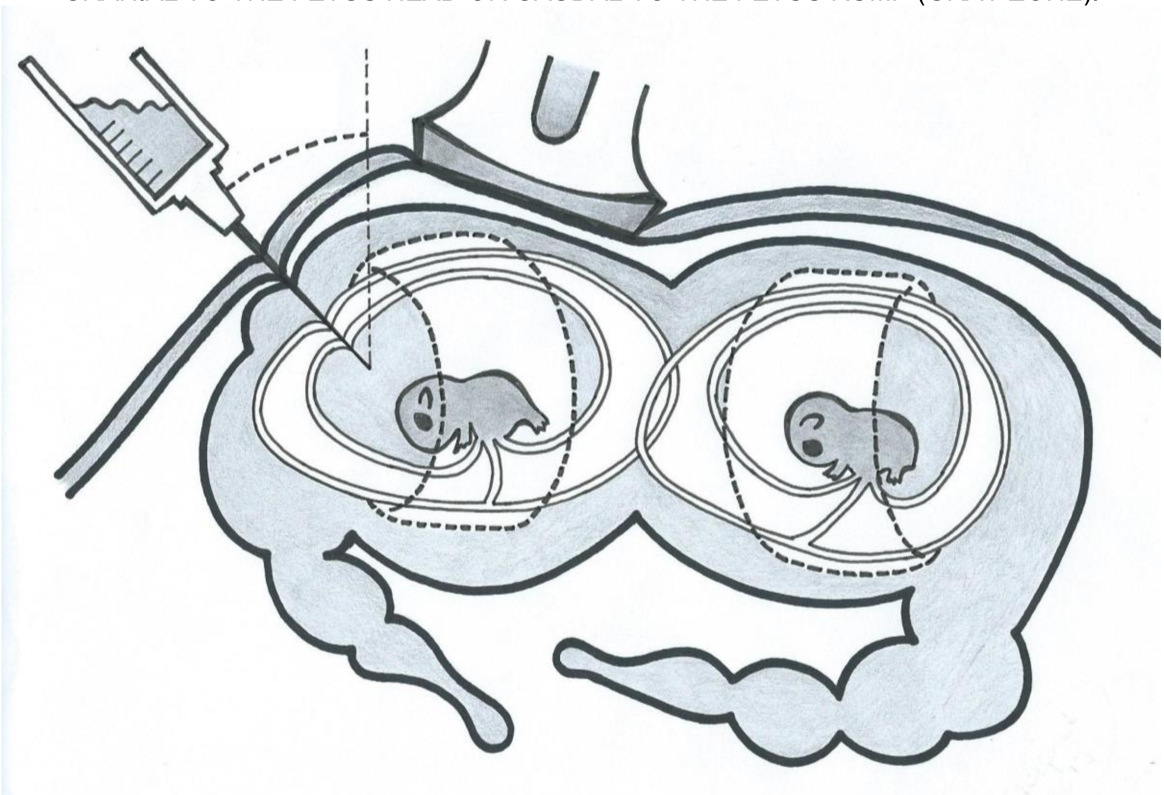
3.3.2 PUNCTURE OF THE ABDOMINAL SKIN

The needle was introduced laterally to the transducer, directly under the middle of the ultrasound cluster at an angle of 45º to the longitudinal plane of the female, contralateral side to the probe. The result was that the transducer and the needle were at an angle of 45º to each other (FIGURE 4). The needle is advanced about 1 cm, and the initial puncture of the skin requires limited pressure while advancing the needle through the animal's subdermal tissue.

3.3.3 UTERINE PUNCTURE

At the moment of uterine puncture can be painful, due to the visceral peritoneum that covers the uterus, so the needle orientation must be confirmed immediately before the uterine puncture, as it can be altered by maternal movements or contraction of the abdominal muscles (FIGURE 5A).

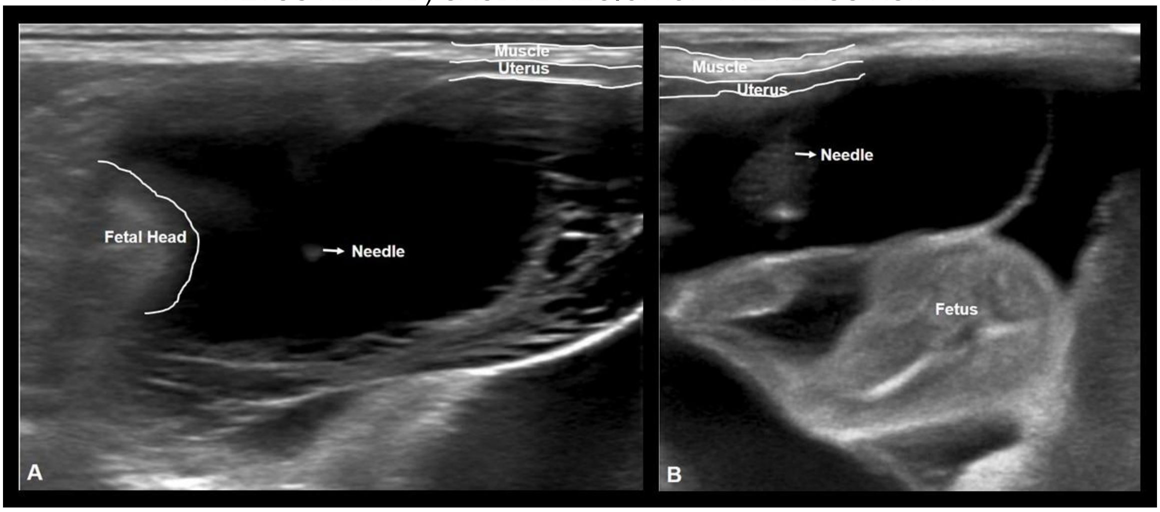
FIGURE 4. SCHEMATIC DRAWING DEMONSTRATING THE LOCATION OF PLACED 22G NEEDLE DURING THE ULTRASONOGRAPHIC-GUIDED AMNIOCENTESIS IN PREGNANT BITCHES. THE NEEDLE WERE INSERTED AN ANGLE OF 45º IN RELATION TO THE FEMALE'S ABDOMEN SURFACE, LONGITUDINAL PLANE. THE BETTER INSERT POINT TO ADVANCE THE NEEDLE WAS IN THE PERIPHERY OF THE GESTATIONAL VESICLE, CRANIAL TO THE FETUS HEAD OR CAUDAL TO THE FETUS RUMP (GRAY ZONE).



3.3.4 ENTRY INTO THE AMNIOTIC CAVITY

Before entering the amniotic cavity, the needle must be located and visualized accurately. The transducer should be slightly away from the needle because the needle identification is best when the angle between them is perpendicular. Partial visualization of the needle can cause puncture of the placenta or uterine wall when the needle leaves the gestational vesicle. The entrance to the amniotic cavity must be reached with a fast and precise movement to avoid the bulging of the membranes, which would result in a false entry, and no fluid will be obtained. If bulging occurs, a small push or twist movement of the needle helps to pierce the protruding membrane, allowing entry into the amniotic cavity (FIGURE 5B).

FIGURE 5. ULTRASONOGRAPHIC IMAGE PRESENTING PART OF THE GESTATIONAL VESICLE WITH FETUS OF APPROXIMATELY 43 TO 45 DAYS OF AGE. THE IMAGE WAS DEMONSTRATING THE PLACE OF THE NEEDLE (ARROW) WHEN IT WAS INTO THE VESICLE - ANECHOIC FLUID. A) CRANIAL REGION OF THE AMNIO ADJACENT TO THE FETUS HEAD. B) CAUDAL REGION OF THE FETUS RUMP.



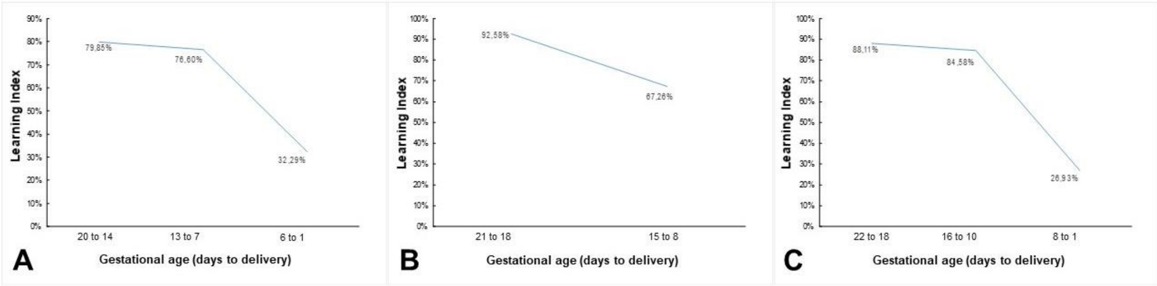
3.3.5 ADVANCE OF THE NEEDLE

Once inside the amniotic cavity, the advance of the needle should stop approximately 2 cm far. This manuver is to prevent any contracture in the wall from displacing the needle and to prevent the fetus from being hit with any sudden or unexpected movement. In the event of a failed puncture, a new puncture site must be chosen, and the needle replaced to avoid contamination.

When the needle is correctly located in the gestational vesicle, the vacuum created by the syringe draws in the fluid automatically, without additional manipulation. Approximately 1 mL of amniotic fluid should be obtained from the procedure, ideally without contamination by the bitch's blood cells.

The learning curves, according to the size (small, medium and large) of pregnant bitches, of the puncture attempts in each gestational period are shown in FIGURE 6. They show that regardless of the size of the bitch, success rate tends to fall as pregnancy progresses and this reduction is more evident at the end of pregnancy.

FIGURE 6. GRAPHICS PRESENTING THE LEARNING CURVES BETWEEN PUNCTURE ATTEMPTS OF AMNIOCENTESIS AND THE GESTATIONAL AGE ACCORDING TO THE SIZE OF THE PREGNANT BITCH IN A) SMALL, B) MEDIUM AND C) LARGE.



The relationship between the puncture attempts and the size of the females and the puncture attempts and the gestational age were not statistically significant, however, the correlation coefficient between the puncture attempts and the litter size are shown in TABLE 3 according gestational age and the size of the pregnant bitch. TABLE 3 shows that only large females who were in the gestational periods between 22 to 18 days and between 16 to 10 days for parturition had strong and very strong correlation coefficients, respectively demonstrating that at these gestational ages the size of the litter influences the number of puncture attempts.

TABLE 3. CORRELATION OF PEARSON (R) BETWEEN THE QUANTITY OF PUNCTURE ATTEMPTS FOR AMNIOCENTESIS AND THE LITTER SIZE ACCORDING TO THE SIZE OF THE PREGNANT BITCH AND THE PREGNANCY PERIOD, IN DAYS FOR DELIVERY.

Bitch Size	Gestational period	r
Small	20 to 14	0.1394
	13 to 7	0.2345
	6 to 1	0.2696
Medium	21 to 18	0.4082
	15 to 8	0.0000
Large	22 a 18	0.8660
	16 a 10	0.9878
	8 a 1	0.5774

3.4 DISCUSSION

The low zootechnical value associated with the short duration of pregnancy in bitches allowed obstetricians and owners to choose to wait for the birth of the pups to verify the presence of malformations or congenital diseases and, from that moment, to plan the treatment. Nevertheless, currently, the high emotional value added to dogs, has stimulated researchers to look for early diagnostic methods to analyze these diseases within and whitout ultrasound findings. A recent report shows the possibility of diagnosing malformations in canine fetuses by ultrasonography after 42 days of gestation (Freitas et al, 2016). However, no other research pointed on how to perform an ultrasonographic-guided amniocentesis in dogs until this moment.

Amniocentesis is an invasive diagnostic method that was first described in human medicine in 1956 to determine sex (Fuchs & Riss, 1956). Since then, the evolution of tissue culture techniques, two-dimensional ultrasonographic and, new molecular genetic tests have expanded the indications and improved the safety and reliability of this procedure (Ralston & Craigo 2004). In veterinary medicine, this technique has been described in horses and reported in studies of other species since 1991 (Schmidt et al, 1991); however, in dogs, it has not been described yet. Thus, the ultrasonographic-guided amniocentesis technique in pregnant bitches was described in detail in this research at different gestational ages and sizes of pregnant females, enabling its use in the practical routine of the reproductive clinic and future studies.

The puncture technique and material (FIGURE 1) used in this research were similar as described for ultrasonographic-guided puncture for abdominal organs (Barr, 1995; Penninck & Finn-Bodner, 1998) in which the methodology is well consolidated in the veterinary clinical routine. It is worth remembering here that care must be taken not to position the transducer obliquely on the female's abdomen wall (it must be perpendicular to the maternal abdominal surface), avoiding creating a false image of the area below the transducer. The freehand technique (FIGURE 2) was the method of choice due to its low cost and versatility of approach, considering that the puncture guide was not an available option, although it would allow the operator to predict the needle path with better precision. On the other hand, the adapter on the transducer would limit the accessibility of the location to be punctured (Hoppe et al, 1986).

The needle size was selected according to the distance it needs to travel towards the amniotic cavity considering the bitches' sizes. This distance was estimated by the ultrasound calipers, enabling the appropriate choice of the needle length from the various commercial options available. In this study, 22G (30x0.7

mm) was chosen for all gestational ages and bitch size, but the ultrasonographer is free to determine any size according to the distance it needs to travel until it reaches the amniotic fluid pool.

The abdomen location defined for the puncture was closely related to the liquid pool and the location of the fetus, always avoiding transplacental insertion and cross. Bonte et al. (2017) describes that they gave preference to the midline of the abdomen avoiding the bitch's breast tissue however, this maneuver was not followed in this study. Thus, the needle crossed the breast tissue if the optimal location indicated this need. We believe that when the location of interest was found, we have to introduce the needle, regardless of the location on the bitch's abdomen surface, in relation to the probe. Usually, the point to collect is a cranial or caudal region into the amnion that is visible on the equipment screen, normally close to the head or rump of the fetus (FIGURE 5A and 5B). At this point, care should be taken minimize lateral movements of the needle to avoid overlying fetal vessels by passing the needle through at a site distant to the umbilical cord insertion.

The transplacental route was performed when necessary in pregnant women, but they noted that half of their patients with complications had required a transplacental amniocentesis (Stark et al, 2000). At this way, the insertion of the needle through the transplacental route of the canine gestational vesicle, in this research, was not recommended as an attempt to eliminate the possibility of placental detachment. However, it is worth remembering here that the placenta of primates is the discoid type in which the villi are concentrated in a region with disc format (Carter & Enders, 2016) which could facilitate the possibility of detachments different from the type of placenta of bitches (zonary placenta) in which the villi are identified just in a regular zone (Carter & Enders, 2016), which facilitated the definition of the appropriate place for needle insertion.

The puncture attempts were unlimited and only considered successful when 1 mL of amniotic fluid was collected, providing to our study a high number of attempts (118) and consequently a low success rate (49.15%). We believe that if the volume of fluid collected was lower, our success rate would be higher and the mean of attempts per bitch size (TABLE 2), approximately two, would be lower. Although there is no limit on the number of puncture attempts, only the sample of large bitches had a maximum number of puncture attempts greater than five due to the presence of a pregnant bitch who had an abnormally sized litter (18 puppies) in the which made it difficult to locate the appropriate puncture site and consequently increased the number of attempts. Even so, we were able to show that the technique of amniocentesis ultrasonographic-guided can be used in pregnant bitches of any size.

The learning curves constructed according to the size of the dogs and at specific gestational periods (FIGURE 6) proved that at the end of pregnancy, there is a greater difficulty in succeeding in ultrasonographic-guided amniocentesis and obtaining 1 mL of a sample of the amniotic fluid. We hoped that this fact could be justified due to the small free space between the fetus and gestational vesicle and, consequently, a smaller amount of amniotic fluid available at the end of pregnancy. However, statistical tests were applied and we were able to prove that the pregnant female' size and the advancing gestational age are not related to the number of unsuccessful puncture attempts. It was believed that smaller pregnant bitches that have smaller gestational vesicles compared to larger pregnant bitches, and advanced gestational age that has a smaller pool of fluid were factors that explained the difficulty of the procedure. On the contrary, the litter size according to the pregnant female size was the only factor that influenced the difficulty in puncture, being only in large bitches with gestational age of up to 16 to 10 days for delivery that was proved by high correlation rate (TABLE 3), in which we could not find an explanation for this fact.

The operator's experience was not tested as a risk factor in our research, but Gordon et al, (2002) showed in his study with amniocentesis in pregnant women that the number of attempts at puncture was not related to the operator's experience. We believe that if the ultrasonographer has experience in performing biopsies with a fine-needle guided by ultrasonography, he will be able to perform the puncture of the fetal amniotic fluid in the future.

The only complication identified in our study with a rate of 9.52% was the miscarriage. We think that these complications were not related to the invasive procedure described in this study because the perinatal mortality rate can reach 24.6% and is more related to litter variables than to breed or breed size, it is more frequent in the first litter and three times higher when the bitch has her first delivery over 6 years of age (Tønnessen et al., 2012).

The volume of fetal fluid to be collected was established at random and the procedure was repeated by at least twice in each bitch at different gestational ages. As a precautionary measure, the same vesicle in consecutive gestational ages was not punctured. Even so, as it is not known what is the total volume of fetal fluid (allantoid or amniotic) in the gestational vesicle of bitches that can be extracted, in this pregnant biches, we have to consider that 1 mL aspiration amniotic fluid could cause the complications. The same volume removed was injected by saline solution in an experimental study with amniocentesis performed on rats to avoid the possibility of oligohydramnios (deficient volume of fetal fluid - Serriere et al., 2006); such conduct could be an alternative in bitches as an attempt to avoid miscarriages.

We believe that the indications for ultrasonographic-guided amniocentesis in bitches are similar to those recommended for pregnant women as well as for other animal species, which are: polyhydramnios (excess fluid in the amniotic sac), advanced maternal age, history of congenital family disease, fetal abnormalities identified by ultrasonography (Jummaat et al, 2019), pulmonary maturity assessment (Bonte, et al, 2017), fetal sex determination (Kamimura et al, 1996) and biochemical assessment of amniotic fluid (Prestes et al, 2001).

The technique of amniocentesis ultrasonographic-guided was described and standardized in our study, and the procedure demonstrated that most collections are safe because 90.48% of pregnant females continued with the pregnancy until delivery. However, our study had some limitations: (1) the population sample was small; (2) the presence of contamination in the collected fluid was not assessed despite the objective here being only the validation of the technique. Future studies that assess the accuracy of the technique, the degree of contamination in the fluid caused by the technique and the risk of premature delivery related to the procedure are suggested.

3.5 CONCLUSIONS

The ultrasonographic-guided fine-needle puncture technique for amniocentesis can be performed on pregnant bitches of various sizes and at different gestational ages, however at the end of pregnancy fetal amniotic fluid collection is more complicated. The collection of fetal amniotic fluids at certain gestational ages will allow the veterinary obstetrician clinician to make an early diagnosis and prenatal planning of potential maternal-fetal management.

4 FINAL CONSIDERATIONS

The present research detailed the ultrasonographic morphology characteristics of specific canine fetal organs and correlated that with gestational age. Moreover, promote the standardization and validated the ultrasonographic-guided fine-needle puncture technique for amniocentesis in pregnant bitches.

We believe that the results obtained could offer the obstetrician and the imaginologist more options to assist in the prediction of the date of delivery in bitches, and provides, in the future, a prenatal diagnosis of potential congenital diseases, enabling the planning of maternal-fetal management in canine species.

5. REFERENCES

Akar H, Konuralp C, Baysal K, Kolbakir F. Ellis-van Creveld syndrome associated with thymic hypoplasia. **Asian Cardiovasc Thorac Ann** 2002; 10(4): 336-338.

Aksakal SE, Kandemir O, Altınbas S, Esin S, Muftuoglu KH. Fetal thymus size as a predictor of histological chorioamnionitis in preterm premature rupture of membranes. **J Matern Fetal Neonatal Med** 2014; 27(11): 1118-1122.

Banzato T, Zovi G, Milani C. Estimation of fetal lung development using quantitative analysis of ultrasonographic images in normal canine pregnancy. **Theriogenology** 2017; 96: 158-163.

Barella G, Lodi M, Sabbadin LA, Faverzani S. A new method for ultrasonographic measurement of kidney size in healthy dogs. **J Ultrasound** 2012; 15(3): 186-191.

Barr F. Percutaneous biopsy of abdominal organs under ultrasound guidance. **J Small Anim Pract** 1995; 36(3): 105-113.

Bataeva R, Bellsham-Revell H, Zidere V, Allan LD. Reliability of fetal thymus measurement in prediction of 22q11.2 deletion: a retrospective study using four-dimensional spatiotemporal image correlation volumes. **Ultrasound Obstet Gynecol** 2013; 41(2): 172-176.

Beccaglia M, Luvoni GC. Prediction of parturition in dogs and cats: accuracy at diferente gestational ages. **Reprod Domest Anim** 2012; 47(Suppl 6):194-196.

Beccaglia M, Alonge S, Trovo C, Luvoni GC. Determination of gestational time and prediction of parturition in dogs and cats: an update. **Reprod Domest Anim** 2016; 51(Suppl 1): 12-17.

Bolis B, Prandi A, Rota A, Faustini M, Veronesi MC. Cortisol fetal fluid concentrations in term pregnancy of small-sized purebred dogs and its preliminary relation to first 24 hours survival of newborns. **Theriogenology** 2017; 88: 264-269.

Bonte T, Del Carro A, Paquette J, Charlot Valdieu A, Buff S, Rosset E. Foetal pulmonary maturity in dogs: Estimated from bubble tests in amniotic fluid obtained via amniocentesis. **Reprod Domest Anim** 2017; 62(6): 10251029.

Britt T, Clifford C, Barger A, Moroff S, Drobatz K, Thacher C, Davis G. Diagnosing apendicular osteosarcoma with ultrasound-guided fine-needle aspiration: 36 cases. **J Small Anim Pract** 2007; 48(3): 145-150.

Carter AM, Enders AC. Placentation in mammals: Definitive placenta, yolk sac, and paraplacenta. **Theriogenology** 2016; 86(1): 278-287.

Cho J, Min J, Lee Y, McCrindle B, Hornberger L, Yoo S. Diameter of the normal fetal thymus on ultrasound. **Ultrasound Obstet Gynecol** 2007; 29(6): 634-638.

Concannon PW, Whaley S, Lein D, Wissler R. Canine gestation length: variation related to time of mating and fertile life of sperm. **Am J Vet Res** 1983; 44(10): 1819–1821.

da Costa RC, Parent JM, Dobson H, Ruotsalo K, Holmberg D, Duque MC, Poma R. Ultrasound-guided fine Needle aspiration in the diagnosis of peripheral nerve sheath tumors in 4 dogs. **Can Vet J** 2008; 49(1): 77-81.

Cruz-Lemini M, Parra-Saavedra M, Borobio V, Bennasar M, Goncé A, Martínez JM, Borrell A. How to perform an amniocentesis. **Ultrasound Obstet Gynecol** 2014; 44(6): 727-731.

Crystal MA, Pennink DG, Matz ME, Pearson SH, Freden GO, Jakowski RM, Use of Ultrasound-guided fine-needle Aspiration Biopsy and Automated Core Biopsy for the Diagnosis of Gastrointestinal Diseases in Small Animals. **Vet Radiol Ultrasound** 1993; 34(6): 438-444.

Dall'Ara P, Meloni T, Rota A, Servida F, Filipe J, Veronesi M C, Immunoglobulins G and lysozyme concentrations in canine fetal fluids at term of pregnancy. **Theriogenology** 2015; 83(4): 766-771.

D'Anjou M-A, Penninck D. Kidneys and Ureters. In: Penninck D, D'Anjou M-A, editors. **Atlas of Small Animal Ultrasonography**. Second edition. Iowa: John Wiley & Sons, Inc; 2015, p. 331-362.

Devriendt A, Cassart M, Massez A, Donner C, Avni FE. Fetal kidneys: additional sonographic criteria of normal development. **Prenat Diagn** 2013; 33(13):1248-52.

Dias T, Sairam S, Kumarasiri S. Ultrasound diagnosis of fetal renal abnormalities. **Best Pract Res Clin Obstet Gynaecol** 2014; 28(3): 403-15.

Driscoll DA, Budarf ML, Emanuel BS. Antenatal diagnosis of DiGeorge syndrome. **Lancet** 1991; 338(8779): 1390-1391.

Eisenbrandt DL, Phemister RD. Postnatal development of the canine kidney: quantitative and qualitative morphology. **Am J Anat** 1979; 154(2): 179-193.

England GCW, Allen WE, Porter DJ. Studies on canine pregnancy using B-mode ultrasound: Development of the conceptus and determination of gestational age. **J Small Anim Pract** 1990; 31(7): 324–329.

Ewald SJ, Frost WW. Effect of prenatal exposure to ethanol on development of the thymus. **Thymus** 1987; 9(4): 211-215.

Feeney DA, Ober CP, Snyder LA, Hill SA, Jessen CR. Ultrasound criteria and guided fine-needle aspiration diagnostic yelds in small animal peritoneal, mesenteric and omental disease. **Vet Radiol Ultrasound** 2013; 54(6): 638-645.

Felker R, Cartier M, Emerson D, Brown D. Ultrasound of the fetal thymus. **J Ultrasound Med** 1989; 8(12): 669-673.

Felsburg PJ. Overview of imune system development in the dog: comparison with humans. **Hum Exp Toxicol** 2002; 21(9-10): 487-492.

Fleming KL, Howells EJ, Villiers EJ, Maddox TW. A randomised controlled comparison of aspiration and non-aspiration fine-needle techniques for obtaining ultrasound-guided cytological samples from canine livers. **Vet J** 2019; 252: 105372.

Freitas LA de, Mota GL, Silva HVR, Carvalho CF, Silva LDM da. Can maternal-fetal hemodynamics influence prenatal development in dogs? **Anim Reprod Sci** 2016; 172: 83-93.

Fuchs F, Riis R. Antenatal sex determination. **Nature** 1956; 177(4503): 330.

Garcia A, Salaheddine M. Bovine ultrasound-guided transvaginal amniocentesis. **Theriogenology** 1997; 47(5): 1003-1008.

Giannico AT, Gil EM, Garcia DA, Froes TR. The use of Doppler evaluation of the canine umbilical artery in prediction of delivery time and fetal distress. **Anim Reprod Sci** 2015; 154: 105-112.

Giannico AT, Gil EM, Garcia DA, Sousa MG, Froes TR. Canine fetal echocardiography: correlations for the analysis of cardiac dimensions. **Vet Res Commun** 2016; 40(1): 11-19.

Giannico AT, Garcia DA, Gil EM, Sousa MG, Froes TR. Assessment of umbilical artery flow and fetal heart rate to predict delivery time in bitches. **Theriogenology** 2016; 86(7): 1654-1661.

Gil EM, Garcia DA, Giannico AT, Froes TR. Canine fetal heart rate: do accelerations or decelerations predict the parturition day in bitches? **Theriogenology** 2014; 82(7): 933-941.

Gil EM, Garcia DA, Froes TR. In-utero development of the fetal intestine: Sonographic evaluation and correlation with gestational age and fetal maturity in dogs. **Theriogenology** 2015; 84(5): 681-686.

Gil EM, Garcia DA, Giannico AT, Froes TR. Use of B-mode ultrasonography for fetal sex determination in dogs. **Theriogenology** 2015; 84(6): 875-879.

Gil EMU, Garcia DAA, Giannico AT, Froes TR. Early results on canine fetal kidney development: Ultrasonographic evaluation and value in prediction of delivery time. **Theriogenology** 2018; 107: 180-187.

Gordon MC, Narula K, O'Shaughnessy R, Barth WH Jr. Complications of third-trimester amniocentesis using continuous ultrasound guidance. **Obstet Gynecol** 2002; 99(2): 255-259.

Groppetti D, Martino PA, Ravasio G, Bronzo V, Pecile A. Prognostic potential of amniotic fluid analysis at birth on canine neonatal outcomes. **Vet J** 2015; 206(3): 423-425.

Hager DA, Nyland TG, Fisher P. Ultrasound-guided biopsy of the canine liver, kidney, and prostate. **Vet Radiol Ultrasound** 1985; 26(3): 82-88.

Haley PJ. Species differences in the structure and function of the immune system. **Toxicology** 2003; 188(1): 49-71.

Hershkovitz R, Amichay K, Stein GY, Tepper R. The echogenicity of the normal fetal kidneys during different stages of pregnancy objectively. **Arch Gynecol Obstet** 2011; 284(4): 807-811.

Hoppe FE, Hager DA, Poulos Jr PW, Ekman S, Lindgren PG. A comparison of manual and automatic ultrasound guided biopsy technique. **Vet Radiol Ultrasound** 1986; 27(4): 99-101.

Jummaat F, Ahmad S, Mohamed Ismail NA. 5-Year review on amniocentesis and its maternal fetal complications. **Horm Mol Biol Clin Investig** 2019; 40(2).

Kamimura S, Nishiyama N, Ookutsu S, Goto K, Hamana K. Determination of bovine fetal sex by PCR using fetal fluid aspirated by transvaginal ultrasound-guided amniocentesis. **Theriogenology** 1997; 47(8): 1563-1569.

Karl K, Heling KS, Sarut Lopez A, Thiel G, Chaoui R. Thymic-thoracic ratio in fetuses with trisomy 21, 18 or 13. **Ultrasound Obstet Gynecol** 2012; 40(4): 412-417.

Kim C, Oblak ML, Nykamp S. Efficiency of ulrasound-guided aspiration of medial retropharyngeal lymph node in dogs. **Can Vet J** 2019; 60(6): 619-624.

Konde LJ. Sonography of the kidney. **Vet Clin North Am Small Anim Pract** 1985; 15(6): 1149-1158.

Kutzler MA, Yeager AE, Mohammed HO, Meyers-Wallen VN. Accuracy of canine parturition date prediction using fetal measurements obtained by ultrasonography. **Theriogenology** 2003; 60(7):1309–17.

Lawson TL, Foley WD, Berland LL, Clarck KE. Ultrasonic evaluation of fetal kidneys. **Radiology** 1981; 138(1): 153-156.

Li L, Bahtiyar MO, Buhimschi CS, Zou L, Zhou QC, Copel JA. Assessment of the fetal thymus by two- and three-dimensional ultrasound during normal human gestation and in fetuses with congenital heart defects. **Ultrasound Obstet Gynecol** 2011; 37(4): 404-409.

Lopate C. Estimation of gestational age and assessment of canine fetal maturation using radiology and ultrasonography: A review. **Theriogenology** 2008; 70(3): 397–402.

Lopate C. Gestational Aging and Determination of Parturition Date in the Bitch and Queen Using Ultrasonography and Radiography. **Vet Clin North Am Small Anim Pract** 2018; 48(4): 617-638.

Lovell KL, Sprecher DJ, Ames NK, Jones MZ. Development and efficacy of ultrasound-guided fetal fluid aspiration techniques for prenatal diagnosis of caprine beta-mannosidosis. **Theriogenology** 1995; 44(4): 517-527.

Luvoni GC, Beccaglia M. The prediction of parturition date in canine pregnancy. **Reprod Domest Anim** 2006; 41(1): 27–32.

Machlitt A, Tennstedt C, Körner H, Bommer C, Chaoui R. Prenatal diagnosis of 22q11 microdeletion in an early second-trimester fetus with conotruncal anomaly presenting with increased nuchal translucency and bilateral intracardiac echogenic foci. **Ultrasound Obstet Gynecol** 2002;19(5): 510-513.

Mattoon JS, Nyland TG. Ovaries and uterus. In: Mattoon JS, Nyland TG, editors. **Small Animal Diagnostic Ultrasound**. Third edition. Missouri: Saunders Company; 2015, p. 634-654.

McGeady TA, Quinn PJ, Fitzpatrick ES, Ryan MT, Cahalan S. Urinary system. In: McGeady TA, Quinn PJ, FitzPatrick ES, Ryan MT, Cahalan S, editors. **Veterinary embryology**. Second edition. Oxford: John Wiley & Sons, Ltd; 2017, p. 240–250.

Mohamed N, Eviston DP, Quinton AE, Benzie RJ, Kirby AC, Peek MJ, Nanan RK. Smaller fetal thymuses in pre-eclampsia: a prospective cross-sectional study. **Ultrasound Obstet Gynecol** 2011; 37(4): 410-415.

Nyland TG, Wallack ST, Wisner ER. Needle-tract implantation following us-guided fine-needle aspiration biopsy of transitional cell carcinoma of the bladder, urethra and prostate. **Vet Radiol Ultrasound** 2002; 43(1): 50-53.

Nyland TG, Widmer WR, Mattoon JS. Urinary Tract. In: Mattoon JS, Nyland TG, editors. **Small Animal Diagnostic Ultrasound**. Third edition. Philadelphia: Saunders Company; 2015, p. 557-607.

Olearo E, Oberto M, Oggè G, Botta G, Pace C, Gaglioti P, Todros T. Thymic volume in healthy, small for gestational age and growth restricted fetuses. **Prenat Diagn** 2012; 32(7): 662-667.

Penninck DG, Finn-Bodner ST. Updates in interventional ultrasonography. **Vet Clin North Am Small Anim Pract** 1998; 28(4): 1017-1040.

Pierini A, Millanta F, Zanforlin R, Vannozzi I, Marchetti V. Usefulness of cytologic criteria in ultrasound-guided fine-needle aspirates from subcentimeter canine mammary tumors. **J Vet Diagn Invest** 2017; 29(6): 869-873.

Prestes NC, Chalhoub MC, Lopes MD, Takahira RK. Amniocentesis and biochemical evaluation of amniotic fluid in ewes at 70, 100 and 145 days of pregnancy. **Small Rumin Res** 2001; 39(3): 277-281.

Ralston SJ, Craigo SD. Ultrasound-guided procedures for prenatal diagnosis and therapy. **Obstet Gynecol Clin North Am** 2004; 31(1): 101-123.

Ripley AM, Penedo MCT, Grahn RA, Martinez de Andino EV, Walbornn SR, Serafini R, Love CC, Hinrichs K. Equine fetal genotyping via aspiration of yolk-sac fluid at 22-28 days of gestation. **Theriogenology** 2020; 142: 34-40.

Salazar I, Yllera MM. Development of the mesonephros, metanephros and gonads in the foetal dog. **Funct Dev Morphol** 1991; 1(1): 59-63.

Sciaky-Tamir Y, Hershkovitz R, Mazor M, Shelef I, Erez O. The use of imaging technology in the assessment of the fetal inflammatory response syndrome-imaging of the fetal thymus. **Prenat Diagn** 2015; 35(5): 413-419.

Serriere S, Nadal-Desbarats L, Seguin F, Pourcelot L, Tranquart F. Ultrasound-guided collection of amniotic fluid in pregnant rats. **J Am Assoc Lab Anim Sci** 2006; 45(4): 49-53.

Shille VM, Gontarek J. The use of ultrasonography for pregnancy diagnosis in the bitch. **J Am Vet Med Assoc** 1985; 187(10): 1021-1025.

Sinowatz F. Development of the urogenital system. In: Hyttel P, Sinowatz F, Vejlsted M, Betteridge K, editor. **Essentials of Domestic Animal Embriology**, London: Elsevier Health Sciences; 2010, p. 252-85.

Smith KC, McGladdery AJ, Binns MM, Mumford JA. Use of transabdominal ultrasound-guided amniocentesis for detection of equid herpesvirus 1-induced fetal infection in utero. **Am J Vet Res** 1997; 58(9): 997-1002.

Stark CM, Smith RS, Lagrandeur RM, Batton DG, Lorenz RP. Need for urgent delivery after Third-Trimester Amniocentesis. **Obstet Gynecol** 2000; 95(1): 48-50.

Sumner JA, Lacorcia L, Rose AM, Woodward AP, Carter JE. Clinical safety of percutaneous ultrasound-guided fine-needle aspiration of adrenal gland lesions in 19 dogs. **J Small Anim Pract** 2018; 59(6): 357-363.

Thiemeyer H, Taher L, Schille JT, Harder L, Hungerbuehler SO, Mischke R, Hewicker-Trautwein M, Kiełbowicz Z, Brenig B, Schütz E, Beck J, Murua Escobar H, Nolte I. Suitability of ultrasound-guided fne-needle aspiration biopsy for transcriptome sequencing of the canine prostate. **Sci Rep**, 2019; 9(1): 13216.

Topie E, Bencharif D, Briand L, Tainturier D. Monitoring the Foetal Phase of Gestation in the Queen With a 12.5-MHz Ultrasound Probe and Prediction of the

Parturition by Combining the Measurements of Head and Abdominal Diameters. **Reprod in Domest Anim** 2015; 50(4): 560-566.

Tønnessen R, Borge KS, Nødtvedt A, Indrebø A. Canine perinatal mortality: a cohort study of 224 breeds. **Theriogenology** 2012; 77(9): 1788-1801.

Wang KY, Panciera DL, Al-Rukibat RK, Radi ZA. Accuracy of ultrasound-guided fine-needle aspiration of the liver and cytologic findings in dogs and cats: 97 cases (1990-2000). **J Am Vet Med Assoc** 2004; 224(1): 75-78.

Watson AT, Penninck D, Knoll JS, Keating JH, Sutherland-Smith J. Safety and correlation of test results of combined ultrasound-guided fine-needle aspiration and needle core biopsy of the canine spleen. **Vet Radiol Ultrasound** 2011; 52(3): 317-322.

Wood AK, McCarthy PH. Ultrasonographic-anatomic correlation and an imaging protocol of the normal canine kidney. **Am J Vet Res** 1990; 51(1): 103-108.

Wood EF, O'Brien RT, Young KM. Ultrasound-guided fine-needle aspiration of local parenchymal lesions of the lung in dogs and cats. **J Vet Intern Med** 1998; 12(5): 338-342.

Yeager AE, Mohammed HO, Meyers-Wallen V, Vannerson L, Concannon, PW. Ultrasonographic appearance of the uterus, placenta, fetus, and fetal membranes throughout accurately timed pregnancy in Beagles. **Am J Vet Res** 1992; 53(3): 342–351.

Zalel Y, Gamzu R, Mashiach S, Achiron R. The development of the fetal thymus: an in utero sonographic evaluation. **Prenat Diagn** 2002; 22(2): 114-117.

APPENDIX I – PAPER: EARLY RESULTS ON CANINE FETAL KIDNEY DEVELOPMENT: ULTRASONOGRAPHIC EVALUATION AND VALUE IN PREDICTION OF DELIVERY TIME

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Early results on canine fetal kidney development: Ultrasonographic evaluation and value in prediction of delivery time

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ABSTRACT

To date there have been no studies that describe the ultrasonographic evaluation of kidney development in canine fetuses. The aim of this prospective and longitudinal study was to monitor fetal kidney development with ultrasound and use fetal kidney measurements as a complementary biometric index for estimation of gestational age. Ultrasonographic examinations were performed on 15 clinically healthy pregnant bitches every four days from 30th day of pregnancy, until visualization of the fetal renal pelvis was no longer possible. Four distinct periods of ultrasonographic canine fetal kidney development were defined. Kidney length and renal pelvis distention were measured on longitudinal plane images. The fetal kidney ranged from 0.40 cm to 2.30 cm in length, and diameter of the pelvis ranged from 0.06 cm to 0.17 cm, however by the end of gestation the renal pelvis was no longer dilated and so its diameter could not be measured. Statistical analysis confirmed a relationship between gestational age and fetal kidney growth. Ultrasonographic evaluation of fetal kidney development is simple to perform. There is a strong correlation between gestational age and kidney length, which allowed generation of an equation to estimate delivery date with high sensitivity between 48 and 52 days of pregnancy. Fetal organ development can be considered complete when the renal pelvis is no longer dilated, this finding can assist the ultrasonographer in staging the gestation by prompting examination for fetal intestinal motility which begins at the same gestational age. Measurement of fetal kidney length can be used in conjunction with other methods to estimate gestational age and predict delivery time.

1. Introduction

Accurate determination of gestational age allows estimation of the time of delivery, such that breeders and veterinarians can plan for an assisted delivery, if required, thus helping to reduce peri-partum losses [1,2]. If hormonal assays and determination of ovulation time are not possible, estimation of gestational age can be based on the time of first ultrasonographic appearance of specific embryonic and fetal structures [3–6]. However, this method is only accurate to within one or two days. Therefore, new methods for more accurate determination of the gestational age in dogs would help to reduce the risk of death as consequence of premature C-section.

Several ultrasonographic examinations, including identification of: fetal liver, stomach, bladder, and heart have been reported to accurately estimate gestational age [5,7]. Recently, high-definition ultrasound visualization of the renal pelvis in feline fetuses was reported [7], and this technique allows the final stage of gestation to be identified more accurately.

Mammalian kidney formation (nephrogenesis) begins in the intermediate mesoderm with early development of the kidney: pronephros, mesonephros, and metanephros. Pronephros and mesonephros are transient excretory systems and disappear without contributing to a permanent renal system and metanephros involves formation of a permanent kidney [8]. Depending on the species, nephrogenesis ceases shortly after delivery but, in the dog, the kidney continues to develop in the first weeks post-partum [9]. Therefore, it has been hypothesized that due to the different embryological stages of renal development the kidneys might appear ultrasonographically different during distinct stages of pregnancy.

The kidneys are first visible ultrasonographically in the canine

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ANNEX I – ANIMAL USE ETHICS COMMITTEE (N°35)

UNIVERSIDADE FEDERAL DO PARANÁ
SETOR DE CIÊNCIAS AGRÁRIAS
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o protocolo número 035/2016, referente ao projeto **"ANÁLISE DO LÍQUIDO AMNIÓTICO OBTIDO POR AMNIOCENTESE GUIADA POR ULTRASSOM PARA AVALIAÇÃO DA MATURIDADE FETAL CANINA"**, sob a responsabilidade de **Tilde Rodrigues Froes**– que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de Outubro, de 2008, do Decreto nº 6.899, de 15 de julho de 2009,e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), efoi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DO SETOR DE CIÊNCIAS AGRÁRIAS DA UNIVERSIDADE FEDERAL DO PARANÁ - BRASIL, com grau 2 de invasividade, em reunião de 14/09/2016.

Vigência do projeto	Setembro/2016 até Setembro/2019
Espécie/Linhagem	*Canis familiaris* (cão)
Número de animais	50
Peso/Idade	10 a 50 kg / 1 a 8 anos
Sexo	Fêmea
Origem	Proprietários particulares, pacientes da rotina do Hospital Veterinário da Universidade Federal do Paraná (UFPR) e de alunos e/ou funcionários da instituição

CERTIFICATE

We certify that the protocol number 035/2016, regarding the project **"ANALYSIS OF NET AMNIOTIC OBTAINED BY AMNIOCENTESIS GUIDED BY ULTRASOUND TO CANINE FETAL MATURITY ASSESSMENT"** under **Tilde Rodrigues Froes** supervision – which includes the production, maintenance and/or utilization of animals from Chordata phylum, Vertebrata subphylum (except Humans), for scientific or teaching purposes – is in accordance with the precepts of Law nº 11.794, of 8 October, 2008, of Decree nº 6.899, of 15 July, 2009, and with the edited rules from Conselho Nacional de Controle da Experimentação Animal (CONCEA), and it was approved by theANIMAL USE ETHICS COMMITTEE OF THE AGRICULTURAL SCIENCES CAMPUS OF THE UNIVERSIDADE FEDERAL DO PARANÁ (Federal University of the State of Paraná, Brazil), with degree 2 of invasiveness, in session of14/09/2016.

Duration of the project	September/2016 until September/2019
Specie/Line	*Canis familiaris* (dog)
Number of animals	50
Wheight/Age	10 to 50kg / 1 to 8 years
Sex	Female
Origin	Private owners, routine patients of Veterinary Hospital at the Federal University of Paraná (UFPR) and students and/ or staff of the institution

Curitiba, 14 de setembro de 2016.

Simone Tostes de Oliveira Stedile

Coordenadora CEUA-SCA

Comissão de Ética no Uso de Animais do Setor de Ciências Agrárias - UFPR