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ULTRASONOGRAPHIC ASSESSMENT IN PREGNANT QUEENS: STUDY OF FETAL HEART RATE AND LYMPHOID ORGANS

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MARCO ANTONIO FERREIRA DA SILVA

ULTRASONOGRAPHIC ASSESSMENT IN PREGNANT QUEENS: STUDY OF FETAL HEART RATE AND LYMPHOID ORGANS

Dissertação apresentada ao Programa de Pós-graduação em Ciências Veterinárias da Universidade Federal do Paraná para obtenção do título de Mestre.
Orientadora: Profª. Drª. Tilde Rodrigues Froes.

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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

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PARECER

A Comissão Examinadora da Defesa da Dissertação intitulada **"ANÁLISE ULTRASSONOGRÁFICA EM GATAS GESTANTES: ESTUDO DA FREQUÊNCIA CARDÍACA E ÓRGÃOS LINFÓIDES FETAIS"** apresentada pelo Mestrando **MARCO ANTONIO FERREIRA DA SILVA** declara ante os méritos demonstrados pelo Candidato, e de acordo com o Art. 79 da Resolução nº 65/09–CEPE/UFPR, que considerou o candidato Apto para receber o Título de Mestre em Ciências Veterinárias, na Área de Concentração em Ciências Veterinárias.

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To my mother, who fought with all her might in order to give me the best and to my father who, even being at an advanced age, has a jovial attitude.

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To all, my sincere thanks.

“Insanity: doing the same thing over and over again and expecting different results...” – *Albert Einstein*

ABSTRACT

The main of research is based on ultrasonographic analysis in the final third of gestation in cats, addressing evaluations that may support the veterinarian's radiologist in the fetal heart rate changes and new data of organogenesis. Therefore, the study was divided into two chapters. In the first chapter, the focus of the research was on the fetal heart rate monitoring in the final third of gestation to detect accelerations and decelerations, a phenomenon described in humans and recently described in dogs. These changes in fetal heart rate may be related to uterine contractions and are strongly connected to the prepartum time. This work has demonstrated that such fetal heart rate oscillations occur in feline fetuses and can be used as a tool in predicting delivery. Due to the evolution of ultrasound devices, the high-resolution image improves the evaluation of tiny structures in the fetus, which would allow the identification of organs not yet described in the literature. For this reason, the second chapter describes in which gestational phase of the cat there is the appearance of lymphoid organs. We made a descriptive evaluation of the thymus and spleen, which may contribute to the assessment of these organs in other species.

Keywords: cat, pregnancy, fetal ultrasound, fetal heart rate, organogenesis

RESUMO

O presente estudo baseia-se na análise ultrassonográfica no terço final da gestação em gatas, abordando avaliações que possa dar suporte ao médico veterinário imaginologista frente às mudanças da frequência cardíaca fetal e novos dados sobre à organogênese. O trabalho foi dividido em dois capítulos independentes. No primeiro capítulo o enfoque da pesquisa foi no monitoramento da frequência cardíaca fetal no terço final da gestação para detecção de acelerações e desacelerações, fenômeno este descrito em humanos e recentemente descrito em cães. Essas mudanças causadas na frequência cardíaca fetal podem estar relacionadas às contrações uterinas e estão fortemente conectadas ao momento pré-parto. Esse trabalho demonstrou que tais oscilações da frequência cardíaca fetal acontecem em fetos felinos e podem ser utilizados como uma ferramenta na previsão do parto. Devido a evolução dos aparelhos ultrassonográficos, a alta resolução melhora a avaliação de estruturas muito pequenas no feto, o que possibilitaria a identificação de órgãos ainda não descritos na literatura. Por esse motivo, o segundo capítulo descreve em qual fase gestacional da gata há o aparecimento de órgãos linfoides. Fizemos uma avaliação descritiva do timo e do baço, o que pode contribuir para avaliação desses órgãos em outras espécies.

Palavras-chave: gata, gestação, ultrassonografia fetal, frequência cardíaca fetal, organogênese

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LIST OF ABBREVIATIONS AND ACRONYMS

bpm	beats per minute
Bw	Bowel
CA	California
CAPES	Coordenação de Aperfeiçoamento de Pessoal do Nível Superior
d	days
et al.	and others
Fc	Focus
FHR	Fetal Heart Rate
FIRS	Fetal Inflammatory Response Syndrome
GUT	Gut-associated Lymphoid Tissues
HR	Heart Rate
Ht	Heart
kg	kilogram
Lg	Lung
LH	Luteinizing Hormone
Lk	Left Kidney
Lv	Liver
MALT	Mucosa-associated Lymphoid Tissues
MHz	Mega-hertz
M-mode	Movement mode
N	Number
RI	Resistive Index
SALT	Skin-associated Lymphoid Tissues
Sp	Spleen
St	Stomach
Tm	Thymus
USA	United States of America
WA	Washington

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CHAPTER 1

Fetal heart rate assessment in the final stage of pregnancy in cats

Abstract

The Ultrasonographic exam is an effective and non-invasive technique for fetal analysis, including evaluation of fetal cardiac activity and consequently fetal viability. Recent studies describe the occurrence of acceleration and deceleration in the fetal heart rate (FHR) in the antepartum period in dogs, an event already established in human beings, probably associated with uterine contractions. The present study investigated whether these variations also occur in cat fetuses and its relationship to parturition. A cohort study was conducted in 19 pregnant mixed breed queens undergoing ultrasonographic examination performed during the final stage of gestation with high resolution machine equipped with 7.5 to 12.0 MHz transducers. Measurements were made using M-mode to pick up the FHR. We grouped the following antepartum periods, 15-13 days, 12-10 days, 9-7 days 6-4 days, 3 days, 2 days and 1 day or less than 24 hours before parturition for the analysis purpose. All data first underwent the Shapiro-Wilk test, and subsequent ANOVA or Kruskal-Wallis test and Turkey's or Dunn's test for analysis of FHR variations; P-value less than 0.05 was considered significant. Acceleration and deceleration of FHR occurs in domestic cats, but not with the same intensity as in dogs to predict 12 hours to delivery, but we found data about eminent delivery in 48 hours.

Keywords: cat, fetal ultrasonography, heart rate

1.1 Introduction

Gestational ultrasonography in dogs and cats can be used to determine fetal age and detect early signs of fetal distress [1, 2, 3]. These assessments help determine the optimal time to perform a cesarean section and avoid conceptus losses [3].

Ultrasonography is an effective and non-invasive technique for fetal analysis, including evaluation of fetal cardiac activity, which can be performed after gestational day 16 or 17 in domestic cats [4, 5, 6]. The fetal heart rate (FHR) is an important parameter used for the analysis of the viability of the conceptus [1, 2-5]. The basal feline FHR has been established at an average of 228.2 ± 35.5 beats per minute (bpm), showing a low variation during pregnancy in domestic cats [1].

Domestic female cats are induced ovulators [4] in which a luteinizing hormone (LH) surge occurs after mating, with ovulation following after 24 to 40 hours [7, 8], facilitating the calculation of gestational age. Echobiometry, alone or in combination with high-resolution ultrasound analysis of fetal organogenesis can also assist in determining the gestational age. However, this method is only accurate to within one or two days [5, 9] with unknown-age pregnancies. This could make it difficult to determine the best time for the caesarean section if necessary, especially in brachiocephalic cats [10, 11].

Recent studies describe the occurrence of FHR variation in the antepartum in dogs [3, 12], alike in humans [13, 14]. This variation of the FHR is probably due to the effects of uterine contractions during labor. In dogs, it is typically observed between 72–24 hours pre-delivery, which intensifies as the delivery approaches 6–1 hour. FHRs as low as 119 bpm have been observed as a result of this natural variation, which could be misinterpreted for signs of fetal distress [2, 3].

Our hypothesis is that the FHR oscillation may occur in the pre-delivery period in domestic cats. This study focused on the last stage of pregnancy in queens. This study had four objectives, namely: 1. To determine if oscillation of FHR can occur in domestic cats with normal delivery; 2. To identify the time of onset in the pre-delivery period (days); 3. To identify the degree of the variation (acceleration versus deceleration); 4. To assess whether these measurements can help decide the optimum time for delivery.

1.2 Materials and Methods

1.2.1 Patient selection

Nineteen pregnant mixed breed queens between 1 and 3 years of age, each weighing 3–3.9 kg were selected for this cohort study. Most queens were adopted as adults, therefore, data regarding previous deliveries is unknown. The fetal ultrasonographic and echocardiographic examinations were performed during the last stage of gestation. All procedures were done in accordance with the Animal Use Committee guidelines. All owners provided written consent for the subjects to be included in the study. The exclusion criteria were as follows: queens that were unavailable for hospitalization, thus, making ultrasound sequential examinations unfeasible, queens that underwent caesarian section, and queens that presented with concomitant disease.

1.2.2 Ultrasonography equipment

The ultrasonographic evaluation of the fetal characteristics and FHR were performed using a high-resolution ultrasound machine (Esaote MyLab^{TM} 30Gold VET, Genova, Italy) equipped with a 7.5–12 MHz sector array transducer (model LA523). The frequency was adjusted between 10 to 12 MHz to improve the image when necessary, as well as to adjust the gain, focus, and depth of penetration for each fetus during examination.

1.2.3 Subjects procedures

All queens were sent to the trichotomy room, and abdominal hair was clipped to optimize the ultrasonographic image. In the ultrasonography room, the queens were restrained in dorsal recumbency using a sponge trough, and an acoustic gel was applied to the transducer. The exams were performed by two trained and experienced sonographers (Silva, M.A.F. and Monteiro, C.L.B.). The examination protocol included scanning the whole abdomen by circling clockwise, starting with the fetus in the left uterine horn (cranial to caudal), followed by the right horn (caudal to cranial), preventing us from examining the same fetus more than once. The analysis of FHR was performed in fetuses positioned in a way that allowed good visualization of the cardiac chambers, and image acquisition in M-mode with adequate waves (Figure 1.1). A period of three minutes was established for the analysis of each fetus, and in every period ten values of FHR were tabulated. In pregnancies with four or less fetuses, each one was evaluated. In large litters, the assessment of a maximum of four fetuses was standardized, so as not to prolong the examination period. A total of 12 to 20 minutes was spent per exam.

Whenever possible, the pregnant queens were examined ultrasonographically once a week throughout pregnancy, from the estimated 20th day of gestation, and probable gestational time was estimated by organogenesis and fetal biparietal diameter as suggested by Topie et al. [9]. Queens that entered the project in more advanced gestation followed the same protocol, with weekly evaluations to estimate the gestational age. At approximately

gestational day 58, the subjects were housed in single cages (dimensions 200 cm × 95 cm) in an animal facility with twelve hours of artificial light, using commercial feeding and water *ad libitum*. After hospitalization, sonographic examinations were carried out twice a day until gestational day 62 and then every four hours to evaluate the FHR until delivery. Antepartum time was calculated after collection and data analysis, by counting backwards from the date of delivery, considering birth as zero hour. For the analyses, data were grouped into the following periods: 15–13 days, 12–10 days, 9–7 days, 6–4 days, 3 days, 2 days, and 1 day or less than 24 hours before parturition.

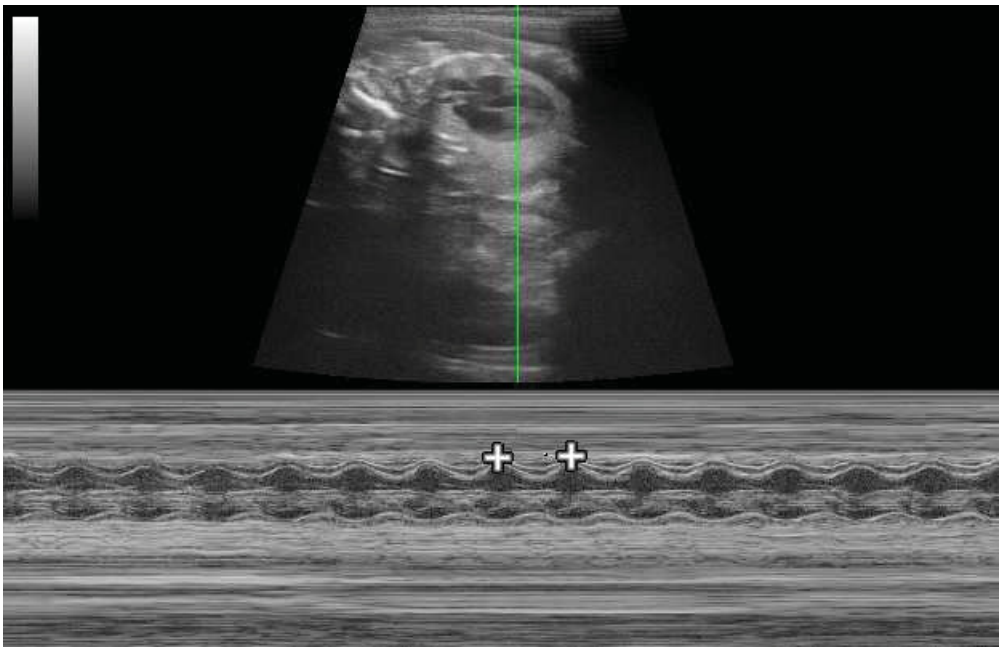

Figure 1.1 - Representative fetal heart M-mode ultrasonography to measure the heart rate. Heart rate, 225bpm.

In line with the study by Giannico et al. [12], we calculated three parameters: the heart rate (*HR*) *Average*, obtained by measuring ten values of FHR per fetus; the *HR Gradient*, calculated by the difference between the highest and lowest FHR in bpm (*HR Gradient* = $HR_{maximum}$ - $HR_{minimum}$); the *HR Variation*, obtained by the percentage variation of FHR in relation to the maximum heart rate recorded (*HR Variation* = *HR Gradient* x 100 / $HR_{maximum}$).

1.2.4 Statistical analysis

All statistical tests were selected and performed by one of the authors (Sousa, M.G.). The Shapiro-Wilk test was used to check for normality. For normally distributed data, analysis of variance (ANOVA) was used to check for differences between the prepartum periods, while data that were not normally distributed were analyzed with the Kruskal-Wallis test. Once differences had been documented, they were further investigated using either Tukey's or

Dunn's test. The analyses were performed using the GraphPad Prism software (Version 5.0 - San Diego, CA, USA) using default settings. A *P*-value < 5% was considered significant.

1.3 Results

All 19 queens included in this study had 1 to 7 kittens, with an average of 4.5 kittens (queens/kitten(s): 2/1, 2/3, 7/4, 3/5, 4/6, 1/7). As measurements were performed in up to four fetuses in each pregnant cat, a total of 68 fetuses were evaluated.

Three queens were primiparous, two queens were multiparous, and the other queens had unknown pregnancy history due to the fact that they were adopted as adults. All fetuses were born healthy and all queens had normal delivery. The fetal hearts were analyzed for three minutes to detect changes in HR. The *HR Average* values, *HR Gradient*, and *HR Variation* in different periods, as well as the results of the Kruskal-Wallis tests and ANOVA, are reported in Table 1.1.

Most notable in the *HR Average* at the 1–0 day period is the contrast between the minimum and maximum values compared to those at 15–13 days, showing a decreasing trend near the delivery. Due to the difference between the maximum and minimum values, the results are best demonstrated using the *HR Gradient* and *HR Variation*. Table 1.1 shows that these parameters progressively increase as delivery approaches.

Table 1.1 - Data of fetal *HR Average* in beats per minute (bpm), *HR Gradient* in bpm, *HR Variation* in percentage, and the *P*-value of the test applied, in 19 queens with normal pregnancy assessed in days before delivery.

Parameter	***Data***	***15-13d***	***12-10d***	***9-7d***	***6-4d***	***3d***	***2d***	***1-0d***	***P-value***
	Minimum	243.6	223.8	225.6	203.6	192.7	169.9	154.3	
	25% Percentile	243.6	236.5	236.5	220.3	210.7	179.0	195.5	
	Median	249.3	240.2	243.4	232.2	225.0	189.1	207.8	
	75% Percentile	263.8	251.5	247.3	243.5	245.3	238.6	226.5	
HR	Maximum	263.8	273.8	248.9	266.7	259.2	258.6	260.5	
Average	Mean	252.2 ^{A}	244.1 ^{A}	241.2 ^{A}	232.6 ^{A}	226.4 ^{AB}	204.9 ^{B}	209.3 ^{B}	<0.0001
(bpm)	Standard deviation	10.41	13.31	7.396	16.62	21.24	30.80	26.77	
	Lower 95% CI	226.4	236.8	235.5	224.6	213.5	188.5	200.8	
	Upper 95% CI	278.1	251.5	246.9	240.6	239.2	221.3	217.7	
	Coefficient of variation	4.13%	5.45%	3.07%	7.14%	9.38%	15.03%	12.79%	
	N	3	15	9	19	13	16	41	
	Minimum	30.00	32.00	32.00	25.00	26.00	21.00	25.00	
	25% Percentile	30.00	36.00	37.00	39.00	41.50	42.25	41.00	
	Median	44.00	43.00	47.00	56.00	52.00	60.00	57.00	
	75% Percentile	45.00	51.00	51.50	66.00	68.00	72.25	77.50	
HR	Maximum	45.00	60.00	76.00	118.0	90.00	85.00	131.0	
Gradient	Mean	39.67	44.33	47.33	57.53	55.08	58.19	63.20	0.0924*
(bpm)	Standard deviation	8.386	8.699	12.93	25.72	18.63	19.08	27.82	
	Lower 95% CI	18.83	39.52	37.39	45.13	43.82	48.02	54.41	
	Upper 95% CI	60.50	49.15	57.27	69.92	66.33	68.35	71.98	
	Coefficient of variation	21.14%	19.62%	27.32%	44.71%	33.83%	32.78%	44.03%	
	N	3	15	9	19	13	16	41	
	Minimum	11.76	12.21	12.21	9.800	9.920	11.76	9.800	
	25% Percentile	11.76	14.12	14.29	15.32	16.39	18.74	16.86	
	Median	15.38	16.41	18.43	20.25	20.31	27.82	25.33	
	75% Percentile	16.67	19.85	18.89	25.85	25.88	30.80	32.97	
HR	Maximum	16.67	22.22	25.76	41.26	37.19	34.11	50.20	
Variation	Mean	14.60 ^{A}	16.59 ^{A}	17.55 ^{AB}	21.82 ^{AB}	21.87 ^{AB}	25.07 ^{A}	25.92 ^{B}	0.0019
(%)	Standard deviation	2.545	3.153	3.944	8.894	7.840	7.922	9.980	
	Lower 95% CI	8.280	14.84	14.52	17.53	17.13	20.85	22.77	
	Upper 95% CI	20.93	18.33	20.58	26.11	26.60	29.29	29.07	
	Coefficient of variation	17.43%	19.01%	22.48%	40.76%	35.86%	31.60%	38.50%	
	N	3	15	9	19	13	16	41	

d, day(s); N, number (set of ten assessments of fetal heart rate). Values with exponent A, B or AB show equality or differences between them in the same line (A = AB; AB = B and B ≠ A). *Kruskal-Wallis test.

The coefficient of variation for *HR Average* tends to increase, starting at 4.13% in the 15–13 day period, and reaching 12.79% in the 1–0 day period (Table 1.1). Figure 1.2 shows a decrease in the mean of *HR Average*; Figure 1.3 shows an increase in the median of *HR Gradient*, and Figure 1.4 shows an increase in the average of *HR Variation*; all these trends occur as delivery approaches.

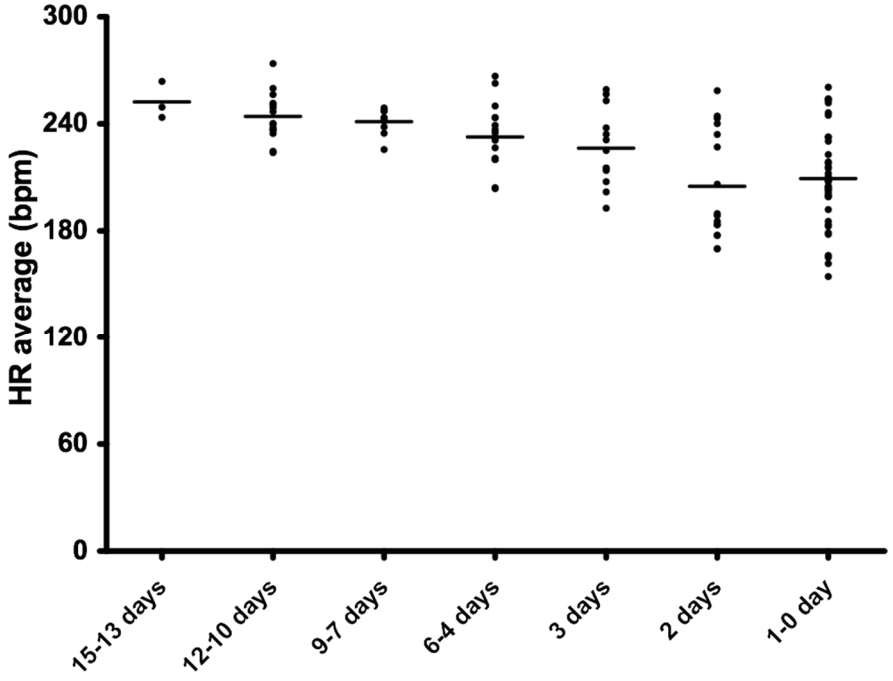

Figure 1.2 - Graphic of the distribution of fetal *HR Average* (in beats per minute) showing a decrease of the mean during the final third stage of pregnancy in the 19 queens with normal delivery. HR, heart rate.

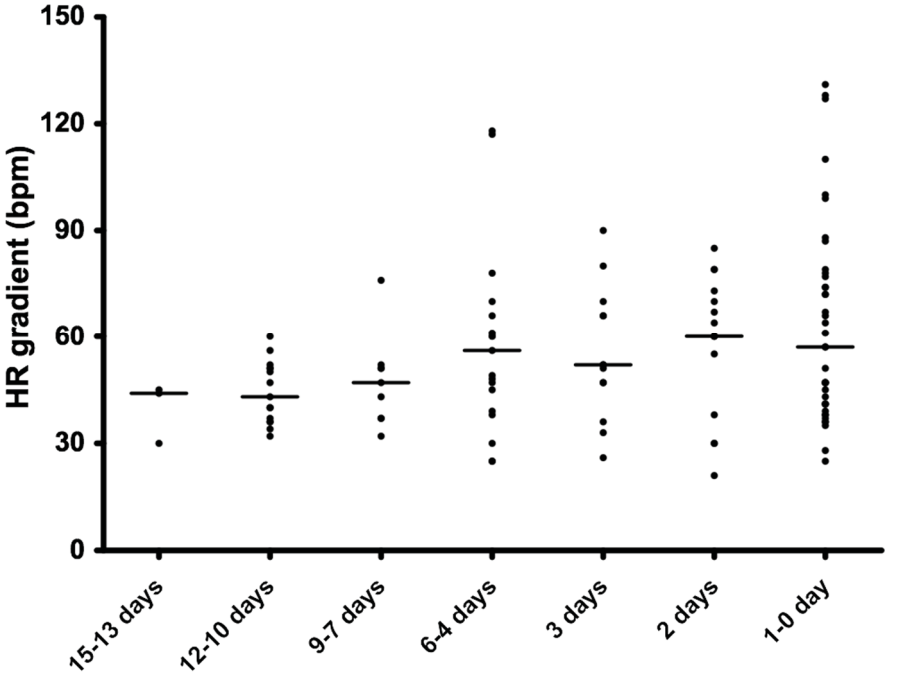

Figure 1.3 - Graphic of the distribution of fetal *HR Gradient* (in beats per minute), showing a decrease of the median during the final third stage of pregnancy in the 19 queens with normal delivery. HR, heart rate.

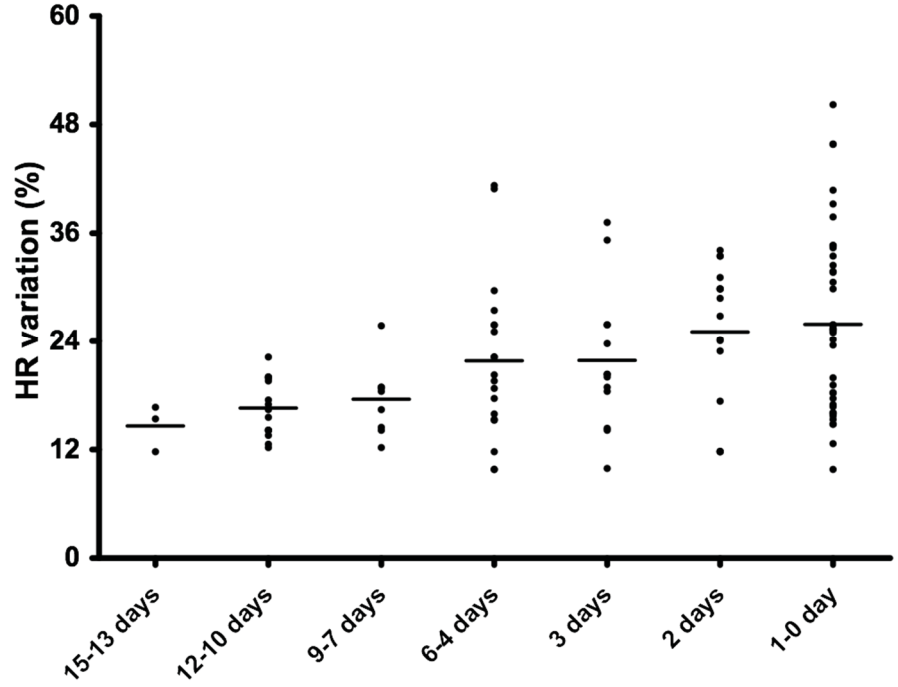

Figure 1.4 - Graphic of the distribution of fetal *HR Variation* (in percentage), showing an increase of the mean during the final third stage of pregnancy in the 19 queens with normal delivery. HR, heart rate.

The 95% confidence intervals of *HR Average*, *HR Gradient,* and *HR Variation* in each of the prepartum periods are shown respectively in Figure 1.5, Figure 1.6 and Figure 1.7. These figures show the overlap of confidence intervals. The *HR Average* parameter indicates that values less than 170 bpm are only seen in the 2 day or 1–0 day periods, but the maximum values of these periods can also be observed in the 3 days or more (Figure 1.5).

Despite the fact that the confidence interval from the minimum value of 54.41 bpm of *HR Gradient* in the 1–0 day period overlaps all the periods evaluated, a gradient above 71.98 bpm may indicate the 1–0 day period (Figure 1.6). However, there is a close proximity with the maximum value detected in the period of 6–4 days (69.92 bpm) (Figure 1.6). Note that in some fetuses, *HR Variation* values greater than 22.77% may occur in the 1-0 day period, but this value is not indicative, as it was also observed in all other periods (Figure 1.6). However, *HR Variation* values higher than 26.60% occurred only in the period of 2 days and 1–0 day (Figure 1.7).

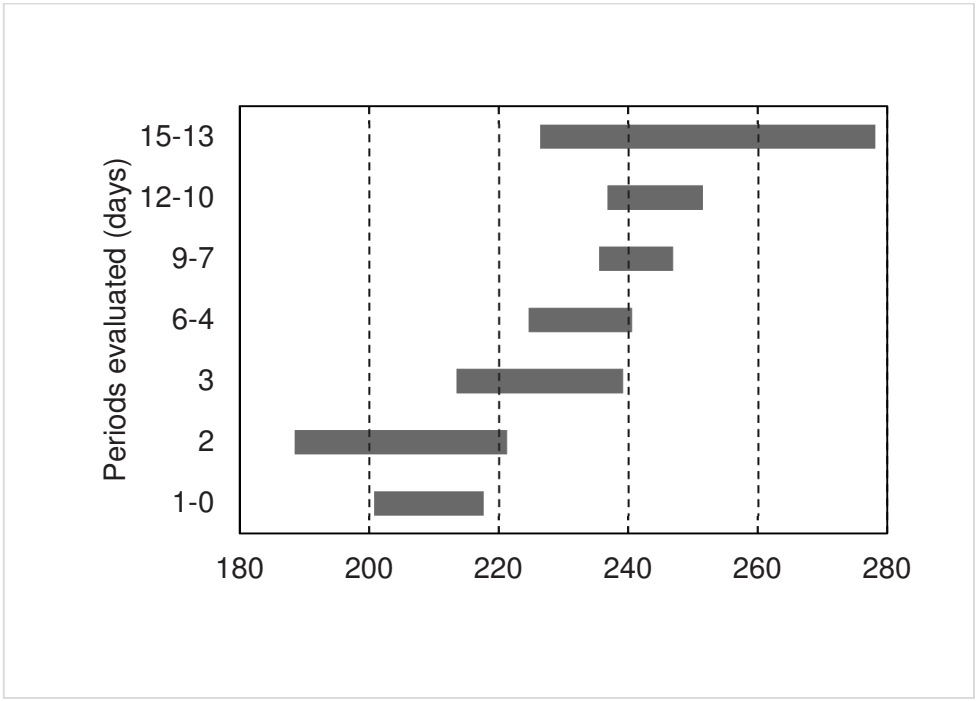

Figure 1.5 - Confidence interval of 95% for *HR Average*. Notice the overlap of these confidence intervals in the final third stage of pregnancy in the 19 queens with normal delivery. HR, heart rate; bpm, beats per minute.

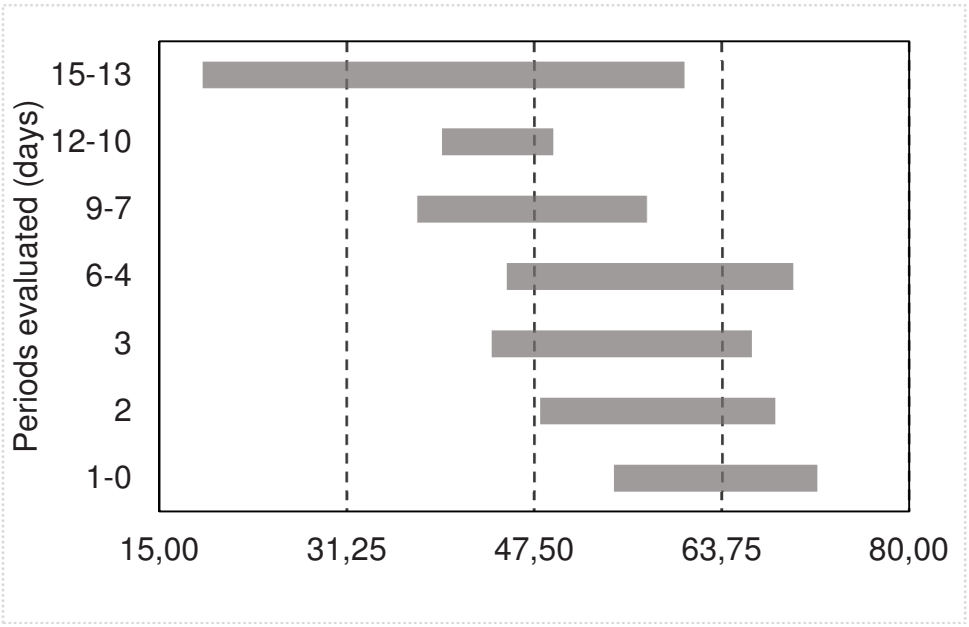

Figure 1.6 - Confidence interval of 95% for *HR Gradient*. Notice the overlap of these confidence intervals in the final third stage of pregnancy in the 19 queens with normal delivery. HR, heart rate; bpm, beats per minute.

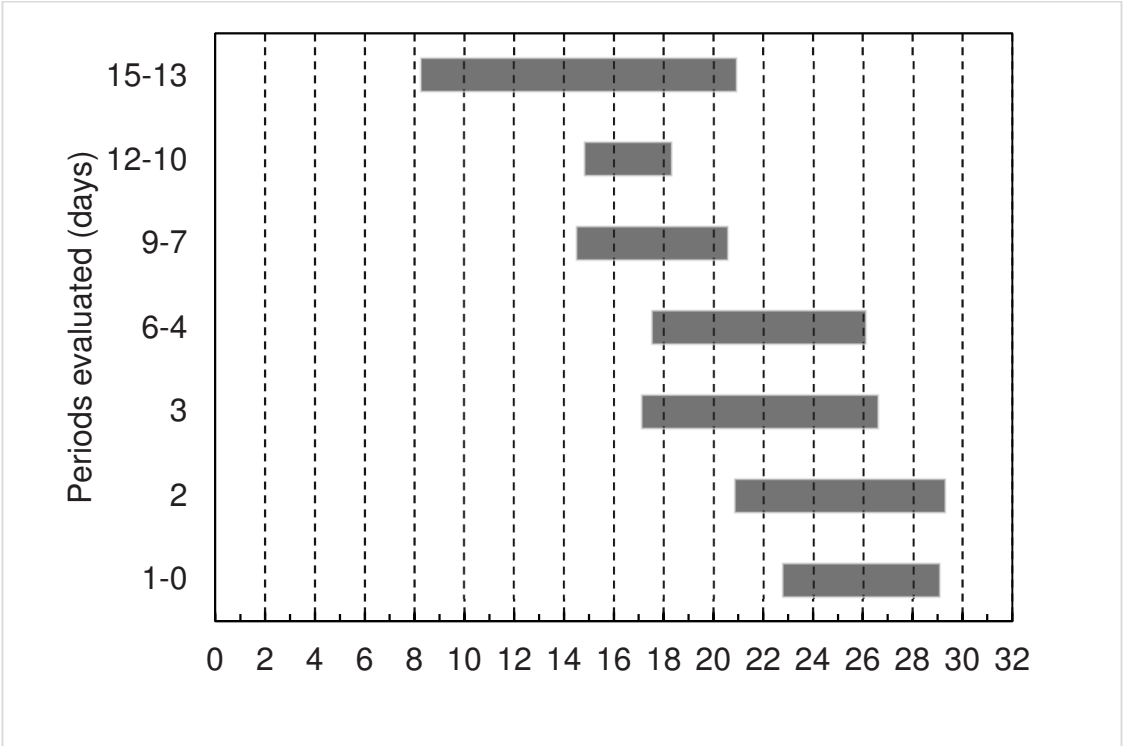

Figure 1.7 - Confidence interval of 95% for *HR Variation*. Notice the overlap of these confidence intervals in the final third stage of pregnancy in the 19 queens with normal delivery. HR, heart rate.

1.4 Discussion

In this study, it was observed that FHR oscillation occurs in pregnant domestic cats, as described in other species, and primarily reported in human fetuses by Caldeyro-Barcia et al. [13] and Hon [14]. These authors also found that humans suffer decelerations in FHR, as well as so-called dips or falls, and that these changes are associated with the peak of the uterine contractions during labor and delivery.

The maximum and minimum *HR Average* values showed a decrease with a good association near to the delivery considering the gradient and variation. After 48 hours pre-partum, the maximum *HR Average* did not rise above 225 bpm (Figure 1.5). Verstegen et al. [1] measured the basal FHR in the queen from the 25th day of gestation until delivery and reported values of 228.2 ± 35.5 bpm; these minimum values are distant from those absolute values of FHR found in our study, which was 127 bpm. However, that study had a smaller sample size and measured the FHR over a shorter window. The 3 minutes measurement duration in this study may have allowed us to capture more changes in FHR. Gil et al. [3] showed that the absolute value of FHR reached a minimum of 119 bpm in the last 12 hours before parturition in dogs.

The changes in FHR are probably caused by uterine contractions, which cannot be detected or differentiated by ultrasonographic examinations [14]. It should be noted that the minimum FHR values detected in this study should not be associated with fetal distress, especially because the lower limit of values in the sample was 127 bpm. Older data suggest that FHR values below 180 bpm indicate fetal distress [2]. However, also in cats, if a subsequent acceleration in FHR is detected, values below 180 bpm should instead be considered physiological effects correlated with uterine contractions in the prepartum period [3, 13, 14]. Conversely, if the FHR stays below 180 bpm, the diagnosis of fetal distress is confirmed, probably linked to fetal hypoxia signals, making it necessary to perform a cesarean section [3, 15]. In the present study, we observed FHR values below 180 bpm in all fetuses analyzed (Table 1.1).

The FHR oscillation in dogs begins to increase five days before birth and intensifies as delivery approaches, with large amplitudes of acceleration and deceleration. These changes in the FHR in dogs have helped decide the best

moment for cesarean or other surgical intervention [3, 12]. However, as shown is this study, the oscillation of the FHR in queens is better analyzed with the values of *HR Gradient* and *HR Variation*, allowing the birth time to be determined with higher accuracy. However, the overlaps between the confidence intervals must be interpreted with caution, because the FHR changes are different compared to those in dogs. In cats, it is therefore advisable to monitor the FHR decrease and *HR Variation* in routine pregnancy ultrasound examinations.

In the present study, *HR Gradient* did not have significant statistical value, but this may be related to the non-parametric test used or the small study population. The coefficient of variation of both *HR Average* and *HR Variation* tended to increase as delivery approached (Table 1.1). A *HR Variation* confidence interval value greater than 26.61% was indicative of delivery occurring within 48 hours (Figure 1.7). Topie et al. [9] combined the measurements of head and abdominal diameters and were able to estimate the time of birth to within ± 2 days. Our results were similar in that, regardless of the estimated gestational age, we could predict the delivery to within 48 hours; the gestational period in the cat is between 63 to 67 days [16].

Breukelman et al. [17] found more episodes of acceleration than deceleration in cattle fetuses in the two weeks before birth, but there is a large bias with the possibility of calving occurring up to 20 days after the estimated date. A study conducted by Jonker et al. [18] had similar findings and also found larger differences between the maximum and minimum value of FHR. In the present study, the maximum FHR remained constant until the period closest to parturition, while the minimum values were of more clinical significance. In other words, the episodes of deceleration seem to predict the uterine contractions, while the maximum values reveal the basal HR.

This study was limited due to the small number of cats evaluated, which may have interfered in the statistical analysis. Future studies might investigate the umbilical artery resistance index, which is another promising parameter in dogs and possibly in cats.

1.5 Conclusion

In summary, the oscillation of FHR occurs in domestic cats, but not with the same amplitude as that observed in dogs within the 12 hours before delivery. The *HR Gradient* and *HR Variation* values described in our study are useful for monitoring the gestation and predicting the delivery within the 48 hours before delivery.

1.6 Acknowledgements

The authors would like to thank the owners who agreed to cooperate with this study and CAPES for the scholarship granted. Additionally, our special thanks to the DVM Fabiano Montiani-Ferreira to lend his animal facility in Veterinary Hospital of the Federal University of Paraná to our subjects.

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CHAPTER 2

Intrauterine identification of lymphoid organs in cats' fetuses by ultrasonographic exam: characterization of spleen and thymus

Abstract

The aim of this study was to describe in the ultrasonographic exam minor structure like the lymphoid organs in cat fetuses these not yet described in the literature. We believed that due to the high-resolution devices, the spleen and thymus identification is possible. Sixteen pregnant mixed breed queens were selected to this study. The fetal ultrasonography exam was performed from 30 days of fetal age until delivery, corresponding to organogenesis and parametric data. The thoracic and abdominal fetal ultrasonography were performed with high-resolution ultrasound machine equipped with 7.5-12.0 MHz linear transducer. The pattern of echogenicity, echotexture, shape of the spleen and thymus, with ultrasonographic windows in the respective gestational age were described. A detailed evaluation of the thymus and spleen was made, and both fetal lymphoid organs were identified starting from the 40 days' gestational age. The fetal spleen have a homogeneous echotexture and could be hyperechoic or isoechoic to liver, and has a hyperechoic line capsule surrounding all organ. The fetal thymus has an almost trapezoidal shape, more mottle echotexture, composed by a hypoechoic and logitudinal hyperechoic lines; and hypoechoic compared to the adjacent lung. Our results may help futures studies to data of normal size of these lymphoid organs.

Keywords: cat, fetal ultrasonography, organogenisis, fetal spleen, fetal thymus

2.1 Introduction

The lymphoid system produces specialized cells which are responsible for the immunological activity. Primary lymphoid tissues are found in the thymus, bone marrow, and the ileal aggregated lymphoid nodule (Peyer's patch), while the secondary tissues include the spleen, lymph nodes, mucosa-associated lymphoid tissue (MALT), gut-associated lymphoid tissue (GUT), and skin-associated lymphoid tissue (SALT) [1].

Recent ultrasonography studies with high-frequency probes have produced highly detailed images of fetal structures [2] [3] and facilitated in depth studies of organogenesis [2] [3] [4] [5]. However, some minor fetal organs and glands in cats, including fetal lymphoid organs such as the spleen and thymus, are difficult to visualize via ultrasonography.

The spleen is a hematopoietic organ until the late fetal phase and its lymphoid responsibilities, i.e., lymphocyte and monocyte production continue throughout life [6]. Ultrasonographic evaluation of the fetal human spleen is possible from 20 weeks of gestation. The data that can be assessed include the size and weight of the spleen, as well as sonographic conformation, with homogenous and hypoechoic presentation of the parenchyma [6] [8] [9].

The thymus is an important organ of the cellular arm of the mammalian immune system [10]. This lymphatic component is responsible for the development and maturation of T cells in fetuses and neonates [11]. The first ultrasound study of the thymus in human showed the possibility of intrauterine evaluation from 14 weeks of gestation. In a study with 251 sonograms, the fetal thymus presentation was hypoechogenic, isoechogenic, and hyperechogenic in relation to the lung [7].

The new ultrasound devices with high-resolution probes bring new opportunities for the evaluation of small structures in fetal life. In the absence of ultrasonographic description of the spleen and thymus of cats' fetuses, this study contains descriptive data on the appearance of these organs through evaluation *in utero*.

2.2 Materials and Methods

2.2.1 Patient selection

Sixteen pregnant mixed breed queens between 1 and 3 years of age, weighing 3–3.9 kg each, were included in this study. The fetal ultrasonography examinations were performed at the second and third trimester of gestation until delivery. All procedures were carried out in accordance with the Animal Use Committee guidelines. All owners provided written consent for their animals to be included in the study. The exclusion criteria were cats that were unavailable for serial examinations, cats that underwent caesarian section, and cats that presented with concomitant disease.

2.2.2 Ultrasonography equipment

The ultrasonographic evaluation of the thorax and abdomen fetal structures were performed using a high-resolution ultrasound machine (Esaote MyLab^{TM} 30Gold VET, Genova, Italy) equipped with a 7.5–12 MHz sector array transducer (model LA523). The frequency was adjusted between 10 and 12 MHz to improve the image when necessary, as well as to adjust the gain, focus, and depth of penetration for each fetus during the examination.

2.2.3 Subjects procedures

All queens were sent to the trichotomy room, and abdominal hair was clipped to optimize the ultrasonographic image. In the ultrasound room, the queens were restrained in dorsal recumbency using a sponge trough, and acoustic gel was applied to the transducer. All exams were performed by one sonographer (Silva, M.A.F.). The examination protocol included scanning the whole abdomen by circling clockwise, starting with the fetus in the left uterine horn (cranial to caudal) followed by the right horn (caudal to cranial), as described by Gil et al. [12].

The ultrasonographic examinations were performed in the pregnant queens from 30 days of fetal age, using organogenesis and parametric data to estimate gestational age [2]. After 36 days of fetal age, ultrasonographic evaluations were performed every other day. The true gestational age was confirmed after delivery (defining delivery as day 65 of gestation [2]).

A preview fetal longitudinal cardiac examination was performed to locate the cranial mediastinum, the most probable site of the thymus [14]. For the spleen characterization, the gastric chamber was used as an anatomical reference on the fetus' left side, using the left kidney as well as the stomach for the probable spleen site [15].

2.2.4 Data analysis

The data were tabulated using the Microsoft Office Excel 2016 program (Redmond, WA, USA) for descriptive analysis. The day of birth was defined as the 65th day of gestation [2] to indicate the age of ultrasonographic appearance of both the thymus and spleen [2]. We tabulated the number of queens in which it was possible to individualize the spleen and thymus independent of the number of fetuses. We also tabulated the number of detected spleens and thymus corresponding to the litter size in each queen.

Fetuses in which the lymphoid organs could not be identified were classified as "unseen." The fetuses that had the spleen and thymus identified were tabulated with their respective gestational age, as well as the number and percentage of fetuses evaluated in relation to the size of the litter, confirmed in the postpartum delivery period.

The pattern of echogenicity of the fetal spleen was compared to that of the liver and the pattern of echogenicity of the thymus was compared to that of the lung. Changes in echogenicity during gestation were also recorded.

2.3 Results

The mean duration of pregnancy of the 16 pregnant mixed breed queens included in this study was 65 days (± 2 days). There were no post-delivery complications in any of the queens evaluated. We verified that it is possible to identify the fetal lymphoid organs (spleen and thymus) via ultrasound monitoring of fetal organogenesis and developmental evaluation of fetal intra-abdominal and intra-thoracic organs. Both fetal lymphoid organs were identified from 40 days' gestational age (± 2 days).

After identifying the fetuses, longitudinal, transverse, and dorsal sonographic sections of the fetal abdomen were made for the spleen search. The fetal spleen was identified when the fetus' abdomen was located in the middle of the screen aligned with the focus in a dorsal plane section (Figure 2.1).

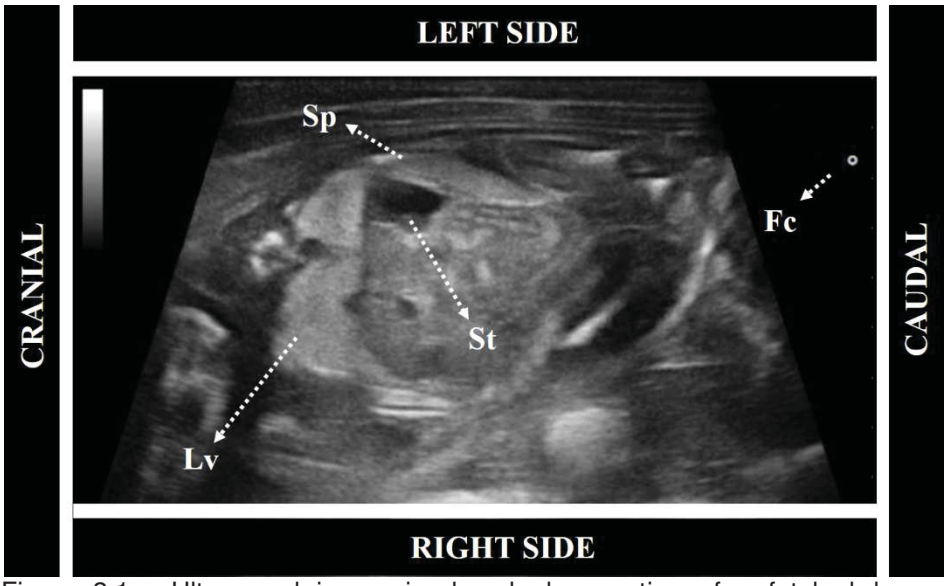

Figure 2.1 - Ultrasound image in dorsal plan section of a fetal abdomen evidencing the stomach (St) like an anechoic chamber its located mediocranial to the spleen (Sp). Cranial to these both organs are possible to visualize the liver (Lv). A line focus (Fc) is positioned to the target organ (Sp).

The main anatomic reference structure used to identify the fetal spleen was the stomach. The fetal stomach was easily identified and provided a reference on the left side of the abdomen, creating an acoustic window for the spleen visualization. The left kidney could also be used as an anatomic reference point for fetal spleen visualization (Figure 2.2). The fetal spleen was most often positioned caudolateral to the stomach (Figure 2.1) and lateral or craniolateral to the left kidney (Figure 2.2).

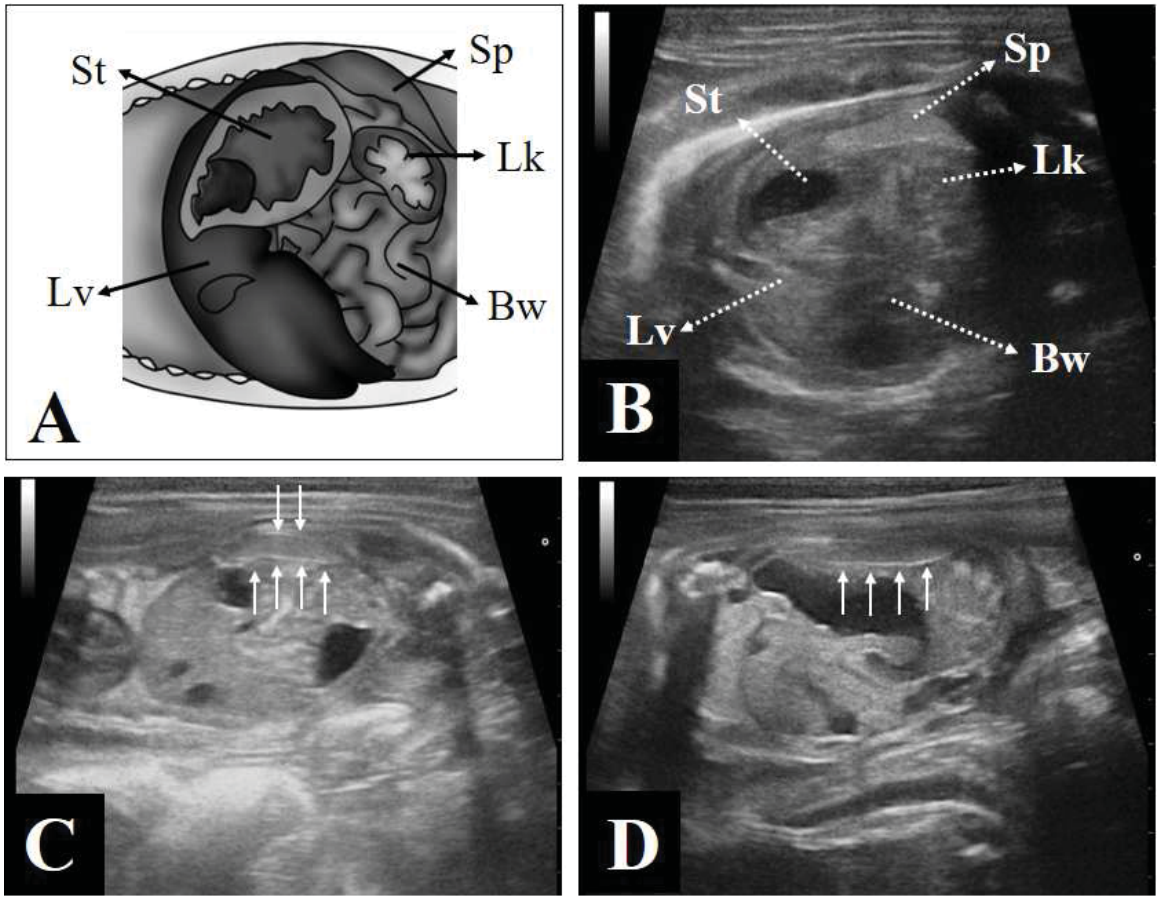

Figure 2.2 - Schematic design (A) punctuating the fetal abdominal organs and their correspondents in ultrasound image (B). The stomach (St) makes an acoustic window to identification the spleen (Sp) as well as the left kidney (Lk) can enable this same function; in this height of ultrasound section is possible to view the bowel (Bw) and the liver (Lv). Ultrasounds images (C and D) demonstrating a very thin hyperechoic line bypassing the spleen (capsule) pointed by the arrowheads.

The fetal spleen was sonographically visualized as a homogeneous structure (Figure 2.3), more hyperechoic than the liver in 10/16 queens (62.5%) and isoechoic in 4/16 (25%). Both echogenicities were found in different fetuses in 2/16 queens (12.5%) (Table 2.1). The spleen's capsule was seen as a very thin hyperechoic line surrounding the organ (Figure 2.2).

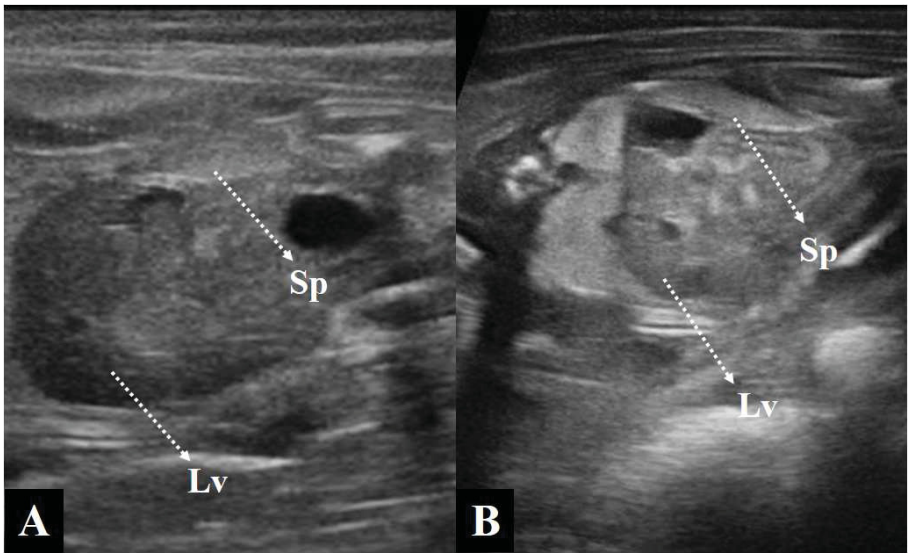

Figure 2.3 - Ultrasounds images demonstrating a homogeneous aspect of the spleen (Sp) parenchyma. There are differences in echogenicity between the liver (Lv) and spleen. The spleen could be more hyperechoic than liver (A) or it could be isoechoic (B).

Table 2.1 - Fetal spleen and fetal thymus ultrasonographic echogenicity characteristics compared to the liver and lung respectively. Total of 16 queens with 29 fetal splenic evaluations and 61 fetal thymic evaluation.

Queen	***Splenic Echogenicity Compared to Liver***	***Thymic Echogenicity Compared to Lung***
A	Hyperechoic	Hypoechoic
B	Hyperechoic	Hypoechoic
C	Hyperechoic	Hypoechoic
D	Isoechoic	Hypoechoic
E	Isoechoic/Hyperechoic	Hypoechoic
F	Hyperechoic	Hypoechoic
G	Hyperechoic	Hypoechoic
H	Isoechoic	Hypoechoic
I	Isoechoic	Hypoechoic
J	Hyperechoic	Hypoechoic
K	Isoechoic	Hypoechoic
L	Isoechoic/Hyperechoic	Hypoechoic
M	Hyperechoic	Hypoechoic
N	Hyperechoic	Hypoechoic
O	Hyperechoic	Hypoechoic
P	Hyperechoic	Hypoechoic

From the 40th day of gestation, it was possible to individualize the fetal spleen. As development progressed, the identification became easier, especially from the 49th day until delivery (Table 2.2) with an increased number of fetuses with this organ observed (Table 2.3). Of the 16 queens evaluated, in only four (25%) was it possible to identify the fetal spleen during the early period of 40–43 days of gestation, while that number increased to 12 queens (75%) from the 49th day of gestation (Table 2.2). The percentage of identification of the spleen was calculated in relation to the litter size of each queen (Table 2.3).

Table 2.2 - Periods of ultrasonographic characterization of the fetal spleen divided in an early period and a late period in 16 queens.

	Early Spleen Characterization Period (40-43d)	***Better Spleen Characterization Period (≥49d)***
Queen	***Gestational age in days/Days to delivery/Number of fetus(es)***	
A	40d/25d/2	49d/16d/3
B	Unseen	49d/16d/2
C	Unseen	51d/14d/2
D	Unseen	51d/14d/3
E	Unseen	53/12d/3
F	Unseen	Unseen
G	43d/22d/1	51d/14d/1
H	42d/23d/2	53d/12d/2
I	Unseen	Unseen
J	Unseen	51d/14d/2
K	Unseen	53d/12d/1
L	Unseen	51d/14d/1
M	Unseen	Unseen
N	Unseen	Unseen
O	40d/25d/1	49d/16d/1
P	Unseen	50d/15d/2

d, days.

Table 2.3 - Number and percentage of fetus(es) with splenic ultrasonographic characterization in an early view and a late period in 16 queens.

	Period 40-43d		***Period ≥49d***		
Queen	***Number of fetus(es)***	***%***	***Number of fetus(es)***	***%***	***Litter Size****
A	2	50	3	75	4
B	.	.	2	33.33	6
C	.	.	2	33.33	6
D	.	.	3	75	4
E	.	.	3	50	6
F	.	.	.	.	7
G	1	33.33	1	33.33	3
H	2	100	2	100	2
I	.	.	.	.	6
J	.	.	2	40	5
K	.	.	1	25	4
L	.	.	1	100	1
M	.	.	.	.	4
N	.	.	.	.	5
O	1	100	1	100	1
P	.	.	2	50	4

*Postpartum count number of kittens.

Similarly, longitudinal, transverse, and dorsal sonographic sections of the fetal thorax were made for the thymus search. The fetal thymus is better visualized with a focus aligned in the center of the thorax in the dorsal plane section, at a height that allows a total view of the heart (Figure 2.4). The fetal lung promotes an acoustic window for visualization of the thymus lobes as well as the great vessels of the heart (Figure 2.4 and Figure 2.5). A good maneuver is to slide the probe laterally until the thymus appears immediately cranial to the heart and in the center of the cranial mediastinum (Figure 2.4).

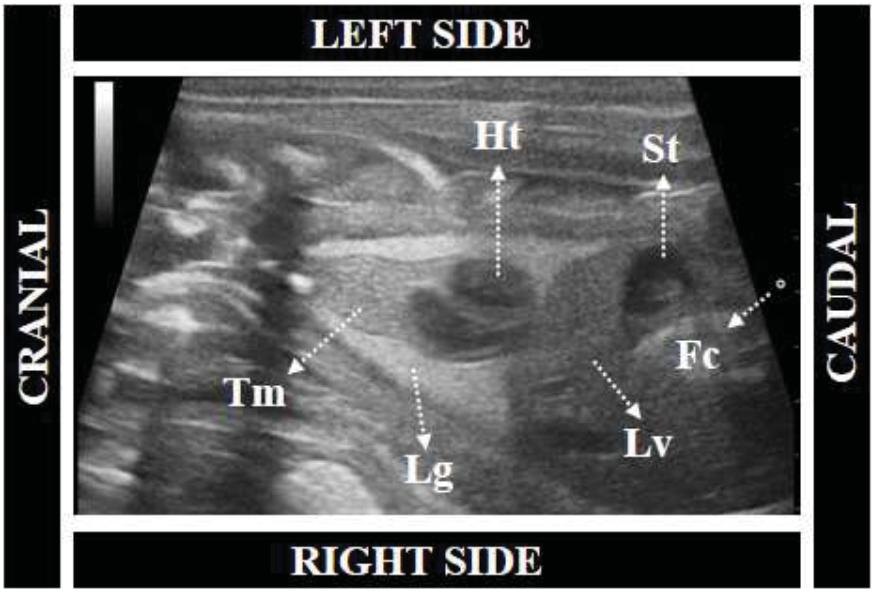

Figure 2.4 - Ultrasound image in dorsal plan section of a fetal thorax evidencing the lung (Lg), heart (Ht) and thymus (Tm). Part of the cranial abdomen region is visible facilitating the liver (Lv) identification. The left side determination is possible due the stomach (St) individualization. A line focus (Fc) is positioned to the target organ (Tm).

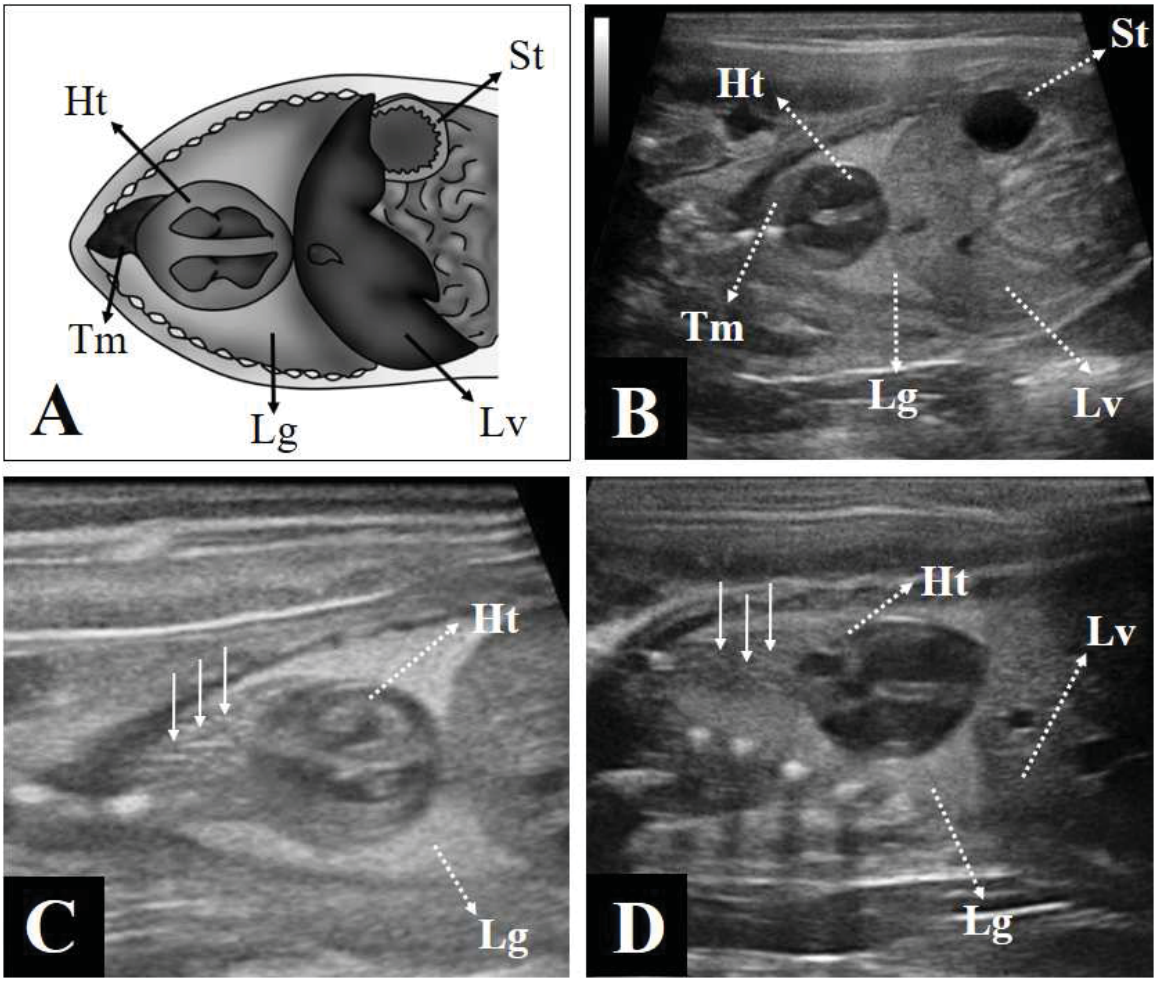

Figure 2.5 - Schematic design (A) punctuating the fetal thorax organs and their correspondents in ultrasound image (B). The lung (Lg) makes an acoustic window to identification the thymus (Tm) as well as the heart (Ht) can enable this same function. In this height of ultrasound section the cranial abdomen region allows the visualization of the liver (Lv) and stomach (St). Ultrasounds images (C and D) demonstrating longitudinal hyperechoic lines in the thymic parenchyma composed by a hypoechoic and heterogeneous echotexture (solids arrows). The thymus is immediately positioned cranial to the heart (Ht) and it is hypoechoic compared to the lung (Lg).

The fetal thymus has an almost trapezoidal shape (Figure 2.4) with smooth and rounded edges, and is composed of a hypoechoic and heterogeneous parenchyma with longitudinal hyperechoic lines (Figure 2.5 C and D). The thymus has a more hypoechoic appearance compared to the lung in the fetus (Table 2.1).

An early visualization of the fetal thymus was possible from the 41st day of gestation. After the 45th day of gestation, organ characterization became easier and more frequent (Table 2.4). In the period of 41–43 days of pregnancy, the thymic organ visualization was possible in 10 queens (62.5%) with a significant increase in this value to 16 queens in the late period (≥ 45 days) (100%) (Table 2.5). The percentage of identification of the thymus was calculated in relation to the litter size of each queen (Table 2.4).

Table 2.4 - Number and percentage of fetus(es) with thymic ultrasonographic characterization in an early view and a late period in 16 queens.

	Period 41-43d		***Period ≥45d***		
Queen	***Number of fetus(es)***	***%***	***Number of fetus(es)***	***%***	***Litter Size****
A	3	75	3	75	4
B	4	66.67	4	66.67	6
C	3	.	5	83.33	6
D	2	.	2	50	4
E	.	.	4	66.67	6
F	.	.	3	42.86	7
G	2	66.67	1	33.33	3
H	2	100	2	100	2
I	.	.	2	33.33	6
J	2	40	3	60	5
K	1	25	3	75	4
L	1	100	1	100	1
M	.	.	2	50	4
N	.	.	2	40	5
O	1	100	1	100	1
P	.	.	2	50	4

*Postpartum count number of kittens.

Table 2.5 - Periods of ultrasonographic characterization of the fetal thymus divided in an early period and a later period in 16 queens.

	Early Thymic Characterization Period (41-43d)	***Better Thymic Characterization Period (≥45d)***
Queen	***Gestational age in days/Days to delivery/Number of fetus(es)***	
A	41d/24d/3	45d/20d/3
B	41d/24d/4	45d/20d/4
C	42d/23d/3	45d/20d/5
D	41d/24d/2	45d/20d/2
E	Unseen	47d/18d/4
F	Unseen	47d/18d/3
G	43d/22d/2	45d/20d/1
H	42d/23d/2	45d/20d/2
I	Unseen	49d/16d/2
J	43d/22d/2	45d/20d/3
K	43d/22d/1	45d/20d/3
L	41d/24d/1	51d/14d/1
M	Unseen	49d/16d/2
N	Unseen	48d/17d/2
O	41d/24d/1	49d/16d/1
P	Unseen	50d/15d/2

d, days.

2.4 Discussion

In the present study, we could identify the lymphoid organs, the spleen and thymus, via ultrasonographic examination in cat fetuses from 40 days' gestational age. There have been no previous descriptions of the organogenesis or specific ultrasonographic aspect of these structures in queens' fetuses, although there are multiple studies that detail the fetal organogenesis of cats [2] [3] [4] [5].

Pioneering ultrasonographic studies in the 1980s revealed the organogenesis of the spleen [6] and thymus [7] in human fetuses. Recently, there has been a greater attention to the size of these structures, mainly the thymus, due to its correlation with the fetal immune system [10]. The reduction in human fetal thymus size has been described as a parameter for the early detection of intrauterine infection during gestation, as well as the diagnosis of preterm premature rupture of membranes [16] [18] [19] and chorioamnionitis [20] [21], demonstrating the importance of analyzing these structures in the fetus.

The spleen is a secondary lymphoid organ, located dorsolaterally in the abdomen, attached to the gastric primordial chamber by the gastrolienal ligament, part of the mesogastrium. It functions as a specialized filter in antigen-presenting

cells, restricting the inflow of antigens. The spleen is inserted into the bloodstream separately from the lymph nodes [1]. Ultrasonographically, the most important anatomical reference points for the identification of the fetal spleen in cats were the stomach and left kidney (Figure 2.1 and Figure 2.2). In humans, the stomach is also used as a reference structure for the fetal spleen, as well as the left adrenal gland [6] and the lateral surface of the vertebral column [8] [9] [22].

Ultrasound identification of the spleen in cat fetuses was possible from 40–43 days (Table 2.2 and Table 2.3). There are no specific data regarding the organogenesis of the spleen in cats, but in dogs, the macroscopic primordia of the spleen and thymus are detected between 27 and 28 days of gestation and the lymphoid organs are mature between 45 and 52 days when the lymphoid infiltration occurs [23]. The best ultrasonographic plane section for detection of the organ was the dorsal plane, using the stomach and left kidney as references (Figure 2.1 and Figure 2.2).

We believe that the ultrasonographic identification of the spleen is facilitated after 49 days of gestation in the queen by the caudal displacement of the stomach in relation to the liver, which was reported by Zambelli et al. [4] to occur between the 48th to 50th day of gestation (Table 2.2 and Table 2.3). The obstacles to fetal spleen identification in the queens were excessive fetal movements and hiccups [4] [5] (Table 2.3), unlike the limitations in human fetuses, which consist of poor fetal positioning and maternal fat [22].

In the present study, the presentation of the fetal spleen was homogeneous. Regarding hepatic echogenicity, 62.5% were hyperechogenic, 25% isoechogenic, and 12.5% isoechogenic/hyperechogenic (Table 2.1), with an elongated and flattened shape, similar to that in the adult cat, described as a "tongue-like" shape in the dorsal plane section [14]. In the rare cases in which the spleen was identified in the abdominal transverse section, a triangular appearance was seen, in agreement with the appearance of the adult cat spleen [15] and the human fetus [22] in this plane.

The thymus is a primary lymphoid organ in which T lymphocytes mature [1]. The thymus size is greatest in the newborn [1], which is probably why it is easy to identify in fetuses. In the queens' fetuses, the most cranial region of the mediastinum contained the thymus, similar to human fetuses [7]. We confirmed that the best ultrasonographic plane for visualization of the thymus was the dorsal

section of the fetal thorax, with centralization of the cardiac silhouette and the adjacent pulmonary lobes (Figure 2.4).

The anatomical reference points for the fetal thymus identification in cats were the great vessels at the heart's base, as well as the contrast of the echogenicity caused by the pulmonary lobes with the thymic gland, generating an acoustic window (Figure 2.4 and Figure 2.5). There are similar descriptions of anatomical reference points for the fetal thymus in other studies, which used three of the great vessels of the heart's base (aorta, pulmonary trunk, and superior vena cava) [10] [18] [24] [25] [26] [27].

It should be noted that the thymus was observed in all 16 queens included in our study when the fetal age was greater than 45 days, and in some queens, visualization was possible in all fetuses of the litter (Table 2.4 and Table 2.5). The best visualization of the fetus in both tabulated periods was made in the dorsal plane. In human fetuses, it is similarly difficult to locate the thymus at early gestational ages (14 weeks). However, there are reports of a rate of thymus identification of 100% at 18 weeks [28]. In other words, the growth in size of the thymus facilitates its identification.

In the present study, the obstacles to identifying the thymus included the inadequate positioning of the fetus, excessive fetal movement, and fetal hiccups. In the human fetus, visualization is typically impeded by acoustic shadowing caused by the ribs and clavicles [7] [28] [29], which were not seen in the current study.

The shape of the thymus has been variously described as quadrilateral with a rounded/pyramidal [7] or oval form [18] [24] [27] in humans. Based on our analysis, we believe that the best description of the fetal thymus shape in queens is "trapezoidal" (Figure 2.4). The lack of consensus in human medicine is probably due to the differences in the patients' physical characteristics and the imaging plane. To resolve this, other features such as contour, echogenicity, echotexture, and position should also be evaluated. In the fetuses of the analyzed queens, the thymus presented a regular contour with rounded borders, located in the cranial mediastinum, located in the cranial mediastinum, adjacent to the cranial aspect of the cardiac base (Figure 2.4 and Figure 2.5).

The fetal thymus has a hypoechoic appearance compared to the surrounding lung, and a heterogeneous echotexture with horizontal hyperechoic

lines inside. In human fetuses, the thymus presents with various echogenicities when compared to the pulmonary lobes, although hypoechogenicity is typical in the later stages of normal pregnancies [7].

Unfortunately, despite being able to identify the fetal spleen and thymus, we were unable to measure the dimensions of these organs. Previous reports in human fetuses have also reported such difficulties [6] [33].

2.5 Conclusions

Our results may help futures studies to provide parametric data of the normal characteristics of these lymphoid organs. Using the high-quality images that can be obtained using modern ultrasound devices, it is possible to observe the fetal thymus and spleen from 45 days of gestation. The observations of organogenesis can contribute to calculations of gestational age.

2.6 Acknowledgements

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ATTACHMENTS AND APPENDICES

3.1 Approval Certificate of “Comissão de Ética no Uso de Animais” from “Setor de Ciências Agrárias” - Federal University of Paraná.

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

CERTIFICADO

Certificamos que o protocolo número 044/2015, referente ao projeto “**Análise ultrassonográfica na gestação em gatas: revisão da organogênese e estudo do fluxo da artéria umbilical e da frequência cardíaca fetal**”, 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), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DO SETOR DE CIÊNCIAS AGRÁRIAS DA UNIVERSIDADE FEDERAL DO PARANÁ - BRASIL, em reunião de 27/05/2015

Vigência do projeto	Agosto de 2015 a Outubro de 2016
Espécie/Linhagem	Gato
Número de animais	50 (cinquenta)
Peso/Idade	Variado / 1 a 6 anos
Sexo	Fêmeas
Origem	Animais de proprietários trazidos ao Hospital Veterinário - UFPR

CERTIFICATE

We certify that the protocol number 044/2015, regarding the project “**Ultrasound examination during pregnancy in cats: a review of organogenesis and study of the flow of the umbilical artery and fetal heart rate”,** under **Tilde Rodrigues Froe** 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 the ANIMAL USE ETHICS COMMITTEE OF THE AGRICULTURAL SCIENCES CAMPUS OF THE UNIVERSIDADE FEDERAL DO PARANÁ (Federal University of the State of Paraná, Brazil), in session of 05/27/2015

Duration of the project	August 2015 to October 2016
Specie/Line	Cat
Number of animals	50 (fifty)
Wheight/Age	Miscellaneous / 1 to 6 years
Sex	Females
Origin	Veterinary Hospital – UFPR

Curitiba, 27 de Maio de 2015.

Ananda Portella Félix
Presidente CEUA-SCA

Simone Tostes de Oliveira Stedile
Vice-Presidente CEUA-SCA

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

3.2 VITA

Médico Veterinário formado pela Universidade Anhembi Morumbi, no ano de 2013.

Cursou residência médica veterinária com área de concentração em Diagnóstico por Imagem pela Universidade Federal do Paraná no período de março de 2013 a março de 2015.

Cursou o Programa de Pós-graduação em Ciências Veterinárias da Universidade Federal do Paraná no período de março de 2015 a março de 2017.

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