

High-definition ultrasonography in the evaluation of the reproductive tract of bitches during the follicular phase of the estrous cycle

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ABSTRACT

The aim was to sonographically evaluate the reproductive tract of bitches during the follicular phase of the estrous cycle using High Density (HD) ultrasonic techniques. Females ($n = 8$) were evaluated at five different times throughout the follicular phase, as determined by vaginal cytology and blood progesterone concentrations. Ultrasonic exams were performed using the ACUSON S2000/SIEMENS device utilizing a multifrequency HD transducer (5.5–18 MHz). Videos of the ovaries were obtained and recordings were evaluated using a DICOM viewer software for counting and measuring the ovarian structures, which were assigned to groups based on diameter in mm: G1: ≤ 1 ; G2: from 1.01 to 3.5; G3 from 3.51 to 5.5; G4: from 5.51 to 10. There was a greater uterine thickness with the progression of the follicular phase ($P < 0.05$). Six distinct regions were identified in the uterine wall. The ovarian dimensions increased ($P < 0.05$) as stage of the follicular phase advanced. There was fluid detected around the ovaries after ovulation. There was a characteristic fat tissue hyperechogenicity around the ovaries at all timepoints. There was a difference in the number of ovarian structures of each dimension group at each time there were assessments ($P < 0.05$). There was a difference in diameter of the largest ovarian structure and in average value of wall thickness at all timepoints when there were evaluations ($P < 0.05$). The HD ultrasonography technique provides for excellent image resolution, allowing for a more precise characterization of the bitch's reproductive structures and changes occurring during the follicular phase of the estrous cycle.

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1. Introduction

The complexity of the bitch's reproductive physiology is a great challenge for appropriate breeding management, because correct identification of the periovulatory changes is one of the determining factors for a successful determination of the physiological state of bitches (Groppetti et al., 2015). Monitoring the changes in reproductive tract structures related to the periovulatory period of bitches can be performed in many ways, such as observing clinical symptoms, vaginal cytology, vaginoscopy, hormonal assays and ultrasonography (Lindsay, 1983; Silva et al., 1996; England and Concannon, 2002; Moxon et al., 2012; Lévy, 2016). Due to physiological variations and individual characteristics of each bitch, however, such as differences in clinical manifestations among bitches, duration of each phase of the estrous cycle, interval between estrous cycles and findings as a result of conducting each of these examinations, such as correlation between vaginal cytology and blood progesterone concentrations, knowledge and experience is required, as well as cautious interpretations (Wilborn and Maxwell, 2012).

The utility of ultrasonography in small animal reproduction has evolved from safe and early pregnancy diagnosis to applications in reproductive management, allowing for obstetric evaluations, diagnosis of urogenital disorders and visualization of ovarian and uterine changes related to the female reproductive status (Davidson and Baker, 2009; Barbosa et al., 2013), however, to properly apply and interpret this imaging modality for evaluation of reproductive processes, the sonographer must have adequate knowledge regarding the reproductive physiology of the bitch, as well as extensive training and ability to properly conduct the procedures (Wilborn et al., 2012).

The development of new technologies for ultrasonic imaging has allowed acquisition of images with more precise resolution of the reproductive tract, fetal development and organogenesis (Davidson and Baker, 2009). Several studies have been conducted to elucidate the changes in the sonographic aspects of reproductive tract of bitches throughout the estrous cycle (Boyd et al., 1993; England and Allen, 1989; Renton et al., 1992; Wallace et al., 1992; Lévy and Fontbonne, 2007; England et al., 2009; Kim et al., 2009; Freitas et al., 2017). Nevertheless, with the advances in ultrasonic imaging quality, there is a need to evaluate the usefulness of these new technologies in reproductive evaluation, to study the reproductive system and correlate findings with physiological processes to improve breeding management procedures.

A new technology termed HD (High Density) ultrasonography, also known as high-definition ultrasonography, has gained interest and applicability for diagnostic imaging and has been determined to be effective for human medicine peripheral nerves (Karmakar et al., 2013) and carpal tunnel evaluations (Tan et al., 2011). In veterinary medicine, there has been results from only one study reported regarding applicability of this technique in gestational ultrasonography and fetal organogenesis in brachycephalic breeds (Maronezi et al., 2021), but there are no reports regarding the usefulness of this technique in the evaluation of the reproductive tract and about changes occurring throughout the estrous cycle in non-pregnant bitches.

The HD technique is based on the use of transducers with a large number of piezoelectric elements (which can reach up to 4096 elements), separated by a small pitch, differing from conventional transducers (which normally contain around 128 elements), resulting in sound-waves with smaller width, thus proportioning greater axial, lateral and elevational spatial resolution, besides providing for a more precise contrast and more detailed images due to the larger number of pixels, generating images with greater quality than those resulting with use of conventional transducers (Lieu, 2010; Szabo, 2014; Merritt, 2018).

The aim of the present study was to sonographically evaluate the dog uterus and ovaries during the follicular phase of the estrous cycle using B mode HD technologies and to standardize findings with blood progesterone concentrations. The hypothesis is that the high-definition images resulting with the use of the HD transducer will allow for more precise evaluation of the reproductive tract, especially the ovaries and ovarian structures throughout the estrous cycle, providing further information regarding the physiological changes of utero-ovarian structures.

2. Materials and methods

2.1. Animals

This study was approved by the Ethics Committee on the Use of Animals (CEUA) of the host institution (protocol No. 003074/19). The animals were from an experimental kennel of the Institution or from external tutors. All details of the experiment were explained and permission for participating in the study was obtained by means of a consent form.

All patient data and history were obtained, and physical examinations were performed to evaluate each female, as established in the literature (Johnston et al., 2001; Grundy et al., 2002). Inclusion criteria were: patients of reproductive age (peripubertal to 6 years of age), no history of reproductive disease, no gestational anomalies, no changes at the time of physical and obstetric examinations, no sonographic evidence of morphological abnormalities of the reproductive tract. Animals that did not meet the criteria were not included.

Seven Beagle bitches (87.5%) and one (12.5%) mongrel (totaling eight animals) were evaluated, weighing between 7.4 and 11.3 kg. Age ranged from 9 months to 5 years, but the majority of bitches (seven) were 9–11 months old. All bitches were considered healthy based on criteria previously established. Of the eight bitches, seven were nulliparous and pubescent, with expression of the first estrous cycle subsequent to puberty. The oldest bitch (5 years) was primiparous, with history of a previous pregnancy, normal parturition with no complications and no fetal nor neonatal abnormalities.

2.2. Timepoints of evaluations

All bitches were sonographically evaluated at five different timepoints. The first timepoint (M1) was established as the time of onset of proestrus (vulvar swelling and bloody discharge), with confirmation occurring based on vaginal cytology and serum progesterone concentration. After this period, all bitches were monitored by observation of clinical symptoms, behavior changes and findings using serial vaginal cytology examinations (2-day intervals or when considered necessary based on clinical symptoms or findings in vaginal smears). Vaginal smears were assessed and when it was determined the bitches were in transition from proestrus to estrus as confirmed by serum progesterone concentration, the second timepoint of evaluations was established (M2).

Two days after the M2 timepoint, all bitches were evaluated at three additional timepoints (M3, M4 and M5), with there being 2-day intervals between evaluations of vaginal cytology and quantifications of serum progesterone concentrations.

2.3. Vaginal cytology and hormonal concentrations

All bitches were monitored for onset of vulvar swelling and bloody discharge. The first day there were detections of these marker characteristics was considered as the first day of proestrus. From that timepoint, vaginal cytology was performed every 2 days to monitor the progression of the phase and to identify changes related to the transition of the estrous cycle phases. Samples obtained using vaginal cytology techniques were placed on microscope slides and stained using rapid panoptic stain. Evaluation of vaginal smears was made using a microscope (Nikon Eclipse E200) at 100x magnification and interpreted according to the percentage of superficial cornified cells. When 80–90% of the cells present were detected to be superficial cornified cells, this was defined as the timepoint when there was onset of estrus and when there was an abrupt decrease of cornified cells, associated with an increase in parabasal, intermediate cells, as well as neutrophils, this was defined as the timepoint when there was onset of diestrus based on findings in a previous study (Johnston et al., 2001).

Blood progesterone concentrations were quantified to confirm each reproductive phase at the timepoints previously described. To quantify the progesterone concentration (P₄), blood samples were obtained by venipuncture of the jugular or cephalic veins. Blood samples were subsequently transferred to a vacuum tube with no anticoagulant for subsequent centrifugation to obtain the serum, which was used for quantification of serum progesterone concentration using Chemiluminescence procedures (Immulite® 1000 Progesterone - SIEMENS). The results were interpreted according to data established in the literature for this technique (Gloria et al., 2018): proestrus: 0.09–1.04 ng/mL; preovulatory period: 1.29–2.7 ng/mL; ovulatory period: 3.18–12.28 ng/mL; oocyte maturation period (post ovulatory period): 17.17–24.7 ng/mL.

2.4. Ultrasonographic evaluation

For ultrasonographic assessment, abdominal hair was clipped and bitches were transferred to the examination room. All patients were fasted for 6 h prior to the evaluation. Bitches were positioned in dorsal or lateral recumbency, parallel to the equipment, with their heads towards the monitor. Acoustic gel was applied to allow evaluation of the structures in the abdominal area.

Ultrasonic evaluations were performed using an ACUSON S2000/SIEMENS, equipped with a linear multifrequency transducer (5.5–18 MHz) utilizing the HD technology, associated with tissue harmonic imaging. Frequency was standardized from 12 to 16 MHz to allow adjustments for complete visualization of the reproductive tract, as necessary. All evaluations were performed by a single operator.

The evaluation was initiated in the caudal abdomen area, identifying the uterine body between the urinary bladder and the descending colon in a sagittal view. Technical adjustments, such as gain, dynamic range, focal zone and frequency were performed as needed to maximize image quality. Qualitative (echogenicity and echotexture) and values for quantitative (uterine body thickness in sagittal view) variables were assessed.

Ovarian evaluations were performed caudal to the ipsilateral kidney in sagittal and transverse views, subtly positioning the transducer lateromedially and craniocaudally and using a fanning motion to assess the organ and evaluate the parenchymal structures (follicles and corpus luteum). Technical adjustments regarding image quality were performed as needed. Qualitative (echogenicity, echotexture, presence or absence of fluid around the ovary) and quantitative (height and length in sagittal view) variables were evaluated. Cine-loop clips of the ovarian images were stored in DICOM format for subsequent analysis using medical image visualization software (RadiAnt DICOM Viewer 2020.1.1 Version 64bits).

Frames that allowed complete analyses of the ovarian parenchymal structures were selected to determine the diameter of each structure. These numbers of these structures were determined and categorized into one of four groups based on size in mm of structures: G1 ≤ 1; G2: from 1.01 to 3.5; G3: from 3.51 to 5.5; G4: from 5.51 to 10. To determine wall thickness of each structure, the thickest portion of the wall was identified and the width was determined. The values of wall thickness of each structure were then used to calculate mean wall thickness for structures in each ovary.

2.5. Statistical analysis

Statistical analysis was performed using the R® software (R Foundation for Statistical Computing; Vienna, Austria). Initially, the Bartlett test of homogeneity of variances and Shapiro-Wilk test for normality of residuals were performed on the data for quantitative evaluations. The values for real or transformed data obtained by conducting ultrasonographic evaluations were compared at all timepoints using the ANOVA test and correlations were determined using the Spearman test. There were considered to be mean

differences when there was a $P < 0.05$.

3. Results

There was a difference in blood progesterone concentrations for each timepoint ($P < 0.05$). Mean values of blood progesterone concentrations at each timepoint are provided in Table 1. Serum progesterone concentrations at each timepoint, associated with vaginal cytology indicated that at the M1 timepoint encompassed the early to mid-proestrus period. Concentrations of progesterone at the M2 timepoint were those during the preovulatory and ovulatory periods. Concentrations of progesterone at the M3 timepoint were those during a portion of the ovulatory and postovulatory periods. The concentrations of progesterone at subsequent timepoints (M4 and M5) represented those during the postovulatory period, as established by Gloria et al. (2018) based on progesterone concentrations that were quantified using the Chemiluminescence technique. In two bitches, the concentrations at the M5 timepoint were those collected during diestrus based on cytological evaluations.

3.1. Uterine ultrasonography

It was possible to identify the uterine body and the ovaries of all patients during the sonographic evaluation relatively easily (eight uteri, 16 ovaries). The uterine horns were partially identified due to the position of these structures in the abdomen and overlapping of the intestinal loops. Images of the cervix, uterine body, uterine bifurcation and uterine horn are included in Fig. 1.

The uterus had a homogenous echotexture but portions of distinct echogenicity of the uterine walls were observed at all timepoints in all bitches, resulting in a “multilayer” aspect. These areas were more prominent in the uterine horns as compared with the uterine body. In total, six areas were distinguished in the uterine walls of all bitches.

Starting from the lumen (fine echogenic line at the center) to the serous surface, a hypoechoic layer was identified, closely related to the next layer, a fine hyperechoic image. The next layer external from the lumen was a small hypoechoic zone and lying next to this zone was a well-defined hyperechoic layer. There was another hypoechoic region lying outside to the hyperechoic layer, and the outer layer was a fine echogenic layer (Fig. 2). There was no anechoic fluid detected in the uterine lumen at any timepoint when evaluations were conducted.

The uterine body lining was thicker at the M1 and M2 timepoints. The uterine body was of similar thickness at subsequent timepoints as the M1 and M2 timepoints when evaluations were conducted (Table 1).

3.2. Ovarian ultrasonography

The sonographic appearance of the ovaries was markedly different at the different timepoints (Fig. 3). At the M1 timepoint, ovaries were oval-shaped with well-defined and regular contours and was hypoechoic compared to the adjacent tissue and predominantly homogenous parenchyma, with the presence of small thin-walled ovarian structures filled with anechoic content. After the M2 timepoint, ovarian qualitative characteristics were markedly different (Table 2). Furthermore, there was a difference in ovarian height and length at the timepoints when evaluations occurred ($P < 0.05$), as indicated by information included in Table 1. Ovarian dimensions were positively correlated with serum progesterone concentration (height: $r = 0.775$; length: $r = 0.624$) and uterine thickness (height: $r = 0.411$; length: 0.384).

At the M1 timepoint, there was no fluid around the ovaries in any patient. At the subsequent timepoint (M2), there was fluid accumulation around 12 ovaries (75%; Fig. 4). After the M2 timepoint, there was lesser fluid quantities than at the M1 timepoint with there being fluid detected around 50% (8) of the ovaries. At the M3 timepoint there fluid around 31.3% (5) of the ovaries at the M4 and M5 timepoints there was no fluid around the ovaries of any bitches (Table 2).

Another qualitative finding was a characteristic increase in fat tissue echogenicity adjacent to the ovaries at all timepoints when there were evaluations (Fig. 4), with a varied occurrence at each timepoint. There were these findings in 75% (12) of the ovaries evaluated at the M1 timepoint. At the M2 timepoint, there was this characteristic echogenicity for all ovaries (100%). At the M3 timepoint, the tissue characteristic echogenicity was visualized at 87.5% (14 ovaries) evaluations. At M4, there was this characteristic in 81.3% (13 ovaries) and at the M5 timepoint, this occurred with only 25% (four ovaries) of the evaluations. When present, this characteristic echogenicity was observed bilaterally in all bitches, except at the M4 timepoint, where a mongrel bitch had this characteristic echogenicity only around the left ovary.

Table 1

Mean \pm SD of serum progesterone concentration, uterine thickness and ovarian dimensions (height and length in sagittal view) obtained using HD ultrasonography procedures of bitches at each timepoint of evaluation.

	Progesterone (ng/mL)	Uterine thickness (mm)	Height (mm)		Length (mm)	
			Left Ovary	Right Ovary	Left Ovary	Right Ovary
M1	0.756 \pm 0.904 ^a	10.495 \pm 1.479 ^a	7.413 \pm 0.546 ^a	8.2 \pm 1.948 ^a	11.563 \pm 2.243 ^a	13.762 \pm 1.545 ^a
M2	6.03 \pm 4.32 ^{ab}	12.35 \pm 1.269 ^b	8.588 \pm 1.502 ^{ab}	9.425 \pm 1.35 ^{ab}	14.725 \pm 1.1 ^b	16.512 \pm 1.716 ^{ab}
M3	10.81 \pm 6.23 ^{bc}	12.21 \pm 1.371 ^b	10.475 \pm 1.724 ^{bc}	10.475 \pm 2.491 ^{ab}	15.6 \pm 1.546 ^b	19.037 \pm 2.385 ^b
M4	16.96 \pm 6.29 ^{cd}	12.875 \pm 1.314 ^b	10.475 \pm 1.861 ^{bc}	11.463 \pm 2.326 ^b	15.1 \pm 1.552 ^b	19.325 \pm 1.952 ^b
M5	20.69 \pm 5.12 ^d	13.163 \pm 0.944 ^b	10.563 \pm 1.115 ^c	10.721 \pm 1.654 ^{ab}	16.563 \pm 2.467 ^b	19.2 \pm 2.368 ^b

Different letters in the same column indicate differences ($P < 0.05$).



Fig. 1. Sonographic aspects of the uterus using HD ultrasonography. A. Longitudinal view of the cervix (between white arrows) and the uterine body (between hollow arrows). B. Longitudinal view of the uterine body, where the region of the uterine bifurcation (*) is identified and two tubular structures can be detected cranially, one dorsally and the other ventrally, corresponding to the uterine horns. C. Longitudinal view of the left uterine horn (between electronic calipers), identified as a tubular structure with differentiation of the uterine layers and sublayers due to hormonal functions.

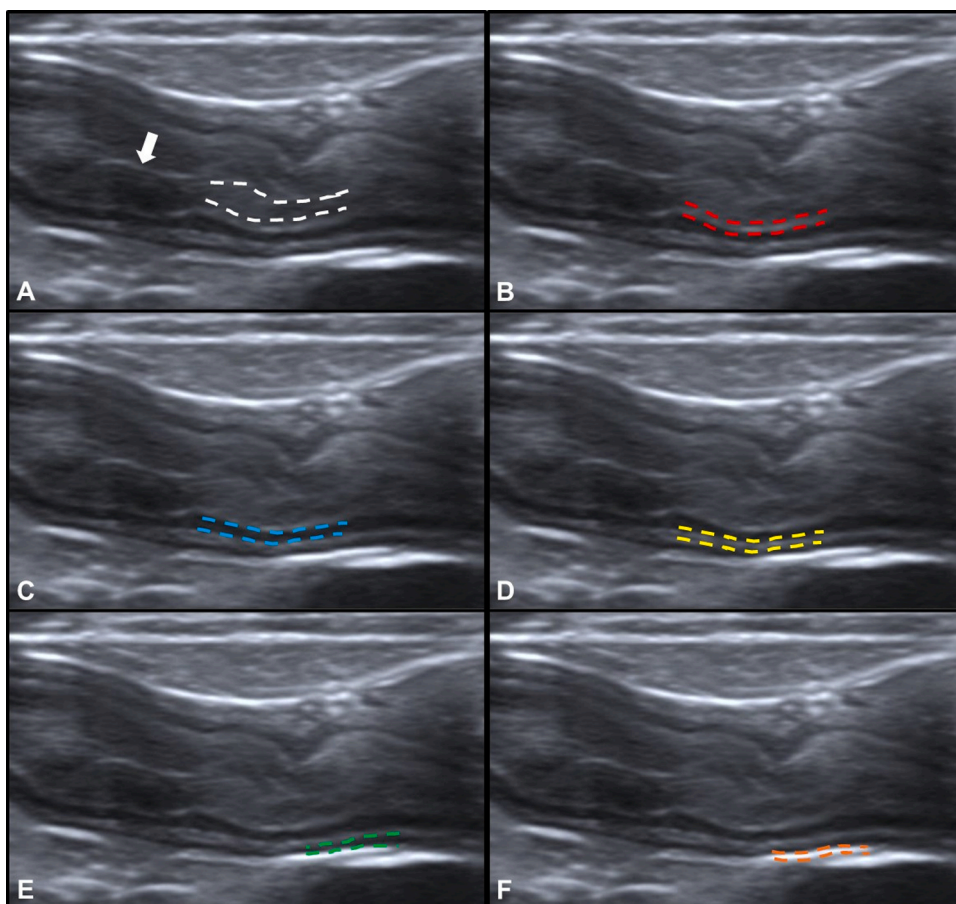


Fig. 2. Longitudinal ultrasonographic view of the left uterine horn of a bitch during estrus ($P_4 = 8.9$ ng/mL), demonstrating a “multilayer” aspect of the uterine walls. A. A relatively hypoechoic layer (between the white dashes) was detected, close to the luminal interface (white arrow). B. A hyperechoic region is visible (between the red dashes) right next to the previous layer. C. Hypoechoic portion (between the blue dashes). D. Linear hyperechoic area (between the yellow dashes). E. Hypoechoic region delimited by the green dashes. F. Fine echogenic region between the orange dashes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Ovarian structures

At all timepoints, it was possible to note that subtle movements in a fanning motion of the transducer were required for proper evaluation of the ovaries, allowing for identification of the ovarian structures. Because ovaries and the parenchymal structures increased in size, it was more difficult to select a single frame in which it was possible to visualize the entire organ at its maximum height and length and identify all ovarian structures. Several frames were selected for complete identification of the ovaries at the maximum dimension in the sagittal view and to localize ovarian structures at the maximum diameter.

At the M1 timepoint, all bitches had small thin-walled cavitary rounded structures filled with anechoic content, indicative of ovarian follicles. At this timepoint, bitches had a mean of five follicles per ovary (mean of 4.5 on the left ovary and 5.5 on the right ovary). The number of follicles per ovary at this timepoint differed in five bitches (62.5%).

At the M2 timepoint, in seven bitches (87.5%), there was a smaller number of ovarian structures when compared to the M1 timepoint and, among these, in five (74.43%) bitches there were fewer ovarian structures in both ovaries. In only one bitch (12.5%), was there a subsequent detection of fewer structures at the subsequent timepoint (M3). The other patients (87.5%) had a consistent number of structures per ovary after the M2 timepoint.

Only one structure smaller than 1 mm was detected in all bitches. The number of ovarian structures as compared with the number at the previous timepoint differed when there were some evaluations. Among these, the number of G2 structures differed in both ovaries ($P < 0.05$). The number of G4 structures was different only in the right ovary ($P < 0.05$; Table 3).

Although the number of G4 structures were different only in the right ovary, the number of G4 structures was positively correlated with serum progesterone concentration (left ovary: $r = 0.451$; right ovary: $r = 0.647$) and uterine thickness (left ovary: $r = 0.368$; right ovary: $r = 0.281$).

There was a difference in size of the largest ovarian structure at the different timepoints of evaluations ($P < 0.05$; Table 3). In the left ovary, the size of the largest structure was positively correlated with serum progesterone concentration ($r = 0.505$), height

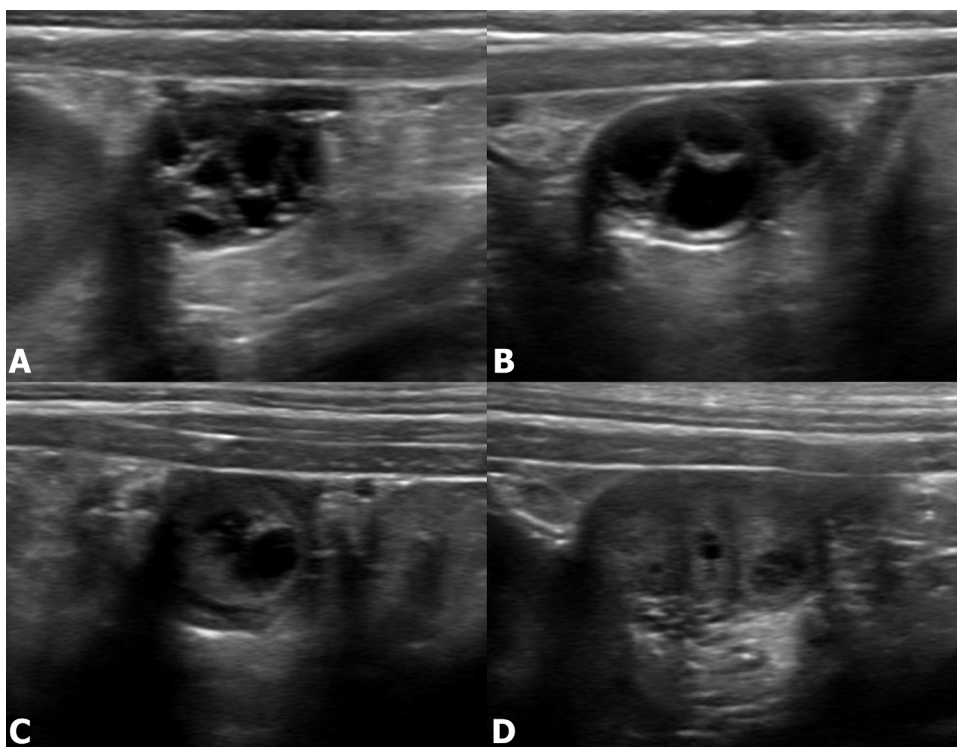


Fig. 3. Sonographic images of the ovaries and the modifications throughout the follicular phase. A. Longitudinal view of the left ovary of a bitch during early proestrus, where several cavitary thin-walled and fluid-filled structures are observed in the ovarian parenchyma. B. Longitudinal view of the left ovary of a bitch during early estrus, before ovulation. It was possible to observe that follicular structures with greater dimensions compared to early proestrus and follicular walls became slightly thickened. C. Longitudinal view of the left ovary of a bitch during the postovulatory period. One ovarian structure with markedly irregular internal margins is identified, corresponding to a corpus hemorrhagicum. D. Longitudinal view of the right ovary of a bitch during early diestrus. Ovarian contours are slightly irregular in shape with three almost completely solid ovarian structures, with very small cavitary areas and markedly thickened regular walls, corresponding to corpora lutea.

($r = 0.664$), length ($r = 0.386$) and number of G4 structures ($r = 0.726$), but was negatively correlated with size of G2 structures ($r = 0.386$). In the right ovary, there was positive correlation with serum progesterone concentration ($r = 0.784$), uterine thickness ($r = 0.34$), height ($r = 0.742$) and length ($r = 0.765$).

Mean ovarian structure wall thickness could also be easily assessed. There was a difference ($P < 0.05$) in mean wall thickness at the timepoints evaluated (Table 3). The values for this variable in both ovaries were positively correlated with serum progesterone concentration (left ovary: $r = 0.749$; right ovary: $r = 0.633$).

The sonographic identification that ovulation had occurred was based on the presence of fluid around the ovaries and/or decrease in the number of follicular structures when compared to the values at the M1 timepoint, as well as values for cytological and hormonal correlations. In six bitches (75%), the first detection of fluid around the ovary occurred at the M2 timepoint, and in the other two bitches this was detected for the first time at the M3 timepoint. There was a marked reduction of the cavitary ovarian structures after ovulation, being almost completely non-detectable subsequent to when ovulations occurred (Fig. 5).

4. Discussion

To the best of our knowledge, this is the first study regarding the application of HD ultrasonography for reproductive assessment of bitches during the follicular phase of the estrous cycle. Ultrasonography is the imaging method of choice for reproductive evaluation in small animals because it is non-invasive and allows for characterization of the changes of follicular and luteal structures (England et al., 2009). Results from the present study are indicative of the potential of the HD technology in complementing findings obtained with conventional B mode ultrasonography evaluations as evidenced by the greater definition of resolution in the images obtained, facilitating the understanding of the morphophysiological changes that occur during the estrous cycle of bitches.

While the first manifestation of proestrus generally occurs at puberty, many bitches may express behavioral estrus without there being occurrence of ovulation during the peripubertal period (Johnston et al., 2001). Gobello (2014) reported puberty in the bitch was a complex process and that the first manifestation of a typical follicular phase, evidence of ovulation and normal luteal phase could be a more precise estimate of sexual maturity. Considering that all seven pubescent bitches had regular proestrus and estrous occurrences, as evidenced by vaginal cytology and serum progesterone concentrations, as well as expected physical characteristics, behavior

Table 2
Percentage of qualitative findings observed in the ovaries of eight bitches (16 ovaries) using HD ultrasonography.

	Ovarian Structures' Content			Fluid in Ovarian Bursa		Focal Reactivity		Contours		Echogenicity		Echotexture	
	An	Mix	Ec	Pr	Ab	Pr	Ab	Re	Ir	Hypo	Hyper	Ho	Co
M1	100%	0%	0%	0%	100%	75%	25%	81.25%	18.75%	100%	0%	100%	0%
M2	50%	50%	0%	75%	25%	100%	0%	43.75%	56.25%	100%	0%	6.25%	93.75%
M3	0%	87.5%	12.5%	50%	50%	87.5%	12.5%	18.75%	81.25%	68.75%	31.25%	0%	100%
M4	0%	50%	50%	31.25%	68.75%	81.25%	18.75%	31.25%	68.75%	37.5%	62.5%	0%	100%
M5	0%	31.25%	68.75%	0%	100%	25%	75%	37.5%	62.5%	0%	100%	0%	100%

Legends: An: anechoic; Mix: mixed echogenicity; Ec: echogenic. Pr: present; Ab: absent; Re: regular; Ir: irregular; Hypo: hypoechoic; Hyper: hyperechoic; Ho: homogenous; Co: coarse

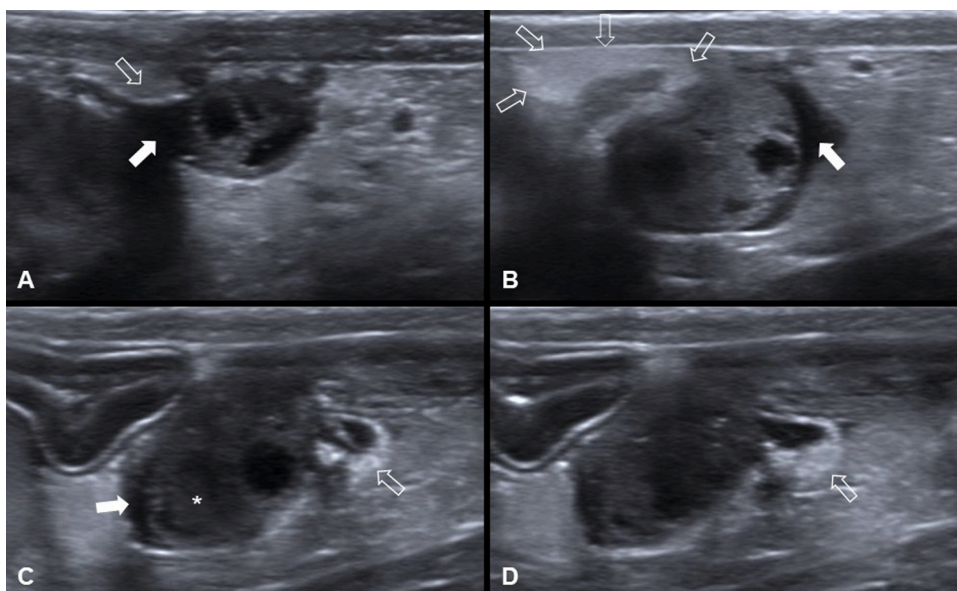


Fig. 4. Sagittal ultrasonographic view of ovarian structures and modifications of the ovaries and adjacent tissue throughout the evaluations. Presence of fluid around the ovary (white arrow) located in the ovarian bursa, as detected in A, B and C was more commonly observed at the M2 timepoint, but was also present at the M3 and M4 timepoints. Tissue reactivity (hollow arrows in A, B, C and D was present at all timepoints of evaluation. There was greater echogenicity of the cavitory ovarian structure content (asterisk) as noted in C.

changes and evidence of ovulation, the bitches in the present study met the criteria for methodological bias regarding experimentation with pubescent bitches. More studies, however, are warranted with different age groups for further assessment of the changes in the reproductive system of bitches during the follicular phase of estrous cycles.

The uterine body could be identified in sagittal view in all patients. There have been no previous studies where there was identification of six distinct portions of the uterine wall of the bitch, therefore, this is an unprecedented finding. The “multilayer” aspect of the uterus when there is hormonal stimulation has already been described in bitches (England and Allen, 1989; Davidson and Baker, 2009; Kim et al., 2009; Freitas et al., 2017) and queens (Gatel et al., 2016), however, in the present study there was identification of a larger number of distinct regions of the uterine lining wall than previously reported.

Histologically, the uterus of the bitch is composed of three distinct layers, the outermost is the serous layer (perimetrium), overlying the muscular layer (myometrium) and inner region nearest to the uterine lumen, the mucosa/submucosa layer (endometrium), all of which have sublayers (Augsburger and Kürzi, 2004; Priedkalns and Leiser, 2006). The uterine tissue changes with fluctuations in hormonal concentrations and in some studies this has been reported to occur in the edematous aspect of the endometrium, sublayer thickening, as well as there being distinct proliferative phases occurring (Galabova et al., 2003; Van Cruchten et al., 2004). The findings in the present study are directly related to image quality that could be obtained using the equipment and transducer due to improvement in axial, lateral and spatial resolutions resulting from utilization of the HD transducers (Lieu, 2010; Szabo, 2014; Merritt, 2018).

In the present study, the luminal interface was observed as a fine hyperechoic region, followed by a relatively hypoechoic layer and a fine echogenic region immediately below the luminal surface (Fig. 2). In women, during the late proliferative period of the menstrual cycle, with transvaginal ultrasonography evaluations, the endometrium is detected as a “trilaminar” structure, where the fine echogenic luminal interface is centrally located, followed by the hypoechoic functional layer of the endometrium and the echogenic basal layer of the endometrium (Forrest et al., 1988; Lenz and Lindenberg, 1990; Bakos et al., 1993; Tetlow et al., 1999; Nalaboff et al., 2001), similar to findings in the present study.

Due to the similarity of findings in the present study with those in women, as well as the previous identification of the central hyperechoic region as the luminal interface, it is hypothesized that the bitch endometrium also has a “trilaminar” aspect during the follicular phase of the estrous cycle, the central portion corresponding to the lumen, the hypoechoic layer corresponding to the functional layer of the endometrium and the fine echoic region corresponding to the basal layer of the endometrium. Further studies are, however, necessary to determine if there is verification of these uterine tissue characteristics using the techniques utilized in the present study.

Next to this “trilaminar” aspect, there was a hypoechoic layer detected in all patients. In women, a hypoechoic layer between the myometrium and the endometrium was identified using transvaginal ultrasonography and was described as the “sub-endometrial halo” or “junctional zone” (Fleischer et al., 1988; Tetlow et al., 1999). Histologically, however, this layer corresponds to a highly vascularized distinct myometrium portion, in which muscle cells are more compact, being consistent with hypoechoic characteristics (Tetlow et al., 1999).

This hypoechoic layer observed in all bitches also had similarities to the findings observed in women, thus, it is hypothesized that

Table 3

Mean \pm SD number of ovarian structures from each group, diameter of the greatest ovarian structure and wall thickness at each timepoint when there were evaluations as assessed by HD ultrasonography in bitches.

	G1 (≤ 1 mm)		G2 (1.01–3.5 mm)		G3 (3.51–5.5 mm)		G4 (5.51–10 mm)		Greatest structure diameter (mm)		Mean wall thickness (mm)	
	E	D	E	D	E	D	E	D	E	D	E	D
M1	0 ^a	0.125 \pm 0.354 ^a	3.375 \pm 2.387 ^a	3.25 \pm 3.2 ^a	1 \pm 1.069 ^a	2.125 \pm 2.357 ^a	0.125 \pm 0.354 ^a	0 ^a	3.85 \pm 1.291 ^a	3.812 \pm 0.583 ^a	0.6112 \pm 0.122 ^a	0.5513 \pm 0.1343 ^a
M2	0 ^a	0.125 \pm 0.354 ^a	1 \pm 1.309 ^b	1.5 \pm 0.756 ^b	2 \pm 1.69 ^a	1.625 \pm 1.188 ^a	0.5 \pm 0.535 ^a	0.875 \pm 0.835 ^{ab}	5.919 \pm 1.646 ^{ab}	5.944 \pm 1.376 ^{ab}	1.241 \pm 0.404 ^a	1.466 \pm 0.523 ^b
M3	0 ^a	0.125 \pm 0.354 ^a	0.75 \pm 1.035 ^b	0.125 \pm 1.246 ^b	1.5 \pm 1.512 ^a	1.625 \pm 1.188 ^a	0.75 \pm 0.886 ^a	1.5 \pm 1.195 ^b	5.944 \pm 2.157 ^{ab}	6.75 \pm 2.108 ^b	2.356 \pm 0.496 ^b	2.199 \pm 0.695 ^{bc}
M4	0 ^a	0 ^a	0.75 \pm 1.035 ^b	1 \pm 0.756 ^b	1.25 \pm 1.165 ^a	2.125 \pm 0.835 ^a	0.875 \pm 0.835 ^a	1.125 \pm 0.835 ^{ab}	6.519 \pm 1.915 ^b	7.169 \pm 1.997 ^b	2.574 \pm 0.697 ^b	2.446 \pm 0.847 ^c
M5	0 ^a	0 ^a	1.375 \pm 1.188 ^{ab}	0.875 \pm 1.126 ^b	0.75 \pm 0.886 ^a	2.25 \pm 1.488 ^a	0.625 \pm 0.744 ^a	1.125 \pm 0.641 ^{ab}	5.681 \pm 2.1 ^{ab}	7.431 \pm 1.415 ^b	2.976 \pm 0.998 ^b	2.753 \pm 0.591 ^c

Different letters in the same column indicate differences ($P < 0.05$).

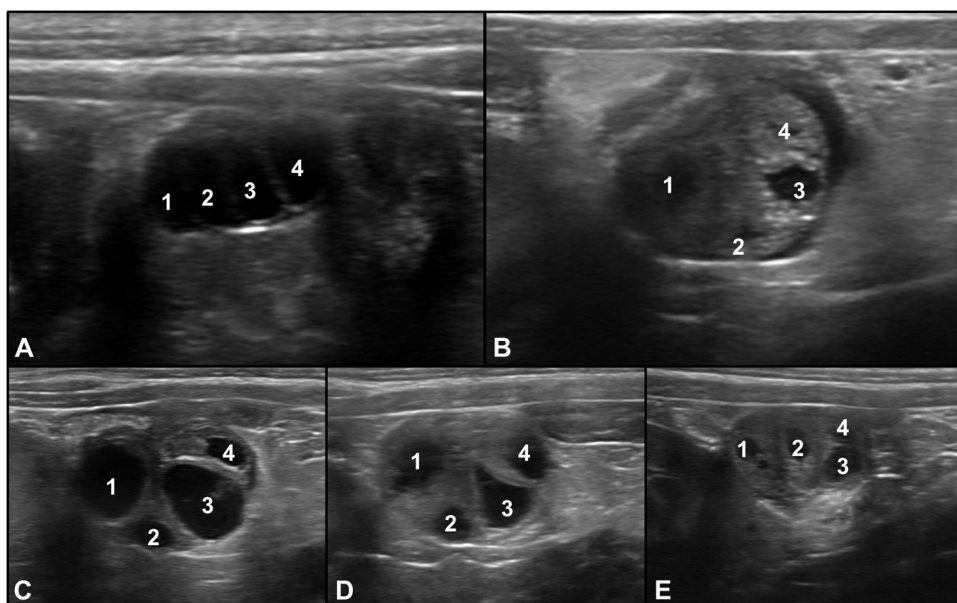


Fig. 5. Sagittal view of the ovarian structures and modifications of the right ovary of a bitch (Beagle, nulliparous, 10 months-old) at the different timepoints of evaluation. A. At the M1 timepoint, the ovary was oval-shaped, with regular contours, hypoechoic parenchyma and presented four thin-walled cavitory structures (1, 2, 3 and 4), filled with homogenous anechoic fluid ($P_4 = 0.56$ ng/mL). B. At the M2 timepoint, it is possible to observe that the ovarian parenchyma has a coarse echotexture, and the content of the ovarian structures has greater echogenicity. Each structure had thickened walls and the cavitory area was smaller, especially structures 2 and 4, even though there was a small amount of fluid in the ovarian bursa and adjacent tissue reactivity ($P_4 = 8.9$ ng/mL). C. At the M3 timepoint, there is an increase in the structure's dimensions, with hyperechoic walls and are filled with homogenous anechoic fluid ($P_4 = 14.6$ ng/mL). D. At the M4 timepoint, there was a thickening of the walls of each structure, which had some irregular internal margins and the ovary had a slight increase in echogenicity of the parenchyma. The contents of structures are still predominantly anechoic, but a small amount of debris can be identified in structure 1 ($P_4 = 22.6$ ng/mL). E. At the M5 timepoint, the walls of the structures were thicker than at earlier timepoints and the cavitory areas were smaller with a nearly solid appearance to each structure. Vaginal cytology assessments indicated bitches were in cytological diestrus at this timepoint ($P_4 = 23.3$ ng/mL).

this “junctional zone” observed in the uterus of the bitch during the follicular phase of the estrous cycle corresponds to the inner circular layer of the myometrium. The hyperechoic region might correspond to the vascular layer of the myometrium and the next hypoechoic might correspond to the outer longitudinal layer of the myometrium. The outermost hyperechoic layer corresponds to the serous layer. During the follicular phase, hormones promote endometrium and myometrial thickening due to edema, development of the uterine glands, blood vessel enlargements and increased blood flow (Groppetti et al., 2010). The findings in the present study are possibly due to changes occurring during the estrous cycle, as well as the greater extent of resolution resulting from use of HD ultrasonography, considering there are no previous reports using conventional B mode ultrasonography.

With the progression of the estrous cycle, there was an increase in uterine thickness between early proestrus (M1) and estrus (M2), and after these timepoints, there were no changes in uterine thickness. Uterine thickening is related to the estrogenic functions during proestrus (England and Allen, 1989), which was also observed in the present study. After ovulation, however, followed by the decrease in concentrations of estrogens, the uterus begins to respond to progesterone which gradually increases during the luteal phase of the estrous cycle and there were no differences in the uterine characteristics during the postovulatory period, as evidenced in the present study which corroborates results of Kim et al. (2009), who performed conventional B mode assessments in bitches to evaluate the sonographic aspect of the uterus during the different hormonal phases.

The sonographic identification of distinct uterine wall tissues during the follicular phase of the estrous cycle might provide for a greater understanding of physiological changes during the estrous cycle of bitches, as well as provide important information for reproductive assessment, provide for a means for early detection of uterine pathologies and correct identification of the affected region. Further studies are warranted to explore the capacity of this technology for these purposes.

During the preovulatory and ovulatory periods, all bitches had focal reactivity around the ovaries as evidenced by the increase in echogenicity of the adjacent fat tissues. Different from the present study, a hypoechoic “halo” surrounding the ovaries has been described at some timepoints during the perioovulatory period using conventional B mode ultrasonography (Bergeron et al., 2013), which was considered to correspond to the ovarian bursa. There are no previous reports regarding the observation of tissue reactivity around the ovary similar to that detected in the present study.

The ovulatory process has already been compared to the inflammatory process, because angiogenic activity, increased vascular permeability, vasodilation and edema are essential characteristics of both processes (Duffy et al., 2019). In one study conducted with human ovaries, it was possible to observe that ovulation involves a cytokine signaling pathway, where the gonadotropin surge release initiates an inflammatory process in the granulosa cells (Poulsen et al., 2019). Considering that these tissue characteristics were more

frequently observed during the preovulatory and ovulatory periods, it is hypothesized that this reactivity might be secondary to the inflammatory process in the ovaries around the time of ovulation.

The presence of fluid around the ovaries in this study has already been described and attributed as the accumulation of intra follicular fluid in the ovarian bursa after follicular rupture and ovulation (Lévy and Fontbonne, 2007). In a study conducted by Lévy and Fontbonne (2007), 39.6% of bitches had this fluid accumulation after ovulation, whereas in a study conducted by Barbosa et al. (2013), all bitches had this fluid accumulation 3 days after ovulation. In the present study, this fluid accumulation was detected at the M2 to M4 timepoints. Because of the anechoic fluid can remain for a few days, this finding should be interpreted cautiously when determining the time when ovulation occurs. Daily evaluations, as well as correlation with other values for variables (clinical manifestation, vaginal cytology and hormone concentrations) are required to confirm the day of ovulation.

In the present study, there was identification of an increase in follicular dimensions from early proestrus (M1) to estrus (M2), corroborating England et al. (2009), therefore, the capacity to detect follicular population dynamics. There is no definitive information regarding the chronology of processes in bitches regarding follicular recruitment, dominance, atresia or even if follicular growth is continuous or in waves (Evans, 2003). Due to the considerable preciseness of determinations using HD technology, it is believed that further studies with this technique could aid in allowing for a more precise understanding of the mechanisms related to the ovarian follicular dynamics in bitches.

In the present study, there was determination of when there was an increase in the wall thickness of the ovarian structures as the duration of the follicular phase of the estrous cycle advanced, where structures with wall thickness greater than 1 mm were observed during the preovulatory period. Wall thickening of ovarian structures has already been attributed to preovulatory luteinization of follicles (Lévy and Fontbonne, 2007) and to the initial formation of corpus luteum (England et al., 2009), where structures with wall thickness of 1 mm are considered preovulatory follicles and those greater than 1 mm are considered to be a corpus luteum. Due to the fluid-filled cavity of the corpus luteum during the initial developmental stages, it might be difficult to sonographically differentiate this structure from preovulatory follicles or a corpus hemorrhagicum (Bergeron et al., 2013; Hollinshead and Hanlon, 2019). Considering that, in the present study, some ovarian structures that corresponded to preovulatory follicles had a wall thickness greater than 1 mm, it was possible to realize that measurements alone cannot be used to differentiate preovulatory follicles from the early developing corpus luteum, thus, differing from the study conducted by England et al. (2009).

Groppetti et al. (2015) conducted a histological analysis of the ovary of the bitch and there were early indications of luteinization of the large follicles during proestrus, characterized by cumulus mucification in the granulosa cells and that, in the ovulatory period, large follicles can coexist with corpus hemorrhagicum, the latter persisting until the end of the fertile period, developing into corpus luteum at the onset of diestrus. The findings of the present study indicated wall thickening after the M2 timepoint and these structures had changes in echogenicity of the content inside the antrum.

This indicates that the thick-walled ovarian structures during the postovulatory period might correspond to corpora hemorrhagica, considering that, histologically, corpora lutea are only present with the onset of diestrus (Groppetti et al., 2015). In this context, HD ultrasonography provides for the capacity to have greater resolution for enhancing the understanding the ovarian changes during the postovulatory period. It, however, is noteworthy that daily evaluations were not performed in the present study, thus, limiting the comprehension of the changes regarding corpora hemorrhagica formation and the modification into corpora lutea. Further studies with HD ultrasonography, therefore, might be necessary for gaining a greater understanding of the sonographic aspect or the ovaries during the postovulatory period and diestrus.

5. Conclusion

The results of the present study provide unprecedented information regarding qualitative findings of the reproductive system of the bitch, where it was possible to detect distinct regions of the uterine wall during the follicular phase, as well as ovarian modifications in the follicular and periovulatory phases using high-definition ultrasonography, providing a new perspective in small animal reproductive ultrasonography. The technological advances in ultrasonography have allowed for great image resolution, consequently providing for a greater understanding of the sonographic anatomy and new applicability of this imaging technique, providing basic knowledge for further identification of physiological changes during different reproductive states. With the use of the HD technology, there were superb images obtained, allowing for more accurate assessment of the reproductive tract, which might aid in breeding management of dogs. Additionally, these findings provide opportunities for future studies utilizing the HD technique for improving reproductive management, as well as the application of this technique for early detection of morphophysiological changes that can lead to abnormalities of the estrous cycle.

CRedit authorship contribution statement

Luiz Paulo Nogueira Aires: Conceptualization, Investigation, Visualization, Writing – original draft, Writing – review & editing, Project administration **Beatriz Gasser:** Methodology, Investigation, Data curation, Writing – review & editing. **Priscila Silva, Priscila Del Aguila da Silva, Marcus Vinícius Silveira, Rafael Kretzer Carneiro and Diego Iwao Yamada:** Investigation, Data curation. **Luciana Cristina Padilha-Nakaghi, Ricardo Andrés Ramirez Uscategui and Marcus Antônio Rossi Feliciano:** Conceptualization, Methodology, Supervision, Resources, Formal analysis, Writing – review & editing, Project administration, Funding acquisition. **Stefano Spada and Marco Russo:** Visualization, Writing – review & editing.

Conflict of interest

The authors report no conflicts of interest.

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References

- Augsburger, H.R., Kürzi, M., 2004. Histomorphologic and morphometric evaluation of the uterine horns in nulliparous and multiparous Beagles. *Am. J. Vet. Res.* 65, 552–558. <https://doi.org/10.2460/ajvr.2004.65.552>.
- Bakos, O., Lundkvist, Ö., Bergh, T., 1993. Transvaginal sonographic evaluation of endometrial growth and texture in spontaneous ovulatory cycles—a descriptive study. *Hum. Reprod.* 8, 799–806. <https://doi.org/10.1093/oxfordjournals.humrep.a138145>.
- Barbosa, C.C., Souza, M.B., Scalercio, S.R.R.A., Silva, T.F.P., Domingues, S.F.S., Silva, L.D.M., 2013. Ovarian and uterine periovulatory doppler ultrasonography in bitches. *Pesq. Vet. Bras.* 33, 1144–1150. <https://doi.org/10.1590/S0100-736X2013000900016>.
- Bergeron, L.H., Nykamp, S.G., Brissou, B.A., Madan, P., Gartley, C.J., 2013. An evaluation of B-mode and color Doppler ultrasonography for detecting periovulatory events in the bitch. *Theriogenology* 79, 274–283. <https://doi.org/10.1016/j.theriogenology.2012.08.016>.
- Boyd, J.S., Renton, J.P., Harvey, M.J., Nickson, D.A., Eckersall, P.D., Ferguson, J.M., 1993. Problems associated with ultrasonography of the canine ovary around the time of ovulation. *J. Reprod. Fertil. Suppl.* 47, 101–105.
- Davidson, A.P., Baker, T.W., 2009. Reproductive ultrasound of the bitch and queen. *Top. Companion Anim. Med.* 24, 55–63. <https://doi.org/10.1053/j.tcam.2008.11.002>.
- Duffy, D.M., Ko, C., Jo, M., Brannstrom, M., Curry, T.E., 2019. Ovulation: parallels with inflammatory processes. *Endocr. Rev.* 40, 369–416. <https://doi.org/10.1210/er.2018-00075>.
- England, G., Concannon, P.W., 2002. Determination of the optimal breeding time in the bitch: basic considerations. In: Concannon, P.W., England, G., Verstegen III, J., Linde Forsberg, C. (Eds.), *Recent Advances in Small Animal Reproduction*. International Veterinary Information Service, Ithaca.
- England, G.C., Allen, W.E., 1989. Real-time ultrasonic imaging of the ovary and uterus of the dog. *J. Reprod. Fertil. Suppl.* 39, 91–100.
- England, G.C.W., Russo, M., Freeman, S.L., 2009. Follicular dynamics, ovulation and conception rates in bitches. *Reprod. Domest. Anim.* 44, 53–58. <https://doi.org/10.1111/j.1439-0531.2009.01416.x>.
- Evans, A., 2003. Characteristics of ovarian follicle development in domestic animals. *Reprod. Domest. Anim.* 38, 240–246. <https://doi.org/10.1046/j.1439-0531.2003.00439.x>.
- Fleischer, A.C., Mendelson, E.B., Bohm-Velez, M., Entman, S.S., 1988. Transvaginal and transabdominal sonography of the endometrium. *Semin. Ultrasound CT MR* 81, 81–101.
- Forrest, T.S., Elyaderani, M.K., Muilenburg, M.I., Bewtra, C., Kable, W.T., Sullivan, P., 1988. Cyclic endometrial changes: US assessment with histologic correlation. *Radiology* 167, 233–237. <https://doi.org/10.1148/radiology.167.1.3279455>.
- Freitas, L.A., Mota, G.L., Silva, H.V.R., Silva, L.D.M., 2017. Two-dimensional sonographic and Doppler changes in the uteri of bitches according to breed, estrus cycle phase, parity, and fertility. *Theriogenology* 95, 171–177. <https://doi.org/10.1016/j.theriogenology.2017.03.012>.
- Galabova, G., Egerbacher, M., Aurich, J.E., Leitner, M., Walter, I., 2003. Morphological changes of the endometrial epithelium in the bitch during metoestrus and anoestrus. *Reprod. Domest. Anim.* 38, 415–420. <https://doi.org/10.1046/j.1439-0531.2003.00459.x>.
- Gatel, L., Gory, G., Chalvet-Monfray, K., Saunders, J.H., Rault, D.N., 2016. Intra- and inter-observer variability in ultrasonographic measurements of the uterus and ovaries in healthy, non-pregnant queens. *J. Feline Med. Surg.* 18, 110–117. <https://doi.org/10.1177/1098612x15574317>.
- Gloria, A., Contri, A., Carluccio, A., Robbe, D., 2018. Blood periovulatory progesterone quantification using different techniques in the dog. *Anim. Reprod. Sci.* 192, 179–184. <https://doi.org/10.1016/j.anireprosci.2018.03.006>.
- Gobello, C., 2014. Prepubertal and pubertal canine reproductive studies: conflicting aspects. *Reprod. Domest. Anim.* 49, 70–73. <https://doi.org/10.1111/rda.12414>.
- Groppetti, D., Aralla, M., Bronzo, V., Bosi, G., Pecile, A., Arrighi, S., 2015. Periovulatory time in the bitch: what's new to know? Comparison between ovarian histology and clinical features. *Anim. Reprod. Sci.* 152, 108–116. <https://doi.org/10.1016/j.anireprosci.2014.11.008>.
- Groppetti, D., Pecile, A., Arrighi, S., Di Giancamillo, A., Cremonesi, F., 2010. Endometrial cytology and computerized morphometric analysis of epithelial nuclei: a useful tool for reproductive diagnosis in the bitch. *Theriogenology* 73, 927–941. <https://doi.org/10.1016/j.theriogenology.2009.11.019>.
- Grundy, S.A., Feldman, E., Davidson, A., 2002. Evaluation of infertility in the bitch. *Clin. Tech. Small Anim. Pract.* 17, 108–115. <https://doi.org/10.1053/svms.2002.36601>.
- Hollinshead, F., Hanlon, D., 2019. Normal progesterone profiles during estrus in the bitch: a prospective analysis of 1420 estrous cycles. *Theriogenology* 125, 37–42. <https://doi.org/10.1016/j.theriogenology.2018.10.018>.
- S.D. Johnston, S.D., Root, M.V., Kustritz, Olson, P.N.S., 2001. *Canine Feline Theriogenology*, WB Saunders.
- Karmakar, M.K., Shariat, A.N., Pangthipapai, P., Chen, J., 2013. High-definition ultrasound imaging defines the paraneural sheath and the fascial compartments surrounding the sciatic nerve at the popliteal fossa. *Reg. Anesth. Pain Med.* 38, 447–451. <https://doi.org/10.1097/AAP.0b013e31829ffcb4>.
- Kim, J.-H., Mun, B.-G., Kim, B.-S., Park, I.-C., Suh, G.-H., Son, C.-H., 2009. Serial ultrasonographic appearance of normal uterus during estrous cycle in miniature schnauzer dogs. *J. Embryo Transf.* 24, 109–113.
- Lenz, S., Lindenberg, S., 1990. Ultrasonic evaluation of endometrial growth in women with normal cycles during spontaneous and stimulated cycles. *Hum. Reprod.* 5, 377–381. <https://doi.org/10.1093/oxfordjournals.humrep.a137106>.
- Lévy, X., 2016. Videovaginoscopy of the canine vagina. *Reprod. Domest. Anim.* 51, 31–36. <https://doi.org/10.1111/rda.12785>.
- Lévy, X., Fontbonne, A., 2007. Determining the optimal time of mating in bitches: particularities. *Rev. Bras. Reprod. Anim.* 31, 128–134.
- Lieu, D., 2010. Ultrasound physics and instrumentation for pathologists. *Arch. Pathol. Lab. Med.* 134, 1541–1556. <https://doi.org/10.1043/2009-0730-RA.1>.
- Lindsay, F.E.F., 1983. The normal endoscopic appearance of the caudal reproductive tract of the cyclic and non-cyclic bitch: post-uterine endoscopy. *J. Small Anim. Pract.* 24, 1–15. <https://doi.org/10.1111/j.1748-5827.1983.tb00407.x>.
- Maronezi, M.C., Simões, A.P.R., Usategui, R.A.R., Pavan, L., Rodrigues, M.G.K., Mariano, R.S.G., Santos, V.J.C., Feliciano, M.A.R., 2021. Gestational echobiometry in brachycephalic bitches using high-definition ultrasonography. *Pesq. Vet. Bras.* 41, e06650 <https://doi.org/10.1590/1678-5150-PVB-6650>.
- Merritt, C.R.B., 2018. *Physics of ultrasound*. In: Rumack, C.M., Levine, D. (Eds.), *Diagnostic Ultrasound*. Elsevier, Philadelphia, pp. 1–34.
- Moxon, R., Batty, H., Irons, G., England, G.C.W., 2012. Periovulatory changes in the endoscopic appearance of the reproductive tract and teasing behavior in the bitch. *Theriogenology* 78, 1907–1916. <https://doi.org/10.1016/j.theriogenology.2012.07.002>.
- Nalaboff, K.M., Pellerito, J.S., Ben-Levi, E., 2001. Imaging the endometrium: disease and normal variants. *Radiographics* 21, 1409–1424. <https://doi.org/10.1148/radiographics.21.6.g01nv211409>.
- Poulsen, L., la, C., Englund, A.L.M., Wissing, M.L.M., Yding Andersen, C., Borup, R., Grøndahl, M.L., 2019. Human granulosa cells function as innate immune cells executing an inflammatory reaction during ovulation: a microarray analysis. *Mol. Cell. Endocrinol.* 486, 34–46. <https://doi.org/10.1016/j.mce.2019.02.014>.

- Priedkalns, J., Leiser, R., 2006. Female reproductive system. In: Eurell, J.A., Frappier, B.L. (Eds.), *Dellmann's Textbook of Veterinary Histology*. Blackwell, pp. 256–278.
- Renton, J.P., Boyd, J.S., Harvey, M.J.A., Ferguson, J.M., Nickson, D.A., Eckersall, P.D., 1992. Comparison of endocrine changes and ultrasound as means of identifying ovulation in the bitch. *Res. Vet. Sci.* 53, 74–79. [https://doi.org/10.1016/0034-5288\(92\)90088-J](https://doi.org/10.1016/0034-5288(92)90088-J).
- Silva, L.D.M., Onclin, K., Verstegen, J.P., 1996. Assessment of ovarian changes around ovulation in bitches by ultrasonography, laparoscopy and hormonal assays. *Vet. Radiol. Ultrasound* 37, 313–320. <https://doi.org/10.1111/j.1740-8261.1996.tb01236.x>.
- Szabo, T.L., 2014. *Diagnostic Ultrasound Imaging: Inside Out*, second ed. Elsevier.
- Tan, T.C., Yeo, C.J., Smith, E.W., 2011. High definition ultrasound as diagnostic adjunct for incomplete carpal tunnel release. *Hand Surg.* 16, 289–294. <https://doi.org/10.1142/S0218810411005564>.
- Tetlow, R.L., Richmond, I., Manton, D.J., Greenman, J., Turnbull, L.W., Killick, S.R., 1999. Histological analysis of the uterine junctional zone as seen by transvaginal ultrasound. *Ultrasound Obstet. Gynecol.* 14, 188–193. <https://doi.org/10.1046/j.1469-0705.1999.14030188.x>.
- Van Cruchten, S., van den Broeck, W., D'Haeseleer, M., Simoens, P., 2004. Proliferation patterns in the canine endometrium during the estrous cycle. *Theriogenology* 62, 631–641. <https://doi.org/10.1016/j.theriogenology.2003.11.015>.
- Wallace, S.S., Mahaffey, M.B., Miller, D.M., Thompson, F.N., Chakraborty, P.K., 1992. Ultrasonographic appearance of the ovaries of dogs during the follicular and luteal phases of the estrous cycle. *Am. J. Vet. Res.* 53, 209–215.
- Wilborn, R.R., Maxwell, H.S., 2012. Clinical approaches to infertility in the bitch. *Vet. Clin. North Am. Small Anim. Pract.* 42, 457–468. <https://doi.org/10.1016/j.cvsm.2012.01.016>.