

ORIGINAL ARTICLE

Comparison of two-dimensional (2D) and three-dimensional (3D) ultrasonography for gestational ageing in the early to mid-pregnant bitch

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Abstract

Accuracy of prediction of the day of parturition in the bitch decreases with advancing pregnancy. We hypothesized that three-dimensional (3D) volume ultrasonography may be superior for gestational ageing than 2D measurements. Thirty-two pregnancy examinations were performed in 25 bitches of different breeds 21–34 days after the first mating. Ovulation date from serum progesterone concentrations was estimated for 15 bitches, and parturition date was recorded for all dogs. Inner chorionic cavity (ICC) diameters and ICC length of the conceptus were measured by 2D B-mode, and ICC volume by 3D ultrasonography (Voluson[®] i, GE Healthcare). Linear or exponential regressions between ICC measurements and time, i.e. days after estimated ovulation or days before parturition, were performed with significance set at $p < .05$. ICC volume increased exponentially over time, while ICC diameter showed a linear growth. ICC volume had similar accuracy ($R^2 = .718$, $p < .001$) to ICC diameter and ICC length ($R^2 = .707$ and $R^2 = .728$, $p < .001$) when analysed in relation to days after estimated ovulation or in relation to days before parturition ($R^2 = .818$, $R^2 = .800$ and $R^2 = .810$, $p < .001$ respectively). There were strong ($R^2 \geq .875$) significant associations between all ICC measurements. In conclusion, ICC volume did not improve canine gestational ageing accuracy, which supports the continued use of the ICC diameter, particularly in light of the difficulty in obtaining 3D measurements. On the basis of ICC volume, the growth of the canine conceptus appeared to be exponential already during the first half of pregnancy.

KEYWORDS

3D ultrasound, canine, dog, embryo, parturition prediction, pregnancy

1 | INTRODUCTION

Ultrasonography is a well-established diagnostic tool for pregnancy examination in small animals and provides a great variety of information without any side effect to the dam or the foetuses (Root

Kustritz, 2005). Besides confirmation of pregnancy, it is also used for gestational ageing, estimation of litter size (with an accuracy of 36% (Toal et al., 1986)), assessment of foetal vitality and detection/evaluation of gestational pathologies (Beccaglia & Luvoni, 2012; England, 1998; England et al., 1990; Lopate, 2008, 2018; Toal

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et al., 1986; Yeager et al., 1992). In the bitch, gestational ageing by two-dimensional (2D) ultrasonography is primarily used when ovulation timing is inadequate or lacking in order to estimate due date and prepare for whelping, especially in situations where veterinary assistance may become necessary due to anticipated dystocia or for planning a caesarean-section (C-section) (Beccaglia et al., 2016; Beccaglia & Luvoni, 2012; Lopate, 2008, 2018). Furthermore, gestational ageing is a valuable asset during any pregnancy evaluation to confirm adequate development and growth of the conceptuses. Gestational ageing includes the ultrasonographic evaluation of foetal organ development and measurement of specific foetal and extra-foetal structures (Beccaglia et al., 2016; England et al., 1990; Kutzler et al., 2003; Lopate, 2008, 2018; Luvoni & Beccaglia, 2006; Nyland & Mattoon, 2002; Yeager et al., 1992). While it is a great tool in the hands of clinical practitioners, the accuracy of these measurements depends on pregnancy stage and the structures evaluated, with the size or breed of the bitch having to be considered and adjustments made depending on the formula being utilized (Beccaglia et al., 2016; Beccaglia & Luvoni, 2012; Kutzler, Yeager, et al., 2003; Luvoni & Grioni, 2000).

The most suitable parameters for predicting the day of parturition are the inner chorionic cavity (ICC) diameter during early pregnancy and the biparietal diameter (BP) during late pregnancy (Beccaglia & Luvoni, 2006; Kutzler, Yeager, et al., 2003; Lopate, 2008; Luvoni & Beccaglia, 2006; Luvoni & Grioni, 2000). From 16–17 to 35 days after ovulation, the ICC diameter, the outer uterine diameter, placental thickness and measurements on the embryo/foetus as they become ultrasonographically visible, i.e. crown-rump length, biparietal diameter (BPD, measured in the sagittal plane at the widest point of the parietal bones) and body diameter (BD, two perpendicular measurements in the transverse plane at the widest portion of the abdomen at the level of the liver and stomach), can be used for gestational ageing. Foetal biparietal diameter and body diameter become increasingly more evident as gestation progresses and can be best measured 35 days after ovulation (Beccaglia et al., 2016; Beccaglia & Luvoni, 2012; Kutzler, Yeager, et al., 2003; Lopate, 2008; Luvoni & Beccaglia, 2006; Nyland & Mattoon, 2002; Yeager et al., 1992). However, gestational ageing performed in early pregnancy is more accurate than late pregnancy estimations (Beccaglia & Luvoni, 2006; Lopate, 2018; Luvoni & Beccaglia, 2006). Gestational ageing in early and mid-pregnancy was predictive of parturition date (estimated to be 65 days after the pre-ovulatory LH peak) with 75% and 66% accuracy with 1 day of error, respectively, and 88% and 82% accuracy with 2 days of error, respectively, compared to 43%–53% between 39 and 62 days after the LH peak (Kutzler, Yeager, et al., 2003). Of all these parameters, ICC diameter is recommended during early pregnancy for parturition date prediction based on its reasonably high accuracy, which was reported to be around 77% within 1-day error, and 85%–88% within 2-day error in small- and medium-size bitches (Beccaglia & Luvoni, 2006). A later study showed ICC diameter's accuracy to be 81% within 1 day and 93.1% within 2 days of error during week 4 of pregnancy, which decreased, although non-significantly, to 67.7% and 85.9%, respectively, during week 5

(Beccaglia & Luvoni, 2012). Later in pregnancy (i.e. beyond day 40), as the conceptus grows and the gestational sac loses its spherical shape, ICC diameter measurements can no longer be used for gestational ageing (Beccaglia et al., 2016; Beccaglia & Luvoni, 2006, 2012; England et al., 1990; Luvoni & Beccaglia, 2006). While biparietal diameter is the most suitable parameter for parturition date prediction during late pregnancy, its accuracy within 1-day error was around 79% during weeks 5 and 6 of gestation, decreased to around 64% by weeks 7 and 8 and to 51% by week 9, while predictions within 2 days of error remained high between 82% and 95% from weeks 5 to 8 of gestation and decreased to 70% by week 9 (Beccaglia & Luvoni, 2012). This may be partly due to the high variability of biparietal diameter values both within and among litters as shown in English bulldog and Boerboel neonatal puppies immediately after term C-section (de Cramer & Nöthling, 2018). Overall, the most accurate prediction of parturition date can be obtained around day 28 after ovulation (i.e. day 30 post-LH peak) using ICC diameter or BPD during weeks 5–6 of gestation (Beccaglia & Luvoni, 2012; Kutzler, Yeager, et al., 2003). Litter size does not seem to have an influence on accuracy of gestational ageing and parturition date prediction (Kutzler, Yeager, et al., 2003). On the other hand, body size and/or breed-specific formulas or corrections to maternal body weight should be used to achieve the most accurate prediction of gestational age or parturition day (Cecchetto et al., 2017; Lopate, 2008, 2018; Luvoni & Grioni, 2000; Sananmuang et al., 2021; Siena & Milani, 2021; Socha & Janowski, 2018; Son et al., 2001; de Albuquerque Vieira et al., 2020; Yeager et al., 1992).

Three-dimensional (3D) and four-dimensional (4D) ultrasonography have also been used for pregnancy evaluation in queens and bitches in one study (Hildebrandt et al., 2009), but gestational ageing was not performed. In general, images generated from 3D ultrasonography are created starting from sequential and parallel 2D scans, which are built by moving crystals inside a specific transducer driven by a linear-step motor (Hildebrandt et al., 2009; Kotoyori et al., 2012; Mattoon et al., 2002). Through specific computer software, the acquired images can be displayed, reconstructed and manipulated to allow detailed and thorough examination of ultrasound images (Hildebrandt et al., 2009; Kotoyori et al., 2012; Mattoon et al., 2002). Real-time 3D ultrasound incorporates time as the fourth dimension (i.e. 4D ultrasound), generating continuous 3D volume data sets and providing functional and dynamic data in three dimensions (Hildebrandt et al., 2009; Mattoon et al., 2002). Both 3D and 4D volume ultrasonography are well established in human obstetrics and gynaecology (Merz & Welter, 2005) and have been reported to offer the advantage to allow the analysis of a number of clinical conditions including congenital disorders or foetal deformities even allowing the demonstration of subtle foetal defects, foetal gender determination, gestational age and birth weight assessment (Kotoyori et al., 2012; Merz & Pashaj, 2017; Wu et al., 2021; Youssef et al., 2011). Their establishment in human medicine is also based on an important emotional aspect for the parents, allowing them to see a concrete 3D reconstruction of their babies, helping them to understand

and accept possible existing malformations, or to reassure them of the absence of foetal abnormalities (Merz & Pashaj, 2017; Merz & Welter, 2005). The use of 3D ultrasound for pregnancy evaluation in animals is still scarce (Becsek et al., 2019; Hildebrandt et al., 2009; Kotoyori et al., 2012). As pioneers in veterinary 3D ultrasonography, Hildebrandt et al. (2009) used nine different volume imaging modalities and obtained 3D volume data and 4D sequences in eight bitches between 26 and 60 days of pregnancy. Their conclusion was that 3D/4D ultrasound could be an additional tool to obtain detailed structural evaluation (e.g. visualization of structural changes such as bone abnormalities, evaluation of blood flow and assessment of foetal movement patterns) of the canine or feline embryo and foetus (Hildebrandt et al., 2009). A recent study reported the use of transrectal 3D ultrasonography for foetal volume analysis in early pregnant mares (Becsek et al., 2019). The authors found good intra- and inter-operator reliability and agreement for 3D foetal volume measurements using two different rotational angles and concluded that 3D ultrasound imaging can be successfully applied during early equine pregnancy for the measurement of both foetal volume and crown-rump length, and that it probably reflects with more accuracy the true crown-rump length compared to measurements obtained by 2D ultrasonography (Becsek et al., 2019). The most important limitations for the establishment of 3D/4D ultrasonography in small animal pregnancy evaluations include the relatively high costs, imaging artefacts due to breathing, and the lack of knowledge and experience of veterinarians with this new technology (Hildebrandt et al., 2009).

The accuracy to predict gestational age or the date of parturition in the bitch by various measurements obtained by B-mode 2D ultrasonography has already been widely studied. However, 3D ultrasonography has only been used in a limited number of bitches by one research group (Hildebrandt et al., 2009) and not with the purpose of gestational ageing. We hypothesized that during early to mid-pregnancy, measuring the volume of the canine conceptus (ICC volume) using 3D ultrasonography would give a more accurate estimate of gestational age than ICC diameter, and that ICC diameter would be comparable to ICC length. The objective of this study was to compare the accuracy of ICC volume obtained by 3D volume ultrasonography with ICC diameter and ICC length from 2D ultrasound measurements for gestational ageing during the first half of pregnancy in bitches, and to assess whether 3D ultrasonography could bring significant benefits.

2 | MATERIALS AND METHODS

2.1 | Animals

Twenty-five bitches of 19 different breeds were included in the study. Six bitches were small (≤ 10 kg), 12 were medium (>10 kg and ≤ 25 kg), five were large (>25 kg), and two were giant (>40 kg) breeds with body condition score (BCS) between 4/9 and 6/9, and age from

1.5 to 7.5 years. Twelve bitches were nulliparous, and 13 were multiparous. Litter size varied between two and 11 puppies.

All bitches were examined by ultrasonography once between 21 and 34 days after the first breeding, and seven dogs were also re-examined 4–10 days after their initial examination. In 15 bitches, the day of ovulation was estimated based on serial serum progesterone measurements using a chemiluminescence immunoassay (Immulite-2000, Siemens Healthcare AG). Ovulation was estimated to occur when serum progesterone concentration first reached approximately 5 ng/ml (Bouchard et al., 1991; Concannon, 2011; Concannon et al., 1977; Johnston et al., 2001a; Lévy & Fontbonne, 2007). At this time, most bitches went to the stud for mating, excluding further progesterone testing. Of those bitches with ovulation timing, ultrasound examinations were performed between 22 and 36 days after the estimated day of ovulation. The actual parturition date, i.e. the day the first puppy was born, was recorded for each dog. Three bitches received exogenous progesterone supplementation during pregnancy due to suspected hypoluteoidism, and five underwent C-section due to dystocia ($n = 4$) or as an elective procedure ($n = 1$). Descriptive data on the bitches are presented in Table 1.

2.2 | Ultrasound examinations

Two-dimensional B-mode ultrasound examinations were performed in a standard fashion including confirmation of pregnancy and conceptus vitality, estimation of litter size and measurements of structures of interest. The abdomen was systematically scanned starting from the urinary bladder as orientation point, continuing on with identification of the cervix and the uterine body, followed by scanning the full length of both uterine horns. All gestational ageing measurements were performed on two different embryos during each examination. For ICC diameter, the optimal transverse plane was chosen where the gestational sac appeared at its largest diameter and was as round as possible. The ICC diameter was measured as previously described (Beccaglia & Luvoni, 2006, 2012) by taking two perpendicular measurements from one side of the inner wall of the conceptus (trophoblastic decidual reaction) to the other (Figure 1). For ICC length, the optimal sagittal plane was chosen where the gestational sac was fully elongated and at maximal height. ICC length was measured on the midline at the maximal length of the gestational sac (Figure 2). Each gestational sac was scanned twice for both ICC diameter and ICC length. All 2D and 3D ultrasonographic examinations were performed with a portable Voluson i ultrasound machine (GE Healthcare) using a RealTime 4D convex transducer (RAB4-8-RS, bandwidth: 2–8 MHz; FOV: 70°). A total of 32 ultrasound examinations were performed on 25 pregnant bitches in either lateral or dorsal recumbency without sedation. Before scanning, ventral abdominal hair was clipped if owners agreed. In order to improve image quality, acoustic coupling gel and/or medical alcohol were applied to the skin of the scanned area.

TABLE 1 Relevant information about experimental dogs: Breed, age, body weight, breed size, number of previous pregnancies, timing of ultrasound examinations during current pregnancy, litter size and complications during pregnancy or delivery (e.g. progesterone supplementation, dystocia) are shown for each bitch

Bitch ID	Breed	Age (years)	Body weight (kg)	Breed size	Number of previous pregnancies	Ovulation timing with progesterone	Pregnancy days from estimated ovulation	Days before parturition	Litter size	Comments
1	Groenendael	6	23.65	Medium	3	No	-	38	7	
2*	Australian Shepherd	4	21.55	Medium	0	Yes	26 and 33*	-	7	Progesterone supplementation
3	Entlebucher Mountain Dog	5	21.9	Medium	3	Yes	25	38	5	
4	Boxer	4	27.1	Large	1	Yes	28	37	10	
5	Doberman	3.5	32.5	Large	1	Yes	26	37	10	
6*	Cairn Terrier	6	8.65	Small	2	No	-	40 and 35*	7	
7	Groenendael	5.5	20.25	Medium	1	Yes	24	38	7	
8	Cairn Terrier	3	9.1	Small	1	No	-	32	5	
9	Continental Bulldog	1.5	24	Medium	0	Yes	27	37	6	
10	Whippet	3	14.4	Medium	0	No	-	28	10	
11	Barsoi	5	37.3	Large	0	Yes	36	29	4	
12	Dutch Shepherd	4	26.4	Large	0	No	-	37	4	
13*	Miniature Poodle	5.5	6	Small	1	Yes	22 and 32*	40 and 30*	3	C-Section (Dystocia)
14	Beagle	4	14	Medium	0	Yes	25	39	7	C-Section (Dystocia)
15*	Leonberger	6	49.7	Large	2	Yes	24 and 28*	-	6	Progesterone supplementation
16	Bedlington Terrier	4	11.9	Medium	1	No	-	27	3	
17	Irish Setter	3	22	Medium	0	No	-	33	11	
18*	Australian Shepherd	3	19.5	Medium	0	Yes	26 and 33*	41 and 34*	6	
19*	Boxer	2	26	Large	0	Yes	25 and 27*	-	2	Progesterone supplementation, C-Section (Elective)
20	Papillon	2.5	3.5	Small	0	No	-	29	3	
21	Papillon	7.5	4.1	Small	3	No	-	36	5	
22	Border Collie	7	15.75	Medium	1	Yes	25	38	5	C-Section (Dystocia)
23	West Highland White Terrier	2	8.3	Small	0	Yes	25	37	4	
24*	Miniature Bull Terrier	1.5	12.8	Medium	0	No	-	35 and 29*	4	C-Section (Dystocia)
25	Leonberger	4.5	53.7	Large	1	Yes	25	37	9	C-Section (Dystocia)

Note: Asterisks depict dogs with two ultrasound examinations.



FIGURE 1 B-mode scan of inner chorionic cavity (ICC) in a bitch 35 days before parturition. Yellow lines denote ICC diameter measured in the transverse plane. Two measurements perpendicular to each other were taken at the largest diameter of the gestational sac from one side of the trophoblastic decidual reaction to the other with the embryo proper centred in the field of view

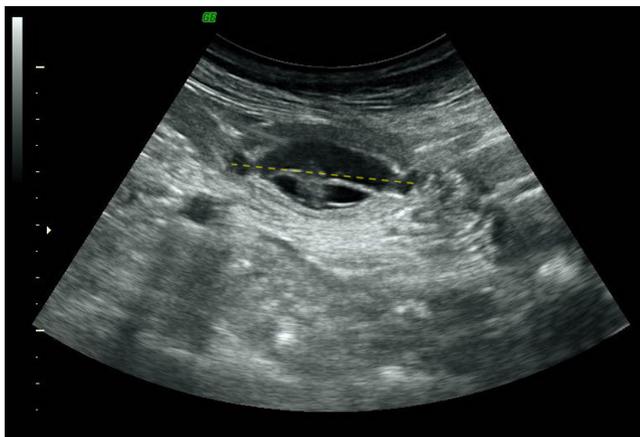


FIGURE 2 B-mode scan of inner chorionic cavity (ICC) in a bitch 38 days before parturition. Yellow line denotes ICC length measured in the sagittal plane at maximal length and height of the gestational sac

Three-dimensional ultrasound scans were performed using the 3D mode function on the same gestational sacs, which were chosen for ICC diameter and ICC length measurements. The sector angle and penetration depth were optimized for each conceptus. Two to three 3D images were acquired of each gestational sac, after confirmation that the view contained the whole conceptus. Recorded 3D scans were imported into the *4D View!* Software (v.14 Ext.2, GE Healthcare) where three projections of each scan could be seen. Determination of ICC volume was achieved with the extended software *Virtual Organ Computer-aided AnaLysis (VOCAL™)*, GE Healthcare). Quality of the stored 3D images was evaluated for definition of the contour of the ICC, and the best quality image was chosen for each gestational sac for further evaluation (Becsek et al., 2019). In five examinations, only one gestational sac could be measured due to decreased image quality noted during analysis with the VOCAL software, which

occurred secondary to breathing artefact or patient movement during the ultrasound scan. Every gestational sac was positioned in the sagittal plane B of the multiplanar view, so that the rotational axis could be set along its longest axis. Subsequently, the gestational sac was rotated 180 degrees with a rotational angle of 30 degrees. A rotational angle of 30 degrees was used, as a previous study did not find any differences between equine foetal volume measurements performed with either 6 degrees or 30 degrees rotational angle (Becsek et al., 2019). During the 180 degrees rotation, the ICC was manually contoured in each plane (6 planes for 30 degrees rotational angles), and the software calculated ICC volume in cm^3 giving rise to a 3D representation of the gestational sac, which could be rotated and observed from all directions (Figure 3).

The protocols and procedures in this study conform to Directive 2010/63/EU and were approved by the Zurich Cantonal Veterinary Office (ZH040/18).

2.3 | Data analysis

Inner chorionic cavity diameter and ICC length measurements were taken on two different conceptuses, each scanned twice during the same examination, i.e., a total of four perpendicular measurements taken for ICC diameter and two measurements for ICC length per gestational sac, and measurements were averaged for statistical calculations. Calculated ICC volume data from two different conceptuses during each examination were averaged for statistical evaluation except in five examinations, where ICC volume was available from only one conceptus. Each examination was considered an independent observation similarly to previous studies (Beccaglia & Luvoni, 2006; Kutzler, Yeager, et al., 2003; Son et al., 2001). The associations between the dependent variables of ICC diameter, ICC length and ICC volume, and the independent variable of time expressed as either 'days after estimated ovulation' or 'days before parturition' were analysed by linear or exponential regression. Goodness of fit of the regression line was determined based on the value of the coefficient of determination (R^2) with significance at $p < .05$. Regression analysis for 'days after estimated ovulation' was performed on data from the 15 bitches with serum progesterone testing and included 20 ultrasonographic examinations. In our dataset, the day of estimated ovulation was assigned to be when serum progesterone concentration first reached ≥ 4.7 ng/ml. Regression analysis for 'days before parturition' was performed on data from 22 bitches and included 26 examinations; three dogs were excluded from this analysis because of progesterone supplementation during pregnancy and/or planned C-section. Bitches that were treated with an emergency C-section due to dystocia were included in the data analysis. The relationships between gestational sac measurements, i.e., ICC diameter, ICC length and ICC volume, were evaluated in all 25 bitches comprising 32 examinations by regression analysis. Statistical significance was set at $p < .05$. Data recording and statistical analysis were carried out with the software Microsoft® Excel Version 15.20 for Mac Os X and IBM® SPSS® Statistics Version 26.0.0.0 for Mac Os X.

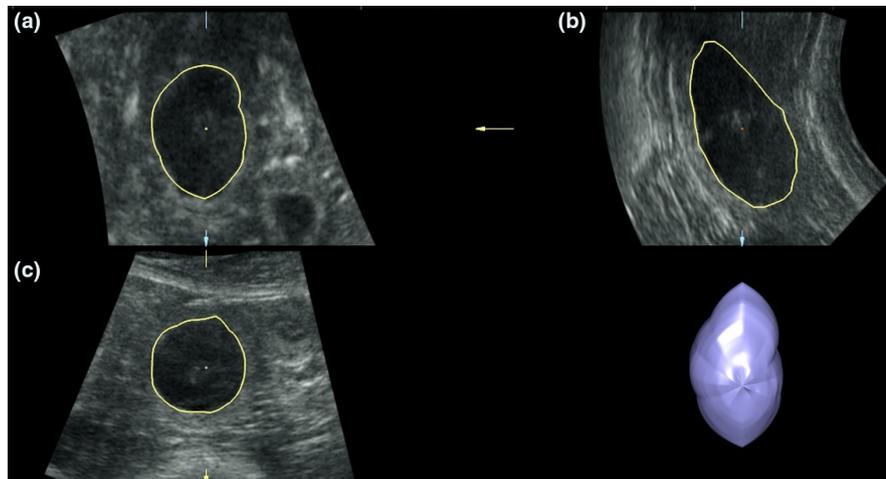


FIGURE 3 Three-dimensional (3D) ultrasonography scan and multiplanar view of the 3D conceptus in a bitch 37 days before parturition. The left upper panel (a) shows the frontal plane, the right upper panel (b) the sagittal plane, the left lower panel (c) the transverse plane and the right lower panel the reconstructed 3D image

3 | RESULTS

3.1 | 2D and 3D measurements in relation to days after estimated ovulation

Inner chorionic cavity diameter showed a strong positive ($R^2 = .707$, $p < .001$) linear association with gestational age when calculated based on time depicted as days after estimated ovulation (Figure 4a). A similar linear relationship ($R^2 = .728$, $p < .001$) was observed between ICC length and time (Figure 4b). ICC volume, on the contrary, showed an exponential relationship ($R^2 = .718$, $p < .001$) with gestational age based on the day of estimated ovulation (Figure 4c).

3.2 | 2D and 3D measurements in relation to the days before parturition

A strong positive ($R^2 = .800$, $p < .001$) linear association was observed between ICC diameter and gestational age based on the days before parturition (Figure 5a). Both ICC length (Figure 5b) and ICC volume (Figure 5c) had an exponential relationship with days before parturition ($R^2 = .810$ and $R^2 = .818$, $p < .001$, respectively).

3.3 | Associations between ICC diameter, ICC length and ICC volume

We found a positive ($R^2 = .932$) and significant ($p < .001$) exponential relationship between ICC diameter and ICC volume (Figure 6a). The associations between ICC length and ICC volume ($R^2 = .940$, $p < .001$) (Figure 6b) and between ICC diameter and length ($R^2 = .875$, $p < .001$) (Figure 6c) were linear.

4 | DISCUSSION

Parturition is a critical event, and accurate prediction of the delivery date may allow better planning and management of whelping to

decrease neonatal losses (Beccaglia et al., 2016). In the last decades, several studies had been published on gestational age determination and parturition date prediction in dogs, based on determination of ovulation by serum progesterone, on the first appearance of embryonic/foetal structures by ultrasonography or by abdominal radiograph, and on ultrasonographic measurements of extra-foetal or foetal structures (Beccaglia et al., 2016; Cecchetto et al., 2017; Kutzler et al., 2003; Kutzler, Yeager, et al., 2003; Lopate, 2008, 2018; Luvoni & Grioni, 2000; Nyland & Mattoon, 2002; Sananmuang et al., 2021; Siena & Milani, 2021; de Albuquerque Vieira et al., 2020; Yeager et al., 1992). Among the various parameters, ICC diameter was found to be a relatively accurate parameter in early to mid-pregnant bitches (Beccaglia & Luvoni, 2012) and is extensively used in daily clinical practice. However, accuracy of ICC diameter decreased from around 81% during week 4 to 67.7% during week 5 of gestation (Beccaglia & Luvoni, 2012). As pregnancy advances and the conceptus grows, the shape and size of the ICC change, rendering ICC diameter less accurate, and ultimately impossible to measure and use for gestational ageing (Beccaglia & Luvoni, 2012). Therefore, we hypothesized that calculating the volume of the ICC using 3D ultrasonography would give us a more accurate estimation of conceptus age by more reliably describing the size of the ICC than ICC diameter. Our results showed strong positive ($R^2 \geq .707$) and statistically significant associations between gestational age and all ICC parameters (diameter, length and volume). This finding suggests that ICC volume measured by 3D ultrasound represents a new parameter for determination of gestational age in pregnant bitches. However, as only minor differences were seen in the coefficients of determination (R^2) between ICC diameter, ICC length and ICC volume when plotting against days of pregnancy, conceptus volume does not seem to substantially increase accuracy of gestational ageing during early-mid-pregnancy compared with the widely used ICC diameter. This finding supports the continued use of ICC diameter for gestational ageing in the bitch as a good and reliable parameter. The coefficients of determination of the regression lines fitted on ICC length measurements were similar to ICC diameter, and thus, ICC length, which can easily be determined by routine 2D ultrasonography without the need for expensive equipment and laborious calculations, is a

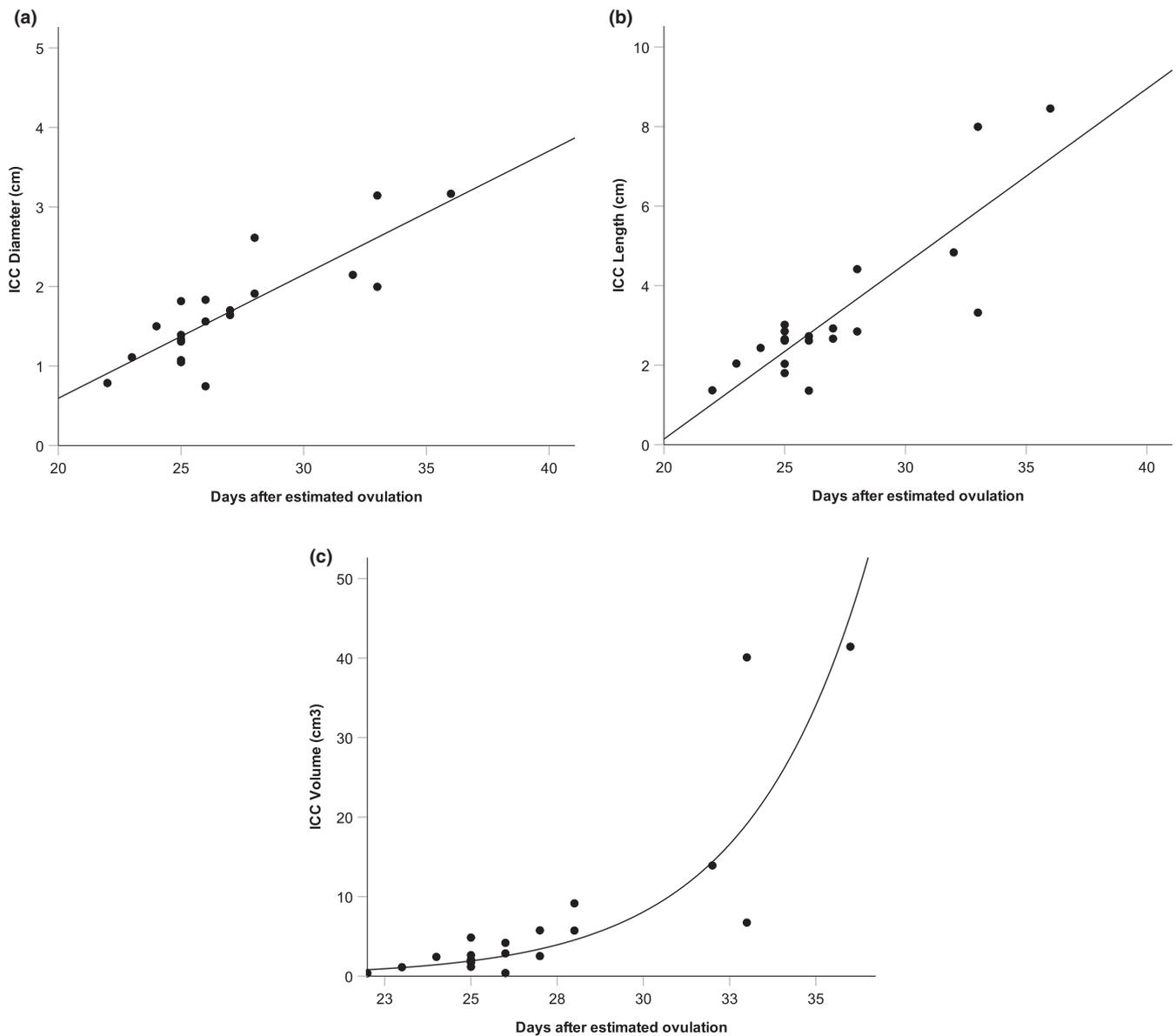


FIGURE 4 (a) Linear regression of inner chorionic cavity (ICC) diameter measured by 2D ultrasonography in relation to days after estimated ovulation ($R^2 = .707$; $p < .001$); (b) Linear regression of ICC length measured by 2D ultrasonography in relation to days after estimated ovulation ($R^2 = .728$; $p < .001$); (c) Exponential regression of 3D ICC volume measured by 3D ultrasonography in relation to days after estimated ovulation ($R^2 = .718$; $p < .001$)

promising alternative and should be further explored for canine gestational ageing in future studies. Limitations in its application may be bitch size and pregnancy stage.

Although using 'days before parturition' yielded slightly better fit of ICC measurement data ($R^2 = .800$ – 0.818 ; $p < .001$) compared with 'days after estimated ovulation' ($R^2 = .707$ – $.728$; $p < .001$), this finding needs to be confirmed in a larger number of dogs, preferably by breed and/or body size, and with more complete ovulation timing. We estimated ovulation to occur when serum progesterone first reached ≥ 4.7 ng/ml, and due to breeding arrangements, it was not possible to follow the majority of bitches until progesterone concentrations reached ≥ 10 ng/ml. This may have introduced a bias into our ovulation timing, even though whelpings occurred within the

expected range of gestation length at 63 ± 2 days after the estimated date of ovulation (Johnston et al., 2001a; Lopate, 2008, 2018; Mir et al., 2011) in all but one bitch with available ovulation timing and parturition data meeting our inclusion criteria. The day of ovulation cannot be assigned by one value of serum progesterone concentration but rather it falls within a range of progesterone values and is based on a rising trend. At the time of ovulation, plasma or serum progesterone concentrations were reported in the range of 4–10 ng/ml and around 5.4 ± 0.6 ng/ml (Johnston et al., 2001a, 2001b), between 3.4 to 6.6 ng/ml and at 4.9 ± 1 ng/ml (Bouchard et al., 1991), between 4.8 ± 0.9 ng/ml and 7.2 ± 1.3 ng/ml (Hollinshead & Hanlon, 2019), or initially reaching ≥ 4 ng/ml (Hahn et al., 2017). Despite the considerable continuous advances in refining canine ultrasonographic

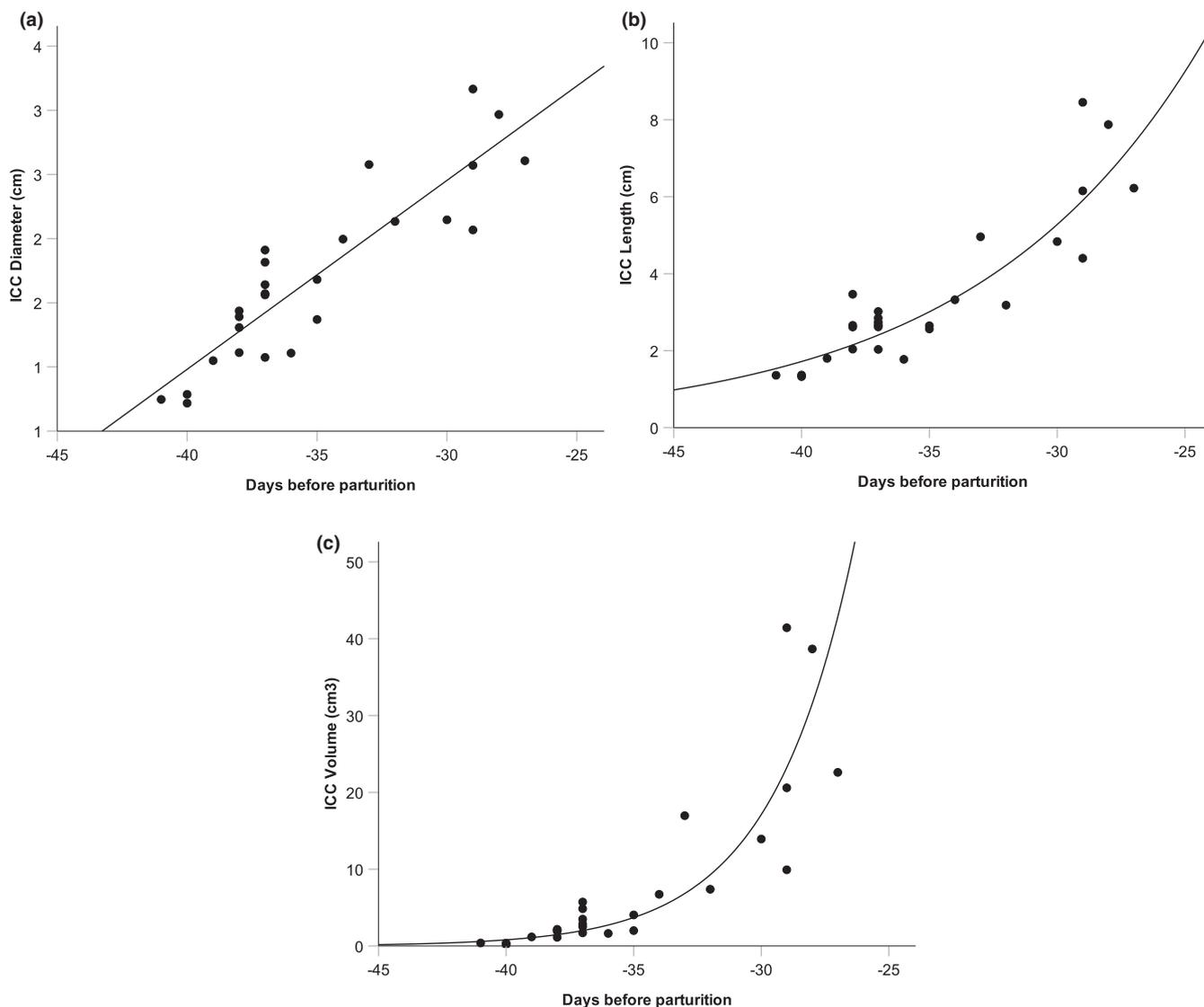


FIGURE 5 (a) Linear regression of inner chorionic cavity (ICC) diameter measured by 2D ultrasonography in relation to days before parturition ($R^2 = .800$; $p < .001$); (b) Exponential regression of ICC length measured by 2D ultrasonography in relation to days before parturition ($R^2 = .810$; $p < .001$); (c) Exponential regression of ICC volume measured by 3D ultrasonography in relation to days before parturition ($R^2 = .818$; $p < .001$)

gestational ageing, currently 'there is no substitute for accurate ovulation timing at the onset of the estrous cycle to determine gestational age' (Lopate, 2018), and the best recommendation is to use a combination of these diagnostic methods (Kim et al., 2007).

Previous reports showed that the increase in conceptus (ICC) diameter in relation to gestational age was significant and almost linear at a rate of approximately 1 mm per day between days 17 and 30 or 1.1 ± 0.1 mm per day between days 19 and 32 (in Labrador bitches) from the pre-ovulatory LH surge, after which the growth pattern was exponential (England, 1998; England & Russo, 2006). Similar results were obtained by other research groups depicting a linear equation between gestational age and ICC diameter (Beccaglia & Luvoni, 2006, 2012; Kutzler, Yeager, et al., 2003; Yeager et al., 1992), which is also consistent with this study's finding of a linear relationship between ICC diameter and time. On the contrary, conceptus length does not have a linear relationship with gestational

age (England, 1998). Our data showed an exponential relationship between ICC length and 'days before parturition', but a linear relationship to 'days after estimated ovulation'. Interestingly, ICC volume was also found to have an exponential relationship with gestational age independent of the time parameter used. Therefore, our results support that the growth of the canine conceptus is probably exponential already during the early stages of pregnancy, which is best shown by ICC volume or ICC length. More frequent measurements of ICC volume and ICC length in a larger group of bitches during early pregnancy could further confirm these findings.

To date, only one study described the use of 3D volume ultrasonography in dogs but without gestational ageing (Hildebrandt et al., 2009). The authors reported that one of the limitations of the 3D ultrasound technology is breathing and movements of the bitches during the scan, which is consistent with our experience and the reason why we excluded 3D measurements on the second embryo in five

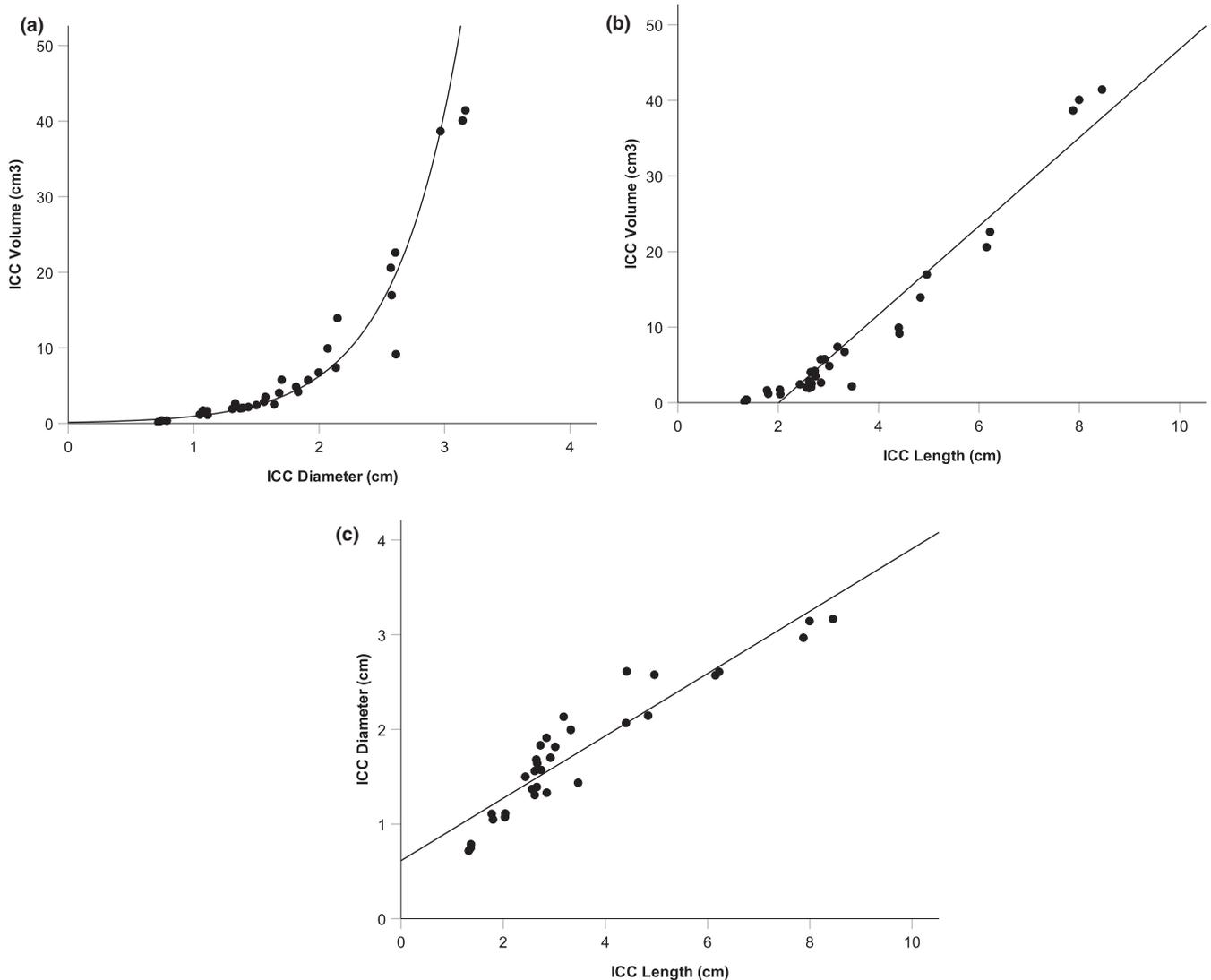


FIGURE 6 (a) Exponential regression of 2D and 3D ultrasonographic measurements of inner chorionic cavity (ICC) diameter and ICC volume, respectively ($R^2 = .932$; $p < .001$); (b) Linear regression analysis of ICC length and ICC volume ($R^2 = .940$; $p < .001$); (c) Linear regression of 2D ultrasonographic measurements of ICC length and ICC diameter ($R^2 = .875$; $p < .001$)

examinations due to motion artefacts. For 3D volume calculations, we used a 30° rotational angle with the rotational axis positioned through the longest axis of the gestational sac, which was chosen based on the results of (Becsek et al., 2019). Their study showed that application of a small rotational angle does not increase reliability, if the rotational axis was placed through the longest axis of the measured parameter (i.e. the foetus), probably because the changes between successive planes are less notable (Becsek et al., 2019). Moreover, reducing the rotational angle would also result in higher time investment.

One of the limitations of our study was the small size of the examined group (25 bitches) and the number of ultrasound examinations ($n = 32$). Furthermore, we included a heterogeneous group of dogs from small to giant breeds. Nevertheless, this study was successful in using 3D volume ultrasonography for gestational ageing with meaningful findings that open new research opportunities in this area of canine reproduction.

In conclusion, measurement of ICC volume by 3D ultrasonography did not improve gestational age accuracy during early-mid-pregnancy. ICC length and ICC volume yielded similar results to ICC diameter for gestational age determination, which supports the continued use of ICC diameter for gestational ageing and parturition date prediction in the bitch. Nevertheless, ICC length is a promising alternative and should be further explored for canine gestational ageing. Finally, in contrast to the ICC diameter, ICC length and volume showed exponential growth of the canine conceptus already during the first half of pregnancy.

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CONFLICT OF INTEREST

None of the authors of this article have any conflicts to declare.

AUTHOR CONTRIBUTIONS

SP contributed to study design, collected, analysed and interpreted the data, and drafted the manuscript. NT contributed to study design and data analysis, and critically revised the manuscript. IMR contributed to study design and data interpretation, and critically revised the manuscript. OB designed the study, collected, analysed and interpreted the data, helped in drafting and revised the manuscript. All authors have approved the submitted version.

DATA AVAILABILITY

The data that support the findings of this study are the authors' unique data and available from the corresponding author upon reasonable request.

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