Author's accepted manuscript

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Published in:

TheriogenologyVolume 141, Pages 1 – 8
1 January 2020
DOI: 10.1016/j.theriogenology.2019.08.017

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Abstract

Puppy neonatal mortality may be related to low birth weight, which has been shown in humans to be linked to placental factors. The relation between the newborn puppy and the placental characteristics has been poorly investigated in dogs. Twenty bitches, 9 toy-sized (i.e. < 5Kg) and 11 small-sized (i.e. 5 to 10 Kg), were included in this study. During natural delivery or c-section, 82 puppies were identified and their order of birth, sex and weight were recorded. Puppy weights were registered at birth and daily until Day 6. Placentas were weighed after removal of extraplacental adnexa, after which a photo was taken and morphometrically assessed. The Total Placental Area (TPA) and the Transfer Zone Area (TZA) were calculated and their surface expressed in mm^2. Immunohistochemistry with monoclonal mouse anti CD31 antibody was used to identify fetal and maternal vessels in the placental labyrinth zone. A vascularization index (VI) was determined for each placenta and the Total Vascular Area (TVA) was estimated. Puppies' birth weight correlated positively with placental weight ($P < 0.001$, $r = 0.689$). A positive correlation was found between the puppies' birth weight and TPA ($P < 0.001$, $r = 0.786$), TZA ($P < 0.001$, $r = 0.772$), and TVA ($P < 0.001$, $r = 0.482$). A positive correlation was also found between placental weight and TPA ($P < 0.001$, $r = 0.661$), TZA ($P < 0.001$, $r = 0.583$), and TVA ($P < 0.001$, $r = 0.333$). In the small-sized breeds, the placentas of low-weight puppies were lighter and had a smaller TZA and TVA ($P < 0.05$). The VI was higher in the placentas of the toy-sized compared to small-sized bitches ($P < 0.01$). No effect of parity, litter size, or sex of the puppy was observed on birth weight or growth rates on Day 6. The growth pattern of low-weight puppies did not differ from that of the other puppies during the first 6 days of life. As in humans, placental weight, the extension of the transfer zone and placental total vascular area correlates closely with the puppies' birth weight in normal pregnancies. Our data could represent reference values for placental weight, TZA, TVA and VI in toy and small-sized dog breeds.

Key words: dog, placenta, puppy, birth weight, CD31

1. Introduction

Neonatal mortality in dogs is a relevant problem, having an impact on both emotional and economical expectations of owners and breeders. Mortality rate is influenced by a large number of factors, including breed, size and age of the dam, or factors such as litter size and birth weight of the neonate. Several studies show how breed and size have a significant effect, with Toy and Small breeds showing the higher mortality [1, 2]

Low birth weight is a disadvantageous condition for the newborn puppy and is associated with higher mortality rates during the neonatal period [1, 3, 4]. Low-birth-weight puppies have a higher body surface area/mass ratio and a lower ability to maintain the correct body temperature and glucose homeostasis compared to their littermates [5].

Some of these puppies are not only small in size: the adverse uterine environment affects their growth, leading to pathological consequences. In human medicine this condition is known as intrauterine growth restriction (IUGR) and its incidence in newborns accounts for 5% of the total population [7]. Gross placental features, such as thickness and surface area, are well-known predictors of birth weight in humans and placental weight alone accounts for 36.6% of birthweight variations [7]. Concerning the canine species, little information is available on the determinants of low birth weight, however the placenta may play a role.

In mammals, placenta plays a key role in the intrauterine growth and development of the fetus. It mediates the interchange of nutrients and O_2/CO_2 across the barrier separating the dam and the developing conceptus. In addition, the placenta removes metabolic breakdown products from the fetus, mediates maternal immune tolerance, protects the embryo/fetus from potentially dangerous xenobiotics, and acts as a major endocrine organ throughout the gestation period.

Before day 20 of the pregnancy, the canine conceptus is supplied by a choriovitelline placenta, which is then replaced by a chorioallantoic placenta. In dogs, the placenta is classified macroscopically as belonging to the zonary type; consisting of a central girdle, the transfer zone, and two lateral bands, the marginal hematomas [8, 9]. The transfer zone forms a complete band surrounding the fetus and the maternal-fetal interface, composed of a complex system of trophoblastic lamellae interdigitating with maternal folds. The intimate placental connection between the dam and the fetus causes the placenta to shed maternal uterine tissue at the time of expulsion, hence the placenta of the dog is deciduate. Canine placenta histologically is of the endotheliochorial type, where the trophoblast directly surrounds the endothelia of the maternal blood vessels [9]. At the same time, the fetal vessels deeply indent the trophoblasts, and the placenta at the end of pregnancy has an enormous number of blood vessels [8]. An evaluation of the transfer zone area and of its vascularity may reflect the efficiency of transplacental diffusional substance transfer.

The main aim of this study was to evaluate the relationship between puppies' weight at birth and placental characteristics in toy- and small-sized dog breeds kept in normal breeding conditions. Possible causes of low birth weight and the relationship between birth weight and neonatal mortality and puppies' growth rate during the first six days of life were also investigated.

2. Materials and methods

2.1. Animals and pregnancy management

Twenty bitches were included in the study (9 toy-sized dogs, i.e. < 5Kg, and 11 small-sized dogs, i.e. 5 to 10 Kg) which in 2017 and 2018 were presented at the Veterinary Teaching Hospital of the University of Pisa for breeding and pregnancy management.

Bitches belonged to nine different breeds, the toy breed group (TOY) included Toy Poodle (n = 5), Russian Toy (n = 2), Yorkshire Terrier (n =1), and Maltese (n =1), while the small breed group (SMALL) included Miniature Pinscher (n = 3), Standard Poodle (n = 2), Jack Russell Terrier (n = 2), Miniature Schnauzer (n = 2), Dachshund (n = 1), and Boston Terrier (n =1).

All bitches included were clinically healthy at physical examination and no reproductive problems were reported in their history. The age of the bitches ranged from 1.3 to 6.3 years (median: 3 years). For breeding management, progesterone concentrations were evaluated using a fluorescence enzyme immunoassay (AIA 360, TOSOH Corp., Minato-ku, Tokyo, Japan), and the day of ovulation was considered when serum progesterone values during cytological estrus ranged between 4 and 8 ng/ml [10]. The normal evolution of the pregnancy was assessed by ultrasound monitoring.

From day 58 of pregnancy, dogs were regularly observed for parturient behavior and rectal temperature was monitored. Parturition was monitored throughout, but no direct intervention unless necessary was performed, respecting the natural course of whelping. If parturition did not proceed, an obstetric and pharmacological intervention was carried out, applying a standard intervention protocol. When bitches did not show any abdominal straining efforts for 1 hour after the expulsion of the last puppy, digital vaginal exploration was performed. If no puppy was present in the birth canal, additional straining was evoked by massaging the dorsal vaginal wall. A second digital vaginal exploration took place after 30 min and, at that time, if no puppy had entered the birth canal, the bitch was treated with 1 IU oxytocin im (Neurofisin®, Fatro s.p.a, Italy). This procedure was repeated after 1 hour if no puppy had been expelled. Obstetric interventions such as a gentle manual traction exerted on the pup and lubrication with gynecological gel were performed to aid the birth whenever necessary. When medical treatment was not successful or when bitches had shown intense abdominal straining efforts regularly for 45 min without any externally visible progress, an emergency cesarean section was performed.

In four cases, the cesarean section was performed immediately after the first abdominal straining efforts of the bitch. No complications were occurred, but a c-section was carried out to avoid any possible problem in bitches that had delivered via c-section at the time of the previous pregnancy.

Fourteen bitches were primiparous and six bitches were multiparous. Overall, 82 puppies were born, 26 belonged to TOY (median litter size: 3 puppies, range: 2-4) and 56 to SMALL (median litter size: 4 puppies, range: 3-8). Sixty-one puppies were delivered by natural whelping, 21 puppies by cesarean section. Three puppies were stillborn and one puppy died within 24 hours of birth. All puppies were born at term and no signs of prematurity were observed.

The Ethical Committee of the University of Pisa reviewed and approved the study protocol (Prot. n. 11045/2017). A written informed consent was obtained by the owners.

2.2. Whelping assistance and puppy weight monitoring

During each natural whelping or cesarean section, puppies were identified either by coat color and/or spot distribution, or by colored woollen collars. The weight of each puppy was recorded before the first suckling using a calibrated analytical scale in 1 g increments. In addition, puppy sex and order of birth were registered. A five parameter-APGAR scoring was performed five minutes after birth to assess newborn viability [11].

Placentas belonging to each puppy were identified, placed in labelled plastic bags, and stored in a refrigerator at 4°C until the end of whelping or C-section.

After parturition, the puppies were weighed daily until Day 6. Weight monitoring was always performed using the same calibrated analytical scale, which was supplied to the breeder in order to complete the follow-up. Their growth rate was calculated as follows: Growth rate (%) = [(weight at day x – weight at birth)/ weight at birth × 100] (Mila et al. 2015) .

When puppies' early growth rate (0-48 h) was below – 4% or weight gain since Day 3 was lower than 10% of puppies' birth weight, additional feeding 2–4 times a day with a commercial milk replacer was started. Mortality between birth and Day 21 was recorded, and necropsy was performed for stillborn puppies or that died after parturition.

2.3. Evaluation of the placenta

2.3.1. Macroscopic evaluation of the placenta

Each placenta was cut transversally, along the opposite side of the umbilical cord insertion site and gently stretched flat fante A single photo was taken, making sure that the camera lens was parallel to the plane on which the placenta rested, with a ruler placed next to it as size reference. Measurements of the placenta were performed by a physician who was not aware of the patients' clinical data, using NIS-Elements Br Microscope Imaging Software (Nikon Instruments; Calenzano, Italy). The Total Placental Area (TPA) and the Transfer Zone Area (TZA), which excluded the hematomas, were marked out manually and their surface expressed in mm^2 (Fig. 1a).

2.3.2. Microscopic evaluation of the placenta

Tissue sampling was performed within 24 h of the end of parturition. Placentas were trimmed of extraplacental membranes and umbilical cord, and placed on absorbent paper for five minutes before being weighed. A sample of allantochorion was dissected at the site where chorionic vessels separate from the surface of the placental belt, and immediately fixed by immersion in 10% neutral formalin solution (pH 7.4) for at least 48 h. Samples were then routinely processed, paraffin embedded and 5µm thick slices were cut. Immunohistochemical staining was performed to identify fetal and maternal vessels in the placental labyrinth using monoclonal mouse anti CD31 antibody (PECAM-1, Cat. n° sc-37674, Santa Cruz Biotechnology; TX, USA), a marker of endothelial cells.

Immunohistochemistry was performed as follows: epitope retrieval was carried out at 120°C in a pressure cooker for 3 min with a Tris/EDTA buffer pH 9.0. The endogenous peroxidases were quenched with incubation in 1% H_2O_2 in Phosphate Buffered Saline (PBS) for 6 minutes. Non-specific binding was blocked by incubation with 5% normal horse serum (Cat. N° S-2000; Vector Labs, CA, USA) in PBS for 45 min at room temperature (RT). Slides were then incubated overnight at 4°C with the primary antibody. After washing in PBS (3 × 10 min), a secondary antibody (biotinylated anti-mouse IgG, Cat N° BA-2001, Vector Labs, CA, USA) was applied for one hour at RT, then slides were washed again n PBS (3 x 10 min), followed by incubation with Avidin-Biotin complex (ABC Kit, Cat. N° PK-6100; Vector Labs, CA, USA) for 30 min at RT. After another washing in PBS (3 x 10 min), sections were incubated for 120s with DAB peroxidase substrate (3,3'-Diaminobenzidine, Cat. N° SK-4105; Vector Labs, CA, USA), a chromogen that produces a brown precipitate. Sections were then dehydrated in increasing gradients of alcohol, cleared in xylene, and mounted with permanent mounting medium.

Image analysis was performed by an experienced physician unaware of the patient's clinical data: five images of the placental labyrinth (resolution 2560x1920 pixel, 0.09 µm/pixel) were randomly acquired for each placenta with the NIS-Elements Br Microscope Imaging Software (Nikon Instruments; Calenzano, Italy), using a 40x objective.

For the quantitative evaluation of the CD31 immunoreactive area, color thresholds of the immunohistochemical signal were optimized for each image and five serial fields of known dimensions were measured (Fig. 1b-c). Two measurements were obtained: "area", representing the area where the software detected the presence of CD31 staining, and "fill-area" representing the "area"-value automatically corrected by the software. For the following evaluations only the "fill-area" value was used. The values obtained on the five images were averaged to obtain a single mean value corresponding to the total CD31 immunoreactive area of a single placenta. The percentage ratio between total CD31 immunoreactive area and the whole image area was calculated as the vascularization index (VI) for each placenta.

The total vascular area (TVA) was estimated by multiplying the vascular index and the transfer zone area (TZA).

2.4. Statistical analyses

Puppy birth weight values were classified into quartiles, separately for toy and small breeds. The first quartile (Q1) represents the lowest 25% of registered values, the second and third quartiles (Q2 and Q3, respectively) represent the 25% of values below and above the median, and the fourth quartile (Q4) represents the highest 25% of registered values. Puppies belonging to Q1 were considered low-weight, while those belonging to Q4 were considered high-weight puppies.

Before analyses, the data distribution was tested using the Anderson-Darling test. Depending on the data distribution of each independent variable, the Pearson test or Spearman test was used to assess the correlation between puppy birth weight and, respectively, placental weight, TPA, TZA, VI, TVA; or the correlation between placental weight and, respectively, TPA, TZA, VI, TVA.

Differences in placental parameters (placental weight, TPA, TZA, TVA) between TOY and SMALL puppies, placentas of Q1 and non-Q1 puppies, placentas of Q4 and non-Q4 puppies, were examined using the independent-sample t-test.

The non-parametric Mann-Whitney test was employed to evaluate differences in birth weight, newborn/dam weight ratio, and feto-placental weight ratio between TOY and SMALL puppies and in VI between placentas of Q1 and non-Q1 or Q4 and non-Q4 puppies. The Mann-Whitney test was also used to evaluate differences in growth rate at 1, 2 and 6 days of life between Q1 and non-Q1 puppies, Q4 and non-Q4 puppies, puppies with weight loss in the first 48 h and puppies with a constant positive growth rate. The effects of breed size (TOY or SMALL), parity (primiparous or multiparous), litter size (normal or large) and sex of the puppy on puppy birth weight and the growth rate during the first six days of life, were evaluated by analysis of variance (General Linear Model). Litter size was considered large when there were four or more puppies for the TOY breeds and seven or more puppies for the SMALL breeds. Values are shown as medians and interquartile range (IQR). The statistical analysis was performed using the statistical program package Minitab 18 (Minitab Inc., State College, PA) and differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Studied population and neonatal mortality (il titolo del paragrafo lo ritiene sempre adeguato? TOGLIEREI PLACENTA RECOVERY)

OF THE 82 puppies born, four (4.9%) died AT BIRTH OR DURING THE 6 DAYS OF OBSERVATION. ALL OF THEM belonged to SMALL. and anatomopathological lesions were not found at necropsy. Among the three stillbirths (meglio stillbirths or stillborn? NON SO, PENSO SIA MEGLIO STILLBORN), two were delivered by c-section and the THIRD by natural whelping. The puppy which died within 24 hours of birth was also from a natural whelping. Two of the dead puppies belonged to the low weight group.

The mean 5 min-APGAR score of the puppies born alive was 9.5 (range 7-10), and the puppy that died within 24 hours had a 5 min-APGAR of 9 at birth.

The puppy sex ratio was 1.05 males to 1.00 females (42/82 males and 40/82 females). Placenta recovery rates were 100% (26/26) and 82.1% (46/56) for TOY and SMALL, respectively.

3.2. Puppies' birthweight and placental characteristics

The median values and quartile classification of puppies' birth weight are reported in Table 1. Puppies' birth weight and placental weight were higher in SMALL compared to TOY ($P < 0.001$). The ratio between puppy birth weight and dam weight was higher in TOY compared to SMALL (5.38% vs 2.94%; $P < 0.05$), while no differences were observed in the feto-placental weight ratio (7.85% vs 6.89%; $P > 0.05$).

Overall, puppies' birth weight correlated positively with placental weight ($P < 0.001$, $r = 0.689$). However, considering the two dog sizes separately, this correlation was found in SMALL ($P < 0.01$, $r = 0.492$), but not in TOY ($P = 0.558$, $r = 0.120$).

A positive correlation was also found between puppies' birth weight and TPA ($P < 0.001$, $r = 0.786$), TZA ($P < 0.001$, $r = 0.772$), and TVA ($P < 0.001$, $r = 0{,}482$). Positive correlations were found between placental weight and TPA ($P < 0.001$, $r = 0.661$), TZA ($P < 0.001$, $r = 0.583$), and TVA ($P < 0.001$, r

= 0.333). Low, although statistically significant, negative correlations were found between VI and puppy weight ($P < 0.01$, $r = -0.314$) or placental weight ($P < 0.01$, $r = -0.307$).

Parity, litter size and sex of the puppy showed no statistically significant effects on puppy birth weight. The median values of puppies' placental weight, TPA, TZA, VI, TVA are reported in Table 2: TOY placentas were significantly lighter and smaller than SMALL placentas.

Placentas in Q1 puppies were lighter compared to non-Q1 puppies in SMALL ($P < 0.05$), but not in TOY ($P = 0.760$). Placenta TPA, TZA and TVA were smaller in Q1 puppies compared to non-Q1 puppies in SMALL (Table 3, $P < 0.05$) but not in TOY. However, TVA was higher in the placentas of Q4 compared to non-Q4 puppies both in SMALL and TOY (Table 3, $P < 0.01$ and $P < 0.05$, respectively). The VI was higher in TOY compared to SMALL placentas (Table 2, $P < 0.01$).

3.3. Puppies' growth during the first six days of life

For the 78 puppies still alive on Day 2, median growth during the first two and six days of life were +16.8% (IQR: 8.5-22.7) and +72.7% (IQR: 57.6-94.1), respectively. Five puppies (5/78) showed a loss of weight in the first 48 hours after birth (range: -2.0, -6.7) and only two were Q1 puppies. The median growth rate of these five puppies during the first six days of life was significantly lower than the median growth rate of the other puppies (10.5% vs 73.1%; $P < 0.05$).

Median growth rates were higher in SMALL compared to TOY on Day 1 and Day 2 ($P < 0.05$), however, no differences were observed on Day 6. There was no significant difference in growth rates between Q1 and non-Q1 puppies and, therefore, Q1 puppies remained significantly smaller compared to the other puppies on Day 6 ($P < 0.05$) (Fig. 2). Growth rates of Q4 puppies were higher compared to non-Q4 puppies on Day 2 ($P < 0.001$), however, no differences were observed on Day 6.

On Day 1, puppies born from multiparous bitches and large litters grew relatively slower than those born from primiparous bitches and normal litters ($P < 0.05$), and such differences were not observed on Day 2 and Day 6. No effect of sex of the puppy was observed on growth rates during the first six days of life.

4. Discussion

Neonatal mortality in dogs is a significant problem, both for the animals' well-being and the breeders' income [3, 4]. The mortality rate ranges from 10 to 13% within the first three weeks after birth [1, 3, 12], exceeding 20% when there is no supervision at delivery, and additional nursing or hand rearing are not performed [2].

The causes of neonatal mortality in dogs are multi-factorial and several risk factors have been reported [2, 13]. In our study, neonatal mortality during the first week of life accounted for 4.9% of all delivered puppies, lower than previously reported findings [1, 3, 12, 14]. Whelping assistance, APGAR scoring system and weight monitoring facilitated the identification of puppies with a particular nursing need, thus decreasing their mortality rates during the neonatal period. Similarly, Veronesi et al. showed how the detection of puppies with poor APGAR scores improves their survival rates [11]. Among the criteria used to evaluate the health of newborns, birth weight is a low cost and easy-to-evaluate parameter; and is also a valuable predictor of neonatal mortality in domestic animals [2, 15, 16, 17].

To identify puppies with a low birth weight, cutoff values for small-, medium-, and large-sized dogs have been reported [2]. However, the significant difference in birth weight between toy- and small-sized dogs observed in this study suggests the need for a further distinction between these two different breed sizes. Low birth weight values for toy-sized dogs and small-sized dogs were provided by this study and could be used as reference values for future investigations.

In human medicine, small for gestational age (SGA) fetuses or newborns are those whose birth weight is less than the 10th percentile for gestational age. When a SGA newborn is not only smaller than normal in size, but there is a pathological restriction of genetic growth potential, this is referred to as intrauterine growth retardation or restriction (IUGR) [18]. IUGR is a common complication in human pregnancy and is associated with a variety of adverse perinatal outcomes [19].

In our study, low weight puppies did not show a higher mortality rate or a different early growth rate compared to the other puppies. The monitoring and support provided by the breeder was sufficient to reduce the mortality of low weight puppies. This suggests that the risk associated with a lower birth weight in our case was mostly due to the disadvantage in size when competing for teats with littermates.

The low weight puppies observed in our study were more recognizable in the SGA definition; this as IUGR newborns, in fact, normally show severe neonatal adaptive problems and their survival rates are low without proper medical care [20]. Some IUGR infants do not manifest acute life-threatening symptoms during the neonatal period (e.g. hypoglycemia and hypoxemia), but the consequences related to their condition may be revealed later in life [21]. In fact, human IUGR is also associated with a higher risk of degenerative diseases in adulthood such as adult-onset diabetes, hypertension, and cardiovascular disease [22].

The long-life negative impact of IUGR has also been reported in domestic animals [23]. In pigs [24]. and sheep [25]., IUGR can affect body composition and meat quality: a decrease in the number of skeletal muscle fibers and an increase in intramuscular fat mass and connective tissue content have been reported. In horses, IUGR can impact on growth and athletic performance [26]. Further studies are required to clarify whether “true” IUGR puppies will develop negative sequelae in their lifetime. The relationship between birthweight and puppy mortality rates has been widely studied, however, very little is known concerning the determinants of birthweight. Several factors influence fetal growth and newborn birthweight including the genetic potential for growth, maternal metabolism, endocrine factors and placental perfusion [27]. In humans, placental function is a determinant of fetal growth and hence of newborn’s birthweight [28].

In our study, placental weight reflected placental size, transfer zone area and total vascular area, and all these parameters correlated closely to puppies’ birthweight. This finding is not surprising, since placenta is the organ responsible for feto-maternal exchange, and placental factors could play an important role as a determinant of puppies' birth weight. In a study by Salafia et al. (2008), six placental measures (placental disc shape, umbilical cord length, distance from cord insertion to nearest margin, large diameter, small diameter and placental thickness) and placental weight were assessed in over 2400 human placentas, in order to define their role as a determinant of birthweight. Placental weight alone accounted for 36.5% of birthweight variance, whereas the six gross placental measures alone accounted for 28.2%. In contrast, the multivariable model proposed by Salafia revealed that only 13.9% of birthweight variation could be explained by maternal factors such as age, height, weight, parity, socioeconomic status, cigarette use, and race [7]. Similarly, Roland et al. showed how placental weight alone explained 39% of variation in infant birthweight [28]. In addition, the direct relationship between fetal weight and both placental weight and placental macroscopic surface area has been demonstrated in several domestic species such as pigs [29], horses [30] and cows [31].

In the small-sized breeds, low-weight puppies had a lighter placenta and a smaller total placental area, transfer zone area, total vascular area compared to the other puppies. As expected, high weight puppies showed diametrically opposite placental features. Thus, there was a clear relationship between low birth weight puppies and gross placental measures in small-sized breeds, but the only significant difference observed in toy-sized breeds regarded the placentas of high weight puppies which were heavier than the placentas of the other puppies. In toy-sized breeds, the apparent absence of a correlation between puppies’ birthweight and placental weight could be related to the use of an insufficiently sensitive analytical scale to evaluate the differences in placental weights, which showed a narrow range of variability in this breed size.

Toy-breed puppies were proportionally bigger in relation to maternal weight compared to small-breed puppies. Interestingly, this difference was not related to a proportional increase in placental size, in fact, although only numerically, the feto-placental ratio was higher in toy-breed puppies compared to small-breed ones. The higher VI observed in toy-sized breed placentas may promote the mother-foetus exchange, hence the correct development of the fetus despite a proportionally smaller placenta.

In our study, parity did not influence the puppies' birth weight. Similarly, no effect of parity was observed in the birthweight of 514 puppies belonging to small-, medium- and large-sized breeds [2]. Maternal parity is a well-recognized predictor of newborn birthweight in humans [32], horses [33] and cows [34], with lighter birthweights observed among newborns from primiparous females, however, the effect of parity is controversial in domestic polytocous species [35, 36].

Litter size did not seem to affect puppies' birth weight, although in a previous study, the proportion of low weight puppies was higher in large litters compared to small ones (37.1 % vs 8.6%) [2]. In piglets, the mean birth weight decreased by approximately 33 g for each additional piglet in a litter [37] and a similar effect has also been demonstrated in kittens [38, 39].

Puppies' growth pattern during the first weeks is determined by several factors such as colostrum/milk intake and environmental conditions, and it is similar in different breeds of dogs [5]. Although a 10% loss of birth weight is commonly considered to be physiological in the first 48 hours after birth [30], a growth rate below –4% has been associated with a higher mortality risk [2].

In our study, 6% (5/78) of puppies showed a loss of weight in the first 48 hours after birth, but only three puppies reached weight values below the threshold of -4 % and none of these died in the first week. No influence of birth weight on growth during the first six days of life was observed in this study, confirming previously reported findings [2]. In contrast, a lower birth weight has been shown to be rapidly compensated for during postnatal growth in kittens [40].

The assistance provided by the breeder, including supplementation with a milk replacer when needed, seems to have helped puppies with a low birthweight or puppies that experienced a weight loss in the first 48 hours to have normal growth rates during the first six days of life, in fact, growth rate did not differ from those of the other puppies. Ultimately, we can assume that the breeders' assistance and puppy monitoring contributed to the reduction in the neonatal mortality rates observed in this study.

Our findings did not reveal any difference between male and female puppies in birth weights or growth rates in the first six days of life, although a sexual dimorphism in postweaning growth curves has been described in dogs [41] and in cats [40].

Growth pattern was not affected by litter size on Day 2 and Day 6, however puppies born from large litters showed lower growth rates compared to puppies born from normal litters on Day 1. In pigs, litter size does not seem to impact on morphological characteristics or fiber type distribution within the muscle, however, preweaning growth performance and weaning weight have been shown to be higher for pigs from small litters than from large ones [37]. Similar findings have been observed in rats and rabbits, in which a negative correlation between litter size and newborn growth rates has been reported [42]. In carnivores, results are controversial and further studies are necessary to clarify the effects of litter size on growth rate [2].

5. Conclusions

This study showed how placental weight and size were closely related to placental vascular area, which, similarly to other species, could be a significant determinant of puppies' birth weight in normal pregnancies.

Reference values for placental weight, total placental area, transfer zone area, total vascular area and index of vascularization in toy and small-sized dog breeds were reported. However, these values need refining for different breed sizes. Further studies are needed to better clarify the impact of placental characteristics on the status of the puppy at birth, as well as their indirect influence on neonatal growth and development.

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Table 1. Classification into quartiles of puppies' birth weight in relation to breed size

Breed size	Number of puppies	Median birth weight (g)	Quartiles of birth weight (g)			
			Q1	Q2	Q3	Q4
Toy	26	134[a]	<131	131-134	135-154	>154
Small	56	193[b]	<173	173-193	194-209	>209

[a–b] Median values within a column with different superscripts are significantly different (P < 0.05).

Table 2. Median values of parameters evaluated in placentas in relation to breed size

Parameter	Breed size	Median	IQR [c]	Minimum value	Maximum value
Placental Weight (g)	Toy	18[a]	6	12	25
	Small	26[b]	11	15	46
Total Placental Area (TPA) (mm^2)	Toy	4278[a]	1068	2822	6288
	Small	5611[b]	1537	3102	7721
Transfer Zone Area (TZA) (mm^2)	Toy	1254[a]	2422	846	3617
	Small	1508[b]	3138	968	5041
Vascularization index (VI)	Toy	0.09[a]	0.15	0.03	0.20
	Small	0.08[b]	0.14	0.03	0.19
Total Vascular Area (mm^2)	Toy	367[a]	84	215	539
	Small	436[b]	156	118	718

a≠b : Within parameter, different superscripts denote a statistically significant difference between size ($P < 0.05$).
[c] Interquartile range

Table 3. Differences between Q1 and non-Q1 puppies, Q4 and non-Q4 puppies depending on breed size. Median (IQR).

Parameter	Breed size	Q1 puppies	Non-Q1 puppies	Q4 puppies	Non-Q4 puppies
Placental Weight (g)	Toy	17 (6)	18 (6)	19 (3)	17 (7)
	Small	22 (4)^{a}	29 (10)^{b}	29 (9)^{c}	25 (12)^{d}
Total Placental Area (TPA) (mm^2)	Toy	3912 (638)	4509 (1045)	4765 (584)	4009 (1077)
	Small	4530 (955)^{a}	6013 (1186)^{b}	6514 (734)^{c}	5354 (1253)^{d}
Transfer Zone Area (TZA) (mm^2)	Toy	2260 (1091)	2422 (774)	2702 (627)	2388 (867)
	Small	2507 (637)^{a}	3457 (893)^{b}	4140 (952)^{c}	3015 (876)^{d}
Vascularization index (VI)	All	0.152	0.146	0.147	0.146
Total Vascular Area (mm^2)	Toy	360 (82)	372 (86)	397 (93)^{c}	356 (67)^{d}
	Small	370 (98)^{a}	462 (162)^{b}	530 (194)^{c}	391 (136)^{d}

a≠b : Within parameter and size, different superscripts denote a statistically significant difference between Q1 and non-Q1 puppies ($P < 0.05$).
c≠d : Within parameter and size, different superscripts denote a statistically significant difference between Q4 and non-Q4 puppies ($P < 0.05$).

Fig. 1. (a) Macroscopic image of post-birth canine fetal adnexa; (b, c) photomicrographs of canine placental labyrinth. a) The Total Placental area and the Transfer Zone area, are encircled by the purple and white lines respectively; TZ=Transfer Zone, MH=Marginal Hematoma, AM=Allantoic Membrane, UC=Umbilical Cord. b) CD31 expression pattern; c) image showing the semi-automatic procedure used for identification and measurement of placental labyrinth vascular area. Scale bar is equal to 2mm in a and to 100 µm in b and c.

Fig. 2. Mean growth rates during the first six days of life for: A) TOY and SMALL puppies; B) Q1 and Non-Q1 puppies.