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Canine and feline reproduction

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Canine and feline reproduction

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Editorial: Canine and feline reproduction

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Editorial on the Research Topic
Canine and feline reproduction

Felidae and Canidae are two families of the order Carnivora with both domestic and wild representatives. Felidae has been reproductively classified as seasonal polyestrous with induced ovulation. According to the IUCN Redlist of Threatened Species, ∼75% of wild felids are listed as threatened or endangered. Canidae reproductive physiology is unique, for example, preovulatory luteinization of follicles, delayed oocyte maturation after ovulation, uterine-independent prolonged luteolysis, and long obligate anestrus between cycles, compared to other mammals. This has caused dog reproductive management to lag behind that of other species. Domestic dogs and cats have served as valuable models for developing reproductive technologies for managing rare, endangered carnivores.

Until the eighties, in most veterinary colleges, animal reproduction was mainly focused on farm animals. Although over the last 25 years there was an increase in canine and feline reproductive studies, further knowledge about physiology, pathology, diagnostic techniques, and therapeutics is still necessary to improve both breeding and contraception in these species.

The goal of this Research Topic is to publish high-quality peer-reviewed articles which update basic and applied knowledge on reproduction in domestic and wild carnivores. In this issue, there are 11 papers including three reviews, two clinical trials, and four questionnaire studies covering some of these topics.

The use of additives or micronutrients represents a feasible approach to improve sperm quality. There seems to be evidence linking gut microbiota and fertility. Mahiddine et al. test the effect of the oral administration of three commensal Lactobacillus spp. on canine sperm quality. They find that total and progressive motility, acrosome integrity, and other kinematic parameters were enhanced after the commensal lactobacilli administration.

Similarly, Aiudi et al. test the antioxidative activity of a mix of polyphenolic substances derived from the hydroxylation of Pinus Taeda lignin (PTHL) on canine blood and semen. PTHL improved the antioxidant status of animals as well as semen volume, concentration, and motility. These studies contain results useful for dog owners and breeders as well as for pet food producers in order to include substances with a potential beneficial effect on health and semen quality.

Prostatic diseases are very common in male dogs, accounting for up to 10% of cases submitted to veterinary practitioners. Palmieri et al. present a comprehensive and updated review describing the gross, cytological, and histological features of prostatic hyperplasia, prostatitis, prostatic cysts, and prostatic carcinoma in both canine and feline species.

Canine corpus luteum (CL) function is quite different from that of most other species. It has been hypothesized that estradiol, as well as its target genes, regulate canine CL lifespan, from formation through maintenance until regression. Pereira Bonfim Neto et al. develop an approach to uncover the relationship between 17b-estradiol and ESR1/ESR2 ratio in the regulation of

canine CL throughout diestrus. ESR1 targets were greater at the beginning of diestrus, while the abundance of ESR2 targets was greater in the end. ESR1/ESR2 ratio shifted from an increasing to a decreasing pattern during the second half of the luteal phase. ERa-mediated positively regulated CL function at the beginning of diestrus and ERb-mediated effect contributed to luteal regression.

Vaginal cytology is a routinely used diagnostic tool in canine gynecological examinations which is useful for estrus cycle staging and the diagnosis of diseases. Vaginal cells vary under the influence of systemic estrogens. Attributing some subjectivity to this method, Reckers et al. standardize the identification of the different vaginal cell types through a tutorial flowchart. The use of this chart will lead to a high agreement among practitioners.

An accurate prediction of parturition day is of high importance to minimize neonatal death when planning labor assistance or cesarean sections (C-sections). However, the prediction of parturition day is still challenging when a pregnant bitch presents in the last week of gestation and the ovulation day is unknown. Although fetal gastrointestinal motility (FGM) has been shown to be useful to assess fetal maturity, there are still many aspects that should be unveiled. Siena et al. quantify FGM in relation to days before parturition (dbp), maternal size, and the sex ratio of pups. FGM increased in the last 5 dbp and it also was higher in small bitches. Conversely, parity and sex ratio did not seem to have an effect on FGM.

Dystocia in dogs is a frequent problem and C-sections, both as an emergency or planned, are a routine practice in small animals. Schrank et al. evaluate, through questionnaires, the incidence of C-sections and contributing factors comparing elective vs. emergency C-sections. These authors find that bitches with either small litters or which had prior C-sections have an increased risk. Primiparous bitches of advanced age and stillbirths presented higher emergency C-sections. A less popular breed, the Norwich Terrier, had an incidence of C-sections of more than 50%.

Conze et al., using the same methodology, compare fertility after C-section and compare it with natural parturition in dogs. They show that more than 90 % of the bitches became pregnant at the *first* breeding attempt either after C-sections or natural parturition. Bitches which underwent C-sections were more likely to have this intervention and more than 50% of bulldogs require C-sections at their *first* parturition.

The early experiences and environment during an animal's development can influence the appearance of specific diseases in adulthood. Factors such as nutrition, stress, or exposure to different microbes during critical windows of development can predispose the animal to certain diseases. An understanding of these factors is needed to guarantee health throughout life. In the review by Gaillard et al., the authors identify and discuss these factors with respect to adult obesity, chronic enteropathy, and behavioral disorders in dogs and cats, and areas of future research are suggested.

The popularity of brachycephalic dogs has been increasing in recent years. The extreme homozygosity of these breeds has led to an increase in the manifestation of deleterious genes that may lead to congenital malformations. There has been no data on the incidence of malformations in brachycephalic dogs compared with other breeds. Estevan et al. compare the frequency of malformations in brachycephalic dogs vs. other breeds. Overall malformations had an incidence of 6.77%, of which 87.5% were represented by brachycephalic breeds. The most common malformations in these breeds were cleft palate and anasarca.

Canids occupy the top of the food chain and are fundamental for the wild environmental balance in South America. On this continent, there are 11 species of canids and although some of them are threatened, little is known about their reproductive biology. Candido Carvalho et al. compile the current knowledge of South American wild canid estrous cycles, pregnancy, and parturition, as well as sexual behavior and ejaculate characteristics, identifying gaps in the knowledge which should be studied to facilitate the development of conservation programs.

Concluding, the results of the above-mentioned studies and reviews of this issue represent an enormous amount of new relevant data on andrological, gynecological, obstetrical, developmental, and genetic topics in both domestic and wild carnivores.

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Canine Vaginal Cytology: A Revised Definition of Exfoliated Vaginal Cells

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Vaginal cytology is an important examination method in the context of gynecological disorders and cycle staging in the bitch. While collection and preparation of samples are easy, the evaluation appears to be challenging. Inconsistent definitions of cell attributes such as size, cornification and the appearance of the nucleus have been published. The aim of the project was to develop a tutorial for vaginal cell determination. To get a deeper insight into the use of cytology in practice, an online survey was distributed to veterinarians interested in small animal reproduction. Participants were asked to define eight cells and answer questions. The agreement of the 16 participants, working in eight different countries, determining the cells was poor ($\kappa = 0.412$). Eleven respondents stated that vaginal cytology has a low reliability. Nevertheless, 13 participants use this tool regularly. The tutorial was developed as a flowchart based on the survey results, scientific literature and own measurements. It guides the user systematically through the evaluation of specific cell characteristics. An evaluation of the results of five raters with difference experience levels led to a high agreement ($\kappa = 0.858$). Vaginal cytology is a useful diagnostic tool, but it seems helpful to standardize the determination of cell types.

Keywords: exfoliative vaginal cytology, vaginal smear, estrus cycle, vaginal cell tutorial, digital microscopy, female dog

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INTRODUCTION

Gynecological examinations belong to the standard procedures in veterinary medicine. The findings are important in the context of breeding management (1). Finding the day of ovulation during estrus is essential for an appropriate timing of mating or insemination and, thus, a high fertility (2). Symptoms such as acceptance of a male for mating (3), the sanguineous vulvar discharge (3), and the edema of the vulva (4) are signs of proestrus and estrus. The symptoms which can be observed via vaginoscopy are color, the intensity of edema and moisture of the vaginal mucous membrane (4, 5). Since some bitches do not show overt behavioral signs (6) it can be helpful to determine other stages of the estrus cycle, i.e. proestrus or diestrus. Also abnormal estrus cycle patterns, such as early or late ovulation, silent heats or split heats (7) can be diagnosed. External symptoms of proestrus are turgid vulvar swelling and sanguineous vulvar discharge (8). The discharge contains a large number of erythrocytes due to diapedesis through the uterine capillaries due to estrogen effect (9). The erythrocytes can also be found in the vaginal smear (10). The diestrus is characterized by slight mucoid discharge which often contains a large number of neutrophils in early stage. The bitch will not allow mounting or breeding of male dogs anymore (8). Furthermore, vaginal cytology belongs to the standard gynecological examinations because it enables insights into potential estrogen influences and the health status of the vagina (11). Sampling of cells, preparing and staining of a smear for vaginal cytology requires only few

skills (12) and can be performed rapidly and inexpensively in daily practice (13, 14). However, the analysis and interpretation of the smears may be affected by several factors. Different sampling and staining procedures may bias evaluation results (15). In addition, the skills of the observer, transition between the different cell stages as well as individual characteristics of the bitch, such as a massive occurrence of erythrocytes, may impede the evaluation of vaginal smears (5).

To the best of our knowledge, no uniform standardized definition of canine vaginal cell types has been published, yet. A closer look into the literature reveals that authors suggest different parameters and definitions of the type of cells, for example regarding the diameter of the different cell types (**Table 1**).

Besides the diameter, the presence and extent of cornification are important aspects for the determination of vaginal cells. The cornification refers to the degenerative process of cells in higher layers in stratified squamous epithelia (16). Different authors give different definitions of cell types based on the extent of cornification (**Table 2**). A majority of the authors agree that basal, parabasal and intermediate cells are not cornified at all (16, 20).

Basal, parabasal and intermediate cells have a round unaltered nucleus (11, 19, 21). According to Johnston et al. (16) the area of the nucleus of an intermediate cell is $>90\ \mu m^2$. Other authors specify the diameter for the nucleus of an intermediate cell to be 7–11 μm (22), what corresponds to an area between approximately 38.5 μm^2 and 95.0 μm^2. Most authors agree that the nucleus is large and clearly discernible (19), prominent and appears normal (16). Superficial cells are described unanimously as cells having a pyknotic nucleus (12, 22, 23), which becomes eventually karyorrhectic (18) or karyolitic (17).

For most authors, the shape of the vaginal cells is an important aspect (22, 24, 25). However, other authors state that intermediate and superficial cells can be confused if they are defined by their shape, only (5, 16). The definition of squamous cells is also not explicitly clear. While some authors state that the nucleus is not visible (19, 20, 24), others observed that these cells are anucleated but often remainings of the disintegrated nucleus are still visible (18). Based on these heterogeneous definitions, it is likely that different evaluators come to different conclusions when interpreting vaginal smears (1). This has also been shown by Arlt (15).

An often-used method for the determination of the cycle stage is the determination of specific percentages of cell types. Some authors claim that a typical vaginal smear in estrus has 100% superficial cells and >80 % cells with pyknotic or absent nuclei (11, 26). Another definition of estrus is the presence of more than 90% superficial keratinized epithelial cells (13). Others define the cytological estrus by 100 % cornification with more than 50% anuclear squames (20). For the determination of diestrus based on specific percentages of exfoliated vaginal cells, the definitions by different authors show a high agreement. The onset of diestrus

TABLE 1 | The table shows the diameter of the different cell types according to several authors.

	Type of cell				
Author	**Basal cell**	**Parabasal cell**	**Intermediate cell**	**Superficial cell**	**Squamous cell**
Johnston et al. (16)	Usually not exfoliated	10–20 μm	20–30 μm	30–75 μm	-
Dreier (17)	10–20 μm	15–25 μm	20–30 μm	35–60 μm	Same size as superficial cells
Wehrend (5)	10–20 μm, usually not exfoliated	10–20 μm	20–50 μm	No diameter measurable because of folds	-
Bostedt et al. (18)	10–15 μm	15–30 μm	25–30 μm	40–60 μm	Same size as superficial cells
Antonov (11)	10–20 μm, usually not exfoliated	15–25 μm	20–30 μm	30–75 μm	-

TABLE 2 | Presence and extent of cornification of different types of vaginal cells according to different authors.

		Definition of the presence and extent of cornification			
Author	**Basal cell**	**Parabasal cell**	**Intermediate cell**	**Superficial cell**	**Squamous cell**
Schutte (19)	-	Not cornified	Small intermediate cells: none Large intermediate cells: Not necessarily found	"not necessarily found"	"With a few exceptions always keratinized"
Johnston et al. (16)	Not cornified	Not cornified	Not cornified	Cornified	Cornified
Perez et al. (12)	-	Not cornified	Not cornified	"Partially cornified"	"Fully cornified"
Root Kustritz (20)	-	Not cornified	Not cornified	Cornified	Cornified
Wehrend (5)	Not cornified	Not cornified	Not cornified	"Increasing cornification"	Not specified
Bostedt et al. (18)	Not cornified	Not cornified	Not cornified	"Are subject to cornification"	"final stage of cornification"
Antonov (11)	-	-	-	-	Cornified

"Not cornified" means that this cell type does not show signs of cornification. "-" means that the author did not specify the appearance of cornification of this cell type. Descriptions in hyphens are quotes from the respective author.

occurs when the number of superficial and squamous cells has decreased by at least 20% (10, 11, 13) and when a higher number of neutrophil granulocytes are present (23, 25). The heterogenous definitions of cell types and percentages of cells characteristic for specific cycle stages by different authors may impair cycle stage diagnoses and lead to a suboptimal agreement between different evaluators. Aim of this study was to learn more about how small animal reproduction experts define vaginal cells. In a second step, we wanted to develop more robust definitions of vaginal cells and evaluate a cell determination tutorial.

MATERIALS AND METHODS

Preparation and Digitalization of Vaginal Cell Slides

In total, 52 vaginal smears were taken from 39 bitches for which health of the genital tract was confirmed. All bitches were presented for gynecological examination in the context of ovulation timing or routine gynecological health check. Bitches were aged between 1 and 14 years (median 5 years, interquartile range 3 and 6 years) and belonged to the breeds Siberian Husky, Continental Bulldog, Great Dane, Rottweiler, Golden Retriever, Afghan Hound, Dobermann (n = 2), Miniature Schnauzer (n = 3), Pomsky, Manchester Terrier (=2), French Bulldog (n = 2), Dachshund, Smooth Collie, Cross Breed (=5), Swiss Cattle Dog, Miniature Bullterrier, Pug (n = 2), Labrador (n = 3), Cairn Terrier, Kangal Shepherd Dog, Bernese Mountain Dog, Border Collie, Shiba Inu, German Shepherd, Leonberger, Monkey Terrier, and Berger Picard. The weight of the dogs ranged between 5 to 56 kg (median 20 kg, interquartile range 10 and 30.25 years). All smears used for this project were leftovers from clinical examinations.

All specimens were taken via an inserted sterile speculum (Proctovision, Karl Storz SE & CO. KG, Tuttlingen, Germany) as described by (11). The samples were collected by using a saline moistened, sterile cotton swab (12). The cotton swab (Medical applicator, Heinz Herenz Medizinbedarf GmbH, Hamburg, Germany) was introduced through the speculum (5) to collect cells from the caudodorsal surface of the vagina (23). After swabbing by gently rolling the dorsal vaginal wall, the swab was removed and rolled onto a glass slide (Objektträger ELKA, Glaswarenfabrik Karl Hecht GmbH & Co KG, Sondheim, Germany) (14). Routine Diff-Quick staining (Haema-Schnellfärbung, LT- Sys Eberhard Lehmann GmbH, Berlin, Germany) was performed after air drying (14). A coverslip (Deckgläser 32*22 mm, Glaswarenfabrik Karl Hecht GmbH & Co KG, Sondheim, Germany) was permanently fixed (Roti Histokitt II, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) to the

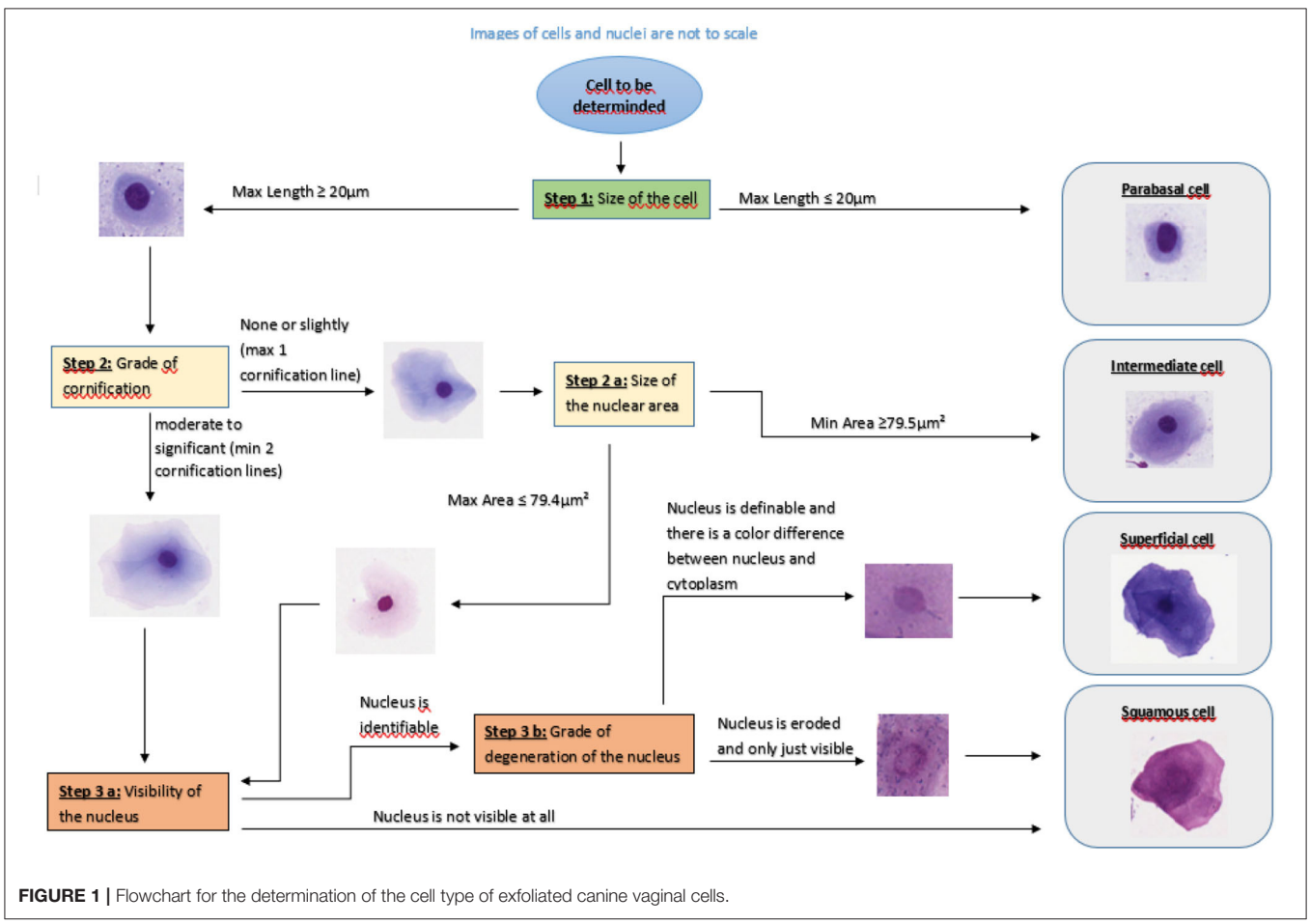

FIGURE 1 | Flowchart for the determination of the cell type of exfoliated canine vaginal cells.

specimen (16). Bitches in different stages of the estrus cycle were chosen, so that all types of vaginal cells were represented.

For digitization the stained slides were scanned with Aperio CS2 (Leica Mikrosysteme Vertrieb GmbH Mikroskopie und Histologie, Wetzlar, Germany). The data were converted into Aperio Scan Scope Virtual Slide (svs) files and analyzed using the software program QuPath®. QuPath® is an open-source software platform for whole slide image analysis (27). The program is able to show the slides in more than 400× magnification and allows measurements of the area and diameter of nuclei or whole cells. It can also be used to label specific cells.

Structure of the Survey

To reach veterinarians who are specialized in small animal reproduction the international Email list "Café Reprod" with around 150 members (personal information from the list administrator, spring 2021) worldwide was used for the distribution of the survey. No reminder was sent. The survey was open for 3 weeks.

The survey consisted of three parts: In the first part, eight pictures of different vaginal cells were illustrated. Participants were asked to define cells as basal cell, parabasal cell, intermediate cell, superficial cell and squamous cell by ticking a box (see **Figure 1**).

In the second part, the participants were asked to answer the following questions by typing a short free text: How do you differentiate a parabasal cell from an intermediate cell? How do you differentiate an intermediate cell from a superficial cell? How do you differentiate a superficial cell from a squame?

Finally, questions about the practical work with vaginal cytology and about some personal information were asked.

Development and Validation of a Tutorial for Vaginal Cell Determination

The information from the survey results and from scientific literature were analyzed. Based on this information and the digitized smears, definitions including specific parameters such as cell size and shape as well as nucleus size and shape were revised and evaluated for all vaginal epithelial cell types.

The tutorial was designed as a flowchart which aims to support determination of canine vaginal cells. For a first validation, five vaginal smears were used. The slides were chosen by the authors in order to ensure that all epithelial cell types were present.

On each slide, the first author assigned the numbers one to 100 to 100 cells by using QuPath®. Inclusion criteria for the selection of the cells were a clear and well visible structure of the cell. Cells which overlapped with other cells or structures were excluded. With the help of a random number generator (https://www.zufallsgenerator.net) 20 cells per slide were selected for the validation. This resulted in 100 cells in total.

All 100 cells were evaluated independently by five persons using the same computer and the same supporting material. No further information e.g., about the dog or stage of sexual cycle were given. The respondents were selected in order to represent raters with different levels of experience. Therefore, the raters were two students of veterinary medicine in their 5th year, two veterinarians working in the field of small animal reproduction for 12 and 24 months, respectively, and one diplomate (ECAR) for small animal reproduction. The first author supervised the process, did not take part in the evaluation and did not influence the evaluation. All evaluators received a list with the numbers of the cells to determine, a colored Din A4 version of the tutorial (**Figure 1**) and a form with a table in which they were asked to document the cell type according to the slide number and cell number.

Statistical Analysis

Fleiss' Kappa was used, which measures the inter-rater reliability between more than two raters. For the calculation of Fleiss' Kappa in this project, R programming language was used (https://www.r-project.org/).

If n_{ij} is the number of raters who assigned the i-th subject ($i = 1, \ldots, N$) to the j-th category ($j = 1, \ldots, k$), then Fleiss' kappa is defined as

$$\kappa = \frac{\overline{P} - \overline{\underset{e}{P}}}{1 - \overline{\underset{e}{P}}}$$

$$Where\, \overline{P} = \frac{1}{N}\sum_{i=1}^{N} P_i, P_i = \frac{1}{n\,(n-1)}\sum_{j=1}^{k} n_{ij}\left(n_{ij}-1\right),$$

$$\overline{P}_e = \sum_{j=1}^{k} p_j^2, p_j = \frac{1}{Nn}\sum_{i=1}^{N} n_{ij}.$$

RESULTS

The Results of the Survey

In total, 16 respondents completed and returned the survey. The definition of the eight presented cells by the respondents are presented in **Figure 2**.

Cell number one was defined as intermediate cell by nine respondents or as parabasal ($n = 6$) or basal cell ($n = 1$). Cells number two, three and four were identified by the most respondents as superficial cells. Cell number five had the highest accordance: all but one respondent defined it as a squamous cell. The definition of cell number six gave a more ambiguous result: nine respondents defined it as intermediate cell but seven were of the opinion that it is a parabasal or basal cell. Both, cells number seven and eight, were defined as intermediate cell by around half of the participants and as superficial cell by the other half. The calculated Fleiss' Kappa for the accordance of all 16 raters was $\kappa = 0.412$.

In the second part, the respondents were asked how they discriminate between cell types. For each of the three scenarios the participants named at least two parameters.

The first question was how they differentiate a parabasal cell from an intermediate cell. The most named parameters were size ($n = 10$) and shape ($n = 10$) of the cells. Nine respondents mentioned the ratio between the nucleus and the cytoplasm. The appearance of the nucleus (size and shape) was named by seven and cornification by one respondent.

Image	1	2	3	4
Definition	Basal cell: 1 Parabasal cell: 6 Intermediate cell: 9 Superficial cell: 0 Squamous cell: 0	Basal cell: 0 Parabasal cell: 0 Intermediate cell: 1 Superficial cell: 11 Squamous cell: 3 No information: 1	Basal cell: 0 Parabasal cell: 0 Intermediate cell: 0 Superficial cell: 14 Squamous cell: 2	Basal cell: 0 Parabasal cell: 0 Intermediate cell: 0 Superficial cell: 13 Squamous cell: 3

Image	5	6	7	8
Definition	Basal cell: 0 Parabasal cell: 0 Intermediate cell: 0 Superficial cell: 1 Squamous cell: 15	Basal cell: 3 Parabasal cell: 4 Intermediate cell: 9 Superficial cell: 0 Squamous cell: 0	Basal cell: 0 Parabasal cell: 0 Intermediate cell: 8 Superficial cell: 8 Squamous cell: 0	Basal cell: 0 Parabasal cell: 0 Intermediate cell: 7 Superficial cell: 9 Squamous cell: 0

FIGURE 2 | Results of the determination of eight canine vaginal cells by 16 veterinarians.

The second question was how to differentiate an intermediate cell from a superficial cell. Shape ($n = 11$) and appearance of the nucleus ($n = 11$) were the main parameters for differentiation of the cells, followed by the size of the cell ($n = 7$). Parameters of minor importance seem to be the cytoplasm: nucleus ratio ($n = 5$), the cornification ($n = 3$) and the color of the cell ($n = 2$).

Finally, the participants were asked how they differentiate a superficial cell from a squamous cell. Mostly named parameters were the presence and appearance of the nucleus ($n = 11$). The shape ($n = 3$), the grade of cornification ($n = 2$) and the size ($n = 1$) had only a minor influence for the differentiation of superficial and squamous cells. Some of the participants stated there was no difference between the two cell types ($n = 3$).

The third part of the survey referred to the practical work and experience of the participants. The number of bitches the participants stated to see in the context of ovulation timing per year is median 125 (interquartile range 50–300).

The majority of the respondents indicated that they perform vaginal cytology in the context of ovulation timing ($n = 13$). Several respondents stated, however, that vaginal cytology is "Not reliable at all" ($n = 5$) or "Not reliable" ($n = 6$) in the context of ovulation timing. The minority chose the answer "Moderate" ($n = 3$) or the answer "Reliable" ($n = 2$). All participants agreed that progesterone measurement for ovulation timing is "Very reliable" ($n = 8$) or "Reliable" ($n = 8$). The respondents use immuno assays for progesterone measurement: Immulite® ($n = 8$), Minividas®

($n = 3$), TOSOH® ($n = 1$), Hormonost® ELISA kit ($n = 3$). In one case no answer was given.

Finally, the respondents were asked about their experience and person. The participants have worked as veterinarian for 24.1 (±10.2) years in mean. One respondent did not specify the years in practice but wrote that she or he has practiced "too long". Asked for the highest degree they have achieved, the participants chose "Veterinarian" ($n = 7$), "Diplomate" ($n = 6$), "PhD" ($n = 2$) or "Specialist of reproduction" ($n = 1$). They work in the USA ($n = 7$), Sweden ($n = 2$), Germany ($n = 1$), Hungary ($n = 1$), Thailand ($n = 1$), Belgium ($n = 1$), Portugal ($n = 1$) and Australia ($n = 1$). One participant stated to work "worldwide".

The Structure of the Tutorial

The tutorial (**Figure 1**) was developed as a flowchart which enables following flowlines and assessing cell aspects step by step. The aim was to guide the user through the evaluation of relevant cell parameters. To support the decision process, sample images were included. These images were derived from the digitalized slides. The tutorial for the evaluation of a vaginal cell starts with the determination of the cell diameter. According to several authors, the maximum diameter of a parabasal cell is 20 µm. To validate this definition, vaginal smears from 10 bitches in anestrus or early proestrus were used. On each specimen 20 cells with a diameter smaller than 20 µm were measured and analyzed with QuPath®. This resulted in 200 cells in total. Not one cell showed cornification or changes in the appearance of the nucleus. Hence, the given definition is suitable and was, therefore, included into the tutorial.

If the cell diameter exceeds 20.0 µm the user follows the flowline to the decision box "Step 2: Grade of cornification". At step 2, the user can decide between the flowlines "none or slightly" and "moderate to significant". These flowlines lead to decision box "Step 2.a.: Size of the nuclear area" or decision box "Step 3.a.: Visibility of the nucleus", respectively. To standardize this decision about the grade of cornification, all cells with no or only one cornification line should be determined as cells with no or slight cornification (**Figure 3**).

The decision box 2.a. refers to the size of the nucleus. According to the above-named authors the area of the nucleus of an intermediate cell is large, round and unaltered. The nucleus of superficial cells becomes smaller. Our aim was to define an area which allows a suitable differentiation of intermediate from superficial cells in at least 95% of cases. To define a precise threshold, vaginal smears from 10 bitches in late proestrus or estrus were selected. On each specimen, the nuclei of 20 cells with at least two cornification lines and a definable and demarcated nucleus = defined as superficial cells (see **Figure 2**) were measured and analyzed. This resulted in 200 nuclei in total. The measurements revealed a mean value of 57.7 µm² (±13.8 µm²) for the area of the measured nuclei. The results of the nucleus area measurements are shown in **Figure 4**. The majority (95 %) of nuclei of cornified vaginal cells is smaller than 79.5 µm². Therefore, the value of 79.4 µm² was selected as threshold for the maximum area of the nucleus of a superficial cell. Thus, if the area of the nucleus is 79.5 µm² or larger the cell is classified as intermediate cell. If the nucleus has an area of 79.4 µm² or smaller the user should follow the flowline to decision box 3.a. and the cell is excluded from being an intermediate cell.

Decision box 3.a. refers to the visibility of the nucleus. If the cell has no visible nucleus, the cell is classified as a squamous cell. If the nucleus is still identifiable, the flowline leads to decision box 3.b. In this case, the grade of degeneration of the nucleus has to be determined. If "the nucleus is definable, demarcated and there is a color difference between nucleus and cytoplasm" the cell it is classified as a superficial cell. Otherwise, if "nucleus is eroded and only just visible" the cell is classified as a squamous cell.

The independent evaluation of 100 randomly selected vaginal cells by five persons using the tutorial led to a Fleiss' Kappa of $\kappa = 0.858$.

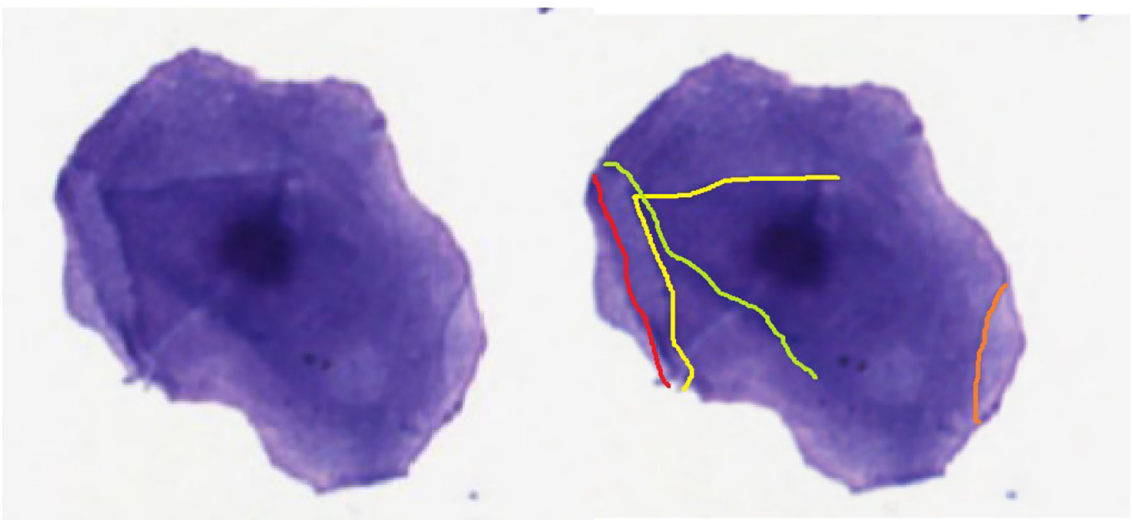

FIGURE 3 | Cornification lines of a vaginal epithelial cell.

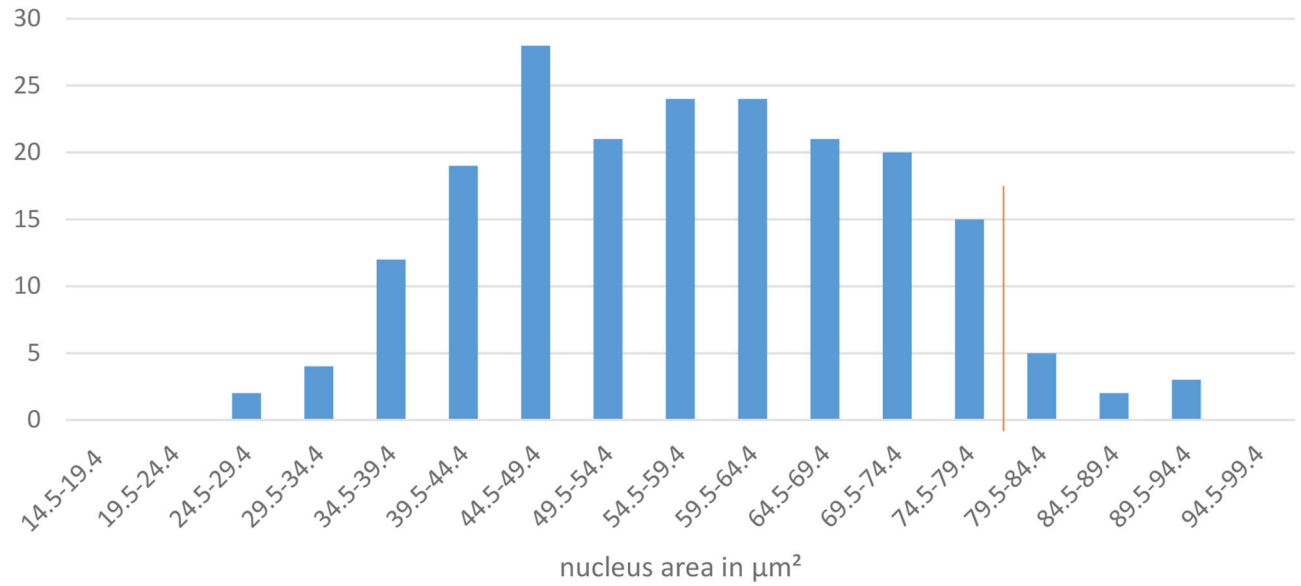

FIGURE 4 | The diagram shows the area of the measured nuclei of cornified vaginal cells in µm² and the frequency of measurements. The red line marks the 95 percentile.

DISCUSSION

Vaginal cytology in dogs and its usefulness and limitations have been controversially discussed recently (15). On the one hand, this diagnostic method has been described as a valuable clinical tool in the context of gynecological examinations (12) but on the other hand, usability of this method may be low for detection of the ideal time for mating or insemination (5, 16). Other authors state the vaginal cytology allows ovulation timing only retrospectively (13, 26). In regard to these different opinions, it seems worthwhile to critically assess this alleged proved and tested method as suggested for many well-proven procedures by Fontbonne (28).

Several factors may impede the reliability of vaginal cytology, which include individual cellular characteristics of the bitch such as a variable percentage of anuclear cells at the time of ovulation (29) or an influx of other cells like neutrophils (1) or erythrocytes (5).

In addition, different methods of taking and staining the smears, as well as no standardized evaluation methods may lead to variability in the interpretation (15, 26). Some authors recommend sampling from the vaginal vestibulum. Advantages of this procedure may include the reduced risk of contamination of the vagina or trauma as well as less defense reactions of sensible bitches if the swabs are not inserted into the more cranial parts of the vagina (19). Other authors do not recommend taking the specimen from the vaginal vestibulum because its cells do not react as quickly to an increase in the blood estrogen concentration as the vaginal mucous membrane (11) and are, therefore, not as indicative of the stage of the estrus cycle (7). Whether the use of a speculum improves the reliability of vaginal cytology and which staining method leads to the most robust evaluation results, has yet to be clarified (15). Also the staining of the cells with different stains need to be discussed. Diff Quick is a rapid, modified Wright- Giemsa stain that is easy to use in a clinical setting (16) and widely used for vaginal smears (15). Therefore, this stain was used for this project. Other stains such as Papanicolaou or Shorr (modified Papanicolaou) are able to detect eosinophilic cells by staining them orange- red (4). This simplifies the identification of superficial cells (4). These stains, however, are not widely used in practice because of the high costs and time requirements (15). If the results of our project would have been different with other staining remains open.

Furthermore, it seems that an important reason for the low reliability is the above-named ambiguity of definitions of different authors for vaginal cells. Arlt (15) has showed highly variable results of vaginal smear assessment probably caused by subjective evaluation.

For preparation of new cell definitions, scientific literature and experts' opinions were analyzed which revealed interesting insights into the perils and pitfalls of vaginal cytology. The Café Reprod E-mail list was chosen for the distribution of the survey to reach participants with a high level of experience in small animal reproduction. Indeed, 16 respondents cannot be regarded as representative. Nevertheless, according to their statements, most were quite experienced veterinarians in the field of small animal reproduction. It can be assumed that a selection bias needs to be considered, namely that people more interested in vaginal cytology were probably more likely to participate in the survey. In that regard, the results of the survey are even more surprising. The agreement of the raters regarding the definition of the vaginal cells was around $\kappa = 0.4$. This means poor agreement.

A value of 0.0 indicates an agreement not better than chance, values lower than 0.40 indicate poor agreement, higher than 0.75 good agreement and 1.0 perfect agreement (30).

The participants stated that cell determination has a low and progesterone measurement has a high reliability in the context of ovulation timing. Nevertheless, still 13 out of 16 routinely perform vaginal cytology. Arlt (15) found similar results in a similar survey. When using both methods, veterinarians may rely more on the progesterone measurement than on vaginal cytology because it is easier and quicker to interpret. The effort on evaluating and interpreting vaginal smears and, therefore, the experience and routine to do so may have decreased among practitioners. This, in turn, may be a reason why the determination of exfoliated vaginal cells is considered to be unreliable. The question arose if more experienced raters may have a lower variation. Therefore, only raters who stated to examine more than 100 bitches per year were included in a subgroup evaluation. This led to a Fleiss' Kappa of $\kappa = 0.533$, meaning moderate agreement. This analysis suggests that variations between the raters may decline with growing experience.

Nevertheless, it is to mention that the raters did not evaluate the stage of estrus cycle based on a whole smear in this project. They were asked to name single vaginal cells.

It seems that some types of cells are easier to determine than other cells. Angular, cornified cells with a definable nucleus or no visible nucleus are better assignable for the raters, almost regardless of the level of experience. However, cells with an oval to polygonal shape, a vesicular and big nucleus and without or little cornification seem to be difficult to define, even for experts. A possible explanation for this result could be that the evaluation based on cornification and a disappearing or pyknotic nucleus is easier to recognize than the evaluation based on the size of a cell and nucleus since there is a scale necessary. In addition, the determination of cells as basal cell by various respondents, independent of their level of experience, seem noteworthy because of their questionable occurrence in a vaginal smear. The characterization of basal cells seems obsolete according to several authors since these cells build the lowest layer of the mucosal membrane (5, 10, 16)and usually cannot be collected with a swab without harming the vaginal epithelium. Therefore, this type of cell is not included in this project.

To improve the reliability of vaginal cytology, standard operating procedures for the interpretation of vaginal smears may be helpful (15). In 1967, Schutte published a classification of vaginal cells. He assigned the cells to groups A to D (19). Limitation of this classification include the widespread thresholds for the nuclear diameter. For example, Schutte stated that the diameter of a large intermediate cell ranges from 7 to 11 µm and that the diameter of a superficial cell is smaller than 6 µm. A definition of cells with a nuclear diameter between 6.0 µm and 6.9 µm was not given. In addition, some definitions such as "small intermediate cell have a relatively large nucleus" were not precise. Noteworthy is, however, that the classification of Schutte has been published 55 years ago and he had not the same technical possibilities for scanning and measuring vaginal cells as we have today. In this project, opinions and experiences of different authors and experts as well as measurements made with the program QuPath® were combined. The tutorial aims to support evaluators analyzing and defining vaginal cells. The evaluation of the tutorial with experienced and unexperienced raters led to a high inter observer agreement. The Fleiss-Kappa $\kappa = 0.858$ can be interpreted as good agreement. A limitation of this project is that only five raters tested the tutorial so far. Since two students used the tutorial with good results, it can be postulated that the tutorial is user-friendly and supports determination of the cells also for non-experts. A debatable point is that the cells in the evaluation were not determined by raters beforehand without the tutorial. However, one can assume that a certain training effect would have biased a control examination, especially if the tutorial would have been offered before a non-tutorial evaluation. Another limitation of this project is the applicability and practicality of the tutorial in daily practice which needs to be tested in future studies. The effort of measurements of cells under the microscope has been described as time-consuming and unsuitable (22). This also applies for the process of digitalization. The measurement of the size of cells or nuclei can be difficult and time consuming. Nevertheless, the regular use of the tutorial may lead into a certain training effect, which was also observed during the evaluations during this project.

If the tutorial is useful in the context of ovulation timing needs to be further assessed in future studies. In the context of this project, the focus was set on the definition of cells. The cell patterns in relation to ovulation or in the context of specific gynecological disorders was not assessed. To limit a breed–related bias the authors strived a preferably wide diversity of breeds ($n = 27$).

To date, the scan process of a slide for this project lasted about 20 min. The file size of the data of one scanned smear was two to three Gigabytes. The quality of the scan highly depends on the quality of the smear. Especially specimens from bitches in anestrus or early proestrus often include only few, small cells compared with specimens from bitches in estrus and early diestrus. In consequence, the scanner has less areas to focus on and less sharp digital scans can be the result. Other variables, such as inconsistent staining and low color contrast, folded cells, air bubbles and particles may also lead to scans of low quality. While human evaluators can compensate these limitations to a certain extent by "reading through" them (31), an automated slide evaluation might not lead to appropriate results. Therefore, accurate collection of cells and preparation of slides, including the proper application of cell material, correct fixation of the cover slips, wiping the slides before scanning, are important requirements for a successful scanning process result (32). Thus, to date the scanning and measurement procedures presented are not usable in daily practice. In practice, scales in the ocular of microscopes may omit the need for digitization of the smears. Parameters from the tutorial can still be used. If a rough assessment of diameters and sizes leads to good agreements needs to be tested in future studies.

Advantages of digital microscopy like remote and off-site access to digitalized slides, easy handling, improved ergonomics, and quantitative measurements are evident (33). New microscopes, which allow easy digitalization and connection

to computers or mobile devices, are already available and it can be expected that they will undergo a rapid further development. Therefore, it is likely that new measurement procedures or even automated cell evaluations will be possible in the near future. Partial scanning of smears with a minimum number of cells to decrease the duration of the scan process and the file size could raise the practicality. More standardized determination of vaginal smears and the emerging digitalization technologies may lead to a more reliable use of vaginal cytology. Another scenario could be an automated evaluation of vaginal smears by artificial intelligence (AI). There is a strong public interest and market forces that are driving the rapid development of such diagnostic procedures (34). Also in challenging on-site staffing situations, such as the COVID 19 pandemic, digital microscopy may be an important tool to keep histology workflows running smoothly (35). Some studies have shown that AI is even partially superior to human experts in cytology, namely in determining neoplastic vs. normal cells (36). Based on AI, standardized diagnostic procedures are possible which minimizes bias from the experience of the evaluators, laboratory equipment and other factors. Potential positive consequences may include reduced costs and earlier diagnosis (37). In digital pathology it is already stated that computerized analysis of specimen based on AI has the potential to reduce laborious tasks while minimizing interobserver variability and maximizing reproducibility (38). AI nowadays can also be used routinely for fecal screening for parasitic infections. Scanners automatically capture images from specimens and upload them into a cloud where the images are processed and analyzed for intestinal parasite eggs. The whole process is comparable or even quicker than the preparation time for conventional fecal flotation tests and led to agreeable results on comparison between the scanner system and parasitologists' examinations (39). Based on these developments, it seems realistic that computer-based analysis of vaginal smears in conjunction with reliable definitions of cell types are possible in the near future. Potentially, the usefulness of vaginal cytology may be improved and should be re-evaluated in the context of detection of gynecological disorders and ovulation timing. Further research is required to study if AI is helpful for the evaluation of vaginal smears. In addition, it needs to be tested if this will allow a more precise determination of the cycle stage. Whether the exact ovulation is predictable by an objective evaluation based on this tutorial has to be assessed in further studies.

CONCLUSION

Vaginal cytology is a useful tool for cycle staging and breeding management of female dogs because of its quick results and easy application. Nevertheless, the evaluator needs to follow standardized determination procedures to obtain objective and repeatable results. In that regard revised cell definitions and a tutorial were developed. In future steps we aim to develop methods for computer-based analysis of vaginal smears.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Dr. Mechthild Wiegard; Ethics Commissioner of the Freie Universität Berlin. Written informed consent for participation was not obtained from the owners because written informed consent was waived for this study, because all vaginal smears were taken during gynecological examination in the context of ovulation timing or routine gynecological health check by trained experts.

AUTHOR CONTRIBUTIONS

FR and SA contributed to conception and design of the project and wrote the manuscript. FR organized the database, the survey, and the flowchart. RK had a consultative role and digitized the vaginal smears. VB performed the statistical analysis. All authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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A Review on Canine and Feline Prostate Pathology

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Prostatic diseases are very common in male dogs, accounting for 3–10% of cases submitted to the veterinary practitioners. Commonly reported canine prostatic disorders include prostatic hyperplasia, prostatitis, prostatic cysts and prostatic carcinoma. However, clinical signs may be non-specific, or many cases are asymptomatic, thus leading to a difficult estimation of the actual prevalence of clinical cases. On the other side, because of the rare occurrence of prostate disease in cats, very little is known about pathogenesis, diagnostic approaches and treatment. The goal of this review is to provide detailed clinical and pathological overview of the feline and canine prostatic pathology, including the most up-to-date classification systems and histological findings. Emphasis is places on gross, cytological and histological features that are critical to reach a definitive diagnosis for a proper treatment and prognosis.

Keywords: dog, cat, prostate, pathology, review

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INTRODUCTION

Diseases of the prostate are common in older male dogs, while occasionally reported in cats. The main challenge from a clinical perspective is the overlap of clinical signs referring to dysfunctions of the urinary and/or intestinal tract that may be observed in the majority of the disease processes. Moreover, some cases may be asymptomatic and go unnoticed or different lesions can be present simultaneously. It has been estimated that 75.6% of dogs that die of disease unrelated to the prostate have however prostatic disorders at post-mortem examination (1).

This means that estimating the prevalence of these disorders is quite difficult. This is particularly obvious when examining prostatic lesions in cats, while in dogs it is well known that some disorders (e.g., prostatic hyperplasia) accounts for the majority of cases of prostatic diseases, followed by prostatitis, tumors and squamous metaplasia, although a combination of different disease processes is very common (e.g., prostatitis concurrent with prostatic hyperplasia).

This article provides a comprehensive overview of prostatic lesions in dogs and cats, covering common diseases affecting this accessory gland of the male reproductive tract, as well as updates and terminologies that have been proposed in the last decade. However, it should be taken into consideration that the information reported by the previous and current literature may not be a real representation of the prevalence of different prostatic disorders due to the paucity of available data, the different frequency of castration in the country of origin of the studies that may influence the incidence of hormone-induced prostatic atrophy and the perception of a higher incidence of prostatic tumors in castrated dogs and sensitivity of the diagnostic procedures of choice.

PROSTATE ANATOMY AND HISTOLOGY

The prostate is the only accessory sex gland of the canine male reproductive tract, while cats possess both prostate and bulbourethral gland. The canine prostate is an ovoid-shaped, bilobed organ that completely envelopes the proximal portion of the urethra close to the neck of the bladder (2). The feline prostate is more caudally placed than in dogs, located 2–3 cm from the urinary bladder, behind the cranial border of the pelvic symphysis under the ventral wall of the rectum (3). It covers the urethra only dorsally and laterally (4). The canine prostate is enveloped by a fibromuscular capsule that receives smooth muscle fibers from the wall of the urinary bladder. The gland has a dorso-medial sulcus and a median septum that divides the prostate into right and left lobe. Each lobe is further separated into lobules by capsular trabeculae. The vas deferens enters the cranio-dorsal surface of each prostate lobe, ending up in the urethra by the colliculus seminalis (2).

There is a marked development of the canine prostate after birth between 24 and 32 weeks of age reflecting the increase in number of Leydig cells in the testis after 28 weeks of age and increased plasma testosterone levels (5, 6). The prostate from sexually immature dogs consists of a branching ductular system radiating from the prostatic urethra admixed with not well-developed acini scattered in the external portion of the gland. These small glandular structures lack any lumen until 8 weeks of age. Most of the prostatic tissue is occupied by connective tissue stroma (7). Between 24 and 32 weeks of age, there is a progressive increase in number and size of the glandular alveoli, increased height of the glandular epithelium, formation of small epithelial projections within the lumen and intraluminal accumulation of proteinaceous material that marks the beginning of the secretory function of the prostatic epithelium (7) (**Figures 1A,B**). Sexual maturity in dogs varies according to the breed and even according to the bodyweight within the same breed (6, 8). Therefore, it is recommended to examine the testes together with the prostate to determine the age of sexual maturity. This may be extremely important for toxicologic studies since immature acini could lead to a misdiagnosis of treatment-related effect of glandular atrophy, while it may just be expression of the stage of sexual maturity.

About 63% of dogs develop progressive enlargement of the prostate with age after puberty (9). On the other side, following castration, the canine prostate undergoes an involution because of androgen deprivation with the serum testosterone concentration reaching very low baseline values already in the 1 week post-castration (10). A 70% reduction of the size of the organ is already obvious 7–14 days after castration and this is likely to occur also in cats (11, 12).

Material from the prostate for cytological evaluation may be obtained through the urethra (prostatic massage), ejaculation or direct fine needle aspirate (FNA) (13, 14) and the collection technique influences the morphology of the epithelial cells and the number of other cell types in the cytological samples. Normal prostatic epithelial cells obtained from aspiration from normal dogs usually occur in small to medium clusters, are uniform in shape and size, cuboidal to lowly columnar with small to moderate amount of occasionally vacuolated basophilic cytoplasm, round to oval central nuclei with a reticular chromatin pattern and small to inconspicuous nucleoli (**Figure 2**). Individual or small cluster of mostly round epithelial cells with the same nuclear and cytoplasmic features as described above are instead observed in cytological samples collected by prostatic massage. Other cell types that may be present are spermatozoa, squamous epithelial cells and urothelial cells (13, 14). The number of other cell types in cytologic samples depends on the collection technique: spermatozoa are most frequently found in ejaculated material, while squamous cells deriving from the distal urethra or the external genitalia and urothelial cells can be found in samples obtained by both prostatic massage and ejaculation (13, 14).

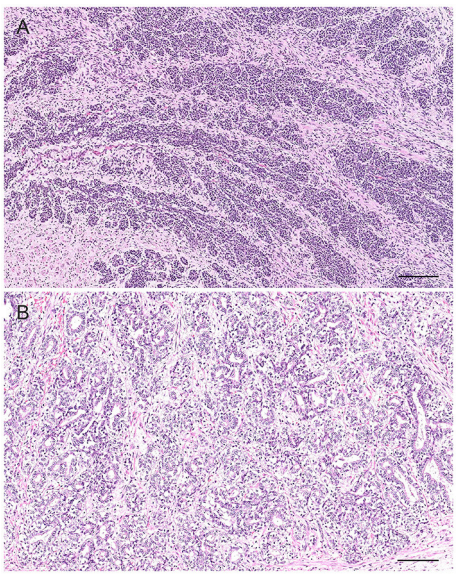

FIGURE 1 | Normal prostate gland from a sexually immature dog. Tissue section. **(A)** Prostate at 12 weeks of age with radiating branching ducts from the prostatic urethra (not visible; on the right-hand side). Hematoxylin-eosin staining. Bar = 400 μm. **(B)** Prostate at 24 weeks of age: irregular glands with a small lumen and moderate amount of stroma. Hematoxylin-eosin staining. Bar = 200 μm.

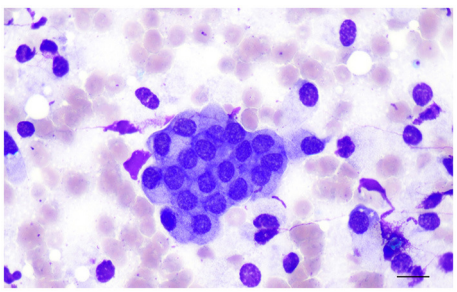

FIGURE 2 | Prostate aspirate showing normal prostatic epithelial cells in small cluster with centrally located nuclei, finely stippled or reticular chromatin pattern and finely granular cytoplasm. Prostate fine needle aspirate (FNA), smear. Wright-Giemsa stain. Bar = 12 μm.

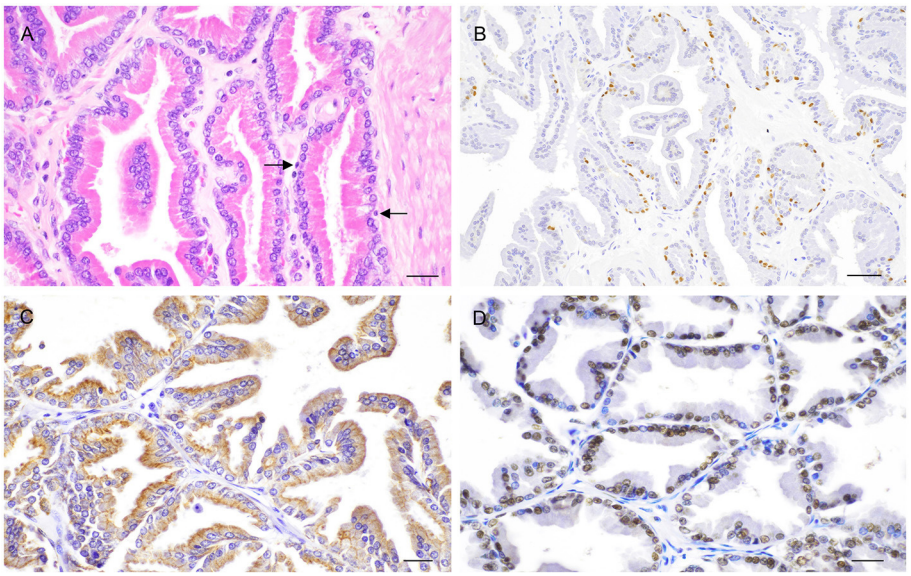

FIGURE 3 | Histological and immunohistochemical features of the normal canine prostate. **(A)** Tubuloalveolar gland lined by secretory columnar cells with low number of basal cells (arrows). Hematoxylin-eosin staining. Bar = 25 μm. **(B)** Nuclear expression of p63 by basal cells. IHC. DAB chromagen. Meyer's hematoxylin counterstain. Bar = 100 μm (mouse monoclonal anti-mouse p63, Dako, 1:150). **(C)** Cytoplasmic expression of CK8/18 by columnar secretory cells. IHC. DAB chromagen. Meyer's hematoxylin counterstain. Bar = 25 μm (mouse monoclonal anti-human Ck8/18, Novocastra, 1:600). **(D)** Nuclear positive staining for androgen receptor (AR) by columnar secretory cells. IHC. DAB chromagen. Meyer's hematoxylin counterstain. Bar = 25 μm (rabbit polyclonal anti-human AR, Santa Cruz Biotech., 1:1,000).

Histologically, the glandular portion of the prostate consists of tubuloalveolar glands producing secretions that are conveyed to the prostatic urethra through small periurethral ducts. Small ducts are also present in the periphery of the prostate, but they cannot be easily discerned without specific staining. The normal epithelium of the prostate consists of two cell layers: a luminal or secretory cell layer and a basal cell layer (**Figure 3A**). A third cell type in the normal human prostatic epithelium is the neuroendocrine (NE) cell (15), whose existence in cats and dogs is controversial (16, 17). In two of the most recent studies on identification of NE cells in dogs and cats, rare serotonin-positive cells have been described in normal and hyperplastic canine prostates with their number increasing after castration (18) and serotonin— and chromogranin A-positive cells in the prostate of sexually mature healthy cats (19). Basal cells are scarce, round to oblong, but occasionally flattened, with scant amount of dense eosinophilic cytoplasm and a small hyperchromatic nucleus. Small nucleoli may be occasionally seen. The immunophenotype of basal cells is distinctive and can be of diagnostic utility. Antibodies directed against high molecular weight cytokeratin (HMWK), cytokeratin 5 (CK5), and p63 react with basal cells (**Figure 3B**) while the same cells are mostly PSA (Prostate Specific Antigen)-, AR (Androgen receptor)-, and CK8/18-negative (20–22). Basal epithelial cells form a discontinuous layer in the canine prostate, while the lack of a continuous basal cell layer in humans is a strong indicator of prostatic carcinoma (23). The precise function of normal prostatic basal cells is unclear in dogs, although it is likely that the basal cell population harbors stem cells (24). The secretory or luminal cells make up the bulk of the epithelial volume. They are cuboidal to columnar with nuclei in the basal or midportion of the cell, an hyperosinophilic granular cytoplasm with occasional vacuolations, and small round nuclei with fine evenly dispersed chromatin. Nucleoli are not usually evident or are pinpoint in size. Secretory cells are PSA-, CK8/18-, and AR-positive (20–22) (**Figures 3C,D**). The columnar cells of the prostatic acini gradually change to a single lining of cuboidal epithelial cells within the ducts.

CANINE PROSTATE PATHOLOGY

Although large-scale epidemiologic studies on the prevalence of prostatic disorders are lacking, prostatic hyperplasia and acute or chronic prostatitis are definitely the most common lesions observed in dogs with an incidence of 46–55.3% and 28–38.5% respectively. These are followed by prostatic cysts (2.6–14%) and prostatic neoplasia (0.2–0.35%) (25–30).

Prostatic Hyperplasia

Prostatic hyperplasia (PH)—traditionally called benign prostatic hyperplasia (BPH)—is the most common prostatic disorder in intact dogs. Approximately 50% of dogs may have histologic changes of PH by 4–5 years of age and more than 90% by 8 years of age (31). Since the term hyperplasia is already defining a benign process, it is preferable to use PH instead of BPH, as recommended by the canine prostate cancer subgroup of the Oncology Pathology Working Group (OPWG), a joint initiative of the Veterinary Cancer Society and the American College of

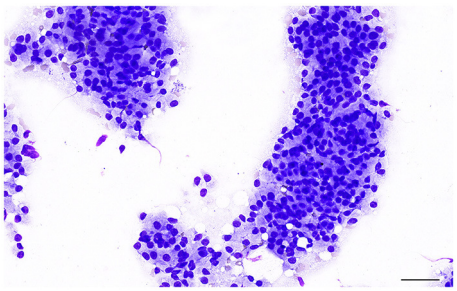

FIGURE 4 | Prostate aspirate showing a large cluster of uniform prostatic epithelial cells from a dog with prostatic hyperplasia. The cells have moderate amount of granular cytoplasm and round, central to eccentric nucleus with finely stippled to reticular chromatin. Note the pink granular material on the background, most likely indicating the secretory proteinaceous activity of the glandular epithelial cells. Prostate FNA, cytospin. Wright-Giemsa stain. Bar = 50 μm.

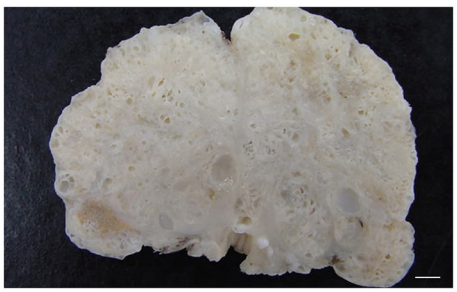

FIGURE 5 | Transverse cross-section of a canine prostate with evidences of prostatic hyperplasia. Enlarged prostate with multifocal small cystic lesions. Bar = 0.35 cm.

Veterinary Pathologists (32). The estrogen: testosterone ratio is increased in affected dogs. Estrogens enhance androgen receptors and acts synergistically with an overproduction of dihydrotestosterone (DHT) in potentiating the hyperplastic process (33). This explains why the administration of androgens in combination with estrogens to orchiectomized dogs induces prostatic hyperplasia (34).

Clinical signs are present only once the prostate is large enough and affected animals may be presented with urethral discharge, tenesmus, hematuria, fertility abnormalities and occasionally a stilted gait caused by prostatic pain (25, 26, 35). Cytologically, the hyperplastic epithelial cells are very similar to the normal prostatic epithelium, although the cellularity of the sample may be higher (13, 14) (**Figure 4**). Mild increase in cell size and anisokaryosis may be noted. Cells can exfoliate in large sheets in a honeycomb pattern (13, 14).

Grossly, PH-affected dogs have a uniformly enlarged prostate and frequently variable-sized cysts (**Figure 5**). PH is a spontaneous morphologic change associated with aging and sex hormone dysregulation and can be histologically classified into glandular and complex hyperplasia (31, 32, 36). A breed predisposition (Rhodesian ridgeback) has been reported by Beining et al. (37), although this finding should be further investigated and confirmed in a large-scale study. Glandular hyperplasia occurs in dogs as early as 2–3 years of age and consists of a uniform glandular enlargement of the prostatic alveoli, increased papillary infoldings and increased number of columnar secretory cells (7, 38) (**Figure 6A**). The hyperplastic epithelial cells have basal nuclei and prominent granules in the apical cytoplasm. It is important to emphasize that early hyperplastic changes are histologically indistinguishable from a normal prostate and the weight of the gland relative to the age, bodyweight and breed may help in most but not all cases. Complex hyperplasia occurs in dogs older than 6 years of age and is characterized by the presence of cystic lesions admixed with areas of glandular hyperplasia and atrophic glands, separated by an increased amount of stroma (collagen, smooth muscle) (36) (**Figure 6B**). Interestingly, DeKlerk et al. (38) induced canine PH in Beagle dogs by administering both 17β-estradiol and either 5α-androstane-3α, 17β-diol or dihydrotestosterone. Young dogs developed glandular hyperplasia, while cystic/complex hyperplasia was never observed in this age group (38). Therefore, aging represents an important contributing factor for the development of cystic hyperplasia. In some cases of PH, chronic inflammation with infiltration of mononuclear cells may be present (5, 38) (**Figure 6B**).

Prostatitis

Prostatitis is a common histologic finding in prostates of intact dogs. Reports in castrated dogs are exceptionally rare and usually associated with a history of recent castration before presentation. This inflammatory process may be subclinical or clinically relevant, especially in the acute stage with the infiltration of large number of neutrophils within the interstitium and in the lumen of the glandular acini (32, 39) (**Figure 7A**). These acute inflammatory forms are usually caused by *Escherichia coli* or *Proteus vulgaris* ascending from the urethra. Other common isolates are *Staphylococcus spp.*, *Streptococcus spp., Pseudomonas spp., Klebsiella spp., Enterobacter spp., Pasteurella, Haemophilus* (11, 33, 40). Other inflammatory cells can also be identified in histological sections such as macrophages, lymphocytes, and plasma cells either in the prostatic acini or stroma (32, 39) (**Figure 7B**). According to the severity of the infection, additional histologic findings may be interstitial edema, hemorrhage and/or necrosis. Cytological samples from cases of bacterial prostatitis contains high number of neutrophils (**Figure 7C**), mostly with degenerative changes, exfoliated hyperplastic prostatic epithelial cells, intracellular and extracellular bacteria (in the absence of previous antibiotic therapy) and occasional macrophages and lymphocytes, especially if the infection is becoming chronic (13, 14). Acute prostatitis is usually associated with fever, anorexia, depression, straining to urinate or defecate, caudal abdominal pain, hematuria, pain on rectal palpation and sporadically edema of the scrotum, prepuce and hindlimb (41).

Prostatitis may be also observed with *Brucella canis* infection (chronic interstitial prostatitis) (42), dissemination of fungal organisms through a systemic infection (*Blastomyces dermatitis, Cryptococcus neoformans, Coccidioides immitis*) causing a granulomatous prostatitis (40, 43, 44) and rarely *Mycoplasma*

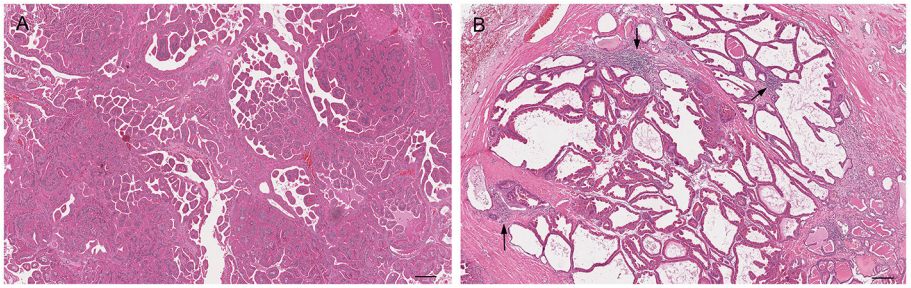

FIGURE 6 | Histological subtypes of prostatic hyperplasia in dogs: **(A)** Glandular hyperplasia: large alveoli with numerous intraluminal papillary projections admixed with a reduced amount of stroma. Hematoxylin-eosin staining. Bar = 100 μm. **(B)** Complex hyperplasia: multifocal lesion with dilated and cystic alveoli. Note the randomly scattered aggregates of inflammatory cells (arrows). Hematoxylin-eosin staining. Bar = 200 μm.

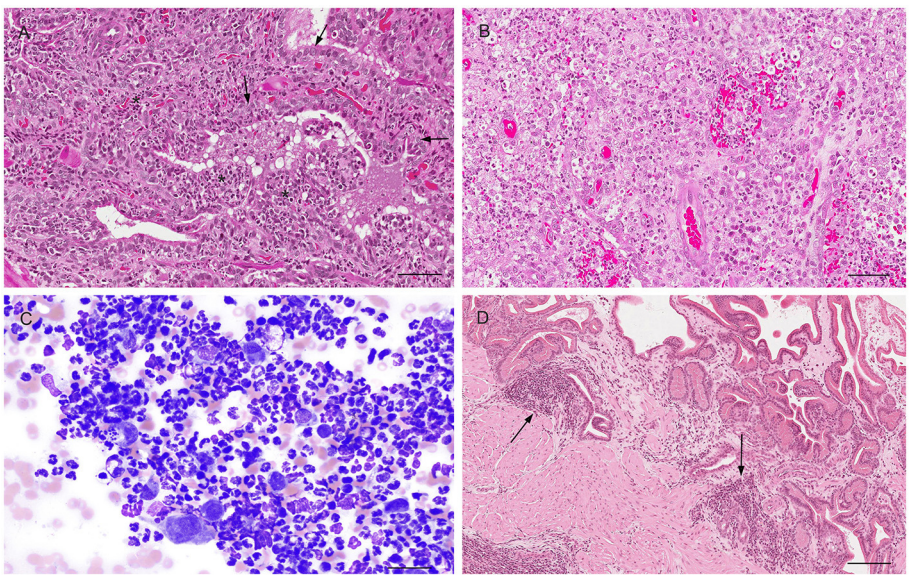

FIGURE 7 | Histological **(A,B,D)** and cytological **(C)** features of three different types of prostatitis in dogs **(A)**. Acute prostatitis with infiltration of high number of neutrophils (asterisks) and occasional reactive changes in the glandular epithelial cells (large nuclei, prominent nucleoli) (arrows). Hematoxylin-eosin staining. Bar = 60 μm. **(B)** Infiltration of high number of neutrophils and macrophages with severe destruction of the gland (pyogranulomatous prostatitis). Hematoxylin-eosin staining. Bar = 60 μm. **(C)** Prostate aspirate showing cytological features of acute prostatitis with high number of neutrophils with minimal nuclear degeneration and rare prostatic epithelial cells. Prostate FNA, cytospin. Wright-Giemsa stain. Bar = 30 μm. **(D)** Mild multifocal chronic lymphoplasmacytic prostatitis (arrows). Hematoxylin-eosin staining. Bar = 120 μm.

canis and *Leishmania* spp. (45, 46). On the other hand, chronic prostatitis might occur in dogs without any clinical signs. When present, clinical signs of chronic prostatitis are non-specific, with affected dogs experiencing recurrent urinary tract infections, poor semen quality, infertility, decreased libido, intermittent urethral discharge (41). Histopathology reveals focal or multifocal infiltration of mononuclear cells in the prostatic stroma and variable degree of atrophy of the prostatic secretory cells and fibrosis (32) (**Figure 7D**).

Prostatic Abscess

Prostatic abscess is usually considered a sequela of chronic prostatitis (26) or can occur in association with acute bacterial prostatitis and cystic hyperplasia (40). Rarely, prostatic abscess may be secondary to bacteremia (26, 28). The diagnosis is usually made by history, clinical examination, ultrasonography of the prostate, laboratory findings and bacterial culture of the urine and/or prostatic fluid (40). Clinical signs may be variable depending on the size of the lesion and whether the infection becomes systemic. They include tenesmus and dysuria caused by the progressive enlargement of the prostate or urethral discharge or systemic symptoms caused by endotoxemia (33). Histologically, prostatic abscesses are characterized by a severe neutrophil infiltration, with gland destruction and necrosis.

Prostatic Cystic Conditions

Prostatic and paraprostatic cysts are grouped under the same paragraph as conditions characterized by the presence of

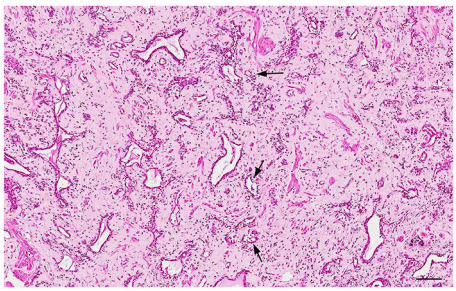

FIGURE 8 | Histology of the prostate from a castrated dog: hormonal atrophy with relative increase of the interstitial stroma and tubules lined by a single epithelial layer. Hematoxylin-eosin staining. Bar = 120 μm.

cystic lesions, although they have different pathogenesis and distribution. Clinical signs are uncommon, unless the size of the cyst becomes large enough to cause dysuria, tenesmus and hematuria (33). Cytologically, they may be acellular or containing few normal epithelial cells, rare non-degenerative neutrophils, macrophages, small lymphocytes and/or erythrocytes and cellular debris (13, 14). Prostatic cysts are traditionally considered a further evolution of a cystic PH with grossly detectable cysts within the prostatic parenchyma and a classical "honeycomb" appearance (26) (**Figure 5**). Histologically, prostatic cysts are large acini with or without intraluminal proteinaceous material lined by atrophic glandular cells. Intraprostatic cysts may also occur in association to squamous metaplasia and prostatitis or when ducts are obstructed leading to accumulation of prostatic fluid, hence the definition of prostatic retention cysts. Some paraprostatic cysts arise from a cystic uterus masculinus, a remnant of the paramesonephric duct, and are commonly located in the craniolateral or dorsal aspect of the prostate (47). Grossly, they are large nodular structures, usually palpable through the pelvic cavity (they can reach up to 30 cm in diameter), enclosed within a fibrocollagenous capsule that might undergo ossification and mineralization (26, 48). Mineralization of paraprostatic cyst is reported to be uncommon (49–51), although Renfrew et al. (52) have observed mineralized cysts in 3 out of 6 affected dogs, concluding that this process is actually more common than implied in the literature.

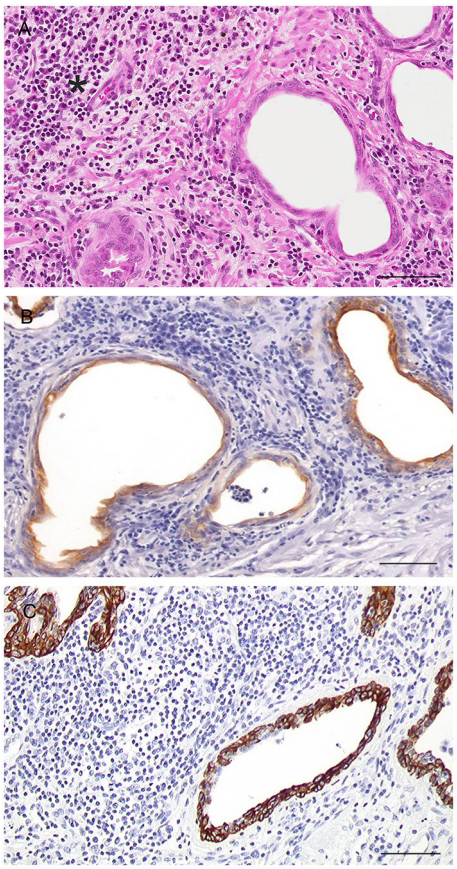

FIGURE 9 | Histological and immunohistochemical features of glandular atrophy associated with chronic inflammation (PIA-like lesions). **(A)** Gland with attenuated epithelium and surrounding chronic inflammatory infiltrate (asterisk). Hematoxylin-eosin staining. Bar = 120 μm. **(B)** Cytoplasmic expression of CK8/18 by epithelial cells of the PIA-like lesions. IHC. DAB chromagen. Meyer's hematoxylin counterstain. Bar = 120 μm (mouse monoclonal anti-human Ck8/18, Novocastra, 1:600). **(C)** Cytoplasmic expression of CK5 by epithelial cells of the PIA-like lesions. IHC. DA chromagen. Meyer's hematoxylin counterstain. Bar = 120 μm (mouse monoclonal anti-human CK5, Novocastra, 1:300).

Prostatic Atrophy

Prostatic atrophy in dogs can be classified into atrophy secondary to neutering (hormonal atrophy) and atrophy associated with chronic inflammation (32). The hormonal atrophy has no clinical significance and may also be secondary to the administration of antiandrogenic drugs (32) and GnRH agonists and antagonists (53). Castration induces a progressive shrinkage of the prostatic acini with relative increase of the fibromuscular tissue until only low number of tubules with a single epithelial lining remains in the advanced stage (54) (**Figure 8**). On the other hand, prostatic atrophy can occur in intact or castrated dogs in association with a focal, multifocal or diffuse lymphoplasmacytic inflammatory infiltrate. At low magnification, these atrophic glands show a hyperchromatic appearance, surrounded by the inflammatory infiltrate. At higher magnifications, the atrophic acini containing at least two layers: (1) basal cell layer and (2) secretory atrophic layer (**Figure 9A**). Both cell populations have reduced cytoplasm and hyperchromatic nuclei. Although these cells are morphologically atrophic, they are also proliferative ("paradox" atrophy) and this specific lesion is defined as Proliferative Inflammatory Atrophy (PIA) in humans. PIA is considered a precursor of Prostatic Intraepithelial Neoplasia (PIN) and prostate cancer (55). Although there are still no studies demonstrating a strict correlation between PIA and progression to cancer in dogs, PIA-like lesions have been described in dogs, both in normal and neoplastic prostates (29, 56–58). The cell population of these lesions in dogs show an intermediate phenotype, expressing both luminal and basal cell markers (59) (**Figures 9B,C**).

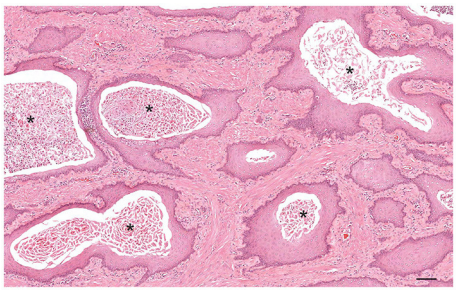

FIGURE 10 | Histology of squamous metaplasia in a dog with Sertoli cell tumor. The glandular alveoli are lined by a pluristratified squamous epithelium with intraluminal accumulation of keratin squames and sloughed epithelial cells (asterisks). Hematoxylin-eosin staining. Bar = 200 μm.

Squamous Metaplasia

When the prostate undergoes squamous metaplasia, the columnar glandular epithelium becomes stratified squamous, with keratin squames shed into the lumen together with sloughed epithelial cells (**Figure 10**). The metaplastic epithelium may be observed in the glandular acini only or even in the ducts. Inflammatory cells (degenerated neutrophils and/or macrophages) may be also present in the lumen, especially if the metaplastic lesion is secondary to chronic prostatitis. In the latter case, squamous metaplasia is a very subtle change and usually a focal finding.

Cytological samples contain aggregates of large, angular and flattened, well-differentiated squamous epithelial cells with abundant pale to light blue cytoplasm and a central, pyknotic or karyorrhectic nucleus. Squamous cells may be admixed with inflammatory cells, bacteria or hyperplastic prostatic cells according to the underlying disease process (13, 14).

This lesion may occur spontaneously in association with the estrogen-producing Sertoli cell tumor (60) or following the administration of estrogens (61). Clinical signs usually related to hyperestrogenism. Short-term exposure to estrogens causes metaplastic changes in the periurethral duct tissue, while changes in the entire gland are secondary to long-term administration of estrogens (62). Interestingly, metaplastic prostates return to the normal morphology once the estrogen stimulation is removed (63, 64). It is likely that estrogens induce proliferation of the basal cells with squamous differentiation rather than androgen-driven differentiation into secretory cells (54). Squamous metaplasia of the canine prostate is not considered a pre-neoplastic change, but it can lead to the formation of cysts and/or abscesses.

Prostatic Tumors

Prostatic tumors are rare in dogs, with variable incidence according to the studies. The lack of specific markers for prostatic cancer in dogs as well as effective diagnostic screening tests makes early diagnosis difficult and therefore the issue of underestimation of cases in the canine population is real.

Prostatic carcinoma is considered androgen-independent and metastasize rapidly in ∼70–80% of cases. Reported sites of metastases are regional lymph nodes, pelvic musculature, vertebral bodies, lung, liver, urethra and urinary bladder, colon and rectum, spleen, heart, kidney, distant lymph nodes and adrenal glands (36). Although there is a common belief that there is an increased risk of carcinoma in castrated dogs (65, 66), carcinoma of the prostate occurs with the same prevalence in sexually intact and neutered dogs (67–71).

Many dogs do not show any clinical signs until late in the course of the disease. When present, the most common clinical signs—excluding those related to potential metastases—are prostatomegaly, painful abdominal palpation, stranguria, dysuria, hematuria, constipation, tenesmus, anorexia, weight loss, pain and paresis of hind limb (26, 28).

Grossly, the prostate may be asymmetrically and irregularly enlarged (**Figure 11A**), with or without invasion of the surrounding organs. In other cases, the prostate present little changes and only a slight enlargement may be detected.

Canine prostatic carcinoma is classified into prostatic adenocarcinoma (AC) and prostatic urothelial carcinoma (UC), although a mixed phenotype can also occur (32). In classical cases, cytological samples consist of variably sized clusters, sheets or scattered single cells with marked criteria of malignancy, including single to multiple nuclei, coarse to clumped chromatin, multiple and prominent nucleoli, severe anisocytosis and anisokaryosis, nuclear molding (**Figure 11B**). Mitoses are common and may be bizarre (13, 14). Prostatic adenocarcinoma and prostatic urothelial carcinoma may be difficult to distinguish cytologically. Some acinar structures may be observed in AC, while urothelial cells show tailed shapes and sporadic cytoplasmic vacuoles with bright-pink material (**Figure 11C**), although these features may not be consistently present in all cases and are not pathognomonic for UC (13, 14).

Prostatic adenocarcinoma (AC) originates from the glandular epithelium and displays a high degree of morphological heterogeneity in terms of growth patterns and histological subtypes, occurring individually or in combination. The simple tubular pattern is characterized by the formation of small acini and tubules (**Figure 12A**). The solid pattern is composed of solid sheets, cords or isolate pleomorphic epithelial cells, that can occasionally be spindle shaped (**Figure 12B**). In the papillary subtypes, cuboidal to columnar cells neoplastic cells form papillary projections with a delicate fibrovascular core within an extended duct (**Figure 12C**). Cribriform prostatic adenocarcinoma shows irregular fenestrated proliferation of the tumor cells filling the lumen of the gland (**Figure 12D**), usually with central necrotic debris (comedonecrosis) (**Figure 12E**) (29, 32). Other less common patterns are micropapillary and sarcomatoid (29, 72).

In all the subtypes, the mitotic index is moderate to high, except for the tubular pattern that usually exhibits a low mitotic rate. It is not unusual to find additional histologic features, such as lymphatic invasion, squamous differentiation, perineurial invasion (**Figure 12F**) (29, 32).

Prostatic urothelial carcinoma (UC) arises from the urothelial cells of the prostatic urethra or the periurethral ducts. Neoplastic cells are extremely heterogeneous, ranging from small polyhedral to large cells, with low to abundant eosinophilic cytoplasm, hyperchromatic or large vesicular nucleus and high

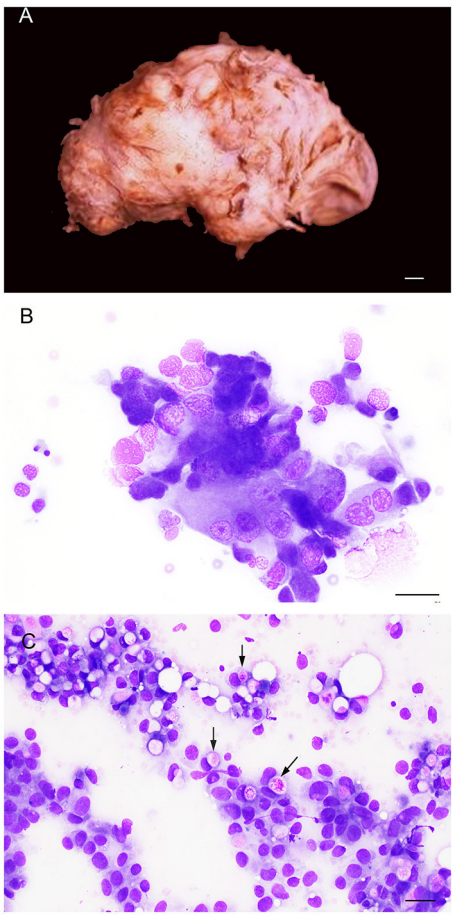

FIGURE 11 | Gross **(A)** and cytological **(B,C)** features of prostatic carcinoma in dogs. **(A)** The prostate is irregularly enlarged, showing a multinodular gross aspect (entire prostate removed from the connections with the urinary bladder and penile urethra). Bar = 1 cm. **(B)** Prostate aspirate showing a small group of neoplastic cells with cytological features of malignancy (pleomorphism, large nuclei, multiple prominent nucleoli). Prostate FNA, smear. Wright-Giemsa stain. Bar = 25 μm. **(C)** Prostate aspirate showing neoplastic epithelial cells from a urothelial carcinoma of the prostate with intracytoplasmic vacuoles containing bright pink material (arrows). Prostate FNA, smear. Wright-Giemsa stain. Bar = 20 μm.

mitotic index. Melamed-Wolinska bodies—intracytoplasmic eosinophilic bodies—can be an important morphologic feature that helps in the diagnosis of urothelial cell tumors (32).

Distinction between urothelial and prostatic origin can be difficult in most cases, except with the tubular histotype that is more aligned with a diagnosis of prostatic adenocarcinoma. The location of the tumor, close to the prostatic urethra, favors a urothelial origin. Neoplastic cells with cytoplasmic vacuoles (signet cells) and absence of tubules and acini would be more supportive of a UC, although signet cells can also be present in areas of urothelial metaplasia within a prostatic adenocarcinoma. Immunohistochemistry (IHC) can be performed if needed (uroplakin III, CK7), although a definitive marker to differentiate urothelial from glandular origin of the tumor is still lacking. It is common practice to rule in a urothelial carcinoma when IHC reveals immunoreactivity of the neoplastic cells to UPIII and CK7 (73). However, cytokeratin 7, previously thought to be expressed by urothelial cells of the prostatic urethra and periurethral prostatic ducts only (74), is actually found even in the glandular epithelium, so that positive staining can be similarly observed in AC and UC (75). Uroplakin III is more sensitive and specific for urothelial tumors in dogs, although it may be negative in 10% of cases, the staining is not uniform within the same tumor and positive regions may be missed and it is consistently negative in undifferentiated tumors (73). As an additional complicating factor, some tumors show a combination of prostatic adenocarcinoma and urothelial carcinoma (73, 75).

Prostatic squamous cell carcinoma shows closely packed non-keratinizing epithelial cells with moderate to large eosinophilic cytoplasm and occasional prominent intracellular bridges and keratin pearls. Primary prostatic squamous cell carcinoma should be differentiated from urinary bladder squamous carcinoma invading the prostate or prostatic urothelial carcinoma with squamous differentiation.

Mesenchymal prostatic tumors, such as fibrosarcoma, leiomyosarcoma, hemangiosarcoma can occur, with features similar to other organs (76–78). Lymphoma has also been described in the canine prostatic gland, mainly in the disseminated disease (79, 80).

In humans, prostate carcinoma may evolve from a well-defined and characterized lesion, called prostatic intraepithelial neoplasia (PIN), that defines a proliferation of atypical epithelial cells within preexisting ducts and acini (15). High-grade PIN (HGPIN) lesions have been described in the prostate of dogs with and without prostate cancer (80–83). Typical HGPIN consists of glands with nuclear crowding and stratification, luminal cells with enlarged nuclei, large and prominent nucleoli and size and shape variation (84). A critical review of the literature describing these lesions in the canine prostate (32) have evidenced the possibility that these lesions may be or (a) expression of a retrograde invasion of the normal glands by adjacent neoplastic glands; or (b) reactive lesions secondary to an inflammatory reaction in the surrounding stroma or (c) atypical/dysplastic changes. Therefore, a certain degree of caution should be applied when reporting these lesions in a diagnostic setting.

Prostatic Calculi, Corpora Amylacea and Polyglucosan Bodies

In the human prostate, prostatic calculi are usually associated with inflammation, prostatic hyperplasia or prostatic cancer (15), while this correlation has not been demonstrated in dogs yet. Prostatic calculi can be infrequently seen in the canine prostate as an incidental finding (85, 86). They can originate from the bladder through the prostatic urethra and then the prostatic duct system (exogenous calculi) or form within the prostate itself, usually within a cyst (endogenous calculi). Exogenous calculi contain constituents of the urine, while endogenous calculi can be formed by the precipitation of element present in the prostatic secretions (87).

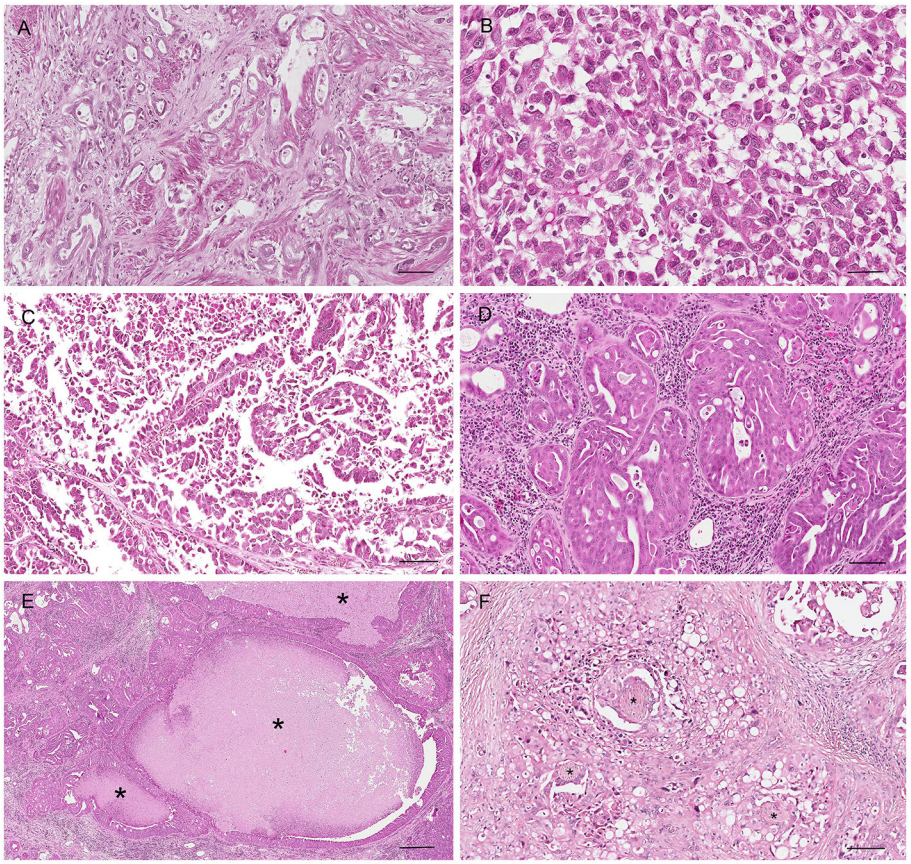

FIGURE 12 | Histological subtypes of canine prostatic carcinoma. **(A)** Tubular carcinoma: small tubules lined by neoplastic epithelial cells. Hematoxylin-eosin staining. Bar = 120 μm. **(B)** Solid carcinoma: pleomorphic neoplastic cells arranged in sold nests without a glandular structure. Hematoxylin-eosin staining. Bar = 40 μm. **(C)** Papillary carcinoma: neoplastic cells forming papillary projection within the lumen. Hematoxylin-eosin staining. Bar = 120 μm. **(D)** Cribriform carcinoma: neoplastic cells filling the lumen with the formation of regular fenestrae. Bar = 120 μm. **(E)** Cribriform carcinoma with central area of necrosis (comedonecrosis) (asterisks). Hematoxylin-eosin staining. Bar = 500 μm. **(F)** Perineurial invasion: neoplastic cells encircle and proliferate around nerves (asterisk). Hematoxylin-eosin staining. Bar = 120 μm.

Corpora amylacea are eosinophilic inspissated secretions that usually assume characteristic concentrical lamellations (15). They are extremely common in the normal human prostate gland, while they are rarely seen in the dog prostate. They can calcify and contributes to the formation of prostatic endogenous calculi. PAS-positive polyglucosan bodies have been described in the smooth muscle cells of the canine prostatic stroma by Kamiya et al. (88), most commonly in aged animals.

FELINE PROSTATE PATHOLOGY

Prostatic Tumors

Prostatic disorders in cats are exceedingly rare, and, despite the lack of studies on the prevalence of prostatic lesions in cats, prostatic carcinoma is the most commonly reported lesion in case descriptions available in the literature (89–95). Affected cats are 6–11 years old and are usually presented with hematuria, dysuria, stranguria, acute urinary obstruction, urinary incontinence, occasionally constipation, inappetence, weight loss and lethargy. Prostatic carcinoma has been described both in intact (91, 94, 95) and neutered (89–93, 96) animals without a specific breed predisposition (domestic shorthair, mixed breed, domestic longhair, Siberian) (97). Definitive risk factors have not been identified due to the sparse number of reports and lack of epidemiologic data. Prostatic carcinoma in cats has not been classified in any histologic subtypes, although most cases are characterized by high degree of cellular pleomorphism, occasional scirrhous reaction, acinar or solid or tubular growth pattern, high mitotic rate and lymphovascular invasion. One case of sarcomatoid carcinoma with neoplastic epithelial cells arranged in acini and tubules surrounded by haphazardly arranged pleomorphic spindle cells has been described by Zambelli et al. (93).

Prostatitis

Bacterial prostatitis is also less common in cats than in dogs, presumably due to the uncommon occurrence of other

predisposing factors, such as primary prostatic hyperplasia and squamous metaplasia. In addition, most of all the cases of bacterial prostatitis begin secondary to the invasion of pathogens in the urinary tract (98), but bacterial urinary tract infections occur much less frequently in cats than in dogs, with only 1–2% of cats affected during their lifetime (99). Prostatitis in cats may be acute or chronic (100, 101), with predominant neutrophilic or pyogranulomatous inflammation. Occasional abscesses may be observed at gross examination in animals with fever, anorexia, and constipation (102). The most common isolate in cats is *Escherichia coli* (100, 102).

Squamous Metaplasia

Squamous metaplasia of the prostate with secondary prostatitis has been described in only one cat with interstitial cell neoplasm in a retained testis (103). The histologic features of this lesion are similar to those observed in dogs with prostatic glandular acini lined by multiple layers of squamous epithelial cells.

Paraprostatic Cyst

Only a single case of paraprostatic cyst has been reported in a 3-year-old neutered male domestic, short-haired cat with a history of pollakiuria. The cyst was lined with transitional epithelium multifocally continuous with the epithelium of the prostatic ducts (104).

CONCLUSIONS

This overview covers all the different disorders affecting the canine and feline prostate providing comprehensive clinical and pathological aspects that may inform pathologists and practitioners in their diagnostic investigation and examination of clinical cases, recognizing the difficulties in making a final diagnosis due to non-specific clinical signs and overlap of different diseases. On the other side, as highlighted by the amount of information included in this review, prostatic diseases are not as infrequent as believed and comparative studies may actually reveal new pathological lesions or pathogenesis that will add another level of complexity to these disorders in domestic animals.

AUTHOR CONTRIBUTIONS

CP, CF-A, and RL-A conceived and designed this review, performed literature review and data collection, wrote the manuscript, and reviewed the drafts. CP supervised the project. All authors have read and approved the final submitted manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.881232/full#supplementary-material

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Effect of *Pinus taeda* Hydrolyzed Lignin on Biochemical Profile, Oxidative Status, and Semen Quality of Healthy Dogs

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Sub-fertility represents a frequent challenge in canine reproduction. The use of micronutrients and/or additives was investigated as an approach to improve sperm quality, which are the main constraints on reproduction in canine species. Although some information is available about the effect of daily supplementation with substances presenting antioxidant/antioxidative activity on semen quality, this study aimed to observe the effect of a polyphenolic mix of substances derived from hydroxylation of *Pinus taeda* lignin (PTHL). For the trial, 40 male dogs were involved, 20 received PTHL for 90 days and 20 were left untreated, serving as a control group. Every 30 days, blood and semen samples were collected and analyzed. The biochemical profile of both groups was not affected by treatment and time ($p > 0.05$). Differently, dogs that received PTHL showed higher blood superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activity ($p < 0.01$). Moreover, the dietary addition of PTHL can significantly increase the semen volume, concentration, and spermatozoa motility ($p < 0.01$) in healthy dogs. PTHL supplementation represents a good way to enhance the semen quality of dogs and improve the antioxidant status of animals.

Keywords: antioxidants, semen quality, oxidative status, dogs, polyphenols

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INTRODUCTION

Several clinical studies suggested that, among nutritional factors, fish-derived n-3 polyunsaturated fatty acids (PUFAs) may exert a positive effect on sperm motility and fertility. Since several PUFAs are essential in many animals, their requirements must be covered by dietary intake (1, 2). Other microelements, such as selenium, copper, and zinc, act directly or indirectly on sperm metabolism (3, 4). Many researchers have shown that nutritional deficiencies can lead to reduced sperm quality through defective spermatogenesis or by generating intense oxidative stress (5). Oxidative damage can cause sperm dysfunction, such as loss of motility and vitality (6, 7). Therefore, antioxidants may play a key role by protecting male stem cells from oxidative damage (8) and by preventing loss of motility and impaired sperm-oocyte fusion capacity (9). However, since the development of canine artificial insemination with frozen semen, various known substances with antioxidant activity, such as vitamins E and C, glutathione, and butylated hydroxytoluene, have been added to the cryopreservation extenders (10–14). Nevertheless, few studies have been conducted on the potential effects of some dietary antioxidant substances on dogs' semen quality. Several vegetable matrices

are rich in bioactive compounds, such as phenolic compounds or tannins with high antioxidant activity, and this makes them potentially suitable for animal feeding (15). Several studies have highlighted the benefits of consuming extracts and foods rich in these compounds (16). Although the mechanisms are unclear, polyphenols and tannins improve the antioxidant status of cells and tissues in humans (17), rats (18), mice (19), livestock (20, 21), and pets (22, 23). One of these natural substances is Pinus taeda hydrolyzed lignin (PTHL), derived from P. taeda, commonly known as loblolly pine, a very common species of tree in Northern America. Lignin is often an agricultural by-product, and is very difficult to be recycled, although it is organic matter. The possibility of a circular economy model where lignin is converted into high added value products also represents an interesting focus from an environmental sustainability perspective. The PTHL is a polyphenol mixture derived from these trees' lignin that proved to have a positive effect on animal welfare (24). The present study aims to evaluate the effect of PTHL oral administration on dogs' plasma oxidative status, biochemical profile, and semen characteristics.

MATERIALS AND METHODS

The protocol for animal research was approved by the Ethics Committee for animal testing–CESA (656/18 – III/13) of the Department of Veterinary Medicine of the University of Bari "Aldo Moro," Bari, Italy.

Animals

The study was performed during the spring of 2021 at the Obstetric, Gynecological, and Andrological Clinic of the Veterinary Hospital of the Veterinary Medicine Department of the "Aldo Moro" University of Bari (Italy). Dogs' experimental management is reported in **Figure 1**. The dogs were clinically examined to ensure their health status 30 days before the commencement of the trial. Each dog has submitted for a clinical examination and blood analyses (hematocrit and total protein) (25, 26). An ultrasound exam of the reproductive tract was also performed, and it was verified that all dogs reacted positively to sperm collection by digital manipulation. Only normospermic male pet dogs kept in the house were included. In the end, forty mixed-breed dogs were included in the present study (**Supplementary Table S1** reported all animals characteristics). Dogs were healthy with a mean age of 3.8 years, a mean body weight of about 27 kg, and a mean body condition score (BCS) of 3. All the dogs started to be fed with the same commercial feed [10 g/kg of body weight (BW) daily, composition reported in **Table 1**] two times a day to ensure a 30-days period of adaptation to the new commercial feed before starting the trial.

Experimental Protocol

The dogs were randomly assigned to one of two equal-sized groups ($n = 20$) using www.randomizer.org (27) (**Figure 1**).

One group was the experimental one (PTHL) and the other was the control group (CON). The PTHL group received the supplement containing PTHL (Oxilem®, I-Green, Padua,

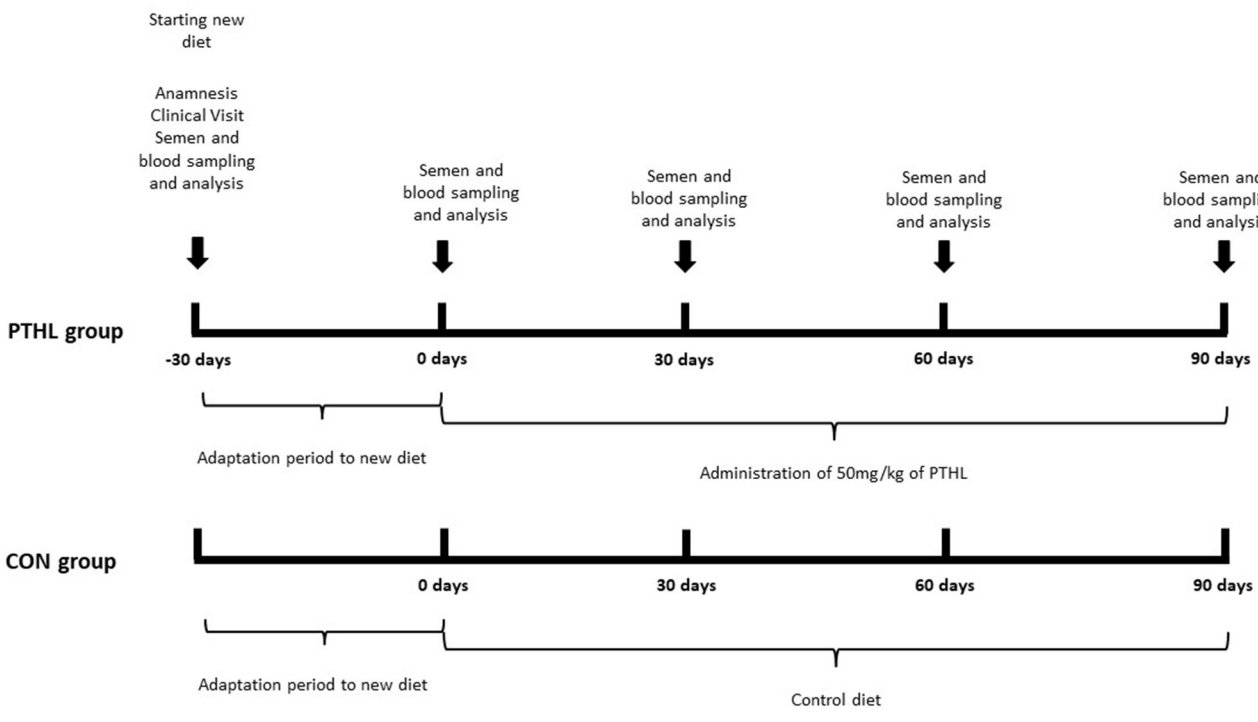

FIGURE 1 | Experimental design. PTHL, Pinus taeda hydrolyzed lignin; CON, Control.

TABLE 1 | Composition of diet commercial feed.

Item	On 100 g of product
Moisture	10 g
Raw protein	28 g
Fat	15 g
Raw fibers	3 g
Ashes	9 g
Vitamin A	1,500 IU
Vitamin D	100 IU
Vitamin E acetate (alpha-tocopherol 91%)	12.5 mg
Vitamin B2	2.6 mg
Vitamin B6 (pyridoxine hydrocloride)	0.5 mg
Vitamin B1 (thiamine mononitrate)	0.6 mg
Choline chloride	75 mg
Iodine (anhydrous calcium iodate)	0.075 mg
D-panthotenic acid	1 mg
Vitamin H (Biotin D)	0.05 mg
Calcium	0.5 g
Vitamin K3 (menadione)	0.125 mg
Vitamin PP (nicotine acid)	2.5 mg
Vitamin B12	0.0035 mg
Folic acid	0.1 mg
Cobalt (basic cobalt carbonate)	0.015 mg
Iron (ferrous carbonate)	2 mg
Manganese (manganous oxide)	4 mg
Copper (Copper sulfate, pentahydrate)	1 mg
Selenium (sodium selenite)	0.01 mg
Zinc (zinc oxide)	3 mg

TABLE 2 | Phenolic composition and antioxidant activity of *Pinus Taeda* hydrolyzed lignin (PTHL)[a].

Item	
Determined composition (g/100 g)	
Vanillin	26.4
Eriodictyol	3.4
Quercetin	2.7
Isorhamnetin	1.6
Rosmarinic acid	1.4
Quercetin ramnoside	13.9
Methylgallate retunoside	42.3
Epigallocatechin-3-methylgallate	1.5
Ferulic acid derivates	6.7
Antioxidant activity (μmol TE[b] g^{-1} DW[c])	
Trolox equivalent antioxidant capacity	23.9
Oxygen radical absorbance capacity	122.4

[a] *According to Gerardi et al. (28) and Blando et al. (29).*
[b] *Trolox equivalents.*
[c] *Dry weight.*
$^{-1}$ *is the measure unit for those parameters calculated on g elevated at* $^{-1}$.

Italy). **Table 2** reports the chemical composition and antioxidant activity of PTHL (24). The PTHL group received 500 mg/kg of PTHL each day during the 90 days of the trial. The dose was orally administered in powder according to the manufacturer's instructions, based on empirical trials (IGreen, Padua, Italy). The supplement was orally administered. During the trial, semen and blood samples collection were performed before starting the trials (day 0) and 30, 60, and 90 days.

Blood Samples and Analysis

Blood was aseptically collected *via* cephalic vein puncture using disposable needles (22G), with a negative pressure 4 ml tube system for serum (without anticoagulant) and plasma (with 15 USP U/ml of heparin) (Becton, Dickinson Canada Inc, Vacutainer 1, Oakville, Canada). Tubes for plasma were immediately centrifuged (1,500 × g for 10 min) while tubes for serum were allowed to clot at a refrigerated temperature for 10 min prior to being centrifuged (1,500 × g for 10 min). All plasma and serum aliquots were stored at −80°C until analyses. Clinical biochemistry parameters were obtained from the serum samples using an automated biochemistry analyzer (CS-300B; Dirui, Changchun, China) as described by De Palo et al. (30). The following parameters were analyzed: alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine phosphokinase (CPK), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), glucose (Glu), blood urea nitrogen (BUN), creatinine (Crea), total serum protein (TP), albumin (Alb), cholesterol (Chol), triglycerides (Trig), non-esterified fatty acids (NEFA), calcium (Ca), phosphate (P), magnesium (Mg), chloride (Cl), (Gesan Production Kit, Campobello di Mazara, Trapani, Italy). Besides, globulins (Glob) and albumin/globulin ratio (Alb/Glob) were calculated starting from total protein and albumin parameters. Standard assay kits were used to calibrate the multi-parameter analyzer (Seracal, Gesan Production Kit, Campobello di Mazara, Trapani, Italy) before each analytical session. After setting the calibration curve, two multi-parameter control sera, a normal and a pathological one (Seracontrol N and Seracontrol P, Gesan Production Kit, Campobello di Mazara, Trapani, Italy), were used to verify internal accuracy, considered satisfactory when the measured value deviated by no more than 3.00% from the manufacturer's declared values. Each sample was analyzed in triplicate, and the value used in the raw dataset was the arithmetic mean of the three recordings for each item. Plasma samples were used for oxidation parameters and antioxidant enzyme activities assays. Thiobarbituric acid reactive substances (TBARS) were determined spectrophotometrically as described by Maggiolino et al. (31), by adding 100 ml of plasma to a 3.7 μl/ml thiobarbituric acid solution. Plasma reactive carbonyl derivative (RCD) levels were determined according to Faure and Lafond (32) using the carbonyl reagent DNPH. Plasma (200 ml) was mixed with 1 ml of water and 2 ml of 200 μl/ml trichloroacetic acid and centrifuged at 1,000 × g for 10 min. The pellet was resuspended in 1 ml of 10 mmol/L DNPH and incubated for 60 min at 37.8°C. For control, 1 ml of 1 mol/L hydrochloric acid was used instead of DNPH. Subsequently, 1 ml of 200 μl/ml trichloroacetic acid was added, and the sample was centrifuged at 1,000 × g for 10 min. The pellet was washed with 1:1 ethanol-ethyl acetate solution and centrifuged at 1,000 × g

for 10 min. The pellet was mixed with 1 ml of 6 mol/L guanidine (diluted in 20 mmol/L dihydrogenphosphate at pH 2.3). Finally, the sample was incubated for 40 min at 37.8°C. The absorbance was measured at 380 nm.

Hydroperoxides (Hy) were determined spectrophotometrically by an iodometric method as described by Maggiolino et al. (33). Aliquots (90 ml) of plasma were put into eight microcentrifuge vials (1.5 ml). Then, 10 μl of 10 mM TPP in methanol were added to four of the vials to reduce ROOHs, thereby generating a quadruplicate of blanks. Methanol (10 ml) was added to the remaining four vials to produce a quadruplicate of test samples. All the vials were then vortexed and incubated at room temperature for 30 min prior to the addition of 900 ml of FOX2 reagent. After mixing, the samples were incubated at room temperature for 30 min. The vials were centrifuged at 2,400 × g for 10 min with a swing-out rotor (Hettich Rotenta/RP centrifuge, Hettich-Zentrifugen, Tuttlingen, Germany). The absorbance of the supernatant was measured at 560 nm using an Ultraspec 2000 spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). The ROOH concentration in the plasma samples was calculated using the mean absorbance difference between quadruplicates of test samples and blank samples.

Protein carbonyls (PC) levels were determined spectrophotometrically as reported by Salzano et al. (21). The superoxide dismutase (SOD) (EC 1.15.1.1) activity was examined according to Misra (34), and the enzymatic activity was based on the 50% inhibition rate of epinephrine auto-oxidation at 480 nm (35). The SOD activity was assessed as the 50% inhibition rate of epinephrine auto-oxidation at 480 nm. The epinephrine autoxidation stimulation by traces of heavy metals present as contaminants in the reagents was prevented by adding 10^{-4} *M* EDTA in the buffer to chelate those ions. The SOD activity was expresses as U/ml.

The catalase (CAT) (EC 1.11.1.6) activity was assayed by the method of Clairborne (36) as described by Tateo et al. (37). The amount of enzyme required to degrade 1 μmol of H_2O_2 in 60 s was defined as 1 unit of enzyme activity by following the decrease in absorbance of H_2O_2 at 240 nm ($e = 40\ M^{-1}\ cm^{-1}$). Its activity was expressed as U/mg of protein.

The glutathione peroxidase (GPx) (EC1.11.1.9.) activity was measured according to Gunzler (38) as described by Dinardo et al. (39). The analysis was based on the measure of the rate of reduced glutathione oxidation by tertbutyl hydroperoxide, catalyzed by GPx. The constant concentration of reduced glutathione was ensured by the addition of exogenous glutathione reductase and NADPH, which converted the oxidized glutathione to reduced glutathione. The rate of oxidized glutathione formation was then measured by the change in the absorbance of NADPH at 340 nm. Its activity was expressed as nmol of NADPH oxidized/min per ml.

Semen Collection and Computer Assisted Sperm Analysis

Dog's semen was collected into an artificial vagina by manual stimulation, while the dogs sniffed swabs of bitches in estrous (using natural estral pheromones). The ejaculate collection was performed using 3 different Falcon tubes, one for each part of the semen: urethral, spermatic, and prostatic (40, 41). The ejaculation analysis was performed as described by Alonge et al. (42). The second seminal part was analyzed by the Computer Assisted Sperm Analyzer (43) (CASA, IVOS-Sperm CASA system, Version 12.3, Hamilton Thorne, MA, USA). The CASA software (IVOS 12.3 version) was set up for canine semen-speficic parameters as reported in **Table 3**. According to the manufacturer's instructions, for each analysis, a 3 μl drop from each sperm sample was diluted 5 times in Tris-Fructose extender and put on a Leja slide 4 chambers of 20 μm (Leja Products B.V. Nieuw Vennep, The Netherlands). The Leja slide was positioned in the dedicated chamber of the microscope, allowing it to settle for a few seconds before analysis. The computerized analyzer scanned five random non-consecutive microscopic fields. The parameters evaluated were: ejaculate volume, concentration, total motility, and percentage of motile spermatozoa (progressive motility), velocity average pathway (VAP), straight-line velocity (VLS), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), beat-cross frequency (BCF), straightness (STR), linearity (LIN), and total number of counted cells (TSC). VAP was elaborated by the software as the average velocity of smoothed cell path, expressed in μm/s. Then, the overall sperm population was divided into 4 groups based on the velocity, according to low VAP cut-off (LVV) and medium VAP cut-off (MVV). Thus, sperms were classified as follows: rapid spermatozoa, with VAP > MVV; medium spermatozoa, with LVV < VAP < MVV; slow spermatozoa, with VAP < LVV; and static spermatozoa, represented by the fraction of those cells not moving during the analysis (44).

TABLE 3 | The IVOS version 12.3 software settings for dog semen parameters.

Parameters	Cut-off value
Frames per second (Fps)	30
Frequency	60 Hz
Temperature of analysis	37°C
Minimum contrast	75
Minimum cell size	4 pixels
Progressive cell cut-off	100 μm/s; 75% STR
Low VAP cut-off	9 μm/s
Low VSL cut-off	20 μm/s

Statistical Analysis

The dataset was tested for normal distribution and variance homogeneity (Shapiro–Wilk test). Afterward, all data were subjected to analysis of variance (ANOVA) using the general linear model (GLM) procedure as reported the following model:

$$Y_{ijkl} = \mu + \alpha_i + O_j + T_k + (N \times T)_{jk} + \varepsilon_{ijkl}$$

where Y_{ijkl} represents the dependent variables, μ is the overall mean; α_i is the ith dog random effect ($i = 1,\ldots 40$), O_j is the effect of the jth oral administration treatment ($j = 1, 2$), T_k is the effect

TABLE 4 | Total protein, albumin, urea, uric acid, creatinine, bilirubin, triglyceride, glycemia, alanine amino transferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP), and cholesterol serum concentration in dogs treated with dietary supplementation of PTHL and untreated dogs (CON) for 90 days.

Parameter	Group	Time (days)				SEM[a]	*p*-value			Reference value[c]
		0	30	60	90		Group	Time	G × T[b]	
Total protein (g/dL)	CON	6.67	6.55	7.00	6.72	0.325	0.049	0.904	0.591	5.4–7.1
	PTHL	6.38	6.10	6.12	6.16					
Albumin (g/dL)	CON	2.98	2.95	3.05	2.95	0.105	0.671	0.748	0.985	2.6–3.3
	PTHL	3.02	2.94	2.98	2.90					
Urea (mmol/L)	CON	59.26	58.95	53.70	60.10	9.890	0.459	0.290	0.919	21–60
	PTHL	52.26	51.02	55.02	57.44					
Uric acid (mg/dL)	CON	0.81	0.92	1.20	1.15	0.190	0.036	0.202	0.534	0.0–2.0
	PTHL	0.62	0.58	0.64	0.86					
Creatinine (mg/dL)	CON	1.42	1.50	1.42	1.42	0.100	0.047	0.473	0.529	0.5–1.5
	PTHL	1.32	1.28	1.34	1.32					
Bilirubine (mg/dL)	CON	0.26	0.40	0.47	0.42	0.135	0.016	0.135	0.426	0.1–0.5
	PTHL	0.22	0.18	0.24	0.38					
Triglyceride (mg/dL)	CON	36.98	32.40	32.57	31.30	9.105	0.337	0.179	0.542	30.1–38.1
	PTHL	38.57	38.62	35.60	37.26					
Glycemia (mg/dL)	CON	86.96	61.20	81.60	71.70	6.100	0.008	0.174	0.163	65–118
	PTHL	89.43	86.74	87.04	88.70					
ALT[d] (IU/L)	CON	31.82	38.50	53.30	36.75	5.750	0.335	0.154	0.230	21–102
	PTHL	34.65	43.20	34.96	34.64					
AST[e] (IU/L)	CON	24.78	27.47	27.15	28.30	2.215	0.442	0.511	0.241	0.0–66.0
	PTHL	21.74	23.86	22.00	23.76					
ALP[f] (IU/L)	CON	22.50	19.05	23.40	16.52	5.115	0.178	0.932	0.649	20–156
	PTHL	23.39	23.78	26.04	28.86					
Cholesterol (mg/dL)	CON	172.46	182.90	206.67	201.40	18.650	0.089	0.374	0.747	135–270
	PTHL	163.06	153.42	168.78	177.32					

[a] *Standard error of the means.*
[b] *Group × Time.*
[c] *Kaneko et al. (45).*
[d] *Alanine aminotransferase.*
[e] *Aspartate aminotransferase.*
[f] *Alkaline phosphatase.*

of the kth time (1,…4), $(O \times T)_{jk}$ is the binary interaction effect of jkth (1,…8) treatment × time and ε_{ijk} is the error term. A Tukey test for repeated measures with respect to time was applied to evaluate the differences among means when the effect of time or the binary interaction of treatment × time was significant. The significance level was set at $p < 0.05$, and the results were expressed as means and mean standard error (SE). Data analysis was performed using SAS software (46).

RESULTS

Tables 4, **5** show serum biochemical-clinical and electrolyte profile results between the two experimental groups (PTHL vs. CON) during the trial. No differences were observed according to time, PTHL inclusion, and their binary interaction ($p > 0.05$).

Results for oxidation metabolites and antioxidant enzymes are reported in **Table 6**. Metabolites derived from lipid (TBARS and Hy) and protein (protein carbonyls) oxidation did not vary over time and between groups ($p > 0.05$). The ferric reducing ability of plasma (FRAP) showed higher values in PTHL groups after 90 days of trial ($p < 0.01$). Observing enzyme activity in the PTHL group, SOD activity constantly increased during the trial ($p < 0.01$), the CAT and GPx activity increased in the first 30 days ($p < 0.01$) and then remained constant. These enzymes did not show any difference in the CON group ($p > 0.05$) during the trial. Moreover, from 30 days to the end of the trial, all these enzymes showed higher values in the PTHL group compared with the CON one ($p < 0.01$).

Table 7 shows semen volume, concentration, VAP, VLS, VCL, ALH, BCF, STR, and LIN results. Not all of them showed variation during the trial in the CON group ($p > 0.05$). Semen volume ($p < 0.05$) and concentration ($p < 0.01$) increased in PTHL dogs during the trial, with higher values in PTHL animals compared with the CON group ($p < 0.01$). The VSL, BCF, LIN ($p < 0.01$), VCL, ALH, and STR ($p < 0.05$) values increased after 90 days of PTHL administration. Only VSL and

TABLE 5 | Chlorine, sodium, potassium, magnesium, phosphorus, and calcium serum concentration in dogs treated with dietary supplementation of PTHL and untreated dogs (CON) for 90 days.

Parameter	Group	Time (days)				SEM[a]	*p*-value			Reference value[c]
		0	30	60	90		Group	Time	G × T[b]	
Chlorine (mEq/L)	CON	112.82	112.62	114.40	110.17	2.850	0.502	0.334	0.653	105.0–115.0
	PTHL	115.14	112.52	113.30	114.90					
Sodium (mmol/L)	CON	150.15	155.22	151.42	147.95	3.035	0.421	0.935	0.199	141.0–152.0
	PTHL	149.68	145.38	151.16	151.04					
Potassium (mg/dL)	CON	4.46	4.44	4.49	4.49	0.135	0.041	0.622	0.659	4.4–5.3
	PTHL	4.19	4.16	4.20	4.22					
Magnesium (mg/dL)	CON	2.08	1.90	2.25	2.25	0.435	0.640	0.065	0.945	1.8–2.4
	PTHL	2.22	1.98	2.20	2.40					
Phosphorus (mg/dL)	CON	4.24	4.70	5.05	3.72	0.425	0.471	0.297	0.447	2.6–6.2
	PTHL	3.96	4.20	4.20	4.20					
Calcium (mg/dL)	CON	10.45	10.40	10.67	10.12	0.340	0.246	0.791	0.791	9–12
	PTHL	10.26	9.88	10.14	10.04					

[a] *Standard error of the means.*
[b] *Group × Time.*
[c] *Kaneko et al. (45).*

TABLE 6 | Thiobarbituric acid reactive substances (TBARs), hydroperoxides (Hy), protein carbonyls, ferric reducing antioxidant power (FRAP), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) serum concentration in dog treated with dietary supplementation of PTHL and untreated dog (CON) for 90 days.

Parameter	Group	Time (days)				SEM[1]	*p*-value		
		0	30	60	90		Group	Time	G × T[2]
TBARs (mmol/mL)	CON	1.14	1.11	1.14	1.02	0.105	0.223	0.042	0.622
	PTHL	1.39	1.37	1.16	1.13				
Hydroperoxides (mmol/mL)	CON	5.18	5.27	5.75	4.53	0.495	0.554	0.307	0.836
	PTHL	4.93	5.33	5.76	5.31				
Protein carbonyls (μmol/mg Protein)	CON	98.95	91.26	92.06	93.47	2.630	0.050	0.192	0.667
	PTHL	96.04	96.76	96.04	99.00				
FRAP (μmol TE/mL)	CON	48.40	46.96	46.77	44.55^{X}	0.380	<0.0001	<0.0001	<0.0001
	PTHL	49.44	48.05	49.81	51.10^{Y}				
SOD (U/mL)	CON	48.41^{A}	49.84^{X}	54.52^{X}	54.07^{X}	1.140	0.522	<0.0001	<0.0001
	PTHL	20.39^{A}	60.05^{BY}	72.40^{CY}	91.91^{DY}				
CAT (U/mL)	CON	2.88^{A}	3.21^{X}	3.33^{X}	3.28^{X}	0.125	0.320	<0.0001	<0.0001
	PTHL	2.12^{A}	4.30^{BY}	4.24^{BY}	4.34^{BY}				
GSPx (nmol NADPH ox/mL)	CON	2.43^{A}	2.43^{X}	2.52^{X}	2.63^{X}	0.035	0.106	<0.0001	<0.0001
	PTHL	2.27^{A}	2.91^{BY}	2.96^{BY}	3.27^{BY}				

[1] *Standard error of the means.*
[2] *Group × Time.*
Different letters on the same line show statistical differences during time in the same group: A, B, C, D = $p < 0.01$; Different letters on the same row show statistical differences between groups at the same aging time: X, Y = $p < 0.01$.

LIN showed higher values compared with CON dogs after 60 days ($p < 0.01$).

Spermatozoa motility results are reported in **Table 8**. Rapid movements increased after 30 days in PTHL dogs ($p < 0.05$), but no differences between groups were observed ($p > 0.05$). Slow and static movements decreased ($p < 0.01$) after 30 days of PTHL assumption, while no variations were observed in the CON group ($p > 0.05$), with lower values in PTHL dogs compared with CON ones ($p < 0.01$).

TABLE 7 | Volume, concentration, velocity average pathway (VAP), straight line velocity (VLS), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), beat-cross frequency (BCF), straightness (STR), and linearity (LIN) of semen in dog treated with dietary supplementation of PTHL and untreated dog (CON) for 90 days.

Parameter	Group	Time (days)				SEM[1]	*p*-value		
		0	30	60	90		Group	Time	G x T[2]
Volume (mL)	CON	4.86	3.65^{X}	3.60^{X}	4.25^{X}	1.770	0.005	0.045	0.284
	PTHL	4.78^{a}	6.80^{bY}	6.18^{bY}	7.06^{bY}				
Concentration (M/mL)	CON	122.86	109.55	120.32^{x}	121.47^{x}	28.805	0.023	0.001	0.163
	PTHL	121.96^{A}	157.56^{A}	191.14^{ABy}	239.44^{By}				
VAP (μm/s)	CON	103.20	106.72	105.67	106.62	9.140	0.023	0.203	0.336
	PTHL	102.96	114.88	125.66	136.70				
VSL (μm/s)	CON	78.60	81.72	83.55^{X}	84.87^{X}	5.755	0.001	0.012	0.143
	PTHL	80.40^{A}	93.04^{AB}	103.52^{ABY}	110.14^{BY}				
VCL (μm/s)	CON	141.98	143.32	145.62	152.67	9.825	0.009	0.055	0.353
	PTHL	143.00^{a}	160.24^{ab}	171.98^{ab}	186.22^{b}				
ALH (μ)	CON	5.24	5.87	5.67	5.80	0.380	0.018	0.016	0.301
	PTHL	5.57^{a}	6.22^{ab}	6.68^{ab}	7.10^{b}				
BCF (Hz)	CON	20.50	22.32	23.65	23.95	1.290	0.044	0.000	0.207
	PTHL	21.05^{A}	23.40^{ABa}	24.92^{ABb}	29.20^{B}				
STR (VSL/VAP)	CON	79.80	32.50	80.75	83.00	5.780	0.011	0.036	0.296
	PTHL	80.78^{a}	85.20^{ab}	88.20^{ab}	90.00^{b}				
LIN (VSL/VCL)	CON	53.80	54.00	56.00^{X}	56.00^{X}	1.755	<0.0001	<0.0001	0.020
	PTHL	54.44^{A}	57.60^{ABa}	65.40^{ABbY}	65.80^{BY}				

[1]*Standard error of the means.*

[2]*Group × Time.*

Different letters on the same line show statistical differences during time in the same group: A, B = $p < 0.01$; a, b = $p < 0.05$; Different letters on the same row show statistical differences between groups at the same aging time: X, Y = $p < 0.01$; x, y = $p < 0.05$.

TABLE 8 | Total motility, progressive motility, rapid, medium, slow, and static movements of spermatozoa in dog treated with dietary supplementation of PTHL and untreated dog (CON) for 90 days.

Parameter	Group	Time (days)				SEM[1]	*p*-value		
		0	30	60	90		Group	Time	G x T[2]
Total motility (%)	CON	73.80	75.02	77.62	76.07	3.750	0.015	0.112	0.306
	PTHL	76.21	81.40	92.80	96.00				
Progressive motility (%)	CON	54.80	58.07	60.67	59.85	4.365	0.007	0.012	0.200
	PTHL	57.44	64.00	71.20	78.20				
Rapid (%)	CON	58.60	60.00	63.00	62.00	2.245	0.002	0.050	0.242
	PTHL	59.89^{a}	72.80^{ab}	79.20^{ab}	82.00^{b}				
Medium (%)	CON	10.00	14.00	12.00	13.00	2.445	0.209	0.462	0.786
	PTHL	12.20	13.00	8.40	8.80				
Slow (%)	CON	18.20	11.50	11.75^{X}	13.00^{X}	2.775	0.005	0.002	0.377
	PTHL	16.58^{A}	7.80^{AB}	6.80^{ABY}	3.40^{BY}				
Static (%)	CON	13.20	12.75^{X}	13.25^{X}	11.75^{X}	2.565	0.009	0.037	0.378
	PTHL	12.04^{A}	6.40^{BY}	6.40^{BY}	5.80^{BY}				

[1]*Standard error of the means.*

[2]*Group × Time.*

Different letters on the same line show statistical differences during time in the same group: A, B = $p < 0.01$; a, b = $p < 0.05$; different letters on the same row show statistical differences between groups at the same aging time: X, Y = $p < 0.01$; x, y = $p < 0.05$.

DISCUSSION

Blood Metabolites and Antioxidant Status

The overall data obtained from the serum biochemical and electrolyte profiles in dogs of both experimental and control groups showed ranges almost comparable with those considered physiological for this species (47, 48). The diet inclusion of PTHL in dogs did not affect the dogs health, considering that all blood constituents alteration makes it possible to hypothesize and potentially diagnose organs functionality alterations (49). For example, ALT activity is strongly related to liver function, AST to muscle and liver cell function (50), and ALP has a widespread tissue distribution, with a lot of isoenzymes isolated from other different tissues, such as liver, bone, intestinal mucosa, kidney, and leucocytes, but all of them showed values within ranges reported in the literature (51), demonstrating that treatment did not cause any tissue damages. Animals' antioxidant defense can be raised *in vivo* (enzymatic) or can derive from the diet (non-enzymatic) (52). For this, TBARs, Hy, and protein carbonyls are considered biomarkers of cell damage: their increase can be generated by oxidative stress and a boost of oxygen reactive substances (ROS). Additionally, FRAP values allow an overall evaluation of plasma antioxidant capacity (53, 54). It is well known that the dietary assumption of high amounts of substances with antioxidant activity results in the transfer of these molecules to different animal tissues, usually followed by a significant increase in their total antioxidant capacity (55), and that several polyphenolic molecules in plants possess antioxidant activities (23). Research is raising interest in these compounds due to their beneficial effects on health as anticarcinogenic (56), anti-inflammatory (57), immune and microbial modulating (58, 59), especially if taken through the diet. Several studies confirm a positive correlation between total antioxidant activity and the total phenol content assumed with diet, for example, spinach and broccoli (60, 61). However, the pharmacokinetics and antioxidant activity of polyphenols are still unclear in dogs (48, 62). Generalized stress induces a systemic increase in oxidation products.

The patterns that do not foresee the activation of the endogenous antioxidant systems, and specifically the catabolites of lipid and protein oxidation (TBARS, hydroperoxides, and protein carbonyls) measured in the plasma, did not show any difference between the two groups of animals during the entire experimental test. However, this does not exclude the hypothesis that the inclusion of PTHL in the diet of dogs with different stressful conditions (work, running, specific pathologies, etc.) may induce a variation in the production of these metabolites. On the other hand, the maximum antioxidant capacity measured by laboratory techniques is different, i.e., the animals that have taken PTHL show greater enzymatic activity and higher levels of FRAP. These results may indicate a potential positive effect of PTHL dietary supplementation against cellular oxidative stress and we can hypothesize that animal under different conditions, i.e., living stress challenges, could show greater differences on these parameters after PTHL assumption. In fact, the enzymatic activity evaluated through the used techniques highlights a capacity to react to an *in vitro* insult and therefore potential greater antioxidant response. These results agree with what observed by other authors studying the inclusion of polyphenols in the diet (62). On the other hand, increased enzymes activity represented a positive result for dog health considering that these enzymes serve as an endogenous defense against oxidative stress phenomena by determining free radical neutralization (63).

Semen Quality and Motility

Infertility is a common problem in the reproduction of dogs. For this reason, several authors suggested different food supplements to improve the quality of the ejaculate (64). According to recent studies (5, 42), the dietary approach by balanced food integration is an effective method for the improvement of fertility in dogs. Present results showed enhancing effects of a 2-month natural antioxidant diet supplementation on canine spermatozoa, as already observed in dogs' sperm quality (42), as well as in other species (65) by other authors. Specifically, the integration of PTHL can significantly increase the semen volume and spermatozoa motility in healthy normospermic dogs. In fact, after only 30 days, the PTHL group generally showed considerable improvements from the feed integration. Furthermore, in the PTHL group, the progressive motility and the percentage of rapid-movement sperms significantly increased, while the percentage of slow and static sperms significantly decreased. Indeed, thanks to PTHL feed supplementation, the sperm quality was significantly improved in all dogs in the treated group. Although the first evidence is evaluable after 30 days of supplementation, the best results have been reached at 60 days of administration, which approximately corresponds to the physiological length of the total duration of spermatogenesis in dogs (61.9 ± 0.14 days; (66)). The improvements in sperm motility depend probably on the antioxidant power of polyphenols contained in PTHL, which is able to improve sperm quality at the beginning of the sperm differentiation and development. It is well known that oxidative stress is one of the major issues associated with sperm function (67, 68) and sperm motility is one of the factors limiting male fertility (69). Although all dogs enrolled were healthy without problems of fertility, the PTHL group showed higher total sperm count, concentration, and progressive motility, compared with the CON group. Moreover, the integration of antioxidants, compared with the normal diet, is associated with a higher number and better motility of spermatozoa (70). In fact, spermatozoa are sensitive to oxidative stress because they are well endowed with polyunsaturated fatty acids, which are vulnerable to free radical attack at the alpha methylene carbons adjacent to the carbon–carbon double bonds (6). In addition, the spermatozoa's capacity for antioxidative defense is relatively low/vulnerable in comparison to other cells/tissues that are susceptible (71). Furthermore, spermatozoa are attacked by the oxidative effect of leukocytes, such as neutrophils, which are present in the male genitals for infection and other causes (67). The decrease in concentration, motility, and function of canine spermatozoa in dogs can be attributed to poor food intake, reduced absorption, increased losses, or augmented demand

for microelements. Recent literature in farm animals show that natural polyphenols are used to reduce oxidative stress because of their antioxidant properties (15, 21, 72, 73). The results of our study are biologically plausible. The production of reactive oxygen species (ROS) causes loss of motility and a decreased capacity for sperm–oocyte fusion, inducing harmful chemical and structural changes to sperm DNA, proteins, and lipids of plasma and mitochondrial membranes (9). ROS induces changes in the sperm membrane altering its fluidity, resulting in loss of motility, and impaired events, such as acrosome reaction (74). Several studies have shown a significant reduction in oxidative stress or DNA damage after treatment with antioxidants (8). Indeed, the literature shows that antioxidants may play a primary role in protecting male germ cells against oxidative action (75). Our results encourage considering the alimentary approach of balanced food supplementation for the solution of dog subfertility. Balanced feed supplementation and integration could mitigate the negative impact of infertility in canine breeding.

CONCLUSIONS

Food supplementation with PTHL can be considered an economic and natural method within an innovative multimodal approach to improve reproductive performances in canine breeding. The benefit of the supplemented diet with PTHL in enhancing semen quality and better supporting the antioxidant status of animals is an important goal for optimizing the male characteristics of reproductive efficiency. Some of the findings allow for speculation on the possibility of planning effect in subfertile and/or pathological male dogs. Therefore, the present article should set the base knowledge for further studies in male dogs with different physiological and pathological conditions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Comitato Etico per la Sperimentazione Animale del Dipartimento di Medicina Veterinaria (CESA-DiMeV). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

GA: investigation, formal analysis, writing—original draft, and writing—review and editing. VC: investigation and formal analysis. AM: conceptualization, investigation, data curation, formal analysis, writing—original draft, and writing—review and editing. MB and AB: investigation. AT: writing—review and editing. PD: conceptualization, investigation, funding acquisition, resources, project administration, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Parameters in Canines After Cesarean Sections

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This study evaluated fertility in canines after cesarean section and compared it with natural parturition. Parameters, such as the time of the next heat after the first parturition or cesarean section, the heat which was used for another breeding attempt, whether it was successful, the number of puppies that were born, and the necessity of another cesarean section were examined. The study relied on questioning patient owners at a University clinic. A Google online form was also used. Information for 261 dogs from different breed groups was included, of which 119 bitches were in the cesarean section group, and 142 were in the natural parturition group. In total, 93 ± 2.7% [LSMeans ± standard error (SE)] and 91.12 ± 3% (LSMeans ± SE) of the bitches became pregnant after cesarean section and natural parturition at the first breeding attempt. There was no significant effect on the breed group or whether the bitch had undergone a cesarean section before ($p = 0.8$ and $p = 0.63$). Bitches, which underwent a cesarean section, were more likely to have further cesarean sections performed ($p < 0.001$). However, neither the breed groups ($p = 0.17$), whether the bitch had undergone a cesarean section ($p = 0.59$), nor the number of previous parities had any effect on the number of puppies born ($p = 0.95$). The breed group bulldogs had a high proportion of cesarean sections. Only 42.11% of the bulldogs had a natural parturition as the first included parturition and only 31% gave birth naturally thereafter.

Keywords: parturition, cesarean section, canine, dogs, fertility

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INTRODUCTION

With the increasing popularity of certain breeds, the incidence of cesarean sections is rising. Unlike other surgeries, cesarean delivery may be both scheduled and predictable intervention (1–4), as well as an emergency surgery (5). Dystocia occurs in ∼5–16% of all bitches during parturition (6), of which ∼60–80% result in a cesarean section (7, 8). Especially brachycephalic breeds are known to have a high risk of cesarean sections and breeds, such as the Scottish Terrier (6, 9, 10).

With the rising incidence of cesarean sections and the increasing desire for planned cesarean sections (11, 12), the fertility and the question if one can expect a natural delivery after a cesarean section are important aspects. Although data from human medicine show varying results (13–16), little is known about the fertility after cesarean sections in dogs (17). In human medicine, the fertility rates show results as no reduction in fertility up to 17% (13–15). In their retrospective study, Conze et al. (17) determined a fertility rate of 100% in 55 bitches, which became pregnant in the 2 years following a cesarean section. No information was included about the breeds, the need

for another cesarean section in subsequent pregnancies, and a possible reduction in the number of puppies that were born. Seyrek-Intas et al. (18) performed a unilateral cornuectomy of the uterus of 18 healthy bitches. Twelve bitches were mated during the first postoperative estrus. Of these, nine delivered puppies. This indicates high fertility, but in the study by Conze et al. (17), only a small population of bitches was examined, which limits the strength of their conclusions. These numbers have to be compared to normal pregnancy rates, which are between 78 and 97% (19–21).

Only a few studies have looked into the fertility parameters of certain breeds (22, 23). Cafaratti et al. (22) examined clinical signs during estrous and mating in the Dogo Argentino and the risk of dystocia and cesarean section. Linde Forsberg and Persson (23) performed a survey on boxers to investigate parameters, such as the estrus interval and the occurrence of dystocia. There is, however, a definite lack in the literature examining and comparing fertility parameters after cesarean section in different breed groups.

MATERIALS AND METHODS

Gathering of Information

Two methods were used for gathering information. Firstly, breeders who were clients of the Clinic for Obstetrics, Gynecology and Andrology of Large and Small Animals with Ambulatory Service, Faculty of Veterinary Medicine were interviewed. They were asked if they would voluntarily provide information about their breeding bitches during registration. No exclusions were made regarding breeds. Bitches having experienced at least two parturitions were included. In the cesarean section group, the first cesarean section to be included had to be an emergency cesarean section. Breeders were interviewed using a standardized questionnaire. A Google form with the same questions was used as a second data collection method. It was sent to clients, who then forwarded it to other breeders. The link to the questionnaire was also accessible *via* the Verein Deutscher Hundezucht (VDH) website and was distributed *via* social media. The same inclusion and exclusion criteria were applied to the Google form. The data were collected between 2017 and 2019.

Parameters

Breed, age, weight, and the physiological inter-estrous interval, which was defined as the time between the non-pregnant bitch's heats, were collected for cesarean section (G1) and natural parturition group (G2). Furthermore, in the cesarean section group following parameters were included (G1): the indication for the cesarean section was assessed. The number of puppies born by cesarean section and the time of the first heat after cesarean section were included. The interval between cesarean section and the following breeding, counted as heat numbers, as well as the outcome of the breeding, was examined. The following parameters were collected in the natural parturition group (G2): questions regarding the number of puppies born in the first included parturition and the time of the first heat after parturition were asked. The interval between parturition and the following breeding, counted as heat numbers, was included, as well as the outcome of the breeding.

If a bitch from G1 or G2 became pregnant again, the breeders were asked whether the bitch was able to give birth naturally and how many puppies were born. If the bitch had undergone a cesarean section, the breeders were asked to state the indication.

Statistical Analysis

The statistical software package SAS 9.4 (24) was used for statistical analysis. Mixed linear models were applied to normally distributed data using the MIXED procedure. The parameters for this analysis were as follows: the time of first heat after cesarean section, difference of puppies born between G1 and G2, and the number of the first heat used for breeding after a cesarean section or natural parturition. Generalized mixed linear models were applied to the analysis of binomial data, such as the variable of a positive pregnancy at the next breeding attempt (0 = no, 1 = yes) and the variable following parturition (0 = no natural parturition, 1 = natural parturition) using the generalized mixed linear models (GLIMMIX) procedure. Due to the fact that Terriers always got pregnant, the model could not represent proper estimates for this parameter. Therefore, it was calculated again without the breed group terrier.

The Akaike's information criteria corrected (AICC) (25) and the Bayesian information criteria (BIC) (26) were used to compare the different models. The model with the smallest AICC and BIC values was chosen. In general, the fixed effects breed group, type of parturition (G1: cesarean section, G2: natural parturition) and the covariate parities (number of parturition which was included as first parturition in the study) were added stepwise to the models. Interactions between the fixed effects were also tested, but none of the interactions was significant. Therefore, the interactions were removed from the model. The pairwise comparisons of the least square means were adjusted by the Bonferroni-Holm correction (27).

The results for the descriptive statistics are illustrated as mean ± standard deviation (SD). The results from the statistical models are illustrated as least square means (LSMeans) ± standard error (SE).

RESULTS

Breeds, Age, Weight, Number of Parturition, and Indication for Cesarean Section

Altogether, 261 bitches were included in the study, of which 119 bitches were in G1 (45.59%) and 142 were in G2 (54.41%). The arithmetic mean age ± SD of bitches at the time the cesarean section was performed and was 3.35 ± 1.15 (1–6 years) in G1 whereas the arithmetic mean age ± SD of the bitches in G2 was 2.69 ± 0.74 years (1.5–7 years) at parturition. The bitches in G1 had a mean weight of 24.93 ± 13.45 kg (2.2–63 kg), whereas the mean weight in G2 was 23.54 ± 12.12 kg (3.3–68 kg). The following breed groups where included: bulldogs (n = 19), herding dogs (n = 38), molossers (n = 41), retrievers (n = 60), shepherds (n = 17), and terriers (n = 28). In

TABLE 1 | Included breed groups.

Number	Breed group	Number of bitches included (*n*)	Median age at first included parturition and first quartil and third quartil (years)
1	Bulldogs	19	2 / 2 / 2.75
2	Herding dogs	38	3 / 2.5 / 3.37
3	Molossers	41	3 / 2.5 / 3
4	Retrievers	60	3 / 2.5 / 4
5	Shepherds	17	3 / 2.5 / 3
6	Terriers	28	2.5 / 2 / 3
7	"Others"	58	3/ 2.05 / 3

total, 58 dogs could not be assigned to the groups mentioned (Schnauzer: $n = 7$; Sennenhund: $n = 7$; European and Asian Spitz: $n = 7$, cocker: $n = 7$, Dalmatiner: $n = 7$, Pudel: $n = 6$, Petit Brabancon: $n = 3$, pug: $n = 3$, Coton de Tulear: $n = 2$, Shih Tzu: $n = 2$, Chihuahua, Dachshound, Weimaraner, Whippet, Papillon, Pumi, and Xoloitzcuintle standard). They were placed in the group "others" (**Table 1**). The breeders stated the following indications for cesarean section: uterine inertia ($n = 48$), malpresentation of the puppy (20), fetomaternal disproportion ($n = 15$), dead fetus (11), singleton ($n = 5$), green vaginal discharge, (2) brown vaginal discharge before the birth of the first puppy ($n = 1$) and uterine convulsion (1), wedged puppies (1), and maternal circulatory problems (1).

Distribution of First Parturition Depending on Breeds

Of the bulldogs, 57.89% ($n = 11$) had undergone a cesarean section during the first parturition included, and 42.11% ($n = 8$) gave birth naturally. Only 42.86% ($n = 12$) of the terriers had given birth naturally as compared to the herding dogs, molossers, and retrievers, where 63.16% ($n = 24$), 60.98% ($n = 25$), and 56.67% ($n = 34$) were able to give birth naturally.

Physiological Inter-estrous Interval

The physiological inter-estrous interval in G1 was 6.56 ± 1.88 months (1–13 months), whereas the physiological inter-estrous interval was 7.21 ± 1.84 months (3.5–13 months) in G2.

Number of Puppies Born

Altogether 1,651 puppies were born in the first parturition. In total, 727 puppies were born in G1 and 924 puppies were born in G2. In G1 6.11 ± 3.18 (1–13) puppies were born per bitch in the first documented cesarean section, whereas 6.51 ± 2.47 (1–12) puppies were born in G2 in the first parturition per bitch.

Following Heat

The LSMean for the first heat after cesarean section (G1) was 6.75 ± 0.19 months and 6.81 ± 0.19 months after natural parturition (G2). Altogether bitches showed their first heat after 1–13 months and 1–14 months after the previous parturition in G1 and G2, respectively. The type of parturition and the covariant parities had no significant effect on the first heat after cesarean section ($p = 0.8$ and $p = 0.35$). The breed group had a significant effect on the first heat after cesarean section ($p < 0.05$). There was a significant difference between the breed group molossers (LSMean 7.48 ± 0.31 months) and the group "others" (LSMean 6.22 ± 0.62 months; $p < 0.05$) and between the breed group retrievers (LSMean 7.65 ± 0.26 months) and the group "others" (LSMean 6.22 ± 0.62 months; $p < 0.05$). Furthermore, retrievers came into heat significantly later post-parturition (LSMean 7.65 ± 0.26 months) than the breed group terriers (LSMean 6.22 ± 0.26 months; $p < 0.05$).

Following Breeding

The LSMean for the interval between cesarean section and following breeding in G1 and parturition and following breeding in G2 counted as heat numbers was 2.3 ± 0.11 in G1 and 2.42 ± 0.1 in G2. The first heat was used in 19.3% ($n = 23$) of the bitches in G1 and 14.08% ($n = 20$) in G2, whereas 47.90% ($n = 57$) of the bitches in G1 and 47.89% ($n = 68$) of the bitches in G2 were bred during the second heat after the previous cesarean section or parturition. The third heat was used for breeding in 21.85% ($n = 26$) and 23.24% ($n = 33$) of bitches in G1 and G2. Looking into the fbreed distribution, none of the bulldog bitches was mated during the first heat, whereas 35.29% ($n = 6$) of shepherds were mated during their first heat (**Table 2**).

The type of parturition had no significant effect on the first used heat after parturition ($p = 0.42$). The fbreed group did have a significant effect on the first used heat after parturition ($p < 0.05$). However, there are no longer any significant effects in the multiple pairwise comparisons for the adjusted values of *p*. Due to the many comparisons between the individual breeds, the alfa level is greatly reduced so that no significant values remain after the adjustment. The covariant parities had no significant effect on the dependent variable ($p = 0.58$).

Following Pregnancy

Altogether 241 bitches became pregnant in the first breeding attempt. Within G1, 112 bitches became pregnant at the first breeding attempt, whereas 129 bitches became pregnant at the first breeding attempt in G2. The LSMean of bitches getting pregnant in the next heat used was 0.93 ± 0.027 in G1 and 0.91 ± 0.03 in G2. With probability values of 93 ± 2.7% and 91.12 ± 3%, bitches became pregnant in G1 and G2 at the first breeding attempt after the previous parturition. There was no significant effect of the fixed effects on breed group and cesarean section ($p = 0.8$ and $p = 0.63$) and the covariant parities ($p = 0.33$).

The pregnancy rate was found to be between 86.5% ($n = 16$) (bulldogs) and 100% ($n = 28$) (terriers).

Following Parturition

Of the original 261 bitches, 246 (94.25%) gave birth again. In G1, 52 of the 112 bitches had undergone a cesarean section, while 60 whelped naturally. As the following indications were stated by the owners: uterine inertia ($n = 23$), fetomaternal disproportion ($n = 6$), no signs of parturition ($n = 3$), dead puppy ($n = 2$), singleton ($n = 2$), weak heartbeats of the offspring (2), physiologically the bitch was not able to give birth naturally ($n = 1$), brown discharge ($n = 1$), the cervix was not dilated (1), planned cesarean

TABLE 2 | Distribution (%) of mated bitches depending on the used heat number and the breed group after a previous parturition.

	Used heat number for breeding						
Breed group	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Bulldogs	0.00	52.63	31.58	5.26	10.53	0.00	0.00
Herding Dogs	15.79	31.58	31.58	13.16	2.63	0.00	0.00
Molossers	29.27	46.34	17.07	4.88	0.00	0.00	2.44
Retrievers	18.33	50.00	23.33	3.33	5.00	0.00	0.00
Shepherds	35.29	47.06	17.65	0.00	0.00	0.00	0.00
Terriers	17.86	53.57	21.43	3.57	3.57	0.00	0.00
"Others"	5.17	53.45	18.97	13.79	6.90	1.72	0.00
Sum	16.48	47.89	22.61	7.28	4.21	0.77	0.34

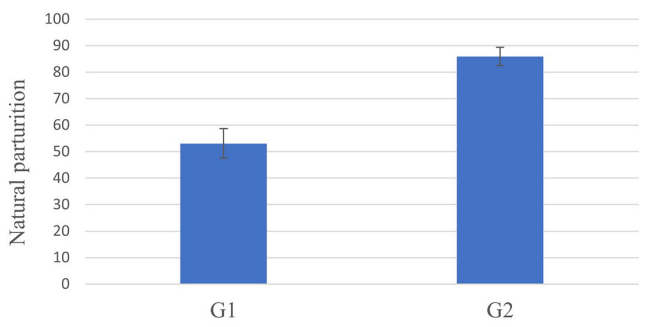

FIGURE 1 | Estimated frequencies [LSMeans ± standard error (SE)] for natural parturition depending on G1 and G2. Animals in G2 had experienced natural parturition more often with 85.94 ± 3.43% as compared to the animals in G1 with 53.08 ± 5.51% ($p < 0.05$).

section (1), and uterine rupture (1). Similarly, 17 (12.69%) of the 134 bitches in G2 had undergone a cesarean section while 117 (87.31%) whelped naturally. The breeders stated the following indications: fetomaternal disproportion (3) and uterine inertia (3), planned cesarean section due to myositis (1) and insufficient amniotic fluid (1), and singleton (1).

The breed group and the type of parturition both had a significant effect on natural parturition ($p < 0.05$). Regarding the cesarean section, animals in G2 had experienced natural parturition more often with 85.94 ± 3.43% (LSMeans ± SE) as compared to the animals in G1 with 53.08 ± 5.51% (LSMeans ± SE; $p < 0.05$; **Figure 1**).

There was a significant difference between bulldogs, where only 31.95 ± 12% (LSMeans ± standard error) had experienced natural parturition compared to herding dogs, where 89.30 ± 5.44% (LSMeans ± standard error) were able to give birth naturally ($p = 0.0079$) as well as between bulldogs (31.95 ± 12%) and "others" (80.07 ± 5.66%) ($p = 0.0285$) (**Figure 2**). All other comparisons showed no significant differences ($p > 0.05$).

Puppies Born During the Following Parturition

Altogether 1,583 puppies were born in the parturition following the first recorded parturition. In G1, 671 and in G2, 912 puppies were born. Each bitch birthed a mean of 6.04 ± 2.86 puppies in G1 (1–13), and in G2, each bitch birthed a mean of 6.8 ± 2.59 puppies (1–15).

The LSMean of the difference in the number of puppies born in G1 was 0.17 ± 0.3 and 0.39 ± 0.28 in G2. The breed group and the type of parturition had no significant effect on the difference in the number of puppies born ($p = 0.17$ and $p = 0.59$). The covariant number of parities had no significant effect on the dependent variable "difference in number of puppies born" ($p = 0.95$).

DISCUSSION

In this study, 261 bitches were included, of which 45.59% ($n = 119$) had a cesarean section and 54.41% ($n = 142$) gave birth naturally. Even if the homogeneity of the groups was not statistically calculated, the bitches in both groups showed similar body masses (G1: 24.93 ± 13.45 kg vs. G2: 23.54 ± 12.12 kg) and similar ages (G1: 3.35 ± 1.15 years vs. G2: 2.67 ± 0.74). The comparison of these groups, therefore, reveals some interesting fertility parameters.

Forsberg and Persson (23) found that the need for veterinary treatment and cesarean sections increased with the bitches' age. Bitches over 4 years old were at an increased risk. In this study, the mean age in G1 was 3.35 ± 1.15 years. A possible explanation could be that only boxers were included in the study of Forsberg and Persson (23), whereas this study examined different breeds.

There was no difference in the first heat's time occurrence regardless of whether the bitches had undergone a cesarean section or experienced natural birth (G1: 6.75 ± 0.19 vs. G2: 6.81 ± 0.19 months). There were differences in the time occurrence of the first heat after parturition between the breed groups, which matches the findings of other studies (28). Thus, cesarean sections do not seem to affect the resumption of the ovarian cyclicity. However, breed group does seem to have an effect on the resumption of the ovarian cyclicity and, therefore, impacts the time of heat occurrence. The first heat after parturition was used for mating in 19.3% ($n = 23$) (G1) and 14.08% ($n = 20$) (G2) of the bitches, whereas the majority of the bitches were bred in the second heat (G1: 47.90% vs. G2: 47.89%). It appears that having a cesarean section does not affect the owner's decision as to when to breed the bitch again. In human medicine, it is

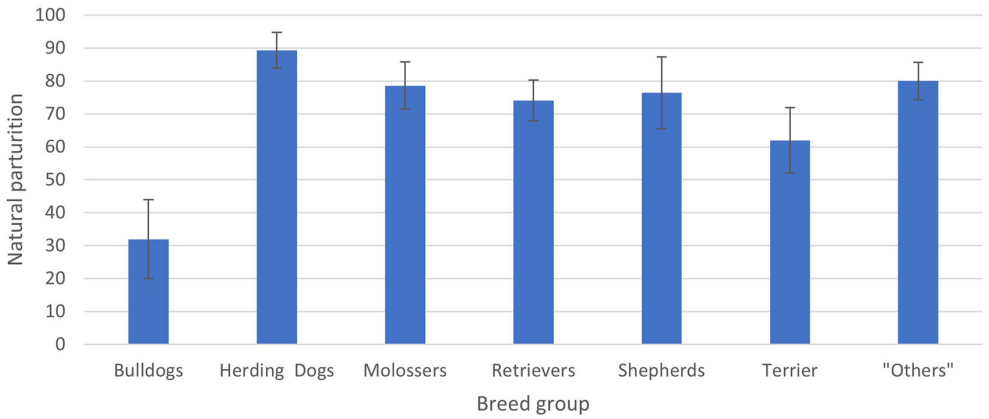

FIGURE 2 | Estimated frequencies [LSMeans ± standard error (SE)] for natural parturition depending on breed groups in case of pregnancy after a previous parturition. Significant differences could be obtained for the comparisons between breed group 1 (bulldogs) (31.95 ± 12.00%) and 2 (herding dogs) (89.30 ± 5.44%; $p = 0.0079$) and between breed group 1 (bulldogs) and 6 (others) (80.07 ± 5.66%; $p = 0.0285$). All other comparisons showed no significant differences ($p > 0.05$).

often recommended that women should only become pregnant again after a certain amount of time if they had a cesarean section (29). Short inter-pregnancy intervals in women are associated with a higher risk of complications during pregnancy and birth (29–31). Corresponding recommendations for dog breeders are not known to the authors. However, considering that dogs have a placenta endochorialis as compared to the placenta hemochorialis in women, the risk factors apparent in human medicine might not play a role in dogs. A factor well known in humans, that might play a role in dogs, is an increased risk of uterine rupture after a cesarean section (29). Nevertheless, only one dog owner reported uterine rupture as an indication for cesarean section.

With a probability of 93 ± 2.7% and 91.12 ± 3%, bitches became pregnant again in G1 and G2 during the first breeding attempt. There was no significant effect on the bitches' breed or whether they had experienced a cesarean before, on the likelihood of this pregnancy ($p = 0.8$ and $p = 0.63$, respectively). However, whether there is an impact on pregnancy success or incidence of complications during pregnancy from using the first or the second heat after the cesarean section remains an open question.

There was no significant difference between breeds ($p = 0.63$) regarding the pregnancy rate. In human medicine, on the other hand, studies indicate a reduction of fertility between 0 and 27% (13–15, 32). This seems to play a subordinate role in dog breeding. In the study by Conze et al. (17) pregnancy rates of 100% were found in 55 bitches within the 2 years following parturition. However, there was a lack of more precise data as to how often the bitches were bred again before getting pregnant. In this study, only the first breeding attempt was used for statistical calculation. In a study by Seyrek-Intas (18), 9 out of 12 bitches that had previously undergone a unilateral cornuectomy were whelped. It has to be taken into consideration that the cornuectomy was performed on healthy dogs. Although one might have expected reduced fertility in bitches with dystocia as compared to healthy bitches, which underwent surgery, this was not confirmed by the study.

Taking a closer look on breed-specific patterns of the first included parturition, 57.89% ($n = 11$) of the bulldogs had a cesarean section, and only 42.11% ($n = 8$) gave birth naturally. A similar proportion can be found in terriers, where only 42.86% ($n = 12$) experienced natural parturition. Therefore, bulldogs and terriers had lower rates of natural parturition as compared to the other breeds: herding dogs, molossers, and retrievers (63.16, 60.98, and 56.67% respectively). This matches the findings of Bergstrom et al. (6) who found that some breeds were at an increased risk of dystocia. Interestingly, they found that the Scottish Terrier is also at a higher risk for dystocia and cesarean sections, which might be in line with the findings of this study regarding the terriers in the first included parturition. However, according to Bergstrom et al. (6), Yorkshire Terriers were not found to be at a high risk for cesarean sections. This suggests that the breeds in the breed groups should be considered individually in subsequent studies. Eneroth et al. (9) reported that the frequency of cesarean sections in French Bulldogs was 43%, which matches the findings of this study.

Altogether, bitches that underwent a cesarean section had a higher likelihood of another cesarean section as compared to bitches, which experienced a first natural parturition. Only 53.08 ± 5.51% ($n = 60$) of the bitches whelped naturally after a cesarean section as compared to 85.94 ± 3.43% ($n = 117$) of the bitches after a natural parturition. The breed group also had a significant effect on the following parturition ($p < 0.05$). A significant difference was recorded between bulldogs, where only 31.95 ± 12% bitches gave birth naturally as compared to the herding dogs, with 89.30 ± 5.44% natural parturition ($p = 0.0079$) as the following parturition. Taking this into account, a future study could evaluate these differences according to individual breeds and not only breed groups. Brachycephalic breeds, in particular, are known to have a high rate of cesarean sections (33).

Furthermore, the question arises whether the subsequent cesarean section had the same indication as the first. Of the 119 bitches in G1, 48 bitches (40.34%) had undergone a cesarean section due to uterine inertia and 20 bitches (16.8%) due to malpresentation of the puppy. In total, 52 of these bitches underwent another cesarean section, where 23 (44.23%) cesarean sections were performed due to uterine inertia. This might indicate that, at least in some bitches, the subsequent cesarean section had the same indication as the first. However, it has to be taken into consideration that the indication for cesarean section was given by an anonymous questionnaire completed by the breeders, so some of the indications may be imprecise. To confirm the result, a retrospective study would be interesting. Never less, our findings match those of Linde Forsberg and Persson (23), who found primary uterine inertia (60%) and malpresentation of the fetus (26%) to be the most common reasons for dystocia.

A mean of 6.11 ± 3.18 (1–13) puppies was born per bitch after the first documented cesarean section (G1) and 6.04 ± 2.86 puppies during the following parturition. Similarly, 6.51 ± 2.47 (1–12) puppies were born in G2 during the first documented parturition per bitch and 6.8 ± 2.59 puppies in the following parturition. This matches the finding of Forsberg and Persson (23). However, the size of the litter is very dependent on the breed, and, as a result, no conclusion can be drawn with regards to the effects of cesareans on litter size. However, neither the breed group ($p = 0.17$) nor whether the bitch had a prior cesarean section ($p = 0.59$) nor the number of previous parturitions had an effect on the number of puppies born ($p = 0.95$). Overall, a prior cesarean section does not reduce the number of puppies born in the following breeding.

CONCLUSION

A prior cesarean section appears to have no negative effect on fertility and the number of puppies born in the following breeding. Nevertheless, dog owners must be aware that the risk of a second cesarean section is high. The breeder should, therefore, consider carefully breeding dogs that underwent cesarean section before, as they seem to have a high risk for dystocia. Our data can support counseling by veterinarians regarding the issue, addressing important ethical aspects.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

KB did the statistical analysis. AW is the head of the study. All authors contributed to the article and approved the submitted version.

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Commensal Lactobacilli Enhance Sperm Qualitative Parameters in Dogs

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Although several methods have been developed to improve male fertility and sperm quality, subfertility remains a primary clinical issue in male reproduction worldwide. The aim of this study was to determine the effects of the oral administration of three commensal *Lactobacillus* spp. on healthy normozoospermic dogs and the qualitative parameters of their sperm. Three weeks of supplementation induced a significant decrease of two phyla, Proteobacteria and Tenericutes, and an increase of phylum Firmicutes. At the species level, the number of *Fusobacterium perfoetens* and *Anaerobiospirillum succiniciproducens* decreased, while *Limosilactobacillus reuteri* increased. Parallel to these results, qualitative sperm parameters such as total and progressive motility, acrosome integrity, and other kinematic parameters were significantly enhanced after commensal lactobacilli supplementation. In addition, we showed that Firmicutes were positively correlated with sperm qualitative parameters, while Proteobacteria, *F. perfoetens*, and *A. succiniciproducens* were negatively correlated. Considering the similarities between the gut microbiome of dogs and humans, these results provide more insight into how gut microbiota regulation could improve male sperm quality in both species.

Keywords: lactobacilli, sperm, canine, gut microbiome, probiotics

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INTRODUCTION

Fertility is influenced by the health and lifestyle of an individual (1). Recent data show that male factor subfertility is responsible for fertility problems in 30% of cases (2). External factors, such as diet (3, 4) and stress (5), as well as internal factors such as aging (6), metabolism (7), and nutrient availability (8, 9), significantly influence reproductive function. These factors could affect the gut microbiome as well as spermatogenesis in males (10), which results in changes in sperm qualitative and quantitative parameters such as sperm morphology, viability, motility, and chromatin integrity. Accordingly, it was hypothesized that the management of gut microbiota could help increase sperm qualitative parameters, and subsequently, a link between the gut microbiome and testicular dysfunction through polyamine metabolism has been established (11). A recent study showed that triptolide-induced testicular dysfunction was successfully reversed by spermine supplementation or transplantation of bacterial strains that enhance spermine production in the gastrointestinal tract (11).

Canine male reproductive function is similar to that of human males, which makes dogs a good model for fertility issues, especially when considering the similarities in diseases, environment, life span, size, genetics, and anatomy (12, 13). Some of the genes involved in infertility and spermatogenesis, are found in both humans and dogs (14), and the morphological and qualitative abnormalities of sperm are similar in both species (15, 16). Further, the gut microbiome of both species is functionally and structurally similar (17, 18). Since their domestication, dogs have been sharing the same food sources as humans (19), and have switched their diet from carnivorous to omnivorous, which gradually modified their digestive and metabolic characteristics, making them closer to those of omnivorous mammals (20). This dietary change further influenced the microbiome of domestic dogs, making it more similar to that of humans. Dogs and humans gut microbiome have similar taxonomic profiles and distributions at the phylum and genus levels (17, 18, 21). The strong similarities between the two species suggest that studying the dog microbiome would help to uncover its roles not just in dogs but also in humans.

Lactobacilli are one of the beneficial commensal bacterial groups that are the most common type of probiotic organisms. They contribute to the maintenance of gut homeostasis by maintaining a beneficial microbial balance. These bacteria defend against colonization by opportunistic pathogens through the production of antimicrobial compounds and their high antioxidant ability (22). As oxidative stress is one of the main causes of subfertility in both dogs and humans (23–25), we hypothesized that commensal *Lactobacillus* spp. could enhance sperm quality in dogs. Although the effects of many dietary supplements on dog sperm have been evaluated before (26–28), probiotics effects have not been evaluated yet. We selected three commensal *Lactobacillus* spp., namely *Lactobacillus acidophilus*, *Limosilactobacillus reuteri* (previously known as *Lactobacillus reuteri*), and *Ligilactobacillus salivarius* (*Lactobacillus salivarius*), based on our previous study (21). All three species are currently used as probiotics and have been approved as feed additives in South Korea.

MATERIALS AND METHODS

Dogs Selection

All the dogs used in this study belonged to the same owner. All experimental procedures in this study were performed with the consent of the owner. Nine poodles kept in the same environment were used in this study. They were fed commercial adult dry food once a day, and water was provided *ad libitum*. The dogs were selected based on different criteria including body condition scores of 4–5 on a 9-point scale, ages ranging from 2 to 9 years, health state, fertility records, and dogs with fertility issues were excluded from the study. All the dogs were healthy, vaccinated, de-wormed, free of diseases, and had no history of medication, diarrhea, or other medical conditions for 4 months prior to the start of the experiment.

Bacteria Culture and Preparation of Supplementation

Three selected *Lactobacillus* spp., *L. acidophilus* KACC 12419, *L. salivarius* KACC 10006, and *L. reuteri* KACC 11452, were obtained from the Korean Agricultural Culture Collection (KACC). All the strains were identified via 16S rRNA sequencing prior to the experiments. Bacterial strains were cultured in De Man, Rogosa, and Sharpe (MRS) broth overnight at 37°C and washed twice using phosphate buffered saline. Then, bacterial pellets were resuspended in water and 20% glycerol and stored at −20°C in aliquoted tubes. Each tube contained the following concentrations: 3.0×10^9 colony forming unit (CFU) of *L. acidophilus*, 2.7×10^8 CFU of *L. salivarius*, and 1.3×10^9 CFU of *L. reuteri*. The tubes were thawed at room temperature before use. All dogs were orally administered a mixture of the three commensal *Lactobacillus* spp., with fructo- and galactooligosaccharide (0.8% of food intake) along with dry food once a day, every day, for 3 weeks.

Sample Collection and Processing

Rectal swab samples were collected at weeks 0 and 3 on the same days as sperm collection, using N-Swab transport (Noble Bio, Hwaseong, Korea). They were then transported to the laboratory at 4°C within 2 h. All the samples were stored at −80°C until further experiments. Microbial genomic DNA was extracted DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). A bitch in estrus was used to stimulate the dogs, and semen samples were collected manually from each dog twice: before the start of dietary supplementation and 3 weeks after the start of the experiment (29). The first fraction was discarded, and the second fraction only, which represents the sperm-rich fraction, was collected and used for analysis. The samples were diluted with Tris-extender 1:1 (v/v) (distilled water, tris (hydroxymethyl) aminomethane 24 g/L, citric acid 14 g/L, fructose 8 g/L, and kanamycin sulfate 0.15 g/L; pH 6.6, 290 mOsm) and centrifuged at 700 ×g for 1 min. Supernatants were collected and centrifuged (500 ×g/5 min), and the pellets were resuspended in Tris-extender and chilling media [54% (v/v) Tris-extender, 40% (v/v) egg yolk, and 6% (v/v) glycerol]. The samples were chilled for 4–6 h at 4°C before being transported to the research facility (Mjbiogen) and processed for analysis (30).

Gut Microbiota Profiling

Library construction, sequencing, and gut microbiome analysis were performed as previously described (21). Briefly, the Illumina 16S Metagenomic Sequencing Library Prep Guide was used for the V3-V4 region. Paired-end sequencing was performed by Macrogen (Seoul, South Korea) using the MiSeq™ platform (Illumina, San Diego, CA, USA). The sequences were trimmed and clustered into operational taxonomic units (OTUs) with 97% identity similarity, and microbial community analysis was performed using Quantitative Insights Into Microbial Ecology (QIIME) 1.9 (31).

Sperm Kinematic Parameters Evaluation

Sperm kinematic parameters were evaluated using a computer-assisted sperm analysis system (Sperm Class Analyzer® System

version 6.4.0.93, Microptic, Barcelona, Spain), which included a Nikon Eclipse ci-L microscope (Nikon, Tokyo, Japan) with a 10× phase-contrast objective, a heating stage at 37°C, with a frame rate set at 25 frames/s. Settings were adjusted according to the manufacturers recommendations (32), and a minimum of 500 cells in 5 random fields per sample were analyzed. For analysis, a sperm drop of 3 μl per sample was placed on Leja 20 μm chamber slides (Leja, Gynotec Malden, Nieuw Vennep, Netherlands) at 37° (33). Total sperm motility, progressive motility, curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR), wobble VAP/VCL (WOB), amplitude of lateral head (ALH), and beat cross frequency (BCF) were analyzed.

Acrosome Integrity Assessment

Fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) was used to assess the acrosome integrity, as described previously (34). Semen smears were fixed in absolute methanol, stained, and mounted with anti-fade mounting medium (VECTASHIELD®, Vector Laboratories, Burlingame, CA, USA). The integrity of the sperm acrosome membrane was evaluated in at least 100 cells per slide, using an epifluorescence phase-contrast microscope (Eclipse Ts 2, Nikon, Tokyo, Japan) and classified as intact (strong green fluorescence) or non-intact (partial or no fluorescence).

Eosin-Nigrosin Staining

Eosin–nigrosin staining was performed to determine the proportion of live sperm cells and morphology defects. Briefly, samples were washed, and equal amounts of semen, eosin, and nigrosin were mixed and smeared onto warm glass slides. The slides were then air-dried, and 200 sperms were examined under a light microscope (Eclipse Ts 2, Nikon, Tokyo, Japan) with an oil immersion objective lens (1,000× magnification). The unstained sperms were counted as alive, stained ones were counted as dead, and the results were expressed as the proportion of live sperm cells (35). Different morphological defects were counted in every slide, according to previous studies (15, 36). Briefly, sperm with a coiling of the mid piece were counted as cells with a coiled tail, the ones with a bending of the mid piece or the entire tail were counted as cells with a bent tail, and sperm with a droplet in the tail were counted as cells with a droplet. Cells with a head defect were counted as well.

Statistical Analysis

Sperm parameters were statistically analyzed using GraphPad Prism 5.0 (GraphPad, CA, San Diego, USA). Prior to the analysis, D'Agostino and Pearson omnibus test was performed. The sperm data were analyzed using a one-tailed paired *t*-test. Alpha- and beta diversity were calculated based on observed OTUs and weighted UniFrac distances, respectively. Other analyses were performed using R software version 3.0.1. The Kruskal–Wallis test or Wilcoxon rank-sum test was used for bacterial relative abundance comparison. Pearson's correlation coefficient was calculated using *cor* function in the R software to measure the association between sperm qualitative parameters and gut microbiome abundance. Plotting was performed using the *Corrplot* function. All values are expressed as mean ± standard error of the mean (SEM), and values of $p < 0.05$ were considered statistically significant.

RESULTS

Sperm Kinematic Parameters

Sperm kinematic parameters were assessed before the start of the experiment (week 0); after 3 weeks of supplementation (week 3), there was a significant improvement in sperm qualitative parameters (**Table 1**; $p < 0.05$). Total and progressive motility were the main qualitative parameters used to evaluate male fertility, and both were significantly enhanced at week 3 compared to week 0 (93.6 ± 3.6% vs. 90.5 ± 2.9% for total motility, and 60.4 ± 7.4% vs. 44.9 ± 7.5% for progressive motility). Other sperm kinematic parameters, such as VCL, VAP, VSL, LIN, STR, and BCF, were also significantly enhanced (**Table 1**).

Viability, Acrosome Integrity, and Morphological Defects

A significant increase in acrosome integrity was seen after commensal lactobacillus supplementation (84 ± 3.4% at week 0 vs. 92.9 ± 1.9% at week 3) ($p < 0.05$), although there was no difference in cell viability (65.7 ± 5.6% at week 0 vs. 73 ± 3.8% at week 3) (**Table 1**). As for the morphological defects, coiled and bent tails proportions were significantly reduced at week 3 (5.6 ± 0.9% and 12.3 ± 5.0% at week 0, 3.0 ± 0.4% and 3.3 ± 1.0% at week 3 respectively). Tail droplets were also reduced at week 3 (2.6 ± 1.2% vs. 0.2 ± 0.0%), but there was no significant reduction in head defects proportion (0.4 ± 0.3% vs. 0.0 ± 0.0%) (**Table 2**).

Gut Microbiota Diversity and Composition

Among the alpha diversity parameters, the Shannon index was statistically different before and after supplementation (**Supplementary Figure 2**). For beta diversity, weeks 0 and 3 tended to be clustered (**Supplementary Figure 2**). Gut microbiota composition at week 3 significantly changed in comparison with that at week 0 (**Figure 1**; **Supplementary Table 1**) ($p < 0.05$). At the phylum level, the relative abundance of Firmicutes significantly increased at week 3 compared to that at week 0, whereas Proteobacteria and Tenericutes significantly decreased at week 3 compared to that at week 0. At the genus level, *Ligilactobacillus* and *Limosilactobacillus* were significantly enhanced after supplementation (**Figure 1**), whereas *Anaerobiospirillum* was significantly decreased. At the species level, *Limosilactobacillus reuteri* was significantly enhanced at week 3, whereas *Fusobacterium perfoetens* and *Anaerobiospirillum succiniciproducens* were significantly decreased (**Figure 1**).

Correlation Between Gut Microbiota and Sperm Parameters

Pearson's correlation coefficients were calculated to evaluate possible correlations between sperm qualitative parameters and gut microbiome populations that were significantly altered

TABLE 1 | Sperm qualitative parameters in the first and last week of supplementation.

Parameters	Week 0	Week 3	*p*-value	Significance
Motility (%)	90.5 ± 2.9	93.6 ± 3.6	0.02	*
Progressive motility (%)	44.9 ± 7.5	60.4 ± 7.4	0.00	*
VCL (μm/s)[a]	95.0 ± 10.1	110.3 ± 11.9	0.03	*
VAP (μm/s)	52.6 ± 5.1	61.1 ± 6.1	0.01	*
VSL (μm/s)	32.2 ± 3.4	40.8 ± 4.1	0.01	*
LIN (%)	33.1 ± 1.6	35.9 ± 2.1	0.04	*
STR (%)	57.1 ± 2.1	62.3 ± 2.2	0.01	*
WOB (%)	56.4 ± 1.0	55.6 ± 1.3	0.79	NS
ALH (μm)	2.5 ± 0.2	2.8 ± 0.3	0.06	NS
BCF (Hz)	10.0 ± 0.8	11.5 ± 1.0	0.03	*
Live cells (%)	65.4 ± 5.6	73.0 ± 3.8	0.1	NS
Intact acrosome (%)	83.8 ± 3.3	92.9 ± 1.9	0.03	*

[a]*VCL, average curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity (average ratio of VSL/VCL); STR, straightness (average value of the ratio VSL/VAP); WOB, wobble; ALH, amplitude of lateral head; BCF, beat cross frequency.*

All results show means ± SEM. Values with a "" are significantly different ($p < 0.05$, $n = 9$), while values with "NS" are non-significant.*

TABLE 2 | Sperm morphological defects in the first and last week of supplementation.

Morphological defects	Week 0	Week 3
Head (%)	0.4 ± 0.3	0.0 ± 0.0
Droplets (%)	2.6 ± 1.2[a]	0.2 ± 0.0[b]
Coiled tail (%)	5.6 ± 0.9[a]	3.0 ± 0.4[b]
Bent tail (%)	12.3 ± 5.0[a]	3.3 ± 1.0[b]

All results show means ± SEM. Values within marked with the letters "a" or "b" are significantly different ($p < 0.05$, $n = 9$).

(**Figure 2**). Firmicutes had a moderate positive correlation with sperm progressive motility and acrosome integrity ($r = 0.50$ and $r = 0.54$, respectively) ($p < 0.05$), whereas Proteobacteria showed a moderate negative correlation with sperm motility, progressive motility, and acrosome integrity ($r = -0.53$, $r = -0.53$ and $r = -0.56$) ($p < 0.05$). Qualitative sperm parameters showed a moderate negative correlation with *F. perfoetens* ($r = -0.54$, $r = -0.59$ and $r = -0.54$) ($p < 0.05$). A weak negative correlation was observed between *A. succiniciproducens* and acrosome integrity ($r = -0.60$) ($p < 0.05$). Other results were not significantly different.

DISCUSSION

Probiotics are used as dietary supplements to improve and regulate the gastrointestinal microbiome by promoting the growth of beneficial bacteria, inhibiting the growth of pathogens and potentially harmful microorganisms (37). Gut commensal lactobacilli such as *L. acidophilus, L. reuteri, L. rhamnosus, L. plantarum, and L. salivariu*s have been used as probiotic supplements in recent years and have a positive effect on the general health and gut microbiome health of the host (38–42). Among the various *Lactobacillus* spp., we selected three of them (*L. acidophilus, L. reuteri*, and *L. salivarius*) as commensal probiotics based on our previous study (21), and a list of approved feed additives in South Korea. Dogs were fed with commensal lactobacilli for 3 weeks as a preliminary study, to evaluate the effects of probiotics after only one cycle of spermatogenesis. A previous study showed that probiotics supplementation significantly improved sperm quality in zebrafish after one spermatogenetic cycle (43). Considering that one canine spermatogenic cycle duration is of approximately 2 weeks (44), and that 4–4.5 cycles are necessary to complete the entire spermatogenesis process in dogs, the observed changes indicate that probiotics supplementation starts to affect sperm quality after one spermatogenesis cycle. Similarly, supplementation of omega 3 and vitamin E significantly improved canine sperm quality after 2 weeks of supplementation (26). Our results could be due to an improvement in epididymal and prostatic fluid, and more research are needed to confirm this.

In the present study, changes in the gut microbiome and sperm qualitative parameters, as well as their correlation with commensal lactobacillus supplementation, were analyzed in normozoospermic dogs. We showed that a combination of three *Lactobacillus* spp., *L. acidophilus, L. reuteri, and L. salivarius*, could improve sperm kinematic parameters, including total and progressive motility (**Table 1**). The enhancement of these parameters might be due to the antioxidant abilities of lactobacillus. It has been reported that *L. salivarius* has antioxidant effects on sperm *in vitro* (45). However, the mechanisms by which it might affect sperm parameters *in vivo* have not yet been reported, and further research is needed to uncover them. The presence of an intact acrosome is required for a successful fertilization, as zona pellucida penetration is only possible after the release of enzymes from the acrosome (46). Although the importance of acrosome integrity cannot be ignored, there are no reports on the effects of lactobacillus supplementation on acrosomes. Here, we report an improvement in acrosome

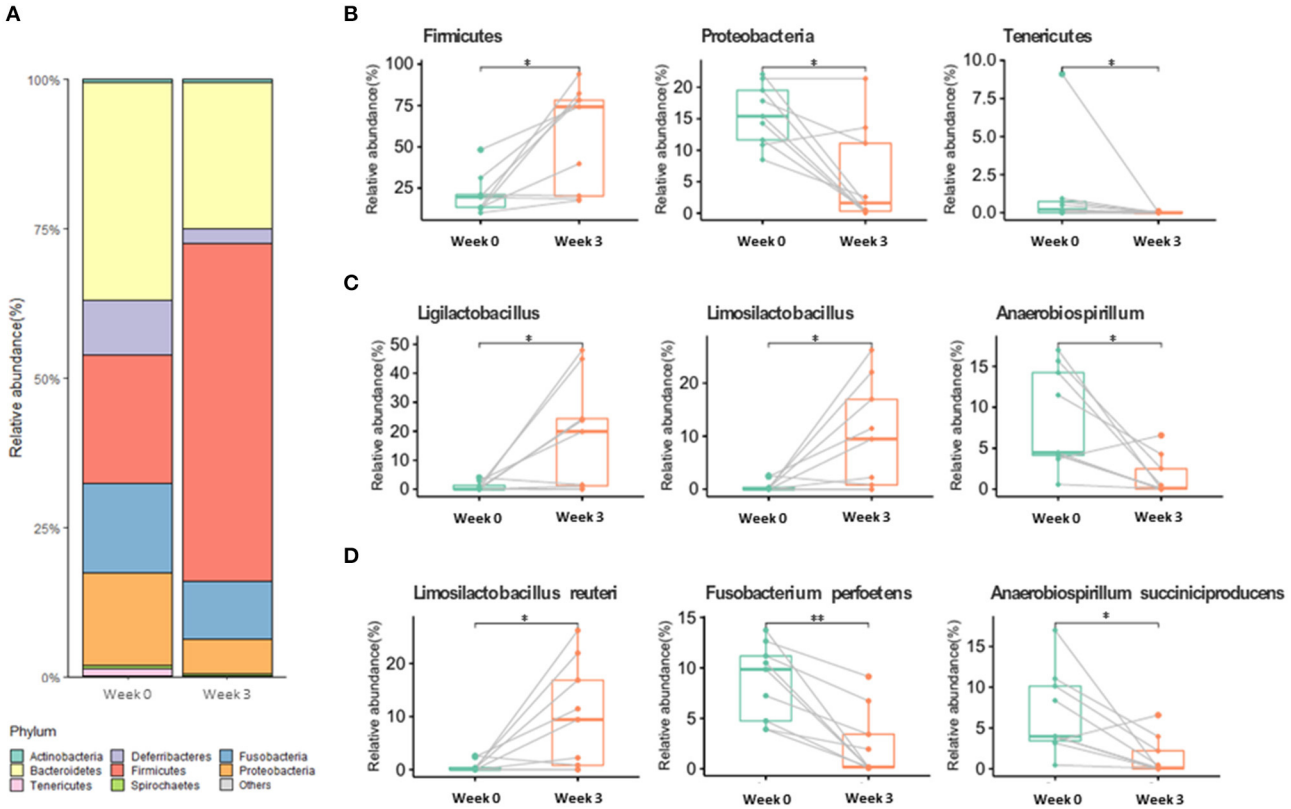

FIGURE 1 | Gut microbiome relative abundance at the phylum **(A,B)**, genus **(C)**, and species **(D)** levels in week 0 and week 3. Values with "*" ($p < 0.05$) or "**" ($p < 0.01$) are significantly different.

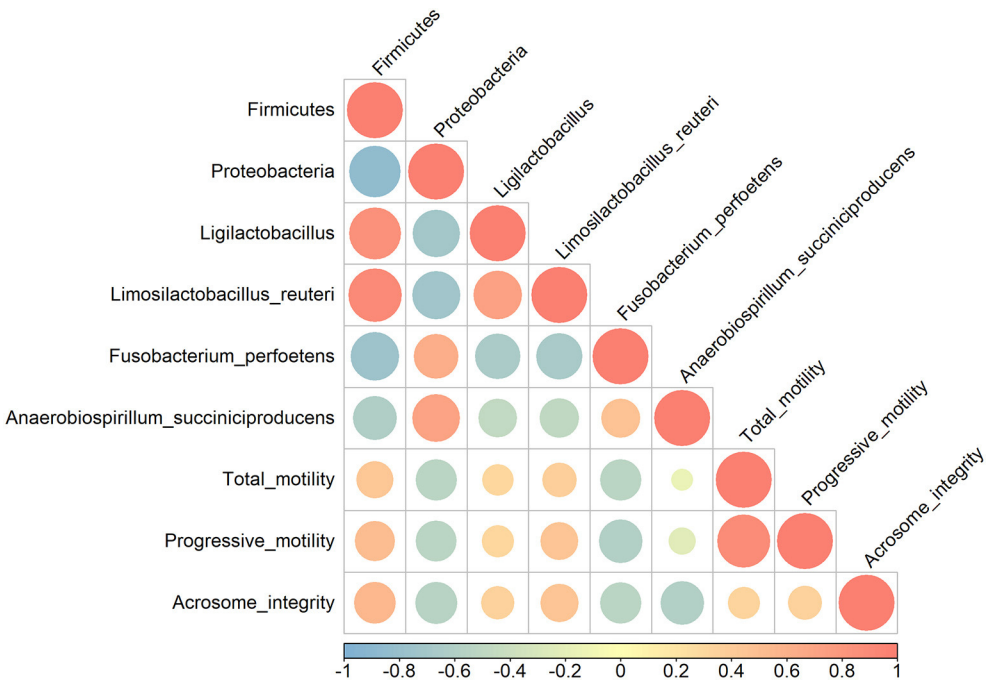

FIGURE 2 | Pearson correlation analysis between relative abundance of bacterial phyla and species, and sperm qualitative parameters. Coefficients are colored from light blue (negative correlation) to light red (positive correlation), and color intensity and circle sizes are proportional to the level of correlation.

integrity after commensal lactobacillus supplementation (**Table 1**).

Sperm tail defects were significantly reduced after the 3rd week (**Table 2**). Although DNA integrity was not assessed in this study, a relationship between DNA fragmentation levels and tail abnormalities has been shown previously (47), which suggests that lactobacillus might have effects on DNA integrity as well. During this experiment, one dog was diagnosed with idiopathic infertility, and 3 weeks of commensal lactobacillus supplementation did not help restore fertility (data not shown). However, a previous study showed that prolonged supplementation with probiotics could restore fertility in infertile human patients; the administration of a combination of *L. acidophilus*, *L. bulgaricus*, *L. rhamnosus*, *L. casei*, *Bifidobacterium breve*, *B. longum*, *and Streptococcus thermophilus* significantly enhanced sperm motility, DNA integrity, and chromatin status in infertile men after 80 days of supplementation (48). Dogs were supplemented with commensal lactobacilli for 3 weeks only, to cover one spermatogenic cycle, while the total duration of the spermatogenesis process in dogs is estimated to last 8–9 weeks, covering 4–4.5 cycles (49). This could explain the absence of effects of probiotics supplementation on the infertile dog and point at the need of longer supplementation periods. Although sperm quality assessment is a fast and accessible procedure in small animal reproduction, it is not a valuable indicator of the fertility state, since fertility is a multifactorial parameter. Therefore, further experiments are required to assess the effects of probiotics on other factors.

Parallel to sperm analysis, gut microbiota composition and changes were also assessed to determine a possible link between changes in sperm qualitative parameters and gut health. Relative abundance changes at the phylum, genus, and species levels are examined in the Results section (**Figure 1**). The numbers of Proteobacteria and Tenericutes significantly decreased after 3 weeks of supplementation, while Firmicutes significantly increased. In other studies, Proteobacteria were linked with diseases and gut dysbiosis (50–52), and our findings further revealed that Proteobacteria are negatively correlated with sperm qualitative parameters (**Figure 2**). These results indicate a possible link between gut dysbiosis and the involvement of Proteobacteria in sperm quality. Additionally, Tenericutes are speculated to be involved in this process, although further confirmatory evidence is needed. Firmicutes contain an important polyamine called spermine (53). Spermine has a role in anti-oxidation and reproductive physiology homeostasis in males (53); it regulates the factors that protect against testicular injury, and oral supplementation with spermine successfully reversed testicular injury (11), which shows a direct link between gut microbiota and testicular tissue repair. In our results, there was a moderate positive correlation between Firmicutes and progressive motility and acrosome integrity. These elements suggest that commensal lactobacilli could be an interesting supplement to patients suffering from testicular trauma, to ensure a fast recovery of testicular function through the involvement of Firmicutes and spermine.

One of the more abundant species, *L. reuteri*, which was also included in our supplementation mix, may have easily settled in the intestine due to its commensal characteristics (**Figure 1**). *L. reuteri* has been reported to be associated with improved gut health (39, 40) and a reduction in the Proteobacteria population (40, 54), which is in accordance with our results (**Figure 1**). Moreover, it efficiently decreased intestinal inflammation (39, 40, 55), and improved digestive comfort (54, 56). However, to this date, there have been no reports on the role of *L. reuteri* in sperm qualitative parameters, and our results show no significant correlation between *L. reuteri* and sperm quality (**Figure 2**). *F. perfoetens* relative abundance decreased after commensal lactobacillus supplementation (**Figure 1**) and showed a strong negative correlation with total motility, progressive motility, and acrosome integrity (**Figure 2**). Considering that *F. perfoetens* is found in aged dogs (21), it can be suggested that commensal lactobacillus supplementation counteracts the effects of aging on reproductive function by regulating the relative abundance of *F. perfoetens*.

In conclusion, supplementation with commensal lactobacilli composed of *L. acidophilus*, *L. reuteri*, and *L. salivarius* in dogs successfully enhanced the viability of sperm kinematic, acrosome integrity, and modified gut microbiota populations, without clinical side effects such as vomiting or diarrhea. Overall, these findings provide insights into the influence of beneficial commensal lactobacillus supplementation on dog sperm quality parameters. Furthermore, some bacterial groups appear to be potential biomarker candidates for sperm quality. Based on the correlation results between gut microorganisms and sperm qualitative parameters, Firmicutes could be used as a positive marker for sperm quality, whereas Proteobacteria and *F. perfoetens* could be used as negative markers. Although this is a preliminary study with a small sample size and a short supplementation period, these results open up new research possibilities in the field of veterinary medicine, and further experiments are warranted to identify other bacterial populations and pathways that influence male reproductive function.

DATA AVAILABILITY STATEMENT

Supplementary data is available in the additional files and further supporting data is available from the authors on request.

ETHICS STATEMENT

Ethical review and approval were not required for this study as noninvasive experiments were performed with the consent of the animal owners. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

MK, FM, and IY: conceptualization, data curation, investigation, methodology, visualization, and writing—review and

editing. MK and FM: formal analysis and validation. MK: supervision. MK and HP: funding acquisition, project administration, and resources. FM: writing—original draft. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: FM, IY, HP, and MK were employed by Mjbiogen Corp.

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An approach to uncover the relationship between 17b-estradiol and ESR1/ESR2 ratio in the regulation of canine corpus luteum

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The canine corpus luteum (CL) is able to synthetise, activate and deactivate 17b-estradiol (E2) and also expresses nuclear estrogen receptors in a time-dependent manner during diestrus. Nevertheless, we are still missing a better comprehension of E2 functions in the canine CL, especially regarding the specific roles of estrogen receptor alpha (ERa) and ERb, encoded by *ESR1* and *2*, respectively. For that purpose, we analyzed transcriptomic data of canine non-pregnant CL collected on days 10, 20, 30, 40, 50 and 60 of diestrus and searched for differentially expressed genes (DEG) containing predicted transcription factor binding sites (TFBS) for *ESR1* or *ESR2*. Based on biological functions of DEG presenting TFBS, expression of select transcripts and corresponding proteins was assessed. Additionally, luteal cells were collected across specific time points during diestrus and specificity of E2 responses was tested using ERa and/or ERb inhibitors. Bioinformatic analyses revealed 517 DEGs containing TFBS, from which 67 for both receptors. In general, abundance of predicted *ESR1* targets was greater in the beginning, while abundance of *ESR2* targets was greater in the end of diestrus. *ESR1*/*ESR2* ratio shifted from an increasing to a decreasing pattern from day 30 to 40 post ovulation. Specific receptor inhibition suggested an ERa-mediated positive regulation of CL function at the beginning of diestrus and an ERb-mediated effect contributing to luteal regression. In conclusion, our data points toward a broad spectrum of action of E2 and its nuclear receptors, which can also act as transcription factors for other genes regulating canine CL function.

KEYWORDS

dog, diestrus, *ESR1*, *ESR2*, 17b-estradiol, progesterone

Introduction

The canine corpus luteum (CL) has been considered as a source and target of steroid hormones, mainly progesterone (P4) and 17b-estradiol [E2, (1)]. The local production and role of P4 has been addressed earlier and there is a consensus that P4 is the primary hormone that maintains corpus luteum function and consequently, pregnancy (2, 3). Regarding E2, it is known that the canine CL produces E2 (4, 5), expresses enzymes for E2 activation (STS, steroid sulfatase) and deactivation [SULT1E1, sulphotransferase 1E member 1 (6)], and that the expression of E2 receptors (ERa and ERb, encoded by *ESR1* and *ESR2* genes, respectively) fluctuates during diestrus in pregnant and non-pregnant CL (1, 6). The ratio between *ESR2*/*ESR1* varies along non-pregnant diestrus, being greater on day 40 post-ovulation (p.o.) compared to days 10, 20, 30 and 60 p.o. (6). The gene *HSD17B7*, encoding 17b-hydroxylase, the enzyme converting estrone to E2, increases from day 10 to 20 and further to day 40 p.o. (6), whereas *CYP19A1*, encoding P450 aromatase, is greater expressed between days 35 and 45 p.o. (1). Expression of *STS* mRNA peaks on day 30 p.o., that of *SULT1E1*, on days 50 and 60 p.o. (6), indicating an established local machinery modulating E2 production and activity, which shows a turning point around day 40 p.o., when early luteal regression starts.

The effect of E2 on luteal function appears to be species-specific. For example, E2 presents a luteotropic role, as observed in rabbits and rats or a luteolytic function, as observed in humans and cattle (7–9). The luteotropic or luteolytic effect depends apparently on which receptor E2 binds to, ERa or ERb, which belong to the nuclear receptor family of intracellular receptors, exhibiting similar structures, but distinct regulatory functions. In general, ERa promotes cell proliferation, whereas ERb appears to have an anti-proliferation role (10). Moreover, in cells that express both receptors, it appears that ERb inhibits the transcriptional activity of ERa (11); consequently, E2 signaling may also depend on the ratio of ERa /ERb (12).

Upon ligand activation, ERs induce genomic and non-genomic effects (13, 14). Non-genomic effects can be mediated through the G-protein-coupled estrogen receptor (GPER), also expressed in granulosa cells and involved in E2 induced VEGF expression (13). The genomic effects result in the regulation of gene transcription and occur through direct binding of ERs to estrogen responsive elements (EREs) in the regulatory regions of E2 target genes. Alternatively, ERs can interact with other transcription factors such as activating protein-1 (AP1) and stimulating protein-1 (SP1) to influence gene expression indirectly (10, 14, 15). Transcription factor binding sites (TFBS) for ERa and ERb have been mapped in MCF7 breast cancer cells through ChIP-PET and ChIP-on-chip analysis (11, 16), which identified 1,234 and 1,457 high confidence ER binding sites, respectively. Around 75% of all ER binding sites can be target by both ERa and ERb receptors. Interestingly, only 5% of ERb binding sites contains exclusively an ERE, but 60% of them contains AP-1 like binding sites combined with ERE-like sites, and 45% among these contains additionally forkhead family binding sites (11). The ratio between ERa and ERb is also able to change the capacity of ERb to bind its specific TFBS (17). Additionally, TFBS can be activated by ERa and ERb independent of ligand (i.e., 17b-estradiol) (18).

E2 can trigger apoptosis in human granulosa cells *via* binding to ERb1 (the only splice variant of human ERb able to bind the hormone) (19), but depending on the cell line, for example in human breast cancer cells, apoptosis can be triggered by E2 binding to ERa (20). Although apoptosis signals are not strong enough to justify regression of a cyclic canine CL (21), E2 has been implicated in human CL regression *via* apoptosis (22). Moreover, a recent study comparing regressing canine CL and pre-partum luteolysis indicated activation of estrogen receptors as one of the main represented functional terms related to structural changes in the regressing CL (23).

Collectively, canine CL expresses both *ESR1* and *ESR2*, and the ratio of *ESR1* /*ESR2* varies throughout diestrus. There is a greater *ESR1* expression in early-luteal phase (1) and greater *ESR2* expression in the late-luteal phase (6). However, the role of E2 and its receptors in regulating canine CL function is still unknown. We hypothesize that E2 binding to ERa and ERb is time-dependent and might activate different E2-responsive genes, and therefore, different biological functions throughout canine CL lifespan. The aim of the present study was (1) to access differentially expressed genes (DEG) in canine CL with over-represented transcription factor binding sites (TFBS) related to *ESR1* and *ESR2* to gather an idea of the presupposed broad action of E2 along diestrus, and (2) characterize luteal cell responses to ERa and ERb inhibition to gain further insights into the role of E2 in specific aspects of the canine CL physiology, particularly on its regression.

Materials and methods

Animals and experimental design

Thirty healthy mongrel bitches were included in this study after approval by the Committee of Ethics in the Use of Animals of the School of Veterinary Medicine and Animal Science of the University of São Paulo (protocol number 2719/2012). After the onset of proestrus bleeding, blood samples were collected on alternate days to determine plasma progesterone (P4) concentrations. An additional blood collection for plasma P4 measurement was made on the day of surgery, prior to anesthesia. Ovulation was considered to have occurred when P4 plasma concentrations reached at least 5 ng/ml (24). The CLs were collected *via*

ovariosalpingohysterectomy on days 10, 20, 30, 40, 50, and 60 post-ovulation (p.o.; $n = 5$ animals per day). After collection, CLs were dissected from the surrounding ovarian tissue and 3 CLs of each animal were frozen immediately in liquid nitrogen for total RNA extraction, qPCR and western blotting analysis; remaining CLs were fixed in 4% buffered formalin for 24 h and used for immunohistochemistry. For cell culture, all CLs collected on days 20, 40 and 60 p.o., from 12 different dogs were immediately washed and kept in sterile phosphate buffered solution prior to processing as described below.

Hormone assay

Plasma progesterone concentrations were measured to define the day of ovulation using a validated chemiluminescence immune assay (Elecsys Progesterone III, Roche Diagnostics). The analytical sensitivity of the P4 assays was 0.10 ng/mL. The inter-assay coefficient of variation (CV) was 7.51% and the intra-assay CV was 6.11% as described by (25). The reagents used for P4 determinations came in the cobas® e pack PROG3 (Roche Diagnostics).

RNA-seq data analysis

RNA-seq data was generated and firstly analyzed as described previously (26), using CL of three different dogs per group. Data are publicly available at NCBI Gene Expression Omnibus under the number GSE89293. A total of 3,300 DEGs resulting from the contrasts 20×10, 30 × 10, 30 × 20, 40 × 10, 40 × 20, 40 × 30, 50 × 10, 50 × 20, 50 × 30, 50 × 40, 60 × 10, 60 × 20, 60 × 30, 60 × 40 and 60 × 50 were converted into their human orthologs using the Mammalian Annotation Database (MAdb: http://madb.ethz.ch), which is a collection of pairwise ortholog groups among human, cow, pig, horse, rabbit, mouse and dog genomes. Finally, we used oPOSSUM3 (27, 28) to identify the overrepresented, conserved TFBS related to ESRs. The same list containing 3,300 DEGs was submitted to oPOSSUM3 twice: in the first run, genes containing predicted TFBS for ERa/*ESR1* were shown, and in the second run, the ones containing predicted TFBS for ERb/*ESR2*. A gene was included in the DEG list if the false discovery rate (FDR) was < 0.01 and the respective p-value < 0.001. Ingenuity Pathway Analyses (IPA, Qiagen, Redwood City, CA, USA), revealed canonical pathways and upstream regulators for DEGs showing TFBS for ERa and ERb. A Venn Diagram was generated (https://bioinfogp.cnb.csic.es/tools/venny/index.html) to visualize upstream regulators related to both ESR1 and ESR2 (intersection).

Quantitative real-time reverse transcription PCR

Total RNA was isolated from CL in different stages of diestrus by Trizol® reagent (Life Technologies, Grand Island, NY, USA) according to manufacturer's instructions. Unless otherwise stated, all reagents and equipment were from Life Technologies. Concentration and quality of RNA were determined using a BioPhotometer (Eppendorf, Hamburg, Germany), and integrity was analyzed by electrophoresis through a 2% agarose gel. Following DNase treatment, 1 μg of total RNA (extracted from CLs) and 0.5 μg of total RNA per sample (extracted from luteal cells in culture) was reverse transcribed using Superscript III reverse transcriptase according to the manufacturer's instructions. DEPC-treated water was used as negative control. PCR reactions were performed with an automated fluorometer (ABI Prism® 7500), using 96-well optical plates. Each sample (25 ng of total RNA) was analyzed at least in duplicate. Negative controls were set up by replacing cDNA with water. Validated genes were selected according to biological processes they participate in (cell proliferation, luteal maintenance, cell death, luteal regression) and the presence of TFBS for ERa and ERb. The gene-specific primers used are listed in Table 1. After evaluation of three different reference genes, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), cyclophilin A (*PPIA*) and ribosomal protein L32 (*RPL32*), we used the NormFinder software (29), which selected *GAPDH* as the best reference gene for our analyses. The relative expression of estrogen receptor 1 (*ESR1*), estrogen receptor 2 (*ESR2*) lymphoid enhancer-binding factor 1 (*LEF1*), catenin-beta 1 (*CTNNB1*), cyclin D1 (*CNND1*), marker of proliferation Ki-67 (*MKI67*), N-Myc downstream-regulated *gene 2 (NDRG2), ATPase* Na^+/K^+ *(ATP1A1),* caspase 3 (*CASP3),* caspase 8 *(CASP8)*, caspase 9 *(CASP9),* cell surface death receptor *(FAS),* and BCL2 associated X apoptosis regulator *(BAX),* cytochrome P450 family 19 subfamily A member 1 *(CYP19A1),* cytochrome P450 family 11 subfamily A member 1 *(CYP11A1),* hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1*(HSD3B1)* and solute carrier family two member *4 (SLC2A4)* was calculated as described previously (30) followed by linear regression (LingRegPCR 7.0) fluorescent analysis (31).

Immunohistochemistry

Luteal tissue protein distribution of caspase 3, caspase 8, caspase 9 and BAX proteins, all involved in cellular death and most likely in canine luteal regression, was evaluated by an immunoperoxidase method on 2 μm tissue sections prepared from four CLs per dog, using one section per CL and four dogs per group to assure accuracy (32). The primary antibodies used

TABLE 1 List of qRT-PCR primers.

Gene	Primer	Sequence	Probe	GenBank N°
LEF1		cf02686726_mh		FJ374770.1
CTNNB1	Forward	5′ ACTGAGCCTGCCATCTGTGC 3′	TTCGTCATCTGACCAGCCGACACCA	FJ268743.1
	Reverse	5′ TCCATAGTGAAGGCGAACAGC 3′		
CCND1		cf02626707-m1		AY620434.1
NDRG2		Cf02722935_m1		XM_858273.1
ATP1A1		Cf02627969_m1		L42173.1
GAPDH		ID cf04419463_gH		AB038240.1
CASP8		ID cf02627553_ml		DQ223013.1
CASP9		ID cf02627331_ml		DQ116956.1
CASP3		ID cf02622232_ml		AB085580.1
FAS		ID cf 02651136_ml		XM_543595.2
BAX		ID cf02622186_ml		AB080230.1
MKI67		ID cf0263588_gl		XM_533319.2
CYP11A1		ID cf02635588_gl		XM_533319.2
HSD3B1	Forward	5′ TCCCCAGTGTTTCTGATTC 3′		AY739720.1
	Reverse	5′ CACCAACAAATGCACGATTC 3′		
SLC2A4	Forward	5′ GCCTGCCAGAAAGAGTCTGAAG 3′	CAGTCCCCAGATACAT	NM_001159327
	Reverse	5′ GCTTCCGCTTCTCCTCCTT 3′		
ESR1	Forward	5′CCTGCAAAGCCTTCAAGAG 3′	TCAATGCTCCCCTGGATGG	AJ313195.1
	Reverse	5′ GGAAGCCGGACAGCTGTAC 3′		
ESR2	Forward	5′ CCTGCAAGGCCTTCTTCAAGA 3′	CATCCAAGGGAACATC	AJ313196.1
	Reverse	5′GGCTGGGCAGCTGTACTC 3′		
CYP19A1	Forward	5′ GTACCGGCCTGACCAGTT 3′	CATGCCAGAGCGCTTC	NM_001008715.1
	Reverse	5′ ACTTAATGATGGAGAAGATGAGCTGACT 3′		

were polyclonal anti-rabbit for caspase 3, caspase 8, caspase 9 and BAX (Table 2). Negative controls were prepared using rabbit IgG (Santa Cruz Biotechnologies, Dallas, TX, USA). Positive controls were mouse lymph node sections prepared according to the manufacturer's protocol [as previously shown by (33)].

Western blotting

CL samples were homogenized in buffer containing 50 mM potassium phosphate (pH 7.0), 0.3 M sucrose, 0.5 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA, pH 8.0), 0.3 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM NaF, and phosphatase inhibitor cocktail (1:100; Sigma-Aldrich). Total protein content was determined spectrophotometrically using the Bradford method (34), and calculated by interpolation of a standard curve constructed with increasing concentrations of albumin, read at 595 nm. For each sample, 50 micrograms of total protein were resolved on 15% SDS–PAGE minigels and electrophoretically transferred onto polyvinylidene difluoride membranes (PVDF, Bio-Rad Laboratories, Hercules, CA, USA). CASP3, CASP8, CASP9, and BAX were detected with specific antibodies (Table 2) and visualized using an Enhanced Chemiluminescence (ECL) kit (Amersham Biosciences, Piscataway, NJ, USA). Images were captured by ChemiDoc MP Image system (Bio-Rad Laboratories) and normalized to the abundance of actin-beta (ACTB; 42 kDa) using ImageJ Software (Bio-Rad Laboratories).

Cell culture

Cell culture was performed to verify the effects of ERa and ERb blockers on canine luteal cells derived from different timepoints in diestrus. Canine luteal cells were isolated from twelve healthy mongrel female dogs at early (day 20 p.o.), mid (day 40 p.o.), and late diestrus (day 60 p.o.; $n = 4$ animals/group). After washing with fresh phosphate buffered saline (PBS) containing 1% antibiotic-antimycotic solution (A5955, Sigma-Aldrich), CLs were minced. Fragments were transferred to 1 ml Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS; Sigma-Aldrich), 1% L-glutamine (Sigma-Aldrich.), 20 mM

TABLE 2 List of antibodies for immunohistochemistry and western blotting (WB).

Antibody	Isotype	Immunogen	Dilution WB	Catalog no
Caspase 3	Polyclonal rabbit IgG	Recombinant catalytically active human caspase-3	1:2500	IMGENEX (IMG-5700)
Caspase 8	Polyclonal rabbit IgG	Recombinant catalytically active human caspase-8	1:2500	IMGENEX (IMG-5703)
Caspase 9	Polyclonal rabbit IgG	Recombinant catalytically active human caspase-9	1:2500	IMGENEX (IMG-5705)
BAX	Polyclonal rabbit IgG	Full length recombinant mouse Bax	1:2500	IMGENEX (IMG-5684)

HEPES (Sigma-Aldrich), 1% antibiotic-antimycotic solution (A5955, Sigma-Aldrich), and 1 mg/ml collagenase type 1 (C0130; Sigma-Aldrich). Samples were incubated for 1 h with shaking (60 shakes/min) at 37°C. The suspension was centrifuged at 200 ×g for 10 min, re-suspended in DMEM, and filtered through a cell strainer (70 μm; BD Falcon; BD Biosciences, Durham, NC, USA). The filtrate was centrifuged at 200×g for 10 min, re-suspended in DMEM (v/v) for 10 min, centrifuged at 200 ×g for 10 min, and re-suspended in DMEM. Subsequently, cells were seeded in 24-well plates and incubated (5% CO2) at 37°C until 90% confluence.

17b-estradiol, MPP and PHTPP treatment

After cultures reached 90% confluence, cells were serum-starved for 24 h. Cultures were divided into six groups: Control, E2 (treated with 100 nM 17β-Estradiol; (Sigma-Aldrich; E2), ERa block (treated with 10 nM methyl-piperidone-pyrazole; TOCRIS Biosciences, Bristol, UK;), ERb block (treated with 10 μM pyrazole (1,5-a) pyrimidine; TOCRIS Biosciences; PHTPP), E2 + ERa block (treated with E2 + MPP), and E2 + ERb block (treated with E2 + PHTPP). To determine which concentration of MPP and PHTPP should be used, dose-response curves were performed and minimal concentrations necessary to achieve stimulation of CYP19A1 expression were chosen. For RNA preparations, culture medium was discarded and 1 ml of TRIzol® was added to the cells, followed by scraping of the cell layer, freezing in liquid nitrogen, and storage at −80°C until further processing by qRT-PCR.

Statistical analysis

Data were tested for homogeneity of variance and normality of residues using the F-test and the Kolmogorov-Smirnov test, respectively. Data are presented as mean ± SEM. The qPCR and hormone data were compared by one-way analysis of variance (ANOVA), for the main effect of day, followed by the Bonferroni correction for normally distributed data. Differences were considered statistically significant when the p-value was <0.05. All statistical analyses of validation procedures were performed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA).

Results

ESR1 and *ESR2* gene expression in the canine CL during diestrus

The *ESR1* (Figure 1A) and *ESR2* (Figure 1B) mRNA expression changed significantly during diestrus ($P < 0.0001$); *ESR1* expression was greater on day 20 than on day 10 p.o. ($P < 0.0001$) decreasing on day 40 and further on day 60 p.o., whereas *ESR2* decreased from day 20 to 30 p.o., increased from day 30 to 40 p.o. and increased further on day 60 p.o. *ESR1*/*ESR2* ratio (Figure 1C) shows an increased from day 20 to 30 p.o. and a decrease from day 30 to 40 p.o., remaining low until day 60 p.o.

Transcription factor binding sites related to the E2 receptors

In a previous work of our group, we compared the temporal gene expression among days 10, 20, 30, 40, 50 and 60 p.o. The analysis revealed the presence of 3300 DEGs in at least one comparison (26). We converted these DEGs into their human orthologs in order to identify the over-represented TFBS related to ERs. In our ortholog approach for the transcription factor (TF) analysis, we assumed that the TF binding sites are evolutionarily conserved, as demonstrated previously (35). Seventy-seven DEGs exhibited TFBS for ERa and 450 exhibited TFBS for ERb (Supplementary Tables 1, 2), whereas TFBS for both ERa and ERb were found in 67 DEGs.

Genes presenting TFBS for ERa were related to several canonical pathways (Supplementary Table 3), among which the most significant, based on -log p-value, were G beta gamma signaling, sulfite oxidation, glycine biosynthesis, retinol biosynthesis, insulin signaling pathway, growth hormone signaling. ERb TFBS were encountered in genes participating in canonical pathways related to epithelial adherens junction signaling, nitric oxide signaling, GABA receptor signaling, signaling by Rho family GTPases, among others (Supplementary Table 3). We found 384 and 778 upstream

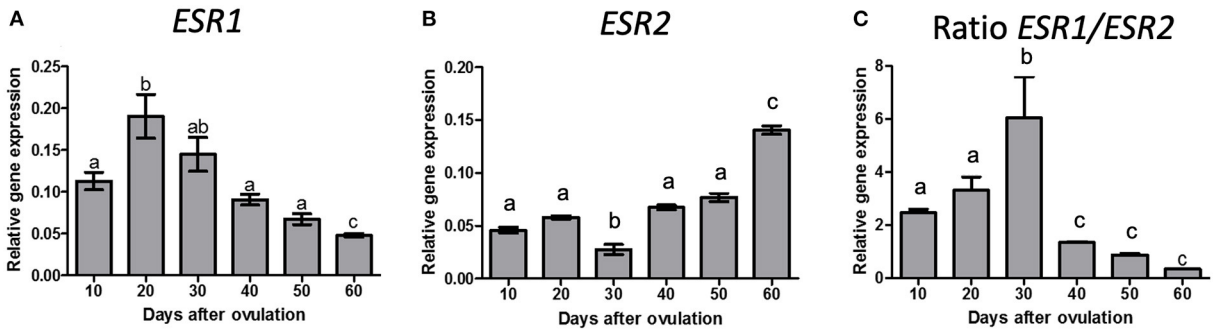

FIGURE 1
Gene expression of ESR1 **(A)**, ESR2 **(B)** and ratio of ESR1/ESR2 **(C)** in canine CL during diestrus (10 to 60 days p.o.). Data are presented as mean ± standard error of relative gene expression ($n = 4$ animals/group). Bars with different letters indicate significant differences among groups ($P < 0.05$).

regulators for ERa and ERb TFBS, respectively, from which 204 were common to both receptors (Supplementary Table 3). Different miRNAs, E2, P4, interleukins, *PPARG*, *MAPK1*and *CTNNB1* were found among the upstream regulators of both ERa and ERb TFBS-containing genes. *CCND1* and *LEF1* were found among the upstream regulators for ERb and ERa TFBS-containing genes, respectively. Moreover, *LEF1, NDRG2* and *ATP1A1* participated as target molecules in several intracellular pathways triggered by ESR2 upstream regulators, including 17b-estradiol.

Validation of mRNA levels of selected genes by qPCR

The selected genes included upstream regulators for *ESR1* (*LEF1*), *ESR2* (*CTNNB1* and *CCND1*), genes regulated by E2 (*NDRG2* and *ATP1A1*), proliferation markers (*MKI67*) and apoptosis markers (*CASP3, CASP8, CASP9, BAX* and *FAS*), which expression has not yet been shown in canine CL along diestrus. Additionally, we measured RNA expression of genes related to glucose uptake (*SLC2A4*) and steroidogenesis (*CYP19A1, CYP11A1* and *HSB3B1)* after E2 receptor inhibition in our cell culture model, from which the *in vivo* expression in canine CL was published elsewhere (1, 36, 37).

No significant differences in mRNA expression were observed for *LEF1*, *CTNNB1*, *CCND1* (Figures 2A–C) or *NDGR2* and *ATP1A1* during diestrus (Figures 2D,E).

MKI67 expression decreased from day 20 to 30 p.o. and remained lower until day 60 p.o. ($P < 0.05$; Figure 3A). *FAS* expression showed highest expression levels on days 40 and 50 p.o. ($P = 0.0002$, Figure 3B). *BAX* expression increased from day 30 to day 40 p.o. ($P < 0.0001$), being greater on the second half of diestrus (Figure 3C).

There was an effect of time ($P < 0.0001$) on *CASP8,* which increased on day 20 and further on day 60 p.o. (Figure 3D). *CASP9* expression increased on day 40 and 60 p.o. in comparison to day 10 p.o. ($P < 0.0001$), reaching maximum values on day 60 p.o. (Figure 3E). *CASP3* expression increased from day 30 to 40 p.o., reaching maximum values on days 40 and 60 p.o. ($P < 0.05$, Figure 3F).

Caspase 3, caspase 8, caspase 9 and BAX protein localization in the canine CL during diestrus

We verified the localization of apoptosis-related proteins (caspase 3, caspase 8, caspase 9, and BAX) in the canine corpus luteum over diestrus. Caspase 3 staining could be observed in the cytoplasm and nucleus of luteal, endothelial, and stromal cells from day 10 to day 60 p.o. (Figure 4). Caspase 8, caspase 9, and BAX followed the same expression pattern as caspase 3; however, the nuclear staining was not evident. Although immunohistochemistry is not a quantitative method, and despite the background staining observed, in particular at later luteal stages, the intensity of signals appeared to increase over time, matching the western blotting results described below.

Caspase 3, caspase 8, caspase 9 and BAX protein expression in the canine CL during diestrus

Western blotting analysis revealed that caspase 3, 8 and 9 as well as BAX expression was increased at the end of diestrus and the highest or greater expression was observed on day 60 p.o. (Figure 5).

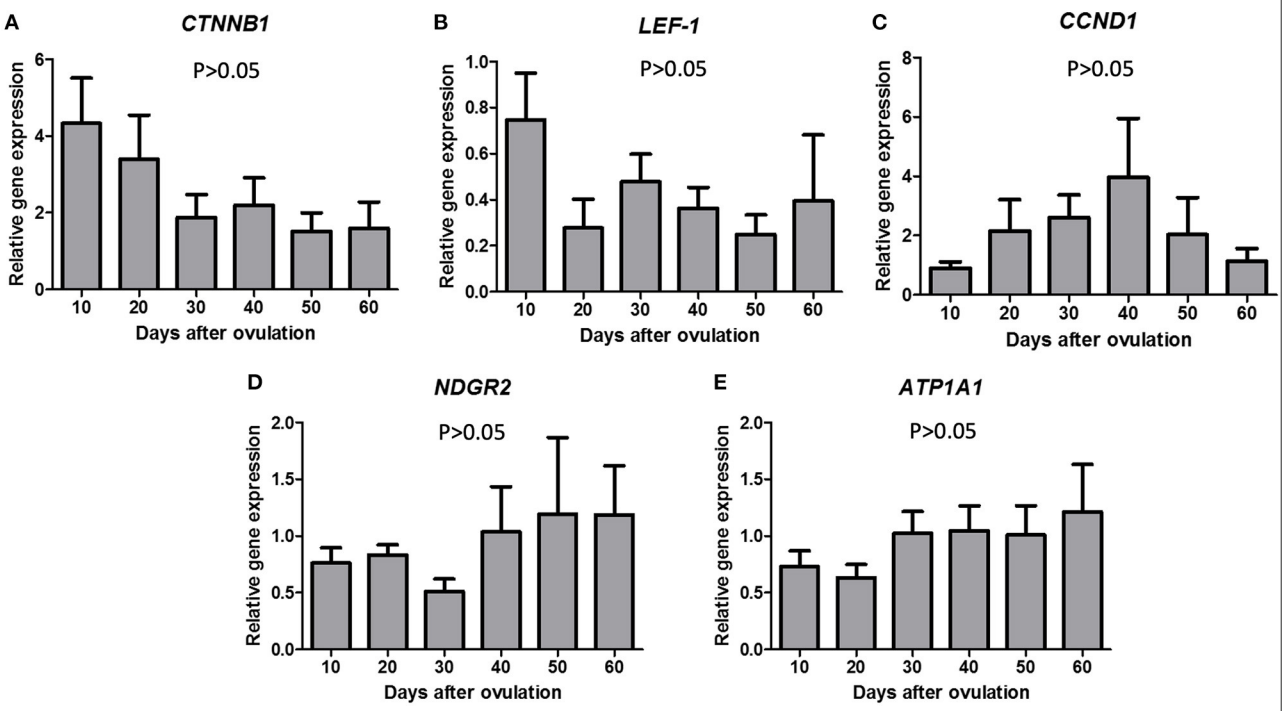

FIGURE 2
Gene expression of CTNNB1 **(A)**, LEF-1 **(B)**, CCND1 **(C)**, NDGR2 **(D)**, and ATP1A1 **(E)** in canine CL during diestrus (10 to 60 days p.o.). Data are presented as mean ± standard error of relative gene expression ($n = 4$ or 5 animals/group). No difference among groups were observed ($P > 0.05$).

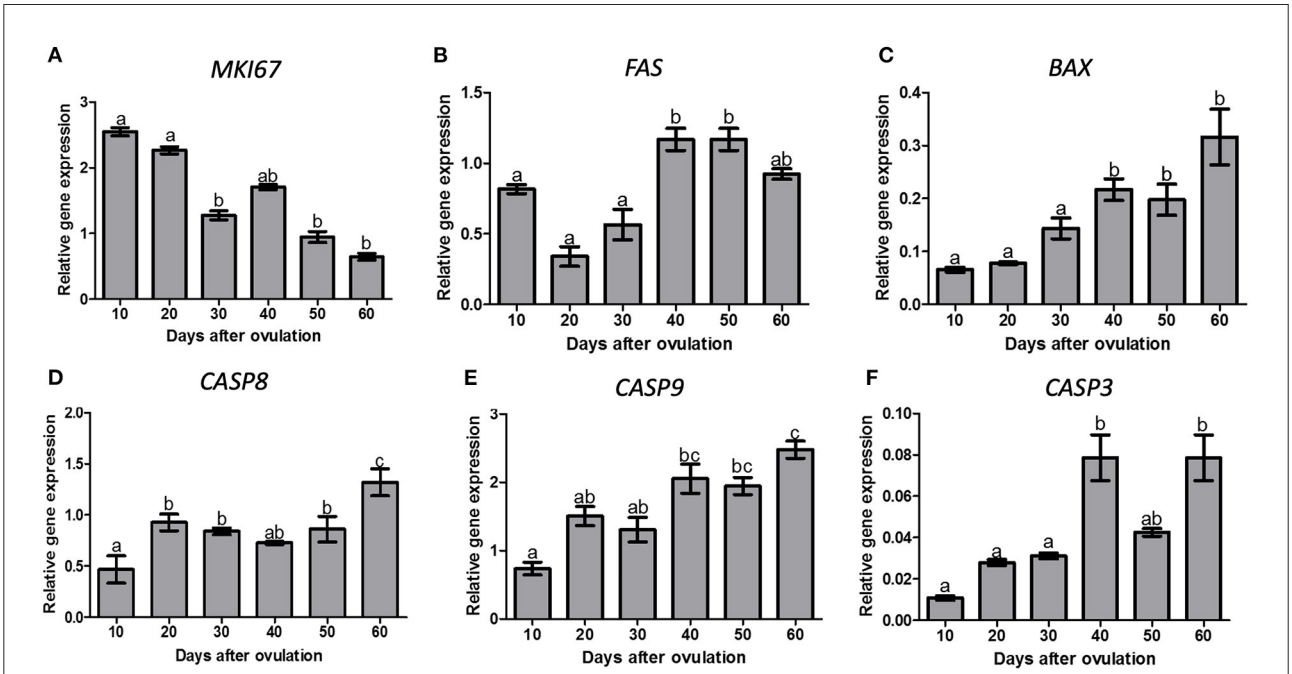

FIGURE 3
Gene expression of, MKI67 **(A)**, FAS **(B)**, BAX **(C)**, CASP8 **(D)**, CASP9 **(E)** and CASP3 **(F)** in canine CL during diestrus (10 to 60 days p.o.). Data are presented as mean ± standard error of relative gene expression ($n = 4$ or 5 animals/group). Bars with different letters indicate significant differences among groups ($P < 0.05$).

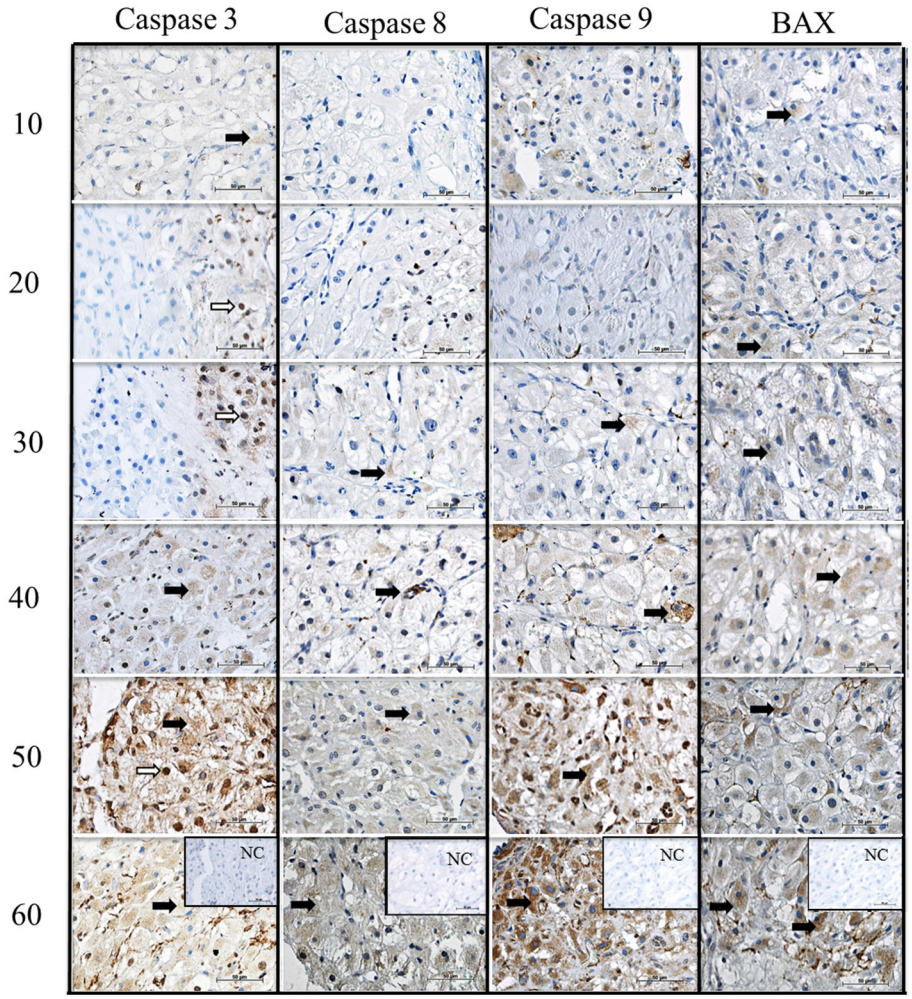

FIGURE 4
Immunolocalization of CASPASE 3, CASPASE 8, CASPASE 9 and BAX in canine CL during diestrus. Lines 10, 20, 30, 40, 50, 60, days after ovulation. NC, negative control. Black arrows indicate the cytoplasmic and white arrows indicate nuclear staining. Scale bar 50 μm.

CASP3, CASP8, CASP9, BAX, FAS, MKI67, CYP19A1, CYP11A1, HSB3B1, and *SLC2A4* gene expression in the canine luteal cells in culture after inhibiting ERa and ERb

We studied genes associated with the specific inhibiting of E2 receptors in luteal cells in three different stages of diestrus: full secretory activity (day 20), early and late luteal regression (days 40 and 60 p.o., respectively). The mRNA expression of genes related to proliferation (*MKI67*), steroidogenesis (*CYP11A1*, *CYP19A1* and *HSD3B1*) and glucose uptake (*SLC2A4*) was evaluated after inhibiting ERa and/or ERb (Figure 6). *MKI67* gene expression was increased when canine luteal cells were treated with 17b-estradiol plus ERb inhibiting; however, it was decreased under 17b-estradiol plus ERa inhibition. This suggests that E2 effects on *MKI67* happened through the ERa receptor. *CYP19A1* and *CYP11A1* followed the same pattern of *MKI67* response to ERa and ERb inhibition.

There was a significant decrease in the expression of *HSD3B1* when canine luteal cells collected on days 20 and 40 were treated with E2, whereas luteal cells treated with E2 plus ERb inhibition showed a significant increase in the relative expression of *HSD3B1*. The expression of *SLC2A4* was identified only in cells collected on day 20 p.o. The glucose transporter 4 transcript was up-regulated in cells treated with E2 + ERb inhibition compared to the E2 + ERa inhibition, emphasizing again the luteotropic effects of ERa.

Expression of apoptosis associated genes in luteal cells after treatment is shown in Figure 7. *CASP3* gene expression was increased when canine luteal cells were treated with 17b-estradiol plus ERa block. However, luteal cells collected on day 60 p.o. also showed an increase in CASP3 when treated with

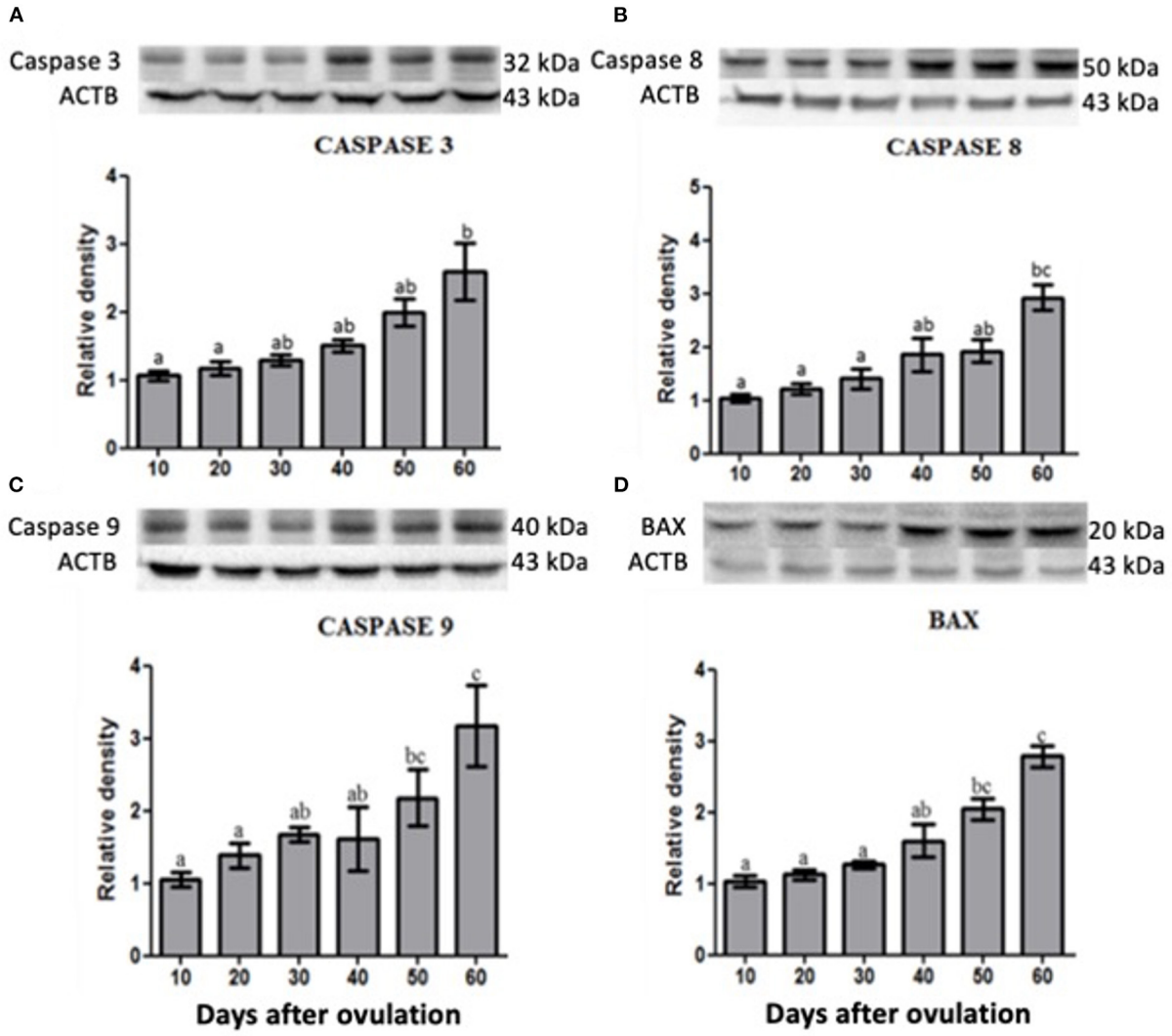

FIGURE 5
Protein expression of Caspase 3 **(A)**, Caspase 8 **(B)**, Caspase 9 **(C)** and BAX **(D)** in canine corpus luteum. Representative blots on top of each column, which represent mean ± standard error ($n = 4$/group). Different letters indicate significant differences among groups ($P \leq 0.05$).

E2 alone if compared to control cells. *CASP8*, *CASP9*, *BAX*, and *FAS* expression followed the same pattern of response to ERa and ERb inhibition, i.e., they always increase under E2 + ERa inhibition but not under ERa inhibition without E2 treatment; moreover, *CASP8* showed a significant increase on day 40 under the stimulus of E2 alone, which was not observed for the other apoptosis-related genes in any studied phase.

Discussion

Due to its long lifespan, as well as some uncertainties regarding the role of 17b- estradiol in its control, the canine CL has been chosen to study diestrus-related 17b-estradiol actions. The present study was based in the concept raised by Papa and Hoffmann (1) that the CL is both source and target of steroid hormones. Our transcriptome results followed by oPOSSUM analysis, revealed predicted DEGs over diestrus with enriched TFBS for the E2-receptor complex, suggesting E2 is involved both in the proliferative and regression phases of the canine CL. Although there are limitations of this analysis based on human ortholog genes, the assumption of evolutionarily conserved TFBS seems to be plausible (35). Such contrasting roles were possibly mediated by the selective binding of E2 to ERa and ERb, as well to the switch on *ESR1*/*ESR2* ratio observed from day 30 to 40 p.o. Moreover, the inhibition of either ERa or ERb in canine luteal cells added to our understanding of the possible roles of both receptors, as described previously for other

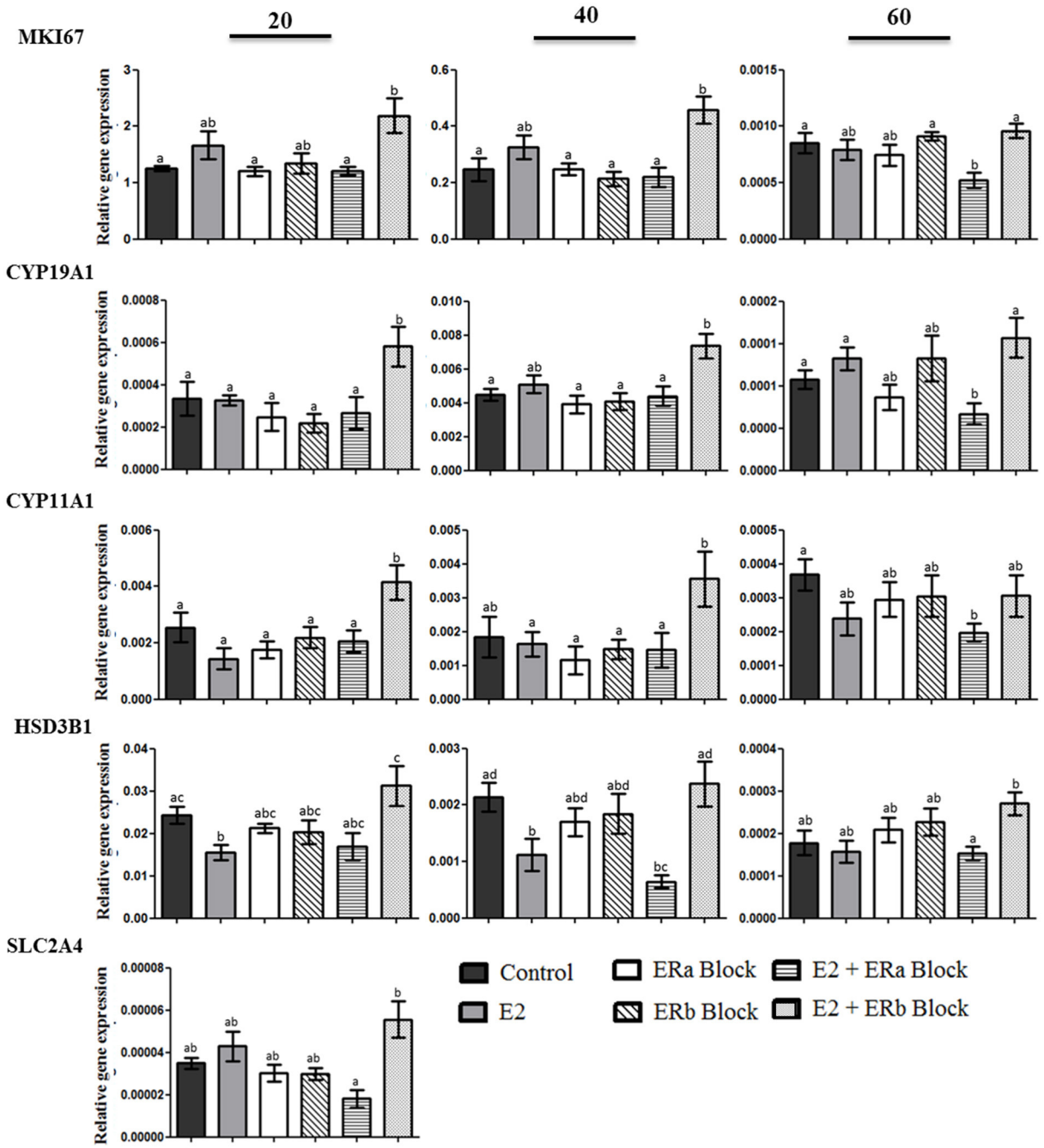

FIGURE 6
Gene expression *MKI67*, cytochrome P450, family 19, subfamily A, polypeptide 1 (*CYP19A1*), cytochrome P450, family 11, subfamily A, polypeptide 1 (*CYP11A1*), 3-β-hydroxysteroid dehydrogenase/Δ-5-4 (*HSD3B1*), solute carrier family 2 (facilitated glucose transporter), and member 4 (*SLC2A4*) in luteal cells collected at 20, 40, and 60 days after ovulation in diestrous bitches. Bars indicate six different groups: Control (no treatment), E2 (treated with 100 nM E2), ERa block (treated with 10 nM methyl-piperidone-pyrazole [MPP]), ERb block (treated with 10 uM (1,5-a) pyrimidine [PHTPP]), E2 + ERa block (treated with E2 + MPP), and E2 + ERb block (treated with E2 + PHTPP). Data represent mean ± standard error of relative gene expression ($n = 4$ animals/group). Bars with different letters indicate significant differences among groups ($P < 0.05$).

species (38) and tissues (39, 40). Comparative aspects of E2 on CL function in dogs and other species have also been recently reviewed in (41).

Increased expression of *ESR1* and consequently of the *ESR1*/*ESR2* ratio has been shown to be associated to aggressive prognostic and worse overall survival in patients with papillary

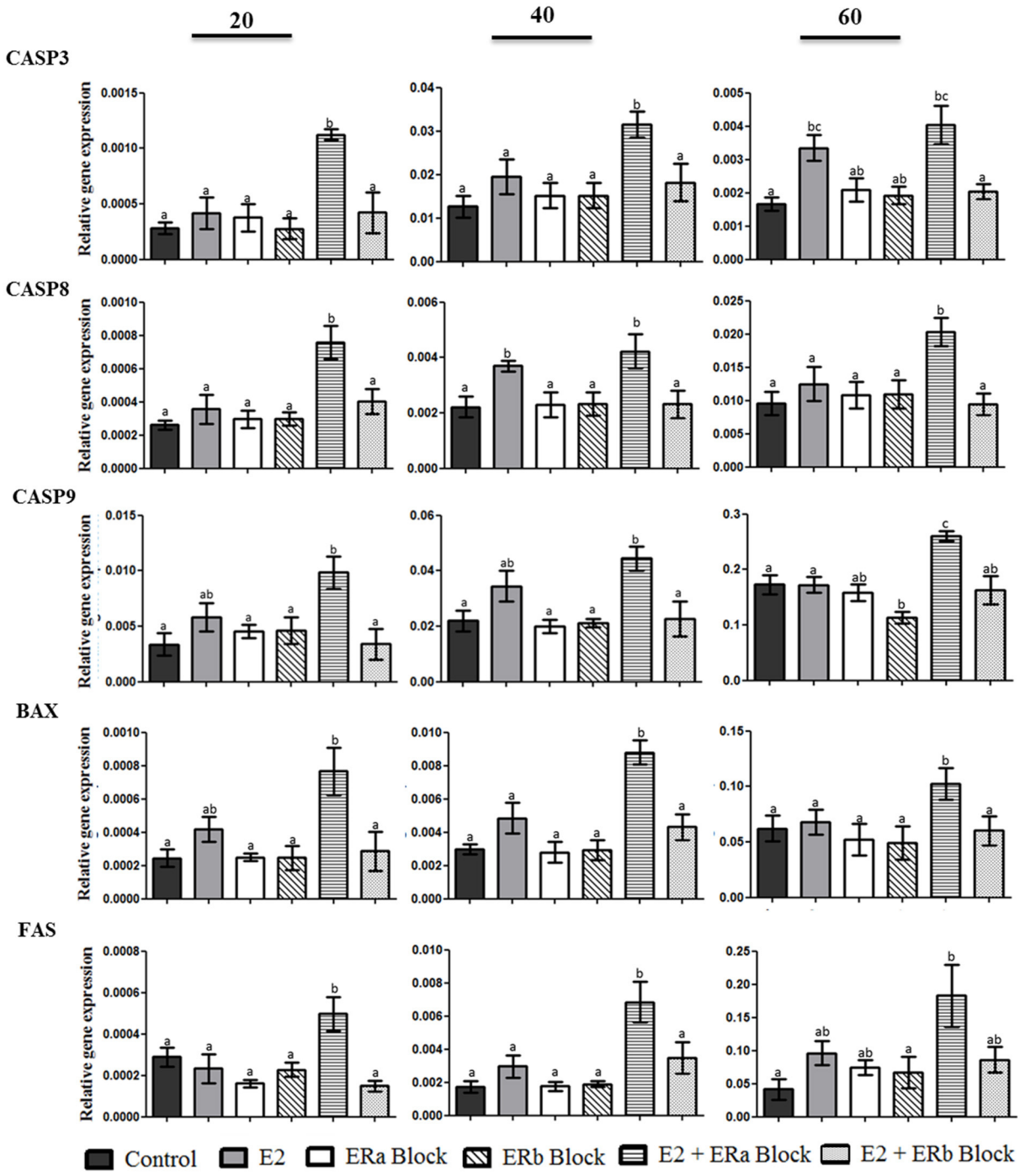

FIGURE 7
Gene expression of *CASP3*, *CASP8*, *CASP9*, *BAX*, and *FAS* in canine luteal cells collected on days 20, 40, and 60 after ovulation and cultivated for 10 days until confluence was reached. Bars indicate six different groups: Control, E2 (treated with E2), ERa block (treated with methyl-piperidino-pyrazole [MPP]), ERb block (treated with pyrazolo (1,5-a) pyrimidine [PHTPP]), E2 + ERa block (treated with E2 + MPP), and E2 + ERb block (treated with E2 + PHTPP). Data represent mean ± standard error of relative gene expression ($n = 4$ animals/group). Bars with different letters indicate significant differences among groups ($P < 0.05$).

thyroid carcinoma (42), whereas decreased *ESR1*/*ESR2* ratio in endometriosis-like phenotype mice lead to low response to P4 and subfertility (43). Moreover, during physiological development of rat Sertoli cells, it has been reported that *ESR1*/*ESR2* ratio decreases with age and this shift seems to be important for termination of proliferation and begin

of differentiation period (44). Also in canine CL the ratio *ESR2*/*ESR1* has been shown to be dependent on the pregnancy and developmental stages (6) (also addressed below). These previous studies, using different species, cells, and organ models, further highlight the different biological effects mediated by the two E2 receptors. Similarly, our results show a switch of *ESR1*/*ESR2* ratio from day 30 to 40 p.o. matching the end of CL maintenance phase and the start of the regression phase. Sonnack (45) used transmission electron microscopy to illustrate morphological changes in canine non-pregnant CL on day 45 p.o. (early luteal regression): the smooth endoplasmatic reticulum of luteal cells shows the first morphological changes and signs of degenerative transformation accompanied by fatty degeneration, mirrored by the deposition of lipid vacuoles in the cytoplasm.

Cancer related research corroborates the idea of ERa acting as a proliferative mediator and ERb as an anti-proliferative agent (42). Moreover, when present in the same cell, as in the case of canine luteal cells, ERb induces the formation of ERa/ERb heterodimers that are less active than ERa homodimers, and thereby work as an ERa repressor (46). Recently, the *ESR2*/*ESR1* ratio was reported for the gestational and non-gestational canine CL (6) and, although not matching completely the ratio reported in the present work, one could observe the mentioned transition in the abundance of transcripts reflected in the increased *ESR2*/*ESR1* ratio (6). More in detail, authors describe a gradual and significant increase in the *ESR2*/*ESR1* ratio between days 10 and 30, and further toward day 40 p.o. followed by a decrease toward the end of luteal phase (6). Interestingly, in the same study (6), the abundance of *SULT1E1* was assessed and was increased during late dioestrus, at days 50 and 60. Based on that, the authors proposed the functional involvement of *SULT1E1* (converts active oestrogens into biologically inactive estrogen sulfates) in the functional withdrawal of E2 in regressing CL (6). Similar conclusions were implied from transcriptomic studies, showing increased expression of *SULT1E1* in regressing CL (23).

Our results from canine luteal cells in culture demonstrated that E2 + ERb block can induce an increase in *MIK67* mRNA expression on days 20 and 40 p.o. in comparison to control group, which cannot be achieved by E2 alone or in combination with ERa block (Figure 6, $p < 0.05$). The same pattern was observed for mRNA expression of steroidogenic enzymes (*CYP19A1* and *CYP11A1*). These mRNAs encode luteotropic proteins (1, 36, 37) aimed to drive luteal function, i.e., progesterone production to its plenitude. Besides LH and prolactin, insulin can also be considered an endocrine luteotropic factor in the canine CL (36) and its contribution to E2 production cannot be ruled out (25). The mechanism of E2 binding to ERa and leading to proliferation is opposed to binding to ERb (47) leading to expression of anti-proliferative genes such as *p-53*, *PI3K* and *Akt*, as well as the increase of stress associated and apoptosis related proteins (48), as also observed in our study.

Although caspase expression is normally associated with apoptosis, other functions in homeostasis have been attributed to them: e.g., caspase 3 participates in bone marrow stromal stem cell differentiation together with caspase 8, which is also involved in T-cell maintenance (49), both mechanisms dissociated from apoptosis. Caspases also participate as regulators of tumorigenesis, since genes involved in cell death have normally a tumor suppressor function (49). This explanation sounds reasonable to justify the increasing amounts of caspases and BAX observed in the second half of diestrus, especially in the phase of late luteal regression (day 60 p.o.), emphasizing the role attributed to E2 binding to ERb, which leads to an anti-proliferative effect. It is worth mentioning that the canine non-pregnant CL does not show signs of apoptotic degeneration during regression (45) and no over-represented biological function was associated with apoptosis when analyzing transcriptomic data (23, 26). On the contrary, the canine pregnant CL shows apoptosis as one of the over-represented biological functions during luteolysis (23).

These findings correlate very nicely with *in vivo* E2 plasma concentration (5) and ERs expression, which acquired an opposite pattern after day 40 p.o. The authors hypothesize that a slight E2 increase as well as the switch of increasing to diminishing *ESR1*/*ESR2* ratio support the canine CL to initiate programmed regression mechanisms, which deserves further experimental confirmation, especially because E2 concentrations used in cell culture, which were able to elicit a response, are above the plasma physiological concentrations. In the absence of E2-ERb drive, the canine diestrous CL could deviate from the physiologic path and go into uncontrolled proliferative conditions, as described for some breast cancers in which ERb expression was dysregulated (50).

In general, inhibition of ERa or ERb in the absence of E2 did not affect gene expression. In contrast, under E2 influence, ERa inhibition permitted upregulation of anti-proliferative factors such as caspases, *BAX* and *FAS,* and ERb inhibition stimulated transcription of proliferative and luteotropic factors such as *MIK67* and steroidogenic enzymes, suggesting the need of the ligand to promote different biological functions. Based on the presented results, it could be hypothesized that manipulating the functionality of ERs could provide a good future tool to regulate luteal life span in dogs, which certainly deserves further research.

No significant difference was observed for mRNA expression validated by qPCR of the selected upstream regulators (*LEF1*, *CTNNB1* and *CCND1*) of ERa and ERb, or E2 target molecules (*NDRG2* and *ATP1A1*). RNAseq analyses were carried out with 3 samples per group, and when adding another three samples for the validation process, no significant difference was observed. Other studies have reported a high variation in gene and protein expression among canine CL samples from the same stage of diestrus (26, 51) not always matching RNAseq data, which made the present observations not surprisingly. Nevertheless, in accordance with the bioinformatic analyses performed for

the above-mentioned genes, which were differentially expressed over canine diestrus, as depicted from RNAseq, and presented a TFBS for ERa and/or ERb concomitantly, it was possible to visualize canonical pathways (Supplementary Table 3) in which *LEF1*, *CTNNB1*, *CCND1*, *NDRG2* and *ATP1A1* might be part during canine CL lifespan.

As target molecule for E2, *NDGR2* was qualitatively (qPCR) and quantitatively (RNAseq) more expressed after day 40 p.o. in canine CL. It is considered a tumor suppressor gene, activated in non-cancer cells under stress situations, leading to suppression of cell proliferation, protein synthesis and inducing cell death (52). Besides being a target molecule for E2, *via* binding to ERb, which in turn regulates *NDGR2* expression *via* transcriptional activation (53), it participates in other canonical and non-canonical pathways, such as *CTNNB1* and *NF-kb*, respectively; both genes also present a TFBS for *ESR2*, which expression in this and other study (6) increases after day 40 p.o. In many malignant tumors, *NGDR2* is able to suppress endothelial cell proliferation and enhance apoptosis by increasing p53 expression (54, 55). The highest p53 gene expression in canine non-pregnant CL was found on day 60 p.o. (26), suggesting a possible contribution of E2 in CL regression mediated by ERb, involving also the apoptotic mechanism and corroborating our functional studies in canine luteal cell culture.

In the present study *ATP1A1* expression increased qualitatively just before the structural regression started and it has already been reported to show a positive correlation with *NDGR2* protein and gene expression (56). In porcine preovulatory luteinized follicles, *ATP1A1* was functionally classified as a cell growth inhibitor (57). A decrease in *ATP1A1* expression has also been observed in several human cancers such as prostate, kidney and bladder, which lead to accelerated proliferation (56, 58, 59). The increased expression of *ATP1A1*, as seen in our RNAseq data, points toward an activation of *ATP1A1* transcription after binding of E2 to ERb, which seems to be necessary to reduce canine CL proliferation and initiate regression.

The *CTNNB1* gene, encoding for catenin b, and *CCND1*, encoding for cyclin D1, are in one hand direct upstream regulators of *ESR1* and *ESR2*. According to their pattern of expression depicted by RNAseq and their function (Supplementary Table 3), they act as repressors of *ESR2* and enhancers of *ESR1* transcription in canine CL in the first half of diestrus. It was also reported that *LEF1* can act as transcriptional repressor for E2 by competing with ERs to bind to DNA (60). LEF1 also participates in several canonical pathways driven by *ESR2* upstream regulators (Supplementary Table 3). Although its mRNA expression did not show time-dependent differences, RNAseq data pointed toward an increased expression in the beginning of diestrus, which corroborates its presumable repressive action on *ESR2*. On the other hand, E2 and related compounds have been shown to exert their proliferative effects by binding to ERa (61), inducing for example *CCND1* transcription, a key regulator of cell cycle progression (62–64). Cell proliferation was reported to be greater in the first half of diestrus and highest until day 15 p.o. in canine non-pregnant CL (1), matching our reported *ESR1/ESR2* ratio, E2 plasma concentrations (5) and cell culture inhibiting assays (Figure 6). It is noteworthy that new approaches we used to unravel E2 mechanisms of action in the canine CL brought complementary data in complete agreement with previously published data.

In conclusion, E2 plays a pleiotropic role in canine corpus luteum, from formation until regression. Several genes and proteins are affected by E2 through its binding to ERs in a time-dependent manner. The number of predicted genes differentially regulated over diestrus in the canine CL showing transcription factor binding sites for *ESR1* and *ESR2* points toward a much broader role of E2. Additionally, the *ESR1/ESR2* ratio associated with E2 fluctuations (5) over diestrus suggests possible underlying regulatory mechanism involved in autocrine and paracrine regulation of canine CL lifespan.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics statement

The animal study was reviewed and approved by Committee of Ethics in the use of Animals of the School of Veterinary Medicine and Animal Science of the University of São Paulo (protocol number 2719/2012). Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

AB: formal analysis, investigation, data curation, and writing-original draft. AC: formal analysis, sample collection, and data curation. RS and LS: formal analysis, investigation, and data curation. IG: sample collection, validation, investigation. MB and SB: investigation, review, and editing the manuscript. MK: review and editing the manuscript. PP: conceptualization, formal analysis, investigation, data curation, writing, funding acquisition, and supervision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.885257/full#supplementary-material

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Incidence and concomitant factors of cesarean sections in the bitch: A questionnaire study

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Dystocia in the canine species is a common problem, and elective cesarean sections (C-sections) have become more frequent in breeds that are at risk. The aim of this study was to evaluate the incidence of C-section and contributing factors and to compare data on elective and emergency C-sections (e.g., regarding stillbirth). Using a questionnaire, a total of 423 bitches of 80 breeds and their 899 litters were included. The mean number of litters per bitch was 2.1 ± 1.1 litters. The overall rate of stillbirth was 6.7%. Of all litters, 194 were born *via* C-sections (21.6%), of which 35 were declared as elective and 159 as emergency due to dystocia. Significantly more C-sections were performed in either small litters (1-2 pups) or large litters (>12 pups) ($p<0.001$). Bitches that have had prior C-sections had a 4-fold increase in the risk of successive C-sections (RR = 4.54 (95%CI 2.56–7.70; $p < 0.001$). Furthermore, primiparous bitches of advanced age had a significantly higher incidence of emergency C-sections ($p = 0.004$). Stillbirth was significantly higher in emergency C-sections compared with that in elective C-sections ($p = 0.003$). Also, timing of intervention had a significant impact on stillbirth in emergency C-sections ($p = 0.025$). Within a breed-specific evaluation, significant differences were observed between breeds regarding incidence of C-section and stillbirth. Lesser-known breeds were represented in the population, and the results showed that the Norwich Terrier had the highest (51.6%) and the Gordon Setter had the lowest (4.8%) incidence of C-section ($p < 0.001$). The inclusion and evaluation of lesser-known breeds regarding incidence of C-section is of importance as it shows that certain breeds without phenotypical traits such as brachycephaly may also have an increased incidence of emergency C-section and stillbirth. We further conclude that more importance may be given to the age at first parturition concerning the occurrence of dystocia and the decision making regarding possible elective C-sections.

KEYWORDS

cesarean section, dystocia, dog, stillbirth, parity

Introduction

Dystocia in the dog is a common problem (1–3), and several risk factors have been described in mixed-breed as well as purebred canine populations (1–5). The medical treatment of dystocia is possible in certain cases, yet the success rate is often low for most cases requiring a cesarean section (C-section) (4, 6). Although certain risk factors

for dystocia are well described, such as breed (7) or singleton pregnancy (8), others are often spoken about within the community of breeders (e.g., the size of the male in relation to the size of the female, prior C-sections of the female and others). Dystocia may have many causes and may in some cases be difficult to identify, especially by unexperienced breeders. It is important to anticipate which pregnancies are at particular risk in order to organize proper medical intervention to ensure the wellbeing of both the mother and her progeny. Elective C-sections are planned ahead of time and may be performed on either a "parturient" (with an open cervix) or "preparturient" (with a closed cervix) bitch (9). Most commonly the term elective C-section refers to a parturient C-section (9) in the absence of evident contractions, expulsion, and/or signs indicating dystocia. This approach has become more popular in small animal reproduction medicine over the last few years (9), particularly in bitches, which are considered to be at high risk of dystocia (8, 9). Various publications evaluated protocols and methods to ensure a successful C-section on the correct date (10–12). Although certain factors such as breed, age, and brachycephaly have been identified as potential risk factors for dystocia (4, 7, 13), there is no standardized and generally accepted rule on how to identify bitches that would benefit from undergoing an elective C-section (14). For this study, we used an online questionnaire distributed through breeders' clubs and social media which gave breeders of different breeds the possibility to respond to specific questions to contribute to the knowledge on potential influencing factors for dystocia and cesarean section in the dog. The aim of our study was to evaluate the incidence of C-section in a population of different breeds, to investigate contributing factors to the occurrence of C-section, and to compare data on elective and emergency C-sections (e.g., stillbirth) as well as to evaluate the impact of C-sections on the rate of stillbirth.

Materials and methods

Data collection was performed *via* a convenience questionnaire distributed to breeders of German-speaking countries (Austria, Germany, Switzerland) as a Microsoft EXCEL® file or as an online questionnaire using the online platform Survey Monkey (https://www.surveymonkey.com) in German language. Executive committees of breeders' clubs were contacted *via* email and offered the opportunity to participate and/or distribute the questionnaire within their community of breeders. Furthermore, social media, in particular Facebook, was used to inform breeders about our research project and to distribute the questionnaire in both its original (Excel) and its online version. Data collection was taken place between October 2020 and May 2021, and no limit was set regarding the date of birth of either the bitches or the litters as long as the breeders were able to communicate the data based on the records they have kept. The collected data for bitches included breed, date of birth, number of litters, number of C-sections, and an evaluation of the breeders regarding housing, the character, the topline, and the body condition (BC) of the dam. The participants were able to classify the topline of the dam as either level, sloping, or high in the rear. The character of the bitch was classified by breeders as either (a) calm and balanced, (b) calm and playful, or (c) fearful, nervous, and/or insecure. Both factors are hardly measurable and based on the breeders impression/evaluation. The breeders were asked to insert all litters a dam had to provide a complete picture of the dam's reproductive career, regardless of the presence or absence of C-sections within the dam's history. Parity of the dam was classified as either primiparous or pluriparous. The exact number of litters in pluriparous bitches was not used in the statistical analysis. Personal information on the breeders was not collected.

The collected data for litters included date of birth, number of pups born, number of pups stillborn, and type of parturition (eutocic/cesarean section). Information collected for cases of cesarean sections included reason for C-section (emergency C-section or elective C-section), time of intervention (prior to or after the birth of the first pup), and number of pups delivered alive or dead during C-section. Parity of the bitch and the number of C-sections that have occurred prior to the date of birth of each litter were recorded. The owner was further asked to classify his experience as either unexperienced (1–2 parturitions), medium level of experience (3–4 parturitions), or high level of experience (>5 parturitions) depending on the number of births she/he had seen and followed.

Microsoft® Excel for Mac (version 16.16.25) was used to create the final dataset for statistical evaluation. All statistical analyses were performed using XLStat (Copyright Addinsoft 1995–2021) and SAS (Copyright © 2002–2022 by SAS Institute Inc., Cary, NC, USA). Descriptive statistics were calculated. Counting data (expressed as percentages) were compared using the k-proportions test, z-test for comparisons of two proportions (with correction for continuity), and chi-square test (or Fisher's exact test when required). Multiple chi-square tests were performed using the Marascuilo approach with Bonferroni's correction. For binary response variable C-section, a logistic regression was performed using the fixed effect of age (five classes) as predictors and the parity (primiparous vs multiparous), prior C-section, and the number of total pups as covariates. The effect of bitch was included in the model as a random effect (PROC GLIMMIX of SAS). Relative risk (RR) and 95% confidence intervals (95%CI) were calculated as the exponential of the estimated coefficients for the binary model. For breeds with at least 20 litters, an analysis of variance was conducted on the total number of pups using a mixed liner model with the fixed effects of breed, type of delivery (C-section vs no C-section), and interaction (PROC MIXED of SAS). The bitch effect was included in the model as a random effect. For stillborn, an ANOVA mixed model was adopted, which included

the fixed effects of breed, experience of the operator, and number of prior C-sections (0,1,2) and bitch as a random effect. For all the analyses, post-hoc pairwise comparisons between least-square means were made using Bonferroni's correction.

Results

General aspects and information on the population

Information on a total of 423 bitches belonging to 138 breeders and their respective litters, accounting for a total of 899 litters, was collected and included in the statistical evaluation. Forty-three bitches were born between January 1, 1980, and December 31, 1999, 129 bitches were born between January 1, 2000, and December 31, 2009, and the majority of bitches (n = 251) were born after January 1, 2010. These bitches belonged to 80 different breeds, of which 79 were acknowledged by the Federation Cynologique Internationale (FCI), whereas one was recognized only by national kennel clubs (Silken Windsprite). The breed distribution is presented in Table 1. No bitches or litters of mixed breeds were included in our population.

The age of the dams at each whelping ranged between 1.1 and 10.6 years, with a mean of 4.3 ± 1.7 years. The bitches included in the sampled population had a minimum of 1 litter and a maximum of 7 litters. The mean number of litters was 2.1 ± 1.1 litters per bitch.

Information on breeders' experience at the moment of birth was available for 896 litters. Breeders described themselves as either unexperienced, medium level of experience, or high level of experience in 21, 17.6, and 61.4% of cases, respectively. No statistically significant differences were observed when evaluating the breeders' experience.

Information on housing was provided for 421 bitches. Indoor housing was defined as indoor (no access to any outdoor area such as a backyard), outdoors (housing exclusively in kennels outdoors), and mixed housing (bitches living inside the house with access to an outdoor area). The majority of bitches were kept in mixed housing (72.9%), 26.4% of bitches were housed indoors, and only 0.7% of bitches were housed exclusively outdoors.

The collected data on the character of the bitch, the BC, and the topline were as might be expected with the majority of bitches being of calm/balanced character (53.1%), normal BC (92.1%), and level topline (92%). Numbers of answers which differed from the majority were few. Therefore, these factors were not included in the statistical analysis. Information on the overall distribution of data as well as the distribution in eutocic parturitions and C-sections of breeders' experience, housing, character, BC, and topline is given in Table 2.

A total of 899 litters were included in the statistical analysis for a total of 5,615 pups. The litter size ranged between 1 and 14 pups with a mean of 6.3 ± 2.9 pups. Of all pups born, 379 were registered as stillborn with a maximum of 9 stillborn pups within one litter and a mean of 0.4 ± 0.9 pups per litter. The overall stillbirth rate was therefore 6.7%. The distribution of total number of pups and number of stillborn pups for the respective breeds is presented in Table 1.

Parity of the dam was recorded as either primiparous or pluriparous at the time of whelping. Of all litters born, 419 were born by primiparous bitches, whereas 480 were born by pluriparous bitches.

Investigation into cesarean section

Of 899 litters, 705 (78.4%) and 194 (21.6%) litters were born *via* eutocic parturition and C-section, respectively. Of these 194 C-sections, 35 (18%) were elective procedures accounting for 3.9% of all litters born and the 159 C-sections due to parturition emergencies correspond to an incidence of 17.7% in our population. Out of the 5,615 pups born in total, 4,652 (82.8%), 116 (2.1%), and 847 (15.1%) pups were born *via* eutocic parturition, elective cesarean sections, and emergency C-section, respectively.

A size in litters born *via* an emergency C-section ranged between 1 and 14 pups with a mean of 5.3 ± 2.9 pups. A litter size in an elective C-section ranged between 1 and 9 pups with a mean of 3.3 ± 2.6 pups. Overall C-sections were significantly more common in litters with 1 or 2 pups (51.6%) than in litters with a high number of pups (>12 pups, 22.7%) or in litters with 3–11 pups (18.1%, $\chi^2_{11} = 75.81$ $p < 0.001$).

When considering the timing of emergency surgery during parturition, C-section was performed in 96 litters (60.4% of emergency C-sections) prior to and in 63 litters (39.6% of emergency C-sections) after the birth of the first pup. Elective C-sections were excluded from the evaluation of this value.

Of the 620 pups delivered during emergency C-sections, 20.5% were stillborn. When emergency C-sections were performed prior to the birth of the first pup, 373 pups were delivered alive, whereas 83 pups were stillborn, accounting for a rate of stillbirth of 18.2%. When emergency C-sections were performed after the birth of the first pup, 120 pups were delivered alive, whereas 44 pups were stillborn, accounting for a rate of stillbirth of 26.8%. This difference in the percentage of stillborn puppies delivered during C-sections performed prior to (18.2%) or after (26.8%) the birth of the first pup was significant ($z = 2.23$; $p = 0.025$). In elective C-sections, 111 pups (95.7%) were delivered alive, whereas five (4.3%) were stillborn. Mortality in elective C-sections (4.3%) was therefore significantly lower than that in emergency C-sections ($z = 2.99$; $p = 0.003$). Stillbirth (at least one stillborn pup) was recorded in 11.4% of all elective C-sections vs. 44.7% of emergency C-sections ($z = 3.54$; $p < 0.001$). A comparison of these results is shown in Figure 1. Elective C-sections were performed

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TABLE 1 Breed distribution within our population.

Breed	N° dams	N° litters	N° C-section	N° pups	N° stillborn pups
American Staffordshire Terrier	2	3	1	26	0
Australian Cattle Dog	1	1	0	7	0
Australian Shepherd	6	11	0	70	2
Austrian Black and Tan Hound	1	2	1	10	0
Bearded Collie	4	12	6	87	6
Belgian Shepherd Dog	7	15	1	111	6
Berger Blanc Suisse	1	2	0	15	0
Bernese Mountain Dog	29	71	19	550	48
Black Russian Terrier	6	11	5	85	8
Border Collie	12	27	5	139	19
Border Terrier	2	5	1	16	0
Borzoi	1	1	1	2	0
Boston Terrier	1	1	1	4	1
Boxer	5	12	2	71	6
Bullmastiff	4	6	3	44	7
Bullterrier (Standard)	1	1	1	10	0
Chihuahua	2	3	0	13	3
Chow-Chow	3	5	0	24	2
Continental Bulldog	1	1	0	13	3
Continental Toy Spaniel	2	4	0	13	0
Dachshund Smooth Haired	1	1	0	5	0
Dachshund Wire-haired	18	47	14	224	4
Deerhound	4	5	0	42	0
Do Khyi (Tibetan Mastiff)	5	12	2	94	17
Doberman Pinscher	1	1	1	4	3
English Cocker Spaniel	4	10	1	48	4
English Setter	5	10	4	62	1
Entlebuch Cattle Dog	3	8	1	42	1
Eurasian	9	19	3	122	4
Flat Coated Retriever	1	2	0	11	0
French Bulldog	4	6	4	31	1
German Shepherd	10	22	2	177	19
German Wolfsspitz	1	1	0	7	0
Golden Retriever	22	49	11	353	18
Gordon Setter	10	21	1	193	8
Great Dane	3	4	2	20	4
Great Swiss	4	10	4	72	26
Groenendael	7	14	2	85	1
Hanoverian Scent Hound	2	3	0	19	7
Havanese	3	9	1	47	5
Hovawart	43	101	13	839	29
Icelandic Sheepdog	1	2	0	10	0
Irish Glenn of Imaal Terrier	3	6	4	32	0
Irish Red and White Setter	1	1	0	6	0
Irish Terrier	5	11	0	66	1
Labrador Retriever	7	12	1	110	5

(Continued)

TABLE 1 (Continued)

Breed	N° dams	N° litters	N° C-section	N° pups	N° stillborn pups
Large Münsterländer	3	7	0	72	3
Manchester Terrier	1	1	0	5	0
Miniature American Shepherd	1	1	0	3	0
Miniature Bull Terrier	11	20	4	105	8
Miniature Dachshund Long-haired	4	9	1	32	1
Miniature Dachshund Wire-haired	2	3	2	6	2
Miniature Poodle	1	1	0	5	0
Miniature Schnauzer	3	7	3	27	1
Newfoundland	12	22	7	119	13
Norfolk Terrier	11	27	3	88	7
Norwich Terrier	12	31	16	99	22
Parson Russell Terrier	8	19	2	87	4
Portuguese Water Dog	1	1	0	14	0
Pug	2	4	2	12	1
Pyrenean Mountain Dog	2	3	1	32	0
Rhodesian Ridgeback	2	5	1	48	1
Rough Collie	11	15	0	87	3
Saarloos Wolfhound	1	1	0	2	1
Schipperke	4	10	2	36	1
Scottish Terrier	9	16	12	73	17
Shetland Sheepdog	8	16	4	55	3
Silken Windsprite*	1	1	0	7	0
Small Münsterländer	1	1	1	2	1
Smooth Collie	3	8	0	57	4
Spinone Italiano	2	4	1	34	1
Staffordshire Bullterrier	3	8	0	65	0
Terrier Brasileiro	6	7	0	52	0
Tibetan Spaniel	8	23	5	126	7
Tibetan Terrier	10	26	4	142	5
Toy Poodle	3	7	1	25	0
Welsh Corgi Cardigan	1	1	0	7	0
Welsh Corgi Pembroke	1	2	1	10	4
West Highland White Terrier	4	8	2	28	0
Whippet	3	3	1	22	0
Total	**423**	**899**	**194**	**5,615**	**379**

N° dams, number of bitches enrolled in the study; N° litters, number of litters included in the study; N° C-section, number of C-sections for each respective breed; N° pups, total number of pups born; N° stillborn pups, total number of stillborn pups; *breed not recognized by the FCI.

more frequently in litters with a single pup (12.9%) than in pregnancies with two or more pups ($z = 14.51$; $p < 0.001$). Of all bitches in our population, 35.5% have had at least one C-section, and prior C-sections were reported in 81 out of 899 litters (9%). Emergency C-sections were performed in 16.7% of all primiparous litters and in 18.6% of all pluriparous litters ($p = 0.518$), whereas elective C-sections were performed in 2.9 and 4.8% of all primiparous and pluriparous litters, respectively. Of all emergency C-sections, 56% have been performed in pluriparous litters. A similar result has been found for elective C-sections as the majority of them (65.7%) were performed in pluriparous litters. In order to further assess the role of age on the incidence of cesarean sections, we divided the bitches into the following five age groups: group 1 < 2 years, group 2 between 2 and 4 years, group 3 between 4 and 6 years, group 4 between 6 and 8 years, and group 5 > 8 years. The observable increase in the incidence of cesarean section with increasing age was not statistically significant ($\chi^2_4 = 6.29$; $p = 0.172$). However, when considering parity of the dam within each age group, there was a significant increase in the incidence of cesarean sections in

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TABLE 2 Description of number of responses and percentages regarding breeders' experience and the dams housing, character, body condition, and topline.

		Overall		Eutocic parturitions		C-sections	
		N	%	*N*	%	N	%
Experience							
	Unexperienced	188	210%	155	22.0%	33	17.1%
	Medium level	158	17.6%	117	16.6%	41	21.2%
	High level	550	61.4%	431	61.3%	119	61.7%
	Total	896	**100.0%**	703	**100.0%**	193	**100.0%**
Housing							
	Only indoors	111	26.4%	72	26.6%	39	26.0%
	Indoors and outdoors	307	72.9%	196	72.3%	111	74.0%
	Only outdoors	3	0.7%	3	1.1%	0	0.0%
	Total	421	**100.0%**	271	**100.0%**	150	**100.0%**
Character							
	Calm/balanced	223	53.1%	139	51.3%	84	56.4%
	Calm/playful	174	41.4%	122	45.0%	52	34.9%
	Fearful/nervous/insecure	23	5.5%	10	3.7%	13	8.7%
	Total	420	**100.0%**	271	**100.0%**	149	**100.0%**
BC							
	Normal	385	92.1%	253	94.1%	132	88.6%
	Underweight	18	4.3%	13	4.8%	5	3.4%
	Overweight	15	3.6%	3	1.1%	12	8.1%
	Total	418	**100.0%**	269	**100.0%**	149	**100.0%**
Topline							
	Level	382	92.0%	249	93.3%	133	89.9%
	Sloping	10	2.4%	6	2.2%	4	2.7%
	High in the rear	23	5.5%	12	4.5%	11	7.4%
	Total	415	**100.0%**	267	**100.0%**	148	**100.0%**

N, number; %, percentage; Experience, breeders' experience; BC, body condition.

primiparous bitches with increasing age (12.2, 17.2, 27, and 50%, respectively, in groups 1–4; $\chi^2_3 = 13.32$ $p = 0.004$). The logistic regression confirmed that age and parity were not significant regarding the C-section when evaluated separately, whereas prior C-section and total number of pups were significant. The presence of prior C-section increased the risk of a C-section in the following whelpings 4-fold (RR = 4.54; 95%CI 2.56–7.70; $p < 0.001$). Regarding litter size, the logistic regression has shown a decrease for the risk of C-section for each pup more up to 11 pups (RR = 0.80; 95% CI 0.75–0.86; $p < 0.001$). The results of the logistic regression remained the same after the exclusion of elective C-sections from the evaluation.

Breed-specific evaluation

To provide a breed-specific evaluation, we included only breeds that were represented with at least 20 litters as given in Tables 3, 4. A significant difference between these breeds was observed in the overall incidence of C-sections ($\chi^2_{12} = 38.46$; $p < 0.001$), with the Norwich Terrier having the highest (51.6%) and the Gordon Setter having the lowest (4.8%) incidence of C-section. The Norwich Terrier was also the only breed in which C-sections in age group 1 (<2 years) have been reported. Bernese Mountain Dogs and Newfoundland bitches had a greater litter size when experiencing eutocic parturitions compared with litters born *via* C-sections ($p < 0.001$). The mean litter size in Bernese Mountain Dogs was 8.5 ± 2.5 pups in eutocic parturitions and 5.6 ± 3 pups in litters born *via* C-section. In the Newfoundland, the mean litter size in eutocic parturitions was 6.8 ± 3.2 pups and 2.4 ± 1.5 pups in litters born *via* C-section. Information on the litter size based on the type of parturition for each breed is given in Table 4. We furthermore observed a significant difference in the number of stillborn pups among breeds ($p < 0.001$), with the Norwich Terrier having the highest (22.2%) and the wire-haired Dachshund the lowest (1.8%) stillbirth rate. When evaluating the incidence of stillbirth in C-sections, we may say that the German Shepherd

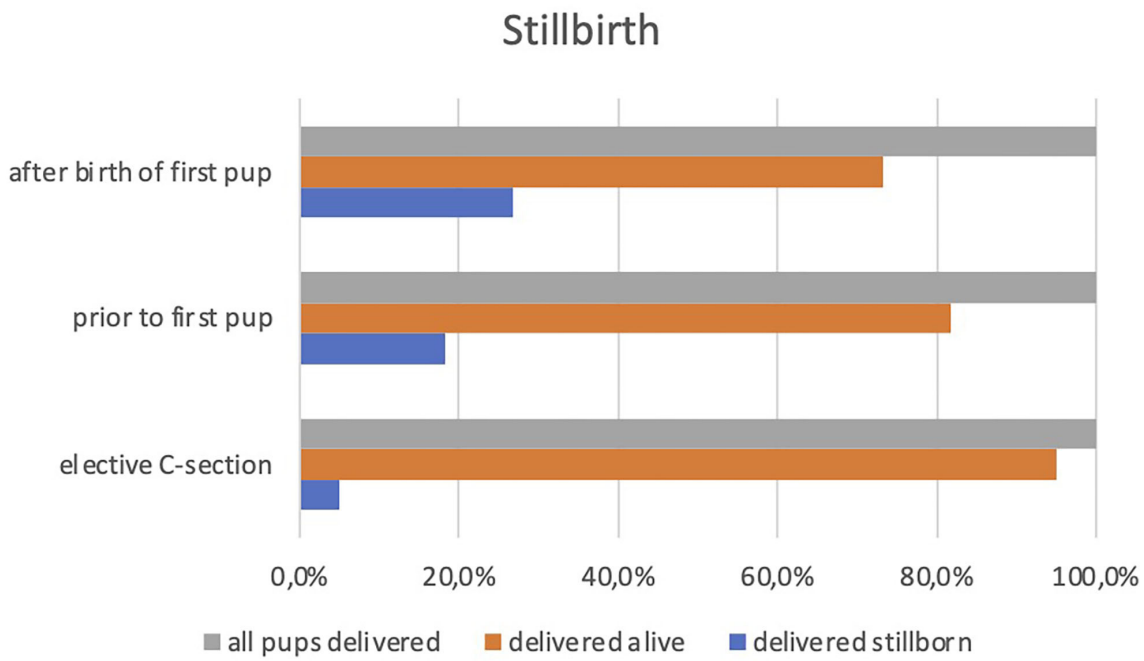

FIGURE 1
Comparison of the percentage of pups delivered alive and pups delivered stillborn during emergency C-sections performed after the birth of first pup and prior to the first pup and elective C-sections.

(71.4%) and the Border Collie (50%) both had a significantly higher number of stillborn pups compared with the other breeds (11.8%, $p < 0.001$). Potential differences between emergency C-section and elective C-section could not be evaluated in a breed-specific manner due to the limited amount of data per breed.

Discussion

Dystocia in the canine species is a frequently encountered emergency (14, 15). Although pharmacological treatments are available, the majority of bitches presenting with dystocia need to undergo a cesarean section (1, 6, 13). Several risk factors for the occurrence of dystocia have been described in various publications (1, 4, 8, 13, 15, 16), including breed, litter size, size of the fetus in regard to the dimension of the pelvic canal, and others. C-section has long been considered an emergency surgery (8, 17); however, the increasing reports on the benefits of elective C-sections have led to the conclusion that in certain cases an elective C-section, regardless of anesthesiological and surgical risks, has more benefits for both the mother and the litter than disadvantages (1, 9, 14). It has been reported that especially breeders of breeds highly at risk opt more frequently for an elective C-section (4, 18). Therefore, different protocols have been described regarding the timing and preparation for elective C-sections as the correct timing is crucial to the survival of the neonates (10–12, 16, 19). Although little information may be found in the literature regarding the incidence of elective C-sections in populations composed of different breeds, an incidence of up to 32% has been reported (14). In populations of breeds at particularly high risk of dystocia, such as the English Bulldog (20), the incidence of elective C-sections may even be approaching 100% (21). Within our population, the incidence of elective C-sections was much lower, accounting for 3.9% of all parturitions. An emergency C-section on the contrary was performed in 17.7% of litters within our population, which is slightly higher than the incidence of 16% reported by Bergström et al. (13) and much higher than the incidence of around 5% reported earlier by Linde-Forsberg (22). Such a difference may be due to the type of data collection as breeders may be more prone to participate in studies like the present if they encountered frequent dystocia and if emergency C-sections are common within their own kennel or breed. Interestingly, brachycephalic breeds were underrepresented within our population contrary to what has previously been reported (7). This lack of representation of brachycephalic breeds within our results does not imply a low risk of emergency C-sections within these breeds, but rather a consequence of type of data collection and a lack of participation

TABLE 3 Breed-specific evaluation.

Breed	Bitches	Litters	CS	CS (%)	ECS	Total pups	Pups stillborn	Stillbirth (%)
Bernese Mountain Dog	29	71	19	26.8	3	550	48	8.7
Border Collie	12	27	5	18.5	0	139	19	13.7
Dachshund Wire-Haired	18	47	14	29.8	3	224	4	1.8
German Shepherd	10	22	2	9.1	0	177	19	10.7
Golden Retriever	22	49	11	22.4	3	353	18	5.1
Gordon Setter	10	21	1	4.8	0	193	8	4.1
Hovawart	43	101	10	9.9	0	839	29	3.5
Miniature Bullterrier	11	20	6	30	1	105	8	7.6
Newfoundland	12	22	7	31.8	1	119	13	10.9
Norfolk Terrier	11	27	3	11.1	1	88	7	8.0
Norwich Terrier	12	31	16	51.6	2	99	22	22.2
Tibetan Spaniel	8	23	6	26.1	0	126	7	5.6
Tibetan Terrier	10	26	4	15.4	0	142	5	3.5

CS, number of C-sections; CS (%), incidence of C-section in percent; ECS, number of elective C-section total pups, number of all pups born; pups stillborn, number of stillborn pups; Stillbirth rate in % without consideration of type of parturition.

of breeders of brachycephalic breeds. The bitches in our study C-sections were performed more frequently in small litters (1 or 2 pups) and large litters of above 12 pups, consistent with the previously reported results (15).

Although emergency and elective C-sections were performed in both primiparous and pluriparous bitches of our studied population, the majority of both elective and emergency C-sections were performed in pluriparous bitches. This result is in disagreement with that of previous reports of a higher risk of dystocia in primiparous bitches (23, 24). One possible explanation is that whenever a bitch may have had problems at her first parturition, the breeders may have decided to request an elective procedure also for the subsequent litter/litters. However, the number of elective C-sections in our study is too low to allow trustworthy conclusions in this regard. Further research with a larger population is needed to better understand and evaluate the motivations breeders might have to request elective C-sections. Furthermore, it is possible that a certain number of bitches within our population experienced dystocia during their first pregnancy which was then resolved without the use of emergency C-section. Further investigation is needed to better understand the incidence of dystocia without the subsequent emergency C-section.

Age has an important impact on pregnancy and delivery in the human and is considered a risk factor for problems during pregnancy as well as for the occurrence of dystocia. Especially primiparous women with an age of >35 years are generally considered as having a geriatric pregnancy with a concomitant increase in risks (25, 26). When age was evaluated within our canine population, the increase in the incidence of C-sections with increasing age was observable, but not statistically significant ($p = 0.172$). Yet, once parity was taken into consideration, we were able to see a statistically significant impact of increasing age in primiparous bitches, leading to a higher incidence emergency C-section ($p = 0.004$). The relationship between age of the dam and incidence of C-sections has previously been investigated with contradicting results (23, 27). The observation of increasing incidence of C-section with increasing age in our population is in disagreement with what has been described previously based on a population composed of different breeds, in which the highest incidence was found within the group of bitches aged between 3 and 5.9 years (4), compared with the highest incidence in bitches aged >8 years in our population. On the contrary, Linde-Forsberg and Persson (27), Bergström et al. (28), and Cornelius et al. (15) are in agreement with our results of increasing incidence of C-sections with increasing age at parturition, regardless of the parity. Münnich and Küchenmeister (1) reported an increased risk of dystocia for singleton pregnancies, which may be characterized by uterine disorders and prolonged parturition in older bitches. Both age and parity are widely known as risk factors for dystocia and subsequent necessary C-section. However, the impact of advanced age at first parturition on the risk of C-section is not described in the recent literature, yet breeders may consider it as an important factor that influences their decision on whether or not to breed and/or perform an elective C-section. Within this study, parity of the bitch was considered as either primiparous or pluriparous without consideration of the exact number of litters born by each bitch. Although parity has shown its importance when combined with the effect of age also in this binary method of classification,

further evaluations will be needed to understand whether such an effect is also influenced by the number of litters a bitch may have had.

Data on history of prior C-sections for the bitches in our study show that a prior C-section increases the risk of the need of C-sections in the subsequent whelpings 4-fold. Despite the high number of studies on risk factors for dystocia in the canine, the role of prior C-sections as a risk factor is rarely reported (9, 23). In a work by Proctor et al. (18), 25 of 149 bitches had a history of prior C-section and nearly half of these bitches underwent an elective C-section at the end of the following pregnancy. Another very important factor driving the decisions of many breeders toward an elective C-section is the fear of stillbirth. As previously mentioned, stillbirth has a high incidence in the canine species, and our results have confirmed that stillbirth is significantly lower in elective C-sections compared with that in emergency C-sections as has been previously described (6, 15). Moreover, the number of litters that did not have any stillborn pups was significantly lower in elective C-sections. Although overall mortality was higher in emergency C-sections, timing was of significant importance. The rate of stillbirth was higher in emergency C-sections performed after the birth of the first pup compared with C-sections performed prior to the birth of the first pup. The incidence of stillbirth within our population of litters undergoing an emergency C-section is slightly lower (if performed prior to the birth of the first pup) or slightly higher (if performed after the birth of the first pup) compared with that of the literature (29). This result may be explained by the fact that C-sections prior to the expulsion of the first pup are usually performed much earlier after the onset of parturition and prolonged birth has been defined as a possible risk factor for stillbirth (1, 15, 30). Our breed-specific evaluation includes breeds that are represented with at least 20 litters. Similar to other publications (13), we did not encounter breeds that have been previously described as being at high risk of dystocia with subsequent C-section with a number of litters above this threshold. On the contrary, some breeds represented with a rather high number of litters such as the Hovawart are rarely mentioned in the literature. On the one hand, the breed with the highest incidence of cesarean section within our population was the Norwich Terrier, which was also the only breed with C-sections recorded in the age group under 2 years. On the other hand, the Gordon Setter had the lowest incidence of C-sections of all included breeds. Such a difference between the Norwich Terrier and the Gordon Setter can be observed also in the study of Evans et al. (20). Eleven of the 13 breeds included in our study were represented in the study of Evans et al. (20). The reported incidences of cesarean section are similar in the Dachshund yet differ in some cases greatly such as in the Miniature Bullterrier and the Norwich Terrier compared with the results of our study. This may be due to the much lower number of litters in our study, as well as due to the difference in questionnaire distribution between the two studies. Although the included breeds have rarely been mentioned in previous studies as at-risk breeds (7, 13), differences in the incidence of C-section between breeds were statistically significant ($p < 0.001$). Although a litter size has been mentioned as a risk factor in the evaluation of the general population, the impact is especially evident in the Bernese Mountain Dog and the Newfoundland. The Norwich Terrier had not only the highest incidence of C-section but also the highest incidence of stillbirth (22.2%) compared with other breeds such as the wire-haired Dachshund that had the lowest incidence of stillbirth with 1.8%. Considering that an emergency C-section has been reported to have an increased rate of stillbirth and that the Norwich Terrier had the highest incidence of C-section, the results may not be reported separately, as the high incidence of emergency C-section may be either cause or consequence of the high rate of stillbirth. Based on the data we collected, a distinction between cause and consequence in the case of the Norwich Terrier is not possible. Although the Norwich Terrier has the highest overall stillbirth rate, the German Shepherd and the Border Collie show a surprisingly high rate of stillbirth in C-sections. Yet, it has to be considered that in both breeds all C-sections were emergency C-sections and that particularly in the German Shepherd the percentage reported is based on two C-sections, which resulted in the birth of 14 pups of which 10 were stillborn. We consider such a result hardly representative for the breed and rather an exception, which may be due to health problems of the dam, the litter, or both. In the case of the Border Collie instead, the number of C-sections is still low with five C-sections due to dystocia accounting for a total of 24 pups of which 12 were stillborn. Further investigation with a higher number of litters and C-sections is needed for these two breeds to evaluate the importance of our results. Furthermore, breeds that were not included in the breed-specific analysis (e.g., Scottish Terrier) had relatively high numbers of emergency C-sections, yet due to their overall low number of bitches and litters, no conclusions may be drawn in this regard (Table 1).

Evaluation on housing, character, BC, and topline of the dam has been merely descriptive, whereas breeders' experience showed no statistical significance. Further data collection with more detailed questions will be needed to increase the number of bitches in order to evaluate the importance of housing, character, BC, and topline on the occurrence of emergency C-section.

All information on our population was collected using a questionnaire without inclusion criteria. This means that breeders were able to participate regardless of the breed they are breeding, the experience they have, or other factors. This fact allowed us to obtain a large number of litters from bitches of 80 different breeds which differ not only in phenotypical appearance but also in other anatomical and behavioral peculiarities. We furthermore included in the general evaluation all breeds to evaluate the overall incidence of C-section in a canine population as well as other factors such as rate

TABLE 4 Breed-specific evaluation of litter sizes depending on type of parturition; litter size is given in mean ± SD of pups per litter.

Breed	C-section (mean ± SD)	Eutocic parturition (mean ± SD)	Overall litter size (mean ± SD)
Bernese Mountain Dog	5.6 ± 3.0	8.5 ± 2.5	7.7 ± 2.9
Border Collie	4.8 ± 2.0	5.2 ± 1.2	5.1 ±1.3
Dachshund Wire-Haired	4.3 ± 1.4	5.0 ± 1.6	4.8 ± 1.5
German Shepherd	7.0 ± 0.0	8.2 ± 2.5	8.0 ± 2.4
Golden Retriever	5.3 ± 3.6	7.8 ± 2.1	7.2 ± 2.7
Gordon Setter	14.0	9.0 ± 3.5	9.2 ± 3.6
Hovawart	7.6 ± 3.3	8.4 ± 2.2	8.3 ± 2.3
Miniature Bullterrier	4.7 ± 1.9	5.5 ± 1.9	5.3 ± 1.9
Newfoundland	2.4 ± 1.5	6.8 ± 3.2	5.4 ± 3.5
Norfolk Terrier	2.3 ± 0.6	3.4 ± 1.3	3.3 ± 1.3
Norwich Terrier	2.8 ± 1.1	3.6 ± 1.5	3.2 ± 1.3
Tibetan Spaniel	5.0 ± 1.3	5.6 ± 1.6	5.5 ± 1.5
Tibetan Terrier	5.0 ± 1.4	5.5 ± 1.4	5.5 ± 1.4

C-section, litter size in litters born *via* C-section; eutocic parturition, litter size in litters born in eutocic parturition; overall litter size, litter size for each breed regardless of type of parturition.

of stillbirth. Although the lack of limitations for participation may be considered an advantage as we were therefore able to enroll also lesser-known breeds, it has also some disadvantages. First of all, it has to be considered that owners and breeders of certain breeds may have been more interested in investing in and in contact with the topic of dystocia and C-section than others, which made them more prone to contribute. This may be due to the fact that their breed has a higher incidence of dystocia, although we do not consider this as the main reason, as few breeders of known at-risk breeds responded to the questionnaire. It may also be due to the fact that the club of these breeders are particularly aware of the risks and consequences of dystocia and motivate their breeders more to contribute to studies such as ours. We have seen such an increased interest and motivation by the breeders' club in particular in the Hovawart breed which contributed a total of 101 litters to this study. It is a known fact that information obtained by questionnaires has to be considered with a certain caution as none of the reported information has been verified by a veterinary professional in the moment of birth. Yet, the contribution was completely voluntary, and no reward was granted for participation. All breeders included in this study are from German-speaking countries (Germany, Switzerland, Austria). Although policies vary between clubs, it is common practice within breeders' clubs of these countries to obligate the breeder to provide detailed information on the mating, the birth, and the litter itself, which is also controlled by the club itself. Therefore, it is of interest to the breeder to provide and keep clean and detailed records. We therefore consider the information collected and the population evaluated as an overall good trustworthy and representation of the reality.

Conclusion

Our study provided an insight into possibly influencing factors on the occurrence of C-section, its overall incidence, and information on the rate of stillbirth in emergency and elective C-sections in the general population. Furthermore, less frequently studied breeds such as the Hovawart and the Norwich Terrier have been investigated in a breed-specific manner, which makes our contribution valuable. An increase in the number of dams and number of litters will be necessary to provide further insight into an emergency C-section in these breeds. The results of this study further indicate an important influence of age of primiparous bitches on the incidence of C-sections. The rate of stillbirth was significantly higher in emergency C-sections when compared with that in elective C-sections. Furthermore, the timing of intervention in the cases of emergency C-sections had an impact on the rate of stillbirth within our population. We therefore conclude that elective C-section in primiparous bitches of advanced age and close monitoring of the parturition particularly in the early stages may therefore be advised to allow fast intervention in the case of dystocia and to assure an increase in puppy survival. A similar conclusion can be drawn regarding bitches that have already undergone C-section in prior parturitions as the risk of the necessity of emergency C-section in successive whelpings is increased.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical review and approval was not required for the animal study because the study is based on a collection of retrospective data without the necessity of performing any type of experiments or actions on the animals.

Author contributions

MS and AM were involved in conceptualization and writing—review and editing. MS and BC were involved in methodology and data curation. BC was involved in formal analysis. MS was involved in investigation and writing—original draft preparation. AM was involved in supervision. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Congenital malformations in brachycephalic dogs: A retrospective study

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The popularity of brachycephalic dogs has increased in recent years due to their docile temperament and peculiar features. The historical inbreeding and consequent lack of genetic diversity involved in the development of these breeds led to an increase in the manifestation of deleterious genes that may lead to malformations. In addition, there are serious health issues intrinsic to the conformation, mainly attributed to these extreme characteristics. Therefore, this retrospective study aimed to observe the frequency of malformations in brachycephalic dogs compared to the pure and mixed breeds (MB). The medical records of pregnant bitches admitted at the Service of Obstetrics and Animal Reproduction (SORA) from January 2017 to December 2021 were retrieved from the hospital's computer system and analyzed one by one. Seven hundred sixty-eight neonates born from 168 litters were included in this study. Of these litters, 72.6% (122/168) were brachycephalic. Malformations were found in 52 puppies, with an incidence of 6.77% (52/768). Of the 32 litters that produced malformed puppies, 28 were brachycephalic (87.5%). In total, 23 types of malformations were registered, the most common being cleft palate (1.30%) and anasarca (1.17%). Ten of the puppies (10/52; 19.23%) presented two or more associated malformations. Bitches above 7 years were more prone to present malformed puppies in their litters. Brachycephalic breeds were 3.03 times more likely to present malformed neonates when compared to other breeds; the odds ratio increased to 5.07 when modern brachycephalic was compared to ancestral brachycephalic. Regarding the mode of delivery, elective cesarean sections accounted for 66.6% of births while 19.64% were eutocic vaginal deliveries, and 13.69% were dystocic. The presence of malformed puppies in a litter causes suffering for the owner, the bitch and for the puppy itself, therefore, the veterinarian plays a key role in this scenario. Knowledge about congenital abnormalities, their causes, diagnosis, and approach is essential to reduce the incidence of malformations and improve the quality of life of these animals.

KEYWORDS

neonate, congenital defects, inbreeding, anasarca, palatoschisis

Introduction

A congenital defect is a deviation from normal morphology or function that occurs during pregnancy and is severe enough to interfere with viability or the physical well-being of the offspring and represents one of the main causes of neonatal mortality (1, 2). A study conducted in Australia suggests that the incidence of congenital defects is about 2.8% and the mortality among malformed puppies can reach 15% (1). In Brazil, a study described 27 types of malformations in 23 dog breeds, in which 24.7% of the litters presented some type of malformation, and the incidence among the neonates was 6.7% (3). The most common malformation described was cleft palate (2.8%) and hydrocephalus (1.5%) (3).

The causes may be genetic, iatrogenic, nutritional, or infectious (2, 4). Griseofulvin, corticosteroids, antibiotics, and even aspirin have been listed as teratogenic, as well as hypervitaminosis A and D (2, 4). Some authors point out the influence of geographic regions, which directly impact exposure to pathogens, as well as differences in nutritional management and different genetic lines (4). Indeed, geographic regions can be an important factor, once infectious diseases related to malformations can be endemic in certain areas and very rare in others. Differences in breeding methods, such as the use of supplements and nutritional support, can also compose the geographic factor since the availability of ingredients varies between regions. The geographical issue also includes different genetic lineages: micro populations of the same breed are genetically isolated from each other reducing genetic variability.

There are three critical periods in fetal development (5): the first one is preimplantation (Days 2–17), in which serious injuries may result in embryonic loss. The second is when organogenesis occurs (Days 19–35) and is an important period when birth defects develop. The third is the fetal period (Days 35 to birth), during which the growth and maturation of organ systems occur. Gross structural defects are uncommon in this phase, except in structures undergoing rapid growth and maturation such as the palate, the cerebellum, and parts of the cardiovascular and urogenital system (2).

Recent studies report a higher incidence of orofacial and vertebral malformations in purebreds, especially in brachycephalics such as English and French Bulldogs, Pugs, and Boston Terriers (2, 4, 6, 7).

Although we have observed an increase in the notification of these cases in recent years (8–10) and many cases in clinical routine, there is no data on the real incidence of malformations and no studies comparing the incidence in brachycephalic dogs with other breeds.

The objective of this retrospective study was to describe the most common congenital anomalies found in litters born in our veterinary hospital from January 2017 to December 2021 and to determine if there is an association between the incidence of neonatal malformations and brachycephalic breeds.

Materials and methods

Data collection and analysis

All medical records of patients admitted to the veterinary hospital "Governador Laudo Natel" have been contained in a computerized system since 2017. Pregnant patients are attended exclusively at SORA (Service of Obstetrics and Animal Reproduction) regardless of other concomitant conditions. Patients admitted for other reasons and later diagnosed as pregnant are immediately referred to our service. To carry out this study, all medical records of pregnant bitches were analyzed one by one. Only the medical records of females who underwent pre-natal care and delivery/cesarean section from January 2017 to December 2021 were included in the statistics.

Data such as breed, age, breeder, and previous medical history were obtained from the records contained in the system. Data obtained from physical, laboratory, and imaging examinations, mode of delivery, and the presence of malformed fetuses were recorded. Other complementary information obtained from the zootechnical records of the breeder was also collected.

The dams included in this study were primiparous and multiparous, from different breeds, and were between 6 months and 9 years old.

The data obtained from the bitches' medical records were cataloged according to the breed and age, mode of delivery, number of puppies, number and type of malformations, and previous history. Although each animal has a medical record with the data on all attendances, the information collected was tabulated in a way that each row corresponded to a different litter, so if one bitch was attended in two different pregnancies, each birth and litter was counted separately. Twenty-nine medical records of females who underwent cesarean section did not contain enough information so could not be included. Puppies that were not born at the hospital, but were later brought in for care due to malformations were also excluded from this study.

Pre-natal care, delivery, and neonatal care

Pre-natal care at SORA encompasses physical examination (heart rate, respiratory rate, blood pressure, body temperature, and blood glucose), laboratory tests (blood count and serum biochemistry—ALT, creatinine, total protein, and albumin), ultrasounds performed on days 30, 45, and 55 of pregnancy, and abdominal radiography for the fetal count on day 55. Additional tests were made when necessary.

The litters considered in this study were followed up and born in SORA by vaginal delivery or c-section. Neonates were evaluated at birth by Apgar score (11) and inspected

for the presence of malformation and those with alterations were included in the statistical analysis. The diagnosis of malformations was made by inspection in most cases, but also using other methods such as laboratory and imaging (Ultrasound, radiography, and echocardiography) when necessary. Malformed puppies amenable to interventions were monitored and treated by the SORA staff and those with conditions incompatible with life were euthanized. Puppies that died were sent for necropsy whenever possible.

Statistical analysis

First, the data obtained from the medical records were placed in an Excel table [Microsoft Office Excel 2019 (16.0); Microsoft Corp.] and a descriptive statistic was performed, in which the frequency of attendance between pure breeds and mixed breeds was observed, in addition to the frequency and types of malformations among the breeds. The mode of delivery were also analyzed concerning breed conformation and the presence of malformations.

In the descriptive statistics, we noticed a high frequency of malformations among brachycephalic breeds, especially in the modern ones, much higher than that observed in other purebred dogs. For this reason, in a second moment, the odds ratio was calculated comparing brachycephalic to other breeds and extreme brachycephalic to ancestral brachycephalic breeds.

The odds ratio, as well as the confidence interval, were calculated using the R program (R i386 4.1.3) Fisher's test was performed to test the association between the analyzed variables. Values were considered as significant at $p < 0.05$.

TABLE 1 Type of neonatal malformation and frequency by breed.

Type of malformation	Frequency	Breed
Palatoschisis	10/768 (1.30%)	French Bulldog, English Bulldog, mixed breed
Anasarca	9/768 (1.17%)	English Bulldog, mixed breed
Mitral dysplasia	6/768 (0.78%)	Pug
Omphalocele	4/768 (0.52%)	French Bulldog, English Bulldog,
Gastroschisis	4/768 (0.52%)	French Bulldog, English Bulldog, Shih Tzu, mixed breed
Renal dysplasia	4/768 (0.52%)	Pekingese
Hydrocephalus	3/768 (0.39%)	French Bulldog, Pug, German Spitz
Cheiloschisis	3/768 (0.39%)	English Bulldog, Shihtzu
Hypospadia	2/768 (0.26%)	Shih Tzu, French Bulldog
Swimming puppy syndrome	2/768 (0.26%)	Shih Tzu
Anencephaly	2/768 (0.26%)	Mixed breed
Atresia ani	2/768 (0.26%)	Shih Tzu
Rectourethral fistula	2/768 (0.26%)	Shih Tzu
Arthrogryposis	2/768 (0.26%)	MB
Eyelid aplasia	2/768 (0.26%)	MB
Macroglossia	2/768 (0.26%)	MB
Amelia	1/768 (0.13%)	French Bulldog
Lateralized anus	1/768 (0.13%)	French Bulldog
Urachus persistence	1/768 (0.13%)	French Bulldog
Spina bifida	1/768 (0.13%)	French Bulldog
Flexural deformity	1/768 (0.13%)	English Bulldog
Aplasia cutis	1/768 (0.13%)	Pinscher Miniature
Portosystemic shunt	1/768 (0.13%)	Pug

Results

A total of 168 medical records of parturition/c-section were eligible for enrollment, and 152 corresponded to purebred litters (21 AKC recognized breeds) and 16 to mixed breeds. Brachycephalic breeds accounted for 72.6% (122/168) of the litters.

Congenital anomaly (CA) was present in 19.04% (32/168) of the litters and 87.5% of these (28/32) were brachycephalic. Twenty-one litters (21/32; 65.6%) with malformed neonates corresponded to commercial breeders and two of the dams already had a previous history of malformed puppies. Two mixed-breed neonates presented multiple congenital malformations: anencephaly, palatoschisis, eyelid aplasia, and macroglossia; the dam was a Lhasa apso accidentally mated to an American Pit Bull. Seventeen litters had stillborn with no apparent malformations. Unfortunately, not all of them could be referred for necropsy, and those that were did not show any macroscopic alterations.

The total number of neonates delivered was 768 and 6.77% (52/768) of them were presented with CA. Overall, 23 different malformations were observed (Table 1). The most common malformations observed were palatoschisis (1.30%, Figure 1) and Anasarca (1.17%, Figure 2). Forty-two (80.7%) of the CA were single/isolated, whereas 10 (19.2%) were associated (Table 2). Of the 52 malformed cases, 27 (52%) died or were electively euthanized because of lethal malformations, with no difference of isolated and associated CA (Figure 3). Core variables such as birth weight and newborn sex could not be evaluated due to the missing data on medical records.

Breeds having at least one malformed puppy were the English Bulldog (15/52), French Bulldog (12/52), ShihTzu (7/52), Pug (7/52), Pekingese (4/52), Miniature Pinscher (1/52), German Spitz (1/52) Lhasa Apso (1/52), and mixed breed (4/52). Table 3 summarizes the frequencies and percentages of congenital malformations stratified by breed.

Brachycephalic breeds were 3.03 times more likely to have malformed neonates than other breeds; modern brachycephalic breeds are 5.07 times more likely to have malformed neonates than ancestral brachycephalics. Age seems to be a risk factor:

FIGURE 1
Cleft Palate in a French Bulldog neonate.

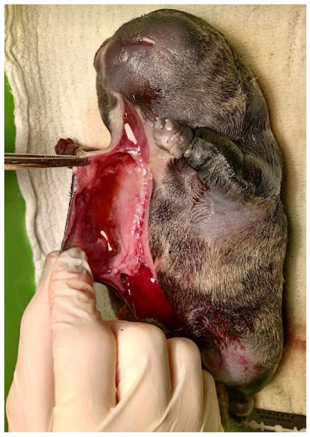

FIGURE 2
Anasarca in an English Bulldog neonate. Note the accumulation of fluid in the subcutaneous tissue, a remarkable characteristic of this type of malformation.

mature bitches (above 7 years old) are 5.71 times more likely to have malformed puppies than young ones. On the other hand, when litter size was compared, no relation was found between the incidence of CA and the number of offspring in a litter (Table 4). Parity could not be evaluated as there were missing data on the medical records.

Breeds with no congenital malformation reports in the present study were the American Bully, Blue Heeler, Border Collie, ChowChow, Boxer, Dachshund, Brazilian terrier, Siberian Husky, Labrador, Maltese, Poodle, Rottweiler, and Yorkshire. Regarding mode of delivery, 66.6% were elective c-sections (112/168), 19.64% (33/168) were eutocic vaginal deliveries, and 13.69% (23/168) were dystocic. There was no report of dystocia due to neonatal malformations; the bitches presented uterine inertia and were submitted to c-section.

Discussion

According to the kennel club, the following breeds can be considered brachycephalic: Pug, French Bulldog, English Bulldog, Boston Terrier, Shih Tzu, Pekingese, Affenpinscher, Cavalier King Charles Spaniel, Lhasa Apso, and Griffon Bruxellois' (12). Many of these breeds were in our medical records. These dogs are characterized basically by short noses and wide heads. Associated with this conformation, problems such as brachycephalic airway syndrome (BAS), stenotic nares, elongated soft palate, narrowed trachea, visual problems, malocclusion, allergies, skin disorders, problems with heart performance, exercise intolerance, distichiasis, protrusion of the third eyelid gland, dystocia, neurological and spinal congenital malformations (7, 13, 14) are reported, including hemivertebrae and spina bifida, described in the present study.

Regarding the mode of delivery, there is a high incidence of elective cesarean sections (66.6%), due to the number of brachycephalic bitches attended at our veterinary hospital. Canine dystocia is a very common problem in clinical practice and can lead to the death of the dam and stillbirth (15). Risk factors for dystocia include breed, age of the dam, parity, litter size, and body size of the bitch. Fetal causes include fetal monster, anasarca, cephalopelvic disproportion, true fetal oversize or disproportion between fetal size and dam size, and fetal death (16). Knowing the risks of dystocia allows the best preparation for the intervention to be carried out at the right time and in the right way (15).

Besides the high rates of Cesarean section, the Pugs, Boston Terriers, and Bulldogs also seem to be most susceptible to neonatal mortality (14). The two most common causes of fetal depression associated with a cesarean section are hypoxia associated with dystocia and depression from medications given to the dam as part of the anesthetic protocol (16) thus, it is expected that breeds that are more likely to experience dystocia or that underwent elective c-sections have high rates of stillborn compared to breeds that can give birth without intercurrences. This information was confirmed by Cornelius et al. (15) who found out that puppies from litters in which a cesarean section was performed had 1.37 odds of being stillborn compared with litters that were delivered by vaginal route. Moreover, they reported that litters that experienced dystocia were 2.35 times more likely to have stillborn compared to the eutocic puppies. Within the last group, they notice that the management of dystocia also influences survival rates

Many studies have described the high incidence of dystocia in brachycephalic patients due to the physical conformation of the puppies (such as head size and chest width), the presence of oversized fetuses, and also fetal alterations such as anasarca and monstrosities, which justify elective c-section as the method of choice for these patients (13, 15, 17). In this study, 23 (13.69%) cases of dystocia ended up in c-sections. Of the 32 litters with malformed puppies, three were born

TABLE 2 Frequency, outcome, treatment, and description of associated congenital malformations by breed.

Multiple congenital malformations	Breed	Frequency (%)	Newborn outcome	Treatment
Amelia + hydrocephalus	French Bulldog	1/52 (1.92%)	Discharge	Clinical treatment for hydrocephalus
Omphalocele + palatoschisis	French Bulldog	1/52 (1.92%)	Dead	-
Palatoschisis + cheiloschisis	English Bulldog	1/52 (1.92%)	Discharge	Surgical correction
Anencephaly + palatoschisis + eyelid aplasia + macroglossia	Mixed breed	2/52 (3.84%)	Dead	-
Hydrocephalus + portosystemic shunt	Pug	1/52 (1.92%)	Discharge	Clinical treatment for both conditions
Atresia ani + hypospadias + rectourethral fistula	Shih Tzu	1/52 (1.92%)	Dead	-
Atresia ani + rectourethral fistula	Shih Tzu	1/52 (1.92%)	Dead	-
Artrogriposis + gastroschisis	MB	2/52 (3.84%)	Dead	-

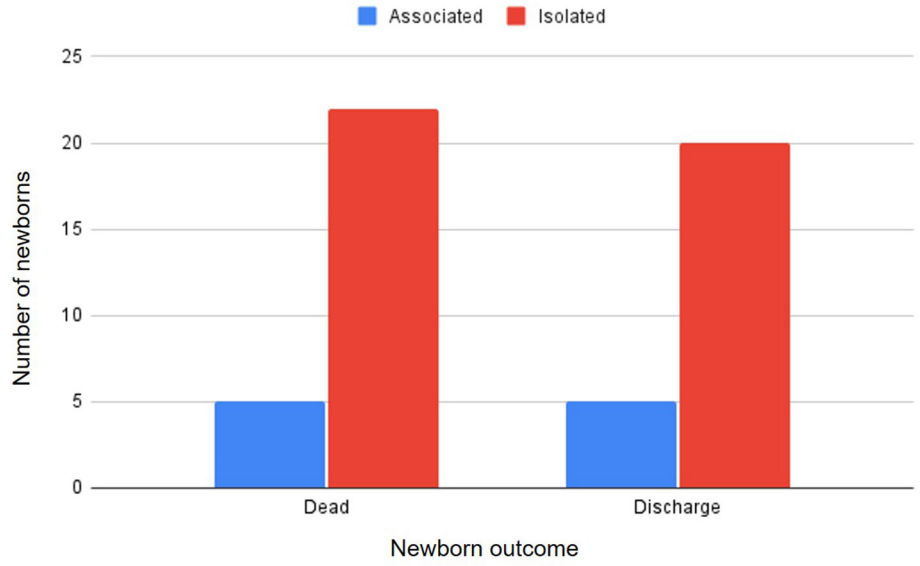

FIGURE 3
Type of congenital anomaly (isolated or associated) and neonatal outcome.

from natural whelping, twenty-six from elective c-section, and three experienced dystocia (two due to uterine inertia and one caused by fetal malpresentation); none of them were caused by malformation.

Regarding the age of the dam, our data showed that mature bitches (over seven years of age) are more likely to deliver malformed puppies compared to young ones. To the authors' knowledge, this is the first study to show an association between aging and malformation in the canine species. In humans, on the other hand, there are robust data associating maternal age with the appearance of malformations: as age increases, it also increases the chance to develop birth defects (18). Whether or not there is a biological cause similar to that described in women must be further elucidated.

Many studies have been published reporting the frequency of specific malformations in dog populations (4, 7, 19, 20) but only a few presented an overview of the prevalence of these conditions in this species. Data obtained in the present research showed that 19.04% of the litters were affected and the incidence of malformations was 6.7%, rates notably higher than the 11.4 and 2.8% reported in the literature, respectively (1).

Among the thirty-two litters that presented malformations, 87.5% were brachycephalic: English Bulldog (10/32), French Bulldog (9/32), ShihTzu (5/32), Pug (2/32), Pekingese (2/32) and Lhasa apso (2/32); moreover, 19 of the 21 types of malformations were recorded in brachycephalic dogs.

Twenty-three types of malformations were observed, the most common being cleft palate (1.3%) and anasarca (1.17%).

TABLE 3 Number and (%) of litters, offspring, and malformed puppies by breed, attended in SORA (Service of Obstetrics and Animal Reproduction) from January 2017 to December 2021.

Breed	N of litters (%)	N of offspring (%)	N of malformed puppies (%)
French Bulldog	43/168 (25.59%)	193/768 (25.13%)	12/768 (1.56%)
English Bulldog	37/168 (22.02%)	187/768 (24.34%)	15/768 (1.95%)
Shih Tzu	18/168 (10.71%)	74/768 (9.63%)	7/768 (0.91%)
German Spitz	9/168 (5.35%)	27/768 (3.51%)	1/768 (0.13%)
Chow chow	8/168 (4.76%)	32/768 (4.16%)	0
Pinscher Miniature	8/168 (4.76%)	23/768 (2.99%)	1/768 (0.13%)
Pug	4/168 (2.38%)	18/768 (2.34%)	7/768 (0.91%)
American Bully	4/168 (2.38%)	27/768 (3.51%)	0
Lhasa Apso	3/168 (1.78%)	17/768 (2.21%)	1/768 (0.13%)
Border Collie	3/168 (1.78%)	21/768 (2.73%)	0
Maltese	2/168 (1.19%)	5/768 (0.65%)	0
Dachshund	2/168 (1.19%)	9/768 (1.17%)	0
Pekingese	2/168 (1.19%)	7/768 (0.91%)	4/768 (0.52%)
Rottweiller	2/168 (1.19%)	2/768 (0.26%)	0
Boxer	1/168 (0.59%)	9/768 (1.17%)	0
Poodle	1/168 (0.59%)	3/768 (0.39%)	1/768 (0.13%)
Blue Heeler	1/168 (0.59%)	6/768 (0.78%)	0
Labrador	1/168 (0.59%)	2/768 (0.26%)	0
Siberian Husky	1/168 (0.59%)	6/768 (0.78%)	0
Fox paulistinha	1/168 (0.59%)	4/768 (0.52%)	0
Yorkshire	1/168 (0.59%)	6/768 (0.78%)	0
Mixed breeds	16/168 (9.52%)	95/768 (4.55%)	4/768 (0.52%)

A large study conducted in Brazil that included 27 types of malformations and 23 breeds showed that 24.7% of litters had some type of malformation, with 6.7% of the neonates affected. The most common condition was cleft palate (2.8%) and hydrocephalus (1.5%) (3), which are in accordance with our findings. A study conducted in Australia reported that 11.4% of litters were born with congenital defects, which represented 2.8% of the neonates (1).

Orofacial clefts are fissures of oral or facial structures that occur during embryonic development. Several studies suggested that the phenotypic manifestation of the cleft in dogs is associated with skull type (4). In these species, the alteration is clinically relevant because of the associated morbidity and high mortality rate (4). The mastiff/terrier genetic group includes most brachycephalic breeds such as Boston Terrier, English Bulldog, and French Bulldog and they seemed to be more predisposed to orofacial clefts (4, 21). Interestingly, the morphology of the cleft appears to be different according to the type of the skull: compared to other breeds, brachycephalic dogs seem to be more prone to cleft lip or cleft lip associated with cleft palate than to cleft palate (21).

A study focused only on orofacial clefts found a phenotypic distribution as follows: 26% of Cleft Lip (CL), 59% of Cleft Palate (CP), and 15% of Cleft Palate and Lip (CPL) (4). Our study shows a similar distribution: of the 12 puppies that had orofacial clefts, 2 presented only CL (16.6%), 9 presented only CP (75%) and just one presented CPL (8.3%). Although our frequencies are different, the authors found out that Brachycephalic breeds were at increased odds of orofacial clefts of any type (4). Indeed, in our study, of 12 puppies with orofacial clefts, only two were Mixed breed and had other associated malformations. All the others were brachycephalic: two Shih Tzu puppies (CL), seven French Bulldogs (CP), and One English Bulldog (CPL). The frequency of orofacial clefts observed in other studies varied according to phenotype (CL vs. CP vs. CLP) and reinforces the hypothesis that the CP phenotype in dogs is more common compared to CL and CLP (4, 21). However, results also showed that this pattern may only apply to certain breeds (e.g., Labrador Retriever, Pembroke Welsh Corgi, and French Bulldog) and not to others (e.g., Boston Terrier, Cavalier King Charles Spaniel, English Bulldog). Accordingly, our results showed that in French Bulldogs, CP is the most common orofacial cleft, while in English Bulldogs, CLP was the most prevalent. Specifically, the odds of CP, CL, and CLP were consistently and significantly higher in the brachycephalic group compared to the reference skull type group (i.e., mesaticephalic) (4). Even though our sample for CPL was too small, we observed a similar distribution and tendency. The treatment of oral clefts is mostly surgical (21) and so our patients were referred to general surgery service and we had no further information about their outcome.

Some authors pointed out a strong influence of the genetic factor on the pathogenesis of orofacial clefts, (4, 21) however, one may not exclude the influence of environmental factors such as geographic region (considering exposure to pathogens) and rearing method (nutritional factors, folic acid deficiency, trauma, and drug use) (4).

The comparison of our results with other Brazilian studies (especially those conducted in the same microregion) is essential due to the geographic issue. Studies conducted in other countries may take into account cultural differences in breeding (such as nutritional management, and infectious diseases) that may affect the incidence of malformation. There was no record of trauma, contact with teratogens, or marked nutritional characteristics in any of the cases of cleft palate observed in this study, but the high incidence in brachycephalic patients (8/10) once again highlights the importance to consider genetic traits.

Anasarca was the second most frequent malformation (9/52) recorded in the present study, with a high incidence in English Bulldogs (8/9). This condition, also called congenital edema or hydrops fetalis, is known to be a heritable recessive trait

TABLE 4 Odds ratio, confidence interval, and significance according to independent variables of the dam.

Independent variables of the dam	Description	Association with malformation	Odds ratio	Confidence interval	*P value*
Breed	Brachycephalic Others purebred Mixed breed	Brachycephalic	3.03	95%	0.009
Age	Young (1-2y) Adult (3-6y) Mature (7-9)	> 7 years	5.71	95%	0.021
Mode of delivery	Elective c-section Therapeutic c-section Eutocic vaginal birth	Not related	-	-	-
Parity	Primiparous Multiparous	Missing data	-	-	-
Litter size	Small (1-4) Medium (4-6) Large (>7)	Not related	-	-	0.5996

that involves generalized subcutaneous edema with or without visceral effusions (2, 10, 22).

The cause is unknown, but some studies point out associated cardiac malformations, infectious causes, and, again, the genetic factor (2, 22). It is important to notice that five of the nine puppies that presented anasarca were from different litters, but originated from the same commercial breeder, which once again denotes inbreeding. The condition often causes dystocia due to a typically enlarged fetus and is usually associated with a high neonatal mortality rate and a greatly increased rate of cesarean sections. The English Bulldog, the Pug, the Boston Terrier, and the French Bulldog all have increased incidence of fetal anasarca and this condition may involve the whole or part of the litter (2, 10, 22). In this study, two of the English Bulldog litters presented more than one anasarca. Although it is associated with high neonatal mortality, there are scarce reports of successful treatment of anasarca's fetuses (22). Unfortunately, all anasarca in this study have some lethal condition (such as pulmonary hypoplasia or bilateral hydronephrosis), so they were euthanized immediately after birth. It is important to point out that puppies who go through treatment and survive, must be neutered.

The present study also described a high frequency of mitral dysplasia (0.78%). However, it is important to consider that the six affected Pug puppies were from the same litter whose mother also presented the condition, suggesting a possible hereditary condition. The same malformation was described in a similar study in a French Bulldog puppy, but the reported incidence was 0.12% (1). Among the cardiac malformations, Mitral dysplasia is one of the most common and has a high incidence in Terrier dogs, with low evidence in Bulldogs (19, 23) and no data available in Pugs. The exact prevalence of heart disease is difficult to estimate because not all patients have heart murmurs.

It is well known that during pregnancy, there are many adaptations in cardiac function, probably due to hemodynamic changes. Blanco et al. (24) described the systolic cardiac function and peripheral circulation changes in pregnant bitches. In this species, maternal cardiac adaptation during gestation plays a major role in uterine perfusion to support fetal development. Comparing echocardiographic parameters in normal and abnormal pregnancies, Blanco et al. (25) reported differences in left ventricular dimension in diastole (LVD), HR, FS, Systolic Volumes (SV), and CO between the two groups. This may point to a possible cardiac maladaptation to pregnancy in bitches with the abnormal gestational course. For this reason, it would be interesting to carry out preventive cardiological examinations in predisposed breeds (Boxer, Newfoundland, French Bulldog, English Bulldog, German Shepherd, Golden Retriever, and Labrador Retriever) before the animals are mated (19).

Melandri et al. (26) described an increase of functional and diastolic parameters and the decrease of systolic parameters as pregnancy progresses in healthy Great Dane bitches, a breed particularly predisposed to dilated cardiomyopathy and Subaortic Stenosis (23, 26). This finding reinforces the importance of echocardiographic examination as part of prenatal follow-up in breeds predisposed to cardiac diseases. The authors also consider Breed as a source of variation in echocardiographic values, therefore the cardiac performance during pregnancy may also be susceptible to a breed-related variation. Once again, pugs are not predisposed to mitral dysplasia and the bitch had no clinical signs, so this result was an incidental finding.

Hydrocephalus was reported as the second most common malformation in a recent study (3), but the rate of this condition in our study was notably lower. This difference might be attributed to the absence of a diagnosis since part of the data was retrieved from medical records. Dogs and cats with congenital hydrocephalus may have signs from birth such as a large, dome-shaped head, persistent fontanelles, and bilateral ventrolateral strabismus that may be the result of either orbital skull malformation or vestibular dysfunction, however, more commonly signs become apparent in the first few months of life (27). Moreover, hydrocephalic puppies often show a retarded growth, so comparison with littermates can be very helpful (28). Unfortunately, the progression of symptoms is variable, so affected puppies may not show clear neurological signs, especially when they are very young (28). Therefore, those puppies who did not present apparent malformations or clinical signs at birth may not have been registered as malformed in the medical records and so the incidence rate may be underestimated.

Reduced cranial capacity impairing cerebral compliance and malformations of the craniovertebral junction (atlantoaxial instability, occipital-atlantoaxial overlap syndrome, and "Chiari-like malformation") are the most common causes of impaired CSF flow and communicating hydrocephalus in a high number of brachycephalic breeds (28).

Congenital hydrocephalus is frequently diagnosed in toy breed dogs including Pomeranians but is also overrepresented by the brachycephalic group (Boston terrier, English Bulldog, Maltese, Pug, Pekingese, and Chihuahua) (27, 28). The latter is the most commonly affected breed, which might indicate that genetics play a role in the pathogenesis of hydrocephalus in this breed (28). Once again, our sample was too small to identify a breed predisposition, but the affected puppies belonged to breeds that seem to be more prone to present that malformation according to the literature. Medical therapy involves decreasing the production of CSF with diuretics, omeprazole, and glucocorticoids associated with antiepileptic drugs, and the most common surgical treatment is the placement of a ventriculoperitoneal shunt (27). When surgical treatment is indicated, patients are referred to the General Surgery Service.

Congenital vertebral malformations are so common findings on diagnostic imaging of the vertebral column in "screw-tailed" brachycephalic patients that 51% of the dogs evaluated in a study had evidence of one or more lumbosacral congenital vertebral malformations. These alterations had a high prevalence even in neurologically normal French Bulldogs, English Bulldogs, and Pugs (6).

In our study, only one French bulldog puppy was diagnosed with spina bifida (0, 13%), and one female that gave birth to one CP puppy already had a previous litter with one hemivertebrae puppy.

Although these changes are diagnosed in animals without clinical signs, Vertebral body malformations are suggested to change spinal biomechanics that can lead to premature degeneration of adjacent intervertebral disk (6). In one study, French Bulldogs showed 0.89 times the odds of being diagnosed with at least a disease compared to other breeds (29).

Of the 32 affected litters, 28 were purebred, from which 21 were from commercial breeding kennels and two of these dams had already records of previous litters with malformations. Breeding for a commercial purpose is usually controlled, to modify specific physical or behavioral traits, or aim at improving health and genetic diversity; however, multiple generations of inbreeding to fix a specific trait can result in offspring with a lack of genetic diversity (30). In addition, dogs that are not wanted for breeding purposes are commonly neutered, so the reproductive population is much smaller than the census population, decreasing genetic variability (30). Genetic effects of inbreeding can be attributed to the fact that the inbred individual may carry two copies of a gene (31), including the deleterious alleles. For this reason, inbreeding influences the incidence of some inherited diseases (31).

Considering only the gross defects previously presented in our study, brachycephalic breeds are 3.03 times more likely to have congenital malformations than other skull types, especially if they have extreme traits.

Many modern brachycephalic breeds are characterized by a shortening of the muzzle bones of the skull without an equivalent reduction in the volume of the associated nasopharyngeal soft tissues that predisposes to clinical upper respiratory tract disorders (32) known as brachycephalic airway syndrome (BAS). Studies showed that the frequency of BAS was higher in modern compared to ancient breeds. This confirmed the findings that boxers are not as susceptible to BAS as many other brachycephalic breeds belonging to the same phylogenetic cluster (33).

Comparison between extreme brachycephalic breeds (English Bulldog, French Bulldog, ShihTzu, and Pugs), known as modern breeds, with ancestral brachycephalic breeds showed that the odds ratio rises to 5.07. This finding highlights the importance to re-evaluate animal selection and breeding management.

The study of Njikam et al. (33) on BAS reported differences between ancestral and modern brachycephalic breeds, which might indicate that some traits are not derived from the same ancestral characteristics. Although one might assume that dog breeds constitute homogenous entities, the popularity of some breeds may have created isolated subsets of dogs within some breeds (33).

A study that estimated the inbreeding coefficient, found that brachycephalic breeds (including those previously cited) had a high degree of homozygosity (30). They also noticed that nearly all of the dogs with high inbreeding coefficients belonged to brachycephalic breeds, which are likely to have reduced lifespans due to specific pathologies imposed by their skull morphology (30).

The same pressure that selects these extreme phenotypic characteristics and brings the intrinsic problems of the conformation (such as respiratory, locomotor, and reproductive difficulties) also selects for serious congenital malformations that worsen the quality of life and increase neonatal mortality. Even malformations that have treatment, cause a lot of suffering for both the puppy, who may need careful and stressful handling, surgeries, and medication, and for the owner, who will spend a lot of money and time for the treatment, not to mention the potential suffering for the bitch in cases of dystocia of fetal origin.

To improve the quality of life for dogs, breeding purebred dogs must be carefully thought out. Due to their extreme characteristics, many brachycephalic patients only reproduce through artificial insemination and cesarean sections. One of the ways to select healthier animals is not to cross animals that only reproduce with the use of biotechniques (34). Breeding standards should be revised and not be left open to interpretation allowing the perpetuation of traits with a negative impact on the health and welfare of the dogs (34).

Limitations inherent to retrospective studies and present in this one includes data loss and unknown confounding factors, which did not allow us to correlate the incidence of the malformations and the sex of the neonates, as well as to calculate the mortality rate. Unfortunately, many puppies born in the sector without apparent malformations, died after being discharged from the hospital and breeders did not always get in touch to report the death or to request a necropsy study.

Conclusion

In summary, the incidence of malformations in brachycephalic breeds is notably higher when compared to other morphological types, especially when considered the ones presenting with extreme traits, which can be attributed, most likely, to the high level of inbreeding. Anasarca and orofacial clefts are the most common malformations, which prompted the need for additional studies about their pathophysiology and treatment. Moreover, investigations focusing on the determination of the genetic role in malformations are of pivotal importance for the veterinarians to be able to accurately instruct breeders in the selection of animals to be acquired and reproduced, as well as to choose the appropriate prenatal care, delivery, and neonatal approach.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Ethical Committee of the São Paulo State University. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Ultrasonographic changes in fetal gastrointestinal motility during the last ten days before parturition in dogs

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Fetal gastrointestinal motility (FGM) was suggested as useful to assess fetal maturity. Our study aimed to quantify FGM in relation to days before parturition (DBP), maternal size, and sex ratio of pups. During the last ten days of pregnancy, 23 clinically healthy pregnant bitches of 16 different breeds ranging in age from 2 to 9 years and body weight from 3.5 to 56.8 kg were monitored twice. The fetal intestine was observed in longitudinal and transversal scan on 3 of the most caudal fetuses in both uterine horns. Gestational age was counted backward from parturition day. The number of fetuses showing FGM was recorded in time in I (−11/−5 DBP) and II (−4/0 DBP). A Mann–Whitney test was performed to analyze variations of FGM% in relation to time and parity. A Kruskal–Wallis test was performed to identify variations of FGM% in relation to maternal size and sex ratio. Statistical significance was set at $\alpha = 0.05$. A total of 147 FGM observations on 50 ultrasonographic monitoring points were performed. The FGM% was higher during time II compared to time I (median: 33%, range 0–100% vs. 100%, range 33–100%; $P < 0.0001$). FGM% was higher in small compared to large size bitches (median: 100%, range 67–100% vs. 67%, range 0–100%; $P = 0.01$). FGM% was not affected by parity and sex ratio. As previously reported, a significant increase in FGM% was observed in the last five DBP. FGM observation may be influenced by the maternal size, with easier evaluation in small size bitches, as well as ultrasound equipment and positioning.

KEYWORDS

pregnancy, bitch, gastrointestinal motility, fetal development, peristalsis, gestational age, ultrasound

Introduction

The prediction of the canine parturition date is of primary importance in preventing neonatal death and ensuring assistance at parturition or planning a C-section. Many clinical, behavioral, and ultrasonographic parameters may be used at different stages of pregnancy (1–3). Fetal parameters such as inner chorionic cavity (ICC), crown-rump length (CRL), body diameter (BD), and biparietal diameter (BP) may be measured by ultrasound (US) throughout pregnancy. ICC is the most accurate fetal parameter in early pregnancy, whereas, in late pregnancy, the most accurate parameter is reported to be

BP (1, 2, 4). CRL and BD are useful for evaluating gestational age in clinical practice (4, 5). Nowadays, many fetal parameters have been described and characterized as useful for the determination of the parturition day (4, 6–8) by calculating specific formulas, depending on the size of the bitch (4, 7, 9–12) and on the breed (13–16). However, all formulas based on fetal measurements are not accurate enough during the last week of pregnancy (17, 18). For this reason, predicting the parturition date when pregnant bitches are examined for the first time during this week is still challenging (1–3).

The US observation of fetal intestinal development is a useful tool for monitoring pregnancy progression, and the appearance of fetal gastrointestinal motility (FGM) indicates the conclusion of organogenesis. For this reason, FGM was suggested as a useful parameter to assess fetal maturity (19). Gil et al. (19) proposed four different developmental phases, starting from the first US visualization of the organ at 23–19 days before parturition (DBP). The first observation of FGM was reported in the third developmental phase from 13 to 9 DBP when FGM was visualized only in some intestinal portions after a prolonged US observation of the organ. During the fourth developmental phase (from 4 to 1 DBP), the intestinal wall layers, as well as dilation of some portions of the intestine and intraluminal mucous and fluid content, were easily evident. FGM was reported after a few seconds of US monitoring of the intestine (19).

In a previous study, we reported the daily observation regarding the percentage of fetuses with recognizable FGM (FGM%) during the last 10 days of pregnancy (from −9 to 0 DBP) (20). In that study, a weak negative correlation was found between FGM and vaginal temperature. In contrast, a correlation was not found between FGM and serum progesterone concentration, fetal heart rate, and rectal temperature. We also observed an increase in FGM% from 17.1 to 63.3% in the last 5 days prepartum (20). Assessing the magnitude and timely order of such an increase was complicated as the study was not designed to investigate differences in FGM over time as a repeated measure (each measure was not systematically performed with a standard frequency and time interval). Based on this study, we hypothesized that the observation of FGM may differ during the last 5 days of pregnancy compared to the previous 5 days.

Moreover, as described by others in the literature, we hypothesized that this parameter could be affected by factors other than the approaching of parturition day. Therefore, the present study aimed to quantify the amount of FGM in relation to DBP, considering and comparing two specific time intervals in which the US was performed twice in the same dam. The effect of maternal size and sex ratio of pups on FGM was also assessed.

Materials and methods

This study was approved by the University of Padova Ethics Committee (Project nr. 69/2018), and written consent was obtained by the owner of each bitch. Twenty-three healthy pregnant bitches of 16 different breeds, ranging in age from 2 to 9 years and in weight from 3.5 to 52.2 kg, were included in the study (Table 1). Bitches were presented to the Veterinary Teaching Hospital of the University of Padova and enrolled for estrous or pregnancy monitoring from July 2020 to May 2021. No treatments were administered to induce estrus and/or ovulation. The bitches were monitored by US using an 8–5 MHz convex transducer connected to a US unit (Philips Affiniti 50G, Italy) in dorsal or lateral recumbency, following hair clipping and application of a contact US gel to the abdominal region. Each bitch was examined at least twice on two non-consecutive days during two intervals: −10/−5 (time I) and −4/0 (time II) DBP. A collection of reproductive history and a clinical examination were performed during each consultation. Moreover, a complete cell blood count (CBC) was performed at time II to monitor the health status of the bitch. The included bitches were monitored by US without any need for hospitalization.

The days of monitoring were calculated based on ovulation day, when progesterone concentration was between 4–10 ng/mL (1, 21). Natural breeding or artificial inseminations (using fresh, chilled, or frozen semen) were performed based on the estimated ovulation day by our team or a referring veterinarian. When the ovulation day was not available, the days of monitoring were based on fetometry measures and formulas for calculating the parturition day reported in Table 2 (3, 6, 7, 22). Depending on the gestational period in which US monitoring was performed, the most suitable parameters were used, such as ICC (4, 7, 12), CRL (4, 6), BD (4, 6), and BP (4, 7, 12). The result was used to calculate gestational age or DBP using the related formulas described in the literature. These results were used to calculate the day on which US monitoring should be performed to assess FGM during the defined time ranges (Table 2). After parturition, gestational age was counted backward from the day of parturition (day 0), and the actual US monitoring day was confirmed based on this calculation.

Fetal intestine of the 3 most caudal fetuses in both uterine horns was observed for at least 30 seconds in a longitudinal and transversal scan. The number of fetuses showing FGM was recorded for each US monitoring point (videos available as Supplementary material). FGM was evaluated for each monitored fetus using a dichotomic score: absent when no bowel movements were evident during the US observation or present when evident intestinal peristalsis was assessed. The US observations were then reported as FGM% (percentage of fetuses

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TABLE 1 Bitch identification number (n.), breed, age, body weight (BW), maternal size, parity, litter size, number of male and female pups, the sex ratio of pups, and days of consultation (day 0 = day of parturition) of bitches included in the study.

Bitch n.	Breed	Age (years)	BW (kg)	Size	Parity	Time I (DBP)		Time II (DBP)		Litter size	Male pups	Female pups	Sex ratio of pups (%)
1	Whippet	5	17.5	Medium	Primiparous	−6		−2		7	3	4	57
2	Whippet	3	16.7	Medium	Primiparous	−10		−3		7	3	4	57
3	Labrador retriever	3	38	Large	Primiparous	−8		−4		12	6	6	50
4	Flat coated retriever	3	33.5	Large	Primiparous	−7		−2		7	5	2	29
5	Kurzhaar	2	33.2	Large	Primiparous	−6		−3		10	6	4	40
6	Bull terrier	3	21.6	Medium	Primiparous	−9		−4		6	3	3	50
7	Norfolk terrier	4	8.8	Small	Pluriparous	−7		−4	−1	3	1	2	67
8	Flat coated retriever	8	38.5	Large	Pluriparous	−9		−4		10	6	4	40
9	Dachshund	3	10.6	Small	Primiparous	−5		−2		5			
10	Crossbreed	7	12.1	Small	Pluriparous	−7		−3		5	4	1	20
11	Epagneul breton	4	20	Medium	Primiparous	−8		−2		7	3	4	57
12	Flat coated retriever	4	30.8	Medium	Primiparous			−3	0	9	3	6	67
13	Flat coated retriever	5	34.8	Large	Pluriparous	−7		−4		7	2	5	71
14	Australian shepherd	8	26	Medium	Primiparous	−11	−7	−4		7	4	3	43
15	Flat coated retriever	7	33.5	Large	Pluriparous	−8		−4		10	3	7	70
16	Australian shepherd	9	26.9	Medium	Pluriparous	−6		−1		9	6	3	33
17	Flat coated retriever	4	30.5	Medium	Pluriparous	−10	−6	−2		9	6	3	33
18	Flat coated retriever	3	36.5	Large	Primiparous	−5		−1		11	5	6	55
19	Bloodhound	4	52.2	Large	Primiparous	−6		−1		14	8	6	43
20	Pomeranian	3	3.5	Small	Primiparous	−10		−2	−1	2	0	2	100
21	Bouvier des Flandres	5	34.5	Large	Primiparous	−11	−5			10	5	5	50
22	Maremmano-abruzzese sheepdog	2	40.3	Large	Pluriparous	−8		−4		6	4	2	33
23	French bulldog	2	11.7	Small	Primiparous	−6		−2		5	2	3	60

Days of consultation are divided into two time intervals: −11/−5 (time I) and −4/0 (time II) days before parturition (DBP). The sex ratio of pups was calculated as the percentage of females present in each litter.

TABLE 2 Formulas for calculation of days before parturition (DBP) or gestational age (GA: days after LH peak) in bitches of different sizes (small ≤ 10 kg, medium 11–25 kg, large ≥ 26 kg) (10–12) for the inner chorionic cavity (ICC), crown–rump length (CRL), body diameter (BD), and biparietal diameter (BP).

Parameter	Maternal size	Formula	Reference	Time of measurement
ICC	Small	DBP = (mm – 68.68)/1.53	(4, 7)	39–29 DBP
	Medium	DBP = (mm – 82.13)/1.8	(4, 7)	
	Large	DBP = (mm – 105.1)/2.5	(12)	
CRL	All sizes	$GA = 24.64 + 4.54 \times cm - 0.24 \times cm^2$	(4, 6)	39–25 DBP
BD	All sizes	$GA = 22.89 + 12.75 \times cm - 1.17 \times cm^2$	(4, 6)	29–7 DBP
BP	Small	DBP = (mm – 25.11)/0.61	(4, 7)	26–3 DBP
	Medium	DBP = (mm – 29.18)/0.7	(4, 7)	
	Large	DBP = (mm – 30)/0.8	(12)	

Measurements (scale either in mm or cm) are the mean of ICC, CRL, BD, and BP measurements in all measured fetuses. Time of measurement refers to the DBP at which each formula was used to estimate the parturition day. References are also reported.

showing FGM). The health of the examined pups was assessed up to 2 weeks of age.

Observing any abnormalities or clinical signs of systemic pathologies and administering hormonal interfering drugs (e.g., corticosteroids, sex hormones) during the 6 months before the estrous phase or pregnancy were considered exclusion criteria. Singleton pregnancies, fetal heart rate alterations with values lower than 160 bpm (23), fetuses with US evidence of morphological abnormalities, a stillbirth rate $\geq$ 30%, the neonatal death of the monitored fetuses or elective C-section, performed before serum progesterone drop (progesterone $>$ 2 ng/ml) were also considered as exclusion criteria. For progesterone assay, the serum samples were analyzed with a fluorescence enzyme immunoassay (FEIA) method using an Automated Immunoassay Analyzer 360 (AIA® 360, TOSOH Corp., Japan) (24). Therefore, bitches in which the US monitoring days were based on fetometry were excluded if, after delivery, the US monitoring days counted backward from the parturition day (day 0) were not in the defined time range.

Statistical analysis was performed using XLSTAT (2017.1.1.62936). Normality was checked using the Shapiro–Wilk test. A Mann–Whitney test was performed to analyze the variations of FGM% observed in the selected fetuses in relation to time intervals (time I–II) and the parity of dams (primiparous vs. pluriparous). A Kruskal–Wallis test was performed to identify variations of FGM% in relation to maternal size (small $\leq$ 10 kg, medium 11–25 kg, and large $\geq$ 26 kg) (10–12) and sex ratio of pups (percentage of females classified as $\leq$ 40%, 41–60%, or $>$ 60%). Sex ratio classes were created using the mean $\pm$ ½ of the standard deviation. Data are reported as median and range; for all the analyses, the level of statistical significance was set at $\alpha = 0.05$.

Results

A total of 23 bitches were included in our study. The ovulation day was unknown in 8 out of 23 bitches. Based on parturition day, the duration of time interval I was extended backward by one day (from −11 to −5 DBP) as two bitches whelped one day later than expected. Twenty-one bitches were monitored during both time intervals. In two bitches, the two US monitoring points were performed only in one time interval (bitch n. 12 and 21 were monitored on days −3 and 0 and −11 and −5 before parturition, respectively). Nineteen bitches were monitored once for each time interval. In contrast, four bitches (n. 7, 14, 17, and 20) were monitored three times during the period of interest: two of them were monitored twice

TABLE 3 Cell blood count results: Bitch identification number (*n.*), days before parturition (DBP) in which the exam was performed, hematocrit (HCT, normal range of 38–49.5%), eosinophils (normal range of 130–530/uL), platelet (normal range of 211–384*10^3/uL), hemoglobin (normal range of 13.3–17.2 g/dl), and white blood cells (WBC) (normal range of 8.53–16.61*10^3/μl).

Bitch n.	DBP	HCT (%)	Eosinophils (/uL)	Platelet (*10^3/uL)	Hemoglobin (g/dl)	WBC (*10^3/μl)
1	−2	42	310	577	14.1	7.52
2	−3	42.8	100	464	14.6	9.08
3	−4	37.1	1,080	516	12.3	16.31
4	−2	36.8	1,020	653	12.4	10.39
5	−3	38.7	1,100	410	13.4	10.81
6	−4	46.3	50	393	15	14.88
7	−1	37.8	220	173	12.8	7.12
8	−4	34.7	1,930	258	11.5	14.05
9	−2	38.7	1,100	456	12.8	13.50
10	−3	37.7	860	480	12.7	11.07
11	−2	44	1,210	718	15	11.06
12	0	39.3	1,970	800	13	15.15
13	−4	32.7	1,720	743	11.2	13.59
14	−4	38.2	157	385	12.7	7.85
15	−4	34.4	870	647	11.5	13.76
16	−1	41	240	457	13.1	8.34
17	−6	40.4	1,520	541	13.7	11.44
18	−5	33.9	30	521	11.1	11.96
19	−1	35.9	670	535	12	15.08
20	−1	43.3	310	198	14.4	9.34
21	−5	34.2	260	621	11.4	11.45

during time interval I, while the other two were monitored twice during time interval II. Five bitches were small size, while eight and ten were medium and large size, respectively. Fifteen bitches were primiparous, and eight were pluriparous (Table 1). Eleven bitches had natural parturition, whereas a C-section was performed in the other 12 cases. Litter size was in the interval of 2–14 pups. In 21 bitches, the US observation of FGM was performed on three of the most caudal fetuses in both uterine horns. In one bitch, US observations were made on two fetuses (as she carried a pregnancy of just two fetuses), and in one bitch on two out of three fetuses because the third one was excluded due to a US diagnosis of fetal anasarca. All the fetuses in which FGM was examined were healthy and alive at birth as well as 2 weeks after birth. The sex ratio of pups was reported in 22 out of 23 bitches. A total of 173 pups (85 females and 88 males) were born. In the monitored pregnancies, seven bitches were in the group carrying $\leq$ 40% of female pups, ten had a percentage of female pups in the interval of 41–60%, and five had $>$ 60% of female pups.

A CBC was performed in 21 out of 23 bitches: on time I in two bitches and on time II in all the other 19 bitches. No abnormalities were found except for a high concentration of platelets (516*10^3 /uL, range 173–800*10^3 /uL), low hematocrit (38.2%, ranging between 32.7–46.3%), low concentration of hemoglobin (12.8 g/dL, range 11.1–15 g/dL), and an increase in the concentration of eosinophils (860 /uL, range 30–1970 /uL) (Table 3). No abnormal clinical signs were reported in any bitch.

A total of 147 FGM observations on 50 US monitoring points (25 on time I and 25 on time II) were performed. A significant difference for FGM% between time I and II was found, with a higher median FGM% during time II compared to time I (33%, range 0–100% vs. 100%, range 33–100%; $P <$ 0.0001) (Figure 1). Considering maternal size, a higher FGM% was observed in small compared to large size bitches (100%, range 67–100% vs. 67%, range 0–100%; $P = 0.01$) (Figure 2). The FGM% was not affected by the parity of dams and sex ratio of pups ($P = 0.607$ and $P = 0.419$, respectively) (Figures 3, 4).

Discussion

In the canine species, the US evaluation of fetal intestine development was performed by Gil et al. (19). They described for the first time four different phases of intestinal development

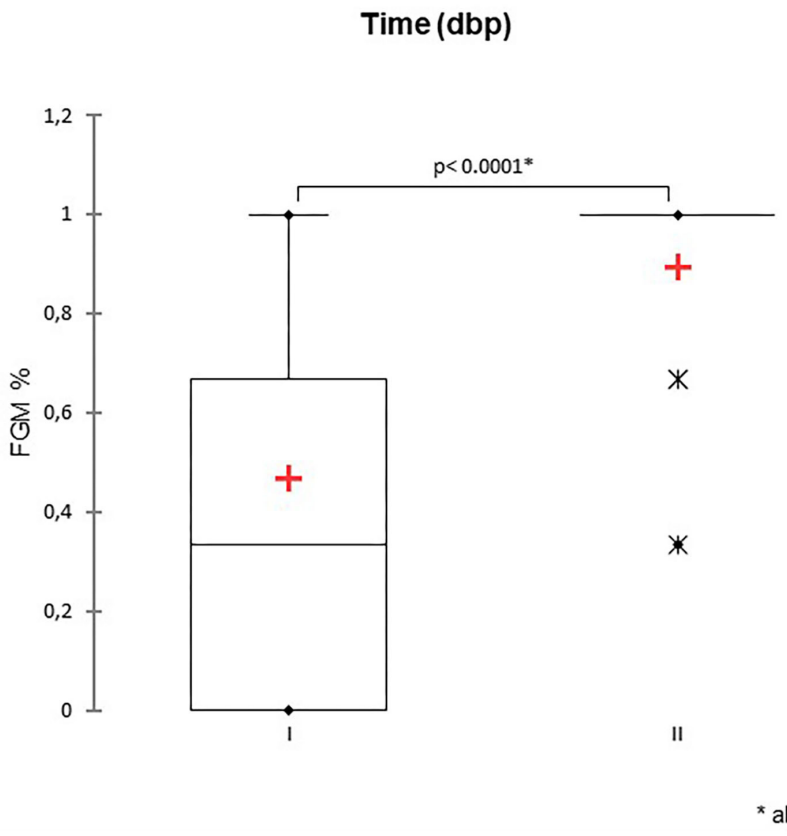

FIGURE 1
The box plot reports the percentage of fetuses showing fetal gastrointestinal motility (FGM%) during two-time intervals: time I −11/−5 days before parturition (DBP) and time II -4/0 DBP. Asterisk (*) indicates a statistically significant difference between classes ($P < 0.0001$). The mean is indicated as a red cross (+).

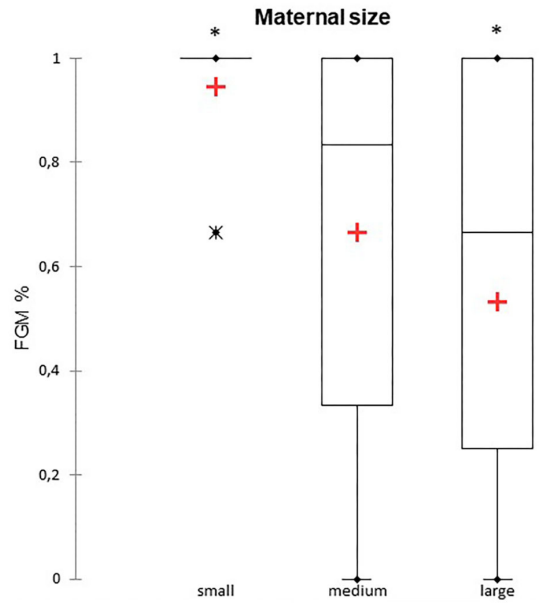

FIGURE 2
The box plot reports the percentage of fetuses showing fetal gastrointestinal motility (FGM%) in relation to maternal size in small (≤ 10 kg), medium (11–25 kg), and large size (≥ 26 kg) bitches (10–12). Asterisk (*) indicates a statistically significant difference between classes ($P = 0.01$). The mean is indicated as a red cross (+).

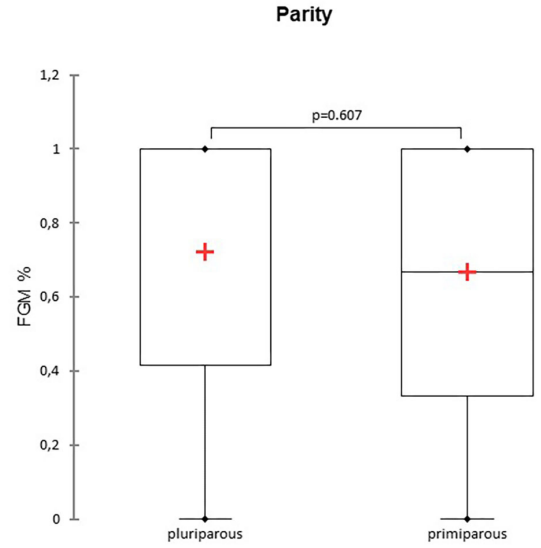

FIGURE 3
The box plot reports the percentage of fetuses showing fetal gastrointestinal motility (FGM%) in pluriparous and primiparous bitches. The mean is indicated as a red cross (+).

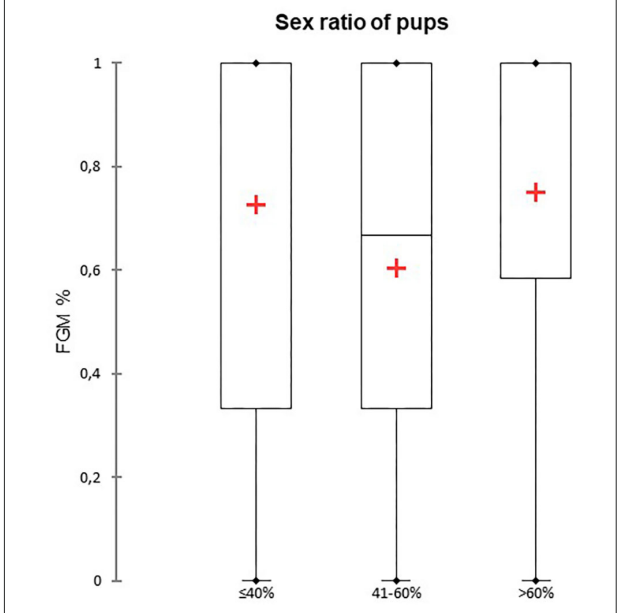

FIGURE 4
The box plot reports the percentage of fetuses showing fetal gastrointestinal motility (FGM%) dependent on the sex ratio of pups. Sex ratios were divided into three different classes depending on the percentage of females: ≤ 40%, 41–60%, and > 60%. The mean is indicated as a red cross (+).

detected by US. Pregnant bitches were divided into two groups, depending on the type of parturition (natural vs. C-section). Based on these two groups, each phase of intestinal development was defined with a specific time range. The day of the C-section was established based on fetal heart rate (< 160 bpm), which was used as a marker of fetal distress (emergency C-section) (19, 23). In our study, all C-sections were elective (based on progesterone concentration < 2 ng/ml); thus, we considered that our study was performed during the period described for natural parturition.

In an earlier study, we monitored FGM during the last 10 days of pregnancy (−9/0 DBP), describing a progressive increase in FGM% as parturition approaches. A marked increase (from 17.1% to 63.3%) was observed during the last ten DBP (20). In that study, each bitch was checked only once, which made it impossible to assess the significance of the increase in FGM% over time (20). Therefore, we decided to determine whether or not FGM% is higher during the last 5 days compared to the previous 5 days of pregnancy, as the last decade of gestation is a period in which accurate parameters to predict the delivery day are still lacking (1–4).

In our study, we observed a lower FGM% (33%) during time I (−11/−5 DBP) compared to 100% during time II (−4/0 DBP). Moreover, US detection of FGM was easier to observe in small compared to large size bitches during the same observation period. Maternal size may influence the ease of detection of FGM by the clinician, as it could be easier to observe fetal intestinal peristalsis when the size of the dam is small compared to large size. Furthermore, it should be considered that the US unit and

probe characteristics, the dam's temperament, her positioning during the US, and the operator's experience influence the evaluation of FGM (25). Further studies with a larger sample size are needed to confirm a correlation between maternal size and the US detection of FGM in the canine species as well as to assess the importance of other environmental and managerial factors.

In our study, FGM% was not influenced by the sex ratio of pups. In human medicine, sex affects fetal maturity by acting as a regulatory factor for the surfactant system, influencing the Na+ transport channel expression and type II pneumonocyte maturation. The difference between male and female development seems to be related to sex hormones and sex-related genetic differences (26, 27). Moreover, in the canine species, fetal maturity is reported to be reached at different time points depending on fetal sex: female fetuses are mature at 59 days post-ovulation, whereas male fetuses mature one day later (28–30). In dogs, pregnancy length seems not to be affected by sex ratio when related to litter size (29).

In our study, no abnormalities were observed in CBC results except for those related to a physiological pregnancy at term (31, 32) and an increase in eosinophils, which may be due to the presence of intestinal parasites. Unfortunately, a fecal exam was not performed on the bitches of our study who showed an increase in eosinophils.

In horses, FGM is highly correlated with DBP, increasing the frequency of FGM observation as parturition approaches. A model was developed considering the combined use of FGM and a maternal parameter, such as tail head relaxation (33). In human medicine, FGM increases progressively from 16 to 40 weeks of pregnancy, and after week 28 FGM is more closely correlated to gestational age than BP or femur length (34). In humans, the combined use of FGM, intraluminal echogenicity of the colon, development of colonic haustra, and colon diameter may be used alone to estimate gestational age. These parameters are useful, especially during the last trimester when the US estimation of gestational age is less accurate than in the other pregnancy periods (34).

Further studies are needed to identify a panel of parameters useful for predicting parturition day during the last ten days of canine pregnancy. In human reproduction, the assessment of persistent US abnormalities in the fetal intestine (dilation or hyperechogenicity) is predictive of the presence of neonatal abnormalities (35). In canine reproduction, assessing FGM during the last decade of pregnancy could also be useful for assessing fetal gastrointestinal abnormalities.

In conclusion, in the pregnant bitches of our study, a marked increase in fetuses showing fetal gastrointestinal motility (from 33 to 100%) was observed in the last 5 days compared to the previous five days before parturition. Peristalsis is a useful tool to assess fetal maturity in the prepartum period in the bitch. Maternal size also influenced the detection of the percentage of fetuses showing fetal gastrointestinal motility, with higher values in small compared to large size bitches. Further studies are needed to assess whether fetal gastrointestinal motility may be used as the only parameter to plan a C-section in clinical practice. In the meantime, fetal gastrointestinal motility may be of help in the estimation of days before parturition as well as in deciding the best day to start monitoring prepartum progesterone, especially when ovulation day is unknown. Further studies are needed to better quantify this parameter variations in correlation with fetal maturity around parturition time.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by University of Padova Ethics Committee (Project nr. 69/2018). Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

CM, MD, and SR: study conception. GS, CM, and FN: practical execution. BC and MD: analysis and interpretation of results. CM and SR: supervision. GS: writing—original draft. GS, SR, MD, BC, CM, and FN: editing. CM: funding acquisition. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.1000975/full#supplementary-material

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Reproduction in South American wild canids—A review

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Canids occupy the top of the food chain and are fundamental in sustaining a wild animal/environmental balance. South America, the most biodiverse continent, has 11 species of canids inhabiting diverse biomes, with or without overlapping territories. Although several species are threatened, little is known about their reproductive biology. Remarkably, basic knowledge regarding ejaculate characteristics, sexual behavior, female reproductive cycles, pregnancy and management, and parturition are scarce or absent. These gaps complicate or preclude development of conservation programs. This review compiles the current knowledge of the reproductive biology of South American canids and discusses implications of this scenario.

KEYWORDS

biotechnology, reproductive physiology, conservation, carnivore, Canidae

Introduction

Scientific interest regarding the Class Mammalia began in the sixteenth century, motivated by livestock predation by members of the Order Carnivora, always searching for food and threatening rural populations (1). Included in the Order Carnivora, the Family Canidae, which includes 13 genera and 35 species (2), aroused great attention due to its great diversity and worldwide distribution (3).

Despite their skulls, teeth, and muscles adapted to capture and kill prey, similar to other members of Order Carnivora, canids have evolved to acquire specific features, including more teeth and longer skulls (4), legs, and feet. Also, they usually have five toes on the forefeet and four on the hindfeet, long un-fused metapodials, no retractile claws, and digitigrade stance. In addition, all male canids have a well-developed penis bone (os penis) (5).

It is noteworthy that canids are extremely diverse and can survive under the most extreme environmental conditions, from scorching deserts to severe winters (6). Consequently, wild canids that arrived from North America, *via* the Isthmus of Panama (7), have occupied very diverse South American biomes, including the Caatinga, Atlantic Forest, Pampas, Cerrado, Amazon, Pantanal, and Chilean Desserts (8). Currently, South America, the continent with the most extraordinary canid biodiversity (2), is home to 11 wild canine species (1), with incredible potential for unique scientific discoveries (3).

Environmental challenges of South American biomes triggered a series of profound genotypic and phenotypic changes (7, 9), readily apparent by the large interspecific ecomorphological varieties in eating habits, predation behaviors, and reproductive

physiology (10, 11). For example, small Bush dogs form packs, inhabit semiaquatic environments, and are carnivores (12). In contrast, the large Maned wolf, with long legs and large ears, is omnivorous and solitary (13).

Wild canids have great ecological importance in South America. Besides being sentinel species for emerging canine diseases (14), they are active participants in maintaining and balancing South American ecosystems (15), including maintenance of flora. For example, while consuming a specific fruit from the lobeira tree (*Solanum lycocarpum*), a Maned wolf (*Chrysocyon brachyurus*) acts as a seed disperser (16). Similarly, the Crab-eating fox (*Cerdocyon thous*) is a secondary disperser of *Eugenia umbelliflora* seeds (17).

Despite their importance, wild canids are seriously threatened due to loss, degradation, and fragmentation of their habitats, forest fires, hunting, and being struck by motor vehicles. Consequently, they are subject to geographic isolation, inbreeding, loss of genetic variability, decreased reproductive efficiency, and reduced population numbers (18, 19). Inbreeding is a critical cause of the increased proportion of morphologically defective sperm in ejaculates (20–25) and increased incidence of congenital reproductive conditions (e.g., monorchidism, anorchidism, cryptorchidism, and testicular hypoplasia) (21). Additionally, geographic isolation can hybridize phylogenetically similar species, such as Culpeo (*Lycalopex culpaeus*), Darwin's fox (*Lycalopex fulvipes*), and Chilla (*Lycalopex griseus*) (26, 27), generating further reproductive impacts.

A possible strategy to mitigate the loss of genetic variability and increase the wild canid population in fragile ecological niches would be to develop and optimize reproductive biotechnologies aimed at establishing germplasm and developing assisted reproductive programs in vulnerable populations at-risk, or seriously threatened species (28). However, despite establishment of the International Convention on Biological Diversity (CBD) during the UN Rio-92 meeting (29), little has been done in South America for conservation of its fauna (30). Specifically, for canids, there are virtually no consistent programs for their preservation, mainly due to no or limited financial support from funding agencies (31).

With this review, our objective is to characterize the state-of-the-art knowledge regarding reproductive biology of the wild South American canids and how this scenario influences development of reproductive biotechnologies.

General features

Like other members of the Mammalia Class, canids are homeothermic, sexually reproducing species, with internal fertilization and intrauterine embryonic/fetal development. After birth, individuals feed predominantly on milk produced by the maternal mammary glands and maintain a close relationship with their mothers until puberty (32).

All South American canids belong to a subfamily Canidae (4). Of them, six occur predominantly in Brazil: Crab-eating fox (*C. thous*), Maned wolf (*C. brachyurus*), Hoary fox (*Lycalopex vetulus*), Pampas fox (*Lycalopex gymnocercus*), Short-eared dog (*Atelocynus microtis*), Bush dog (*Speothos venaticus*) and five in other countries, except Brazil, Culpeo (*L. culpaeus*), Darwin's fox (*L. fulvipes*), Chilla (*L. griseus*), Sechuran fox (*Lycalopex sechurae*), and Gray fox (*Urocyon cinereoargenteus*) (33).

Due to its wide geographic adaptability, the Crab-eating fox is present in most of South America (4, 34, 35), and lives as far north as Panama, in Central America (36). This great adaptability is mainly attributed to their eating habits, including insects, rodents, reptiles, birds, river crabs, eggs, and fruits (34).

The Crab eating-fox, the only species of the genus *Cerdocyon,* is medium-sized and has a predominantly gray and light brown brindle coat, except on the tips of the ears, back of the legs, and between the jaws, where it is black (35). There is a longitudinal strip of darker hair along its dorsal line, and in the chest and belly, the coat is lighter (4). Adults have a body 60–70 cm long and a characteristic voluminous tail of ∼30 cm. Members of this species live and hunt in pairs or extended family groups formed by parents and three juvenile animals, with larger groups being rare (11, 37).

The Maned wolf, the only species of the genus *Brachyurus*, is widely distributed in South America (38). The species has been progressively adapting to agricultural regions, due to high rodent densities. It is also present in areas of previously dense forests transformed into pastures, if there are residual niches of original vegetation (39–41). The Maned wolf, one of the most threatened South American canids (42), is considered the flagship species for preserving the Cerrado, with many efforts devoted to its preservation (43).

The Maned wolf is the largest wild canid in South America. It has a body and tail that are 94–115 and 38–50 cm long, respectively, reddish-brown ruffled fur, characteristic mane, singular ears with well-developed bursae, and a narrow skull, similar to coyotes (*Canis latrans*) (39, 44). The slender body, long limbs, and paced gait are evolutionary evidence of its adaptation to savannas (39). Since 1985, the Maned wolf has been in the Species Survival Plan (SSP) of the Association of Zoos and Aquariums (AZA), with publication of manuals describing development of conservation strategies (45, 46).

The Short-eared dog, the only species of the genus *Atelocynus* (4), is medium-sized (47) and considered the only endemic canid of the Amazon region, where it inhabits low-anthropic-disturbed plains near the margins of the rivers (4), living in burrows or hollow tree trunks together with the young (47). They are solitary animals. They tolerate living in pairs in captivity but do not have social interactions with their peers (4). Due to its low population density, it is rarely seen, and it is the least studied wild canid in the world. Consequently,

its geographical distribution is poorly documented (48), and little is known about its habits, ecology, reproduction, or behavior (47, 49). The Short-eared dog differs from other South American canids in having an elongated head, small ears, dense fur (4), and partial interdigital membrane, compatible with its aquatic habitats (50). Its coat is dark gray or reddish, mixed with white hairs, including the long and hairy tail (50). It has a varied diet, predominantly fruits, insects, small and medium-sized mammals, fish, crabs, amphibians, and carrion (47).

The Bush dog, the only species of the genus *Speothos*, is rarely observed in the wild despite having diurnal habits. It is present in several Central and South American regions, including southern Panama, Guyana, Suriname, Venezuela, Colombia, eastern Peru, eastern Bolivia, Paraguay, and northeast Argentina. In Brazil, it occurs in the Amazon, Cerrado, Atlantic Forest, and Pantanal (51). Despite being short, the size of its members exceeds those of the animals of the genus *Aletocynus*, which is probably an evolutionary adaptation to its habitat of humid forests, gallery forests, and places close to watercourses. Unlike other neotropical canids, the Bush dog has a broad head, elongated body, short snout, short limbs and tail, and small, rounded ears (52). It also has a characteristic footprint with five pads, due to the first palm digit imprint (53). Adults have a reddish coat, which can vary from darker to yellowish tones, whereas puppies are grayish (52). They are gregarious animals that live and hunt in family groups of up to 10 individuals (54). They have a strictly carnivorous diet and, due to their group organization, can prey on medium and large animals, including pacas (*Cuniculus paca*), coatis (*Nasua nasua*), and even small deer (*Mazama* spp.) (52). They have a vast vocal repertoire, with an ability to mimic the sounds of their prey, thereby attracting them (55, 56).

The Gray fox, the only canid of the genus *Urocyon*, is medium-sized and omnivorous. It has a total length of 76–112.5 cm, and a whitish fur coat that covers its body. There is also a blackish band on its back and tail (4). The females' smaller size confers a certain sexual dimorphism to the species (57). Due to its excellent food adaptability, it lives throughout the Americas, from southern Canada to northern South America (58), preferentially inhabiting mixed pine forests (57). In North America, Gray foxes live sympatrically with coyotes and are preyed upon by them (59).

Except for the Gray fox, all South American foxes belong to the *Lycalopex* genus (31). The Hoary fox (*L. vetulus*) is present only in Brazil, predominantly in the Cerrado and in transition areas within the Pantanal and Caatinga (60). It has grayish fur on the back of the head and body and a characteristic yellowish belly. Adult males may have a darker dorsomedial band. Due to the similarity of coat, it can be confused with Crab-eating fox and Pampas fox. Therefore, it is essential to consider its more diminutive size for differentiation (61), as it is the smallest neotropical canid (11). With a predominantly insectivorous diet (62), they hunt alone, in pairs, or even in family groups of three to five individuals (11).

The Pampas fox (*L. gymnocercus*) is a medium-sized fox (63, 64) with sexual dimorphism due to males being larger and heavier than females (49). Due to its generalist eating habits, it is well-adapted to various South American biomes in southern Brazil, eastern Argentina, eastern Bolivia, northern Rio Negro Argentina Province, and western Paraguay (65). This species feeds on small and medium-sized rodents, hares, birds, armadillos, skunks, lizards, native fruits, carrion, and garbage (65), has solitary habits, and shelters in burrows during the day (65). The coat on the head and back is reddish with a darker band in the dorsomedial region, extending to the end of the tail, which is relatively long, thick, and gray; in the ventral area, the coat varies from light gray to white. The ears are relatively large, triangular, wide, and reddish (64).

Culpeo (*L. culpaeus*), the largest fox of the genus *Lycalopex* among wild South American canids, is surpassed in size only by the Maned wolf (66). It is distributed from Ecuador to southern Chile, adapting well from desert to forest (4, 67). It has whitish-gray fur on the dorsal area and above the shoulder, whereas on the head, neck, ears, and legs, the tone can be brown or red. The tail, long and thick, has black fur at its tip. It is omnivorous but, unlike other carnivores, defecates in open spaces and does not cover its waste (13).

The Darwin's fox (*L. fulvipes*) has a short and thin snout that, despite its small size, has a robust appearance, elongated body, and short legs with a fur coat that varies from brown to black (68). It is present in humid forest areas of Chile, with only three known groups, located in the Chiloé Island, coastal mountains of Nahuelbuta National Park (26), and Valdivian Coastal Reserve (69), respectively. Darwin's fox is considered an opportunistic omnivore; its diet varies with region and season of the year (68). It is believed that they can form expanded groups under food scarcity and territorial restrictions (13).

The Chilla (*L. griseus*) is a medium-size fox widespread in plains and mountains on both sides of the Andes in Chile and Argentina (Group, Canid Specialist., 2016). It was introduced to Tierra del Fuego in 1951 to control an excessive population of European rabbits (*Oryctolagus cuniculus*) that were causing environmental imbalance (70). Its body, measuring up to 60 cm with a tail up to 36 cm, is covered by yellowish-gray dorsal fur, with sparse white and black hairs. In the ventral region, the fur is whitish reddish-brown. It preferentially inhibits dry and cold climate biomes close to mountain formations such as the Patagonian steppe (7); however, it is very adaptable and can live in hostile habitats such as the Atacama Desert and the cold and rainy forests of Tierra del Fuego (71). The Chilla is omnivorous and has a varied diet of small rodents, arthropods, birds, and fruits (71).

The Sechuran (*L. sechurae*) fox, present from southwest Ecuador to central-west Peru, occupying the Sechura desert (7), is considered omnivorous and can survive prolonged intervals

TABLE 1 General features of South American canids.

Species	Red list category	Chromosome number	Body weight (kg)	Dental form I; C; P; M = total
Crab-eating fox	Least concern	74 (73)	5.7 (4)	3/3; 1/1; 4/4; 2/3 = 44 (35)
Maned wolf	Threatened	76 (74)	25-30 (75)	3/3; 1/1; 4/4; 2/3 = 42 (39)
Hoary fox	Threatened	74 (74)	03.3 (4)	3/3; 1/1; 4/4; 2/3 = 4 (60)
Pampas fox	Least concern	74 (75)	04.4 (4)	3/3; 1/1; 4/4; 2/3 = 42 (75)
Short-eared dog	Threatened	74–76 (76)	09.5 (4)	3/3; 1/1; 4/4; 2/3 = 42 (76)
Bush dog	Threatened	74 (74)	08.0 (2)	3/3; 1/1; 4/ 4;1/2 = 38 (4)
Culpeo	Least concern	74 (77)	09.7 (4)	3/3; 1/1; 4/4; 2/3 = 42 (77)
Darwin's fox	Endangered	–	03.1 (4)	3/3; 1/1; 4/4; 2/3 = 42 (68)
Chilla	Least concern	74 (73)	03.6 (4)	–
Sechuran fox	Threatened	74 (78)	03.6 (4)	3/3; 1/1; 4/4; 2/3 = 42 (79)
Gray fox	Least concern	66 (80)	09.0 (2)	3/3; 1/1; 4/4; 2/3 = 42 (81)
Falkland Island fox	Extinct	66 (80)	–	–

TABLE 2 Biomes occupied by South American canids.

Scientific name	Species	Biomes
Cerdocyon thous	Crab-eating fox	Atlantic Forest, Cerrado, Pantanal, Caatinga, Pampas, and tropical forest (34, 36)
Chrysocyon brachyurus	Maned wolf	Cerrado and transition between Cerrado and Atlantic Forest (38, 41)
Atelocynus microtis	Short-eared dog	Amazon Region (4)
Speothos venaticus	Bush dog	Amazon Region, Cerrado, Atlantic Forest, and Pantanal (51)
Urocyon cinereoargenteus	Gray fox	Savanna (71)
Lycalopex vetulus	Hoary fox	Cerrado and in transition areas between Pantanal and Caatinga (60)
Lycalopex gymnocercus	Pampas fox	Pampas, Steppe, and tropical rainforest (65)
Lycalopex culpaeus	Culpeo	Tundra and Montane Forest (4, 67)
Lycalopex fulvipes	Darwin's fox	Humid forest areas of Chile (26, 69)
Lycalopex griseus	Chilla	Tundra, Montane Forest and steppe (71)
Lycalopex sechurae	Sechuran fox	Tundra and Montane Forest (7)

of feed and water restrictions (72). It has a small head, with a short snout and long ears, covered on its rostral face with gray fur, except around the eyes where the tones are reddish-brown. They are solitary animals, rarely seen in groups, except when they form couples during the reproductive season.

Additional characteristics of the South American canids are shown in Tables 1, 2.

Reproductive characteristics

Reproductive physiology

Little is known about the reproductive physiology of wild South American canids. The scientific literature on the subject is scarce and full of gaps. For example, compared to the information available for domestic dog, almost nothing is known about their puberty and age at sexual maturity, reproductive endocrinology, estrous cycle, sexual behaviors, mating system, or time of ovulation. Information related to the dynamics of the vaginal epithelium throughout the estrous cycle and stages and duration of delivery is also limited. For Darwin's fox and the Short-eared wild dog, even gestation duration is unknown. Available information, generated predominantly from observational studies, is summarized below.

Seasonality

Reproductive seasonality is a vital strategy to concentrate births in times of greater food availability and environmentally favorable for offspring survival (82–84). Most wild South American canids are considered seasonally polyestrous, with two reproductive phases: anestrus and breeding seasons. Except for the Maned wolf that is a shot-day-breeder (autumn-winter breeder) (39, 85, 86), most of them, such as the Crab-eating fox (87), Chilla (33), Gray fox (33), Short-eared dog (49),

Darwin's fox (68), and Sechuran fox (88), are considered long-day breeders, with a breeding season during late winter and spring. Exceptionally, the Crab-eating fox may give birth twice in the same year, depend on its geographic location and food availability (37). The Hoary fox (89), Pampas fox (90), and Culpeo (52) are monoestrus species, mating during the winter or spring. Of these, the Hoary fox starts its breeding season early, in July (89).

Distinct from the others, the Bush dog is annual polyestrous, although ovarian activity occurs only in the dominant female of the group, being suppressed in subordinate females (84, 91, 92), similar to wolves (81). Unlike other canids, the presence of a male was associated with shortened interestrus intervals and increased numbers of estrous cycles (92).

Little is known about effects of seasonality on male reproduction and sperm production. Based on testosterone data in Bush dogs, it was concluded that sperm production is year-round and not seasonal (84). However, in Maned wolves, blood testosterone concentrations are stable throughout the year, although they have a higher seminal quality index and greater scrotal circumference during the breeding season (2). In Gray foxes, sperm epididymal reserve increases during the reproductive season (93).

During the breeding season, the Maned wolf male increases vocalization and territorial marking with urine (39). The male effect is also described in Bush dogs, where the male presence shortens the interestrus interval (91).

Estrous cycle

Information regarding the estrous cycle of the wild canids is scarce and inconsistent. Most of the information is related to the Maned Wolf, in which the follicular phase of estrous cycle seems to be similar to the bitch, with proestrus lasting ~14 days (94), estrus ranges from 1 to 10 days (95), and mating lasting up to 14 min (94, 96). During proestrus, there is vaginal swelling and secretions, as well as increases in social solicitatious behavior (94). In an ultrasonographic survey, the ovaries were ~1.10 cm long and ~0.7 cm wide, although the uterus was not visualized (97). In this species, there is also evidence that ovulation is induced and only occurs in the presence of the male (98), probably triggered by specific volatile organic compounds present in male urine (99), as several volatile organic compounds present in the urine of male Maned wolf are considered semiochemicals (100).

Regarding the Crab-eating fox, it is only known, from an endocrine and colpocytological study, that the estrous cycle phases are like those of the bitch (*Canis lupus familiaris*), except for the absence of bleeding during proestrus (101). Estrus lasts 3 days and, similar to what happens in the dog, during the mating, the penis is retained for 5–8 min when the breeding pair assumes a back-to-back position (37). Plasma progesterone concentration reaches its maximum value (~46 ng/mL) on the 10th gestational day (101).

The long non-gestational diestrus (~75 days), a particular feature of the bitch estrous cycle (77), is only described in Hoary fox (89) and Culpeo females (102).

Andrology

Knowledge about andrology of South American canids is minimal, and there are no controlled studies regarding male reproductive endocrinology, puberty, and sexual maturity.

Ultrasonographic descriptions of male reproductive tract are only available for Maned wolf and Crab-eating fox. The ultrasonographic appearance of the testes in Maned wolves is quite different from that of the dog, with a hypoechoic coarse echotexture and mediastinum slightly echogenic and poorly defined. The testes are 2.87 cm long and 1.22 cm wide. Echogenicity of the prostate is similar to that of surrounding tissues, making it difficult to visualize (97). In Crab-eating fox, the prostate is localized caudal to the bladder with a bilobed, regular contour, with homogeneous parenchyma and central prostatic urethra. Spectral Doppler ultrasonography revealed that testicular and capsular arteries had biphasic blood flow with evident systolic peaks, followed by a gradual reduction of diastolic flow (low vascular resistance). In contrast, intratesticular arteries have monophasic blood flow patterns without marked flow during diastole (103).

Semen collection techniques and sperm characterization are the subjects most addressed; however, the surveys and species are limited. Electroejaculation, the method of choice for semen collection in wild carnivores (104), has been adapted to various species (105–107). However, electroejaculation seems to be ineffective in most South American canids, as there is almost always contamination of the ejaculate with urine (108, 109). This represents a critical obstacle for semen characterization and obtaining physiologic sperm samples for artificial insemination or cryopreservation. Urinary contamination of semen is known to cause severe abnormal sperm morphological changes and necrospermia.

There are only a few reports of semen collection by electroejaculation in Maned wolf (110, 111) or urethral catheterization in Maned wolf and crab-eating fox (112, 113). Collection of seminal samples by urethral catheterization of males pre-treated with a medetomidine and ketamine combination seems to be a promising technique for obtaining seminal samples from wild canids *in situ* (112, 113). However, all attempts to collect semen from Maned wolf and Crab-eating fox by electroejaculation or urethral catheterization performed in our laboratory were unsuccessful and/or samples were contaminated with urine (unpublished data). In Crab-eating foxes, attempts at urethral catheterization were monitored by abdominal ultrasonography to ensure that the catheter tip was positioned caudally to the bladder neck, at the *colliculus*

TABLE 3 Mean (± SD) of semen characteristics of South American canids.

Semen collection method	Semen characteristics	Species	
		Crab-Eating fox	Maned wolf
Eletroejaculation	Volume (mL)	–	2.0 (0.6) (111)
	Total motility (%)	–	59.8 (4.9) (111)
	Sperm concentration (10^6 × spz/mL)	–	43.4 (18.2) (111)
Pharmacological induced ejaculation	Volume (mL)	0.04 (21.98) (113)	0.1 (112)
	Total motility (%)	40.0 (29.01) (113)	40.0 (112)
	Sperm concentration (10^6 × spz/mL)	277.6 (298.7) (113)	10.0 (112)
Digital stimulation	Volume (mL)	0.4 (178.4) (103)	1.3 (1.2) (83)
	Total motility (%)	68.0 (6.1) (103)	76.1 (23.9) (83)
	Sperm concentration (10^6 × spz/mL)	463.7 (594.4) (103)	73.9 (87.2) (83)
Epididymal harvest	Volume (mL)	–	–
	Total motility (%)	–	95.0 and 62.5* (115)
	Sperm concentration (10^6 × spz/mL)	–	25.4 and 23.5* (115)

Spz, spermatozoids; *Sperm collection during breeding season.

TABLE 4 Gonadosomatic index and tissue proportion of the testicular tubular and intertubular compartments in *Cerdocyon thous* and *Lycalopex vetulus*.

Scientific name	Species	*N*	Gonadosomatic	Seminiferous tubules	
			Index (%)	Intertubular tissue (%)	Seminiferous tubules (%)
Cerdocyon thous	Crab-eating fox	6	0.07 (0.02) (109)	12.7 (5.3) (109)	87.5 (5.2) (109)
		6	–	15.64 (2.84) (108)	86.96 (2.47) (108)
Lycalopex vetulus	Hoary fox	5	–	13.04 (2.47) (108)	84.37 (2.84) (108)

seminalis region, using the median portion of the prostate as a reference.

The difficulty of obtaining ejaculates in South American canids has been overcome in Maned wolf (83, 114) and Crab-eating fox (103) using seminal collection by digital stimulation of the penis, similar to what is done in domestic dogs. This approach has already allowed seminal characterization in captive Crab-eating fox (103) and Maned wolf (83), determination of sperm seasonal changes (83), and cryopreservation of seminal samples (114) in Maned wolf. It is worth noting that this technique required prior conditioning of the Crab-eating fox (103) and safety precautions since the animals are neither sedated nor anesthetized. However, in the Maned wolves, there was no necessity for conditioning. The males were physically restrained with a muzzle and catch pole, and seminal samples were obtained in the first attempt (83). Semen characteristics of Crab-eating fox and Maned wolf are summarized in Table 3.

Other techniques employed to study male reproductive physiology, particularly spermatogenesis, sperm production, and testicular disorders, are histological evaluation of testicular parenchyma, preparation of smears, and epididymal sperm counts. These approaches enabled characterization of the testicular morphology of Crab-eating fox (116, 117) and Hoary fox (116, 118) (Table 4), sperm production in the adult Maned wolf (119), and enumeration of epididymal sperm in Gray fox (120).

There are only two reports of seminal cryopreservation in Maned wolf. In the first study, sperm were frozen with an egg yolk extender containing 1 M dimethyl sulfoxide or 1 M glycerol. The use of dimethyl sulfoxide resulted in higher post-thawing motility (20.0 ± 1.9 vs. 13.5 ± 2.1%) and plasma membrane integrity (51.2 ± 1.7 vs. 41.5 ± 2.2%) than glycerol (111). In the other study, conducted by our group, semen was frozen with a TRIS egg yolk extender containing 7% glycerol; post-thawing sperm motility was >55% (114). In other species of South American canids, there are apparently no reports on sperm cryopreservation.

Sexual behavior and mating systems

Little is known about wild South American canids' sexual behaviors and mating systems; however, most findings indicate

TABLE 5 Pregnancy duration and litter size of South American canids.

Species	Duration of pregnancy (days)	Litter size
Crab-eating fox	52–59 (4, 11)	3–6 (6)
Maned wolf	62–66 (4, 39, 77)	1–5 (4, 39)
Hoary fox	50 (10)	2–5 (7)
Pampas fox	55–60 (4)	3–5 (6)
Short-eared dog	–	2–3 (51)
Bush dog	67 (33); 60–83 (4)	1–6 (6)
Culpeo	55–60 (4, 33)	3–8 (6)
Darwin's fox	56 (118)	2–3 (119)
Chilla	53–58 (4)	2–4 (6)
Sechuran fox	–	–
Gray fox	51–63 (4)	1–10 (6)

that Pampa fox (121), Chilla (71), Culpeo (122), and Maned wolf (39, 123) have solitary habits and males and females only pair during mating season. Crab-eating fox (124), Maned wolf (39), Pampa fox (63), Hoary fox, and Gray fox (81) are monogamous. However, even though the Gray fox generally has monogamous behavior, polyandry is often apparent (125).

Unlike the above-mentioned species, Bush dog (4) and Chilla (13) are polygamous and form harems; however, reproductive activity is suppressed in subordinate females by the presence of the dominant female of the family group. In the Bush dog, the couple separates from the group at the time of copulation (4).

Pregnancy and births

The duration of gestation of wild South American canids and the number of offspring born in each litter are generally similar to those of the bitch, with reported data summarized in Table 5 (4, 6, 7, 10, 11, 33, 39, 51, 86, 126, 127).

In general, pregnancies range from 52 to 60 days, being longer in the Maned wolf, which can be up to 65 days (4, 45), and in the Bush dog, with conflicting reports that it can vary from 65 to 83 days (4, 91). Parturition in Short-eared Dog (49), Crab-eating fox (37), Maned wolf (45, 128), Darwin's fox (68), and Chilla (13) occurs in burrows, where the young are kept during initial development. Like domestic dogs, eyes, and auditory canals of Maned wolf puppies only open around the ninth day of life (1, 45, 129).

In Maned wolf, the first parturition can occur as early as at 1.5 years of age, but predominantly happens at 4 years, and pregnancies can be established until 10-12 yr, although uncommon, being more usual from 3 to 8 years (95).

The causes of embryonic and fetal loss and neonatal deaths are unknown in wild canids. There is only one report that Maned wolf females that experience neonatal losses have lower concentrations of progesterone metabolites in their feces during the second half of pregnancy than those whose offspring survived (130).

Behavior monitoring can be used as a non-invasive tool to distinguish mating success in captive Maned wolf. Although the pair's interactive behavior patterns are similar during estrus, approach behavior is only maintained in the post-estrus period when the female becomes pregnant (131).

Breastfeeding and parenting

Except for the Sechuran, in which births occur without the presence of the male (72), in other wild South American canids, couples established during the breeding season seem to remain together when pups are suckling. This behavior appears to be significantly favored by monogamy and couple stability (92). In Crab-eating fox, the young remain with their parents, forming extended family groups (124). Similarly, the Maned wolf has stable parental relationships, and males can collaborate during parturition and early development of the young (39, 44). Shared parental care is also reported for the Hoary fox (60), Pampas fox (132), Bush dog (56), Chila (71), and Darwin's fox (122). In Darwin's fox, while the father's parenting care increases, maternal care progressively decreases (68). Male parental behavior in canid appears to be stimulated by a seasonal rise in plasma prolactin concentrations. This phenomenon is well-documented in the Gray wolf, but not yet described for other South American canids (92).

After weaning, which occurs at 4 months of age in Hoary fox (11) and in Darwin's fox (68), 2 months in Pampas fox (4), 2.5 months in the Bush dog (4), and 30–37 days in Coupe (33), family groups remain stable. This arrangement lasts until 6–7 months postpartum in Short-eared dog (50) and Chilla (33), 10 months in Bush dog, when the pups reach sexual maturity (52), and 9–12 months in Crab-eating fox (37). In Short-eared dog, females abandon their offspring and settle in a new foraging area (49). In Hoary fox (61), Bush dog (4), Chilla (76), and Crab-eating fox (124), offspring leave their parents and settle in other areas.

There is no information regarding sexual development of the wild South American canids, except for Gray foxes that reach sexual maturity at 10 months of age but do not always become pregnant in the first year (13).

Current situation and perspectives

Physiological knowledge is essential for developing reproductive biotechnologies for establishing germplasm banks for present and future use. However, a broad understanding of the challenges is indispensable to succeed in this task.

The initial objective of this review was to compile the available knowledge regarding reproductive biology

of wild South American canids, assess potential use of this information for development of animal conservation programs, and highlight the main obstacles to realization of these proposals. However, it is clear that this possibility remains remote.

The considerable current gaps in the knowledge of reproductive biology of South American canids and their great diversity are substantial obstacles to developing consistent conservation programs. This situation is aggravated by difficult access to knowledge already produced, as some of it was recorded only in local yearbooks of the zoos or zoo-botanical parks.

The current knowledge predominantly derives from observational and descriptive studies regarding social dynamics (133), ecology (89, 134, 135), diet (17, 34, 67), sharing of foraging areas by sympatric canids (38), diet, habitat use, and home ranges of sympatric canids (38, 59, 65, 136, 137), reproductive season (83, 84, 138), parental care (37, 44, 45, 132), time of birth (92, 127), and ontogeny (12, 37). However, basic female reproductive processes such as endocrine patterns, details regarding the estrous cycle, ovulatory mechanisms, physiology of pregnancy, and causes of infertility are still not understood. Concerning andrology, this scenario is even worse. It was disconcerting to realize, for example, that there are no basic studies about male reproductive anatomy and physiology, except for Maned wolf (83) and Crab-eating fox (103).

There are several reasons for the reduced number of studies on reproductive biology of wild South American canids. Unlike domestic animals, in wild animals, simple procedures such as physical examination, blood collection, and ultrasonographic examination are complex due to the need for pharmacological restraint. This creates potential risks for accidents for the animals and people involved in these procedures. Besides this, restraint procedures invariably trigger endocrine and behavioral stress responses (139) that invalidate many physiological data. One approach to overcome these difficulties is using non-invasive methods of monitoring endocrine function by measuring fecal concentrations of sex steroid metabolites. This method was used to determine endocrine and reproductive cycles in Hoary fox (88), Maned wolf (94) and Bush dog (91), and to study fetal losses in Maned wolf (130). However, this assessment is also difficult as it requires monitoring the animals to verify the sample's origin and rapid retrieval to avoid degradation of sex hormones metabolites by fecal microbiota (140–142). These issues are particularly limiting in free-range and nocturnal animals.

Society does not clearly understand the role of wildlife conservation in planetary balance and global One Health. Our current urban life seems to keep us away from this reality and strongly influences private and public policies. Limited public and private investments in research are blamed for the limited knowledge regarding reproduction in the wild South American canids. The appeal to raise funds for studies involving livestock and companion animals is much more substantial, as they are directly related to food production and human wellbeing.

Difficulties in developing efficient conservation plans go well-beyond just financial issues. These initiatives also depend on specialized labor and are affected by difficulties in priorizing species (143, 144). Wildlife conservation programs require an interdisciplinary team to understand and mitigate the challenges of wildlife sanitary conditions, nutritional requirements, behavioral issues, arrested sexual development, infertility, and low reproductive efficiency (14, 145, 146).

The future of the South American wild canids depends on developing several areas of knowledge. A promising area of study involves development of hormonal protocols for induction of ovarian activity and ovulation. However, on this subject, there is only one report of the use of deslorelin implants (GnRH agonist) for 7–11 days to induce ovarian activity and ovulation in paired Maned wolf females and of the use of recombinant equine luteinizing hormone for ovulation induction in isolated ones (96). Another promising area is the use of knowledge on the reproductive physiology of the bitch (141, 147) to monitor reproductive activity in female wild canids. This approach was used in Maned Wolf females in which the plasma concentrations of anti-Mullerian hormone reflected ovarian activity, being higher in adulthood and during the breeding season (147).

Final considerations

Current knowledge gaps make it impossible to establish consistent conservative programs and develop reproductive biotechnologies for South American Canidis. This reality is worrying and emphasizes the need to alert the scientific community, society, and governments of the urgent requirement for investments and the potential consequences of failing to act promptly, as most of these animals are currently either vulnerable or threatened. Furthermore, in the broader context, preservation of South American biomes is imperative, as it does not make sense to develop reproductive knowledge and reproductive technologies only for captive specimens. Preserving the environmental conditions, coupled with broader knowledge of reproductive function, are critical to increase the genetic variability, number of free-ranging animals, and ultimately promote planetary One Health.

Author contributions

JC and JF conceived of the proposed idea and wrote the manuscript. FS, JK, and JF revised and edited the manuscript.

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Environmental risk factors in puppies and kittens for developing chronic disorders in adulthood: A call for research on developmental programming

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Many dogs and cats are affected by chronic diseases that significantly impact their health and welfare and relationships with humans. Some of these diseases can be challenging to treat, and a better understanding of early-life risk factors for diseases occurring in adulthood is key to improving preventive veterinary care and husbandry practices. This article reviews early-life risk factors for obesity and chronic enteropathy, and for chronic behavioral problems, which can also be intractable with life-changing consequences. Aspects of early life in puppies and kittens that can impact the risk of adult disorders include maternal nutrition, establishment of the gut microbiome, maternal behavior, weaning, nutrition during growth, growth rate, socialization with conspecifics and humans, rehoming and neutering. Despite evidence in some species that the disorders reviewed here reflect the developmental origins of health and disease (DOHaD), developmental programming has rarely been studied in dogs and cats. Priorities and strategies to increase knowledge of early-life risk factors and DOHaD in dogs and cats are discussed. Critical windows of development are proposed: preconception, gestation, the suckling period, early growth pre-neutering or pre-puberty, and growth post-neutering or post-puberty to adult size, the durations of which depend upon species and breed. Challenges to DOHaD research in these species include a large number of breeds with wide genetic and phenotypic variability, and the existence of many mixed-breed individuals. Moreover, difficulties in conducting prospective lifelong cohort studies are exacerbated by discontinuity in pet husbandry between breeders and subsequent owners, and by the dispersed nature of pet ownership.

KEYWORDS

behavior, epigenetics, microbiota, nutrition, obesity, chronic enteropathy, developmental programming

Introduction

There is increasing awareness that aspects of early life in puppies and kittens, especially nutrition during gestation and early growth, impact the risk of neonatal mortality (1–3) and the development of chronic diseases in adulthood (4, 5). In many mammalian species, early-life and parental experiences have been investigated as potential contributors to the developmental origins of health and disease (DOHaD). The concept of DOHaD encompasses the observations that environmental exposures during development can drive epigenetic changes that modify, or "program" the expression of genes, affecting structural and functional development, with rapid or delayed risks to health. In humans, the first 1,000 days of life, which approximates to gestation plus 2 postnatal years, have been identified as a critical period when developmental programming sets the foundations for optimal neurodevelopment, growth and health (6). Much of what is known about DOHaD and the epigenome is derived from laboratory animal models (7), but experimental knowledge has also accrued for ruminants, pigs and even horses (7–9).

In dogs and cats, most research into the etiology of chronic adulthood conditions has focused on adult environmental predictors and risk factors, without investigating whether these have developmental origins. Literature searches in PubMED® (31 July, 2022) with the broad search string (epigenetics OR "developmental programming" OR DOHaD OR "developmental origins of health and disease"), combined with "Dogs" or "Cats" as Medical Subject Headings, retrieved 218 articles. After screening titles and abstracts for relevance, 51 articles relating to dogs and 6 relating to cats remained; the most apparent topics of interest were epigenetic modifications in cancer cells [32 articles (56%)], and epigenetic aspects of breed phenotype. Overall, there has been inadequate consideration in dogs and cats of the extent to which the environment, during different stages of growth and maturation, can influence the subsequent occurrence of adult conditions and behavioral traits, even if these environmental factors are chronologically distant. Research in domestic carnivores has been led mainly by experts in specialized fields of veterinary medicine, including nutrition, reproduction, gastrointestinal microbiology, and behavior, with a paucity of expertise in DOHaD that crosses the relevant disciplines.

This review provides an overview of the main environmental risk factors in puppies and kittens that can affect the occurrence of obesity, chronic enteropathy (CE) and behavioral problems in adulthood. These chronic disorders are common in domestic carnivores, challenging to treat, and have major deleterious effects on health, quality of life and potentially longevity (10–12). Difficult behavior can lead to a break-down in the human–animal bond, and may result in abuse, relinquishment or euthanasia of pets (13–15). It is possible that some of the modifiable variables explored may represent ongoing risks that commence or become apparent in early life, and some may be manifestations of DOHaD, with changes in the epigenome at periods of developmental plasticity. There is also the potential for exposures to unmask the effects of DOHaD. Suggested research priorities are discussed for each condition, based on existing research in puppies and kittens, factors in adult dogs and cats known to be associated with the condition and hypothesized to become established during early life, and on knowledge of developmental programming in other species. Research strategies are proposed to increase our understanding of the long-term impact of early environment and life events for dogs and cats. Such strategies must include studies to determine the role of DOHaD as has been done for other species. These studies might ultimately allow the generation of guidelines to inform disease prevention from as early as preconception. This is not only important for animal welfare, but should be considered in the broader economic and societal context of dog and cat ownership.

Building upon evidence in dogs and cats, humans and laboratory animals, we propose a timeline of key exposures and developmental milestones in puppies and kittens that shape and define "early life." Early life in this review is not intended to relate to a fixed chronologic age or necessarily to the same period of development classically considered in DOHaD studies in other species. It is used to describe the periods preceding adulthood in which the physiological and psychological maturation of puppies and kittens can be affected for good or bad, or modifiable risk factors for later chronic disease emerge. This is intended to help frame future research and to encourage breeders, owners and veterinarians to take a holistic, integrated and proactive approach to promoting the long-term health of pets (10–12).

The context for research on early-life development of dogs and cats

Societal

Dogs and cats are cherished as family members in many households, making their long-term health a high priority for owners. Societal benefits of dog and cat ownership include the promotion of human health and wellbeing (16, 17); dogs also work in a wide variety of service roles. While these factors, combined with a general concern for animal welfare, provide a rationale for advancing our understanding of early-life risk factors for chronic diseases, they also mean that acceptance of invasive research in these species is limited; this is likely to be one reason for the relatively slow advancement of DOHaD knowledge in dogs and cats.

With respect to large-scale observational studies, the dispersion of the pet population makes studying connections between early and late exposures and events particularly

challenging. There is no coherent network of the relevant parties throughout a pet's life. For example, each individual dog or cat may have a different breeder who is responsible for its prenatal and first 2–3 postnatal months of life, and each pet may subsequently be homed with a different owner, who in turn may have a different veterinarian.

Economic

The size of the pet population and the direct economic significance of pets are tangible measures that help to contextualize the importance of pursuing avenues for preventive medicine. There are an estimated 92.9 million dogs in Europe (25% of households; 2021 data) (18), and 83.7–88.9 million in the USA (45% of households; 2020 data) (19). The total population of cats is estimated to be more than 113.6 million in Europe (26% of households; 2021 data) (18) and 60.2–61.9 million in the USA (26 % of households; 2020 data) (19). Sales of pet food products were €27.2 billion in Europe in 2021 (18). In the USA in 2021, market sales were $50.0 billion for pet foods and treats, $34.3 billion for veterinary care and product sales, and $9.5 billion for other services outside of veterinary care, such as boarding, grooming and insurance (20).

Biological

Breed

Large phenotypic variability within the canine species, and to a lesser extent the feline species, contributes to the complexity of research in companion animals. The canine species exhibits the widest morphological and weight differences between breeds of all terrestrial mammalian species. More than 350 breeds of dogs are recognized by the International Cynological Federation (21). Adult weights range from 1 kg, for a Chihuahua, to more than 100 kg, for an English Mastiff. Moreover, many pet dogs (up to 40% in the UK) are a mix of breeds (22). Age at which adult body weight is attained correlates with dog breed size, ranging from ∼9 to 10 months for toy, small and medium-sized breeds, to 11–15 months for large and giant breeds (23). Size diversity is less pronounced in the cat population, in which 45 breeds are recognized (24) and only 5–15% of cats are pedigreed (25). Adult cat weights range from ∼2 kg for a Munchkin to 10 kg for a Maine Coon (25).

Reproductive biology

Understanding early-life risk factors for adult diseases and the potential for developmental programming requires a knowledge of species-specific biology of conception and fetal and neonatal development (summarized for dogs and cats in Supplementary Figure 1, Supplementary Tables 1, 2). This allows the timing of environmental exposures to be related to the differentiation of cell types and the development of specific tissues and organs. Overall embryonic and fetal development is similar between dogs and cats (26) with the exception of oocyte maturation and ovulation. Ovulation in cats is typically induced by coitus (26), although spontaneous ovulation seems to be more than anecdotal (27). Oocytes are released in metaphase II, so fertilization can occur as soon as they reach the oviduct (28). In dogs, there is spontaneous ovulation of immature oocytes at prophase I. Oocyte meiosis resumes after ∼ 48 h in the oviduct, and fertilization occurs from 90 h after ovulation (28, 29). Another difference in dogs is that follicles undergo preovulatory luteinization, so serum progesterone concentrations are already high at ovulation (28, 29).

Milestones of early life

Dogs and cats share many major biological milestones with other species, but the timing and biological details differ. These milestones include embryonic and fetal events (Supplementary Figure 1; Supplementary Tables 1, 2), neonatal survival, transition to solid foods and neutering. Periods of organ and organ/system development and maturation in which external factors can modify its developmental trajectory are numerous. These critical periods represent different windows of opportunity to promote development beneficial to long-term health.

Early-life environmental exposures and events as risk factors for selected disorders in adult dogs and cats

Obesity in dogs and cats

As stated by Kopelman (2000), "obesity can be defined as a disease in which excess body fat has accumulated such that health may be adversely affected" (30). Obesity is defined as a chronic relapsing disease, which itself can predispose to other non-communicable diseases, such as diabetes mellitus, cardiovascular diseases and cancer in dogs and cats (31). In the field of veterinary medicine, over 20 national and international veterinary and associated organizations support the classification of obesity as a disease (32), which is regarded as the number one health problem in companion animals (33).

Overweight and obesity in both dogs and cats is generally measured by determining the body condition score (BCS), which correlates well with adipose tissue mass (32–35). On this basis, a study of dogs at family pet shows in the UK reported that 65% of adult dogs were overweight or had obesity, and 9% had obesity (10). In the 2018 obesity prevalence survey in the USA conducted by the Association for Pet Obesity Prevention

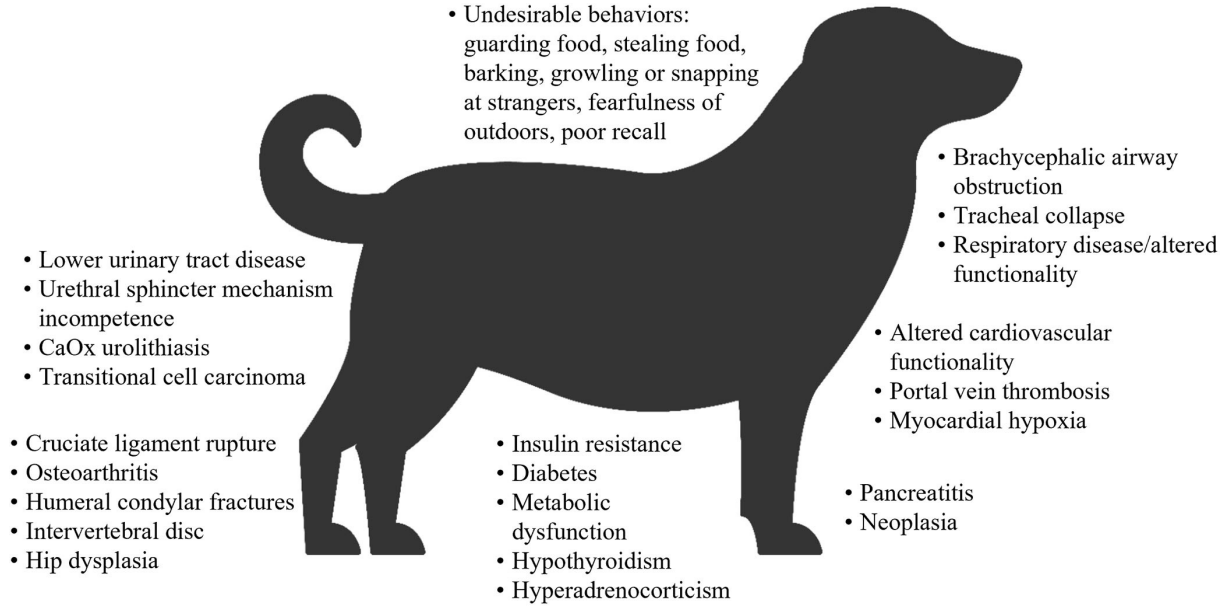

FIGURE 1
Overview of comorbidities associated with obesity or overweight in dogs (31, 37–40, 42, 43, 46–49). Some associations were specific to either obese dogs or overweight dogs. CaOx, calcium oxalate.

(APOP), veterinarians assessed 36.9% of dogs as overweight, and 18.9% as having obesity (36). Obesity and/or overweight in dogs are associated with many comorbidities, functional impairments (37–43), a shorter lifespan (44), and a poorer quality of life (45) (Figure 1). In the APOP 2018 survey for cats, the prevalence of overweight and obesity were 26 and 34%, respectively. The prevalence of overweight or obesity in adult cats at vaccination visits in New Zealand was 22 and 3%, respectively (50). As for dogs, overweight and/or obesity in cats is associated with an increased risk of a wide range of co-morbidities (46, 51, 52), a reduced lifespan and a higher risk of death (severe obesity only) (53, 54), and some, but not all data, suggest a reduced quality of life (55) (Figure 2).

Early-life risk factors for obesity in adult dogs

Risk factors for obesity can be identified in early life, as early as the fetal period. For example, low birth weight in Labrador Retrievers has been associated with overweight in adulthood even after adjusting for age and neuter status: 70% of dogs with birth weights below the median were overweight as adults, compared with 47% of dogs with birth weights above the median (5). No association was found between adult obesity and growth rate between birth and Day 2 or between Day 2 and Day 15. In contrast, in a study of female Beagle colony dogs raised in controlled environmental conditions, birth weight did not correlate with adult overweight status, but fast growth rate from birth to 2 weeks was a predictor for adult overweight at 2 years of age (4). By the age of 7 months, BCS discriminated between dogs that would be overweight as adults and those that would be slightly overweight or ideal weight (4). No significant difference was found between adult weight groups in their energy intake or resting energy expenditure corrected for metabolic bodyweight at the age of 4 months. Resting energy balance between the age of 7 and 10 months was significantly higher in puppies who were overweight compared with ideal weight in adulthood. During this study, dogs were fed *ad libitum* (time-restricted after weaning) with a diet formulated for growth, or for neutered adults, as appropriate. In a different study, a retrospective analysis of veterinary practice records found that dogs that were obese by 3 years of age (127 breeds, 93% neutered) had faster growth in body weight between 12 and 60 weeks of age than that modeled in healthy dogs in ideal body condition (59).

Neutering is common for dogs and cats and is often performed before puberty while they are still developing. The exact timing varies between countries, species and breeds, and is still a controversial issue. Neutering is well established as one of the most significant risk factors for obesity in adults (46, 60–65). Prepuberty or peripuberty neutering in the context of this review is considered an early-life environmental exposure of relevance to the risk of obesity, albeit during the later stages of development. The potential impact of sex (60, 66, 67) and age of neutering on the effects of neutering on adult obesity are unclear because findings differ by study. A prospective cohort study of Golden Retrievers identified an ~ 42% greater risk of obesity in dogs neutered between 6 and 12 months

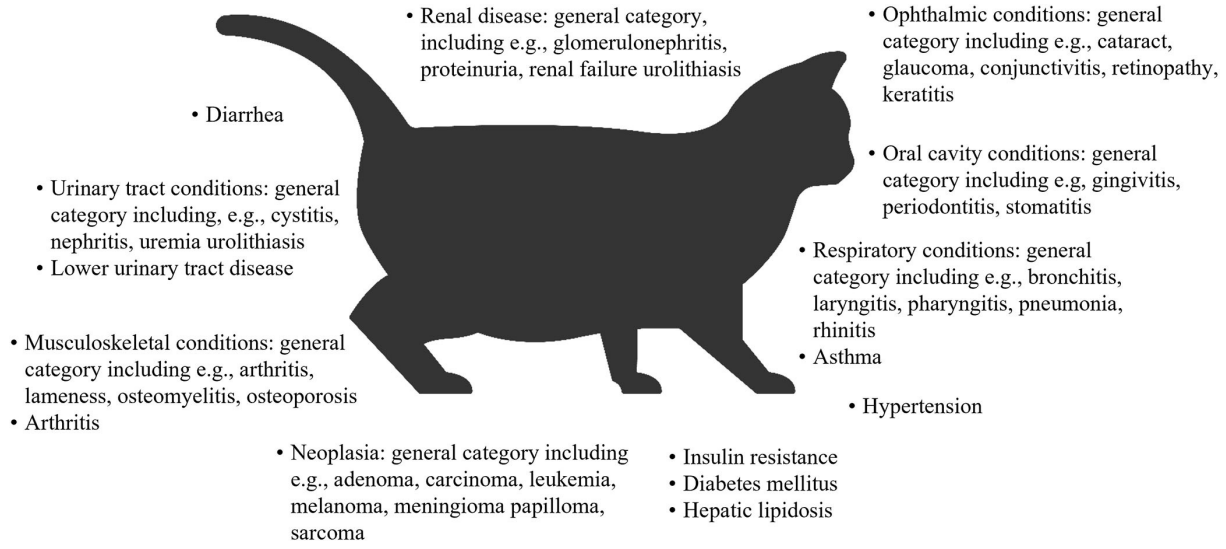

FIGURE 2
Overview of comorbidities associated with obesity or overweight in cats (31, 52, 53, 56–58). Some associations were found only for certain body condition scores within the obese or overweight range. Associations were often found between obesity or overweight and a general category of disease or conditions pertaining to an organ/organ system. In these cases, examples are provided of some of the conditions that the study included in the category definition.

of age compared with those neutered at >1 year of age, but no difference in risk between neutering at <6 months compared with 6–12 months and >1 year (66). Conversely, in a retrospective study of veterinary records, age at neutering (ages ≤6 months, >6 months to ≤1 year and >1 to ≤5 years of age) was not associated with the risk of obesity (67). When growth patterns of pet dogs from the same proprietary data source were examined, neutering before and after 37 weeks was associated with slight upward or downward shifts in growth trajectory, respectively; however, these shifts were small, suggesting limited overall impact on weight gain and, therefore, future obesity (68). Differences in study design and dog breeds might explain apparent inconsistencies between these studies. Indeed, interactions between breed size, age at neutering and number of veterinary visits per year were reported to affect the risk of overweight (67).

Hormonal changes resulting from neutering could have a direct effect on the risk of obesity. Neutered dogs have lower metabolizable energy requirements than sexually intact dogs (69, 70), and neutering can increase indiscriminate appetite (71). Evidence suggests that neutering could also unmask or augment the effects of environmental exposures in the younger animal. Increases in the bodyweight of female Beagle dogs after neutering between the ages of 7 and 10 months were higher in dogs retrospectively identified to be at risk of adult obesity by having a higher neonatal growth rate than their contemporaries of an ideal adult bodyweight (4). The growth of Labrador Retrievers between 2 and 21 days of life was associated with risk of obesity at adulthood, but only in neutered dogs (72).

Early-life risk factors for obesity in adult cats

In humans, breast feeding has a protective effect against childhood obesity compared with feeding formula milk (73), which might in part be associated with the presence of leptin in breast milk. Leptin is a hormone produced by adipose tissue that inhibits food intake and modulates glucose metabolism; the main source for neonates may be maternal milk (74). In a small study in cats, the odds for overweight in adulthood were 3 times less in kittens suckled for >6 weeks compared with <6 weeks, and there was a predisposition for overweight with suckling duration of 11 weeks or less (75). The investigators hypothesized that a short suckling period might lead to perturbations in the development of control mechanisms for fat accumulation and body composition through curtailment of leptin intake. A fast growth rate in cats is a key risk factor for obesity. A comparison of *ad libitum*-fed colony cats that were overweight with those of ideal weight at a median of 8.5 years of age, showed a significant association between growth rate between 3 and 12 months and later overweight status (76). In a different study that modeled the growth of colony cats fed *ad libitum* from weaning, early growth rate indicated by weight at 15 weeks of age was a significant predictor of being overweight at 9 years (77). Hypotheses to explain these associations include genetic, epigenetic and *in utero* factors, in addition to physical activity, food quality, feeding behavior and the gut microbiome (76).

Faster growth rate, smaller litter size, lower birthweight, and maternal overweight before pregnancy were associated with a predisposition of kittens to be overweight at 8 months of age in a study focused on genetic factors, and designed to reduce the

potential for non-genetic confounders and epigenetic differences (78). Despite the study design, some of the findings suggested that developmental programming might have played a role. The authors speculated that epigenetics might underlie the weak but significant negative correlation of litter size with overweight at 8 months. Also, epigenetic differences might have contributed to the observation that, although both overweight mothers vs. lean and variable-weight mothers, and male vs. female sex were associated with faster weight gain of kittens, this relationship became statistically significant for the maternal phenotype later than the sex difference.

As in dogs, neutering of male and female kittens is a risk factor for adult obesity (65, 79, 80). Whilst neutering is associated with increased appetite and food intake (81–83), it is also associated with reduced maintenance energy requirements (70, 81, 84). There is insufficient evidence in cats to know whether the age at neutering is associated with risk of obesity. However, differential changes in appetite have been associated with age of neutering; acute hyperphagia was observed in female cats neutered at 31 weeks of age but not in those neutered at 19 weeks of age (85). These behavioral changes may be associated with the effects of neutering on appetite-related hormones such as ghrelin, leptin, adiponectin and glucagon-like peptide-1 (86). For example, in a study of adult male cats, serum concentrations of adiponectin rapidly decreased after neutering, and within 7 days, there was a significant increase in serum concentration of ghrelin (83).

Potential research priorities

Nutrition

The role of early-life nutrition in the development of adult obesity demands more extensive and diverse research. In puppies and kittens and their parents, nutrition is relatively easy to modify in both research and "real-world" settings, and is likely to have multiple impacts on factors associated with obesity (87–91). At its simplest, chronic excessive calorie intake that starts at a young age results in progressive accumulation of body fat that ultimately manifests as adult obesity. However, a wealth of evidence in other species, including humans, shows that nutritional insults both *in utero* and postnatally can program later obesity and other metabolic disorders (92, 93). Models of obesity in polytocous species demonstrate that poor maternal nutrition (quantitative and/or qualitative) can modulate aspects of fat deposition and energy homeostasis in offspring through epigenetic mechanisms (89, 94, 95). Alterations in the development of the offspring's hypothalamus-adipose tissue axis are believed to be particularly important for obesogenic traits, manifested as structural changes, mal-programming of appetite regulation favoring orexigenic pathways, central leptin and insulin resistance, and alterations in noradrenergic innervation of adipose tissue (96, 97). Research is needed to determine if low birth weight in puppies, as a risk factor for adult obesity, is an example of fetal programming of a "thrifty" phenotype, whereby a metabolic profile set to cope with inadequate nutrition during pregnancy, later becomes a risk factor for obesity in the context of abundant postnatal nutrition. Paternal nutrition in laboratory animal models can also program obesogenic traits in the offspring (98), but this does not appear to have been researched yet in companion animals.

Obesogenic traits can also be sensitive to postnatal nutritional environment as development of organs and hormonal pathways continues after birth in mammals (87). The literature on postnatal maturation of domestic carnivores is limited, and as in other species, the timing depends on the organs involved (Supplementary Tables 1, 2). For example, changes in the morphology of organs such as the adrenal gland can occur during the first year (99), functional maturation of digestive processes may not occur until 3 months (100) and the immune system may not attain all adult characteristics until 12 months (101). Myelination of the neocortex continues to increase until ∼9 months after birth (102). Nevertheless, it is reasonable to hypothesize that developmental plasticity is concentrated in the suckling period. Research is needed in dogs and cats to determine the effects of diet in the pregnant and lactating dam on the quantity and quality of colostrum and milk, and whether these effects have consequences for the offspring's adult body composition and metabolism. The evaluation of the impact of food intake and nutritional interventions of the first days of life is particularly relevant for low birth weight puppies and kittens when considering nutritional interventions; rapid catch-up growth is associated with an increased risk for adult obesity in other species (97).

Growth

In both puppies and kittens, higher growth rates have been associated with adult obesity (59, 77). It is unclear if and how aspects of energy balance regulation during growth predispose adults to be obese or of ideal weight. Postprandial decreases in acylated ghrelin, an orexigenic gut hormone, are delayed in 7-month old female Beagles already identified as being on a trajectory to adult overweight, and this may promote excess food intake (4). The basal plasma concentration of leptin is positively associated with adiposity but does not appear to be an early predictor of weight gain. In humans; evidence suggests that leptin's main role is to signal low body fat stores in situations of negative energy balance (4, 103). Research is needed in larger study populations with different breeds and sexes to characterize further the dynamics of energy balance during growth associated with adult obesity, and to evaluate any role of developmental programming and the environmental triggers. Ideally, studies of growth and obesity should evaluate body composition. However, whilst for practical reasons BCS is most commonly used to evaluate adiposity, the scoring scales have only been properly validated in adult dogs and cats. Puppies and kittens have different body composition profiles

and morphologies compared with adult dogs and cats (104–106), which makes diagnosing overweight status with BCS scales designed for adults unsatisfactory. Greater objectivity and more uniformity between studies might easily be achieved by evaluating growth against growth rate standards now available for a comprehensive range of different breed sizes from 12 weeks of age (68, 107). These standards will be valuable in facilitating DOHaD research in obesity by identifying rapid or slow growth at an early age, and for case ascertainment in body composition and metabolic studies.

Gut microbiota

Differences in the gut microbiota and/or microbiome of obese vs. lean adult dogs and cats have been observed (108–112) and changes characterized in obese dogs and cats during diet-driven weight-loss studies (108, 113, 114). However, associations between diet, gut microbiota, enteroendocrine hormones and metabolic disturbances are complex, with studies reporting contrary findings (113, 115, 116). When the effects of macronutrient ratios in diets fed to both dams and their kittens were evaluated, composition of the pre-weaning diet did not affect the profile of bacterial populations in kitten feces at 8 weeks, but did modulate expression levels of genes in the glucose and metabolic pathways in blood samples taken at 18 weeks (117). The findings were reversed for a comparison between two post-weaning diets. What has not been investigated directly is any association between gut microbiota as it is developing in the puppy and kitten and adult obesity, and the potential for early nutrition to influence this. Research in other species on developmental programming suggests that could be a fruitful avenue of research (118, 119).

In mice, gut microbiota mediate changes in global histone acetylation and methylation of DNA both locally in cells of the colon and distally in tissues such as liver and white adipose tissue (120). These microbe-mediated changes have been demonstrated in species other than dogs and cats during early life at a time when the gut microbiota is developing (121). Microbial metabolites have a direct role in epigenetic modifications, and the composition of the gut microbiota is relevant because the profile of metabolic byproducts of dietary constituents such as short-chain fatty acids (SCFAs) may differ between bacterial species (120, 122). Factors such as suckling vs. bottle feeding, lifestyle, environment and exposure to antimicrobials may also impact obesogenic traits through their effects on the emergent microbiota (123, 124).

Neutering

The strength of neutering as a modifiable risk factor for obesity in both dogs and cats demands a greater understanding of the interactions between sex hormones and diet on appetite-related hormones and blood metabolites (86). The impact of neutering at different stages of development (early vs. late) needs to be dissected to resolve differences between studies and explore sex, species and breed differences. The impact of environmental exposures such as nutrition and growth rate during the first days/weeks/months on the effects of subsequent neutering is under-researched, but existing data warrant further longitudinal prospective studies (4). One question to be addressed is whether neutering unmasks or potentiates the effects of developmental programming puppies or kittens.

Interaction of environmental exposures with genetic susceptibilities to overweight and obesity

The interaction of genetic risk factors with modifiable variables in development can increase or decrease the likelihood of particular phenotypes. In humans, genes enriched or only expressed within the central nervous system have a central role in the biology of obesity (125). Knowledge of genetic susceptibilities can help researchers design studies on developmental programming and interpret their results.

Dog breeds including Pug, Beagle, Golden Retriever, English Springer Spaniel, Border Terrier, Labrador Retriever, and Cavalier King Charles Spaniel are at a higher risk for overweight than crossbred dogs (126, 127), whilst domestic short-hair cats have an increased risk of obesity (127). Candidate genes for genetic variants suspected to increase the risk of obesity in dogs include *POMC, FTO, PPARG, MC4R, and MC3R, INSIG2, GPR120* (127). Genetic variants may be restricted to a small number of breeds, e.g., a 14 base-pair deletion in *POMC* associated with obesity and food motivation found in Labradors and Flat-coated Retrievers (128, 129). Genetic risk factors need to be a consideration in studies investigating the impact of early-life environment on obesity. Genome-wide association studies could help elucidate the genetic background of obesity in companion animals and there is potential value in both within breed and large-scale across-breed approaches.

Chronic enteropathy in dogs and cats

Chronic enteropathy is an overarching term that encompasses subgroups of chronic intestinal disorders based on treatment response: immunosuppressant-responsive enteropathy [IRE, previously known as idiopathic inflammatory bowel disease (IBD)], food-responsive enteropathy, and antibiotic-responsive enteropathy (12). The prevalence of CE reported in different studies ranges from 1 to 18% (12). In cats, IRE frequently coexists with small cell lymphoma (130), which is considered to fall under the umbrella of CE in this species (131). Although the underlying etiology of each subtype of CE is unclear, and may not be the same, they are chronic inflammatory conditions, and the pathogenesis reflects interactions between the gut microbiota and gut immune systems in the context of environmental factors such as diet, and genetic susceptibilities in some breeds.

Early-life risk factors for chronic enteropathy in adult dogs

There are very few data on early-life risk factors for CE in dogs; however, these limited data implicate a diverse range of variables that warrant full investigation. Puppies that had historically presented in the acute stages of canine parvovirus infection at a median of 12 weeks of age, had a greater risk of owner-reported chronic gastrointestinal signs in later life than control dogs that presented at the veterinary clinic either for a routine check or for signs not associated with parvovirus [odds ratio 5.33 (95% CI: 2.12–14.87)] (132). A similar study also found that previous parvovirus enteritis was a risk factor for persistent gastrointestinal signs, and among dogs that had recovered from parvovirus infection, markers of disease severity were associated with that risk (133). In another study, early modifiable risk factors for CE in adulthood included vaccination of the dam during pregnancy, type of solid food fed to puppies during the first 6 months, and the puppy's body condition ("slim" rather than "normal weight") (134). These results should be interpreted with caution because of methodological limitations such as retrospective owner questionnaires, participant bias and broad diet types that were not nutritionally controlled. In a retrospective review of veterinary records from a medical teaching hospital in the USA, neutering was associated with an increased odds of IBD in males and especially female dogs (odds ratios for neutered vs. sexually intact 1.43 and 2.0, respectively, $p < 0.05$ for both) (135). The authors hypothesized that the same anti-inflammatory and antioxidant effects of estradiol demonstrated in murine models could be protective against IBD in dogs.

Early-life risk factors for chronic enteropathy in adult cats

Although chronic enteropathy in cats is well described in the literature (131, 136), no studies were found that have investigated modifiable risk factors in kittens.

Potential research priorities

Disruption to the maturing gut microbiota, which might be due to diet or antimicrobials, is associated with increased risk of later IBD in humans or experimental colitis in animal models (137). Research suggests that epigenetic modifications underlie interactions between diet, the immune system and the microbiota in the development of chronic diseases including IBD (121, 138).

We suggest that investigation of any association between gut microbiota in puppies and kittens and development of CE in adulthood should be a research priority (Figure 3). There are complex interrelationships between gut microbiota, host metabolism, the immune system, intestinal inflammation and gastrointestinal health or dysfunction (139). Is there a relationship between the gut microbiota that develops in puppies and kittens and that found in adult dogs and cats with CE, which differs from that in healthy adults? Do perturbations in the developing gut microbiota affect the maturation of the immune system and acquisition of tolerance in ways that predispose puppies and kittens to later CE? Do the effects of microbiota dysbiosis on the gut metabolome in these pets epigenetically program susceptibility to future CE and/or dysbiosis? Pieces of the puzzle have been characterized in puppies and kittens (140, 141), and separately in adults with CE (139, 142, 143), but the existence of a link between these has not yet been established.

Bacterial dysbiosis is defined as alterations in the composition of the bacterial gut microbiota leading to functional changes in the microbial transcriptome, proteome or metabolome, and/or decreased bacterial diversity (139, 144, 145). It is reported that 72%−79% of dogs and 76% of cats with CE have dysbiosis as evaluated by dysbiosis indices (142, 146, 147). Research in humans and animal models suggest that the role of dysbiosis in the pathogenesis of IBD could be causative (148, 149). This makes the development of the gut microbiota in puppies and kittens, and perturbations of this, of particular interest as a potential risk factor for CE.

The possibility in dogs of intra-uterine bacterial transfer from dam to fetus is controversial, but after birth, data suggest that the dam seeds the initial bacteria and her individual microbial profile plays a fundamental role in shaping the gut microbiota of her litter (150). The richness of bacterial species in the neonatal gut increases from day 2 after birth, and the gut microbiota changes significantly with age during the suckling and weaning period (151). The greatest changes to the microbiota of healthy puppies had occurred by 5–6 weeks of age in one investigation (151), although differences between the microbiota of offspring and dam were still apparent at 8 weeks in another study (152), and small changes might feasibly occur until 1 year of age (153). As with puppies, the gut microbiota of healthy kittens develops substantially during suckling and weaning, although the adult profile might not be fully achieved in those periods (154, 155). In a study in kittens, changes in the microbiome were still evident at 18–30 weeks of age, but had stabilized by 30–42 weeks (156). In another study, the microbiome was relatively stable in kittens aged 8–16 weeks (141, 157). Further longitudinal investigation is clearly needed. It is contended that the microbiota established in puppies and kittens is likely to be generally stable during healthy adult life as for humans, but this needs to be verified (139, 151, 152).

The relevance of dietary influences on gut microbiota and the gut microbiome in puppies and kittens to later gastrointestinal health status is likely to be multifactorial. Existing research needs to be extended to investigate early diet as a potential risk or protective factor for CE. For example, pre- and probiotic supplementation of Great Danes in the last week or last 4 weeks of pregnancy reduces the risk of neonatal gastroenteritis in their offspring (158). It is hypothesized that this protective

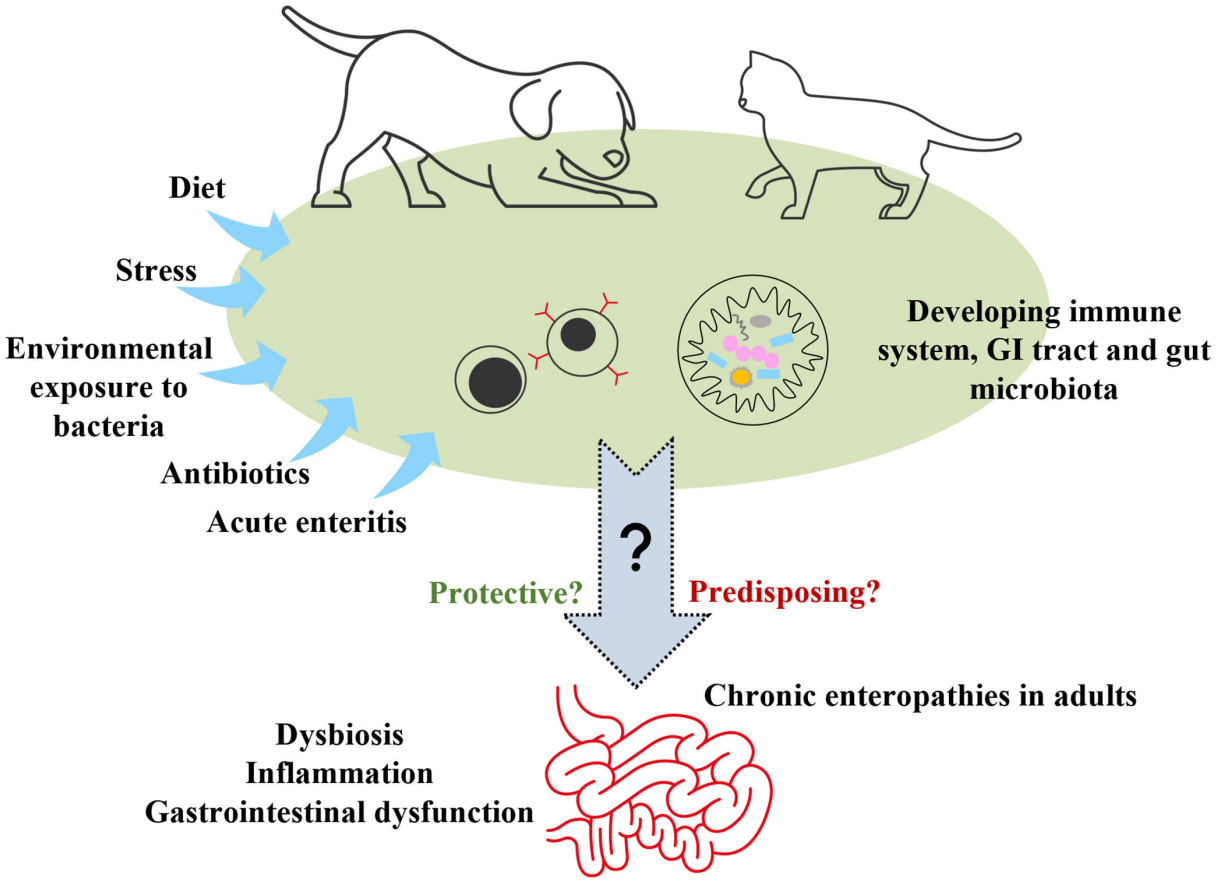

FIGURE 3
Early-life environmental exposures to investigate as potential risk factors for chronic enteropathies in adulthood. GI, gastrointestinal.

effect is conveyed *via* the entero-mammary link, given that in other studies feeding dams with pro and/or prebiotics improved the immune properties of their colostrum (159, 160). Another hypothesis (not mutually exclusive) is that the effect is mediated by the selection of health-promoting bacteria in the dam that then colonize the neonates.

The most profound disturbances to gut microbiota are those caused by antibiotic use. It is hypothesized that antibiotic use could be a major priming factor in puppies for later CE. In humans, antibiotic treatment in the first postnatal year is associated with an increased risk of later development of IBD (161–163). Acute diarrhea is common in puppies, and it is often treated with antibiotics. The fecal microbiota changes in dogs with acute diarrhea and the bacterial groups involved are not consistently reported to be the same as in chronic diarrhea, although reduction in fecal concentrations of SCFAs is a shared finding (139, 145, 164). In a prospective controlled study, metronidazole (a common antibiotic treatment for acute diarrhea) significantly altered the fecal microbiome and metabolome of healthy dogs, including a decrease in the abundance of Fusobacteria, which are key SCFA-producing bacteria, and the main bile acid converting bacterium *Clostridium hiranonis* that was associated with a reduction in secondary bile acids (165). Changes persisted in nearly half of the dogs for at least 4 weeks. The long-term effects of such treatment in puppies still establishing a normal gut microbiota needs to be explored in studies on developmental programming. A course of antibiotic treatment (20 or 28 days) in 2-month old cats with upper respiratory tract disease was shown to delay the maturation of their gut microbiota compared to healthy untreated cats (166). The duration of effects differed between antibiotics; the impact of amoxicillin-clavulanate on the microbiome occurred mainly during treatment, whereas the impact of doxycycline was observed from 1 to 3 months after antibiotic withdrawal (166). Research should extend to the use of antibiotics in pregnant dogs; data in humans and mice suggest that this is a risk factor for gastrointestinal disease in the offspring (167, 168).

It is not known whether the gut microbiota influences early development of the gastrointestinal tract and susceptibility to

chronic disease through epigenetic modifications in puppies and kittens. Data from mouse studies however, point to the importance of gut microbiota in modulating post-natal development of the gut through DNA methylation of genes in intestinal epithelial cells associated with immunity, metabolism, and vascular regulation (122, 162). Changes in bacterial metabolites associated with CE in dogs are known in other species to influence epigenetic modifications affecting immune and inflammatory pathways. For example, decreased fecal abundance of *Fusobacterium* and *Faecalibacterium* in dogs with CE is associated with reduced fecal concentrations of the SCFA propionate (147, 169). Short-chain fatty acids can regulate epigenetic modifications by inhibiting histone deacetylases (HDACs) and contributing acetyl donors for DNA or histone modifications.

Abnormalities or deficiencies in immune responses to environmental antigens, together with genetic susceptibilities, appear to play central roles in the development of CE in dogs (170). The possibility that some of the immunopathogenesis in dogs with CE has origins in epigenetic changes was raised by an investigation of the reduced intestinal expression of mucosal IgA found in these dogs (171). Hypermethylation of the gene for TACI was negatively associated with expression of mucosal IgA; the authors hypothesized that such changes in methylation status might have been induced by inflammatory mediators and exposure of the gut to an altered intestinal microflora (171). Such mechanisms might therefore be a link between environmental exposures during development and risk of later CE.

Across all the avenues of research suggested, developmental periods of particular interest in puppies and kittens include initial colonization of the neonatal gut, weaning, and the transition from breeding facilities to new owners, when diarrhea is common, coinciding with changes in diet, stress and exposure to different microbial environments. Large populations need to be studied to understand interindividual variations in microbiota—there may not be a single "normal," "healthy" microbiota. Robust studies are needed that use nutritionally specific diets and record only veterinarian-diagnosed CE. Those conducting research in developmental programming must of course consider breed susceptibilities and breed-independent genetic associations with disease. Dog breeds susceptible to CE include Weimaraner, Rottweiler, German Shepherd, Border Collie and Boxer (12, 172).

Behavioral problems in dogs and cats

Behavioral problems in dogs and cats are common and can affect their welfare and quality of life (173), their relationship with humans, and their suitability for assistance work (174). Difficult behavior is frequently cited by owners as being at least one of the reasons for them relinquishing their pets to animal rescue centers, being the primary reason for 10% of dogs in a recent Canadian study (13), and the sole reason for 27% of dogs and 19% of cats in a US study (14). They can also drive some owners to seek elective euthanasia for their pets (15). Although the nature of behavioral problems is wide ranging, such as aggression toward humans and other animals, separation anxiety, and soiling in the house, at least some adverse behavior traits detrimental to the long-term future of dogs and cats can be attributed to their early-life environment.

Most neurological development occurs during fetal life; it continues rapidly in the neonate, but myelin formation and maturation continues until at least 36 weeks of age in dogs (175). Regions of the brain develop at different rates throughout early life, potentially therefore remaining susceptible to environmental exposures (175–177). The development of behavioral and cognitive traits can be considered in different phases: gestation, the neonatal period including feeding, neurological stimulation and mothering in the first 3 weeks, early socialization from ~ 3 to 12 weeks of age, late socialization from 12 weeks up to 6 months, and the enrichment period, which may extend to 1 year of age (177, 178). It is believed that experiences during each period have cumulative effects on trainability, health and performance (177, 178).

Early-life risk factors for behavioral problems in adult dogs

No research in dogs investigating the effects of maternal stress or diet during pregnancy on the behavior of offspring was identified, except a mention that puppies of malnourished dams were extremely nervous in addition to displaying physical abnormalities (179).

Poor maternal care and socialization before 3 months of age have been associated with fearfulness in dogs, and poor maternal care alone was also associated with a combination of fearfulness, noise sensitivity and separation anxiety (180). These data were derived from a survey of owners, but other studies with more objective measures show that the level of mothering can affect the performance of dogs in cognition tests, stress responses, and temperament in later life. However, some research findings appear to be contradictory as to whether an environmental exposure has a positive or negative effect. For example, in one prospective study, guide dogs that had experienced more intense mothering had poorer problem-solving abilities and showed higher levels of anxiety at 14–17 months of age, both of which were associated with a significantly greater risk of failing the guide dog training program (174). In contrast, a benefit of greater maternal care was demonstrated in male and female Beagle puppies; the mean duration of daily maternal care in their first 3 weeks was positively correlated with exploration and latency of the first yelp, and negatively correlated with stress in isolation tests at 8 weeks of age (181). A study with long-term follow up found that a higher level of maternal care of male and female German Shepherd dogs was associated

with greater physical and social engagement (e.g., ball retrieval, positive acceptance of handling) as well as aggression in young adults at 18 months of age (182). In summary, stimuli in the suckling period appear to effect some behaviors of adult dogs, but the direction of reported associations is not always intuitive or consistent, perhaps reflecting the complexity of the biology as well as interstudy differences in behavior tests, ages, and breeds (181).

In a review of seven observational studies on dogs originating from high-volume commercial breeding establishments and sold either online or through pets shops, risk factors were highlighted for later behavioral and psychological problems (183). In the largest of these seven studies, UK dogs acquired from sources such as pet stores and the internet were 1.8 times more likely to show aggression toward humans than dogs acquired directly from breeders (183, 184). Across studies, aggression was the most common problem behavior associated with commercial breeding establishments or puppy farms and pet stores. Although causative factors were not investigated, potential causes discussed included stress in the dam, insufficient or excessive neonatal stimulation, early weaning and maternal separation, and social isolation between the age of 3 and 12 weeks.

By the early socialization period, the central nervous system has developed to a stage that allows conditioning and associated learning (177). Socialization of puppies with familiar conspecifics is important for the development of communication competency, and early interactions with non-familiar conspecifics may influence the risk of aggressiveness in adult life (177, 185). For example, restriction of a puppy's contact with conspecifics in the 8 weeks after their first exposure to other dogs in a public setting was found to be associated with aggression toward unfamiliar dogs when they were 1–3 years old (185). Early socialization with humans is important for later responses to handling, leash training and stress tests (186).

Behavioral traits and non-social cognitive abilities continue to develop in puppies during the late socialization and enrichment periods (187–189). In young candidate working dogs, measures of inhibitory control, attention and spatial cognition improved between 3 and 12 months of age (187). In a second longitudinal study, performance of cognitive tasks improved between the age of ∼ 9 weeks and 21 months, and the adult phenotype for some traits could be predicted from test results in puppyhood (188). However, little is known about specific exposures in these periods that might influence the course of brain development, and the general environmental context, breed and sex are also likely to play a role (189). Questionnaires completed by foster carers of puppies from ∼ 2 months of age until the initiation of formal guide-dog training, showed a positive behavioral effect of growing up in a household with another dog and with more experienced puppy raisers (189). Puppies that had been attacked or threatened by an unfamiliar dog showed significantly higher "dog-directed fear" and "stranger-directed aggression" at the age of 12 months old compared with puppies that had not experienced that trauma and had worse training outcomes (189). However the age at which the trauma had occurred was not specified.

A link between epigenetic changes and human-directed social behavior in dogs was found in one study (190). The DNA methylation of the promoter region of the oxytocin receptor gene (*OXTR*) was measured by bisulfite pyrosequencing followed by methylation-specific PCR in mouth epithelial cells obtained from various Canidae. Four differentially methylated 5′-cytosine–phosphate–guanine_3′ (CpG) sites were identified. They were subsequently studied in a large population ($n = 217$) of Border Collies. Not only did DNA methylation status differ between females and males, it was also associated with their response in a "threatening approach" test in a sex-dependent manner. For example, more methylation at a specific CpG site in female dogs tended to correspond with a greater likelihood of appeasing behavior in the test, whereas the opposite relationship was found in male dogs. In addition, CpG sites differed in whether promoter methylation increases or decreased *OXTR* expression levels (190), both neuter status itself and the interaction of sex with neuter status did not predict methylation levels at the three CpG sites investigated. This study highlights the complexity of relationships between epigenetic modifications and behavior in dogs, and the need for research on environmental factors that influence the epigenetics of the *OXTR* gene.

Early-life risk factors for behavioral problems in adult cats

There is a dearth of knowledge on the effect of the maternal environment during pregnancy and subsequent behavioral problems in adult feline offspring. However, one study showed that when the kittens of dams that had been malnourished throughout pregnancy were fostered onto non-food deprived cats, both physical and behavioral development were delayed (179, 191). The behaviors affected included time spent playing and the use of a litter tray. Moreover, in adulthood the cats displayed marked antisocial behavior and alternation between dominant and submissive behaviors. These observations could potentially represent classic DOHaD in cats. In other research, protein restriction of cats during late gestation and lactation adversely affected the attachment processes in both dams and kittens in the first 12 days after birth (192).

There is ongoing neurological development of kittens during the first 3 postnatal months (193). This is observed, for example, in increasingly sophisticated ability to respond to sound, development of visual placing and binocular coordination, and gross behaviors during interactions with siblings (193). Early life experiences of cats affect behavior in adulthood (194). For example, human handling of kittens during the sensitive period for socialization has been associated with more friendly behavior

toward humans at the age of 1 year (195, 196). The extent to which epigenetic modulation and/or genetic differences contribute to these observations is unknown.

Potential research priorities

We suggest that research into the risk factors for behavior problems in dogs and cats needs to diversify to include assessments *in utero* and more studies of environmental influences in animals aged 6–12 months. This should not be at the expense of further work in neonates when brain development is particularly plastic. Neonatal studies are facilitated by the relative ease with which the environment can be controlled for related individuals in the same litter, although this does not allow for the distinction between genetic and environmental effects. Apparently contradictory results for the effect of maternal care on stress responses need to be explored, perhaps by comparing different stress challenges and intensities at various ages in animals kept in standardized environments. More long-term studies are required to determine the durability and reversibility of the effects of early-life environment on adult behavior.

The extent to which epigenetic modulation drives risk factors for behavioral disorders in dogs and cats is not known. The few existing data linking epigenetic changes to dog behavior (190) highlight the complexity of the relationships involved. Environmental factors influencing known epigenetic variation of *OXTR* are especially important to explore. Polymorphisms in the gene for the dopamine receptor 2 (*DRD2*) are associated with fearful behavior in some breeds of dog (197), and a variant haplotype in this gene is associated with anxiety separation in Golden Retrievers (198). *DRD2* could therefore be another gene of interest to study for epigenetic changes affecting behavior.

Studies in other species provide a rich source of developmental behavioral data and hence hypotheses for dogs and cats. Various cognitive, behavioral and emotional disturbances in children have been associated with stress during development (199, 200). Prenatal stress can result in structural and functional changes in multiple regions of the developing fetal brain, including the hypothalamus-pituitary axis (199, 201). DNA methylation of the glucocorticoid receptor gene and *OXTR* are examples of mechanisms believed to link childhood experiences with psychiatric disorders and temperament, respectively (202, 203). Preconception experiences of parents may also be relevant and can affect anxiogenic responses of their offspring and subsequent generations of descendants (204, 205). Rodent studies highlight the importance of the exact timing of environmental exposures and sexual dimorphism in the developmental sequelae (206). Stresses that may affect the behavior of offspring include, for example, preconception psychological trauma of either parent, maternal diet during pregnancy, early separation of offspring from their dam, and mothering behaviors (201, 205, 207, 208). Evidence from various species also links the composition of the gut microbiota with neurocognitive and behavioral development, building the concept of a microbiome-gut-brain axis (138, 209, 210).

Strategies for research to understand early-life risk factors for chronic diseases and behavioral problems in dogs and cats, and the potential role of developmental programming

The developmental origins of health and disease have not yet been confirmed in pets, but the examples discussed suggest that developmental programming is likely to be as important as in other species. We recommend a concerted multidisciplinary approach to explore developmental programming in dogs and cats and to close the large knowledge gaps compared with other species.

Human research and information campaigns on DOHaD are conducted in the context of a species-specific critical window of development. We believe that the overall critical window of time implicated in the development of dogs and cats extends from preconception to the end of growth, comprising five periods: preconception, gestation, the suckling period, early growth pre-neutering or pre-puberty, growth post neutering or post puberty to adult size (Figure 4). Within this window, there will be different and sometimes overlapping critical periods for different aspects of health and disease according to the developmental plasticity of the relevant tissues. The upper age limit of this "window" will depend upon species and breed. Variables of particular interest for DOHaD research in dogs and cats, both individually and in combination, include the maternal and neonatal environment, nutrition and associated weight gain and/or growth rate, the gut microbiota, weaning stress, and neutering (Figure 4). Work in other species highlights the need for studies to consider the periconception period (211), the exact timing of environmental exposure, differences in programming between the sexes (212–214), the role of the placenta (215), paternal influences on the offspring's epigenome, breed and genetic variation (216–218).

It is critical for the whole scientific research community to be able to access large datasets encompassing high quality "whole of life" data and biobanks of tissue samples in order to explore the long-term impact of exposures in early life. Research colonies of cats and dogs with internal breeding programs are useful to address the effect of individual interventions, but they are uncommon and do not reflect the situation of household pets, which are exposed to many non-controlled, interacting environmental factors. Analysis of large prospective observational cohorts of privately owned animals, perhaps spanning up to 15 years to encompass whole lifespan, may identify effects of programming that are small in the individual

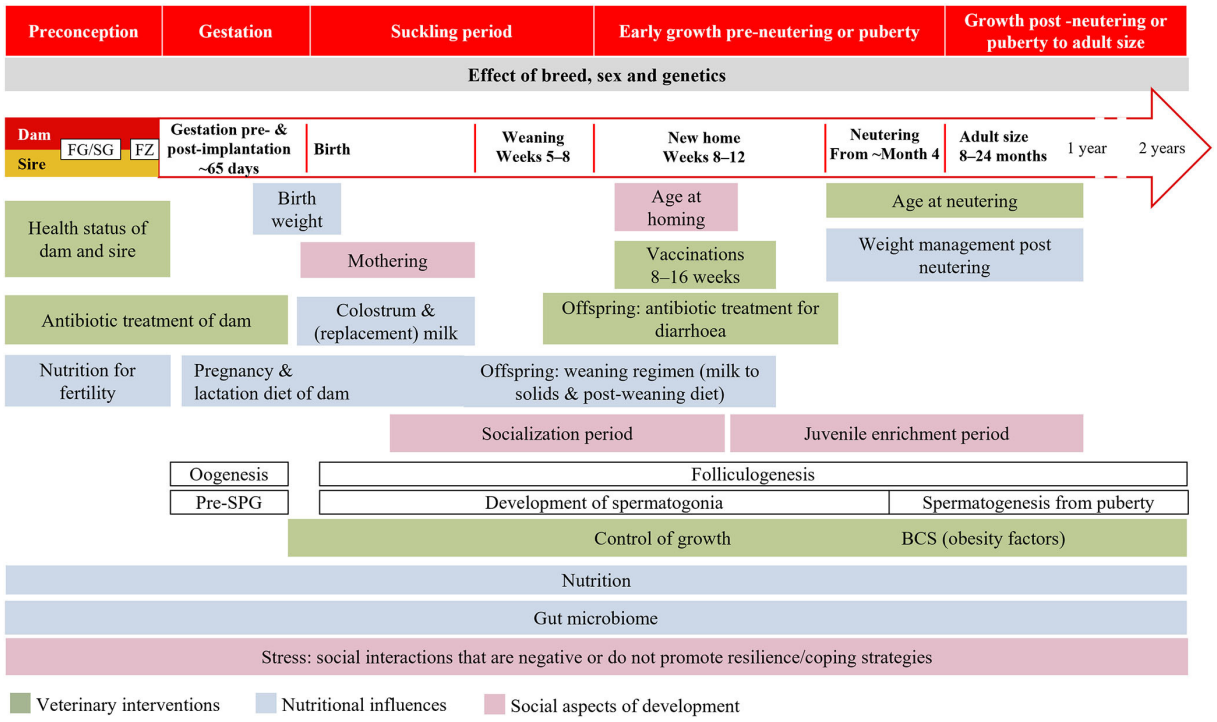

FIGURE 4
Windows of developmental programming proposed in dogs and cats. BCS, body condition score; FG/SG, folliculogenesis/spermatogenesis; FZ, fertilization; Pre-SPG, prespermatogonia.

and very variable between individuals. A particular challenge to be addressed is obtaining longitudinal data for a dog or cat that follows both parents in the preconception period and extends through gestation and neonatal life to adulthood and end of life. This might need data from two breeders (one for the sire and one for the dam), at least one owner, and probably at least three veterinary surgeons (one each for the sire, dam and puppy or kitten). These stakeholders need to be engaged with the potential wide-ranging and long-term implications of DOHaD and the many possible opportunities for intervention. Institutions that breed and train dogs for service roles, and subsequently monitor their progress, e.g., guide dogs, can provide collaborative opportunities for researchers.

Although there are ongoing large, prospective and observational longitudinal studies, these primarily target dogs after they have left the breeder, and so will lack data from the first 2 months after birth. For example, the Generation Pup project operated by the Dogs Trust in the UK was initiated in 2016 to use owner and veterinary data from up to 10,000 dogs to identify modifiable risk factors during development that impact adult health and welfare (219). The research will investigate relationships between genotype, environment, and health and behavior outcomes at different life stages. There are also breed-specific longitudinal cohort studies such as the Golden Retriever Lifetime Study run by the Morris Animal Foundation in the USA, which is collecting data on the lifestyle, environment, behavior and health of 3,000 dogs recruited between 2012 and 2015, including annual biological samples (66, 220, 221). The ongoing Dogslife epidemiological project (University of Edinburgh, University of Manchester, University of Liverpool and the Kennel Club) recruits UK pedigree Labrador Retrievers born after January 2010 ($n = 6{,}084$ dogs by December 2015) (222, 223). Owners complete questionnaires each month for the first year of their dog's life and every 3 months thereafter (222, 223). In the USA, the ongoing Dog Aging Project (University of Washington and Texas A&M University) has recruited tens of thousands of companion dogs to explore aspects of "health-span" i.e., the period of life spent free from disease (224). The Norwegian School of Veterinary Science cohort established to investigate skeletal disease in four large dog breeds ($n = 700$ puppies recruited 1998–2001), is notable as an example of a longitudinal cohort in which dog litters were recruited from the time of the dam's mating, and data were obtained from breeders, owners and veterinarians (225, 226).

Longitudinal cat registries and cohort studies appear to be scarce. The landmark Bristol Cats Study led by the University of Bristol is the first reported birth cohort study of kittens

(227). Cats were registered between the ages of 8 and 16 weeks, and owners complete questionnaires at set intervals. Analyses reported to date include the prevalence of and risk factors for obesity, and owner-reported lower urinary tract signs (228–230). The Cat Phenotype and Health Information Registry in the USA (UC Davis Veterinary Medicine) collects DNA samples from healthy and diseased cats with long-term follow-up where possible (231).

Data on epigenetic mechanisms linking early-life experiences to adult disorders in dogs and cats are scant; they are needed to help confirm and understand developmental programming, and unravel the effects of genetic background. Methods for profiling genome-wide DNA methylation are well established, and much can be achieved before attempting to identify the specific genes responsible for an epigenetically determined phenotype (232, 233). Epigenetic mechanisms other than DNA methylation should also be studied, such as histone modifications, including but not limited to methylation and acetylation, and non-coding RNA that can regulate gene expression during cell differentiation and development (234). A publicly available repository of canine epigenomic data (BarkBase) has recently been established, comprising the results of RNA sequencing and assays determining chromatin accessibility across the genome (235). The database includes 27 different adult tissues and five fetal tissue types at four embryonic timepoints. The Royal Veterinary College has instituted the Companion Animal Brain Bank—a standardized collection of brain tissue and other biological samples from dogs and cats euthanized with neurological conditions, together with appropriate controls. Although such tissue banks could be used to investigate changes in the epigenome, including those associated with disease, without corresponding data on environmental aspects of pregnancy and early life, they will not provide evidence of developmental programming.

The effect of early-life experiences and developmental programming on at least some physiological characteristics will be affected by genotypic differences between the many breeds and mixed breeds. Targeting research to specific breeds on the basis of their propensity for developing the disease or behavior of interest can be advantageous, e.g., the Labrador Retriever for obesity.

There are fewer breeds of cats to contend with in DOHaD research, but overall the knowledge gaps are greater than in dogs. There appear to be fewer longitudinal field data in cats compared with dogs, and there is probably less public awareness of the potential impact of developmental programming on chronic diseases.

Concluding remarks

There is direct evidence for early-life risk factors associated with obesity and behavioral problems in dogs and cats, and to a much lesser extent CE in dogs. However, multidisciplinary prospective long-term research is needed to confirm DOHaD in these species. Extensive data from other species provide a scientific foundation to help prioritize early-life events and exposures for investigation. The diversity of dog and cat breeds, breeding management and lifestyles adds complexity to such research. It is believed that breeders, owners and veterinary surgeons each have a critical window of opportunity in one or more of the life stages from preconception to the end of the dog or cat's growth phase in which to promote programming beneficial to long-term health. An appreciation by each of these groups of the overall window of development may also help to foster shared responsibility, transparency and information sharing.

Dogs and cats are considered to be family members, and yet veterinary medicine struggles to treat common conditions that adversely impact pets' quality of life, the special owner–pet bond, and the health benefits pets can bring to individuals and society. Preventive medicine and husbandry practices from preconception onwards must take a higher priority and be fueled by a better understanding of developmental programming at the population level.

Author contributions

VG, SC, GE, OF, AG, JS, CV, PC-P, and FP contributed substantially to the conception of the article and to interpretation of data presented. All authors critical reviewed the manuscript for important intellectual content. The authors take full responsibility for the scientific content of the paper and they have all approved the submitted version.

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Conflict of interest

FP and VG are employees of Royal Canin SAS. SC is a French Government Agent and head of NeoCare ENVT, a Public Research Unit. NeoCare Unit was partially funded by Royal Canin Research Division from 2012 to 2021. As such, SC has presented research results and clinical recommendations at Royal Canin-sponsored conferences. GE provides veterinary clinical advice to Waltham Petcare Science Institute, Waltham-on-the-Wolds, Leicestershire, UK. OF is an employee of Wisdom Panel (Kinship), a Mars Petcare Company. AG is an employee

of the University of Liverpool, but his post is financially supported by Royal Canin, has received financial remuneration for providing educational material, speaking at conferences, and consultancy work from Royal Canin, and is also a member of the Guide Dogs Scientific Advisory Group. JS is an employee of the Gastrointestinal Laboratory at Texas A&M University, which offers diagnostic testing on a fee-for-service basis, and has received speaker honoraria and consulting fees from Royal Canin SAS, Nestle Purina Petcare, ExeGI Pharma, Nutramax Laboratories, and Hill's Pet Nutrition. CV has done consulting work for a variety of pet food companies (Nestlé Purina PetCare, Royal Canin, Mars Pet Care, and Dechra Specific), has participated as an investigator in clinical trials sponsored by Royal Canin and Affinity Pet Care, develops educational materials for Morris Institute, is part of the Scientific Advisory Board of FEDIAF and a member of the Global Nutrition Committee of the WSAVA, and participates as a speaker or attendee in continuing education events sponsored or organized by Royal Canin, Nestlé Purina PetCare, and Hill's Pet Nutrition.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer AW declared a shared affiliation with the author GE to the handling editor at the time of review.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.944821/full#supplementary-material

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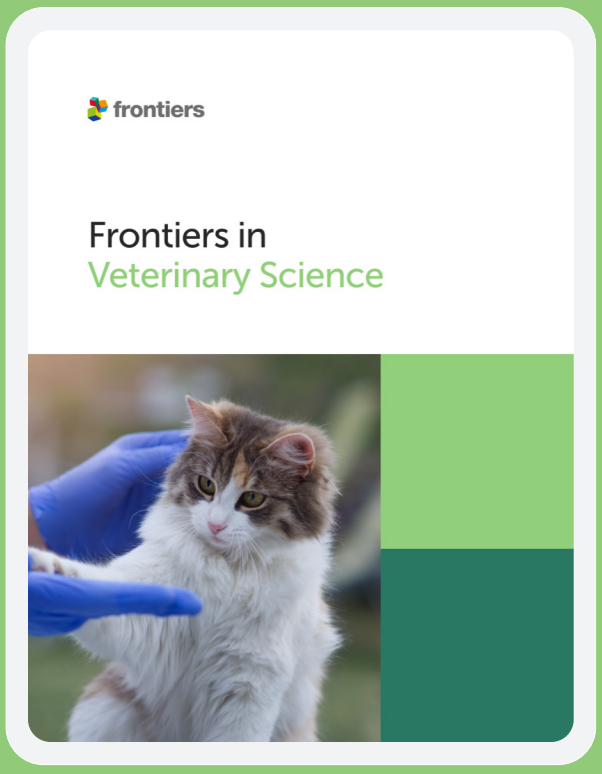

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