PERIOVULATORY TIME IN THE BITCH: WHAT'S NEW TO KNOW?

Comparison between ovarian histology and clinical features

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Abstract

The ability to recognize specific events happening in the ovaries during periovulatory time allows to manage optimally canine reproduction. The actual ovarian status can be faithfully elicited by histological evaluation while clinical and hormonal aspects are indirect effects of gonadal activity. The objective of this study was to assess the efficacy of the vaginal cytology and blood progesterone (P4) assay in accurately identifying histological changes occurring at the ovarian structures, mainly during the fertile period. Tertiary follicles, *corpora hemorrhagica* (CHs) and *corpora lutea* (CLs) from forty healthy bitches undergoing ovariohysterectomy were evaluated by histo-morphometry based on their aspect, number and size. The tertiary follicles distribution (small, medium and large) was statistically different ($P < 0.002$) among all the stages of the reproductive cycle, except for small follicles (< 2 mm) always observed from proestrus to anestrus. Very large follicles (> 4 mm) were predominant ($P = 0.008$) around ovulation when P4 mean was 6.1±1.7 ng/mL. Early postovulatory estrous period was characterized by CHs ($P < 0.002$) and P4 mean of 16.7±5.9 ng/mL. The end of the fertile period - beginning of diestrus - coincided with the development of CLs ($P = 0.001$) associated to a mean P4 of 73.9±9.9 ng/mL. The small ($P < 0.001$) and medium ($P<0.05$) follicles diameter was positively correlated with the bitch size, whereas the number of follicles larger than 4 mm was significantly lower in bitches younger than 4 years ($P < 0.02$). This study might improve the knowledge on some critical steps in the canine reproductive management, mainly periovulatory phase and the end of the fertile period, essential to plan breeding programs.

KEY WORDS: ovary; histology; histometry; ~~progesteronemia~~ progesterone; reproductive cycle; bitch

1. Introduction

Canine breeding is increasingly professional and attentive to the reproductive aspects. The incorrect identification of the fertile period results in apparent hypo-/infertility in the bitch, especially in case of single mating/insemination, chilled-frozen or low quality semen. Changes occurring at the ovary level during the periovulatory period and the transition to the diestrus phase constitute the core of capacity and reproductive success in the bitch. Different techniques for monitoring periovulatory events, such as vaginoscopy, behavioural cues and ovarian ultrasound, have been reported (Johnston et al., 2001; Bergeron et al., 2013). The most rational method applied to routine breeding management is based on vaginal cytology together with blood progesterone concentration measurement (Fontbonne, 2010). Preovulatory blood progesterone concentration allows an indirect estimate of the ovulation and of the LH surge, which is associated in the canine species with a granulosa cells preluteinisation occurring several days before the ovulation (Concannon, 2009).

Although behavioural and hormonal aspects are the obvious effects of the ovarian modifications happening throughout the sexual cycle, little is known about the precise quali-quantitative features of the follicular population and luteal structures and their influence on the hormonal during the fertile period in the bitch. As the knowledge on canine ovarian physiology is far below that in other domestic species, the accurate correlation between the ovarian morphology and hormonal aspects might add novelty, providing specific information relative to crucial events in canine reproduction. To this aim, this study provides a histologic and histometric survey of the paired gonads of 40 healthy bitches undergoing ovariohysterectomy throughout all the stages of the reproductive cycle. Twenty-three of these dogs were evaluated during the periovulatory period. Number, structure and dimension of 414 ovarian structures (follicles, *corpora*

hemorrhagica and *corpora lutea*) were evaluated and compared to the vaginal cytology and blood progesterone outcomes.

2. Materials and methods

2.1. Animals

Gonads were obtained from forty healthy cycling bitches (*Canis lupus familiaris*) attending the Reproduction Unit of the Faculty of Veterinary Medicine, Milan (Italy) for routine spaying. Only bitches undergoing the monitoring of the reproductive cycle before spaying were enrolled in this study. Bitches belonged to different breeds (small sized breeds: < 10 kg, N=13; medium sized breeds: 10-30 kg, N=18; large breeds: > 30 kg, N=9) and ages (< 4 years, N=25; 4-9 years, N=10; > 9 years, N=5). The bitches average weight was 18.1 ± 11.6 kg (from 2.5 to 41.5 kg); average age was 4.1 ± 3.6 years (from 6 months to 13 years). All animals underwent an accurate anamnestic and clinical assessment. Only bitches clinically healthy at the time of the first screening were included in the study. Ovariohysterectomy was performed at specified times depending on the stage of the reproductive cycle, using a standard technique through median laparotomy access in supine bitches (Howe, 2006).

Only bitches having initial plasma progesterone concentration < 2 ng/mL were included in the study. Based on the stage of the reproductive cycle (defined as described below) bitches where included into 7 groups: proestrus (P; N=6), estrus before ovulation (E1; N=6), periovulatory estrus (E2; N=5), estrus after ovulation (E3; N=5), early diestrus (D1; N=7), middle-late diestrus (D2; N=6), anestrus (A; N=5).

2.2. Methods for reproductive cycle monitoring

Reproductive cycle was monitored by vaginal cytology and plasma progesterone (P4) measurement, starting from proestrus. The optimal time for surgery was chosen on the

basis of subsequent monitoring of these two parameters. Vaginal cells were collected from the anterior portion of vagina using a sterile metal spatula after vulvar labia spreading. The spatula was inserted at dorsal commissure of the vulva, avoiding clitoral fossa then advanced at 45° angle and gently rubbed on vaginal vault. Cells from spatula were gently transferred to cytological slides, air-fixed for haematoxylin and eosin staining (Hemaquick; Tektron, Bornheim, Germany) and examined under an Olympus (Olympus, Tokyo, Japan) BX51 photomicroscope at X 100, X 200 and X 400 magnifications. Eosinophilic index (EI) was also calculated (England, 2013) from proestrus to early diestrus as:

EI = number of anuclear cells/total number of epithelial cells, examined at 100x magnification.

Blood samples for analysis of progesterone were collected at the cephalic vein into heparinized tubes. Plasma progesterone concentration was determined using a quantitative test based on ELFA technique (Enzyme Linked Fluorescent Assay; MiniVidas, BioMérieux, France). Assay principle combines an enzyme immunoassay competition method with a final fluorescent detection (Brugger et al., 2011).

2.3. Definition of the stages of the reproductive cycle

On the basis of the available literature, the stages of the reproductive cycle, including the intermediate ones, were classified by vaginal cytology and blood progesterone outcomes as follows:

- Proestrus (P) was defined as the period between the first day of appearance of vulvar bloody discharge and just before the LH surge, that is blood progesterone concentration values below 2 ng/mL associated to a corresponding vaginal cytology characterized by the presence of erythrocytes and a mixture of parabasal, intermediate and superficial cells together with neutrophils and bacteria (Concannon

and Rendano, 1983; Wright, 1990; Simpson et al., 1998; Johnston et al., 2001; Kutzler et al., 2003; Michel et al., 2011).

- Estrus started at the LH surge, which was identified by P4 values above 2 ng/mL (Wright, 1990; Johnston et al., 2001; Kutzler et al., 2003; Michel et al., 2011). Due to the ovulation occurring at blood progesterone concentration between 4 and 10 ng/mL (Wright, 1990; Johnston et al., 2001; Michel et al., 2011), preovulatory (E1) and periovulatory (E2) estrus stages were comprised from 2 to 4 ng/mL and from 4 to 10 ng/mL blood progesterone concentration values, respectively. Postovulatory estrus stage (E3) was characterized by progesterone concentrations above 10 ng/mL. Superficial cells are the predominant epithelial cell type present in vaginal smears during estrus, together with a decreasing number of erythrocytes and usually absent neutrophils (Johnston et al., 2001).
- Early diestrus (D1) was defined as the period between the first day of appearance of cytological diestrus until the ninth day after (Groppetti et al., 2010). The abrupt change in relative number of epithelial cells, mainly superficial *versus* parabasal/intermediate cells, marks the onset of cytologic diestrus (Johnston et al., 2001). After this time and for a further fifty days middle-late diestrus was considered (D2) (Groppetti et al., 2010).
- Anestrus (A) was characterized by basal values of blood progesterone concentration (< 2 ng/mL) and ~~compliant~~ corresponding vaginal cytology characterized by parabasal and small intermediate cells (Johnston et al., 2001; Okkens and Kooistra, 2006; Groppetti et al., 2010). Scant cellularity and no clinical signs of ovarian activity (serosanguineous vulvar discharge and hypertrophy) allowed to distinguish between anestrus and proestrus.

153 **2.4. Histology and Histometry**

154 Immediately after ovariohysterectomy an accurate macroscopical evaluation of the
155 ovarian structures present in both ovaries of each bitch was performed, recording number
156 and size of tertiary follicles, *corpora hemorrhagica* (CHs) and *corpora lutea* (CLs). After
157 fixation by immersion in 10% neutral formalin for 48-72 h at 4°C, each gonad was further
158 sliced to evidence and count ovarian structures in more detail. The resulting fragments
159 were then dehydrated in a graded series of ethanol and embedded in paraffin. Tissue
160 blocks were cut at 4 □m thickness, de-waxed and stained with routinary Haematoxylin-
161 Eosin (H&E) for general morphological purposes and histometry.

162 The sections were observed and photographed under an Olympus BX51
163 photomicroscope equipped with a digital camera and DP software (Olympus, Italy) for
164 computer-assisted image acquirement and managing.

165 Only tertiary vesicular follicles were considered for histometric purposes. These follicles
166 were subdivided into three categories according to their diameter: small (< 2 mm),
167 medium (2-3 mm) and large (> 3 mm) follicles.

168 The number and dimensions of *corpora hemorrhagica* and mature *corpora lutea* were
169 also evaluated.

170 Follicles exhibiting pyknotic granulosa cells and/or degenerated oocyte (highly irregular
171 shape and/or the presence of pycnotic nucleus) were seldom recognized and classified as
172 atretic. Atretic follicles were not considered in the morphometric study as well as
173 primordial, primary and secondary follicles.

174 Histometry was performed on micrographs taken on H&E-stained sections. The diameters
175 of the ovarian structures (two diameters at right angles, with the basement membrane as
176 limit) were measured in the largest cross section utilizing the highest magnification
177 allowed by their individual dimensions (x40 objective for follicle diameter < 300 μm,

178 x20-10 for follicle diameter 300-1000 μm and x4 for follicle diameter >1000 μm). For
179 each ovary, all follicles having diameter >1 mm have been recorded. The diameters of the
180 large and small luteinic cells were also measured in mature *corpora lutea*.

181 **2.5. Statistical analyses**

182 Data were analysed using a commercial statistical software (IBM SPSS 21.0 for
183 Windows, IBM SPSS, Armonk, New York, USA). Descriptive statistics were expressed
184 as mean ± standard deviation (SD).
185 For statistical purposes dogs were stratified in three groups by age (< 4 years; 4-9 years;
186 > 9 years). Number and size of the ovarian structures present in both ovaries of each bitch
187 were compared to the weight, age and stage of the reproductive cycle, after verification
188 of the normality of the data distribution with a Shapiro-Wilk test, by the non-parametric
189 Kruskal-Wallis test and linear regression. The progesterone values were compared to
190 number and size of the ovarian structures by the non-parametric Spearman correlation
191 test. Statistical significance was defined as $P < 0.05$.
192

193 **3. Results**

194 **3.1. Histologic and histometric features of the ovarian structures**

195 A total of 414 ovarian structures (tertiary follicles, CHs and CLs) were examined in the
196 40 paired ovaries collected from 40 bitches.

197 **3.1.1. Morphological aspects**

198 Throughout the reproductive cycle in the cortical ovarian zone it was possible to notice
199 the presence of nests of primordial and/or primary follicles (Fig. 1a, b), which were not
200 however considered in this study as well as secondary, non-cavitated follicles (diameter
201 less than 1 mm). Polyovular follicles containing two or more germ cells, each of them

surrounded by an evident *zona pellucida* were frequently detected (Fig. 1c). Frequently cortical tubules, characteristic of the canine ovary were also observed (Fig. 1a). Atretic follicles were present in growing number in E3, D1 and D2 however, they were not considered in the morphometric study. Multilaminar tertiary follicles (N=293) having an *antrum* surrounded by many layers of granulosa cells were recorded from proestrus to anestrus (Fig. 1b, c, d, e), with slightly different features:

- Small follicles during P were made up by 10-14 layers of small and round, basophilic granulosa cells (Fig. 1b). In E1 some signs of preluteinisation, which are follicular cell hypertrophy and lipid accumulation, could be noticed in granulosa cells and vascularization started to be detected in the inner theca. In E2 and E3 the small follicles appeared quiescent, with no signs of luteinisation in the flat and thin granulosa. Many erythrocytes were observed into the stroma surrounding the follicle, as noticed also in D1.
- Medium follicles during P showed round to columnar granulosa cells and many blood vessels could be seen around the follicle. Theca layers were still indistinct. In E1 the granulosa layer started forming folds into the *antrum*, and early signs of luteinisation could be observed. Blood vessels were more numerous around the follicle. In E2 the granulosa folds were more pronounced and marked signs of luteinisation could be noticed at cellular level (Fig. 1e). Crowding of blood vessels around the follicle intensified. A similar aspect could be seen in E3.
- Large follicles in P and E1 looked like medium follicles in the corresponding phases, except for dimensions (Fig. 1d). In E2 the granulosa was markedly folded, with numerous vessels containing much blood in the fold axis. Even more blood was seen in E3.

CHs formed in E2 and E3 after ovulation when the theca capillaries invaded the layers of the luteinized granulosa, thus starting the obliteration of the follicular *antrum* (Fig. 1f, g, h).

CLs were characterized by hypertrophy and accumulation of large amounts of lipid in the internal theca cells and follicular epithelium (Fig. 1i). The capillary network of the *theca interna* gives rise to the rich vascularization of the CL. The cells of the CLs, intensely replicating, were differentiated into small (SLC) and large (LLC) luteal cells (Fig. 1k) that invaded and gradually obliterated the lumen. SLC were characterized by a low cytoplasmic/nuclear ratio with an eccentrically placed irregular nucleus and paucity of cytoplasmic lipid droplets. LLC were polyhedral or spherical in shape, possessed a higher cytoplasmic/nuclear ratio, centrally located round nucleus and abundance of cytoplasmic lipid droplets.

CL regression was characterized by vacuolization of luteal cells, abundantly filled with lipids (Fig. 1j). Increase and invasion by the surrounding connective tissue induced shrinkage of the involuting CL.

3.1.2. Histometric outcomes

The mean number and size of the ovarian structures at different stages of the reproductive cycle is shown in Table ~~2~~ 1. The number of medium ($P = 0.001$) and large follicles ($P < 0.002$) was statistically different among all the stages of the reproductive cycle, while small follicles were always observed from proestrus to anestrus. During proestrus a mean of 12.5 ± 5.3 tertiary follicles were recorded per each bitch, with a predominance of follicles larger than 2 mm in diameter (medium and large; 82.7%) (Table ~~2~~ 1). In E1 small, medium and large follicles were evenly distributed (Table ~~2~~ 1) with a mean of 14.7 ± 7.6 follicles per bitch. Approaching to ovulation (E2) the mean number of follicles was 14.6 ± 8.2 with a predominance of the large follicles (52.9%). More than a half of the

large follicles (52.4%) had a diameter greater than 4 mm. The number of these very large follicles significantly differed compared to the other phases of the reproductive cycle (P = 0.008). The occurrence of the ovulation was confirmed either by macro- and microscopic detection of CHs in the ovaries (Fig. 1f, g). CHs around 6 mm in diameter (Table ~~2~~ 1) began to be detected (2.4 ± 5.3 per bitch) together with follicles in the same and/or in the opposite ovary during E2. CHs were the predominant ovarian structures (6 ± 1.6 per bitch) during postovulatory estrus (E3) ($P < 0.002$), accompanied by a decreasing number of follicles (8 ± 6.6 per bitch), mainly smaller than 3 mm. The diameter of the medium follicles statistically increased with advancing estrus (E3; $P < 0.03$). Large follicles reached diameters around 5 mm while CL had not yet found in this stage (Table 1). Early diestrus (D1) was characterized by the appearance of the CLs (7 ± 3.2 per bitch; $P < 0.001$) (Fig. 1i) still coexisting with few, mainly small, follicles (2.1 ± 5.7 per bitch) and some CH (3.5 ± 3.5 per bitch). However few large follicles with diameter around 5 mm were still recorded. CLs showed a diameter of above 6 mm (Table 1). The mean number of CLs per bitch in D2 was 5.8 ± 1.5 ($P < 0.001$). Small luteal (SLC) and large luteal (LLC) cells had 15.5 ± 1.9 □m (ranging from 10.6 to 19.9 □m) and 26.5 ± 3.8 □m (ranging from 20 to 38.6 □m) long diameter, respectively. Cellular size was unchanged with the transition to the middle-late diestrus (D2): SLC had 15.5 ± 2.1 □m (ranging from 10.1 to 19.5 □m) and LLC 28.8 ± 4.3 □m (ranging from 20 to 39.2 □m) long diameter. However, in D2 cells showed increased signs of vacuolization due to lipid deposits. Few tertiary follicles (3.2 ± 4.3 per dog), mainly smaller than 2 mm in diameter, were recorded. However, a small percentage (3.7%) of large follicles was detected in D2. Regressed CLs from the current and previous cycles persisted in anestrus (A) and were detectable in all subsequent stage. The mean diameter of regressing CLs was 2.9 □ 0.9

mm with SLC and LLC of 15.8 □ 2.5 □m and 27.1 □ 4 □m long diameter, respectively. Immature and small follicles (4 ± 5.6 per dog) were also observed during anestrus.

3.2. Clinical features

Figure 2 illustrates the vaginal cytology, ovarian macroscopic aspects, and a detail of typical ovarian histology in the 7 ranges of the reproductive cycle. Blood progesterone values, which are analytically reported in Table 1, are also graphically indicated on the bottom of the figure.

During proestrus vaginal smears were characterized by a heterogeneous population of epithelial cells, mainly parabasal and small intermediate cells, EI ≤ 20%, together with erythrocytes, leukocytes and bacteria. Mucous filaments were also observed (Fig. 2a). Vaginal cytologic pattern in E1 was similar to that in proestrus but intermediate cells, small and large, predominated with EI increasing over 20% (Fig. 2b). During E2 numerous large intermediate and nucleated superficial cells with few anuclear superficial cells (EI > 50%), some erythrocytes, rare neutrophils and bacteria, were observed (Fig. 2c). After ovulation (E3) vaginal smears were characterized by anuclear superficial cells (EI ≥ 70%) surrounded by a clear background without bacteria and cellular debris (Fig. 2d). The onset of cytologic diestrus was characterized by an abrupt decrease in the percentage of anuclear superficial cells (EI < 40%) together with an increase in intermediate cells and reappearance of neutrophils (Fig. 2e). Bitches having more or less than 6 recent CLs in the paired ovaries showed a mean progesterone value of 67.5 ± 20.5 ng/mL and 49.9 ± 24.9 ng/mL, respectively. However, no statistical difference in progesterone values was detected in respect to the number and size of the CLs, CHs and follicles. During middle-late diestrus a variable percentage of small intermediate and parabasal cells was detected in vaginal smears with few neutrophils and sometimes mucous filaments. Samples collected during anestrus were characterized by low

cellularity that consisted predominantly of single or small group of cohesive parabasal cells.

3.3. Correlation between the follicular structures and weight and age of the bitches

The diameter of the small ($P < 0.001$) and medium ($P < 0.05$) follicles was positively correlated with the body size of the bitch. The number of follicles larger than 4 mm was significantly lower in bitches younger than 4 years ($P < 0.02$).

4. Discussion

To our knowledge, this is the first detailed description of the histological morphometry of the ovarian structures (tertiary follicles, *corpora hemorrhagica* and *corpora lutea*) through the reproductive cycle of the bitch - with particular emphasis on the periovulatory period - compared to the vaginal cytology and blood progesterone outcomes, in such a way to show what exactly the ovary looks like in a precise periovulatory moment.

Optimal fertility in bitch occurs between two and five days after ovulation when mature oocytes are present (Wildt et al., 1978; Concannon, 1993). The ovulation is assumed to occur approximately two-three days after the LH surge (Wildt et al., 1978; de Gier et al., 2006). Unfortunately, the time of LH surge is highly variable in dogs and takes a relatively long time, ranging approximately from ~~one to five days~~ 24 to 60 h (Wildt et al., 1978; Concannon, 1993 Onclin et al., 2002; Concannon, 2009). The rapid rise in blood progesterone concentration at the LH surge and ovulation makes its measurement, together with vaginal cytology, the most reliable marker to detect periovulatory events in clinical practice (Okkens et al., 2001; Fontbonne, 2010; Moxon et al., 2012). However, the actual ovarian status can be accurately determined only by the histological examination being the clinical, behavioural and hormonal aspects their indirect effects. Little is known about the exact correlate between histological and hormonal aspects in the cycling bitch. Previous studies on ~~the~~ canine ovarian activity are based on the oocytes

evaluation and ultrasonographic measurements of the follicular populations (Payan-Carreira and Pires, 2008; England et al., 2009; Reynaud et al., 2009; Bergeron et al., 2013). However, luteinisation of follicles in bitch occurs before ovulation and follicles do not collapse immediately after ovulation (Bouchard et al., 1991; Concannon, 2009; England et al., 2009), making difficult to distinguish between preovulatory follicles and/or *corpora lutea* by ultrasound (Bergeron et al., 2013). Studying the canine ovary *in situ* is also complicated by anatomical challenges, especially an encapsulating ovarian bursa that interferes with the ultrasonographic view of the gonads (Wildt et al., 1978; England et al., 2009; Songsasen et al., 2011).

The observed changes of vaginal cytology in the transition from proestrus (P) to ovulatory period (E2) were mild, variable and subjective. These appearances became noticeable in the postovulatory period (E3) and in early diestrus (D1). The percentage of keratinized cells recorded in this study was significantly lower than previously reported, mainly in early follicular phase (England and Concannon, 2002). This disagreement could be due to a subjective interpretation, intrinsic to vaginal cytology assessment. A variety of methods of collection, staining and analysis of vaginal smears have been reported. As shown by Moxon et al. (2010), the analysis of vaginal cytology in order to classify different epithelial cellular type and to estimate the stage of the reproductive cycle varies between technicians (Moxon et al., 2010). Indeed, vaginal cytology alone is not able to detect the precise periovulatory events, such as the day of LH surge or ovulation, while together with blood progesterone evaluation allows to accurate define the different stage of the reproductive cycle (Wright, 1990; Johnston et al., 2001; Kutzler et al., 2003; Fontbonne, 2010; Groppetti et al., 2010; Michel et al., 2011).

Before the LH surge (P) the medium follicles with still indistinct theca layers and the large follicles with thickened granulosa folds and wide *antrum* predominated. The LH

surge is stated the first day of the estrus (E1) (Wright, 1990; Johnston et al., 2001; Kutzler et al., 2003; Michel et al., 2011). In the canine species the LH surge is associated with a preluteinisation of the granulosa cells that begin to secrete progesterone (de Gier et al., 2006; Concannon, 2009). In medium and large follicles during proestrus we already observed early signs of luteinisation, which became more marked in E1 together with folded and vascularized granulosa layers. This time is characterized by the mucification of the cumulus in the granulosa cells, the intensity of which depends on the follicular maturity (Reynaud et al., 2006). Preliminary histological studies suggested early canine luteal cells to originate primarily from theca ingrowth, with dispersed mid-cycle luteal cells appearing of a single type as in some rodents (Concannon, 2012). In our sample the mean progesterone value at this time was 2.9 ± 0.4 ng/mL.

The ovulation is regarded to occur when blood progesterone concentration is between 4 and 10 ng/mL (Wright, 1990; Johnston et al., 2001; Michel et al., 2011). In our sample the ovulation was observed when the progesterone reached the value of 6.1 ± 1.7 ng/mL and the number of very large follicles, with diameter more than 4 mm, increased ($P = 0.008$). In the polytocous species not all follicles ripen at the same time. In bitch ovulations of the various follicles are spread over a 24– 36 h period (Boyd et al., 1993). In agreement with these data, we observed very large follicles, with diameter more than 4 mm, coexisting with CHs from ovulatory period (E2) to early diestrus (D1). The occurrence of ovulation was confirmed by CHs development ($P < 0.002$). Although the ovarian assessments were not carried out at different times in the same subject, it is interesting to note that only the 41% of the follicles observed during ovulation (E2) ~~were~~ might have been replaced by CHs after ovulation (E2), while all the CHs (E3) might have become CL after ovulation (D1). These observations should confirm that unovulated follicles persist in the ovary after ovulation as speculated by some authors (Levy and Fontbonne, 2007).

377 The end of the fertile period, which coincides with the beginning of diestrus (D1), namely
378 the development of CLs (P = 0.001), was characterized by a mean value of blood
379 progesterone concentration greater than 36.9 ng/mL. Therefore, taking into account the
380 individual variability and delayed ovulations, the period of greatest fertility in the bitch
381 in our study was restricted between values of 10 and 37 ng/mL of blood progesterone
382 concentration. The precise identification of the range of the fertile period is essential to
383 plan programs of assisted reproduction in the bitch. Development of the tertiary follicles,
384 *corpora hemorrhagica* and *corpora lutea*, appeared to be related to minimal hormonal
385 variations even within the same stage of the reproductive cycle. The tertiary follicles
386 distribution (small, medium and large) was statistically different (P < 0.002) among all
387 the stages of the reproductive cycle, except for small follicles (< 2 mm), always observed
388 from proestrus to anestrus. In agreement with Reynaud *et al.* (2009), the number and
389 distribution of tertiary follicles significantly differed between proestrus (P) and early
390 estrus (E1), but no follicles larger than 5.5 mm in diameter were detected before LH surge
391 in our experiment. The number and distribution of large follicles significantly differed
392 between early estrus (E1), periovulatory estrus (E2) and estrus after ovulation (E3) (P <
393 0.002). Moreover, large follicles with a diameter greater than 4 mm characterized the
394 early estrus up to ovulation (P = 0.008).
395 Diestrus is characterized by the development of CLs the size of which has been reported
396 to be 3-12 mm in diameter (Rehm et al., 2007); we recorded diameters of 5-6 mm and
397 about 3 mm in recent and involved CLs, respectively. Small and large luteal cells have
398 been described in the pig (Richards et al., 1994), ovine (Fitz et al., 1982) and cattle (Cools
399 et al., 2013) to originate from theca and granulosa cells, respectively (Baithalu et al.,
400 2013). The size of SLC (12-23 □m) and LLC (25-55 □m) in cattle increased as the stage
401 of estrus cycle progressed and during pregnancy (Baithalu et al,. 2013). They have never

been described in the dog before. Our results show a similar size in this species with SLC between 10 and 20 μm of diameter and LLC between 20 and 40 μm of diameter while the size was unchanged from early to late diestrus.

During anestrus some studies record only follicles smaller than 1 mm in diameter (Reynaud et al., 2012), or ranging from 4 to 6 mm (Reynaud et al., 2009). In agreement with other authors (England et al., 2009), we observed small follicles of 1.3 ± 0.3 mm of diameter together with involved CLs.

The diameter and the number of growing follicles have been positively correlated with the body size of the female (Reynaud et al., 2010). In our study the diameter of the small follicles was positively correlated with the weight of the bitch ($P = 0.009$), while the number of follicles larger than 4 mm was significantly lower in bitches younger than 4 years ($P < 0.02$). No statistical difference in progesterone values was detected in respect to the number and size of the CLs, CHs and follicles.

In conclusion, histologic and histometric evaluation of the gonads of 40 cycling bitches along with the clinical and hormonal outcomes permitted to appreciate subtle changes that occur through the reproductive cycle, mainly in periovulatory period. Actually, it may be difficult to clinically identify the precise changes that occur in the ovarian tissues. This study allowed to deepen the knowledge on some critical steps in the canine reproductive management, mainly periovulatory phase and the end of the fertile period, essential to plan breeding programs.

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Authors' contributions: **D.G.**, **S.A.** and **M.A.** conceived and designed the experiment. **D.G.** and **A.P.** collected the clinical data, performed surgery and estimated the estrous period of bitches. **M.A.** and **G.B.** collected and processed the canine specimens employed in the study**. M.A.**, **S.A.** and **D.G.** evaluated the slides, and photographed them. **M.A.** performed the histometric measurements, which were statistically evaluated by **V.B.** and **D.G.**. **D.G.** and **S.A.** arranged the figures and wrote the manuscript. All the Authors participated in critical reading and final approval of the manuscript.

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Legends

Figure 1. Microscopic aspects of the canine ovary in the cycling bitch. *(a)* In a subcapsular position primordial follicles (arrows) as well as cortical tubules (empty arrow) can be seen. Primordial follicles contain an oocyte surrounded by a layer of 3-6 flattened pre-granulosa cells. *(b)* A small, multilaminar follicle is present, showing an *antrum* surrounded by many layers of granulosa cells and fully developed thecae. Primary follicles are also present in sub-cortical position (arrow). *(c)* A polyovular antral follicle can be seen, containing two germ cells, each of them surrounded by an evident *zona pellucida*. *(d)* Large tertiary follicle, characterized by an enormous *antrum* filled with *liquor folliculi*. The *cumulus oophorus* containing the oocyte can be seen protruding into the follicular cavity. *(e)* Signs of pre-ovulatory luteinisation can be seen in the granulosa of a tertiary follicle. *(f)* Ovulation of a mature preluteinized follicle. The follicle ruptures in proximity of the ovarian stigma. *(g)* Detail of the luteinized follicle wall just after ovulation, together with first signs of transformation into *corpus hemorrhagicum*, due to invasion of the *antrum* by capillaries from the stroma. *(h)* During the cellular replication and luteinic transformation the *corpus hemorrhagicum* develops into *corpus luteum*. The lumen progressively obliterates. *(i)* Mature *corpus luteum* is a solid gland with the typical histo- and cytologic organization of a steroidogenic endocrine gland. *(j) Corpus luteum* regression is characterized by shrinkage and vacuolated luteal cells, abundantly filled with lipids. *(k)* High magnification of glandular tissue forming the *corpus luteum*. Diameters of the large luteinic cells are signposted by a bar, arrows indicate small luteinic cells. Scale bars:

(a,e,i) =200µm; *(b,c,h)* = 500µm; *(d,f)* = 1000µm; *(g,j)* = 100µm; *(k)* = 100µm.

Figure 2. Vaginal cytology *(a-g)*, macroscopic aspects *(h-n)* and details of ovarian histology *(o-u)* typical of the 7 steps identified in the reproductive cycle in the bitch. The graphic summarizes the corresponding mean blood progesterone concentration throughout the cycle.

(a) A group of cohesive parabasal cells surrounded by some erythrocyte. *(b)* Large numbers of erythrocytes with intermediate cells and some leukocytes (arrow: neutrophil; arrowhead: lymphocyte). *(c)* Superficial cells and rare erythrocytes. *(d)* Superficial, mainly anucleate cells. *(e)* Superficial and intermediate cells with abundant neutrophils. *(f)* Heterogeneous epithelial cells population with some neutrophils. *(g)* A typically little cells sample with small groups of parabasal cell. *(h)* Translucent small structures of vesicular appearance characterize proestrus. *(i)* During preovulatory estrus follicular structures increase in diameter and *liquor* content. *(j)* Very large follicles are evident before ovulation. *(k)* CHs appeared after ovulation. *(l)* At the beginning of diestrus CHs and CLs coexist. *(m)* Mature CLs characterize medium-late diestrus; *(n)* In anestrus ovaries are typically small and inactive. *(o)* Small tertiary follicle. *(p)* Preovulatory large follicle. *(q)* Ruptured large follicle immediately after ovulation. *(r)* Transformation of the collapsed tertiary follicle into *corpus hemorrhagicum*. *(s)* Transformation of *corpus hemorrhagicum* into *corpus luteum*. *(t)* Mature *corpus luteum*. *(u)* Involuted ~~Involved~~ *corpus luteum (corpus albicans).* Scale bars: *(a-g)* = 100µm; *(o,u)* = 500µm; *(p,q)* = 700µm; *(r,s,t)* = 1000µm.

Figure 1

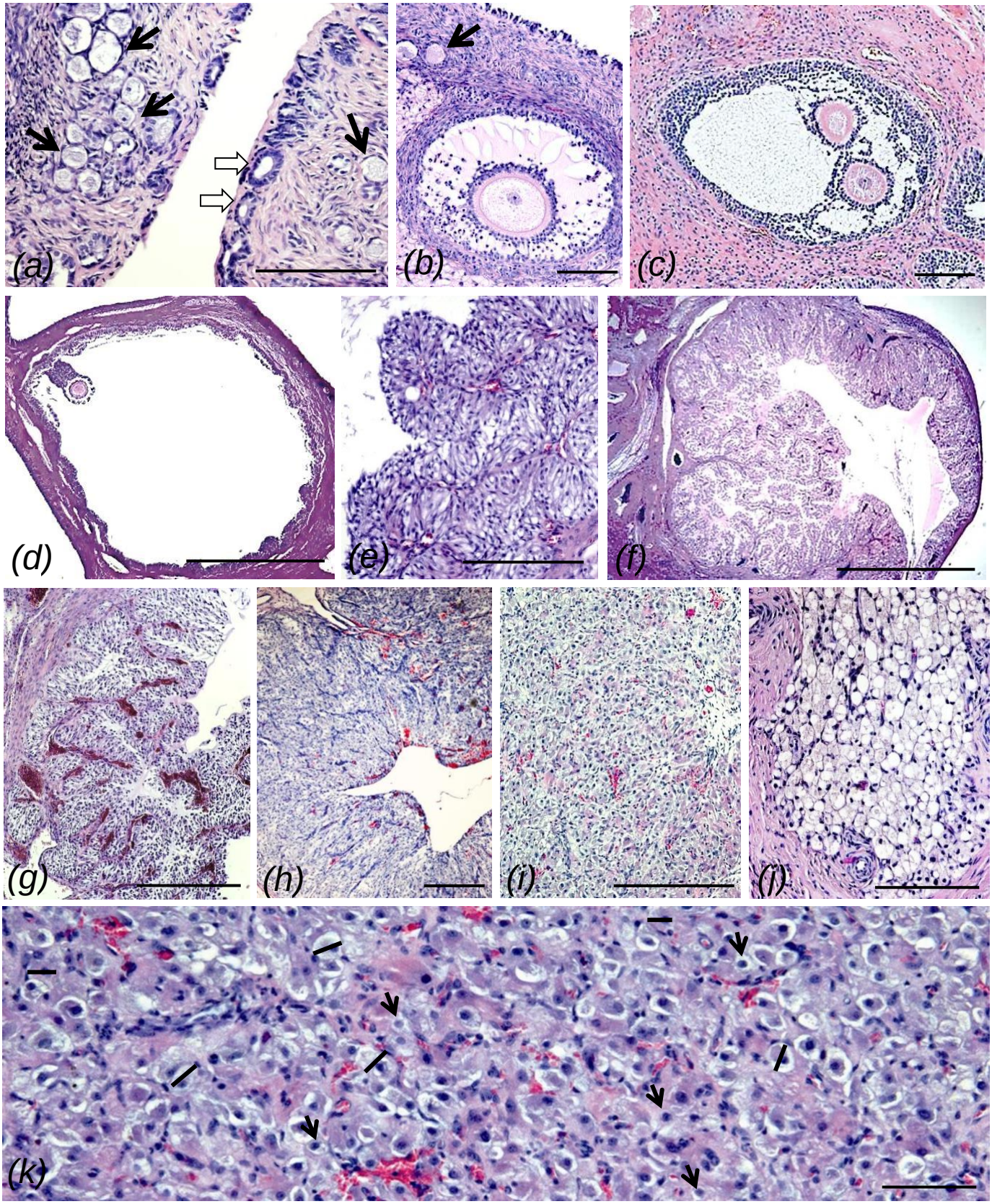

Figure 2 REVISED

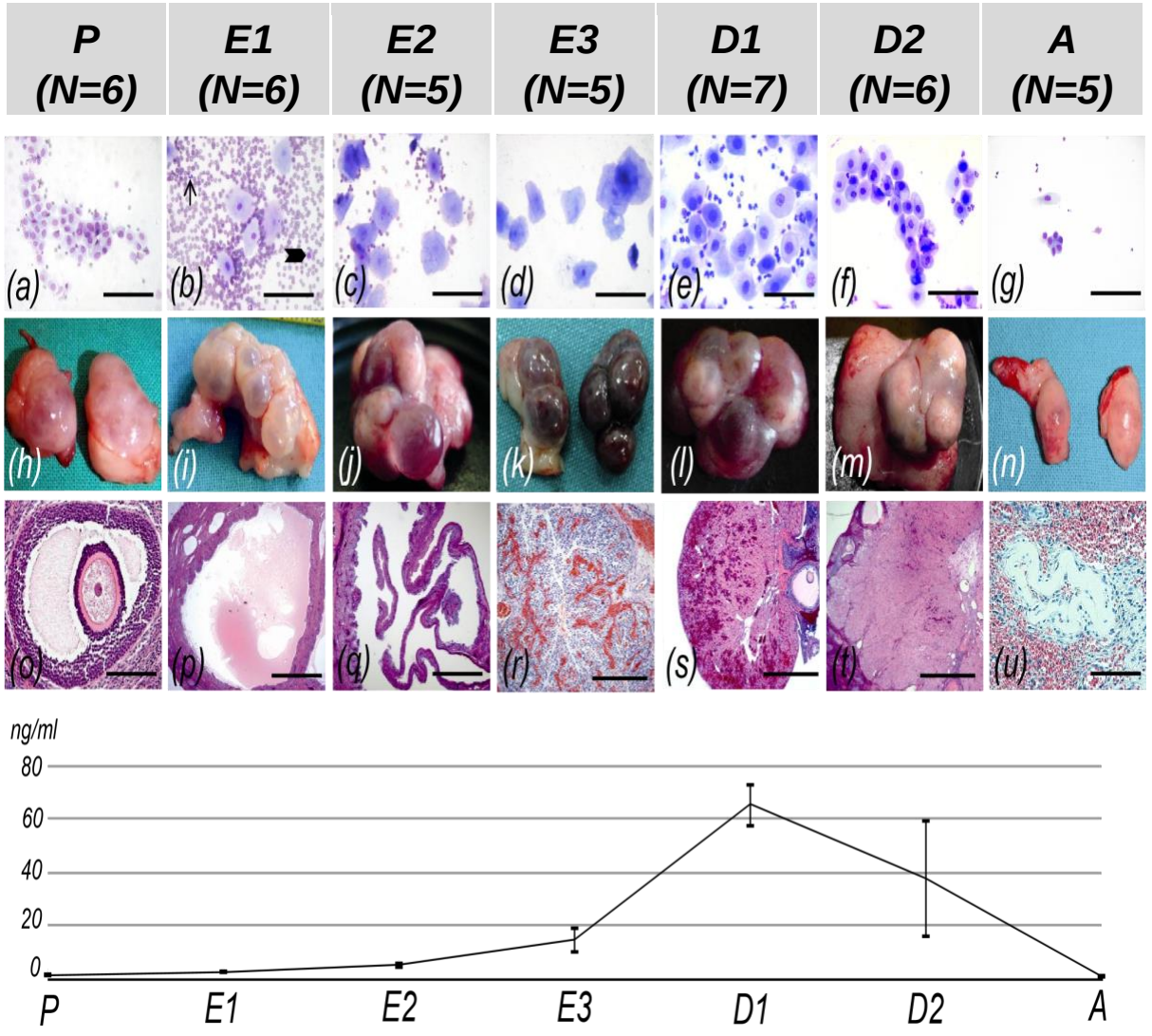

Table 1 - Type, size and percentage of ovarian structures per bitch at the different stages of the canine reproductive cycle. Values are indicated as mean ± SD.

	Progesterone (ng/mL)	Small follicles	Medium follicles*	Large follicles*	*Corpora hemorrhagica*	*Corpora lutea*
Proestrus (P)	1,7 ± 0,3 [1,2-1,9]	1,2 ± 0,4 mm 17,3%	2,4 ± 0,3 mm 44%	3,6 ± 0,4 mm 38,7%	-	-
Preovulatory estrus (E1)	2,9 ± 0,4 [2,5-3,5]	1,5 ± 0,3 mm 33,9%	2,3 ± 0,2 mm 30,5%	3,8 ± 0,1 mm 35,6%	-	-
Periovulatory estrus (E2)	6,1 ± 1,7 [4,2-8,9]	1,5 ± 0,4 mm 11,8%	2,5 ± 0,3 mm 21,2%	4,1 ± 0,7 mm 52,9%	6 ± 1,2 mm 14,1%	-
Postovulatory estrus (E3)	16,7 ± 5,9 [10,6-22,5]	1,3 ± 0,4 mm 23,2%	2,5 ± 0,3 mm 25%	4,9 ± 0,3 mm 8,9%	6,1 ± 1,1 mm 42,9%*	-
Early diestrus (D1)	73,9 ± 9,9 [36,9-80]	1,6 ± 0,2 mm 21,4%	2,7 ± 0,1 mm 4,3%	4,9 ±0,3 mm 2,9%	6,4 mm 10%	5,6 ±1,7 mm 61,4%**
Middle-late diestrus (D2)	42,6 ± 25,2 [10,6-80]	1,3 ± 0,3 mm 31,5%	-	3,1 ±0,1 mm 3,7%	-	6,1 ± 0,7 mm 64,8%**
Anestrus (A)	1,3 ± 0,5 [0,8-1,9]	1,3 ± 0,3 mm 100%	-	-	-	-

* P < 0,002

** P = 0,001

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	[10,6-80]					
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** P = 0,001