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# **The oestrous cycle and inhibition of oestrus in canids**

Lena Lindh

DOCTORAL DISSERTATION

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# Abstract

Dogs (*Canis familiaris*) are distributed globally with an estimated population of around 700 million, including both pet and free roaming dogs. In pet dogs, contraception has traditionally been achieved by surgical removal of the gonads, however, in dogs intended for breeding, reversible contraception is of outmost importance.

In recent times, animal welfare legislation and ethical guidelines for companion animals have been improved, raising a demand for new non-invasive contraceptive methods in dogs. Traditional contraception by removal of the gonads has been criticised and condemned as an invasive procedure, subjecting the animals to not only a painful procedure and recovery but also possible long-term side effects.

To achieve contraception in any species, a thorough understanding of the oestrous cycle is mandatory. In this study, our general aim was to investigate the oestrous cycle of the female dog and possible inhibition of oestrogen synthesis using an aromatase inhibitor (Finrozole). As the use of pet populations for research purposes raises several ethical questions, the first study was performed in farmed blue fox vixens (*Vulpes lagopus*), that present hormonal patterns very similar to those seen in domestic dogs. A successful contraceptive outcome in vixens was achieved with aromatase inhibitor Finrozole, when doses of  $\geq 3.5$  mg/kg were used, and treatment was initiated at least four days before the day of artificial insemination. The first study done in vixens clearly indicated the need for an accurate determination of the stage of the oestrous cycle, leading the second study to assess the critical time-points of transitions between stages of the oestrous cycle in domestic dogs of various breeds. This was done by comparison of clinical, endocrinological, vagino-cytological and histomorphological parameters. Findings of the second study emphasises difficulties in staging of the oestrous cycle in domestic dogs, with clinical and endocrinological parameters not always coinciding with the morphofunctional status of the reproductive organs.

For a deeper insight into synthesis of oestrogen and possible inhibition of oestrogen synthesis, the third study aimed to investigate the activity of aromatase in ovarian tissue during different stages of the domestic dog oestrous cycle. Aromatase activity was confirmed by immunohistochemistry during pro-oestrus, ovulation and early dioestrus, coinciding with the outcome of the first study. Interestingly, immunohistochemical analysis confirmed immunopositive staining for aromatase especially in theca interna derived cells.

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# List of original publications

This thesis is based on the following articles:

- I Lindh L, Lindeberg H, Banting A, Banting S, Sainmaa S, Beasley S, Korhonen HT, Peltoniemi OAT. Administration of aromatase inhibitor MPV-2213ad to blue fox vixens (*Vulpes lagopus*) as a model for contraception in female dogs. *Theriogenology* 2020 Aug; 152:53-63. Epub 2020 Apr 15. <https://doi.org/10.1016/j.theriogenology.2020.04.009>
- II Lindh L, Kowalewski MP, Günzel-Apel A-R, Goericke-Pesch S, Myllys V, Schuler G, Dahlbom M, Lindeberg H, Peltoniemi OAT. Ovarian and uterine changes during the oestrous cycle in dogs. *Reprod Fert Dev* 2023;35(4):321-337. <https://doi.org/10.1071/RD22177>
- III Lindh L, Kowalewski MP, Goericke-Pesch SK, Lindeberg H, Peltoniemi OAT. The spatio-temporal distribution of aromatase cytochrome in ovary throughout the canine oestrous cycle. *Reprod Fert Dev* 2024;36, RD23201. <https://doi.org/10.1071/RD23201>

The publications are referred to in the text by their roman numerals.

## List of abbreviations

BPH	Benign prostatic hyperplasia
E2	Oestradiol-17 $\beta$
GnRH	Gonadotropin releasing hormone
LH	Luteinising hormone
FSH	Follicle stimulating hormone
P4	Progesterone
VER	Vaginal electrical resistance
AI	Artificial insemination
CL	Corpus luteum
CLs	Corpora lutea
CLIA	Chemiluminescent immunoassay
RIA	Radioimmunoassay
PRL	Prolactin

# 1 Introduction

## 1.1 Introduction

The domestication process of dogs (*Canis familiaris*) has been widely debated, with several different theories suggesting how dogs became one of the first animals to be domesticated diverging from their main ancestor, the grey wolf. The wolf (*Canis lupus*) was domesticated between 32,000 and 16,000-11,000 years ago, thousands of years before humans developed from hunter-gatherers to an agricultural way of life (Tancredi and Cardinali 2023). It remains undebatable that dogs represent high variability in their accommodation to human life, acting as pets but also as working dogs for humans with medical conditions, not to mention army, police and border forces (Otto et al. 2019). Dogs are also used to detect health issues like cancer, oncoming seizures and hypoglycemia, having a prophylactic and therapeutic value for people (Wells 2007). To experimentally study the domestication process itself in 1959, Russian geneticist Dmitri Belyaev chose the silver fox (*Vulpes vulpes*) as a canid model, hypothesising that the domestication was based on selection for tamability (Herbeck et al. 2022). The concept of a domestication syndrome in animals has ever since been described widely, describing characteristics such as tamability, loss of reproductive seasonality and changes in appearance (coat colours, ear and tail form, craniofacial morphology) (Lord et al. 2020). Along with the studies of the domestication process itself, ethical and animal welfare legislation have actively been developed and improved recently, setting up new guidelines for domesticated species such as the dog. With these new guidelines, breeding management has become a significant subject, as neutering of dogs is considered common procedure to ensure better accommodation to human household conditions. However, several adverse effects are linked to the surgical removal of gonads, such as urinary incontinence (Reichler et al. 2005), vaginitis (Reichler 2009), transitional cell carcinoma (Knapp et al. 2000), cranial cruciate ligament rupture (Slauterbeck et al. 2004), joint disorders (Hart et al. 2014, 2016), osteosarcoma (Ru et al. 1998), but also behavioural changes (Kim et al. 2006). Since with domestication comes responsibility, we must focus on the development of new possibilities for non-invasive contraception, to ensure painless procedures for reproductive management in this highly variable species domesticated by humans, also known as the man's best friend. As King Frederick II of Prussia said before his

death in 1786; “the only, absolute and best friend a man has, in this selfish world, the one that will not betray or deny him, is his dog”.

### **1.1.1 Two closely related species, the domestic dog and the blue fox**

The domestic dog (*Canis familiaris*) and the farmed blue fox (*Vulpes lagopus*) belong to the same family of *Canidae* exhibiting a very similar reproductive physiology. Both species are mono-oestrous, polytocous and spontaneous ovulators. The blue fox is a seasonal breeder (Mondain-Monval et al. 1993), whereas the domestic dog is by large considered as a nonseasonal species (Abe et al. 2008). In both, female dogs and blue fox vixens, oocytes are ovulated as primary, undergoing their first meiotic division in the oviducts. Maturity and capability to become fertilised is reached in dogs on the 3rd day and in foxes 1 day after ovulation (Farstad et al. 1989).

The tubal transport and preimplantation periods are long in both, female dogs and vixens, with implantation occurring 19 and 17 days post ovulation, respectively, followed by a short post implantation period (Valtonen and Jalkanen 1993). In female dogs, morulae develop into blastocysts in the distal oviducts and enter the uterus 10-12 days after the luteinising hormone (LH) peak (Verstegen-Onclin and Verstegen 2008). In blue fox vixens, blastocysts can be found in the uterus 6-10 days after the peak in vaginal electrical resistance (VER), slowly entering the uterus from day 6 onwards (Lindeberg 2006). Differences between these species, however, can be seen in variable durations of the stages of the oestrous cycle (Table 1) and differing blastogenesis according to Valtonen and Jalkanen (1993). According to their studies, the duration of stages of the oestrous cycle is shorter in vixens compared to female dogs. Also, the length of gestation is shorter in blue fox vixens, lasting 56 days (Valtonen and Jalkanen 1993) compared to pregnancy in female dogs, ending approximately  $65 \pm 1$  days after the LH surge (Concannon et al. 1989). In blue foxes, the litter size is 8-12 pups according to Ahlstrøm and Wamberg (2000). The litter size in domestic purebred dogs is highly dependent on the breed, with litter sizes of  $3.5 (\pm 0.04)$  puppies described in miniature breeds and  $7.1 (\pm 0.13)$  puppies in giant breeds by Borge et al. (2011).

## **1.2 Stages of the oestrous cycle**

The female domestic dog is a mono-oestrous, polytocous, typically a non-seasonal breeder, with exception to the Basenji breed (Fuller 1956). The oestrous cycle can be divided into four stages as shown in Table 1.; pro-oestrus, oestrus, dioestrus and

anoestrus (Concannon 2011), with an interoestrous interval of 7 months (range 3.5-13 months) (Feldman and Nelson 2004). The length of each stage of the oestrous cycle varies within individuals and different breeds, but sometimes also within oestrous cycles of the same individual. The age of sexual maturity is breed related (Concannon 2011) and is most commonly reached at the age of 8-12 months, with the first ovarian cycle occurring 1-6 months after attaining adult height and weight (Feldman and Nelson 2004).

**Table 1** Duration of the stages of the oestrous cycle in female dogs and vixens.

Species	Source	Pro-oestrus	Oestrus	Dioestrus	Anoestrus
Dogs	Concannon 2011	5–20 days	5–15 days	50–80 days	80–240 days
Vixens	Farstad 1992	7–24 days	1–8 days	Not known	9 months
Vixens	Valtonen and Jalkanen 1993	2–14 days	3–5 days		

Farmed blue fox vixens present a very similar hormonal pattern to female dogs, although being mono-oestrous seasonal breeders. Vixens present reproductive activity during the spring months from February to May, reaching sexual maturity at 9 months of age usually coming into oestrus and accepting mating in mid-March to mid-April (Farstad 1992). Clinical signs of oestrus such as increased vocalisation, urination and swelling of the vulva can be detected on average two weeks prior to the LH peak indicating the initiation of pro-oestrus (Farstad 1992). Oestrus in blue fox vixens is shown for 4-5 days (Farstad 1992), duration of each stage of the oestrous cycle presented in Table 1. The onset of oestrus in vixens may vary in relation to the LH peak, and may start simultaneously with the LH peak, a day after the LH peak or even at ovulation two days after the LH peak (Møller et al. 1980, Farstad et al. 1989).

### 1.3 Determination of the stage of the oestrous cycle

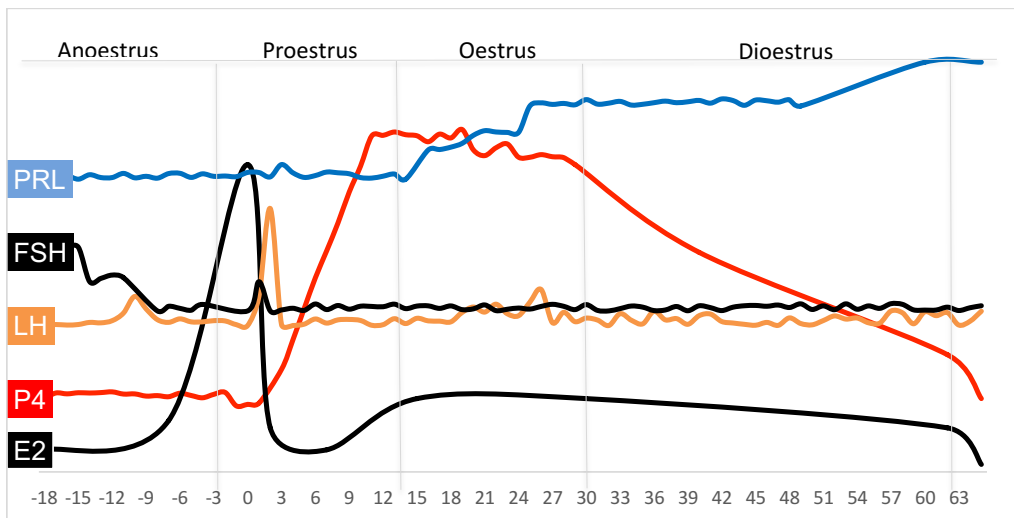
An accurate determination of the stage of the oestrous cycle is essential especially in assisted reproduction as well as in efforts to manipulate the oestrous cycle, for instance induction of oestrus, postponement or contraception. In case of natural mating, pregnancy can be achieved in dogs despite inaccurate timing, due to the longevity of male dog spermatozoa in the female genital tract. Breeders tend to mate their female dogs traditionally on the 12<sup>th</sup> day of visible clinical signs of oestrus, which coincides with the results by Hori et al. (2012), concluding ovulation to occur

on average 11 days (range 3-31 days) after the onset of sanguineous vaginal discharge. In domestic dog reproduction, the most preferred parameters are evaluation of clinical signs, progesterone measurement, vaginal cytology and ultrasonography of the ovaries. Clinical findings such as receptivity towards the male, appearance and oedema of the vulva, amount and quality of vulvar discharge as well as reactivity to vulvar stimulation are parameters that can be evaluated in female domestic dogs. Due to the wild nature of blue fox vixens, similar parameters are not easily evaluated in this species due to handling related stress and aggression of the animals. In blue fox vixens, an accurate evaluation of the stage of the oestrous cycle can, however, be achieved by the measurement of vaginal electrical resistance.

In pro-oestrus of female dogs, elevated oestradiol-17 $\beta$  (E2) concentrations lead to visible external signs, such as vulvar oedema, hyperaemia and sanguineous vaginal discharge (Concannon et al. 1975, Concannon 2011). In female dogs, oestrogen concentrations reach their maximum values in late pro-oestrus, 1-2 days prior to the surge in LH. In vixens, the oestrogen peak almost coincides with the LH surge, with no more than one day in between these two hormonal events (Møller et al. 1984a, L. Jalkanen, unpublished).

After the preovulatory LH surge, E2 decreases, followed by an increase in progesterone (P4) concentrations, leading to onset of behavioural oestrus (Concannon et al. 1975, Kowalewski et al. 2015). This increase in P4 concentrations is caused by preovulatory luteinisation of ovarian follicles, with P4 values reaching concentrations of 5 ng/ml at the time of ovulation (Concannon et al. 1989). The dog is the only domestic animal species not producing placental steroids (Hoffmann et al. 1994, Nishiyama et al. 1999) whereas placental steroid production in vixens has not been studied to our knowledge. The formation of *corpora lutea* (CLs) is mandatory for a successful pregnancy in female dogs, with CLs slowly attaining maximal steroidogenic capacities slowly around days 15 to 30 after ovulation (Kowalewski 2018). P4 concentrations continue to rise after the LH surge, reaching peak values during the first 20 and 35 days of the luteal phase. Peak values in P4 concentrations in the blue fox vixens are found earlier, namely 10-20 days after the LH peak (Møller 1973). After the peak, P4 concentrations in female dogs slowly decline below 1 ng/ml by day 55-90 (mean 70) of the oestrous cycle (Concannon 2011). Interestingly, the luteal life span in pregnant and non-pregnant female dogs differs, with it lasting even longer than 80 days in non-pregnant individuals (Hoffmann et al. 2004). This phenomenon of a prolonged luteal phase in non-pregnant animals has also been described in blue fox vixens by Møller in 1973. In clinical practice, breed and individual variation in P4 values are common, however, P4 concentrations are always increased in both pregnant and non-pregnant female dogs due to a lack of anti-luteolytic agent, resulting in physiological pseudopregnancy (Kowalewski 2018). In pregnant female dogs, P4 values decrease rapidly approximately 60 days after ovulation enabling parturition to occur

(Kowalewski et al. 2010). The hormone prolactin (PRL) is the principal luteotropic factor in female dogs from days 24-28 of pregnancy, reaching maximum values around 50 ng/ml before parturition (Kowalewski 2017). In blue fox vixens, Møller et al. (1984b) have shown that PRL levels rise gradually from around two weeks after the LH peak until parturition. Values were at their highest from one day prior to parturition until day 10 of lactation (Møller et al. 1984b). During pseudopregnancy in female dogs, PRL increases slightly during dioestrus with maximal values around 9 ng/ml at the end of the luteal phase corresponding with the time of parturition (Kowalewski 2018). A schematic illustration of the hormones mentioned is presented in Figure 1.



**Figure 1** Schematic illustration of concentrations of prolactin (PRL), follicle stimulating hormone (FSH), luteinising hormone (LH), progesterone (P4) and oestradiol (E2) during all stages of the oestrous cycle in the domestic dog. Initiation of LH surge is indicated as day 0. Modified from Kowalewski 2018.

### 1.3.1 Vaginal cytology

In domestic dogs, vaginal cytology is a widely used tool for determination of the stage of the oestrous cycle as described by Arlt (2018) and Reckers et al. (2022). The method is based on assessment of vaginal epithelial cells, more specifically the cornification of the vaginal epithelium in reaction to increased oestrogen levels. Vaginal cytology for determination of the stage of the oestrous cycle is a non-invasive and cheap tool in everyday practice, but it requires a skilled practitioner for reliable analysis. For instance, oestrogen-dependent cornification of the vaginal epithelium and presence of anuclear superficial cells does not always correlate with peripheral oestrogen concentrations. The time point when 80-90% of superficial

keratinised cells are observed can vary between 6 days prior to as late as 4 days post the LH surge (Feldman and Nelson 2004). Besides, the cytology appears similar at both increasing and decreasing E2 levels, why late pro-oestrus/oestrus and late dioestrus/anoestrus cannot be distinguished from one another by vaginal cytology (Kowalewski 2018).

### **1.3.2 Vaginal electrical resistance (VER)**

In blue fox vixens, the method of choice for determination of the stage of the oestrous cycle is the measurement of vaginal electrical resistance (VER). This diagnostic tool is based on the electrical conductivity of vaginal mucus. Oestrogen is responsible for histological changes in the vaginal epithelium of red fox vixens (Mondain-Monval et al. 1977), similarly to what can be seen in female dogs. Cyclic changes of E2 are reflected in the electrical properties of the vaginal tract, with a delay of 1-2 days to plasma E2 concentrations (Møller et al. 1984a). Peak values of vaginal electrical resistance can be found during the initiation of oestrus and ovulation in the blue fox (Møller et al. 1984a, Farstad et al. 1989). The declining phase in electrical resistance is considered the optimum time for insemination in the blue fox and serial measurements are required for a reliable evaluation. Interestingly, VER values in the canine species differ from those seen in cows, sheep and pigs (Leidl and Stolla 1976). In sheep, VER values are at their lowest during oestrus, whereas in the female dog (Klötzer 1974) and in the blue and silver fox vixens, values are at their highest during oestrus (Møller et al. 1980). The conductivity of the vaginal tract is not related to sanguineous discharge as assumed earlier by researchers Leidl and Stolla (1976), since no vaginal bleeding is present in the vixen during oestrus (Møller et al. 1984a).

## **1.4 Morphological changes in uterine and ovarian tissue during the oestrous cycle in female dogs**

As pro-oestrus begins, walls of enlarged follicles in ovarian tissue become folded consisting of a vascular theca interna, basement membrane and an avascular mural granulosa (Jöchle and Andersen 1977, Kowalewski et al. 2015). Preovulatory luteinisation is characterised by theca interna cells changing their shape from elongated to rounded (Kowalewski et al. 2015).

At ovulation, ovarian tissue consists of strongly luteinised preovulatory follicles and recently ovulated follicles. After ovulation, ingrowth of luteinising theca interna cells and formation of blood vessels continue (Kowalewski et al. 2015). Different from livestock, no distinction between small and large lutein cells can be made in

the domestic dog, and the CLs are fully formed 20-25 days after ovulation (Kowalewski 2014).

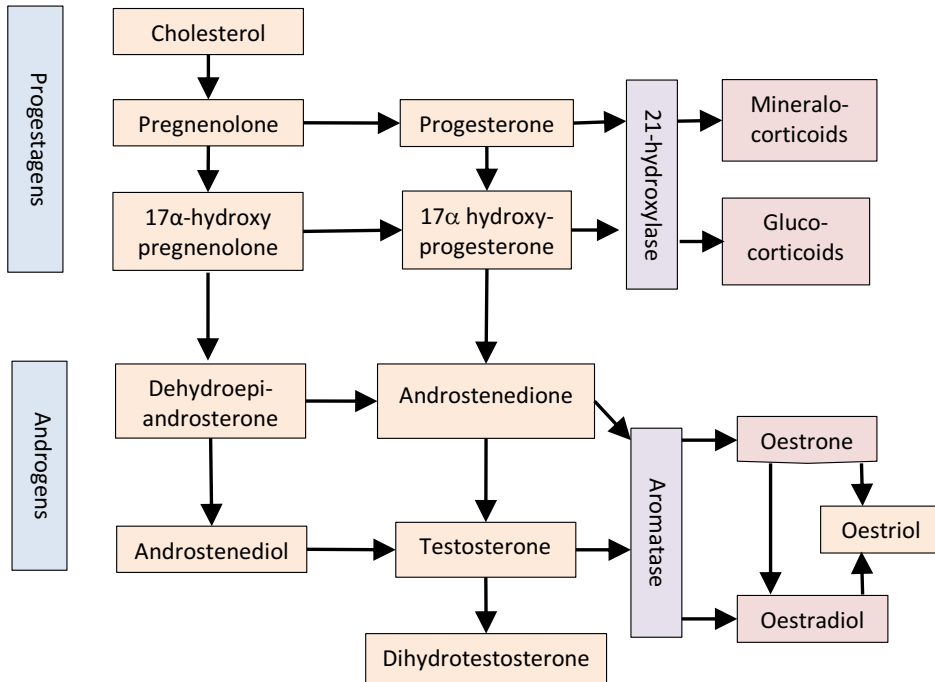
In oestrus, maximal endometrial stromal oedema is present in the uterus with widening of the uterine lumen (Rehm et al. 2007). After ovulation, when developing CLs are present in ovarian tissue, the uterus contains numerous basal glands and luminal crypts with abundant cytoplasm (Rehm et al. 2007). When the luteal phase comes to an end and circulating concentrations of P4 decrease, endometrial epithelial cells often accumulate lipid droplets leading to a foamy morphological appearance that appears to be physiological in female dogs, despite being indicative of pathology in other species (Bartel et al. 2014). After luteal regression, the surface of the endometrium has crypt-like formations, formed by the epithelial lining of foamy cells attached to their basement membrane (Galabova et al. 2003). Afterwards, the endometrial stromal compartment undergoes involution with massive fatty degeneration in the epithelium, resulting in epithelial desquamation and renewal (Galabova et al. 2003).

## 1.5 Aromatase

Aromatase (CYP19) is a cytochrome P450 enzyme which catalyses the conversion of androgens to oestrogens (Ahokoski et al. 2001) as shown in Figure 2. Since aromatase plays a key role in oestrogen synthesis, aromatase inhibitors have been used to treat oestrogen dependent medical conditions such as endometriosis (Pavone and Bulun 2012) and breast cancer in humans (Simpson and Davis 2001) but also benign prostatic hyperplasia (BPH) in male dogs (Gonzalez et al. 2009). In humans, the highest aromatase concentrations are found in granulosa cells of premenopausal women, whereas adipose tissue is the major site for aromatase in women after menopause (Grodin et al. 1973, Bulun et al. 1994). Inhibition of aromatase decreases oestrogen production in the ovaries as well as the peripheral conversion of androgens and local oestrogen production in the brain. This results in induction of ovulation and superovulation when an aromatase inhibitor is administered early in the menstrual cycle of women (Pavone and Bulun 2013). During treatment with aromatase inhibitors in humans, serum levels of androstenedione, 17-hydroxyprogesterone and testosterone will increase (Goedegebuure and Hokken-Koelega 2020). Aakvaag and Eik-Nes concluded in 1965 aromatisation of androstenedione to be the main pathway for the synthesis of oestrogen in the domestic dog ovary. In the red fox (*Vulpes vulpes*), androstenedione is suggested to be related with ovulation or initiation of luteinisation (Mondain-Monval et al. 1979).

Biologically active E2 is produced by human ovarian granulosa cells during the follicular phase.

- Cholesterol is converted to androstenedione in theca cells
- Androstenedione is converted to oestrone in granulosa cells by aromatase (Pavone and Bulun 2013)



**Figure 2** Pathways of human steroid synthesis (Modified from Häggström and Richfield 2014).

Activity of aromatase has been described in many species, such as horses (Mlodawska and Slomczynska 2010), rats (Stocco 2008), bats (Abhilasha and Krishna 1998), squirrels (Li et al. 2012), bears (Araki et al. 1996), cows (Laurincik et al. 1996), cattle, pigs and sheep (Lautincik et al. 1994), pigs (Slomczynska and Tabarowski 2001), goats (Kandiel et al. 2010) and humans (Sasano et al. 1989, Takayama et al. 1996). In domestic dogs, Papa and Hoffmann (2011) described the expression of aromatase in the CLs. Aromatase expression in ovarian tissue seems to be dependent on the stage of follicular development and the species: aromatase immunopositive staining was reported in granulosa cells during breeding season in wild ground squirrels (*Citellus dauricus* Brandt) (Li et al. 2012), in granulosa cells of preovulatory follicles of superovulated heifers, gilts and ewes (Lautincik et al. 1994), whereas theca interna cells stained only in porcine preovulatory ovaries. In rats, no immunostaining was detected in pre-antral follicles, however, theca and granulosa cells in early antral and preovulatory follicles stained immunopositively (El-Maasarany et al. 1991).

## 1.6 Contraception in female dogs

Traditionally, in female domestic dogs, contraception has been achieved by surgical gonadectomy. However, the surgical approach may be complicated by several issues. Firstly, surgical gonadectomy in female dogs can also lead to long-term adverse effects. These side-effects include urinary incontinence (Reichler et al. 2005), vaginitis (Reichler 2009), transitional cell carcinoma (Knapp et al. 2000), cranial cruciate ligament rupture (Slauterbeck et al. 2004), joint disorders (Hart et al. 2014, 2016), osteosarcoma (Ru et al. 1998) and behavioural changes as shown by Kim et al. (2006), underlining the need for new contraceptive methods in female dogs.

New animal welfare legislation encourages dog owners to choose less invasive contraceptive methods and to avoid surgical neutering unless a medical indication is present. Also, reversibility of contraception is of outmost importance, especially for dogs intended for breeding purposes. For instance, in male dogs, chemical castration can be achieved by a GnRH-agonist implant, causing temporary suppression of fertility (Palm and Reichler 2012).

In female dogs, progestins have been used for decades for medical suppression of oestrus, despite the risk of side-effects such as development of cystic endometrial hyperplasia, mucometra, pyometra, mammary gland tumours and diabetes mellitus (Berky and Townsend 1993, Kutzler and Wood 2006), especially after long-term administration. GnRH agonist implants have been tried in prepubertal and adult female dogs for postponement of puberty and oestrus, with satisfying results seen only in prepubertal individuals (Schäfer-Somi et al. 2022). For short-term suppression of oestrus, the GnRH antagonist acyline can be used when administered at the beginning of pro-oestrus (Root Kustritz 2012).

Additionally, androgens have been used for oestrus suppression, causing side-effects such as clitoral hyperplasia, virilisation, heightened aggression and potential liver complications (Maenhoudt et al. 2018) as well as mucoid vaginal discharge and epiphora (Root Kustritz 2012). The lack of safe options for reversible contraception in female dogs underlines the need for new medical alternatives. Inhibition of aromatase could be a potential method for alteration of the oestrous cycle in dogs.

## 2 Aims and hypotheses of the study

This thesis includes research on the oestrous cycle in canids, aiming for a better understanding of the hormonal and physiological changes occurring during oestrus, possibly leading to new ways of contraception and prevention of oestrus.

Our specific aims are listed below:

- I. Inhibition of aromatase and alteration of oestrogen synthesis could be an option for contraception in canids. We aimed to evaluate the contraceptive effect of an orally administered aromatase inhibitor in blue fox vixens (*Vulpes lagopus*), a species that has a very similar oestrous cycle to the one seen in female dogs. We hypothesised that inhibition of oestrogen synthesis by aromatase inhibition at the beginning of oestrus results in contraception.
- II. The research in blue fox vixens underlines the importance of accurate determination of the stage of the oestrous cycle to achieve contraception. Therefore, we wanted to evaluate clinical, endocrinological and vagino-cytological parameters in addition to histomorphology of ovarian and uterine tissue samples from female dogs. Our hypothesis was that clinical, endocrinological and vagino-cytological parameters correspond with histomorphological evaluation of tissue samples.
- III. We aimed to gain further knowledge on oestrogen synthesis in female dogs, by immunohistochemical evaluation of aromatase expression in the domestic dog ovary during all stages of the oestrous cycle. We hypothesised that aromatase is expressed during stages of high oestrogen production mainly in granulosa cells.

## 3 Materials and methods

### 3.1 Experimental design

The first study (I) in blue fox vixens was a placebo controlled open study performed at MTT Agrifood Research Finland facility in Kannus, Finland. All procedures were conducted under approval from the Animal Experimental Board in Finland (ELLA, decision STH904A, license 8.12.2008ESLH-2008-09367/Ym-23). Blue fox vixens were investigated for progression of oestrus to determine the accurate timing for artificial insemination (AI) with fresh semen. To determine the contraceptive effect of aromatase inhibition, all vixens received oral administration of either placebo or the aromatase inhibitor Finrozole. We evaluated pregnancy rates and number of live born pups, possible malformations in pups and the contraceptive effect of aromatase inhibition.

For the second (II) and third (III) study, tissue samples were collected from privately owned female dogs, with consent from dog owners to become research participants. All procedures were conducted under approval from Animal Experiment Board (ELLA; ESAVI/2325/04.10.07/2017/decision 42978) in Finland, and the board considered the protocol acceptable without a standard handling for extensive animal experiments.

In the second study (II), we aimed to gather insight into the oestrous cycle of the female dog, by evaluating how clinical, endocrinological and vagino-cytological parameters correlate with the histomorphological picture in ovarian and uterine tissues at each stage of the oestrous cycle. For endocrinology, peripheral P4 and E2 concentrations were measured. P4 was analysed by two different assays: chemiluminescent immunoassay (CLIA) and radioimmunoassay (RIA). By using two different assays, we were able to compare these assays with each other, giving insight into possible deviations in numeric values of different assessment methods.

In the third study (III), we aimed to study the distribution of aromatase in ovarian tissue of the female dog during all stages of the oestrous cycle. Ovarian tissue samples were analysed for aromatase expression by immunohistochemistry, indicating which cells participate in oestrogen synthesis and at what stage of the oestrous cycle, serving as a basis for targeted inhibition of oestrogen synthesis.

## 3.2 Animals and treatment groups of each study

The number of animals, treatment groups, ages and species are presented in Table 2.

**Table 2** Number of animals, age and species in each study.

Study No.	No. of animals and treatment groups	Age, years	Species				
I	n=80, n=20 per group <table border="1" style="margin-left: auto; margin-right: auto;"> <tr> <td>A - Placebo</td> </tr> <tr> <td>B - 0.5 mg/kg</td> </tr> <tr> <td>C - 3.5 mg/kg</td> </tr> <tr> <td>D - 24.5 mg/kg</td> </tr> </table>	A - Placebo	B - 0.5 mg/kg	C - 3.5 mg/kg	D - 24.5 mg/kg	1.5 - 4.5	Farmed blue fox vixens
A - Placebo							
B - 0.5 mg/kg							
C - 3.5 mg/kg							
D - 24.5 mg/kg							
II	n=39	2 - 9	Female dogs				
III	n=39	2 - 9	Female dogs				

In the first study (I) done in vixens, 80 animals were selected out of 190. All vixens were housed individually at MTT Agrifood Research Finland facility in Kannus, Finland, in cages (length 110 cm x width 105 cm x height 70 cm). Each cage was provided with a whelping nest box and activity objects. Selection criteria included 1.5-4.5 years of age and one to four previous whelpings. Body weight varied between 6.7-10.9 kg in selected animals. All vixens were examined for pathology before inclusion into the study. The total of 80 animals was divided into four groups (n=20 vixens per group) according to the dose of treatment or placebo: group A (placebo), group B (0.5 mg/kg), group C (3.5 mg/kg) and group D (24.5 mg/kg). The aromatase inhibitor was administered orally once a day for 21 consecutive days.

For the second (II) and third (III) study, healthy, sexually mature and privately owned female dogs, aged 2-9 years with no history of reproductive disorders like alterations in the duration and interval of oestrus and abnormal vaginal discharge pointing to uterine disease and without any previous use of contraceptive treatment were included. A total of 39 female dogs underwent ovariectomy and partial hysterectomy at different stages of the oestrous cycle.

In all female dogs, both ovaries and the cranial part of both uterine horns were collected during open abdomen surgery. A conventional protocol for anesthesia was used; sedation by intramuscular (i.m.) injection of levomethadone 0.2 mg/kg (L-Polamivet, MSD Animal Health GmbH, Switzerland) and medetomidine 0.002 mg/kg (Dexdomitor, Orion Pharma, Finland), induction by propofol intravenously 1-3 mg/kg (PropoVet Multidose, Zoetis, Finland) and maintenance by inhalation of

isoflurane (Attane Vet, Piramal Critical Care B.V., the Netherlands). For post-operative analgesia, meloxicam 0.2 mg/kg (Meloxoral, Le Vet B.V., the Netherlands) and buprenorphine 0.02 mg/kg (Bupaq Multidose vet, Richter Pharma AG, Austria) was given i.m. according to body weight. To promote patient safety, ovariectomy and partial hysterectomy were done in all female dogs using a surgical vessel sealing device (LigaSure, Covidien, Medtronic, Minneapolis, MN, USA), which provides a combination of pressure and energy to create vessel fusion. Samples from 39 female dogs were collected at various stages of the oestrous cycle: pro-oestrus (n = 8), oestrus (n = 12), dioestrus (n = 9) (luteal phase) and anoestrus (n = 10). Samples of 27 females (pro-oestrus, luteal phase and anoestrus) were collected during routine neutering without specific timing of surgery, besides the owner's knowledge of the reproductive history. To obtain tissue samples close to ovulation, the remaining 12 female dogs were scheduled for surgery as described in detail below.

### **3.3 Monitoring of oestrus in blue fox vixens**

The onset of oestrus was determined by external clinical signs, in vixens mainly as monitoring of swelling of external genitalia. At the beginning, vulval swelling was monitored twice weekly. When the first signs of swelling were detected, changes of the external genitalia were evaluated daily and rated on a scale from 0 to 3, with 3 indicating maximum swelling. When maximum vulval swelling was detected, measurement of VER was begun using a modified Ohm-meter (SiLi Heat-detector, Lima A/S, Sandnes, Norway).

#### **3.3.1 Blood sampling and hormone assays in blue fox vixens**

Blood samples were collected twice weekly during the first three weeks of aromatase inhibitor administration, thereafter one sample was collected per week resulting in 11 blood samples per animal. All samples were collected during sedation, with anesthesia lasting for 30 minutes by an intramuscular injection of 0.2 mg of medetomidine (Dorbene 1 mg/ml, Laboratorios SYVA S.A., Spain) and 20 mg of tiletamine-zolazepam (Zoletil 100, 250 mg/ml + 250 mg/ml, Virbac, France). Blood was collected from vena saphena after clipping a small area of fur and wiping the skin with 70% EtOH.

Four ml of blood was collected into two lithium heparin tubes (Terumo Europe N.V., Leuven, Belgium) which were centrifuged 3000 rpm for 10 minutes and plasma transferred into two separate tubes, each containing at least 0.5 ml of plasma. Samples were frozen at -20°C before being placed on dry ice for shipment

to the diagnostic laboratory at the National Veterinary School in Lyon, France for hormone analyses.

### **Oestradiol-17 $\beta$ (E2)**

Samples were assayed by using a Packard RIA STAR solid scintillation counter. Oestradiol assays were performed using the kit ESTRADIOL-2 (Clinical Assays) supplied by Diasorin, 92160 - Antony (France), validated for dogs, without any changes to the usual kit protocol. The analytical sensitivity, calculated as the apparent concentration of analyte which was distinguishable from the zero calibrator (two standard deviations below zero) was 5.0 pmol/l, the intra-assay coefficient of variation ranged between 2.6% and 6.1%, the inter-assay coefficient of variation ranged between 4.6% and 6.1%. Samples for which the apparent concentration was below the analytical sensitivity of the assay were assigned to a concentration equal to zero. All the analyses were performed with the same batch of reagents (ref 122 876) and controlled with the same control serum (CE, target values: from 336 to 504 pmol/l).

### **Progesterone (P4)**

Progesterone was measured by a solid-phase, competitive chemiluminescent enzyme immunoassay, using the reagents Immulite 2000 Cortisol L2KPW2 provided by Siemens Healthcare Diagnostics SAS and validated for dogs. The analytical sensitivity (quantification limit) for the assay was 0.7 nmol/l and the working range is from 0.7 to 127 pg/ml. The within-run and total precisions range between 9.5% (21.3 nmol/l) and 21.7% (0.46 nmol/l). Samples for which the apparent concentration was below the quantification limit of the assay were assigned a concentration equal to zero. All the analyses were performed with the same batch of reagents (ref 304) and controlled with the specific steroid controls of Siemens (CON 6, batch 022, three levels).

## **3.4 Monitoring of oestrus in female dogs**

The initial evaluation of the stage of the oestrous cycle was done based on the interoestrous interval and data regarding the previous or ongoing oestrus provided by the owner of the dog. Thereafter, a clinical gynecological evaluation was performed with assessment of the external genitalia (presence of oedema, softness of the vulva, quantity, consistency and colour of vaginal discharge). By applying stimuli to the external genitalia of the female dogs, reactivity to stimulation was

assessed, i.e. did the female dog turn the tail to the side, did the dog raise the vulvar area or gain standing position indicating acceptance for mating. The appearance of the caudal vaginal mucosa was evaluated by vaginoscopy, using an otoscopic speculum (Heine Beta 400 F.O., Heine Optotechnik GmbH & Co. KG, Germany) to assess crenulation, oedema, colour, hyperemia and mucus on the vaginal lining. A sample was collected from each female dog for assessment of vaginal cytology to evaluate the quality and quantity of vaginal epithelial cells. For determination of the stage of the oestrous cycle, reproductive history, gynecological examination, vaginal cytology and measurement of peripheral P4 were assessed.

### **3.4.1 Blood sampling and hormone assays in female dogs**

In all 39 female dogs, blood serum samples were collected at the time of surgery. In the dogs assigned to the group of oestrous females (n=12), 1-3 additional blood samples were collected prior to surgery to determine the stage of oestrus and to adjust the timing of surgery. Peripheral E2 and P4 were measured, P4 by both CLIA and RIA. Measurement of E2 by RIA has been described in domestic dogs by Hoffmann et al. (1992, 1994), Klein et al. (2003) and Jurczak et al. (2020). Determination of P4 by CLIA was performed as validated by Kutzler et al. (2003). Blood samples were collected into two BD Vacutainer SST 3.5 ml tubes containing spray-coated silica and a polymer gel for serum separation. Samples were centrifuged (Eickemeyer Laboratory centrifuge, Tuttlingen, Germany) at 7000 rpm (11 559 g) for 10 min. Then, serum was aliquoted and transferred into cryovials for longer storage at -20°C prior to sending in dry ice for analysis by RIA. One part of serum was used for immediate in-house measurement of P4 by CLIA (Immulite 1000 LKPW1, Siemens Healthcare GmbH, Erlangen, Germany). P4 measurement by RIA and measurement of E2 was performed at the Veterinary Clinic for Obstetrics, Gynecology and Andrology, Justus Liebig University, Giessen, Germany. P4 measured by CLIA was done at Veterinary Clinic Reprovet, Espoo, Finland.

#### **Oestradiol-17 $\beta$ (E2)**

The RIA applied for the measurement of E2 concentrations is well established in domestic dogs (Hoffmann et al. 1992, 1994, Klein et al. 2003, Jurczak et al. 2020). To overcome matrix effects serum samples were extracted twice with toluene prior to radioimmunological determination. The pooled extracts were dried down and redissolved in assay buffer. All measurements were performed in duplicate. The measuring range was between 2 and 128 pg/ml. Intra- and inter-assay coefficients of variation (CV) were 7.1% and 17.1%, respectively.

## **Progesterone (P4)**

P4 concentrations were measured with CLIA and RIA for endocrinological characterisation of the stage of the oestrous cycle. CLIA was performed by using commercially available reagents for the P4 assay (Siemens Healthcare Diagnostics), as validated in the female dog by Kutzler et al. (2003). The measuring range was between 0.2 and 29.9 ng/ml. Intra- and inter-assay CV were 3.2% and 13.2%, respectively.

The measurement of P4 by RIA was basically performed according to the method of Hoffmann et al. (1973), whose application in domestic dogs has been described repeatedly (Hoffmann et al. 1992, 1994, Klein et al. 2003, Jurczak et al. 2020). Hexane was used as the extractant during sample processing. The routinely used procedure covers a concentration range between 0.1 and 8.0 ng/ml. All measurements were performed in duplicate. In case of concentrations above the measuring range, the analysis was repeated applying a lower sample volume. Intra- and inter-assay CV were 8.8% and 8.9%, respectively.

## **3.5 Artificial insemination and pregnancy diagnosis in blue fox vixens**

Semen was collected from fertile males every other day throughout the breeding season. The males and the vixens were transported from their cages to the semen collection and insemination facility using individual cages for each animal. Maximum of three males were collected at a time and the freshly collected non pooled semen was used to inseminate the vixens. If more semen was required for the day's inseminations, then three new males were picked up for semen collection. An unsedated male at a time was placed in a semen collection stand and the second fraction of the ejaculate (0.2 to 1 ml of undiluted semen) was collected by digital manipulation into a glass vial which was stored in a + 30°C incubator prior to semen collection. According to the colour and consistency of the collected semen, the fraction was diluted with EDTA (stored at room temperature in + 20°C) up to a total volume of 2 to 3 ml. Watery fractions were discarded, thick milky fractions were diluted with up to 3 ml of EDTA and less milky fractions with up to 2 ml of EDTA. A small sample of diluted semen was analysed under a light microscope (x 400) to check the motility of the spermatozoa. Sperm concentration was not counted. For AI, diluted semen with at least 70% progressively motile sperm was required. Prior to AI, the diluted semen was stored at + 20°C from 30 min to 3 h between inseminations.

Artificial inseminations were done within eight hours after a 50 Ω decline in VER for 1 to 4 times depending on the progression of the oestrus. A prolonged increase

in VER values resulted in multiple inseminations, with inseminations done every other day until a drop in VER values was confirmed. Ten vixens were not inseminated due to either trauma, technical or human error. Inseminations (n=70) were done using a Norwegian catheter. Sterile catheters and sheaths covering the metal catheters during vaginal introduction were stored at +30°C prior to AI. An unsedated vixen was fixed by the neck and tail in an insemination rack. The vulva was disinfected with 70% EtOH before inserting a sheath into the vagina, through which the Norwegian catheter was guided to the opening of the cervix. After passing the cervix, 1 ml of fresh diluted semen was deposited into the uterus with a syringe.

Pregnancy diagnosis by ultrasonography was done at the time of blood sampling in anaesthetised vixens (n=54) during days 23-46 of pregnancy. Each vixen was placed on an examination table in dorsal recumbency, and the abdominal fur was sprayed with alcohol. Then a 7.5 MHz linear probe of an Aloka SSD-DX Scanner (Tokyo, Japan) was inserted on the abdominal skin and the uterus was scanned for detection of foetuses. In sixteen (n=16) vixens, pregnancy was confirmed at the end of gestation by whelping. Pregnancy rates were calculated based on both ultrasonography confirming pregnancy and pups born.

## **3.6 Statistical analysis**

### **3.6.1 Blue fox vixens**

All data were first subjected to testing for normal distribution. Transformation (log 10) of data was used where appropriate. E2 and P4 profiles and VER were analysed using analysis of variance with repeated measurements (split-plot ANOVA (Gill and Hafs 1971); software: IBM SPSS Statistics version 24, release 24.0.0.0). In the General Linear Model for repeated measures, treatments (n=4) were considered as between subject factors (fixed effect) and periods (n=5) as within subject, repeated factors (days, every two days together forming a period). As a post testing procedure for E2 and P4 concentrations at given time points, the Tukey procedure was used for pairwise comparisons and the Dunnett procedure for timewise comparisons. Statistical testing for pup losses and gender distribution between placebo and treatment groups was done using ChiSquare.

The effect of treatments (n=4) on pregnancy outcome was analysed through ChiSquare testing. The effect of the treatments on litter size was tested through analysis of variance where treatments were used as independent factor and number of pups as outcome variable. Thereafter, multiple comparisons were carried out by Student's t-test where appropriate.

### **3.6.2 Female dogs**

All data were first subjected to testing for normal distribution. E2 and P4 profiles were analysed using analysis of variance with stage of the oestrous cycle as the between group factor and LSD post hoc testing was used for multiple comparisons between stages of the oestrous cycle (ANOVA; software: IBM SPSS Statistics version 24, release 24.0.0.0) (Gill and Hafs 1971). Clinical, vagino-cytological and endocrinological findings were used as test values to calculate positive predictive values that would describe the accuracy of those in terms of the stages of the oestrous cycle.

Histomorphological finding was considered as the true state of the oestrous cycle, against which the test value was calculated. The positive predictive value was calculated as the proportion histomorphologically positive among those that test positive according to clinical / endocrinological / vagino-cytological evaluation. All results are given as mean values  $\pm$  S.D. Significance level was set at  $P < 0.05$ .

## 4 Results

### 4.1 Effect of aromatase inhibition on hormonal profiles and pregnancy rates in blue fox vixens

The administration of aromatase inhibitor Finrozole did not affect the typical developmental pattern of VER nor levels of plasma E2 concentrations. In all four groups, measured E2 levels followed a typical pattern for vixens during oestrus. Interestingly, mean P4 values were found to be significantly increased in all treatment groups compared to the placebo group. 28 days after the first AI, a second peak in P4 occurred in both higher treatment groups, differing from the usually seen profile of P4 during luteal phase in vixens.

Contraception in vixens was achieved when administration of the aromatase inhibitor was begun four days or earlier in relation to AI, and the administered dose was 3.5 mg/kg or 24.5 mg/kg (Table 3). Treatment with 0.5 mg/kg begun 4 days prior to AI resulted in a 50% pregnancy rate, whereas the same treatment begun 3 days prior to AI yielded a pregnancy rate of 85.7%. Any dosage of aromatase inhibitor begun 2 days or less before AI, had no effect on conception and pregnancy rates were the same as in the placebo group. Numbers of pups live at birth in different groups were 151 (placebo), 55 (0.5 mg/kg), 52 (3.5 mg/kg) and 23 (24.5 mg/kg). No deleterious effects on pups (for instance malformations) caused by administration of the aromatase inhibitor could be verified.

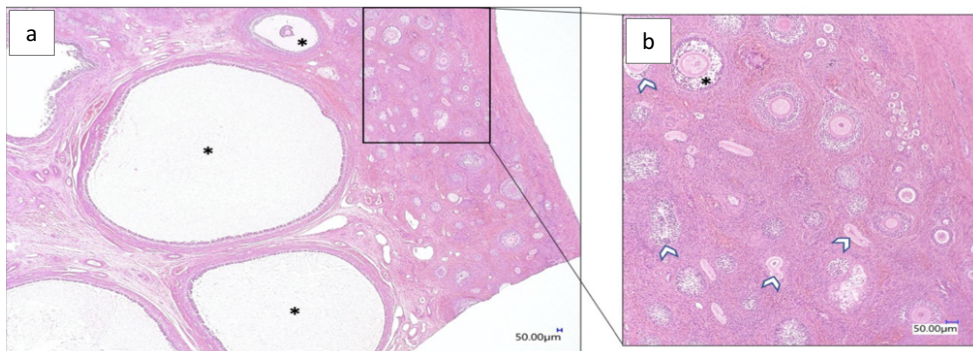
**Table 3** Pregnancy rates (%) for blue fox vixens in the individual treatment groups (A, B, C, D) according to the number of days between initiation of finrozole treatment and first AI.

Days between initiation of treatment and first AI	A n=19	B n=16	C n=18	D n=17
0			100.0	
1		100.0	100.0	100.0
2	100.0	100.0	100.0	100.0
3	100.0	85.7	66.7	50.0
4	100.0	50.0	0.0	0.0
5	100.0		0.0	0.0

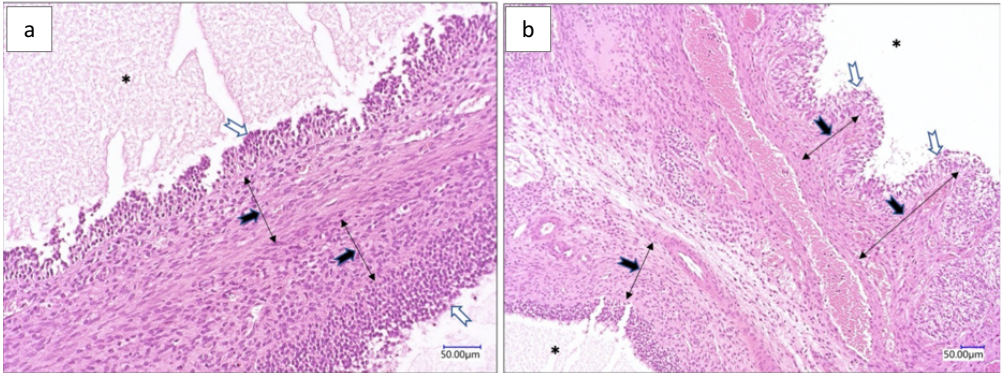
AI = artificial insemination. Group A (placebo), group B (0.5 mg/g), group C (3.5 mg/kg) and group D (24.5 mg/kg).

## 4.2 Morphological changes in ovarian and uterine tissue during the oestrous cycle in female dogs

For determination of the stage of the oestrous cycle in female dogs, clear differences were found between clinical, endocrinological and histomorphological parameters. Females considered to be in oestrus based on clinical and endocrinological evaluation, were histomorphologically determined to be in luteal phase based on developing CLs in ovarian tissue. Morphological changes related to the stage of the oestrous cycle were clearly seen in both ovarian and uterine tissue. At the time of pro-oestrus, increased follicular activity was seen in the ovarian cortex, including growing preantral and antral/tertiary follicles (Fig. 3). Preovulatory luteinisation was visible in larger tertiary follicles as folding of the follicular wall, leading to theca interna cells becoming rounded as a morphological sign of luteinisation (Fig. 4). In the uterus, the increased follicular activity was associated with signs of endometrial glandular proliferation (oestrogenisation) (Fig. 5).



**Figure 3** Pro-oestrus in the domestic dog. Increased follicular activity in ovarian cortex, including growing preantral and antral/tertiary follicles (asterisk). Atretic follicles are observed in earlier stages of follicular phase (white arrowheads in b).

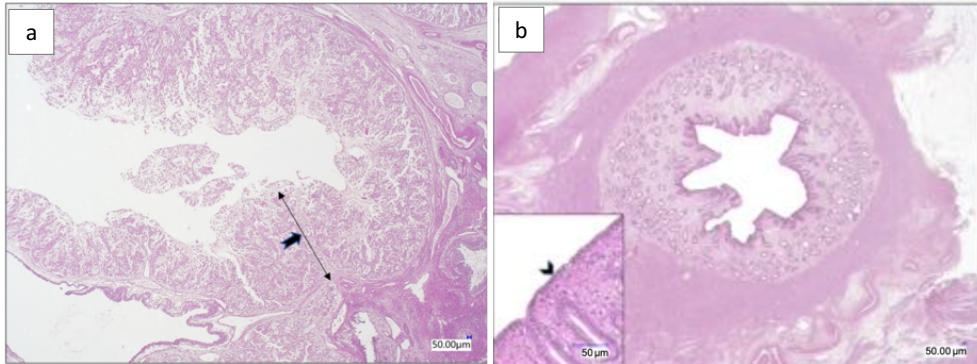


**Figure 4** Preovulatory luteinisation in the domestic dog. Folding of the follicular wall in larger tertiary follicles of the ovary, black arrows indicating theca folliculi layers. Mural granulosa cells are indicated by white arrows in a and b.

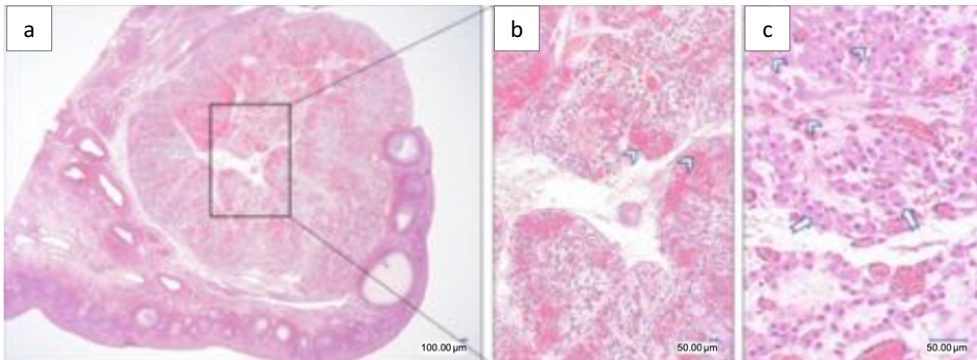


**Figure 5** Pro-oestrus in the domestic dog. Uterine cross sections showed pronounced signs of endometrial proliferation, i.e. oestrogenisation (a and b), with the surface epithelium composed of cuboidal and low columnar surface epithelial cells (black arrowheads in c) and an increasing number of uterine glands.

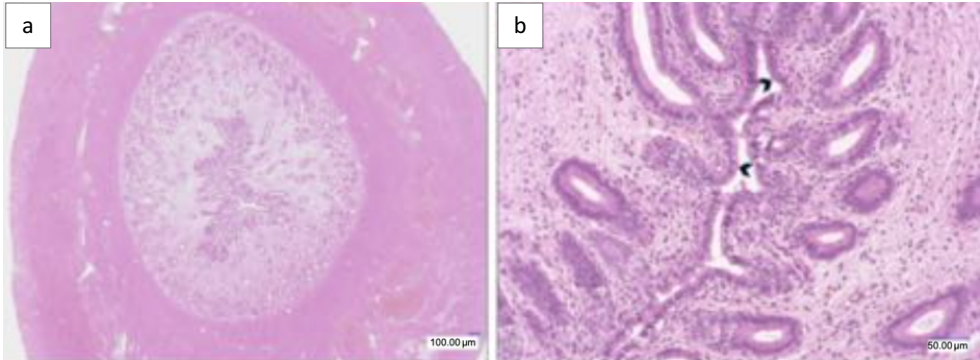
As pro-oestrus progressed into oestrus and ovulation, preovulatory and freshly ovulated follicles showed stronger folding of walls with increased preovulatory luteinisation of thecal compartments (Fig. 6). After ovulation (transition to dioestrus), early dioestrus showed corpora haemorrhagica (Fig. 7) in ovarian tissue and continuing glandular proliferation in uterine tissue (Fig. 8).



**Figure 6** Oestrus and ovulation in the domestic dog. A recently ovulated follicle in the ovary (a), showing strong folding of follicular walls with advanced preovulatory luteinisation of the thecal compartments (black arrow). In the uterus (b), strong glandular proliferation is observed; the insert in b shows the appearance of luminal epithelium (black arrowhead) at higher magnification.

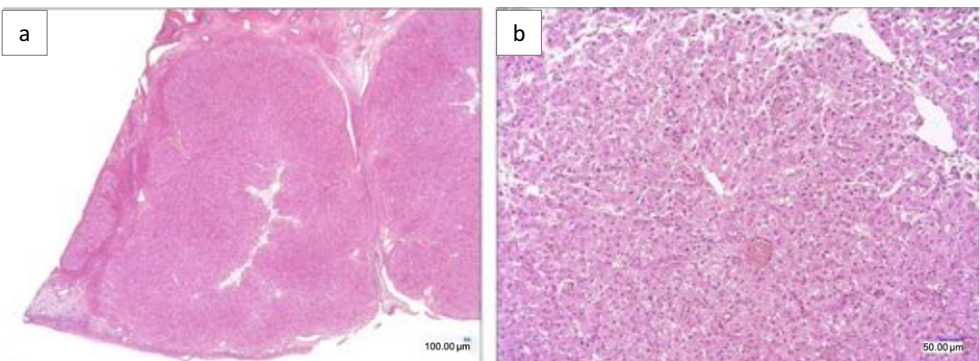


**Figure 7** Early, post ovulatory stage of luteal formation in the domestic dog. Example of a corpus haemorrhagicum is shown in a–c. Corpus luteum is not yet fully formed, with follicular cavity not fully closed and extravasate erythrocytes (open arrowheads in b and c). Lutein cells have homogenous cytoplasm (white arrows in (c)).

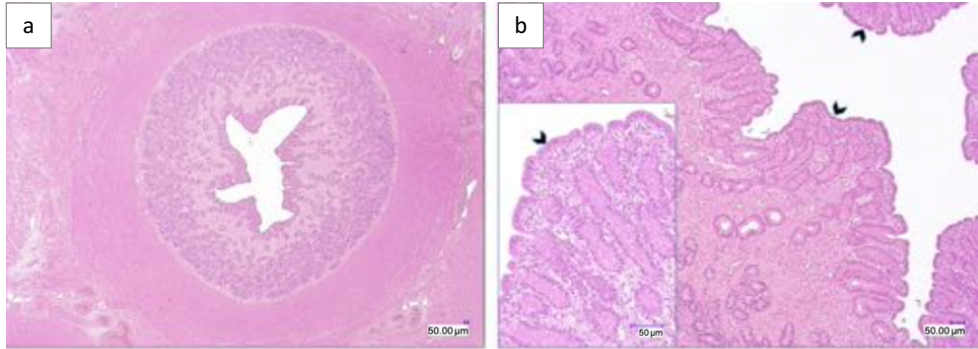


**Figure 8** Early, post ovulatory stage in the domestic dog. In the uterus (a, b) the glandular compartment is strongly proliferated with columnar surface epithelium (black arrowheads in b).

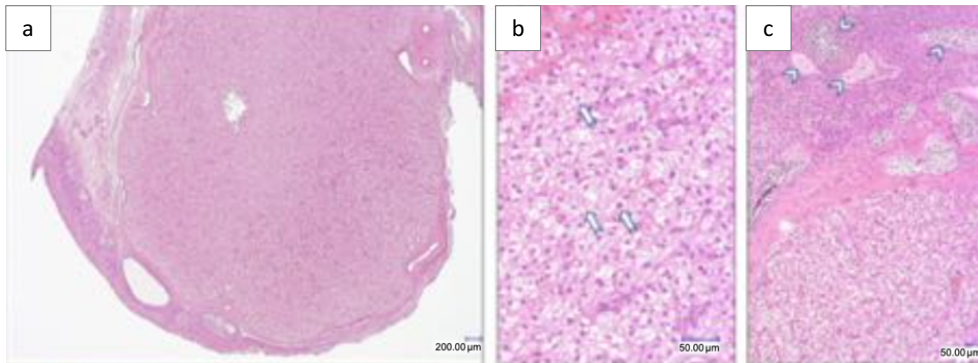
Fully developed CLs (Fig. 9) were confirmed in early/mid dioestrus, with proliferated glands and columnar surface epithelium in uterine tissue (Fig. 10). As dioestrus progressed into late dioestrus, the ovarian tissue showed regressing CLs with luteal cells with signs of cytoplasmic degeneration (Fig. 11). At this stage, the columnar epithelium in the uterus contains large vacuoles indicating cellular degeneration as the vacuolised columnar epithelium is replaced by fresh cuboidal cells (Fig. 12). In anoestrus (Fig. 13), ovarian tissue sections showed corpora albicantia and strongly degenerated vacuolised luteal cells. In the uterus, the surface epithelium is composed of cuboidal cells and at later stages, glandular structures have been replaced by stromal compartments (Fig. 14).



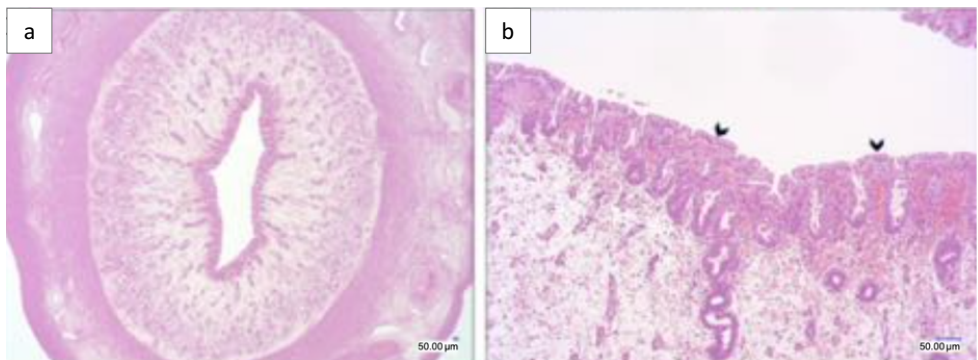
**Figure 9** Early/mid dioestrus in the domestic dog showing a fully developed corpus luteum (a and b).



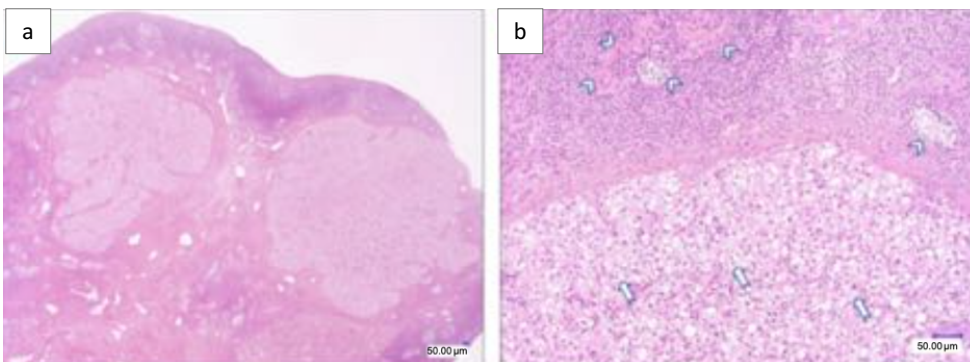
**Figure 10** Early/mid dioestrus in the domestic dog. Uterine tissue (a and b) at its dioestric appearance, with proliferated glands and columnar surface epithelium (black arrowheads in b; the insert in b shows the uterine surface at higher magnification.)



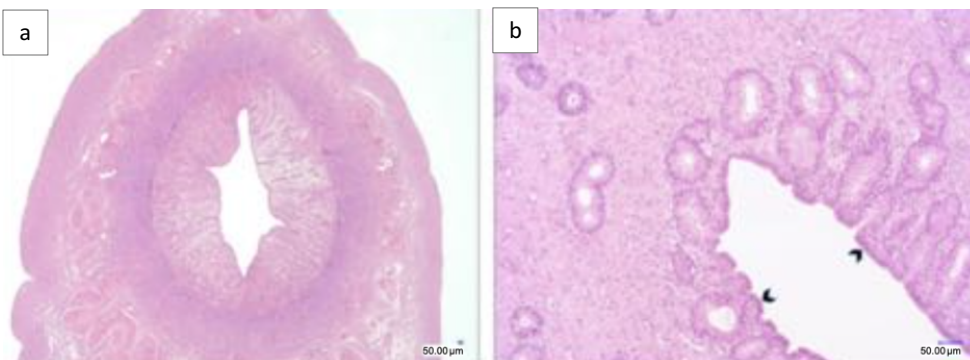
**Figure 11** Late dioestrus in the domestic dog. A regressing corpus luteum is observed (a–c) with luteal cells showing signs of cytoplasmic degeneration (white arrows in b). Atretic follicles are shown (c) by white arrowheads.



**Figure 12** Late dioestrus in the domestic dog. In the uterus (a and b), the surface columnar epithelium shows large vacuoles as signs of cellular degeneration (black arrowheads in b).



**Figure 13** Anoestrus in the domestic dog. Corpora albicantia are visible in ovarian sections (a) with strongly degenerated vacuolised, luteal cells (white arrows in b). In the ovarian cortex, several atretic ovarian follicles, including atretic secondary and small tertiary follicles are visible (white arrowheads in b).



**Figure 14** Anoestrus in the domestic dog. In the uterus (a and b), the surface epithelium is composed of cuboidal cells (black arrowheads in b).

### 4.3 Hormonal parameters during the oestrous cycle in female dogs

For endocrinological evaluation, E2 and P4 values were measured, P4 by both CLIA and RIA as shown in Table 4. The values of these two different assays clearly differed, underlining the need to understand assay related deviations when analysing results. In pro-oestrus, oestrus and dioestrus, RIA values were higher than CLIA values. At lower values in anoestrus, the two assays were more comparable. E2 values were increased during pro-oestrus and oestrus, with decreasing values confirmed in dioestrus and anoestrus.

## 4.4 Vaginal cytology

The results of vaginal cytology are presented in Table 4. Two female dogs in pro-oestrus (n = 8) had vaginal smears with superficial epithelial cells >80%, for six other female dogs the emphasis within early follicular phase was in the occurrence of parabasal and intermediate cells. In oestrus (n = 12), great similarity was found in the evaluation of vaginal smears, with >80% superficial epithelial cells confirmed by all three evaluating clinicians subjectively. During dioestrus (n = 9), three female dogs showed a clear shift from superficial cells towards intermediate cell cytology, with less cornification of cells. In the remaining six female dogs in dioestrus, vaginal cytology still showed >80% superficial epithelial cells. All vaginal smears during anoestrus (n = 10) showed none or very low numbers of superficial epithelial cells, with a clear majority of parabasal cells, indicating low levels of circulating E2.

**Table 4** Results of vaginal cytology, E2, P4 in 39 female dogs.

Stage of the oestrous cycle	Vaginal cytology % (superficial / intermediate / parabasal and basal cells)	E2 pg/ml Min-Max (Mean ± s.d.)	CLIA P4 ng/ml Min-Max (Mean ± s.d.)	RIA P4 ng/ml Min-Max (Mean ± s.d.)
Pro-oestrus	30/40/30	7.0 - 48.5 (20.4 ± 12.5)	0.2 - 1.3 (0.5 ± 0.4)	0.2 - 2.6 (0.8 ± 0.8)
Oestrus	80/20/0	11.7 - 45.3 (20.9 ± 13.9)	1.1 - 7.5 (3.1 ± 2.5)	1.5 - 5.8 (3.6 ± 1.6)
Early dioestrus	50/30/20	8.0 - 23.8 (14.2 ± 5.6)	2.0 - 29.9 (11.3 ± 11.0)	3.7 - 60.1 (19.6 ± 22.3)
Mid dioestrus	10/70/20	7.6 - 20.0 (13.8 ± 8.8)	7.7 - 23.9 (15.9 ± 11.4)	12.2 - 47.2 (29.7 ± 24.7)
Late dioestrus	10/40/60	6.9 - 28.9 (14.0 ± 8.9)	0.2 - 5.3 (1.8 ± 2.0)	0.3 - 9.1 (3.2 ± 3.5)
Anoestrus	0/30/70	3.7 - 13.9 (10.1 ± 3.7)	0.4 - 0.9 (0.5 ± 0.3)	0.1 - 0.9 (0.5 ± 0.2)

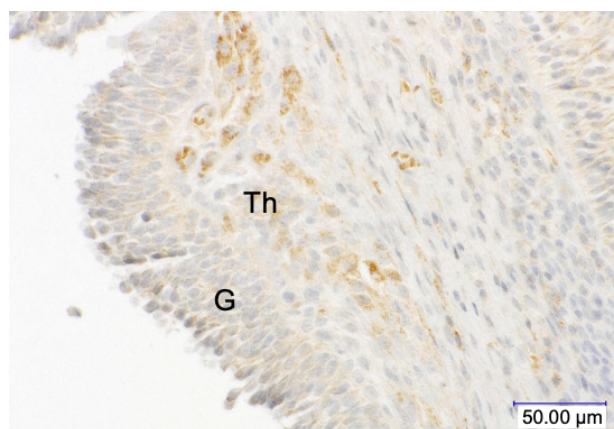
## 4.5 External clinical signs of oestrus in female dogs

The clinical examination and determination of the stage of the oestrous cycle by external clinical signs showed somewhat similar findings for individuals allotted to a specific stage of the oestrous cycle. However, not all female dogs expressed oestrus

in a similar way, some of the dogs having minor/or no vaginal discharge compared to abundant sanguineous discharge during pro-oestrus. The appearance and swelling of the vulva were not prominent in all individuals during oestrus, even though progression of oestrus appeared normal by endocrinological and cytological evaluation. Reaction to stimulation indicated the willingness of the female, with standing position for mounting seen during oestrus. The magnitude of the reaction seemed to be dependent on the breed, with introvert breeds expressing a reaction of smaller magnitude towards the clinician during stimulation. Vaginoscopy, i.e. evaluation of the appearance of the vaginal mucosa, was found to be a reliable parameter for assessment of the stage of the oestrous cycle with only minor variation between individuals.

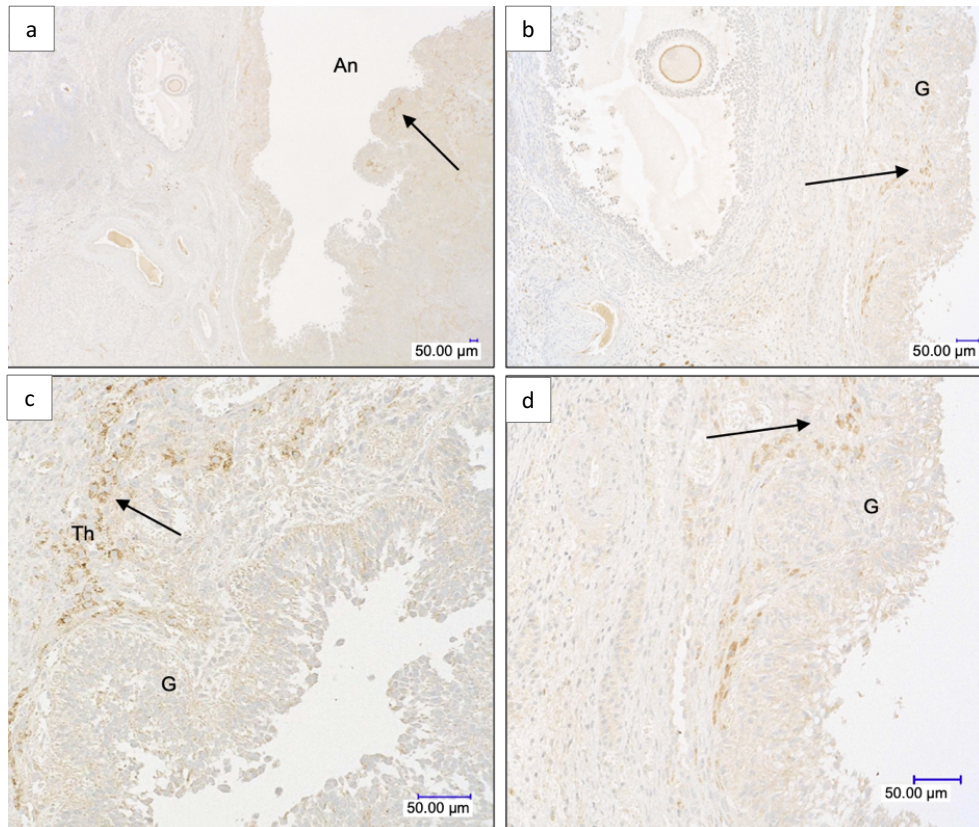
#### **4.6 Activity of aromatase in ovarian tissue during all stages of the oestrous cycle in female dogs**

Staining for aromatase clearly differed during the different stages of the oestrous cycle. During pro-oestrus, aromatase staining was absent in preantral and early antral follicles, with positive staining seen in antral and luteinising follicles. Interestingly, signals appeared stronger in theca interna cells compared to signals in granulosa cells (Fig. 15).



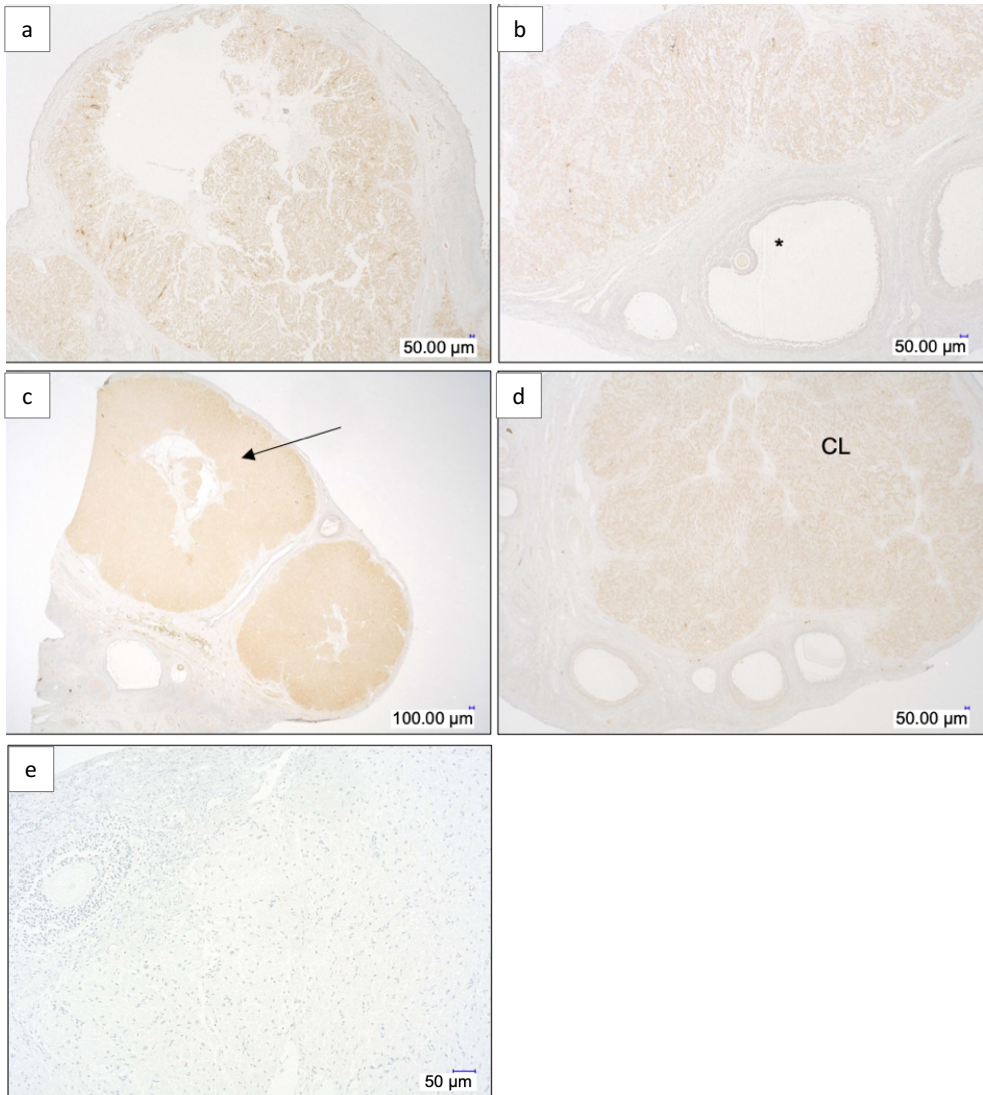
**Figure 15** Aromatase staining of a domestic dog ovary during pro-oestrus. Immunopositive staining was present in mural granulosa cells (G) and luteinising theca interna cells (Th), with theca cells displaying a stronger intensity of signals.

As pro-oestrus turned into oestrus (Fig. 16), immunopositive staining for aromatase decreased with no or weak staining seen in ovaries at the time of ovulation (Fig. 17 a and b).

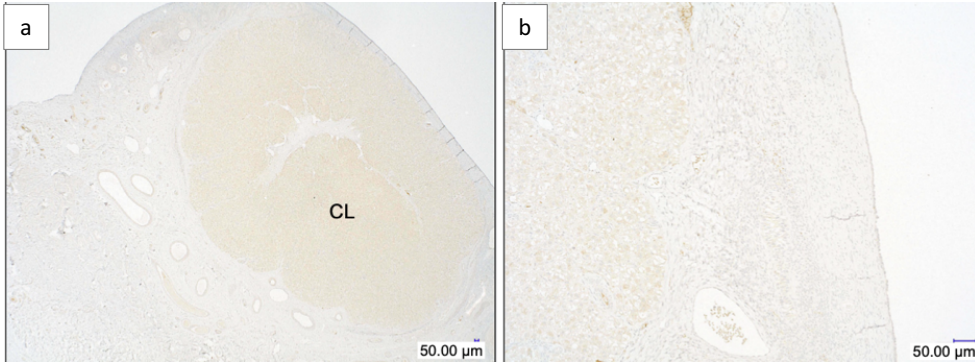


**Figure 16** Aromatase staining of a domestic dog ovary during oestrus and late oestrus. Immunopositive staining for aromatase indicates signs of ongoing luteinisation of antral (An) follicles with staining appearing stronger in theca interna cells (Th) compared to granulosa cells (G), as indicated by the arrow (a–d).

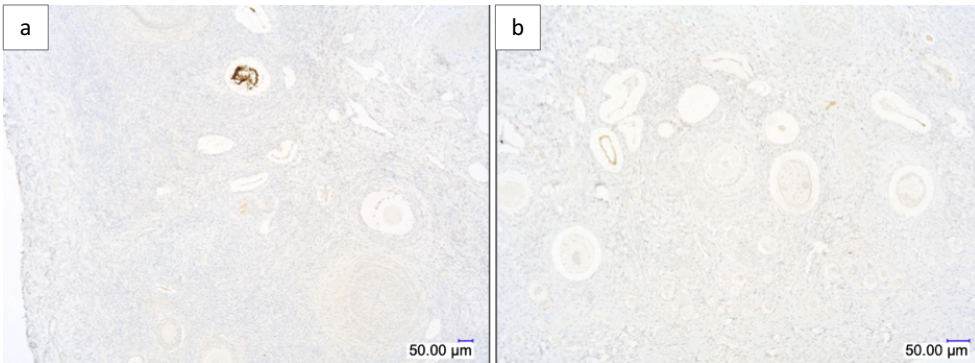
Developing CLs showed clearly detectable staining for aromatase in lutein cells, with strong aromatase expression seen in lutein cells after ovulation (Fig. 17 c and d). Fig. 17 e shows the isotype control. In late luteal phase, immunopositive staining for aromatase weakened (Fig. 18) with negative staining confirmed in anoestrus (Fig. 19).



**Figure 17** Aromatase staining of a domestic dog ovary during early/mid dioestrus. Ovaries at the time of ovulation are presented in a and b. In b, additional small tertiary follicles are visible (\*) displaying no or only weak immunostaining for aromatase. Developing corpora lutea (CLs, one CL in d) at different magnifications are shown in c and d with immunopositive staining for aromatase, indicated by arrow in c. Isotype control is shown in e.



**Figure 18** Aromatase staining of a domestic dog ovary during late dioestrus with representative figures of a regressing corpus luteum (CL) shown at different magnifications (a and b). The CL presents weaker staining for aromatase.



**Figure 19** Aromatase staining of a domestic dog ovary during anoestrus. Ovarian cross-sections remain negative for aromatase staining (a and b).

## 5 Discussion

Contraception is of utmost importance, especially in the globally distributed, domesticated canine species of around 700 million individuals, both free roaming and privately owned pets (Hughes and Macdonald 2013). The management of dog populations is mandatory not only from an animal welfare perspective, but also from a human health standpoint as the World Health Organization indicates feral dogs as the source contributing to 99% of all rabies transmissions to humans (World Health Organization Home Page 2024). This is one of the perspectives why the present issue should be considered as an essential global One Health question. New, efficient ways for contraception are needed to ensure a safe environment and inhibition of dog population overgrowth, by non-invasive and preferably reversible contraception. As new alternative contraceptive methods are investigated, traditional gonadectomy is deemed an invasive procedure, subjecting female dogs to possible long-term side effects. These side effects include joint disorders (Hart et al. 2014, 2016), osteosarcoma (Ru et al. 1998), transitional cell carcinoma (Knapp et al. 2000), cranial cruciate ligament rupture (Slauterbeck et al. 2004), urinary incontinence (Reichler et al. 2005) and vaginitis (Reichler 2009). As shown in our study in vixens, contraception by inhibition of aromatase can be an option for successful contraception, by targeted inhibition of oestrogen synthesis (I). This, however, requires administration of the aromatase inhibitor Finrozole at early pro-oestrus, emphasising the importance of accurate determination of the stage of the oestrous cycle, to avoid pregnancy. Based on our results in vixens, we conclude that contraception in canids can be achieved by inhibition of aromatase, if the treatment is begun during pro-oestrus and the treatment dose is  $\geq 3.5$  mg/kg. Further studies are, however, needed in female dogs, to ensure successful contraception by inhibition of aromatase also in this species.

In the treatment groups of the blue fox vixens, high P4 values were measured in treatment groups 16-17 days after AI with an interesting second peak in P4 occurring approximately 28 days after the first AI. No such peak was observed in the placebo group. The variation in P4 secretion during the luteal phase in vixens has previously been described by Valberg and Farstad in 1992, with P4 values increasing after mating, achieving maximum values on days 8-12 of pregnancy and then declining gradually until delivery around day 52. Yapura et al. (2011) described the potential of non-steroidal aromatase inhibitor letrozole as an alternative for

controlling ovarian function in cattle. Letrozole was found to extend the lifespan of the dominant follicle resulting in a delay of the next follicular wave or in delayed ovulation (Yapura et al. 2013). Letrozole treatment in cattle had a consistent luteotropic effect and treatment was associated with development of a larger CL and/or high circulating concentrations of progesterone (Yapura et al. 2014). We suspect that the second peak in P4 values in blue fox vixens treated with Finrozole, could be explained by the luteotropic effect of aromatase inhibitors that was observed for Letrozole in cattle.

## **5.1 Parameters for accurate determination of oestrus**

In domestic dogs, whether the aim is mating, AI or contraception, a precise determination of the stage of the oestrous cycle is crucial. Especially in many different breeds of domestic dogs, variation can be seen between breeds in sexual behaviour, susceptibility towards the male, length of the stages of the oestrous cycle and clinical signs of oestrus, making exact determination of the stage of the cycle only by clinical observation difficult (own observations). In clinical practice, timing of mating or AI is decided by endocrinological measurements, clinical evaluation of external signs of oestrus, vaginal cytology or a combination of all parameters mentioned.

To further gain knowledge and to assess changes at cell level, histomorphology of the uterus and ovaries was evaluated during all stages of the oestrous cycle (II). From a histomorphological point of view, we considered luteal phase or dioestrus to begin when developing CLs were identified in ovarian tissue. Many female dogs evaluated to be in oestrus by clinical signs, were thereby from a histomorphological point of view already in dioestrus.

As female dogs are known to ovulate immature oocytes (Holst and Plemister 1971), mating or AI is usually done after ovulation. Domestic dog oocytes may have a lifespan of 7 or 8 days after ovulation (England et al. 2006), extending the fertile period in this species from oestrus to luteal phase, when changes at cell level are recognised. Furthermore, longevity of the domestic dog spermatozoa in the female genital tract has been described by Doak et al. (1967), lasting up to 11 days. As the longevity of both the oocytes and spermatozoa are recognised in the domestic dog, conception can be achieved if successful mating occurs during three stages of the oestrous cycle, i.e. pro-oestrus, oestrus and dioestrus.

The findings of our research (II) suggest incomplete agreement between clinical signs of oestrus and the histomorphological status of the reproductive organs. By conclusion, this should be taken into consideration when determination of the exact stage of the oestrous cycle is crucial, for instance when treatment with anti-oestrogenic compounds must be initiated during pro-oestrus.

The monitoring of external signs of oestrus is somewhat similar in foxes and domestic dogs, despite the differences in the nature of the species and especially in their handling. In blue fox vixens, handling has been described as a stressor (Osadchuka et al. 2000), limiting the methods for detection of oestrus into monitoring of the swelling of the vulva and VER measurements. In both species, the appearance and swelling of the external genitalia can be assessed, and in female dogs, also, the sanguinity of the vaginal discharge. In female dogs, assessment of vaginal cytology has been a conventional way for determination of oestrus as previously described by Arlt (2018) and Reckers et al. (2022). The oestrogen-dependent cornification of vaginal epithelial cells seemed to correlate with the histomorphological status of reproductive organs during oestrus in our study (II). However, one need to bear in mind that the day when 80-90% superficial keratinised cells are observed, can occur 6 days prior to the LH surge or as late as 4 days after the LH surge (Feldman and Nelson 2004), excluding vaginal cytology as a marker for ovulation.

In blue fox vixens, vaginal cytology is not used for evaluation of the stage of the oestrous cycle, instead measurement of VER is the golden standard for detection of oestrus and the optimal day of AI in vixens. VER values rise when decreasing oestrogen levels cause changes to the electrical conductivity in the vaginal environment. This coincides with hormonal parameters of female dogs, in which decreasing E2 and increasing P4 levels stimulate the LH surge and trigger ovulation, leading to onset of behavioural oestrus (Kowalewski 2018). In blue fox vixens, Farstad (1992) described that after a rapid decrease in E2 levels, P4 levels increase rapidly simultaneously with the LH peak. This shift from E2 dominance to increasing P4 values, could be used as a tool to confirm transition from pro-oestrus to oestrus in domestic dogs as suggested by our study (II).

Our research (II) also demonstrates variations in endocrinological, vaginocytological and clinical findings, when evaluating the stage of the oestrous cycle in female dogs. For instance, differences in P4 profiles can be seen between different breeds, individuals and even between oestruses of the same individual. It is also important to remember that various methods for P4 measurement exist in clinical practice, and interpretation of measured P4 values requires understanding of the assay used, since values differ according to specific assays (II), as shown also by Gloria et al. (2018).

RIA has been considered the gold standard due to its sensitivity, specificity and reproducibility (Moxon et al. 2010, Tal et al. 2020). However, it is an expensive method linked to a long turn-around time and potential hazards, whereas other methods such as enzyme-linked immunosorbent assays (ELISAs), liquid chromatography-tandem mass spectrometry (LC-MS/MS; Trabert et al. 2015, Patton et al. 2014, Rota et al. 2016), enzyme-linked fluorescence assay (ELFA) and CLIA (Brugger et al. 2011, Chapwanya et al. 2008, Moxon et al. 2010, Nothling and

de Cramer 2018) have been introduced. Two different CLIA systems are available for measurement of canine progesterone, the Immulite (Immulite 2000, Siemens Healthcare, Erlangen, Germany; IS) and the Elecsys (Roche, Mannheim, Germany; ER). Both methods have been compared separately and together to RIA analysis (Chapwanya et al. 2008, Kutzler et al. 2003, Nothling and de Cramer 2018, Schmicke et al. 2016, Volkmann 2006, Tal et al. 2020). P4 values by the Elecsys were described by Tal et al. (2020) as similar to RIA, whereas Immulite values significantly differed to those analysed by RIA. The most used ELFA in veterinary practice is the miniVIDAS® (Biomérieux, Marcy L'Etoile, France), a method showing high agreement to RIA with mean deviations of 15% (Brugger et al. 2011). When comparing ELFA and CLIA, Uhlmann et al. (2016) described good correlation despite ELFA values being higher than CLIA.

We conclude that accurate monitoring of oestrus should be done by assessment of several parameters and an understanding of the used laboratory assays. A combination of P4, vaginal cytology, vaginoscopy, ultrasonography and evaluation of clinical signs, to accurately confirm transition from one stage of the oestrous cycle to another, is recommended.

## **5.2 Spatio-temporal distribution of aromatase in ovarian tissue of the domestic dog**

To further understand the oestrogen synthesis on a cellular level, our study (III) evaluated the spatio-temporal distribution of aromatase in ovarian tissue of the domestic dog. Distribution of immunopositive staining for aromatase was found to correlate with timepoints of high oestrogen secretion in the female dog, with no staining seen at the time of sexual quiescence during anoestrus (Fig. 19). Immunopositive staining for aromatase was confirmed during pro-oestrus (Fig.15), leading to the conclusion that administration of compounds for targeted inhibition of oestrogen synthesis should be aimed at early pro-oestrus, coinciding with results of successful contraception achieved in blue fox vixens by inhibition of aromatase (I).

Our study (III) confirms the oestrogen producing capacity of antral follicles, luteinising follicles and CLs of the domestic dog. In rodents, aromatase is restricted to the gonads and the brain, with aromatase expression limited to the follicle and CLs in ovaries of sexually mature animals (Stocco 2008). Our finding of aromatase activity in antral follicles of the domestic dog coincides with studies done in rodents (Guigon et al. 2003), confirming aromatase expression in large antral follicles and preovulatory follicles.

As described by Kowalewski in 2014, both granulosa cells and theca cells contribute to the formation of the CLs in the domestic dog, however, to which extent

the two cell groups contribute is unknown. Sato et al. (2016) described how luteal formation in female dogs involves folding of the theca and granulosa cell layers before the surge in luteinising hormone. After the LH surge, hypertrophied thecal cells and thecal blood vessels invade into mural folds, leading to complex luteinised folds with vacuolated secretory cells and integration of thecal blood vessels (Concannon and Hansel 1977). Thereafter granulosa and theca cells transform into luteal cells (Concannon and Hansel 1977, Sato et al. 2016), leading to the formation of the CLs. In this study (III), immunopositive staining for aromatase in theca interna cells interestingly prevailed over the immunopositive staining in granulosa cells, leading to the conclusion that the primary cellular source of lutein cells might be of thecal origin.

In rodents, aromatase expression is higher in mural granulosa cells at the outer edge of the follicle compared to granulosa cells closest to the antral cavity, with no expression seen in cumulus granulosa cells (Guigon et al. 2003, Turner et al. 2002). Differences in expression of aromatase in rodent mural and cumulus granulosa cells are not clear from a molecular and physiological basis, however, aromatase in cumulus granulosa cells is suspected to be silenced by oocyte-derived compounds (Stocco 2008). We did not examine the expression of aromatase in the cumulus granulosa cells of domestic dogs and further studies are needed in this area. Understanding of which cells are involved in oestrogen synthesis is of outmost importance, for contraception by targeted inhibition of oestrogen production.

## 6 Conclusions

1. For successful contraception by aromatase inhibitor Finrozole in blue fox vixens, the treatment dose has to be adequately high, and administrations initiated at least 4 days before AI.
2. Accurate determination of the stage of the oestrous cycle in female dogs, requires a combination of several diagnostic tools, such as clinical and vaginocytological evaluation and measurement of progesterone, with mandatory understanding of the assays used for hormonal profiling.
3. Histomorphological evaluation of ovarian and uterine tissue does not always correspond with the clinical, endocrinological and vaginocytological parameters in female dogs.
4. Spatiotemporal distribution of aromatase during the oestrous cycle in female dogs showed immunopositive staining especially in theca interna derived cells, possibly directing oestrogen inhibiting compounds in the future to this specific group of cells.

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