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Histological Changes of the Reproductive Tract of Queens after Treatment with Megestrol Acetate

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SUMMARY

ABSTRACT

Megestrol acetate (MA) is a short-acting progestin marketed as an oral formulation (Estropilll^{TM}, MSD, Italy) for estrus prevention in queens through suppression of the hypothalamic-pituitary-ovarian axis. Historical use of different protocols, including excessive dosages of MA has been associated with endocrine and uterine complications as cystic-endometrial hyperplasia (CEH), uterine enlargement, pyometra, adenomyosis, and mammary signs (hyperplasia, benign and malignant nodules, mammary adenocarcinoma). This study aims to assess the histological features of uteri and ovaries of queens treated with low dosages of MA for a relevant time span to assess/rule out potential utero-ovarian side effects.

Six post-pubertal (9 months-6 years) intact, and healthy female cats (1 Persian, 1 Bengal and 4 European shorthair cats) were presented for reproduction control. Inclusion criteria were absence of past reproductive problems, vaginal cytology indicative of anestrus or postestrus and serum progesterone (P4) concentration below 2.0 ng/ml. Duration of treatment (11.5 µg/kg/day orally, approximately 5 drops/kg/day) ranged from 1 to 6 months (1-month, n=1; 2-months, n=1; 4-months, n=3; 6-months, n=1). Hematology, biochemistry, urinalysis, reproductive ultrasound, vaginal cytology and P4 assay were performed before and after treatment. Ovariohysterectomy was performed on all 6 queens following their first heat post-treatment. Ovaries and uteri were examined histopathologically.

Four/6 queens had P4 <2.0 ng/ml, therefore treatment was immediately initiated. The remaining 2 cats had P4 >2.0 ng/ml indicating they had ovulated. Thus, treatment onset was postponed for the necessary time to rule out pregnancy and avoid P4 overexposure (from to 20-40 days depending on P4 value and date of last heat). During treatment, five queens didn't exhibit signs of heat and all 6 queens remained in good health. All queens returned to heat after the end of the treatment and had basal P4 at their last check-ups, just days after treatment ended, before heat.

The histological examination revealed a well-developed glandular mucosa in all queens and corpora lutea in 3 females. Evidence of hemorrhage in the uterine mucosa was found in 3/6 animals. A cystic and atypical endometrial hyperplasia and purulent endometritis were observed in one subject (indoors without male contact), who showed yellowish and

bloody discharge 2 weeks after the end of heat. Ovarian cysts were detected in another queen, although they were already present before treatment.

The cat who developed pyometra ovulated spontaneously both before and after MA treatment. Spontaneous ovulating queens tend to undergo repeated luteal phase which probably makes them a poor candidate for any (even low dose) progestin treatment. Overall, the results demonstrate that using MA for short-term contraception in healthy adult queens is effective and clinically safe up to 6 months, although histopathological alterations can already be observed. The absence of a control group limits the study's ability to conclusively attribute these changes to the treatment. Further research on the safety of MA treatment in spontaneous ovulating queens (especially consistent ones) and on the reversibility of these histopathological MA-induced alterations is needed.

1. INTRODUCTION

The domestic cat (*Felis catus*) is the most widespread pet in the world. According to "The Ecology Global Network" statistics the total global number of cats currently ranges between 600 million and 1 billion and among that range, domestic cats account for 373 million (Mori et al., 2020).

Considering these numbers, it is easy to understand how the control of the feline population's reproduction is a current issue. In this debate, the role of the veterinarian is fundamental because animal welfare, public health and overpopulation management are part of the veterinarian's responsibilities. In recent years the topic of feline overpopulation has gained global importance, and it is a multifactorial problem.

Cats are characterized by a high fertility. If left free to reproduce in presence of available resources, this species can increase in number very rapidly. During its reproductive life, a freely mating female cat can give birth to two or three litters per year, each averaging three to four kittens (Feldman and Nelson, 1996). To avoid unethical issues in the breeding field, the Fédération Internationale Féline established in its Breeding & Registration Rules a maximum of two litters/queen/year which is valid in Italy and other European countries.

The economic factor also must be considered. Inflation and economic availability can discourage the population from sterilizing their cats and increase the abandonment rate. Sterilized cats are less prone to roam and move across the territory in search of a mate during the breeding season, remaining confined to more delimited territories. Although sterilization is not always sufficient to contain the problem, it could obviously help to manage the overpopulation.

The growing increase of feline colonies has also raised several social and health issues. Stray cats can represent a threat to public health through the transmission of zoonoses (e.g., Toxoplasma, rabies) and diseases to other species or domestic cats. Moreover, a considerable increase in stray cats can be a source of damage to wildlife, especially avian species, to peace (e.g., vocalization during breeding seasons) and to public hygiene (e.g. fecal contamination).

Different countries have attempted to find effective methods for feline birth control. In the United States, for example, euthanasia is used to control the surplus of strays. However, the growing public sensitivity towards animal welfare and the ethical problem of euthanizing healthy subjects have led to the adoption of alternative systems, such as the "trap-neuter-return" (TNR) program and/or education programs against abandonment. In the TNR, community cats are trapped by volunteers, brought to a veterinary clinic to be spayed or neutered, vaccinated, eartipped, and returned to their natural habitat where they have lived.

Surgical sterilization is a means to reduce fertility and the risk of reproductive diseases. If female cats are sterilized before six months of age, there is a reduction of 91% in the risk of developing mammary carcinoma, and an 86% reduction if the procedure is performed within the first year of age (Overley et al., 2005). Gonadectomized female cats are also less susceptible to the incidence of uterine pathologies (pyometra, neoplasia), ovarian cysts or tumors, estrogen-mediated pathologies (vaginal hyperplasia/prolapse, myeloid aplasia) or progesterone-related conditions (pseudopregnancy, mammary hypertrophy), as well as those related to pregnancy and parturition (abortion, dystocia, uterine prolapse).

Sterilized subjects tend also to be less active and aggressive towards humans and their own conspecifics. Sterilization permits also to avoid the manifestation of estrous behavior poorly tolerated by owners (Romagnoli & Sontas, 2010). Nevertheless, sterilization is not the solution to behavioral problems which must be treated by animal behaviorists.

However, surgical sterilization in female cats presents risks such as generic surgical risks, return to heat due to ovarian remnant syndrome, infection of the uterine stump, incidence of Mast Cell Tumors (MCT), lymphoma, carcinoma and sarcoma (Ferrè-Dolcet et al., 2023), obesity (Belsito et al., 2009), and orthopedic problems (Fischer et al., 2004) (Romagnoli et al., 2024).

In the last years this new perspective on surgical sterilization has encouraged the research for reversible and less invasive methods alternative to surgery. An option to surgical sterilization can be chemical sterilization. Chemical sterilization involves the use of hormonal drugs capable of controlling and suppressing the reproductive activity of pets for more or less extended periods. The most studied hormonal treatments in cats are

gonadotropin releasing hormone (GnRH) agonists, progestins, androgens, and melatonin compounds.

The cat is a “long-day breeder”, therefore an exposure of less of 8 hours of light per day induces prolonged anestrus due to the production of melatonin (Prescott, 1973). Exogenous administration of melatonin can potentially simulate this physiological condition (Goericke-Pesch et al., 2014). Subcutaneous melatonin implants (Melovine®, 18 mg/cat) were reported to be effective for 4 months (Alabodi & Almeeni, 2024).

Androgens cause suppression of gonadotropin release in females and inhibition of gonadal function; however, their use in cats has been discouraged because the effective dosage (50 µg/day) is very close to the toxic dosage (60 µg/day) (Shille & Sojka, 1995).

GnRH agonists are widely used in cats. The most popular one is deslorelin, which is officially registered for male dogs and cats and for prepubertal female dogs. Deslorelin (Suprelorin®, Virbac) causes hyperstimulation of the pituitary gland and then hyperproduction of luteinizing hormone (LH) and follicle stimulating hormone (FSH) with consequent folliculogenesis and ovulation. Approximately ten days after administration, the high concentration of GnRH establishes a down-regulation of the pituitary receptors, followed by a prolonged ovarian or testicular quiescence. In postpubertal female cats, the duration of efficacy of deslorelin treatment (4.7 mg and 9.4 mg) is variable and can last from 1.5 to over 3 years in some female cats (Goericke-Pesch et al., 2013).

Progestins are synthetic analogs of progesterone used for the control and temporary suppression of estrus in queens. In the past they had a bad reputation that discouraged their use among veterinarians and breeders. However, it has been clearly demonstrated that side effects occurred only with experimental protocols that used excessive dosages, or patients with clinical characteristics or in phases of the reproductive cycle not suitable for treatment (Romagnoli, 2015).

This thesis will concentrate on a short-acting progestin, namely Megestrol Acetate (MA). The aim of the study is to assess the potential histological effects of the reproductive tract of queens (uteri and ovaries) treated with low dosage of MA.

1.1 MACROSCOPIC REPRODUCTIVE ANATOMY OF THE QUEEN

The reproductive trait of the queen is characterized by ovaries, oviduct, uterus (uterine horns, uterine body, cervix), vagina, vulva and mammary glands.

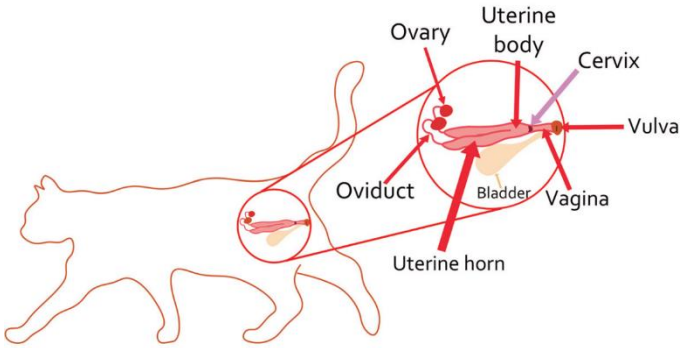

Figure 1. Reproductive anatomy of the queen (Johnson, 2022)

1.1.1 OVARIES

The queen has two ovaries, which typically measure around 1 cm x 0.5 cm.
By using ultrasonography, they can be directly observed caudally to each kidney's caudal pole. A section of the broad ligament known as the suspensory ligament of the ovary suspends the ovaries in the abdomen. The proper ligament of the ovary connects them to the uterine horn. An ovarian bursa, an outgrowth of the mesosalpinx, a peritoneal fold, surrounds each ovary. The ovarian artery begins in the aorta and provides blood to the ovaries. The ovarian vein drains blood from ovary, oviduct, and cranial uterus into the caudal vena cava (Ríos et al., 2023).

1.1.2 OVIDUCTS

The oviducts are lengthy and twisted tubes that are 5–9 cm in size. They consist of fimbria, infundibulum, ampulla, and isthmus, and are held up by the mesosalpinx, which is part of the broad ligament. Fertilization takes place at the junction of the ampullary and isthmus regions, where the sperm from the isthmus meets the oocytes at the ampulla's border. The oviduct enters the uterus and terminates in a small papilla of smooth muscle. Its blood supply consists of anastomoses between the ovarian and tubal branches of the ovarian artery and ascending branches of the uterine artery (Ríos et al., 2023).

1.1.3 UTERUS

The queen's uterus is bicornuate, with a short body and two lengthy horns that vary in size from 7-10 cm based on the queen's maturity, previous litters, and cycle stage. The blood for it comes from the uterine artery, which is a division of the vaginal artery. Blood exits the uterus through the uterine vein, which connects to the internal iliac vein (Ríos et al., 2023). During ultrasound evaluation, the main part of the uterus can be seen above and slightly caudal to the bladder, with the horns extending upwards on either side of the bladder. The uterine horns of a post pubertal queen should have a diameter of 1 cm or smaller and should not contain any fluid. The body of the uterus leads caudally to the cervix. The cervix is positioned in front of the vulva, approximately 40-45 mm, and creates a wide angle with the vagina (Watson & Glover, 1993). Visualizing the cervix using ultrasound is challenging due to its caudal location, despite being a thickened muscular region. The external os of the cervix reaches into the upper part of the vagina and faces ventrocaudally.

1.1.4 VAGINA AND VULVA

In the region the cranial vagina close to the cervical opening there is a structure known as the vaginal fornix. The dorsal median fold is caudal to the cervix and situated on the dorsal aspect of the vagina. The prominence of this fold varies throughout the phases of the estrous cycle, reaching its peak during estrus. This increased prominence contributes to a reduction in vaginal diameter and an extension of the fornix length during estrus. The vestibule serves as a connection between the vaginal vault and the exterior, lacking a distinct hymen. The vestibule's diameter is considerably larger than that of the vagina, measuring approximately 4 mm, and it is about 2 cm in length. Caudally, the vestibule terminates at the level of the vulva, which is composed of two dorsal labia. The urogenital sinus in the queen is 1–2 cm long, resulting in a narrow and non-distensible vagina (Fig. 3). Stimulation of the anterior limit of the urogenital sinus induces vocalization, like the response observed during coitus (Watson & Glover, 1993). The vulvar lips are positioned just below the anus. During estrus, the vulva becomes slightly edematous, though not as prominently as in dogs. The clitoris is located within the vulvar labia, housed in the clitoral fossa on the ventral side of the caudal vestibule. Analogous to the penile tissue in

males, the clitoris may become enlarged due to chronic irritation or certain disorders of sexual development.

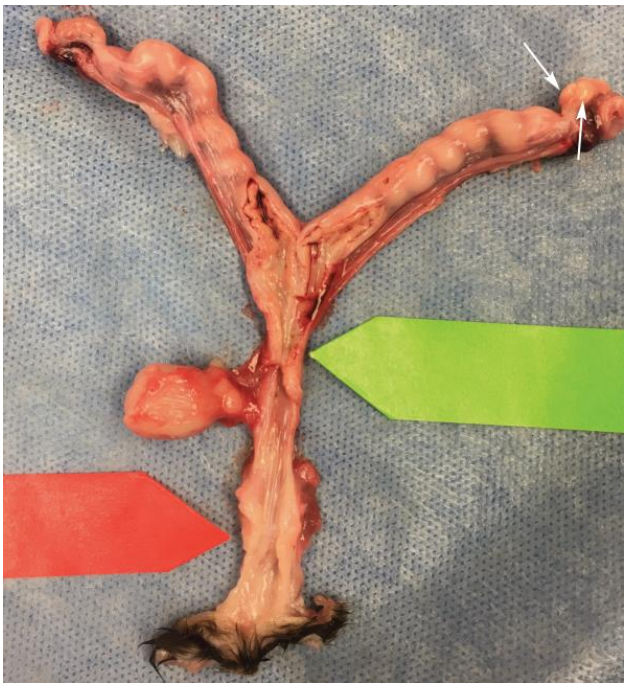

Figure 2. Reproductive tract of a normal queen in diestrus. The green arrow indicates the limit between vagina and cervix. The red arrow indicates the uretral sphincter. The white arrows indicate the corpora lutea (CL). (Johnson AK., 2022)

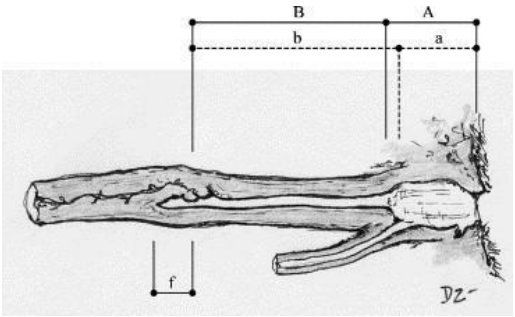

Figure 3. Longitudinal section of the low reproductive tract in the queen: urogenital sinus (A), vagina (B), vestibule (a), vagina (b), fornix (f). (Zambelli & Cunto, 2005)

1.1.4 MAMMARY GLANDS

The mammary glands, unique to mammals, are modified subcutaneous apocrine sweat glands with a critical role in providing nutrition and passive immunity to newborns. Structurally, the mammary gland is a branching ductal system embedded within substantial fibrovascular and adipose tissue. The ductal network starts with the collecting (papillary) ducts of the nipple and culminates in secretory alveoli when the gland is fully differentiated. At birth, the mammary glands are not fully developed; their development begins at puberty and undergoes morphological changes with final modifications occuring only during pregnancy.

The nipple sheath comprises specialized epithelium, forming the raised teat in adults. Typically, each teat in cats has 4–7 papillary duct orifices. Cats generally have four mammary glands per side, identified as axillary (M1), thoracic (M2), abdominal (M3),

and inguinal (M4). These are also termed cranial (T1) and caudal (T2) thoracic, and cranial (A1) and caudal (A2) abdominal. Occasionally, a fifth inguinal (M5) gland may be present.

In cats, the axillary (M1) and thoracic (M2) mammary glands receive blood supply from the perforating branches of the internal thoracic, intercostal, and lateral thoracic arteries. The abdominal (M3) glands are supplied by the cranial superficial epigastric artery, while the inguinal glands (M4) receive blood from branches of the external pudendal artery. The veins of the feline mammary glands closely parallel the arteries, with some veins crossing the midline, facilitating metastatic spread between paired glands. This anatomical feature is unique to the cat's mammary gland, though it is extremely rare (Nickel et al., 1986).

1.2 MICROSCOPIC REPRODUCTIVE ANATOMY OF THE QUEEN

1.2.1 OVARIES

The cortex of the ovary is characterized by a cuboidal epithelium known as germinal epithelium, beneath which lies a layer of dense connective tissue called the tunica albuginea. Within the ovarian cortex there are follicles, stromal connective tissue, and blood vessels. Ova develop within follicles, which are categorized into four types: primordial, primary, secondary, and tertiary. Each developing follicle features an oocyte, several layers of granulosa cells and peripheral thecal connective tissue cells (Figure 4). Ovulation involves the rupture of the follicle, releasing the ovum, followed by the space filling with blood and luteal cells to form the corpus hemorrhagicum and corpus luteum, respectively. In bitches and queens, cords of epithelial cells, known as interstitial glands and consisting of endocrine-type cells, are dispersed throughout the stroma. The medulla, located internal to the ovarian cortex, consists of richly vascularized loose connective tissue, lymphatics, and nerves. This region also contains channels lined with cuboidal epithelium called rete ovarii (Foster, 2007).

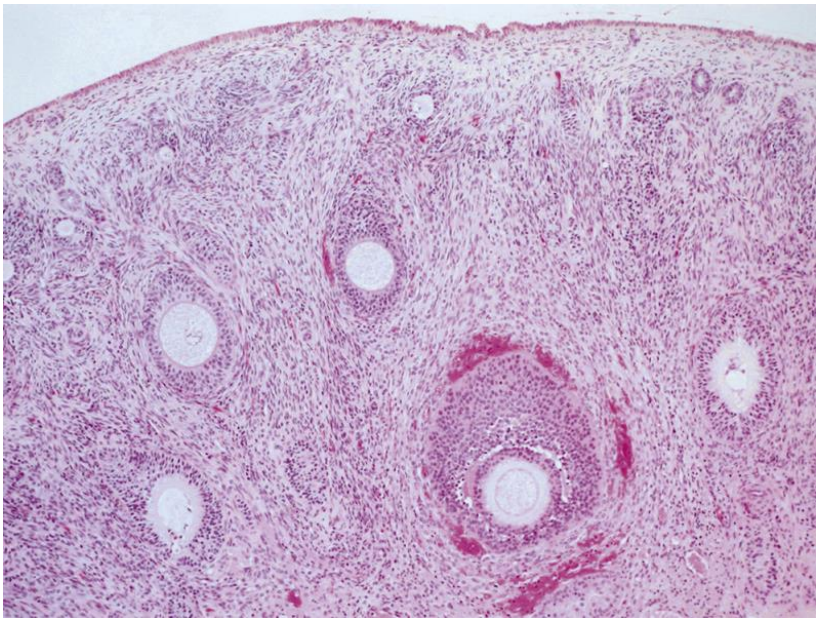

Figure 4. Normal ovary, tissue section. Several developing follicles, each with an oocyte surrounded by a layer of granulosa are present within the stroma of the ovarian cortex. The cortex is lined by a simple layer of cuboidal epithelium. (H&E; LP.)

1.2.2 UTERUS

The uterine tubes are divided into four regions: the infundibulum, ampulla, isthmus, and uterotubal junction. These tubes are supported by the mesosalpinx, which contains a significant amount of fat, and a small opening that connects the bursa to the abdominal cavity. The infundibulum encircles the ovary. The uterine wall is composed of three layers: the outer perimetrium (serosa), the middle myometrium, and the inner endometrium (mucosa) (Fig. 5). The perimetrium consists of loose connective tissue and is covered by peritoneal mesothelium. The myometrium is divided into a thick inner circular layer and a thin outer longitudinal layer, with a richly vascularized and well-innervated stratum usually separating these muscle layers. The endometrium's epithelium is simple cuboidal or columnar in the bitch and queen, depending on the estrous cycle. Simple, branched endometrial glands extend into the lamina propria. The cervix, which separates the external genitalia from the uterus, acts as an effective barrier to the external environment. Unlike other species, the feline cervix lacks transverse folds and typically opens dorsally (Foster, 2007).

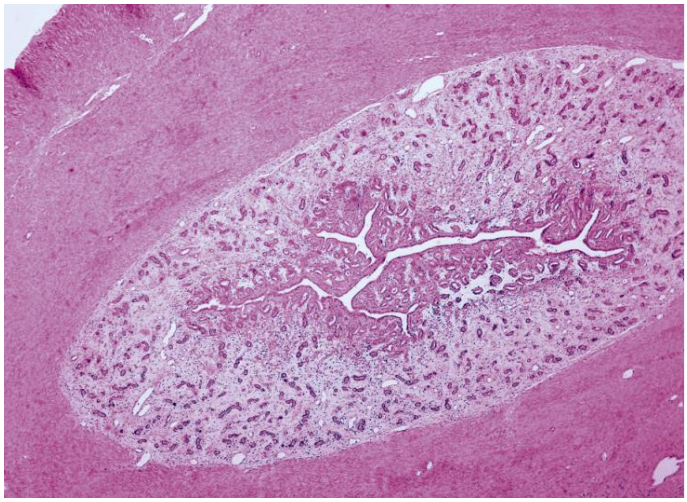

Figure 5. The dense area surrounding the endometrial glands is the myometrium, which consists of two layers of smooth muscle, inner circular and outer longitudinal. (H&E; LP.)

1.2.3 VAGINA AND VULVA

The vagina is a musculomembranous canal that extends from the uterus to the vulva. Its wall includes three layers: an inner mucosal layer, a middle smooth muscle layer, and an outer coat of connective tissue and peritoneum (cranially) (Banks, 1986). The mucosal layer is made up of stratified squamous epithelium, which undergoes specific morphological changes in response to the estrous cycle.

The vulva is anatomically similar to the caudal vagina and includes the vestibule, urethral orifice, clitoral fossa, and labia. The mucosa of the vulva is lined by stratified squamous epithelium, with some keratinized epithelial cells present in the vestibule and clitoral fossa (Allison et al., 2008).

1.2.4 MAMMARY GLANDS

The teat represents the terminal portion of the mammary gland secretory system and is covered by epidermis. In cats, the teat is associated with hair follicles and large sebaceous and apocrine glands. Melanocytes can be found among the basal epidermal keratinocytes. Non-secretory teats in cats are typically very small and often concealed by hair. Each cat teat has 2–3 major papillary orifices at the apex, with the rest positioned more laterally. Within the dermis of the teat and along the larger ductal system, longitudinal and transverse smooth muscle fibers and elastin fibers are present. The teat ducts are lined by stratified squamous epithelium and encircled by a circular smooth muscle sphincter.

Each teat duct opens into the teat sinus, which is lined by bilayered cuboidal to columnar epithelium with flattened myoepithelial cells surrounding the duct. Large interlobular ducts, or lactiferous ducts, empty into the distal teat sinus. These interlobular ducts are bilayered and lined with cuboidal epithelium, featuring an outer layer of fusiform myoepithelial cells. The smaller distal interlobular ducts are lined by a monolayer of cuboidal epithelium with fewer myoepithelial cells. The terminal interlobular ducts transition into intralobular ducts, which are lined by a single layer of cuboidal epithelium and surrounded by discontinuous spindle-shaped myoepithelial cells. The alveoli are lined by a single layer of epithelial cells and externally by star-shaped myoepithelial cells. The secretory alveolar epithelium is tall, ranging from cuboidal to columnar, with intracytoplasmic lipid droplets that accumulate within the alveolar lumina.

Extension and branching during ductal growth occur through the proliferation of epithelial cells that penetrate gaps between myoepithelial cells. Both epithelial and myoepithelial cells reside on and produce a basement membrane, primarily composed of collagen type IV, laminins, and heparan sulfate proteoglycans. The stroma, which supports the epithelial structures of the mammary gland, is derived from specialized mesoderm and includes connective and adipose tissue, blood vessels, lymphatics, and nerves. This interstitial tissue may occasionally contain histiocytes, plasma cells, and small lymphocytes. The stroma is divided into intralobular stroma, composed of loosely arranged and finer collagen bundles, and interlobular connective tissue, characterized by thicker and more tightly organized collagen. The amount of adipose tissue present is variable.

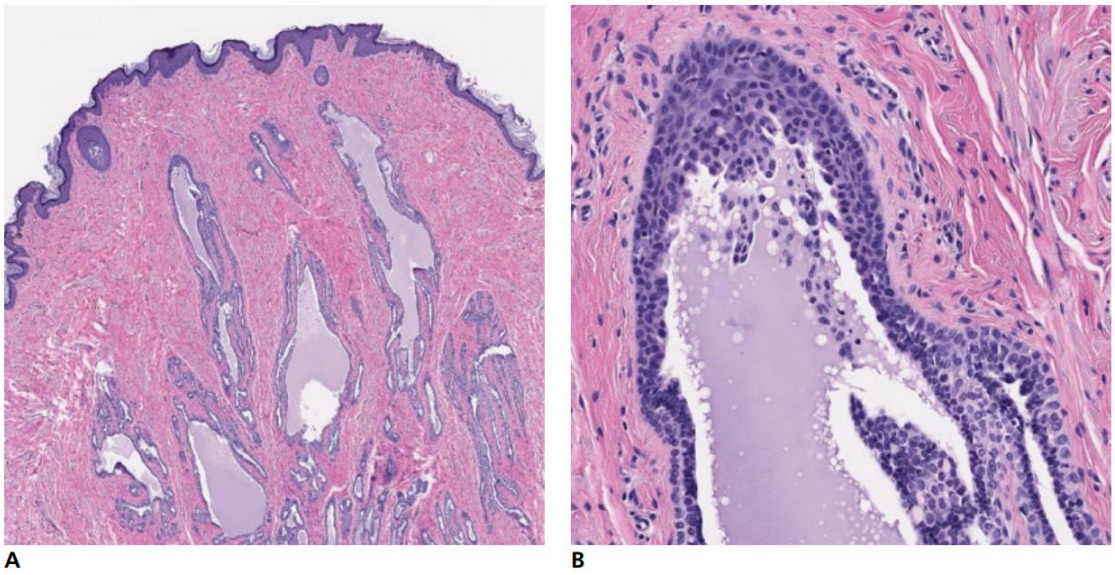

Figure 6. (A) Normal feline nipple. The epidermis is normal, and no adnexa are present. Numerous teat ducts and teat sinuses are ecstatic and contain eosinophilic secretion. The surrounding stroma consists of collagen and smooth muscle bundles. (B) Junction between normal teat duct and teat sinus with intraluminal secretion. (Mauten, 2017)

1.3 PHYSIOLOGY OF THE FEMALE CAT

1.3.1 HYPOTHALAMIC-PITUITARY-OVARIAN AXIS

The hypothalamus is a portion of the diencephalon located ventrally to the thalamus, where it forms the floor of the third ventricle. It consists of numerous clusters of neuronal bodies called hypothalamic nuclei with different and specific functions, from the control of body temperature and blood circulation to the regulation of food and fluid intake, the sleep-wake cycle, sexual behavior, and defense and attack mechanisms. The sites of the two groups of nuclei predisposed to reproductive control are represented by the tonic center (consisting of the ventromedial and arcuate nucleus, and responsible for pulsatile and constant secretions of neurohormones) and the phasic center (also called the pre-ovulatory center, represented by the preoptic, suprachiasmatic nucleus and the anterior hypothalamic area, and responsible for secretory spikes). Neurons in these regions secrete GnRH, a decapeptide hormone that stimulates the production of gonadotropins FSH and LH by the pituitary gland (Senger, 2012).

The hypothalamus is connected to the pituitary gland by a sophisticated network of capillaries called the hypothalamic-pituitary portal system, which is important because it

allows minute amounts of hormones to act directly on target cells in the anterior lobe of the pituitary gland without being diluted by systemic circulation (Senger, 2012).

A common feature of all these neurons is the feedback regulation exerted by the products of secretion on hypothalamic cell activity. Positive feedback stimulates GnRH secretion, while negative feedback suppresses it. High concentrations of progesterone, for example, strongly inhibit hypothalamic neurons allowing only basal production of GnRH (and thus of FSH and LH), which is insufficient for the late stages of follicular development or the preovulatory peak of LH (Kauffman, 2022).

Conversely, high estradiol levels positively stimulate the phasic center resulting in massive release of GnRH causing the release of high amounts of LH, inducing the terminal stages of follicle development and thus ovulation. Hence the belief of many endocrinologists that the tonic center responds primarily to negative feedback, as opposed to the phasic center which is more sensitive to positive feedback (Senger, 2012).

The pituitary gland, is located at the base of the skull, in a bony niche called the sella turcica, surrounded by the dura mater. It is divided structurally and functionally into three portions: a posterior lobe, the neurohypophysis, an anterior lobe, the adenohypophysis and the intermediate lobe. The neurohypophysis is a direct extension of the central nervous system, and it is connected to the hypothalamus via the infundibulum, with the purpose of directly secreting two hypothalamic hormones, such as vasopressin and oxytocin, into the circulation. On the other hand, the adenohypophysis is devoted to the release of trophic hormones such as TSH (thyroid-stimulating hormone), ACTH (adrenocorticotropic hormone), GH (growth hormone), FSH, LH and prolactin. The intermediate lobe role in reproduction control is less important (Senger, 2012).

Pituitary gonadotropins (FSH and LH) are two glycoproteins that play essential roles in promoting gonad development, steroidogenesis, and gamete production. Specifically, in the female, FSH acts on the granulosa cells of the ovary by inducing follicular development and estradiol production; LH, on the other hand, interacts with the theca interna and luteal cells by stimulating ovulation, corpus luteum formation and progesterone secretion. Granulosa cells also produce another hormone, inhibin, which

operates negative feedback on FSH release, thereby controlling follicle development (Senger, 2012).

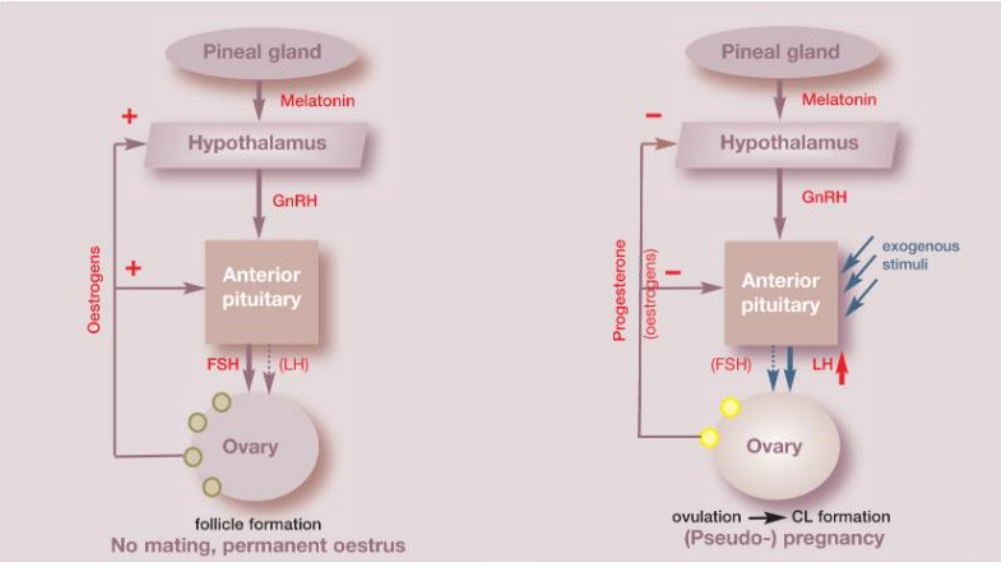

Figure 7. Neuroendocrine control of reproduction in the queen (Goericke-Pesch, 2010)

1.3.1.1 ESTROUS CYCLE IN THE QUEEN

The queen is seasonally polyestrous, experiencing multiple cycles within a breeding season, followed by a period of non-cyclicity known as anestrus. Queens cycle during periods with longer daylight hours, with cyclicity ceasing when day length shortens. This pattern can vary geographically, depending on latitude and photoperiod, with some queens resuming heat cycles as early as December or January. Studies have shown that the seasonality of the cat estrous cycle can be influenced by the length of the photoperiod (Leyva Ocariz et al., 1989). A transition from shorter to longer day lengths induces estrus in cats, mediated by the reduced release of melatonin from the pineal gland, which is regulated by the circadian rhythm. Melatonin, released during periods of darkness, inhibits the production of GnRH and disrupts the hypothalamic-pituitary-gonadal feedback loop. When kept under a 12–14-hour light cycle, domestic cats can cycle year-round (Shille et al., 1979).

1.3.1.2 OVULATION

The queen is an induced ovulator, meaning ovulation occurs following coitus. Without vaginal stimulation, the estrus cycle remains anovulatory. However, spontaneous ovulation (without coitus) can occur in approximately one-third of cats (Binder et al., 2019; Pereira et al., 2024). The incidence of spontaneous ovulation increases with body weight (Binder et al., 2019). The factors that stimulate or predispose queens to spontaneous ovulations or corpus luteum (CL) formation are largely unknown. Suggested factors include tactile contact with other females in cat colonies, stroking of the cats' back and neck by humans, and stress from experimental procedures (Bertschinger, 1997) though there is no experimental or clinical evidence supporting these possibilities.

Ovulation is highly dependent on the number of matings within a short period. One study found that only 1 out of 12 cats ovulated after a single mating on day 1 of estrus, whereas 4 out of 12 ovulated after a single mating on day 4 of estrus (Wildt et al., 1980). In contrast, when multiple matings (three) occurred on a single day, 10 out of 12 cats ovulated on days 1–3 of the estrus period (Wildt et al., 1980). It is generally believed that four or more matings are necessary to provide sufficient stimulation and release of LH for ovulation to occur. The LH surge happens shortly after mating, peaking at 2 hours and remaining elevated for approximately 8–12 hours (Tsutsui & Stabenfeldt, 1993). Ovulation occurs within 36 hours of mating, provided that preovulatory follicles are present.

During copulation, the penis of the tomcat stimulates receptors in the queen's vagina, transmitting a signal to the hypothalamus via an afferent spinal pathway. The hypothalamus is then stimulated to release GnRH, which in turn causes the release of LH and FSH from the pituitary gland (Feldman, 2004). The release of FSH is subsequently inhibited by inhibin from large follicles (Bristol & Woodruff, 2004).

1.3.1.3 PUBERTY

Puberty in queens typically occurs between 6 and 12 months of age, when a weight greater than 2–3 kg (approximately 80% of their adult weight) is attained. The age at which puberty is reached depends on the time of year the kitten was born and specific breed variations. Longhair cats generally reach puberty later than shorthair cats (Tsutsui & Gh,

1993). Additionally, smaller breeds tend to reach puberty earlier than larger breeds. A kitten born in the spring that reaches 6–8 months of age during the shorter days of the year may not enter puberty until the next breeding season, when day length increases.

1.3.1.4 STAGES OF ESTROUS CYCLE

The feline estrous cycle consists of five stages: proestrus, estrus, interestrus, diestrus, and anestrus.

Proestrus is often not observed in cats due to its short duration, typically less than 24 hours, and subtle behavioral signs. During proestrus, follicle size increases, estrogen concentrations rise, and the vaginal epithelium thickens. Vaginal cytology during this stage shows less than 50% cornified cells and mainly small to large intermediate cells. The female cat may exhibit vulvar swelling, rubbing, and vocalization but does not accept the male (Johnson, 2022).

Estrus is characterized by elevated estradiol levels (>20 pg/ml) from the growing follicles, and the queen becomes receptive to males and accepts coitus. This phase, also known as the follicular phase, averages 5–7 days but can range from 2 to 19 days (V. Shille et al., 1979). During estrus, vaginal cytology shows more than 75% cornified epithelium, although intermediate cells may still be present, and complete cornification, as seen in dogs, may not occur. The cells' nuclei become pyknotic but typically do not disappear entirely. Red blood cells are rarely seen in the vaginal cytology unless there is mucosal irritation from obtaining the swab, and neutrophils are uncommon but may appear briefly as the queen enters interestrus. During estrus, the follicles mature, reaching 2–3 mm in diameter, with each ovary generally containing three to six mature follicles per follicular wave.

Behavioral signs of estrus include rubbing the neck and face on walls and furniture, rolling on the back, vocalization, assuming the lordosis position, and pressing with the hind feet while elevating the rear quarters. If mating occurs before follicle maturation, ovulation is unlikely (Little, 2012). There is conflicting evidence about the role of mating followed by ovulation on the length of estrus. Anecdotal and empirical observations point to a shortening of the estrus phase (disappearance of estrus signs) after ovulation.

However, one of the classical studies on the subject found no significant difference (V. M. Shille et al., 1979) (Little, 2012).

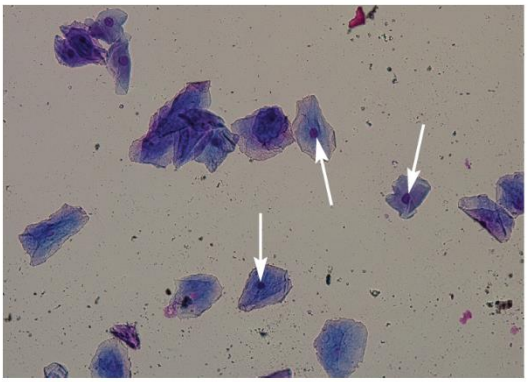

Figure 8. Vaginal cytology of a queen in estrus. In this field, all cells are showing some degree of cornification based on the rough, irregular cell border and flattened appearance. Approximately half of the cells have retained nuclei (arrows), but these are small and dark, indicating pyknosis.

If ovulation does not occur, the estrus phase is followed by **interestrus**. The duration of interestrus varies among individual cats, averaging 8–9 days (V. Shille et al., 1979). During interestrus, the dominant follicles undergo atresia, and estradiol levels decrease. Vaginal cytology during this phase shows less than 50% cornified epithelial cells, with mainly parabasal and intermediate cells present (Figure 9). As the current set of follicles regress, the next wave of follicular development begins. Interestrus then transitions back into estrus.

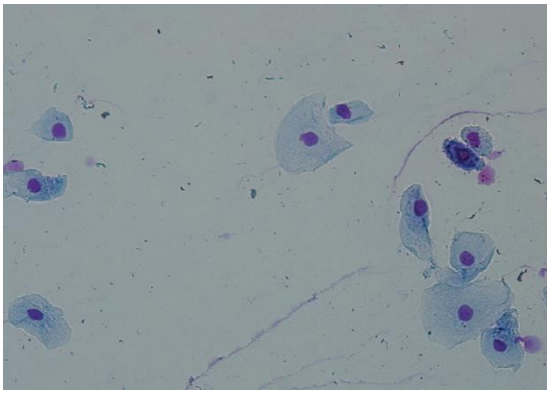

Figure 9. Vaginal cytology of a queen in the interestrus phase of the cycle. Some cells are cornified but make up <50% of all cells and the nuclei are not pyknotic. The remaining cells are smaller, with roundlimits and less cytoplasm (intermediate and parabasal cells).

If ovulation occurs, the queen will enter the **diestrus** phase. The presence of one or more CL on the ovary confirms diestrus (Figure 10). Vaginal cytology during this phase will primarily show small intermediate and parabasal cells, and superficial ones at a smaller extent (Campana et al., 2024) (Figure 11). A study demonstrated that progesterone, secreted by the corpora lutea, begins to rise by day 4 after the first mating, indicating the onset of the luteal phase (Verstegen et al., 1993). The luteal phase is the period where active corpora lutea are located on the ovaries and the queen is exposed to elevated progesterone.

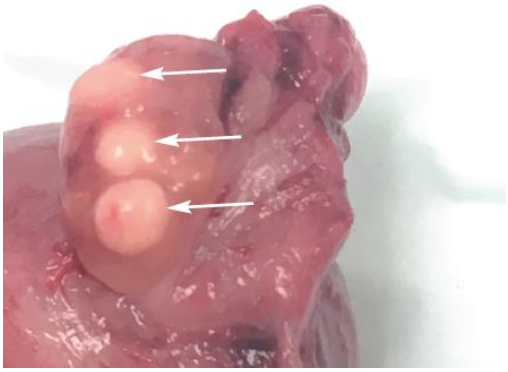

Figure 10. A queen in diestrus at the time of ovariohysterectomy. Three corpora lutea (CLs; arrows) are clearly visible within the ovary. (Johnson, 2022)

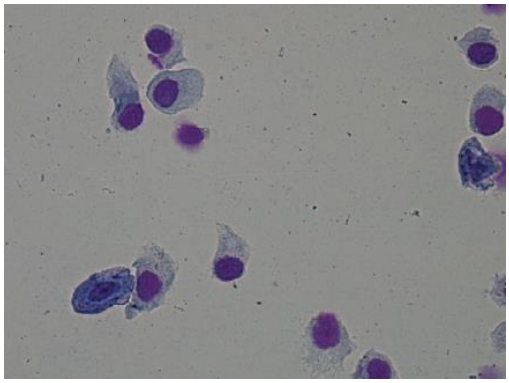

Figure 11. Vaginal cytology of a queen in diestrus, showing predominantly parabasal cells (Johnson, 2022)

Anestrus lasts several months when the days are shorter. The queen doesn't show estrous behavior. Cytologically it is characterized by small intermediate and parabasal cells, but it is also possible to find superficial cells although the reason behind is unclear (Campana et al., 2024). Serum estradiol and progesterone are at baseline levels during this stage and the queen is sexually inactive. There is no ovarian activity (Johnston et al., 2001).

1.3.3 ANATOMIC MODIFICATIONS DURING THE ESTROUS CYCLE

Depending on the stage of the estrous cycle, the uterus's outer diameter and the endometrial lining's appearance may change. The endometrium is lined with a single layer of cuboidal cells and the endometrial glands are dormant during inactive periods. As the queen enters the follicular stage endometrial and myometrial diameters grow, and endometrial glands multiply. Many elongated, active glands are seen in the luteal phase endometrium (Chatdarong et al., 2005). Hysterography, which consists of contrast uterine

radiography, can be used to study the shape of the uterine cavity. The endometrial outline is smooth in a healthy cat. The uterine horns are straight while the cycle is inactive (Figure 12). Due to the growth of the endometrium and increased muscle movements, the uterine horns develop a curved shape with a wavy inner cavity in the follicular phase (Figure 13). The morphology of the lumen changes from a straight to an irregular, wavy, and coiled shape in the luteal phase (Chatdarong et al. 2005). The cervix is typically only open during estrus, regardless of ovulation in queens (Chatdarong et al. 2002). The time when the cervix is open typically aligns with the time when the vaginal smear shows cornification (Chatdarong et al. 2002).

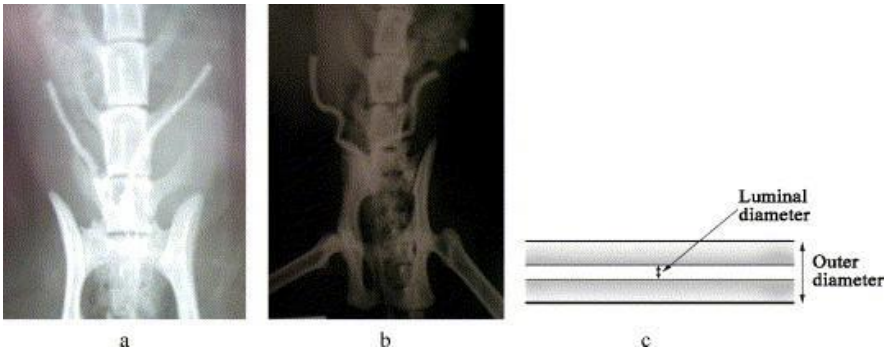

Figure 12. Hysterograms from individual cats: (a) straight uterine shape with a straight luminal cavity and a smooth inner contour is the predominant hysterographic appearance in the inactive stage of the reproductive cycle; (b) moderately curved uterine shape with a straight luminal cavity and a smooth inner contour is the major characteristic of the hysterogram in the postpartum group of cats; (c) schematic illustration demonstrating a straight luminal cavity of the uterus. (Chatdarong et al., 2005)

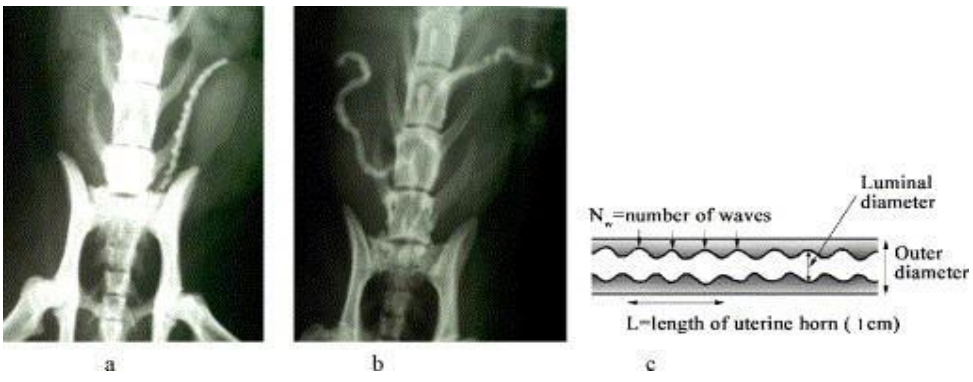

Figure 13. Hysterograms from individual cats: (a) straight uterine shape with a wavy luminal cavity and a smooth inner contour is the predominant hysterographic appearance in the follicular stage of the reproductive cycle; (b) moderately curved uterine shape with an irregular wavy luminal cavity and a smooth inner contour observed in the luteal stage of the estrous cycle; (c) schematic illustration demonstrating a wavy luminal cavity, degree of waviness = Nw/L (Chatdarong et al., 2005).

Zambelli et al. (2004) conducted the initial research on changes in the vagina and cervix throughout the estrous cycle and when not breeding. Genital sizes were acquired by making silicon molds or by extracting reproductive tracts during different stages of the cycle, as well as from queens in different reproductive states. There were no significant differences in total vaginal length and vaginal diameter throughout the estrous cycle ($P > 0.05$) as shown in Table 1. Zambelli et al. (2004) determined that during the follicular phase, the key changes were longer fornix, shorter vagina, and narrower angle between cervical and vaginal axes (Figure 14).

Stage	**Mould dimensions (mm)**				
Empty Cell	**Total vagina length**	**Fornix length**	**Vagina diameter**	**Angle between cervical and vaginal axis (degrees)**	**Vagina height**
Proestrus/estrus	47.0 ± 5.5	3.3 ± 0.9	1.2 ± 0.1	137.2 ± 12.9	0.5 ± 0.1
Diestrus/pregnancy	45.8 ± 7.5	1.5 ± 0.7	1.5 ± 0.1	143 ± 6.3	1.3 ± 0.2
Interestrus	47.5 ± 5.3	2.1 ± 0.9	1.2 ± 0.2	131.3 ± 9.8	0.6 ± 0.2
Anestrus	49.3 ± 4.4	1.3 ± 0.9	1.6 ± 0.4	154.3 ± 8.8	1.5 ± 0.2

Table 1. Mean (±S.D.) vaginal and cervical measurements from molds and formalin-preserved reproductive tracts of domestic queens. (Zambelli et al., 2004).

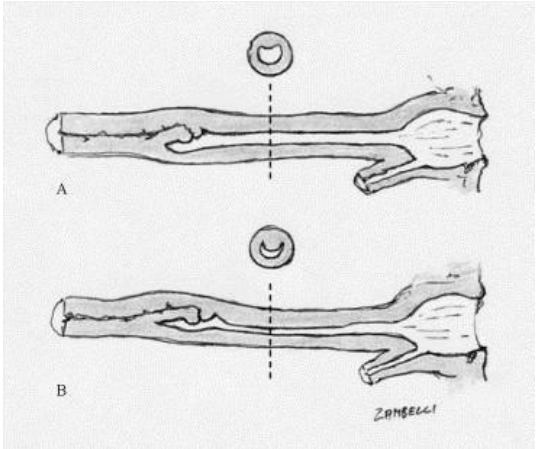

Figure 14. Reproductive tract of two queens during different stages of the estrous cycle: anestrus (A), estrus (B). Some anatomical differences are evident: the dorsal medial fold is more prominent and longer during estrus than anestrus (see the transversal sections); the fornix is longer and flattened during estrus in comparison with anestrus; the cervical axis has a greater slope in estrus than in anestrus. (Zambelli et al., 2004)

1.4 STAGING THE ESTROUS CYCLE IN THE QUEEN

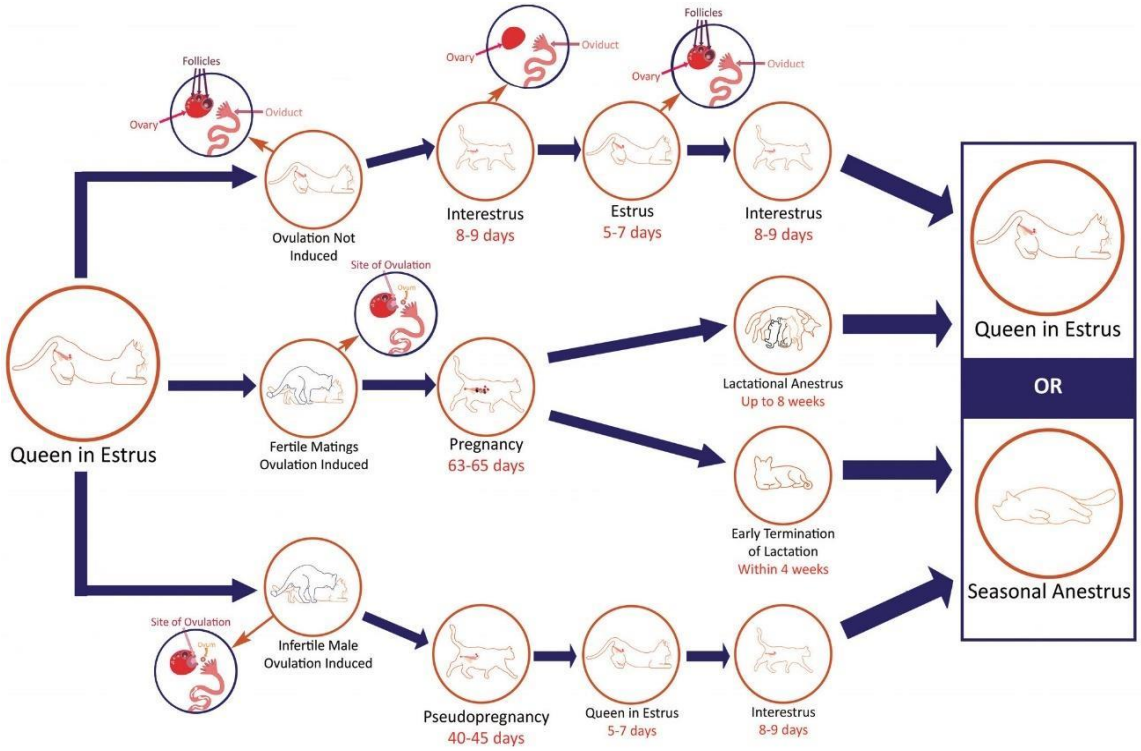

Figure 15. Diagram showing the progression through the feline estrous cycle. Figure designed by Jamie M Douglas. (Johnson, 2022)

1.4.1 HORMONAL EVENTS OF ESTROUS

FSH, produced by the pituitary gland, initiates the development of ovarian follicles. An average of 3 to 7 follicles develops and start producing estradiol (Tsutsui et al., 1989). As follicular activity peaks, blood estradiol levels increase and vary widely, but usually are over 20 pg/ml (Goodrowe, 1992). Estradiol levels stay high for 3 or 4 days and then abruptly fall. The high estradiol levels produce two important effects: explicit estrous behavior and priming of the gonadotropin surge necessary to cause ovulation (Banks & Stabenfeldt, 1982).

Ovulation requires the release of LH from the anterior pituitary gland. Sufficient stimulus, either copulatory or non-copulatory, is required to stimulate increased release of GnRH from the medioventral hypothalamus. GnRH release causes the LH surge. Several days of estradiol priming are required, for most cats, before LH release sufficient to cause

ovulation occurs. This is typically reached by the third or fourth day of estrus (Banks & Stabenfeldt, 1982).

Queens vary considerably in the number of matings required to induce sufficient LH release and ovulation (Schmidt, P.M., 1986). On average, most queens will ovulate after 4 or more matings (Feldman, E.C, et al., 1996). All oocytes are ovulated at once, so all kittens in a litter are the same gestational age.
There are some anecdotal reports of superfetation in queens (Beaver & BG, 1973; Harman, 1917), although this has never been definitively demonstrated to occur in domestic cats. Superfetation is the simultaneous presence of fetuses of differing gestational age in the uterus.

Ovulation occurs approximately 48 hours following the LH surge (Schmidt, P.M. et al, 1983). Progesterone levels rise within 24 hours from the ovulation and may reach values of 60-90 ng/ml by 15 to 25 days post-ovulation (Verstegen, J.P. et al, 1993). Peak progesterone levels are highly variable from queen to queen. Throughout pregnancy, progesterone is maintained at high levels until the last few weeks of gestation, when it falls to under 2 ng/ml at term (Verstegen, J.P. et al, 1993). The CL is considered the main source of progesterone throughout pregnancy. Nevertheless, some queens gonadectomized after 45 days of pregnancy are able to queen normally, which suggests a steroidogenic capacity of the feline placenta (Tsutsui et al., 2009). This fact has been confirmed by several authors (Siemieniuch et al., 2012) (Braun et al., 2012), although progesterone produced by the placenta has not yet been detected in measurable amounts on peripheral blood, probably acting at a local level (Tsutsui et al., 2009)

1.4.2 METHODS TO STAGE THE FELINE CYCLE ESTROUS

Hormonal assays allow the identification of specific moments of the estrous phase. Methods used to measure hormone levels in the blood are radioimmunoassay, immunoassay, and chemiluminescence. Unlike the bitch, in the queen, serum levels of progesterone are not used to determine the most favorable time for mating, as it is mostly an induced ovulatory species. Estrogen assay is not used in veterinary practice due to its pulsatile secretion. If estradiol rises above 60 pg/ml generally a folliculogenic phase is present; values of 10 pg/ml, on the other hand, are associated with interfollicular phases

(Edqvist and Stabenfeldt, 1989). Progesterone measurement 2 or 3 days after coitus can still demonstrate ovulation (if > 5 ng/ml) (G.C.W. England, 2010).

Vaginal cytology is an easy and cheap method with great value in small animal reproduction. Through the type of cells in the smear it is possible to determine the phase of the cycle in queen. Background, cellularity and types of cells must be evaluated in order to correlate the smear with the ovarian cycle. The alterations in % and types of cells are directly related to serum estrogen concentration. Estrogens are secreted by a maturing follicle and cause a thickening of vaginal lining, moving the cells from her blood supply, with the subsequent degeneration and atrophy. Thus, vaginal cytology is always a reflex of estrogen activity on the female.
The preferred method to obtain a good vaginal cytology is the application of a cotton swab which must be of adequate size and introduced only 1 to 1.5 cm in the vagina.

The cells that can be seen in vaginal cytology can be classified in keratinized and nonkeratinized. Keratinization is a reflex of circulating estrogens. The basal cells (near blood supply) are small, round and with a clear nucleus, but in the cornified cells (with no blood supply) the nucleus becomes smaller, pyknotic and finally it is disintegrated leaving an anuclear cell.
There are different types of cells that can be observed on a vaginal smear:

- **Parabasal cells:** small, round, slightly oval, large vesiculated nucleus and small cytoplasm. Good staining.
- **Intermediate cells:** slightly larger than parabasal cells to twice that size. Smooth, oval to rounded irregular borders, nucleus smaller than in parabasal cells. They have more cytoplasm than parabasal cells.
- **Superficial cells:** are the largest in vaginal cytology with a sharp, flat, angular cytoplasmic borders and a small pyknotic, fading nuclei.
- **Anuclear squames:** irregular vaginal cells, no nucleus, smaller than superficial. These are the cells that have also been called "fully cornified."
- **Neutrophils:** inflammatory cells that can be normal or abnormal in vaginal cytology depending on the cycle phase.
- **Red blood cells:** abnormal in the queen.

The correlation between ovarian cycle and vaginal cytology is discussed in chapter 1.3.2. 4.

It is important to remember that vaginal cytology is just a complement of a good clinical examination, anamnesis and hormone assays.

1.5 USE OF REPRODUCTIVE DRUGS TO MANIPULATE CYCLICITY

The queen's reproductive cycle can be temporarily and reversibly blocked without any tangible consequence for her future health and fertility using drugs.

1.5.1 ALTERNATIVES TO SURGICAL CONTRACEPTION

A variety of methods have been studied as alternative to surgical sterilization. The key point is to control the endocrine regulatory mechanisms. These include:

- Progestins causing a negative feedback effect on the pituitary;
- Immunisation against endogenous GnRH or LH;
- Application of GnRH agonist implants, leading to downregulation of the hypothalamus-pituitary-gonadal axis;
- Use of melatonin implants.

1.5.1.1 IMMUNISATION AGAINST ENDOGENOUS GnRH or LH

Active immunisation against LH and GnRH has gained widespread acceptance as a means of controlling reproduction and associated behaviours in farm animals (eg, Improvac; Pfizer Animal Health, anti-GnRH vaccine used in pigs) (Khan et al., 2008). However, until now the efficacy of these vaccines has been shown to be limited in companion animals, especially in cats.

GnRH is a decapeptide with low immunogenicity and it must be bound to an immunogenic compound (e.g. Freund's adjuvant, tetanus toxoid or parts of distemper virus) to increase its immunogenicity. Additionally, some species differences seem to exist. Whereas a vaccine against luteinizing hormone-releasing hormone (LHRH) conjugated to tetanus toxoid was highly effective in reversibly blocking steroidogenesis and spermatogenesis in male dogs, antibody titles in toms showed high individual variation among treated animals and did not correlate with testosterone concentrations or

fertility (Ladd et al., 1995). Bovine LH receptor implants (day 0) and LH receptor protein as repeated injections (days 98, 139, 160, 193) were used for immunogenic stimulation in another study in female cats (Saxena et al., 2003). Hormone concentrations in treated animals were significantly lower than those of control animals, with a duration of effect of about 501 days as indicated by a significant decrease in LH antibody titres. However, currently no GnRH or LH vaccine is registered for use in companion animals.

Immunization against ovarian antigens was first proposed more than 50 years ago when it was discovered that rabbits exposed to hamster ovary tissue produced antibodies that blocked fertilization (Ownby & Shivers, 1972). The zona pellucida (ZP) is a protective layer of proteinaceous, acellular, gelatinous material that covers the outer surface of the ova in mammals. In the dog and cat, secretion of the ZP originates from the oocyte, as demonstrated by transmission electron microscopy and immunogold staining (Barber et al., 2001). Fertilization is a complex process that begins with binding of sperm to the ZP. Sperm specifically bind to zona pellucida 3 receptor (ZP3) and undergo the acrosome reaction, resulting in the release of enzymes that digest the ZP, allowing sperm to enter and fuse with the oocyte. Antibodies against ZP block sperm receptor sites, rendering ova impervious to sperm. The ZP3 receptor has unique epitopes, which make it an ideal target for immunocontraception. However, immunization with ZP proteins from the same species has little effect (B.S. Dunbar, 1983). Porcine ZP3 (pZP3) has been consistently utilized in vaccine development because it is readily available from abattoirs and has antigenic similarities to the ZP3 of dogs and cats (Aitken et al., 1996).

1.5.1.2 GnRH AGONISTS IMPLANTS

Deslorelin (the active ingredient in Suprelorin® 4.7 mg and Suprelorin® 9.4 mg; Virbac) is a synthetic GnRH agonist that is seven times more potent than GnRH. Prolonged stimulation of GnRH receptors by deslorelin leads to desensitization of these receptors. This results in a lack of synthesis and/or lack of release of the gonadotropins LH and FSH, inducing temporary infertility in treated individuals (Junaidi et al., 2007).

The 4.7 mg deslorelin implant is registered under the brand name Suprelorin in the European Union (EU), Australia and New Zealand for the long-term suppression of fertility in adult male dogs and cats, and for puberty postponement in pre-puberal bitches. The EU and Australia have further registered the 9.4 mg implant for chemical castration

of adult male dogs and adult male ferrets; Australia has additionally approved the implant to manage hyperadrenocorticism in ferrets. Suprelorin® F, a 4.7 mg deslorelin implant, is available in the United States as a Food and Drug Administration Indexed Product to manage adrenal disease in sterilized and sexually intact male and female ferrets. Suprelorin 9.4 mg contains a double dose of deslorelin and a matrix without the excipient sodium acetate anhydrous to allow for a longer duration of infertility.

As the GnRH aminoacidic sequence is highly conserved and as desensitization always occurs following continuous exposure to GnRH agonists (such as deslorelin), several research teams have investigated the potential of deslorelin to control reproduction and/or to prevent or suppress sex hormone-related behavior and disease in male and female cats.

Deslorelin implants are inserted subcutaneously through a needle between or in the region of the shoulder blades. If easy removal of the implant is desired (e.g., to shorten the duration of action), the implant may also be inserted into the umbilical area (Romagnoli et al., 2019).

Deslorelin implants have been shown to effectively, safely and reversibly postpone puberty or suppress reproductive function and related behaviors in male and female cats (Fontaine, 2015). In sexually mature cats, the duration of efficacy of a 4.7 mg deslorelin implant has been shown to be approximately 24 months (range 16–37 months) in females (Goericke-Pesch et al., 2012). The effects of deslorelin are reversible, as demonstrated by fertile matings approximately 2 years post-treatment in both male and female adult cats (Fontaine, 2015).

1.5.1.3 PROGESTINS

Synthetic analogues of progesterone, or progestogens, are sexual steroids commonly used to control reproduction in many mammalian species, including humans (Chatterton RT Jr, 2012).

Progestins effectively inhibit ovulation through changing the release of hormones from the ovaries (oestradiol, inhibin, and/or activin), leading to lack of adequate pituitary stimulation and absence of peak levels of FSH and LH that occur before ovulation (Beijerink et al., 2008).

Their negative feedback mechanism on the hypothalamus and pituitary, which triggers a sequence of cascade-type events (inhibition of GnRH release, followed by arrest of pituitary LH and FSH release, and the consequent block of estrogen and progesterone secretion), is harmless (Edwards M & Ahmet S, 2024).
However, progestogens have several target organs within the reproductive and endocrine systems upon which they will inevitably produce an effect while exerting negative feedback on the hypothalamus.

Physiological effects of progestogens at appropriate doses (Romagnoli & Ferre-Dolcet, 2022) are:

- Hyperplasia of the endometrium and mammary parenchyma;
- Mild lowering of adrenocorticosteroid secretion;
- Increased secretion of prolactin and growth hormone;
- Mild insulin resistance;
- Mild behavioural modifications (lack of display of reproductive behaviour, calmer attitude)

Such effects are normally innocuous given appropriate drug dosing and patient choice. Unfortunately, however, in the past both drug dosing and patient choice have been made without adequate knowledge and/or consideration of the scientific evidence. For this reason, progestogens earned a negative reputation as dangerous drugs that induce mammary and uterine pathology.

For example, in 2009 in an 11-year-old domestic shorthair intact queen treated with medroxyprogesterone acetate (MPA) for 9 years, multiple abnormalities were described such as bilateral ovarian cysts, cystic endometrial hyperplasia and pyometra (CEH–P), mammary adenoma, fibrosarcoma and cystic-papillary adenocarcinoma. Histopathologically, follicular ovarian cysts, CEH–P, and benign and neoplastic mammary lesions were identified (Keskin et al., 2009).

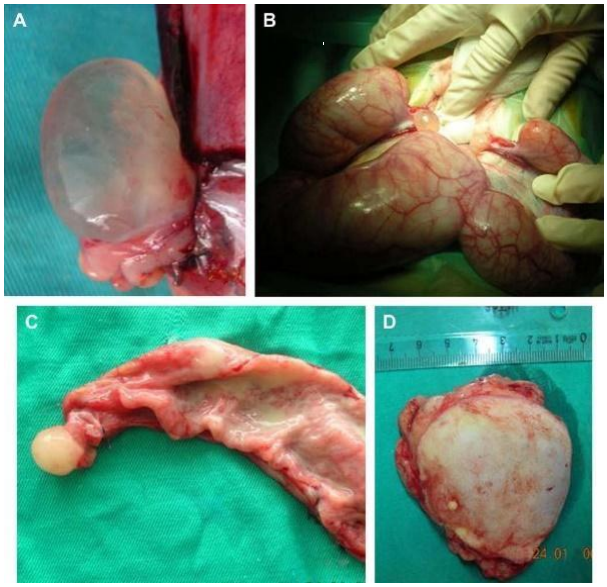

Figure 16. Effects of a 9 years long medroxyprogesterone treatment in the reproductive organs of a female cat: follicular cyst in the right ovary (A), fluid-filled uterus (B), pus in left uterine horn (C) and mammary tumor in the right inguinal gland (D) (Keskin et al., 2009).

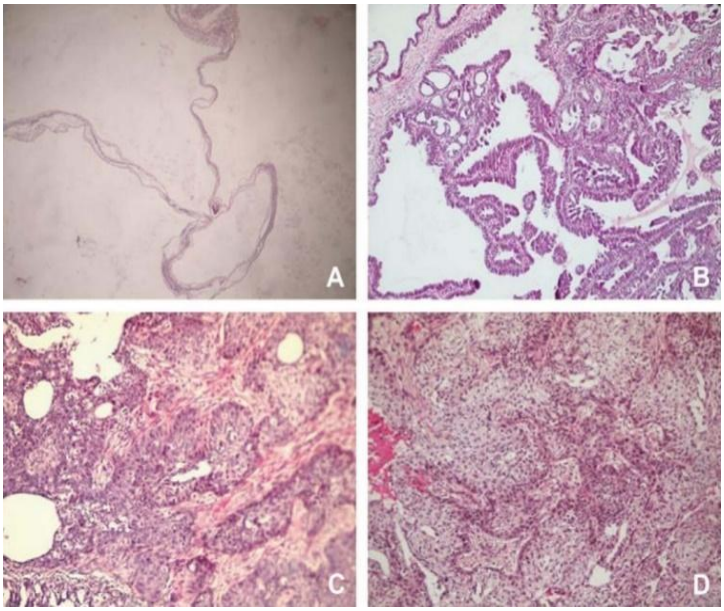

Figure 17. Follicular cyst (A) in ovary. Cystic-papillar areas (B) in uterus. Fibrosarcomatous (C) and hyperplasic areas in alveolar epithelium (D) in mammary glands. (Keskin et al., 2009)

Progestins are used in zoo felids to regulate fertility and also in these species there are possible side effects. A 2002 study demonstrated that melengestrol acetate (MGA) increases the risk of development of advanced CEH and related lesions. In particular, the genital tracts of 212 zoo felids (99 MGA treated and 113 control) representing 23 species were evaluated. Adenomatous and cystic hyperplasia were prevalent in both MGA-treated (85%) and control (61%) groups, and the risk of developing these lesions increased with age (Munson et al., 2002).

Due to the potential harmful side effects of progestins and in order not to intensify the physiological ones, careful patient selection is crucial.
Patients to be avoided in case of treatment with progestins are:

- Prepubertal queens: in the first luteal phase there are rare cases of mammary hyperplasia.
- Pregnant queens: progestogens block the onset of the first stage of labour.
- Queens with mammary conditions (e. g. mammary hyperplasia or mammary neoplasia).
- Queens in diestrus: to avoid overexposure to progesterone
- Queens with uterine discharge: it is a condition that must be treated ad completely resolved before any administration of reproductive hormones. Progestins may worsen a uterine condition (cystic endometrial hyperplasia/ pyometra).
- Queens with prolonged heat: progestins can cause estrous signs to disappear hiding an underlying condition that will be left untreated (Romagnoli, 2015).

The progestogens more commonly used in cats are: megestrol acetate (MA), MPA, proligestone, levonorgestrel and chlormadinone acetate (CMA). MA and levonorgestrel have a shorter duration of action compared to the other drugs.

In queens, long-acting drugs such as medroxyprogesterone acetate and proligestone are normally marketed as depot injections for estrus postponement, while short-acting drugs such as MA (which queens also respond well to when used long-term) are marketed as oral compounds for estrus suppression.

1.6 MEGESTROL ACETATE (MA)

MA is the shortest acting progestin available on the veterinary market. It is currently commercially available as an oral formulation (syrup) in Italy, under the name of Estropill™, MSD, Italy.

1.6.1 PHARMOCOLOGY

MA (6-methyl-6-dehydro-17α-acetoxyprogesterone) is a progestin with an affinity estimated to be several times greater towards progesterone receptors than endogenous progesterone (Janne, et al. 1978). It has antigonadotropic effects, weak partial androgenic activity (Luthy IA, et. al 1988) and weak glucocorticoid activity (Schindler et al., 2003).

The oral bioavailability of megestrol acetate is approximately 100% (Kuhl H, 2005).

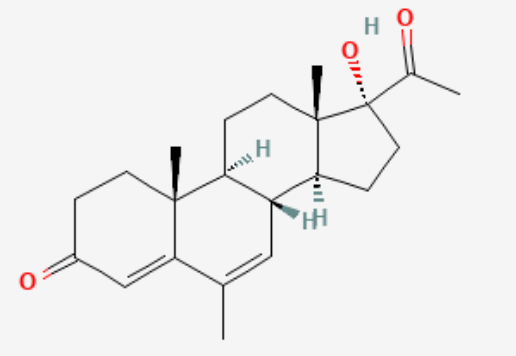

Figure 18. Chemical formula of megestrol acetate (MA)

1.6.2 HISTORY OF MA

In the 1930s it was discovered that progesterone administered by intramuscular injection was capable of blocking ovulation in rabbits. A quest for progesterone-like substances that could control reproduction ensued, leading to the synthesis and characterization of several progestins with potential contraceptive application, including MA.

In the U.S., starting in 1975, MA was sold as a canine contraceptive under the name Ovaban™. Its use in cats was off label. Ovaban™ is no longer produced but generic forms of MA continue to be available in the U.S.

In 1980s and 1990s MA ended up being frequently used for reproductive and non-reproductive indications at dosages of 5 mg/cat 1–3 times/week for months (the so-called 'feline silver bullet'). For a 4.0 kg cat, a 5.0 mg/cat/week dosage is equivalent to 1.25 mg/kg/week, which is an extremely high dosage when considering that satisfactory

control of reproduction with almost no side effects can be obtained in queens using lower doses (Romagnoli, 2015).

Efficacy and safety of MA were reported in some studies published in 1977 (Houdeshell & Hennessey, 1977; Oen EO, 1977). Unfortunately, neither paper was considered and use of MA at extremely high dosages became commonplace. Overdosing was responsible for several reproductive and endocrine side effects, which were widely reported in the world literature during the 1980s and 1990s (Romagnoli, 2015).
An extralabel formulation of MA (FeralStat) was also developed in North America for use in cats and privately marketed from 2008 by a veterinarian (Dr John Caltabiano) outside of regulatory oversight (Greenberg et al., 2013).
FeralStat was popular with some American cat colony managers, although no scientific data exist on this product's efficacy and safety in cats. By 2011, after the passing of Dr Caltabiano, FeralStat orders were no longer being fulfilled (Romagnoli, 2015).

1.6.3 DOSAGE

MA dosages can be labelled as low, medium and high, based on a combination of the dose given in a single treatment, the frequency and the duration of treatment. Research strongly suggests that higher dosages increase the risk and severity of adverse reactions.

Low dosages, despite MA short half-life, are efficacious for prolonged estrous postponement in cats for up to 30 weeks. The low dosage currently recommended is 0.625 mg/kg/week, approximately 11.5 µg/kg/day, which correspond to 5 drops/kg/day (Oen EO, 1977) (Romagnoli, 2015). This protocol can be considered relatively safe for cats.

Intermediate dosage can be defined as > 0.625 mg/kg per day for one week or q48h for 2 weeks. In this case it is possible to see short term reversible side effects like adrenocortical suppression and diabetes mellitus. (Romagnoli, 2015)

Intermediate dosages administered for long periods of time (several weeks, months, years) can be considered high dosages. (Romagnoli, 2015)

Recently published studies reported efficacy and safety of very low (0.010–0.015 mg/kg q24h) doses of MA administered to cats for prolonged periods of time (Grassi et. al., 2024).

1.6.4 SIDE EFFECTS

MA side effects, often associated with excessive dosages, can be categorized as: reproductive and non-reproductive.

1.6.4.1 REPRODUCTIVE SIDE EFFECTS

Reproductive complications can be uterine enlargement, pyometra, uterine adenomyosis (Bulman-Fleming J., 2003), cystic endometrial hyperplasia (CEH) (Bellenger & Chen, 1990), mammary hyperplasia (Hinton M and Gaskell CJ, 1977), mammary nodules (benign and/or malignant) and mammary adenocarninoma.

Pyometra is a pus-producing infection of the uterus, most often in association with a bacterial infection. In a field trial of 244 cats that were administered MA on a weekly basis (2.5 mg/cat), one cat developed pyometra after 3 years of treatment (Oen, 1977).

1.6.4.2 NON-REPRODUCTIVE SIDE EFFECTS

Possible non-reproductive side effects are diabetes (Pukay BP., 1979), increase of appetite, increase of body weight, adrenocortical suppression, cutaneous xanthomatosis (Kwochka & Short, 1984) and blindness, due to hyperlipidaemia causing opacity of the anterior chamber (Herrtage et al., 1985).

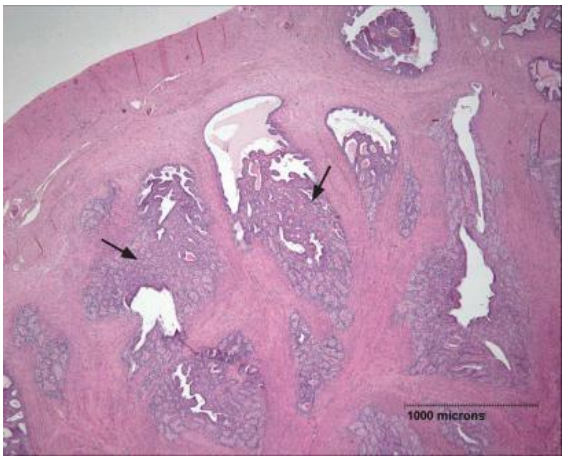

Figure 19. Photomicrograph of a uterine horn section showing endometrial proliferation and invasion of the myometrium characteristic of uterine adenomyosis (arrows). Hematoxylin and eosin. Bar = 1000 μm. (Bulman-Fleming J., 2003)

Patients to be avoided in case of treatment with MA are mentioned in chapter 1.5.3.3.

2. MATERIALS AND METHODS

The aim of this study was to investigate the potential histological effects of the reproductive tract of queens (uteri and ovaries) treated with low dosage of MA. This study was approved by the University of Padova Ethics Committee (Project n ° 57 /2024).

2.1 ANIMALS

This study included female cats according to the following criteria:

- Intact
- Post-pubertal
- Healthy and with no history of past reproductive problems
- Vaginal cytology not compatible with estrus
- Serum progesterone < 2.0 ng/ml (not in diestrus)

2.2 STUDY DESIGN

The study was carried out in the Veterinary Teaching Hospital (VTH) of the University of Padova using the following protocol:

1. Pre-treatment visit
2. Monthly check-up visit
3. Post-treatment visit
4. Confirmation of estrous
5. Surgical sterilization (ovariohysterectomy)
6. Histopathology of the ovaries and uterus

2.2.1 PRE-TREATMENT VISIT

Before the beginning of the treatment, in order to verify all the enrollment criteria, the queens underwent a thorough clinical exam. If the criteria were met, the queen was enrolled in the study and her owners informed about MA effects and how to use it.

The pre-treatment visit included: history, general physical examination, reproductive tract examination, vaginal cytology, blood collection for hematology, biochemistry and progesterone assay (P4), urine collection (urinalysis) and abdominal and reproductive ultrasound.

For history the following set of information was collected: signalment (age, breed), lifestyle (indoor/outdoor), eating habits, attitude of the animal, contact with other conspecifics or other animals, prophylaxis, puberty age, matings, date of last heat and its length, estrous behavior, previous pregnancies and parturition (and litter size), past reproductive problems and previous treatments.

The general physical examination included: skeletal state and body conformation, state of nutrition (measured through the body condition score (BCS)) and muscle tone, weight, level of consciousness and attitude, skin and subcutaneous tissue, mucous membrane color and capillary refill time, lymph nodes, body temperature, respiratory rate and respiratory assessment, heart rate and cardiac auscultation and abdominal palpation.

Reproductive tract examination focused on visual inspection and manual palpation of external genitalia and mammary glands. In that way it was possible to exclude abnormalities, lesions, abnormal discharges and inflammation. The mammary glands were examined to observe their shape, dimension and color and to determine eventual presence of nodules, hyperplasia, swollen areas and abnormal discharges from nipples.

Then, a vaginal smear was collected from queens using non-sterile disposable latex gloves. A cotton swab was damped with running water and introduced horizontally approximately 1-2 cm into the vagina of the queen and then pulled out. The cotton swab was then rolled on a glass microscope slide .

Afterwards, the slide was stained with Diff-Quik stain (MGG quick stain, BioOptica), a commercial Romanowsky-type stain with a methanol-based fixative, an eosin-based acid solution, and a thiazine-based basic solution. The slide was immersed 5 times in all 3 solutions in the above-mentioned order and then washed with running water, being careful not to direct the stream of water directly onto the smear. The slides were left to dry naturally.

The slide was then analyzed under a light microscope (Eclipse CiTM, Nikon, Tokyo), at low magnifications (40x and 100x) to assess cellularity and background “clearing”. Cellular types and the degree of keratinization were examined at higher magnifications (200x and 400x).

The estrus cycle of each queen was staged based on the ratio between cornified/keratinized (superficial and anucleate) and non-cornified/non-keratinized (intermediate and parabasal) cells, along with the presence of polymorphonuclear cells.

Diagnosis of estrus was based on the detection of >70% cornified cells in a slide with moderate to high cellularity and a clear background. Anestrus was characterized by a vast predominance of intermediate and parabasal cells, along with scarce cellularity. Interestrus (postestrus) and diestrus were defined cytologically by a mixed population of cornified and non-cornified cells, tendentially with the latter in higher proportion in interestrus. However, these 2 cycle phases were distinguished through serum P4 assay.

Then, blood and urine samples were collected. If necessary, the animal was sedated to reduce stress during manipulation and to facilitate sample collection. The sedation was carried out through a combination of drugs injected intramuscularly in the thigh: butorphanol (0.2 mg/kg), dexmedetomidine (6 mcg/kg) and ketamine (1 mg/kg).
Blood was collected from the jugular vein with the animal placed in sternal recumbency and neck extended. The sampling zone was shaved and disinfected with alcohol and a 22G needle attached to a 5 ml syringe was positioned at the level of the vein. Then, the blood sampled was transferred into 2 tubes: an EDTA K2 tube for hematology and a plain tube for biochemistry and P4.
The EDTA tube contains an anticoagulant, so after blood filling it was immediately and gently shaken in order to avoid the formation of clots. Whereas the plain tube was left upright to allow coagulation.
Blood samples were analyzed in the laboratory of the VTH. After 1000G centrifugation of the blood contained in the plain tube, serum was utilized for biochemistry (BT 1500TM; Biotecnica, Rome) and P4 assay (Automated Immunoassay Analyser-360TM, Tosoh, Tokyo). This machine determined P4 levels through a fluorescence enzyme immunoassay (FEIA).
Hematology and biochemistry were performed to investigate the health state of the animal. Their results were compared with post-treatment exams to verify MA safety.

P4 assay and vaginal cytology helped to understand the phase of the estrous cycle of the queen because, as aforementioned, the treatment could be started only in anestrous or interestrous to avoid an overload of P4. If the P4 was under 2 ng/ml the queen could start

the treatment with MA, whereas if the P4 level was above 2 ng/ml the treatment was postponed. So, in this case the pet owners were re-called for a new pre-treatment visit after the necessary time to rule out pregnancies, non-fertile matings or spontaneous ovulation.

The last part of the pre-treatment appointment consisted in abdominal and reproductive ultrasound. The ultrasound was conducted using an ultrasound unit and a micro convex probe of 5-8 MHz (Affinity 50TM, Philips, Amsterdam). Queens were positioned in dorsal recumbency in U-shaped pillows and shaved in the abdominal area. To support visualization the area was sprinkled with alcohol and the probe covered with gel ultrasound.
To find the ovaries the first organs searched were kidneys, because ovaries are located caudally to the caudal pole of the ipsilateral kidney. Ovaries examination allow to observe the eventual presence of fluid-filled structures like follicles, corpora lutea or cysts. The length and the height of both ovaries were taken.
The uterine horns were found caudally to the ovaries, and the uterine body dorsal to the urinary bladder and ventral to the colon. The uterus was observed to evaluate the presence of fluid in the lumen (e.g., pyometra, hydrometra), its diameter, the thickness of its wall and any other pathological alteration. Measuring the uterus allowed us to understand if the organ increased its dimensions because of the treatment with MA.
During abdominal ultrasound the bladder was visualized and cystocentesis was conducted to obtain a urine sample through a 22 G needle attached to a 5 ml syringe.

The urine sample was contained in a urine tube (vacUaptacaTM) and sent to VHT Padova laboratory to be analyzed macroscopically, physiochemically and microscopically. Also, protein-creatinine ratio and sediment analyses were done.

At the end of the pre-treatment visit if the queen complied with all the criteria, the owners received indications for administering MA (Estropill^{TM}, MSD Animal Health S.r.l). The posology was 5 drops/kg/day of body weight, and it was suggested to give them mixed with food or directly into the oral cavity at the same time each day. It was told to the owners to ensure that the queen assumed all the dosage.

2.2.2 MONTHLY CHECK-UP VISIT

Each month until the end of the treatment, a check-up visit at the VTH was organized to verify that MA did not cause health problems, and that the dosage administered was effective. The duration of the treatment was decided according to the owner's requests.
These visits included collection of history, general physical examination, reproductive tract examination, vaginal cytology and abdominal and reproductive ultrasound (Annex 1).

2.2.3 POST-TREATMENT VISIT

At the end of the treatment another visit was organized in order to evaluate the general health of the queen and MA safety and effectiveness. The steps done in this visit are the same described in the pre-treatment one.

2.2.4 CONFIRMATION OF ESTROUS

To the owners were told to refer to the VTH when their queens showed their first signs of heat after the treatment. The confirmation of estrous was made through vaginal cytology. Owners were also asked to characterize the heat in terms of duration and behavior.

2.2.5 SURGICAL STERILIZATION

After the first heat post-treatment the queens underwent ovariohysterectomy.
The cats were premedicated with methadone (0.2 mg/kg), dexmedetomidine (8µg/kg) and ketamine (1mg/kg) administered in a single syringe intramuscularly. They were subsequently induced with propofol. The abdominal hair was clipped and the bladder was emptied by manual compression, with the animal in lateral recumbency as the distended bladder may hinder locating and exteriorization of uterus. The queens were intubated, and volatile anesthesia maintained with isoflurane.
A ventral midline approach was preferred. The location of the surgical incision was midway between the umbilicus and pubis following the linea alba.

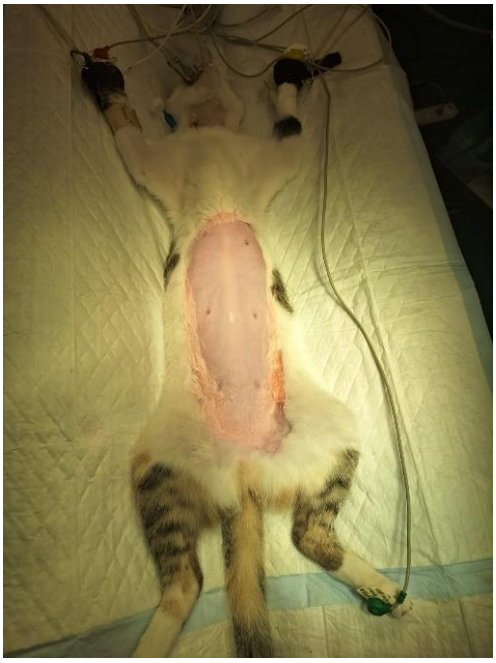

Figure 20. Positioning of the patient after hair clipping, scrubbing and anesthetic induction

A sharp dissection of the skin was made with a scalpel followed by a blunt dissection of the subcutaneous tissue along the midline with Metzenbaum scissors. Once the midline was identified it was elevated with a thumb forceps and punctured with a scalpel blade sharp side facing up.

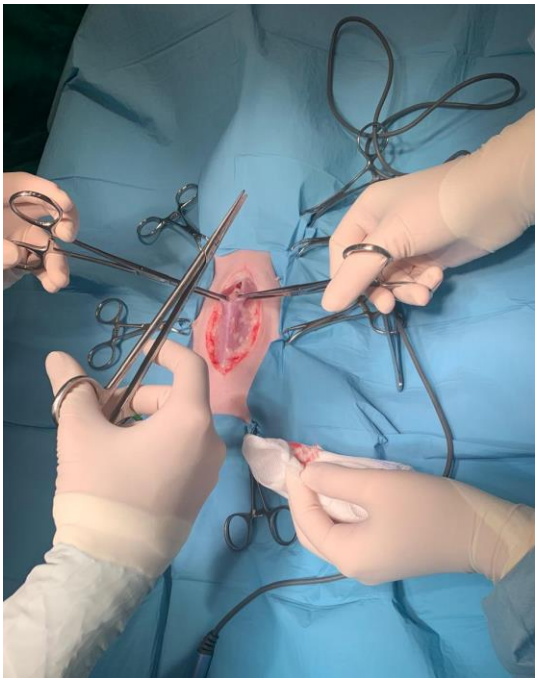

Figure 21. Dissection of the linea alba

After complete dissection of all tissues, ovaries and uterus were identified through palpation by inserting a finger along the abdominal wall. After the uterine horn was individualized, it was gently retracted and followed cranially until the ovary is located. Then, the ovaries were exteriorized.

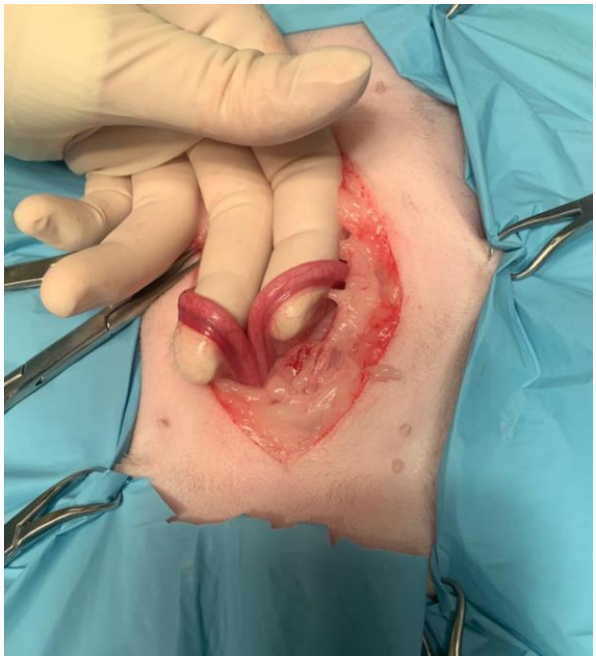

Figure 22. Identification of uterine horns

Forceps were placed on the proper ligament for retraction and the suspensory ligament was broken or stretched with digital pressure without damaging the ovarian vessels. Afterwards a window was made in the mesovarium immediately caudal to the ovarian vessels.

Three forceps (straight or curved conformation) were placed on the ovarian pedicle. A first ligature with absorbable multifilament thread 2/0 (Vicryl, Ethicon) was placed at least 2 to 3 mm distal to the most distal forceps and tightened. The most distal forceps wa s then released, and a second ligature with absorbable multifilament thread 2/0 placed in the crush. The ovarian pedicle was grasped with thumb forceps and cut between the remaining two forceps. The middle forceps were removed and, after confirming lack of bleeding, the pedicle was released into the abdomen.

Subsequently the mesometrium was bluntly dissected lateral to the vessels and electrocoagulated to achieve hemostasis. The uterine arteries were legated with absorbable monofilament 2/0 thread (PDS, Ethicon).

The cervix may be hard to differentiate from the uterine body in cats so determining how much, if any cervix has been removed at surgery, is often impossible. According to the surgeon's preference a Parker-Kerr suture with absorbable multifilament thread 2/0 (Vicryl, Ethicon) was done in the cervix and at least 3 mm cranial to that site the tissue was transected.

In absence of bleeding the abdominal wall was sutured with absorbable multifilament 2/0 thread (Vicryl, Ethicon) through a continuous suture. Subcutaneous and cutaneous tissues were sutured with absorbable monofilament 3/0 (PDS, Ethicon) threads through a simple continuous and an intradermic suture, respectively.

At the end of the surgery, a surgical knot was made in the left ovary in order to the distinguish the two ovaries during histopathological evaluation.

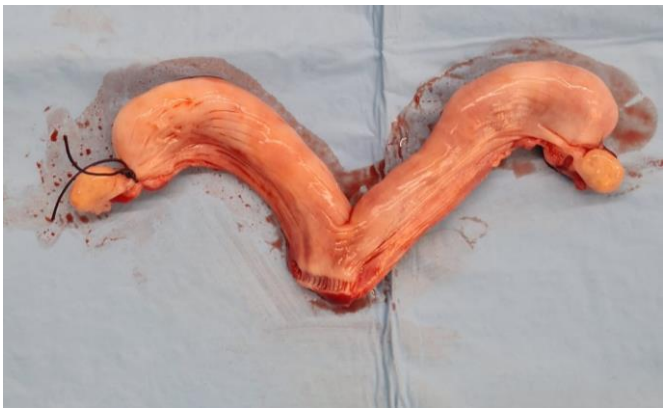

Figure 23. Ovaries and uterus of Subject 6 after ovariohysterectomy

Then the reproductive organs were placed in a closed plastic container and fully covered with 10% neutral buffered formalin solution. Meloxicam (0.1 mg/kg) was administered intravenously right after the surgery. Post-operatory treatment consisted in meloxicam (0.05 mg/kg) orally once a day for 4 days from the day after the surgery. The owners were instructed to control the surgical wound daily and to keep the queen on an Elizabethan collar or a surgical body to avoid complications. A control visit was scheduled after one week from the surgery.

2.2.6 HISTOPATHOLOGY OF OVARIES AND UTERUS

The histopathology of ovaries and uterus was done at the laboratory of histology of the University of Padova.

Ovarian and uterine histological slides were made following these steps:

1. Fixation
2. Trimming
3. Tissue processing
4. Embedding
5. Sectioning
6. Staining
7. Mounting

2.2.6.1 FIXATION

As aforementioned, ovaries and uterus were fully covered with 10% buffered formalin (volume 1:10). This was done to preserve the tissue in its natural state and prevent decay. The tissues were immersed in fixative for several hours to favor formalin penetration.

2.2.6.2 TRIMMING

Under the chemical fume hood using a sharp blade the tissues were trimmed to a thickness that allow them to be completely embedded with paraffin. Serial sectioning was obtained from the samples and placed in the cassettes (Bio-Optica, Milano).

Figure 24. Cassette Bio-Optica used in the study.

2.2.6.3 TISSUE PROCESSING

Tissue processing consists in replacing the water in the tissue with a medium that can be solidified for sectioning. In the laboratory of the University of Padova the cassettes were

processed by an automatic fully enclosed tissue processor (Leica ASP300S, Leica Biosystems, Wetzlar).

The cassettes were put in its pressured chamber to promote the penetration of the reagents. Then, the machine was powered to carry out the process.

The tissues were passed through a series of alcohol baths with increasing concentrations of ethanol (e.g., 50%, 70%, 90%, and finally 100%) to remove water. In this way the tissues were dehydrated. Then the alcohol was replaced with a clearing agent (xylene) which makes the tissue transparent. Finally, the clearing agent, ethanol and water were cleaned and replaced by paraffin wax.

Figure 25. Automatic fully enclosed tissue processor (Leica ASP300S, Leica Biosystems, Wetzlar)

2.2.6.4 EMBEDDING

The tissues after being processed by the automatic tissue processor were placed in a thermic unit (UT200, Bio-Optica, Milano) set at 64°C. It is an instrument used for the paraffin warming during embedding.

Figure 26. Thermic unit (UT200, Bio-Optica, Milano)

Then the sample was placed into a mold and filled with molten paraffin wax through a paraffin distributor (DP500, Bio-Optica, Milano). After that the wax was allowed to cool and solidify, forming a block in a cold plate (PF100, Bio-Optica, Milano).

Figure 27. Paraffin distributor (DP500, Bio-Optica, Milano)

Figure 28. Cold plate (PF100, Bio-Optica, Milano)

2.2.6.5 SECTIONING

The additional layers were removed from the block in order to bring the samples to the surface of the block. After that the blocks were placed in the freezer for 30 minutes to favor their manipulation during sectioning with the microtome.
Then the paraffin blocks were sliced into sections about 4-5 micrometers thick with a rotary microtome (Leica RM2145, Leica microsystems, Wetzlar).

Figure 29. Rotary microtome (Leica RM2145, Leica microsystems, Wetzlar)

The thin sections were then floated on a warm water (56°C) bath to remove wrinkles and transferred to glass microscope slides.
The slides with thin sections were placed in ranks and put into an oven and warmed (70°C) for some days to melt the paraffin.

2.2.6.6 STAINING

To differentiate and visualize various structures in the tissue, as most biological tissues are transparent, the slides were stained with Hematoxylin and Eosin (H&E). The staining was processed by a machine (Leica ST5020 Autostainer XL, Leica Biosystems, Wetzlar) using the program “Hematoxylin and Eosin”. The program consists of 18 step, in which the slices placed in racks were dipped in different reagents for a specific amount of time, as expressed in Table n°2.

STEP	REAGENT	TIME
1	Xylol	2 minutes
2	Xylol	3 minutes
3	Alcohol 100°	2 minutes
4	Alcohol 100°	2 minutes
5	Alcohol 95°	2 minutes
6	Alcohol 70°	1 minute
7	Water	15 seconds
8	Distilled water	20 seconds
9	Hematoxylin	2 minutes and 30 seconds
10	Water	7 minutes
11	Alcohol 95°	15 seconds
12	Eosin-phloxine	50 seconds
13	Alcohol 95°	30 seconds
14	Alcohol 95°	30 seconds
15	Alcohol 100°	1 minute
16	Alcohol 100°	1 minute
17	Xylol	2 minutes
18	Xylol	2 minutes

Table n.2 Staining protocol of Hematoxylin and Eosin (H&E)

The stains utilized were Mayer hematoxylin (DIAPATH s.p.a.) and eosin-phloxine (DIAPATH s.p.a).

Figure 30. Leica ST5020 Autostainer XL, Leica Biosystems, Wetzlar

2.2.6.7 MOUNTING

To preserve the stained tissue on the slide and prepare it for microscopy, a mounting medium (Eukitt®, Sigma-Aldrich, St. Louis) was applied to the slide, and a coverslip was placed over the tissue section. Then the slides were examined with a microscope at different magnifications.

3. RESULTS

3.1 ANIMALS

The study population was composed of 6 healthy post-pubertal female cats of different ages ranging from 9 months to 6 years presented to the VTH of the University of Padova for reproduction control. The subjects belonged to different breeds: European shorthair cat (n=4), Bengala (n=1) and Persian (n=1). Subjects were identified by a number (1-6) corresponding to the chronological moment of enrollment in the study.

The study period went from January 2021 to October 2023.

The duration of the treatment was variable depending on owners' request: 1 month (n=1), 2 months (n=1), 4 months (n= 3) and 6 months (n=1).

The following table (Table n. 3) shows the demographic details of each studied enrolled.

Number ID	Name	Age	Breed	Duration of the treatment
1	Marolly	9 months	European shorthair	2 months
2	Cloe	1 year	European shorthair	1 month
3	Margot	1 year and 8 months	European shorthair	4 months
4	Betty	6 years and 7 months	Persian	4 months
5	Otta	1 year and 4 months	European shorthair	4 months
6	Libellula	1 year and 3 months	Bengala	6 months

Table 3. Name, age, breed and duration of the treatment of the 6 queens enrolled.

3.2 PRE-TREATMENT VISIT

At the pre-treatment visit, four queens had serum progesterone < 2 ng/ml, while two queens had serum progesterone > 2 ng/ml without any mating with males (Subjects 3 and 5). Therefore, treatment was postponed for 20-30 days in these 2 queens to avoid an overload of progesterone. This way, all 6 queens initiated treatment while in interestrus or anestrus.

Subject	Pre-treatment P4 (ng/ml)
1	1,19
2	0,42
3	2,71
4	1,21
5	20,08
6	0,1

Table 4. Serum progesterone of the 6 queens during the pre-treatment appointment.

All queens but one showed an unremarkable reproductive ultrasound prior to treatment. Subject 4, however, presented cysts in both ovaries, which were not associated to any clinical symptomatology.

3.3 MEGESTROL ACETATE EFFECTS DURING TREATMENT

Five/6 queens did not show any sign of estrous, exhibiting behavioral and cytological anestrous. One queen (subject 6) displayed signs of estrous during the treatment: vocalization, lordosis and rubbing against objects.
No reproductive side effects were reported during the treatment nor any ovarian or uterine abnormality were observed during monthly ultrasound examination. Also, post-treatment blood and urine analysis results did not reveal any clinical significant deviations from the pre-treatment ones.

Three/4 queens, for which body weight data was available, increased their weight during the treatment. An increased appetite was reported in 4 queens. Post-treatment weight of subject 1 and subject 2 were not available.

N. subject	Pre-treatment weight (kg)	Post- treatment weight (kg)
1	3.3	-
2	2.8	-
3	4.2	4.2
4	3.2	4.0
5	3.2	3.8
6	3.7	5.6

Table 5. Comparison between pre-treatment and post-treatment weight (kg).

3.4 END OF THE TREATMENT

At the end of the treatment all 6 queens were in anestrous. Behavior, P4 and vaginal cytology were compatible with anestrus.

Subject	Post-treatment P4 (ng/ml)
1	0,57
2	1,42
3	1,5
4	0,78
5	0,78
6	0,45

Table 6 Serum progesterone of the 6 queens enrolled at the end of the treatment (ng/ml)

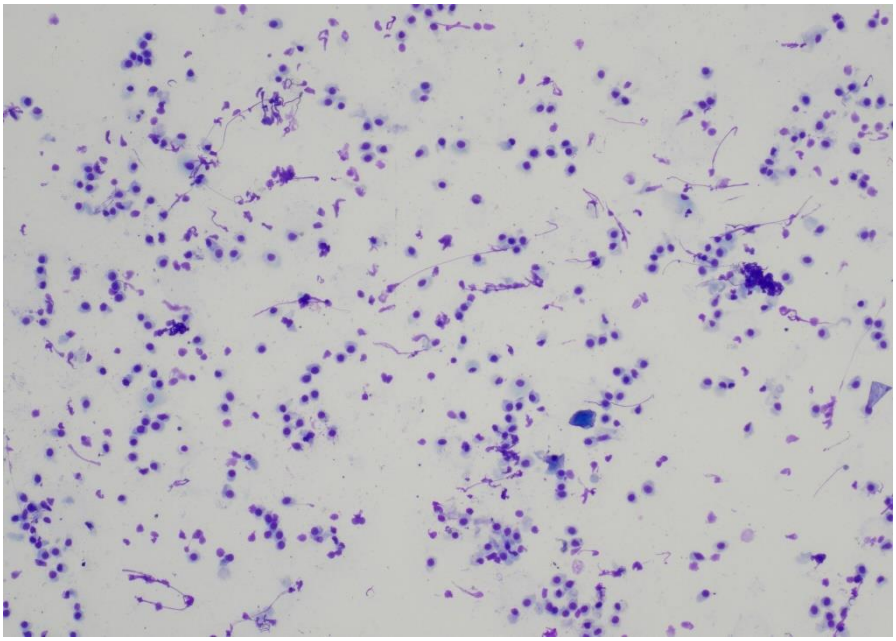

Figure 31. Post-treatment vaginal cytology of subject 3 indicative of anestrus: high cellularity, 100% of non-keratinized cells (predominantly parabasal and small intermediate cells), absence of polymorphonucleate and red blood cells.

3.6 RESUMPTION OF CYCLICITY

All the queens returned to heat after the treatment. Estrus was detected through behavioral manifestations and confirmed with a keratinized vaginal cytology (Figure 28). After the treatment all queens apart from n° 5 remained in good health. After two weeks of her first heat post-treatment, Subject 5 showed decreased appetite, depression and yellowish and bloody vaginal discharge. An ultrasound was conducted in another veterinary facility, due to geographic distancing from the VTH and a pyometra was diagnosed. The queen started antibiotic treatment (amoxycillin + clavulanic acid) and was surgical sterilized at the VTH in the following days.

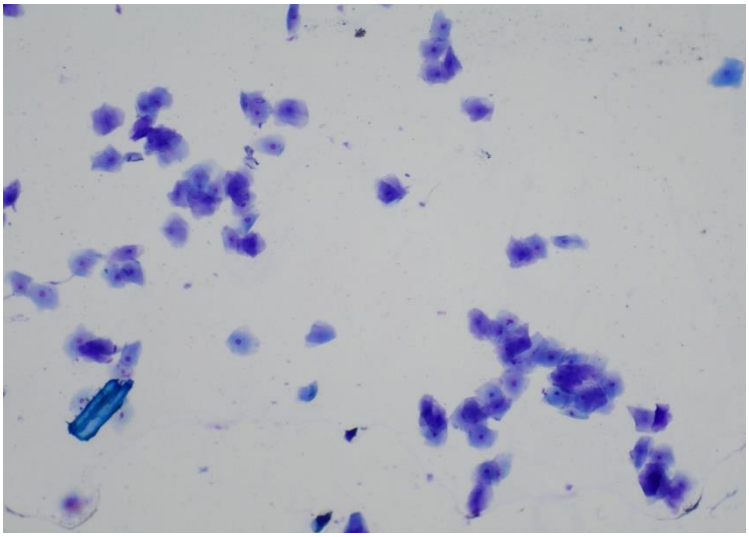

Figure 32. Vaginal cytology of subject 6 indicative of estrus. High cellularity 100% of keratinized cells (predominantly superficial, but also anucleate), absence of polymorphonucleate and red blood cells.

3.7 HYSTOPATHOLOGY

After sterilization, a macroscopic examination of the ovaries and uteri was performed. No alterations were identified in 4/6 queens. Whereas in two queens (Subjects 5 and 6), the uterus appeared hyperplastic.

The histological examination revealed a well-developed glandular mucosa in all queens. Evidence of hemorrhage in the uterine mucosa was found in 3/6 animals. A cystic and atypical endometrial hyperplasia and purulent endometritis were observed in one subject and ovarian cysts were detected in another, although these cysts were already present before treatment.

3.7.1 SUBJECT 1

The left ovary appeared with a parenchyma within physiological limits characterized by ovarian follicles in different stages of maturation and some corpora lutea. The right ovary appeared with a parenchyma within physiological limits characterized by ovarian follicles in different stages of maturation and many corpora lutea. The uterus presented a physiological aspect characterized by a well-developed glandular mucosa was observed.

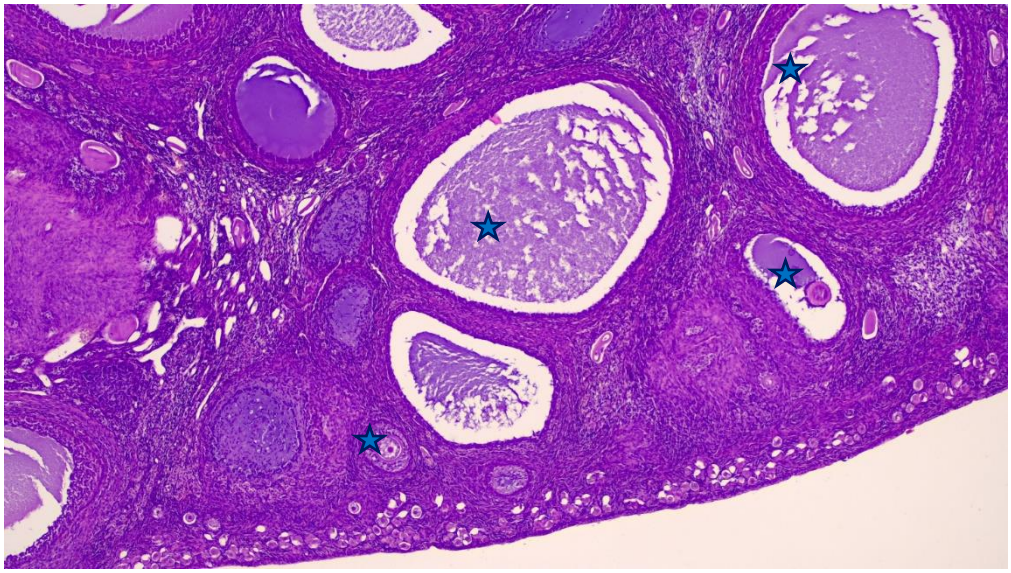

Figure 33. Left ovary (subject 1), 4 x magnification: ovary characterized by follicles in different stages of development (blue stars).

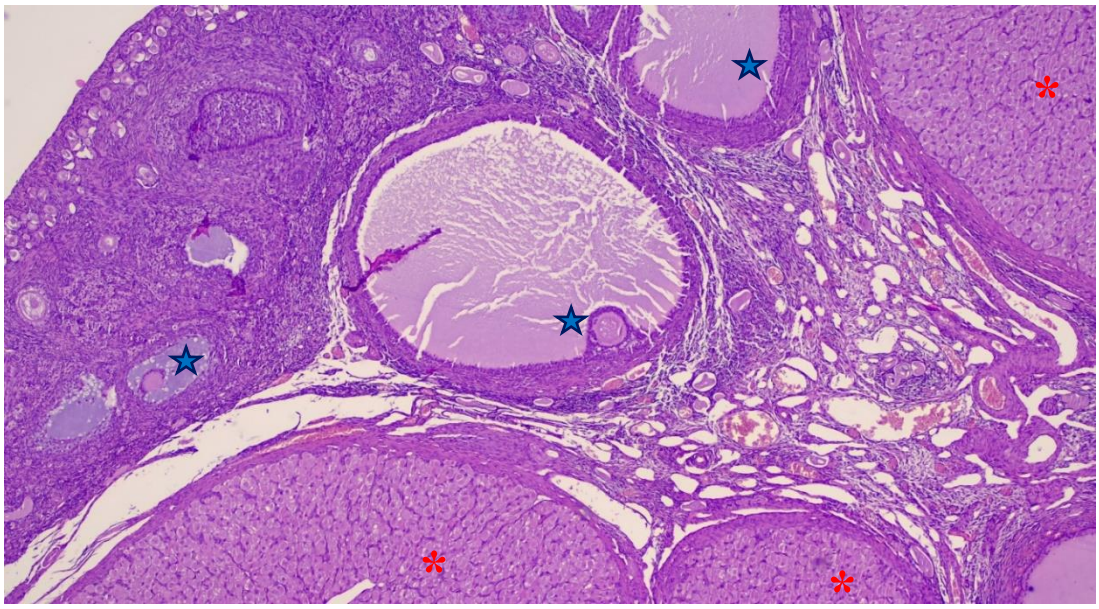

Figure 34. Right ovary (subject 1), 4 x magnification: ovary characterized by follicles in different stages of development (blue stars) and different corpora lutea (red asterisks)

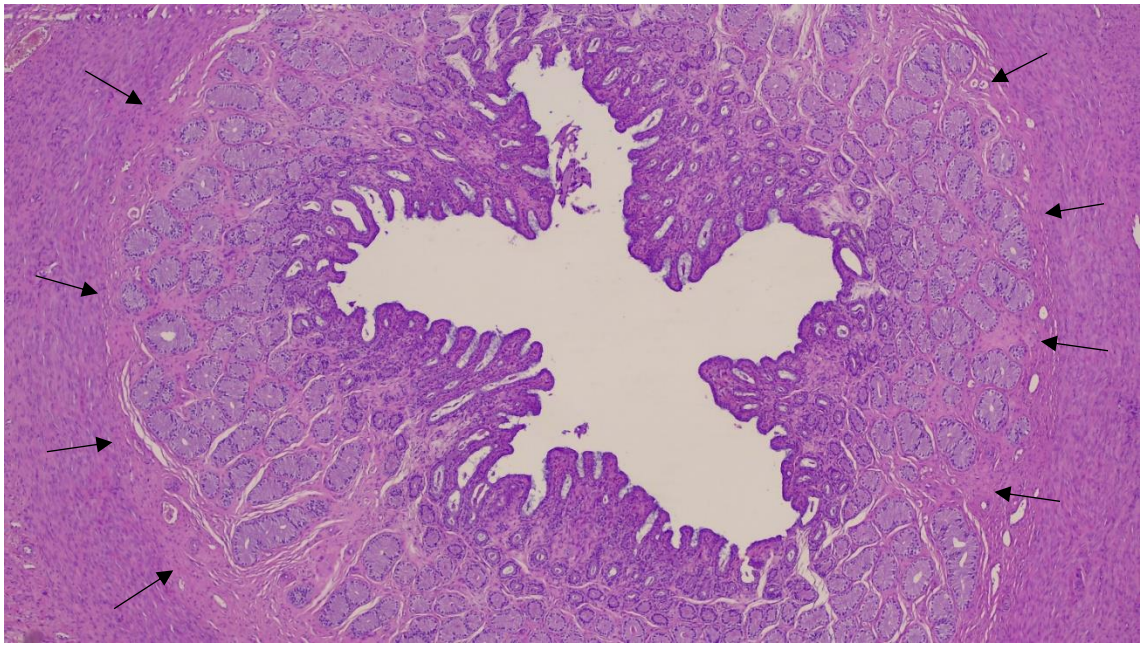

Figure 35. Uterus (subject 1), 4 x magnification: uterus with a well-developed glandular mucosa (black arrows)

3.5.2 SUBJECT 2

The left and right ovary presented a parenchyma within physiological limits characterized by ovarian follicles in different stages of maturation and no corpora lutea.

The uterus presented a physiological aspect characterized by a well-developed glandular mucosa. The mucosa was somewhat hemorrhagic.

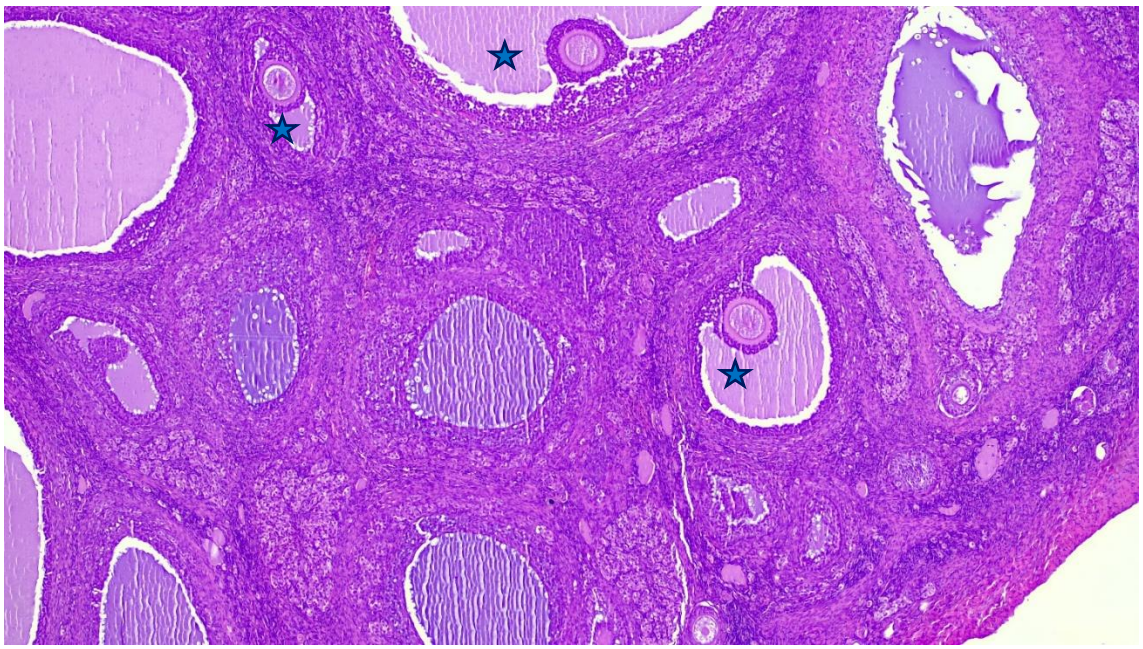

Figure 36. Left ovary (subject 2), 4 x magnification: ovary characterized by follicles in different stages of development (blue stars).

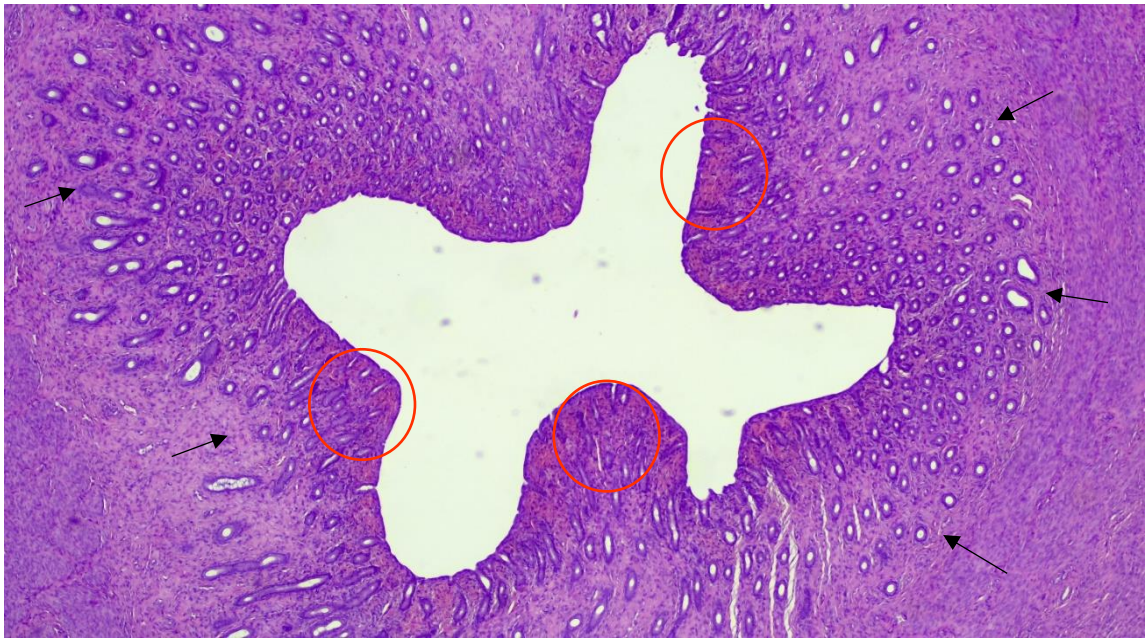

Figure 37. Uterus (subject 2), 4 x magnification: uterus with a mild multifocal superficial hemorrhage of the mucosa (red blood cells within red circles) and well-developed glandular mucosa (black arrows).

3.5.3 SUBJECT 3

The left ovary appeared with a parenchyma within physiological limits characterized by ovarian follicles in different stages of maturation and no corpora lutea. The ovarian vessels were congested.

The right ovary appeared with a parenchyma within physiological limits characterized by ovarian follicles in different stages of maturation and no corpora lutea.

The uterus presented a well-developed glandular and hemorrhagic mucosa.

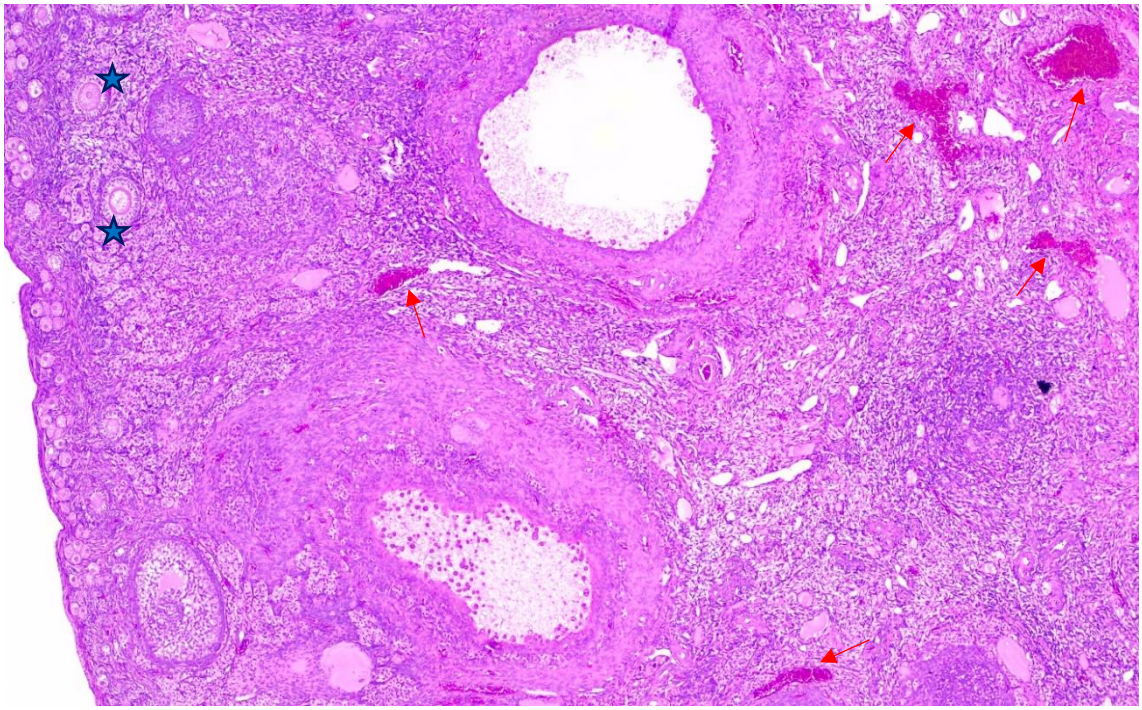

Figure 38. Left ovary (subject 3), 4 x magnification: ovarian parenchyma with congested blood vessels (red arrows) and follicles (blue stars).

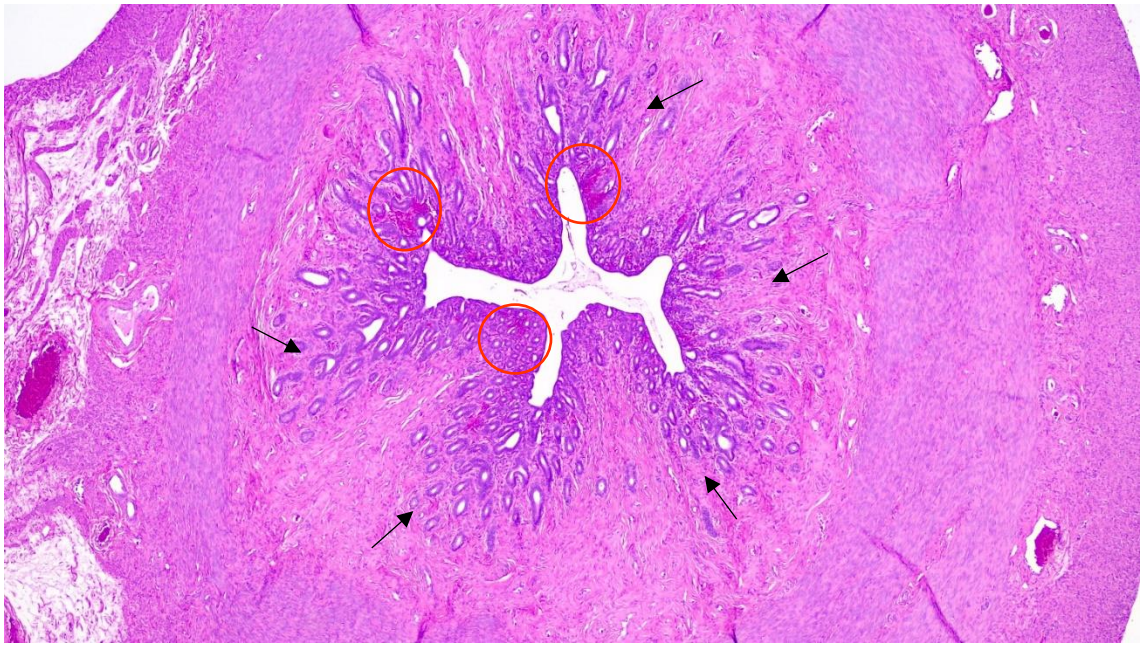

Figure 39. Uterus (subject 3), 4 x magnification: uterus with a hemorrhagic (red blood cells within red circles) mucosa and well-developed glandular mucosa (black arrows)

3.5.4 SUBJECT 4

The left and right ovaries were characterized by ovarian follicles in different stages of maturation and no corpora lutea. No hemorrhages were detected in the right ovary, while they were observed in the left ovary. Cystic formations of variable diameter were reported in both ovaries, some located within the ovarian parenchyma and others at the level of the hilum. The cysts were delimited by a monolayer of cubic or flattened epithelium, multifocally ciliated and devoid of atypical features. The cysts were classified in both ovaries as cysts of the rete ovarii.

In the uterus, mild multifocal superficial mucosal hemorrhages and presence of hemosiderophages deep in the submucosa were reported.

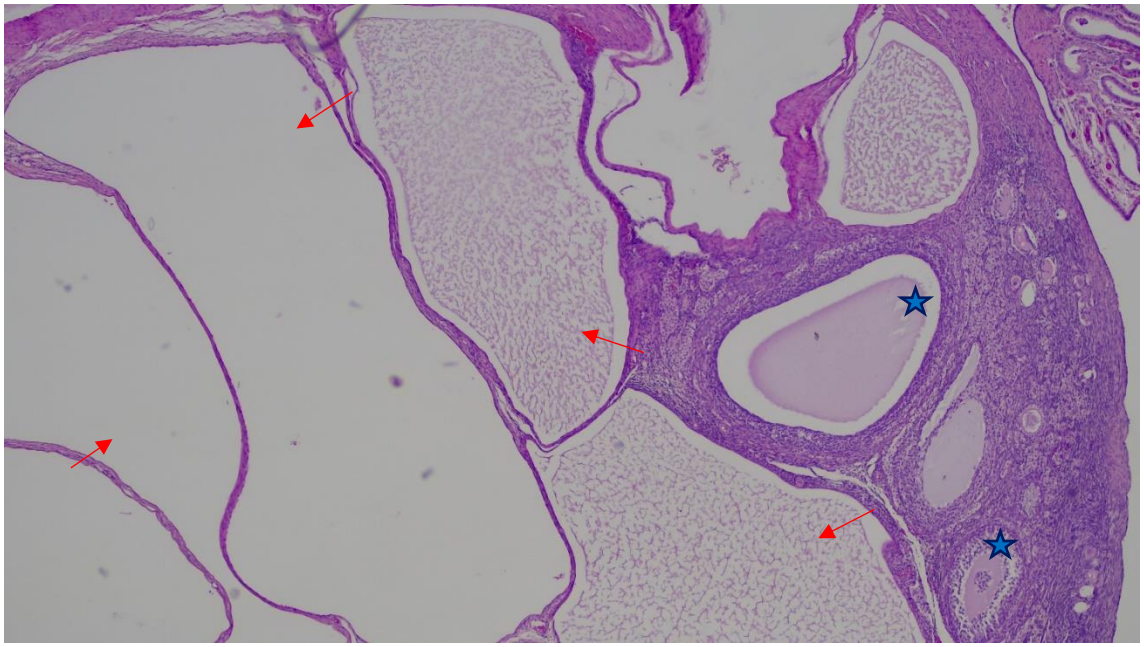

Figure 40. Left ovary (subject 4), 4 x magnification: ovarian parenchyma with follicles (blue stars) and cysts (red arrows)

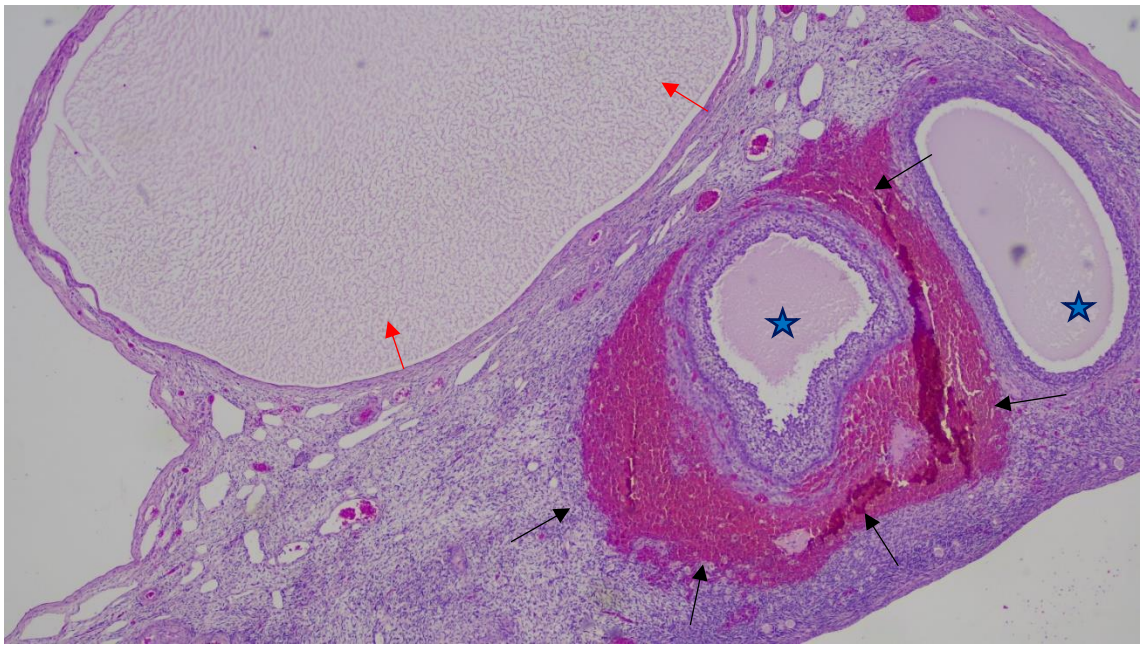

Figure 41. Right ovary (subject 4), 4 x magnification: ovarian parenchyma with follicles (blue stars), hemorrhages (black arrows) and a cyst (red arrows)

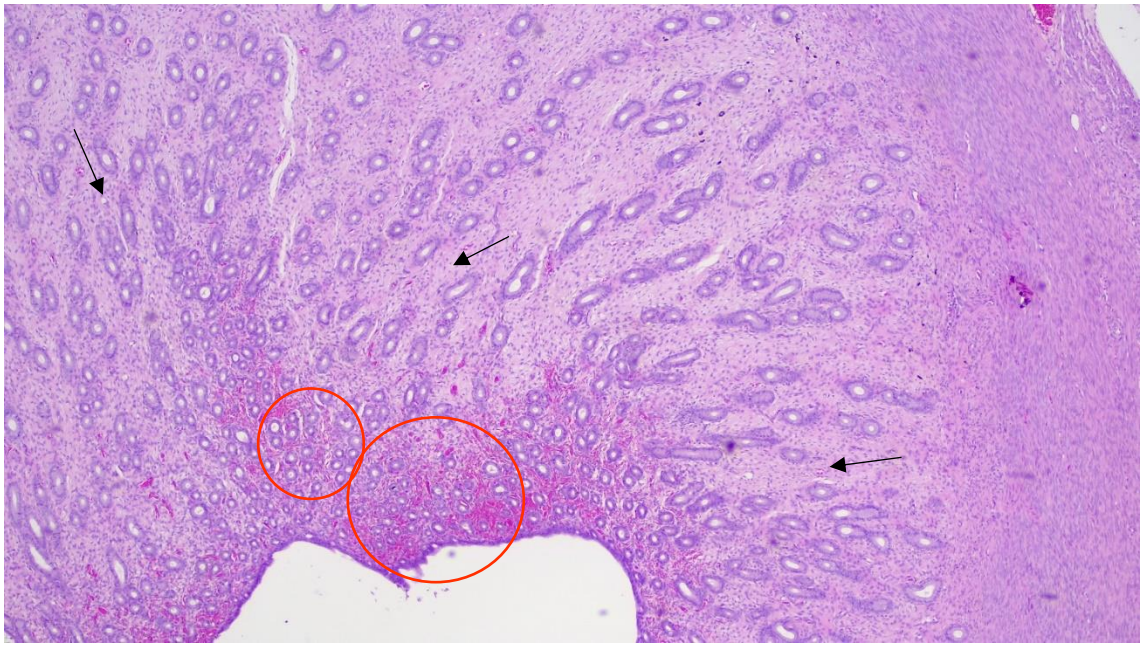

Figure 42. Uterus (subject 4), 4 x magnification: uterus with a hemorrhagic and well-developed glandular mucosa (black arrows)

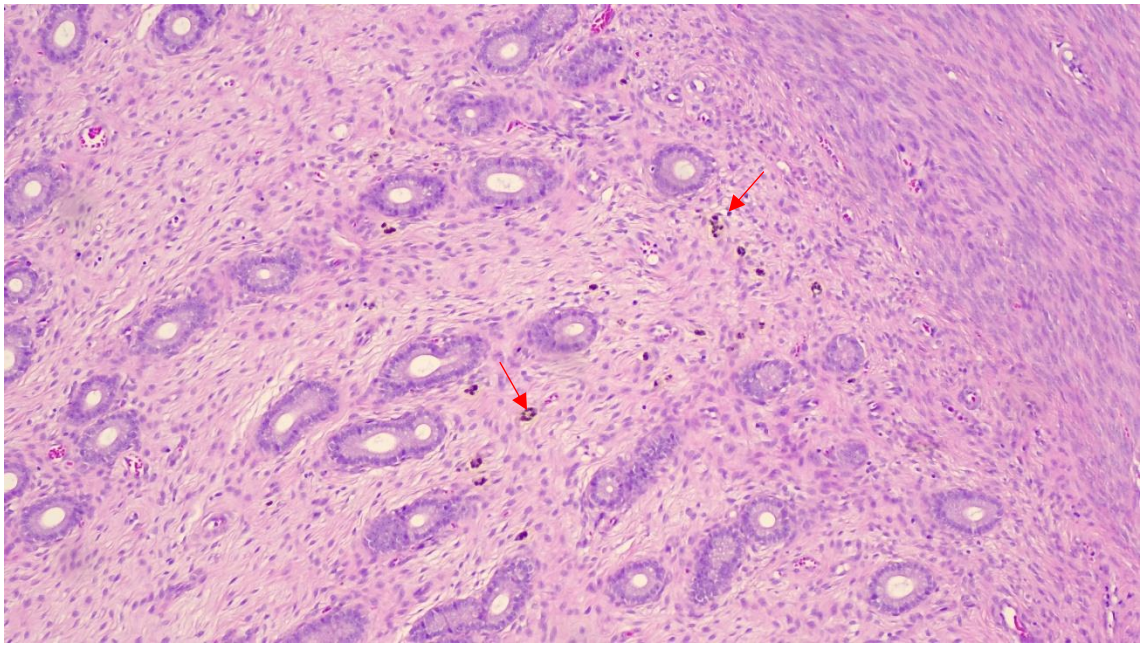

Figure 43. Uterus (subject 4), 10x magnification: deep uterine mucosa bordering the muscular layer, visible hemosiderin deposits (red arrows).

3.5.5 SUBJECT 5

The left and right ovaries appeared with a parenchyma within physiological limits characterized by ovarian follicles in different stages of maturation and corpora lutea of different size.

In the uterus, the mucosa appeared proliferative with the presence of cysts delimited by hypertrophic cylindrical elements and fibrous stroma associated with a moderate lymphoplasmacytic inflammatory infiltrate. At the level of the uterine body, recurrent endometrial micropapillae affecting the lumen were observed. The papillae were made up of elements with mild characteristics of anisocytosis (larger than normal variation of size of cells) and moderate multifocal anisokaryosis (larger than normal variation of size of the nuclei of cells). Many cells were multinucleated with irregular and hyperchromatic nuclei and some of them were in mitosis. The diagnosis was cystic and atypical endometrial hyperplasia associated with chronic moderate endometritis.

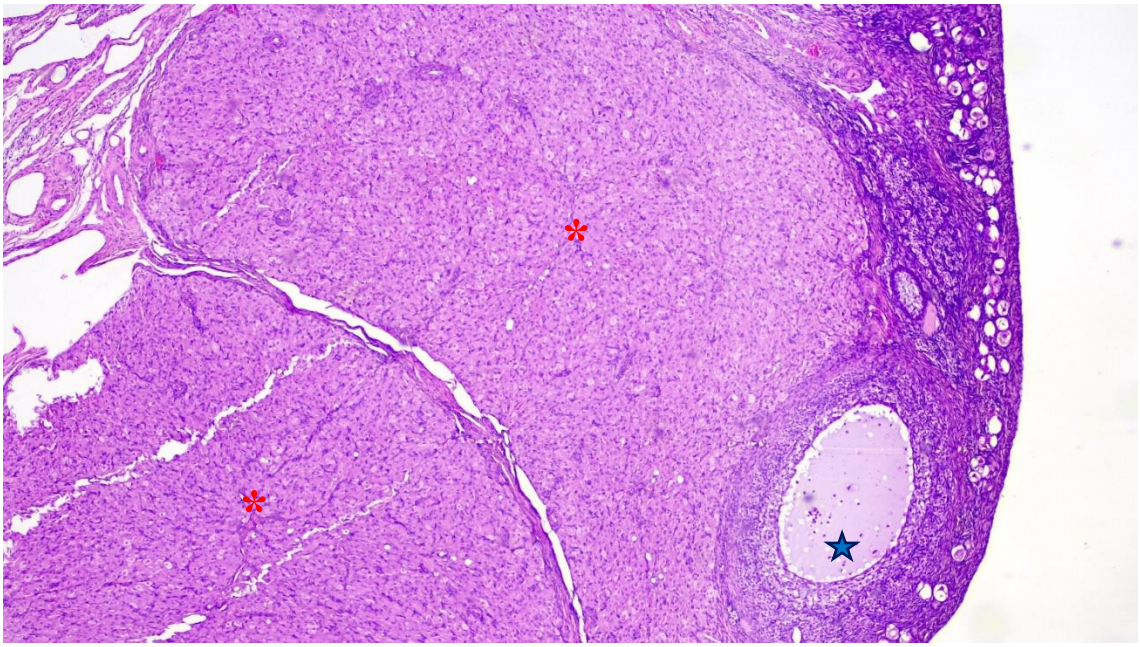

Figure 44. Left ovary (subject 5), 4 x magnification: ovarian parenchyma with corpora lutea (red asterisks) and a follicle (blue star)

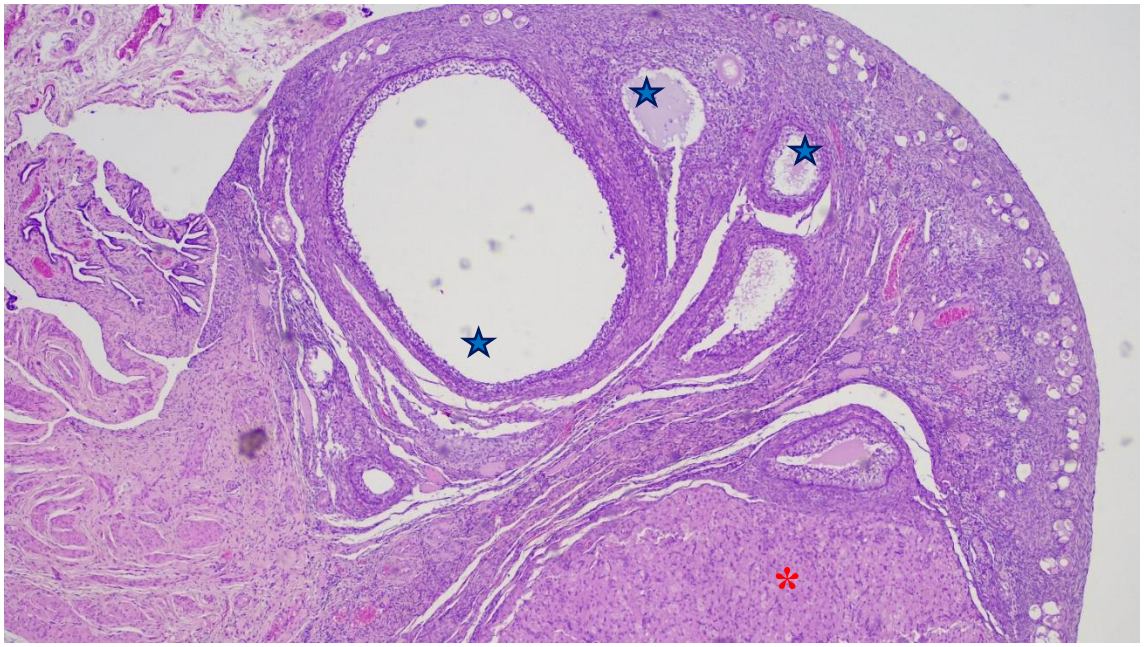

Figure 45. Left ovary (subject 5), 4 x magnification: ovarian parenchyma with corpora lutea (red asterisks) and follicles (blue stars).

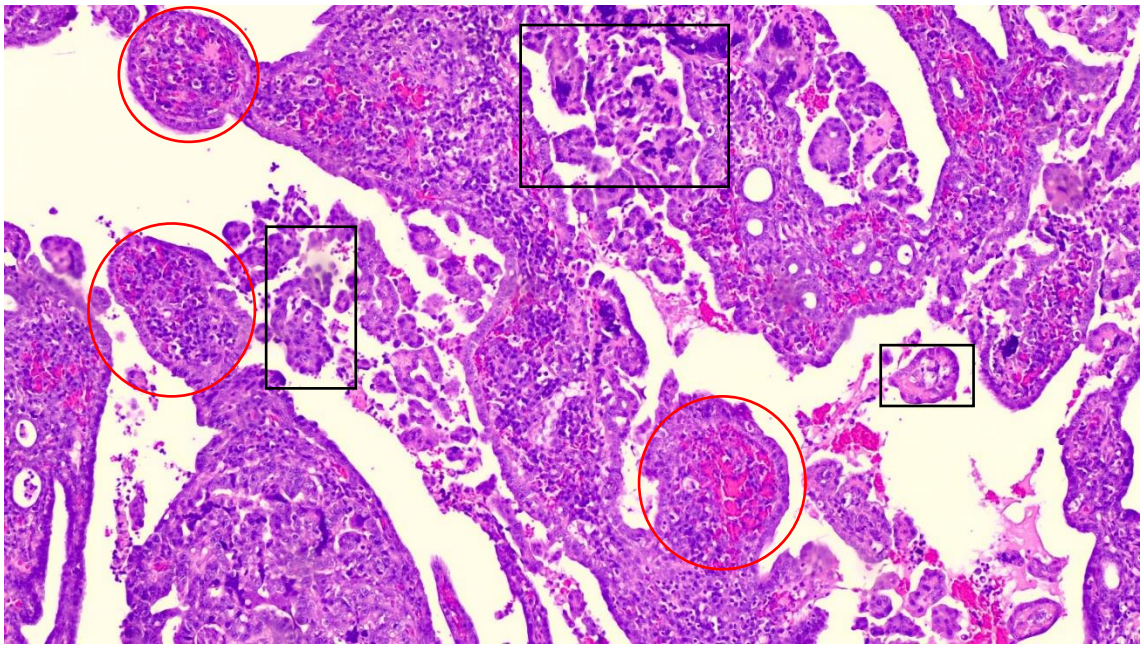

Figure 46. Uterus (subject 5), 10x magnification: cystic endometrial hyperplasia, papillae (red circles), syncytia (multinucleated cells) (black rectangles).

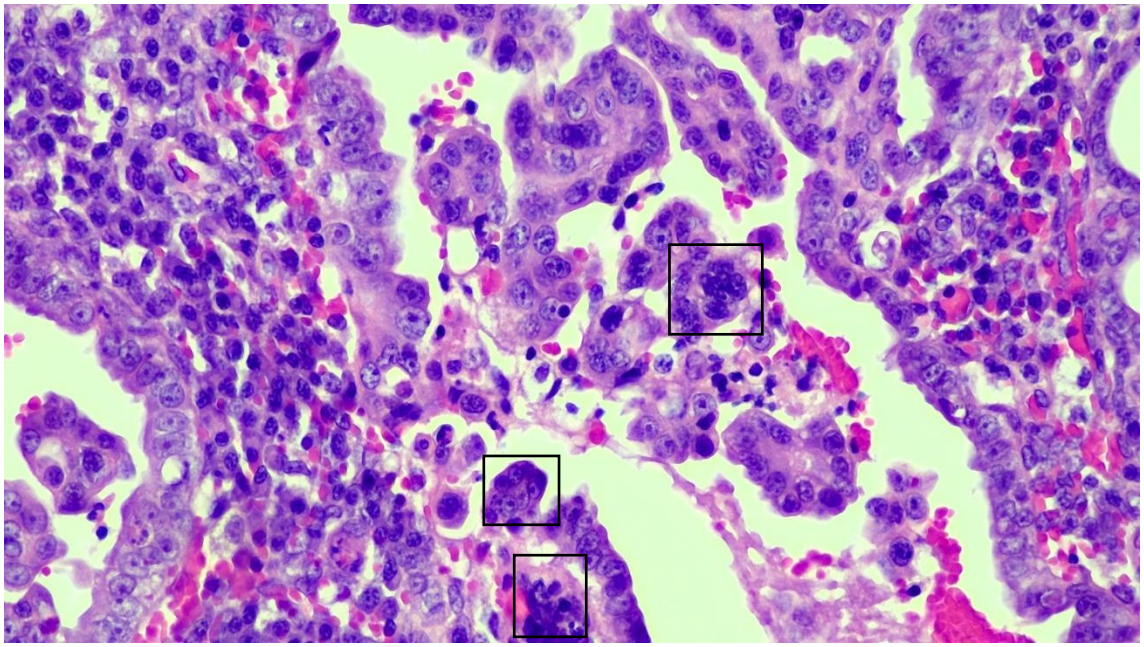

Figure 47. Uterus (subject 5), 40x magnification: moderate anisokaryosis, multinucleated cells-epithelial syncytia (black rectangles).

3.5.6. SUBJECT 6

The left ovary appeared with a parenchyma within physiological limits characterized by ovarian follicles in different stages of maturation and corpora lutea of different size.

The right ovary appeared with a parenchyma within physiological limits characterized by ovarian follicles in different stages of maturation and corpora lutea of different size.

The uterus was characterized by a well-developed glandular mucosa and mild accumulation of mucus in the lumen (mucometra suspect).

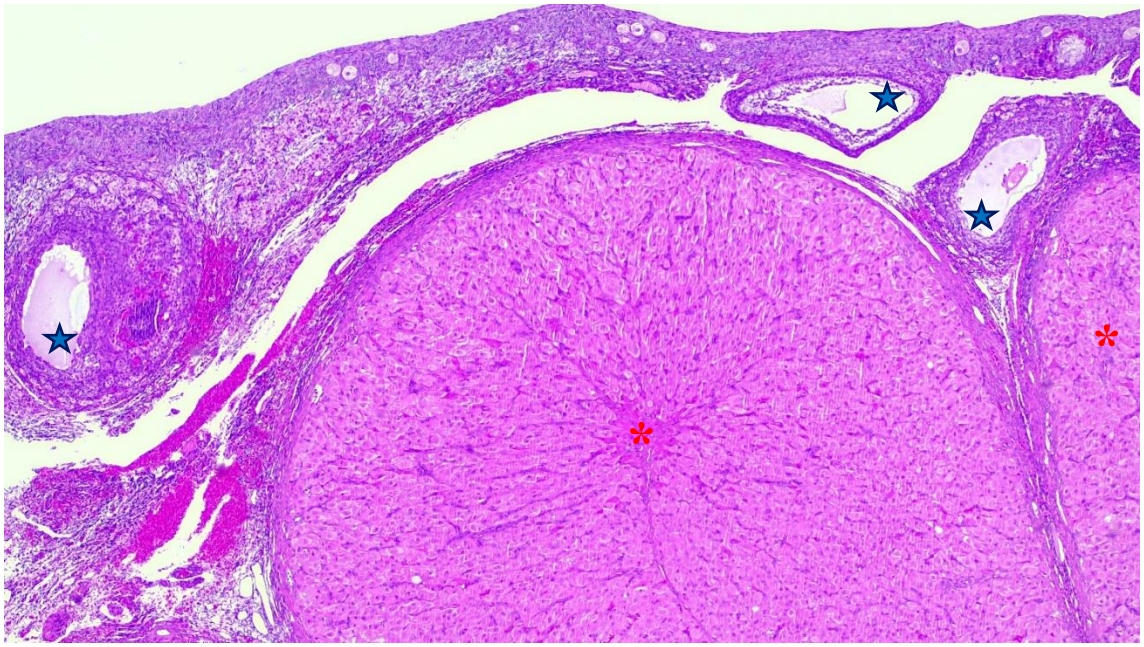

Figure 48. Left ovary (subject 6), 4 x magnification: ovarian parenchyma with corpora lutea (red asterisks) and follicles (blue stars)

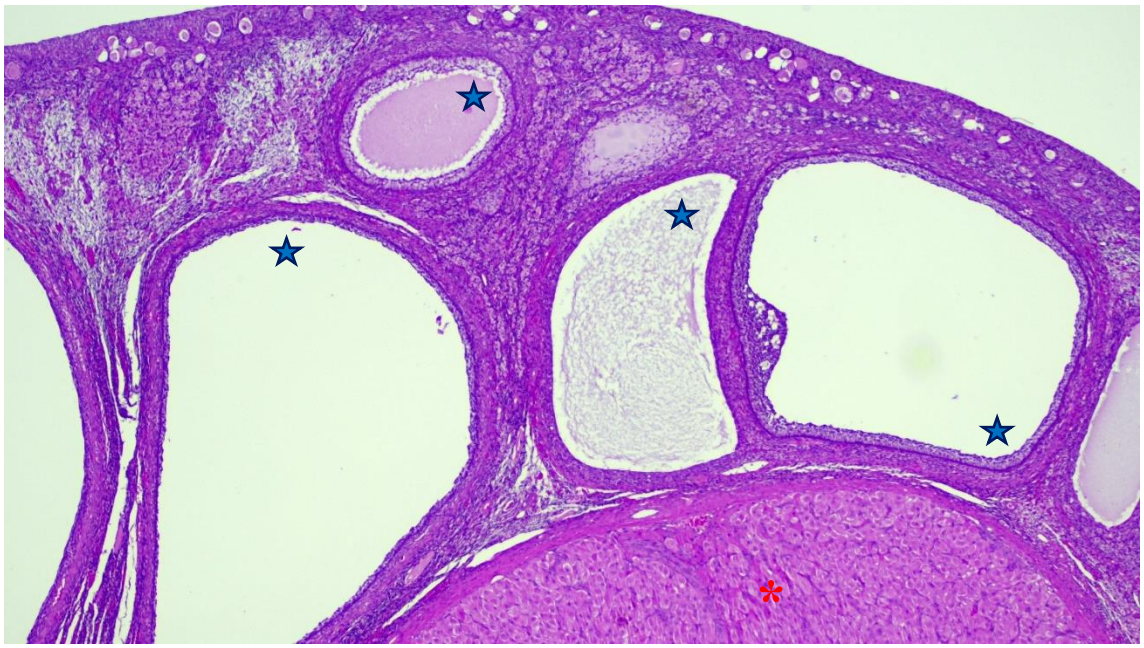

Figure 49. Right ovary (subject 6), 4 x magnification: ovarian parenchyma with corpora lutea (red asterisk) and follicles (blue stars).

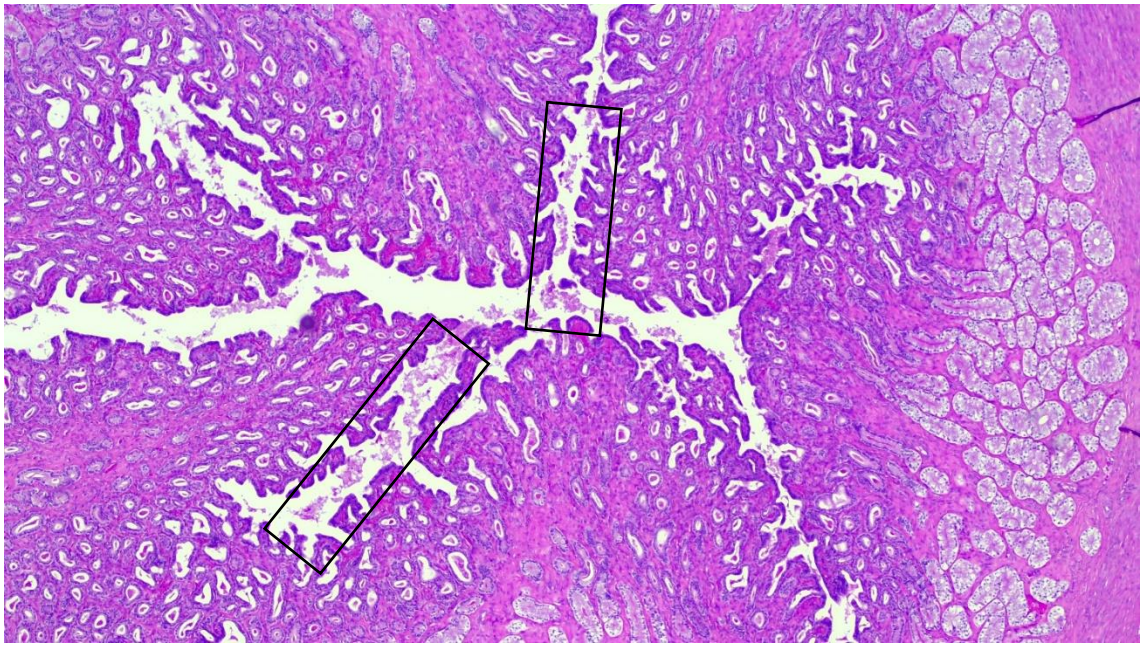

Figure 50. Uterus (subject 4), 4 x magnification: uterus with well-developed glandular mucosa and mucometra (black rectangles)

The following table summarizes all the histological findings detected in each queen (Table n. 7).

Subject	Follicles	Corpora lutea	Ovarian cysts	Endometrial hyperplasia	Pyometra
1	X	X			
2	X				
3	X				
4	X		X		
5	X	X		X	X
6	X	X			

Table n. 7 Histological findings in ovaries and uteri of the subjects enrolled.

4. DISCUSSION

4.1 CLINICAL ASPECTS

Estropill™ is an effective short-term medication for suppressing the estrous cycle in queens, as evidenced by the fact that none of the subjects, except for Subject 6, resumed cyclicity during treatment. The resumption of cyclicity of Subject 6 is presumably explained by her weight gain during the treatment, as illustrated in Table 4. The dosage of the treatment, as previously stated, was calculated based on body weight (5 drops/kg). Subject 6 gained approximately 2 kilograms over six months, and at the time of estrous diagnosis, she had gained 1.2 kg. It is likely that this weight variation resulted in the administration of an inadequate and insufficient dosage. Consequently, it can be inferred that the efficacy of Estropill™ is closely tied to a minimum effective dose, and even slight variations in body weight may reduce its effectiveness.

All queens, except for Subjects 1 and 2, exhibited increased appetite during treatment with MA. However, it should be noted that Subjects 1 and 2 underwent treatment before the other 4 subjects and they were clinically followed by another veterinarian, and the extent to which this aspect was investigated remains unclear.

Subjects 3 and 5 were identified as spontaneous ovulators due to their progesterone levels exceeding 2 ng/ml, despite having had no contact with male conspecifics. It was therefore assumed that they ovulated without any external stimulus. Spontaneous ovulation in queens projects a luteal phase, which may be clinically significant if it happens on several estrous cycles because it implies prolonged exposure to progesterone compared to queens that only ovulate post-mating. This is particularly relevant in the case of domestic queens of little if any reproductive value. Prolonged progesterone stimulation, as observed in Subject 5, should be carefully considered when administering progestins. Consequently, spontaneous ovulators may be unsuitable candidates for progestin treatment.

At the time of sterilization, the phase of the estrous cycle of the queens was unknown, as neither P4 nor cytological evaluations were conducted on that day. The most recent data were obtained one or two months prior to surgery, and it is presumed that the queens were either in diestrus (if spontaneous or mating-induced ovulation occurred), in anestrus or in interestrus. No queen was sterilized while presenting clear signs of heat.

Notably, corpora lutea were observed on histological evaluations of Subjects 1 and 6, which were not initially classified as spontaneous ovulators. Since corpora lutea form after ovulation, this observation warrants further discussion. No information is available regarding Subject 1 , as her clinical follow-up was conducted by a different veterinarian. However, it is known that Subject 6 was a domestic queen with no contact with males, suggesting that she too may have been a spontaneous ovulator, though this was not detected during the pre-treatment evaluation. This finding is crucial, as it implies that three spontaneous ovulators may have been present in this study, with only one displaying clinical manifestations following progestin treatment. Therefore, while spontaneous ovulators should be considered unsuitable for progestin treatment, as the risk of developing reproductive side effects may be higher, it is also essential to recognize that clinical manifestations may arise due to individual susceptibility to such medications.

4.2 HISTOLOGICAL FINDINGS

Based on the histological findings of this study, it appears that progestins induce morphological alterations in the ovaries and uteri. While all queens exhibited such alterations, only Subject 5 experienced clinical repercussions.
Since this study did not include a control group, it is not possible to conclusively determine whether these observed alterations were a direct result of the treatment. In future studies, it would be valuable to compare the histology of untreated queens with those treated in this study.

Nevertheless, the findings seem to be correlated with the potential effects of progesterone on reproductive organs, particularly because sterilization took place within a relatively short time from treatment. Thus, it cannot be excluded and rather it is reasonable to assume that the observed changes could be attributed to MA Progesterone causes modifications in the endometrium, making it more receptive to embryo implantation, increasing vascularization, stimulating the development of endometrial glands, and enhancing their secretion (Cable et al., 2023). Given these effects, it is plausible that MA was responsible for the vascular congestion, well-developed glandular mucosa in several queens the mucometra observed in Subject 6, and cystic hyperplasia of the uterine mucosa in Subject 5.

Ovarian cysts, however, are presumed to be unrelated to progestin treatment, as they were present and unchanged in Subject 1 before, during, and after the treatment. Furthermore, no literature references support a connection between progestins and ovarian cysts. These ovarian cysts were classified as cysts of the rete ovarii. The rete ovarii is a structure formed from the primary sex cords in the female and is the equivalent structure to the rete testis in the male. (Gelberg et al., 1984). In adults it is regarded as a nonfunctional embryonic remnant and the functional significance of these cysts is unknown (Gelberg et al., 1984). This type of cystic structure does not produce sex steroid hormones and the rete ovarii tissue is physiologically unaffected by sex-hormone-regulated differentiation and maturation processes (Songsasen et al., 2009). Considering these facts, the absence of effect of MA on subject 1' cysts seems obvious.

In subject 6 mucometra was reported. Mucometra is an accumulation of sterile mucoid fluid in the uterine lumen without any significant systemic clinical sign (Pretzer, 2008). However, the condition rarely occurs in cats. Causes of mucometra in cats include persistence of corpora lutea (exposure to progesterone), impatency of vulva, vagina, cervix and uterus by congenital abnormalities, neoplasia, inflammation and scarring or accidental ligation (Johnston et al., 2001). The uterus of cat n. 6 was judged as hyperplastic at macroscopic evaluation. Corpora lutea were also found in this queen, indicating that an ovulation took place, even though serum P4 pre and post treatment were below baseline. The nature of this ovulation seems to be spontaneous as in the subjects 3 and 5, because the queen was housed indoors and had no contact with intact tomcats. It is not possible to pin down the role of MA or of this ovulation in inducing uterine hyperplasia and mucometra, as both these factors induce exposure to progesterone. It is only possible to hypothesize that this spontaneous ovulation that occurred after the MA treatment period potentiated the physiological effects of progesterone on the uterine tissue leading to hyperplasia and mucometra. Nevertheless, none of these mild alterations had any clinical implication in subject 6.

In future studies, it would also be beneficial to allow more time (e.g., one year) before sterilization to assess whether the observed morphological changes are reversible, considering that in this study, surgery was performed only a few months after treatment.

Particular attention should be given to Subject 5, as this queen not only presented with open pyometra and clinical sings but also exhibited unusual morphological alterations. Cystic and atypical endometrial hyperplasia are rare diagnoses in queens. In humans, atypical endometrial hyperplasia is considered a precancerous lesion of endometrial adenocarcinoma, from which it must be distinguished. However, precancerous lesions have not been reported in cats with endometrial adenocarcinoma (Suzuki et al., 2021).

Endometrial adenocarcinoma is a rare tumor in cats. While there are a few reports of endometrial adenocarcinoma in younger cats (Cho et al., 2011; Payan-Carreira et al., 2013), the tumor tends to develop in older individuals, is highly metastatic and invasive, and metastasizes or recurs shortly after resection, making the prognosis for this neoplasia poor.In human histopathology, atypical endometrial hyperplasia is classified as either simple or complex atypical hyperplasia. These two types are differentiated by the presence of architectural complexity, including anisokaryosis (similar to what was observed in Subject 5) (Mills & Longacre, 2010).

A comparable pattern (Figure 51) was documented in a 2021 study, which examined animals with hemorrhagic or purulent vaginal discharge, behavioral changes, or during annual check-ups. In these cases, queens were diagnosed with pyometra and papillomatous nodules (Suzuki et al., 2021).

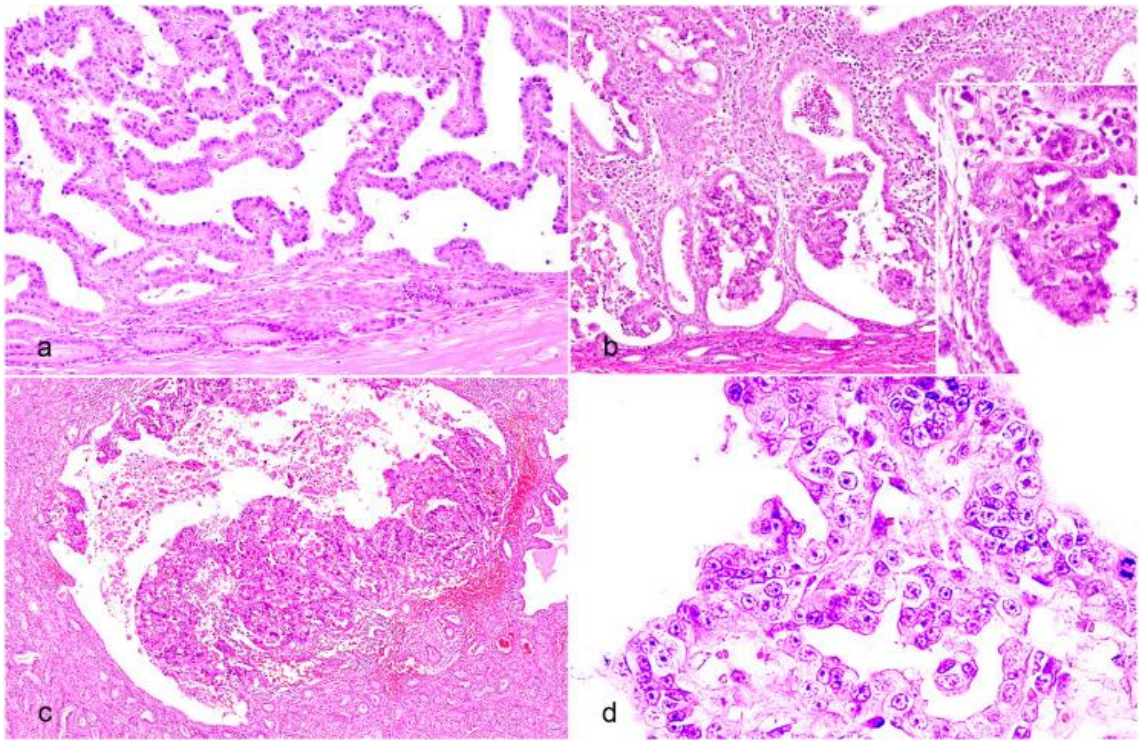

Figure 51. Atypical endometrial hyperplasia, uteri, cats. (a) Papillomatous proliferation of endometrial epithelial cells without atypia, HE. (b) Atypical papillomatous proliferation of endometrial epithelial cells, HE. (c) Atypical papillary proliferation of the endometrial epithelial cells arranged in stratified layers, HE. (d) Anisokaryosis, pleomorphic nuclei, prominent large nucleoli and increased chromatin in hyperplastic endometrial epithelial cells, which have lost orientation, HE. (Suzuki et al., 2021).

Considering the findings of the aforementioned study, it is possible that the lesions observed in Subject 5 may be associated with pyometra, and therefore, these lesions could be characteristic of the condition rather than a result of treatment with MA. This hypothesis warrants further investigation to clarify whether the lesions are related to pyometra or to the effects of MA treatment.

5. CONCLUSION

To conclude, this study provides a new valuable insight into the short-term use of MA as a safe method of estrous suppression in post-pubertal queens. The findings suggest that MA is generally effective in suppressing the estrous cycle, as evidenced by the fact that only one queen (Subject 6) displayed signs of estrous during treatment. The case of Subject 6 highlights the importance of accurate dosage calculations, particularly in light of weight fluctuations during the treatment period, which can significantly affect the drug's efficacy.

Additionally, the histopathological findings from this study point to the potential impact of progestins like MA on the reproductive organs, particularly in inducing morphological changes in the ovaries and uterus. Although these changes did not result in clinical complications for most subjects, the case of Subject 5, which developed pyometra and exhibited atypical endometrial hyperplasia, raises important concerns. This underlines the need for further research to assess the safety of progestin use, particularly in queens identified as spontaneous ovulators, who may be more prone to adverse reproductive effects.

Future studies should consider a longer follow-up period to determine the reversibility of the observed morphological changes and explore the relationship between progestin treatment and potential reproductive side effects. Moreover, the inclusion of a control group would provide a clearer understanding of whether the observed histological changes are directly related to the treatment with MA.

In conclusion, while MA seems to be an effective estrous suppressant in queens, careful consideration of dosage and patient selection is essential to ensure the safety of treated animals.

ANNEX

Annex 1

SOGGETTO N. 1

OWNER

Objective clinical examination and ultrasound	✗ / ✓	Note
Skeletal state and constitution		
Body state of nutrition and muscle tone		
Level of consciousness and particular signs and attitude		
Skin and subcutaneous tissue		
Mucous membrane color and capillary refill time		
Lymph nodes		
Body temperature		
Respiratory rate		
Heart rate		
Great organic functions		
Reproductive tract examination		
Ultrasound evaluation		
Sedazione		

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