

MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

Evaluation of Vaginal Cytology for Estrous Cycle Classification in the Queen (*Felis catus*): Interobserver Agreement and Validation

Avaliação da Citologia Vaginal para a Classificação das Fases do Ciclo Éstrico na Gata (*Felis catus*): Concordância Interobservador e Validação

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Áreas científicas: Ciências Fundamentais; Medicina e/ou Cirurgia de Animais de Companhia.

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ABSTRACT

This internship report describes the activities developed as part of the curricular internship of the Integrated Master's Degree in Veterinary Medicine. During eight weeks at KATZENPRAXIS (Vienna, Austria), I had the opportunity to observe consultations across multiple specialties, participate in clinical case discussions, and follow the treatment and diagnostic imaging of hospitalized cats. During eight weeks at Faculty of Veterinary Medicine, University of Ankara, Türkiye, I followed reproduction cases, particularly queens admitted for elective ovariohysterectomy. This opportunity led to the development of this study.

The queen is a seasonally polyestrous species whose reproductive cyclicity is regulated by the photoperiod. The feline estrous cycle is characterized by hormonal fluctuations that affect the vaginal epithelium, resulting in changes that can be assessed by vaginal cytology. However, interpretation of this technique in queens remains challenging due to subtle transitions between reproductive stages and inter-individual variability.

The aim of this study was to evaluate vaginal cytological patterns in clinically healthy queens undergoing elective ovariohysterectomy and to assess inter-observer variability and accuracy in the classification of reproductive stages.

A total of 32 vaginal cytology samples were collected and classified by two trained researchers. When available, samples were compared with serum progesterone concentrations and verified presence of functional *corpora lutea*. Inter-observer agreement was assessed using Cohen's Kappa test followed by Weighted Kappa test. Sensitivity and specificity for each researcher was also analysed.

Inter-observer agreement was minimal ($\kappa = 0,391$), with a marked improvement ($\kappa = 0,835$), when weighted agreement methods accounting for biological proximity between cycle stages were applied. Sensitivity for diestrus identification was low, whereas specificity was high for both researchers.

In conclusion, vaginal cytology in queens is a subjective but useful complementary diagnostic tool. Its diagnostic accuracy improves when integrated with behaviour anamnesis, hormonal and direct or indirect ovarian assessment, supporting its use within a multimodal approach to feline reproductive management.

KEY-WORDS: estrous cycle; inter-observer variability; queen; vaginal cytology;

RESUMO

Este relatório de estágio descreve as atividades desenvolvidas no âmbito do estágio curricular do Mestrado Integrado em Medicina Veterinária. Durante oito semanas na KATZENPRAXIS (Viena, Áustria), tive a oportunidade de observar consultas de diversas especialidades, participar na discussão de casos clínicos e acompanhar os tratamentos e exames imagiológicos de gatos internados. Durante oito semanas na Faculdade de Medicina Veterinária da Universidade de Ankara, Turquia, acompanhei casos de reprodução, particularmente de gatas admitidas para ovariectomia eletiva. Esta oportunidade levou ao desenvolvimento do presente estudo.

A gata é uma espécie poliéstrica sazonal cuja ciclicidade reprodutiva é regulada pelo fotoperíodo. O ciclo éstrico felino caracteriza-se por flutuações hormonais que afetam o epitélio vaginal, resultando em alterações que podem ser avaliadas através da citologia vaginal. No entanto, a interpretação desta técnica em gatas continua a ser desafiante devido às transições subtis entre os diferentes estádios reprodutivos e à variabilidade interindividual.

O objetivo deste estudo foi avaliar os padrões citológicos vaginais em gatas clinicamente saudáveis submetidas a ovariectomia eletiva, bem como avaliar a variabilidade interobservador e a precisão na classificação dos diferentes estádios reprodutivos.

Foram recolhidas 32 amostras de citologia vaginal, classificadas por dois investigadores treinados. Sempre que disponíveis, as amostras foram comparadas com as concentrações séricas de progesterona e com a presença confirmada de corpos lúteos funcionais. A concordância interobservador foi avaliada através do teste Kappa de Cohen, seguido do teste Kappa ponderado. A sensibilidade e a especificidade de cada investigador também foram analisadas.

A concordância interobservador foi mínima ($\kappa = 0,391$), com uma melhoria marcada ($\kappa = 0,835$) quando foram aplicados métodos de concordância ponderada que consideram a proximidade biológica entre os estádios do ciclo. A sensibilidade para a identificação de diestro foi baixa, enquanto a especificidade foi elevada para ambos os investigadores.

Em conclusão, a citologia vaginal em gatas é uma ferramenta diagnóstica complementar subjetiva, mas útil. A sua precisão diagnóstica melhora quando integrada com a anamnese comportamental e com a avaliação hormonal e ovárica, direta ou indireta, apoiando a sua utilização numa abordagem multimodal da gestão reprodutiva felina.

PALAVRAS-CHAVE: ciclo éstrico; citologia vaginal; gata; variabilidade inter-observador;

CASE REGISTRY AND ACTIVITIES DEVELOPED DURING THE INTERNSHIP

During an eight-week internship at KATZENPRAXIS Dr Hoyer & Dr Kamm, in Vienna, Austria, I had the opportunity to observe and assist in the consultation and clinical management of 104 cats undergoing care for a variety of clinical purposes, including routine health assessments and vaccinations, diagnostic imaging procedures, and therapeutic interventions for an array of medical conditions. As this is a feline specialized veterinary medical facility, all of the patients observed during the internship were cats.

Of the 104 cats observed, 50% (n=52) were females.

The patients' ages ranged from two months to twenty-three years and were classified into four age categories: younger than two months; two to twelve months (inclusive); one to seven years; and older than seven years. Most of the patients were one to seven years of age ($\approx 58\%$, n=60), followed by patients older than seven years of age ($\approx 29\%$, n=30), patients from two to twelve months of age ($\approx 12\%$, n=12) and, lastly, patients younger than two months ($\approx 2\%$, n=2).

The most commonly observed breed was the European Shorthair ($\approx 72\%$, n=75), followed by the Maine Coon ($\approx 10\%$, n=10) and the British Shorthair ($\approx 6\%$, n=6). Other breeds were observed, such as, the Ragdoll ($\approx 3\%$, n=3), the Norwegian Forest Cat ($\approx 2\%$, n=2), the Russian Blue ($\approx 1\%$, n=1), the Birman ($\approx 1\%$, n=1), the Oriental Shorthair ($\approx 1\%$, n=1), the Bengal ($\approx 1\%$, n=1), the American Curl ($\approx 1\%$, n=1) and the Abyssinian ($\approx 1\%$, n=1). Although the breed is not officially recognised by FIFe and its breeding and ownership is prohibited in Austria, one ($\approx 1\%$) Scottish Fold cat was observed.

The age and breed distributions are illustrated, in figures 1 and 2, respectively.

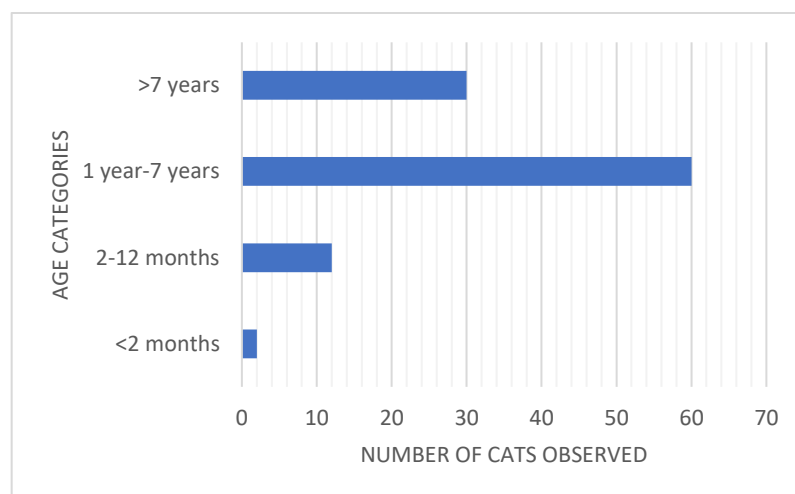


Figure 1. Number of cats observed at KATZENPRAXIS in function of their age.

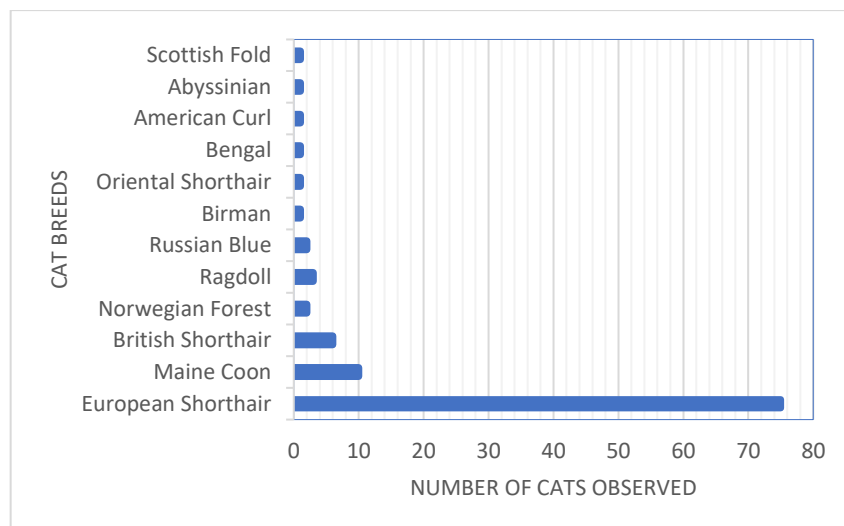


Figure 2. Number of cats observed at KATZENPRAXIS in function of their breed.

In terms of clinical case distribution, the vast majority were related to Internal Medicine ($\approx 65\%$, $n=67$), comprising approximately two-thirds of all observations, followed by Preventive Medicine ($\approx 18\%$, $n=19$), Imagiology ($\approx 10\%$, $n=10$), Surgery ($\approx 5\%$, $n=5$) and Urgent Medicine ($\approx 3\%$, $n=3$). The internal medicine cases were distributed among the following specialties: Ophthalmology ($\approx 3\%$, $n=3$), Endocrinology ($\approx 11\%$, $n=11$), Nephrology ($\approx 6\%$, $n=6$), Stomatology/Dentistry ($\approx 6\%$, $n=6$), Hematology ($\approx 2\%$, $n=2$), Nutrition ($\approx 3\%$, $n=3$), Oncology ($\approx 8\%$, $n=8$), Dermatology ($\approx 6\%$, $n=6$), Orthopedics ($\approx 10\%$, $n=10$), Parasitology ($\approx 3\%$, $n=3$), Otorhinolaryngology ($\approx 2\%$, $n=2$), Urology ($\approx 3\%$, $n=3$) and Gastroenterology ($\approx 4\%$, $n=4$), as illustrated in figure 3.

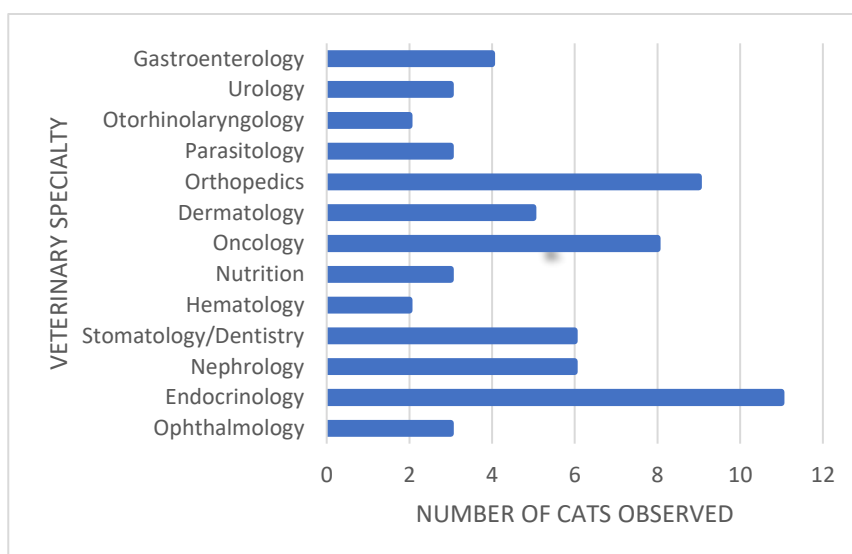


Figure 3. Internal Medicine cases' distribution among the different veterinary specialties.

Tables 1 to 16 present the diagnostics distribution among each veterinary specialty, regarding the patients' sex and age. All Preventive Medicine cases consisted of a physical examination, blood sampling and laboratory analysis, vaccination when duly needed and, in older cats, a routine abdominal ultrasound exam.

Table 1. Surgery diagnostics' distribution regarding the sex and age of the patients.

SURGERY	Female				Male				TOTAL	%
	<2 months	2-12months	1-7 years	>7 years	<2 months	2-12months	1-7 years	>7 years		
Tooth extraction	0	0	1	0	0	0	0	0	1	20%
Radical tooth extraction	0	0	0	0	0	0	2	0	2	40%
Orchiectomy	0	0	0	0	0	1	0	0	1	20%
Complications	0	0	0	0	0	0	1	0	1	20%
TOTAL	0	0	1	0	0	1	3	0	5	100%
%	–	–	20%	–	–	20%	60%	–		

Table 2. Imagiology diagnostics' distribution regarding the sex and age of the patients.

IMAGIOLOGY	Female				Male				TOTAL	%
	<2 months	2-12months	1-7 years	>7 years	<2 months	2-12months	1-7 years	>7 years		
Inflammatory Bowel Disease (IBD)	0	0	4	0	0	0	2	0	6	60%
Hypertrophic Cardiomyopathy	0	0	1	0	0	0	0	0	1	10%
Portosystemic Shunt	0	0	0	0	0	0	1	0	1	10%
Intestinal Intussusception	0	0	1	0	0	0	0	0	1	10%
Gastritis	0	0	0	0	0	1	0	0	1	10%
TOTAL	0	0	6	0	0	1	3	0	10	100%
%	–	–	60%	–	–	10%	30%	–		

Table 3. Emergency Medicine diagnostics' distribution regarding the sex and age of the patients.

EMERGENCY MEDICINE	Female				Male				TOTAL	%
	<2 months	2-12months	1-7 years	>7 years	<2 months	2-12months	1-7 years	>7 years		
Pneumothorax	0	0	0	1	0	0	0	0	1	≈33%
Bite Wound	0	0	0	0	0	0	1	0	1	≈33%
Foreign Body Ingestion	0	0	0	0	0	0	1	0	1	≈33%
TOTAL	0	0	0	1	0	0	2	0	3	100%
%	–	–	–	≈33%	–	–	≈66%	–		

Table 4. Ophthalmology diagnostics' distribution regarding the sex and age of the patients.

OPHTHALMOLOGY	Female				Male				TOTAL	%
	<2 months	2-12months	1-7 years	>7 years	<2 months	2-12months	1-7 years	>7 years		
Cat Flu	0	0	1	0	0	2	0	0	3	100%
TOTAL	0	0	1	0	0	2	0	0	3	100%
%	–	–	≈33%	–	–	≈66%	–	–		

Table 5. Endocrinology diagnostics' distribution regarding the sex and age of the patients.

ENDOCRINOLOGY	Female				Male				TOTAL	%
	<2 months	2-12months	1-7 years	>7 years	<2 months	2-12months	1-7 years	>7 years		
Diabetes	0	0	1	0	0	0	1	1	3	≈28%
Hypoadrenocorticism	0	0	1	0	0	0	0	0	1	≈9%
Hypertiroidism	0	0	0	5	0	0	0	0	5	≈45%
Conn's Syndrome	0	0	0	0	0	0	1	0	1	≈9%
Idiopathic Hypercalcemia	0	0	1	0	0	0	0	0	1	≈9%
TOTAL	0	0	3	5	0	0	2	1	11	100%
%	–	–	≈28%	≈45%	–	–	≈18%	≈9%		

Table 6. Nephrology diagnostics' distribution regarding the sex and age of the patients.

NEPHROLOGY	Female				Male				TOTAL	%
	<2 months	2-12months	1-7 years	>7 years	<2 months	2-12months	1-7 years	>7 years		
Chronical Kidney Disease (CKD)	0	0	1	2	0	0	0	2	5	≈83%
Pyelonephritis	0	0	0	1	0	0	0	0	1	≈17%
TOTAL	0	0	1	3	0	0	0	2	6	100%
%	–	–	≈17%	50%	–	–	–	≈33%		

Table 7. Stomatology/Dentistry diagnostics' distribution regarding the sex and age of the patients.

STOMATOLOGY/DENTISTRY	Female				Male				TOTAL	%
	<2 months	2-12months	1-7 years	>7 years	<2 months	2-12months	1-7 years	>7 years		
Gingivostomatitis	0	0	2	0	0	0	4	0	6	100%
TOTAL	0	0	2	0	0	0	4	0	6	100%
%	–	–	≈33%	–	–	–	≈67%	–		

Table 8. Hematology diagnostics' distribution regarding the sex and age of the patients.

HEMATOLOGY	Female				Male				TOTAL	%
	<2 months	2-12months	1-7 years	>7 years	<2 months	2-12months	1-7 years	>7 years		
Feline Leukemia (FeLV) related anemia	0	1	0	0	0	0	0	0	1	50%
Idiopathic Anemia	0	0	0	1	0	0	0	0	1	50%
TOTAL	0	1	0	1	0	0	0	0	2	100%
%	–	50%	–	50%	–	–	–	–		

Table 9. Nutrition diagnostics' distribution regarding the sex and age of the patients.

NUTRITION	Female				Male				TOTAL	%
	<2 months	2-12months	1-7 years	>7 years	<2 months	2-12months	1-7 years	>7 years		
Obesity	0	0	1	0	0	0	2	0	3	100%
TOTAL	0	0	1	0	0	0	2	0	3	100%
%	–	–	≈33%	–	–	–	≈67%	–		

Table 10. Oncology diagnostics' distribution regarding the sex and age of the patients.

ONCOLOGY	Female				Male				TOTAL	%
	<2 months	2-12months	1-7 years	>7 years	<2 months	2-12months	1-7 years	>7 years		
Lymphoma	0	0	0	0	0	0	0	1	1	12,50%
Pancreatic Adenoma	0	0	0	1	0	0	0	0	1	12,50%
Mammary Carcinoma	0	0	0	0	0	0	0	0	1	12,50%
General Neoplasia	0	0	0	1	0	0	0	0	1	12,50%
Squamous Cells Carcinoma	0	0	0	1	0	0	1	0	1	12,50%
Ocular Melanoma	0	0	0	0	0	0	1	0	1	12,50%
Unspecified liver tumor	0	0	0	0	0	0	1	0	1	12,50%
Unspecified eye tumor	0	0	1	0	0	0	0	0	1	12,50%
TOTAL	0	0	1	3	0	0	3	1	8	100%
%	–	–	12,50%	37,50%	–	–	37,50%	12,50%		

Table 11. Dermatology diagnostics' distribution regarding the sex and age of the patients.

DERMATOLOGY	Female				Male				TOTAL	%
	<2 months	2-12months	1-7 years	>7 years	<2 months	2-12months	1-7 years	>7 years		
Malassezia	0	1	0	0	0	0	0	0	1	≈17%
Food Allergy	0	0	3	0	0	0	0	0	3	50%
Environmental Allergy	0	0	0	0	0	0	1	0	1	≈17%
Psicogenic Alopecia	0	0	0	0	0	0	1	0	1	≈17%
TOTAL	0	1	3	0	0	0	2	0	6	100%
%	–	≈17%	50%	–	–	–	≈33%	–		

Table 12. Orthopedics diagnostics' distribution regarding the sex and age of the patients.

ORTHOPEDICS	Female				Male				TOTAL	%
	<2 months	2-12months	1-7 years	>7 years	<2 months	2-12months	1-7 years	>7 years		
Osteoarthritis	0	0	0	5	0	0	3	1	9	90%
Brachial Plexus Avulsion	0	0	0	0	0	0	1	0	1	10%
TOTAL	0	0	0	5	0	0	4	1	10	100%
%	–	–	–	50%	–	–	40%	10%		

Table 13. Parasitology diagnostics' distribution regarding the sex and age of the patients.

PARASITOLOGY	Female				Male				TOTAL	%
	<2 months	2-12months	1-7 years	>7 years	<2 months	2-12months	1-7 years	>7 years		
Isosporiasis	0	0	0	0	0	1	0	0	1	≈33%
Toxoplasmosis	2	0	0	0	0	0	0	0	2	≈67%
TOTAL	2	0	0	0	0	1	0	0	3	100%
%	≈67%	–	–	–	–	≈33%	–	–		

Table 14. Otorhinolaryngology diagnostics' distribution regarding the sex and age of the patients.

OTORHINOLARYNGOLOGY	Female				Male				TOTAL	%
	<2 months	2-12months	1-7 years	>7 years	<2 months	2-12months	1-7 years	>7 years		
Unilateral Otitis	0	0	0	0	0	0	1	0	1	50%
Laryngeal Paralysis	0	0	1	0	0	0	0	0	1	50%
TOTAL	0	0	1	0	0	0	1	0	2	100%
%	–	–	50%	–	–	–	50%	–		

Table 15. Urology diagnostics' distribution regarding the sex and age of the patients.

UROLOGY	Female				Male				TOTAL	%
	<2 months	2-12months	1-7 years	>7 years	<2 months	2-12months	1-7 years	>7 years		
Idiopathic Cistitis	0	0	1	0	0	0	0	0	1	≈33%
Bacterial Cistitis	0	0	1	0	0	0	1	0	2	≈67%
TOTAL	0	0	2	0	0	0	1	0	3	100%
%	–	–	≈67%	–	–	–	≈33%	–		

Table 16. Gastroenterology diagnostics' distribution regarding the sex and age of the patients.

GASTROENTEROLOGY	Female				Male				TOTAL	%
	<2 months	2-12months	1-7 years	>7 years	<2 months	2-12months	1-7 years	>7 years		
Inflammatory Bowel Disease (IBD)	0	0	0	0	0	0	2	0	2	50%
Pancreatitis	0	0	0	0	0	0	1	0	1	25%
Hepatitis	0	0	0	0	0	0	0	1	1	25%
TOTAL	0	0	0	0	0	0	3	1	4	100%
%	–	–	–	–	–	–	75%	25%		

The most common reason for presentation was routine health check-ups (≈18%, n=19). When stratified by age category, the most frequent reason for presentation in cats older than seven years was osteoarthritis (≈8%, n=8). In cats aged between one and seven years, the most common reasons were routine check-ups (≈8%, n=8) and IBD (≈8%, n=8). For cats aged two to twelve months, routine check-ups were the predominant reason for presentation (≈6%, n=6), whereas in cats younger than two months, toxoplasmosis was the sole reason for presentation (≈2%, n=2). When analysed by sex, routine check-ups were the primary reason for presentation in both females (≈7%, n=7) and males (≈12%, n=12).

During this internship, I also had the opportunity to observe and perform a range of clinical procedures, including blood sampling and ultrasonography, as illustrated in Table 17.

Table 17. Number of procedures observed and performed during the internship at KATZENPRAXIS.

Procedure	Observe	Perform
Ultrasound	16	4
X-Ray	0	21
Blood Draw (jugular vein)	26	3
Blood Draw (saphenous vein)	15	0
Blood Draw (cephalic vein)	7	5
Blood Analysis*	3	67
Urine Analysis**	1	17
Echoguided Cystocentesis	16	0
Venous Catheterization	11	5
Fine Needle Aspiration Cytology (FNAC)	4	0
Diff-Quick Staining***	0	9
Blood Pressure Measurement	10	10
Urinary Catheterization	1	0
Chemotherapy	3	0
Parenteral Administrations (SC/IM/IV)	8	19
General Physical Exam	22	8
Otoscopy	6	4
Ophthalmoscopy	3	3

*Using IDEXX ProCyte®, IDEXX Cathalist One®, Zomedica Truforma® and external laboratory; **Using IDEXX SediVue®, urine test strips, refractometer and microbiological cultures; ***Used to stain blood, urine, FNAC and apposition cytologies.

During an eight-week internship at the Faculty of Veterinary Medicine of the University of Ankara, Turkey, particularly at its Main Hospital, Gynaecology and Obstetrics Clinic, and Reproductive and Artificial Insemination Clinic, I had the opportunity to observe and assist in the consultation and clinical or reproductive management of 47 animals, the majority of which ($\approx 91\%$, $n=43$) were cats. This discrepancy may likely be attributed to the strong cultural appreciation for cats that is deeply rooted in Turkish society.

All the dogs observed ($n=4$) were female as were the majority of cats ($\approx 88\%$, $n=38$). The predominance of female patients can be explained by the fact that a substantial part of my internship was spent in the gynaecology and obstetrics clinic.

The patients' ages ranged from five months to twelve years and were classified into three age categories: five to twelve months (inclusive); one year to seven years; and older than seven years. All the canine patients were in the one year to seven years of age category. Nevertheless, most feline patients were in the five months to twelve months of age category ($\approx 70\%$, $n=30$), followed by patients in the one year to seven years of age category ($\approx 28\%$, $n=12$) and, lastly, patients older than seven years of age ($\approx 2\%$, $n=1$).

All canine patients observed belonged to different breeds, including the Labrador Retriever, German Shepherd, Belgian Malinois Shepherd, and one mixed-breed dog. Among the feline patients, the most commonly observed breed was the European Shorthair ($\approx 63\%$, $n=27$), followed by the British Shorthair ($\approx 30\%$, $n=13$). One British Longhair cat ($\approx 2\%$), as well as, one Persian cat ($\approx 2\%$) and one Ragdoll cat ($\approx 2\%$) were also observed.

The age and breed distributions are illustrated in figures 4 and 5, respectively.

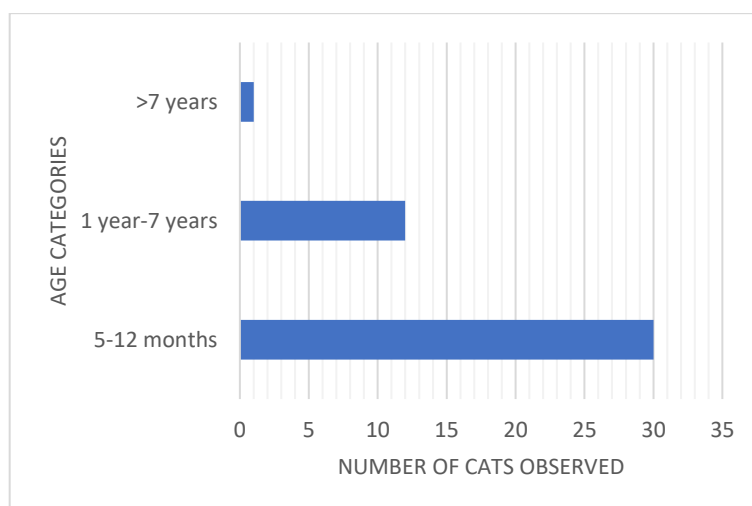


Figure 4. Number of cats observed at Ankara University in function of their age.

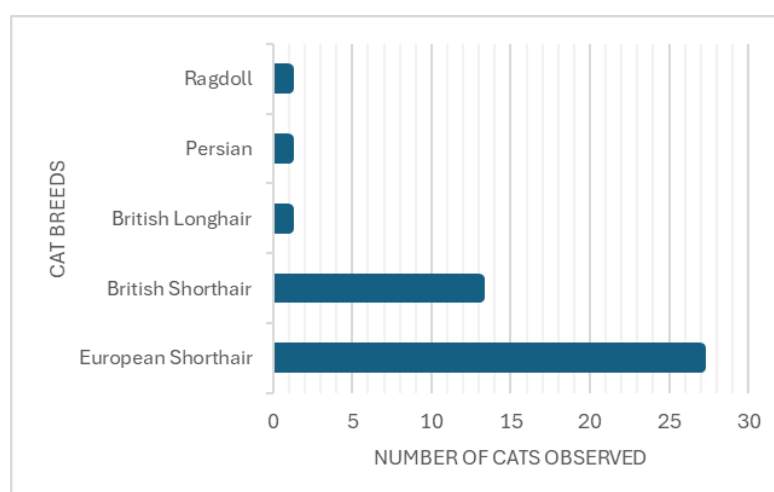


Figure 5. Number of cats observed at Ankara University in function of their breed.

In terms of clinical case distribution, the vast majority were related to Surgery ($\approx 96\%$, $n=45$), followed by Imagiology ($\approx 2\%$, $n=1$), and Infectology ($\approx 2\%$, $n=1$). The surgery cases were distributed among the following specialties: Gynaecology and Obstetrics ($\approx 87\%$, $n=39$), Andrology ($\approx 11\%$, $n=5$) and Orthopedics ($\approx 2\%$, $n=1$). This distribution can be observed in Figure 6. All the canine patients are

included in the Gynaecology and Obstetrics surgeries and all the male patients are included in the Andrology surgeries.

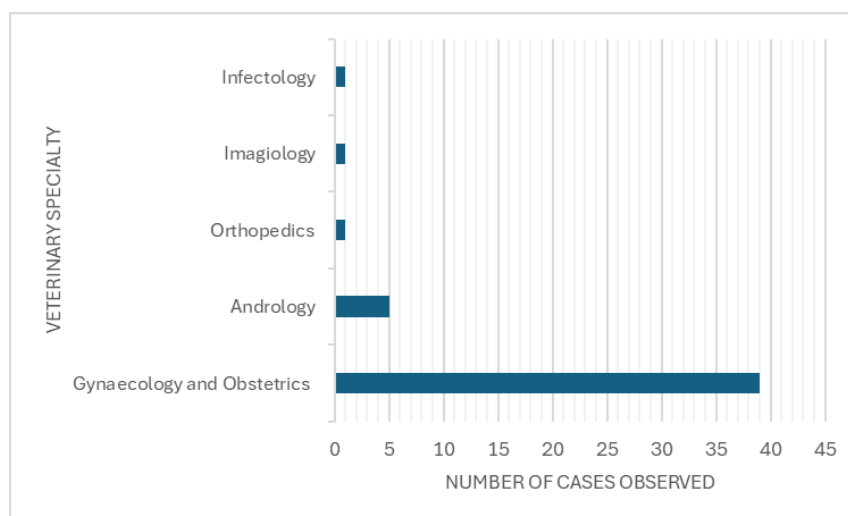


Figure 6. Observed cases' distribution among the different veterinary specialties

Tables 18 to 22 represent the diagnostical distribution among each veterinary specialty, regarding the patients sex and age.

Table 18. Gynaecology and Obstetrics diagnostics' distribution regarding the sex and age of the patients.

GYNAECOLOGY & OBSTETRICS	Female*			TOTAL	%
	5-12months	1-7 years	>7 years		
Elective Ovariohysterectomy (OVH)	24	5	0	29	≈74%
Pyometra (+ Ovariohysterectomy)	0	2	0	2	≈5%
Mastectomy (+ Ovariohysterectomy)	0	1	0	1	≈3%
Hydrometra (+ Ovariohysterectomy)	1	0	0	1	≈3%
Ovarian Remnant Syndrome (ORS)	1	1	0	2	≈5%
Feline Mammary Fibroepithelial Hyperplasia	1	1	0	2	≈5%
C-Section (+ Ovariohysterectomy)	0	2	0	2	≈5%
TOTAL	27	12	0	39	100%
%	≈69%	≈31%	–		

*Gynaecology and Obstetrics is a specialty dedicated to female patients only.

Table 19. Andrology diagnostics' distribution regarding the sex and age of the patients.

ANDROLOGY	Male*			TOTAL	%
	5-12months	1-7 years	>7 years		
Orchiectomy (ORQ)	3	1	0	4	80%
Penectomy	0	1	0	1	20%
TOTAL	3	2	0	5	100%
%	60%	40%	–		

*Andrology is a specialty dedicated to male patients only.

Table 20. Orthopedics diagnostics' distribution regarding the sex and age of the patients.

ORTHOPEDICS	Female			Male			TOTAL	%
	5-12months	1-7 years	>7 years	5-12months	1-7 years	>7 years		
Tibia and Fibula Fracture	0	1	0	0	0	0	1	100%
TOTAL	0	1	0	0	0	0	1	100%
%	–	100%	–	–	–	–		

Table 21. Imagiology diagnostics' distribution regarding the sex and age of the patients.

IMAGIOLOGY	Female			Male			TOTAL	%
	5-12months	1-7 years	>7 years	5-12months	1-7 years	>7 years		
Ovarian Cyst	0	0	1	0	0	0	1	100%
TOTAL	0	0	1	0	0	0	1	100%
%	–	–	100%	–	–	–		

Table 22. Infectology diagnostics' distribution regarding the sex and age of the patients.

INFECTOLOGY	Female			Male			TOTAL	%
	5-12months	1-7 years	>7 years	5-12months	1-7 years	>7 years		
Feline Infectious Peritonitis (PIF)	1	0	0	0	0	0	1	100%
TOTAL	0	0	0	0	0	0	1	100%
%	100%	–	–	–	–	–		

Overall, elective ovariohysterectomy was the most common reason for presentation ($\approx 62\%$, $n=29$). This is also true when looking at feline patients, since this species represented all of the cases observed, while the main reason for presentation in canine patients was C-Section, representing half of this species cases. When stratified by age category, the only reason for presentation in animals older than seven years was ovarian cyst ($\approx 2\%$, $n=1$). For animals aged between one and seven years and animals aged five to twelve months, the most common reasons were elective ovariohysterectomy ($\approx 11\%$, $n=5$ and $\approx 51\%$, $n=24$, respectively). When analysed by sex, elective ovariohysterectomy was the primary reason for presentation in females ($\approx 62\%$, $n=29$) and orchiectomy was the primary reason for presentation in males ($\approx 9\%$, $n=4$).

During this internship, I also had the opportunity to observe and perform a range of clinical procedures, including blood sampling and biopsies, as illustrated in Table 23, as well as, to visit the

International Centre For Livestock research and Training and to observe and participate in experimental activities at the Departments of Pharmacology and Histology of the Veterinary Medicine Faculty, such as, cell culture techniques, toxicity studies, definitive slide preparation, immunohistochemistry techniques and microscopy observation.

Table 23. Number of procedures observed and performed during the internship at Ankara University.

Procedure	Observe	Perform
Ultrasound	2	0
Catheterization	40	0
Blood Draw (jugular vein)	0	1
Blood Draw (saphenous vein)	8	6
Blood Draw (cephalic vein)	15	0
Diff-Quick (for cytologies)	0	32
General Physical Exam	2	4
Vaginal Cytology	0	32
OVH	41	1
ORQ	3	0
Mastectomy	1	0
C-Section	2	0
Biopsy	0	32

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“Life happens wherever you are, whether you make it or not.”

-Uncle Iroh

“O Homem é do tamanho do seu sonho.”

-atribuída a Fernando Pessoa

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LIST OF ABBREVIATIONS

A	Anucleate Epithelial Cell
AMH	Anti-Müllerian Hormone
CEH	Cystic Endometrial Hyperplasia
CI	Confidence Interval
CKD	Chronical Kidney Disease
CL	<i>Corpus Luteum</i>
D	Diestrus
ELISA	Enzyme-Linked Immunosorbent Assay
Er	Erythrocyte
EVSSAR	European Veterinary Society for Small Animal Reproduction
FeLV	Feline Leukemia Virus
FIFe	Federation International Feline
FIP	Feline Infectious Peritonitis
FNAC	Fine Needle Aspiration Cytology
FSH	Follicle-Stimulating Hormone
GnRH	Gonadotropin-Releasing Hormone
I	Intermediate Epithelial Cell
IBD	Inflammatory Bowel Disease
ID	Identification
INH	Inhalation
IM	Intramuscular
IV	Intravenous
LH	Luteinizing Hormone
ND	Non-Diestrus
Ne	Neutrophile
P4	Progesterone
PB	Parabasal Epithelial Cell
ORS	Ovarian Remnant Syndrome
ORQ	Orchiectomy
OVE	Ovariectomy
OVH	Ovariohysterectomy
S	Superficial Epithelial Cell
SC	Subcutaneous

1. INTRODUCTION

The queen is a seasonally polyestrous species with reproductive activity regulated by photoperiod. Throughout the estrous cycle, cyclical hormonal fluctuations induce physiological changes in the reproductive tract (Johnson, 2022; Schäfer-Somi, 2017; Schmidt *et al.*, 1983). These cyclic alterations affect the ovaries, uterus and vaginal epithelium (Eurell & Frappier, 2006; Shille *et al.*, 1979), resulting in cytological patterns, with some degree of differentiation, that may be evaluated through vaginal cytology (Johnston *et al.*, 2001).

The feline estrous cycle presents proestrus, estrus, interestrus, diestrus and anestrus, each associated with specific hormonal alterations and epithelial responses. During the follicular phase, rising estrogen concentrations stimulate proliferation and cornification of the vaginal epithelium, leading to an increased proportion of superficial epithelial cells. Following ovulation, progesterone secretion from the *corpora lutea* promotes a transition toward less cornified cell populations (Reece, 2015; Shille *et al.*, 1979).

Despite these recognized physiological mechanisms, the interpretation of vaginal cytology in queens remains challenging due to the subtle cytological transitions between stages which may result in reduced inter-observer reliability (Arlt, 2018; McHugh, 2012; Moxon *et al.*, 2010; Reckers *et al.*, 2022). Furthermore, spontaneous ovulation in queens has increasingly been reported, complicating the interpretation of cytological findings and highlighting the need for complementary diagnostic methods (Pereira *et al.*, 2025).

Elective ovariohysterectomy (OVH) provides a unique opportunity to perform vaginal cytology and observe the ovaries under clinical conditions. The direct observation of ovarian structures, particularly the presence or absence of *corpora lutea*, combined with progesterone assays, improves the interpretation of cytological findings and contributes to a more accurate characterization of the feline reproductive cycle (Binder *et al.*, 2019; Pereira *et al.*, 2024).

Despite its importance as a minimally invasive, rapid and cost-effective clinical tool for oestrus cycle staging in the bitch, vaginal cytology is still not routinely used in the queen. The limited number of studies available in the literature, together with the absence of standardized feline cytological criteria comparable to those established in canine reproduction, represents a significant research gap (Pereira *et al.*, 2025).

2. LITERATURE REVIEW

2.1 Reproductive Physiology Of The Queen

The queen has bilateral ovaries suspended in the dorsal abdomen connected to the uterine horn. The ovarian cortex may contain follicles or *corpora lutea* (CL). Follicles are structures that may present at various stages of development (primordial, primary, secondary or tertiary). The histologic appearance of the ovary varies with stage of the estrous cycle. During anestrus, the surface of the ovary is smooth and 0,5 mm diameter follicles are visible histologically and, as the estrus approaches, three to seven follicles enlarge up to 2,5 – 3,5 mm in diameter. In older cats, the ovaries may present as shrunken and nodular, but follicles and *corpora lutea* are still present, since true senile atrophy does not occur (Eurell & Frappier, 2006; Johnson, 2022; Johnston *et al.*, 2001).

The uterine tubes are bilateral, long and tortuous structure. Three segments of the uterine tube can be distinguished; the infundibulum, the ampulla, and the isthmus – joining the uterus through a papilla that protrudes towards the uterine lumen (Eurell & Frappier, 2006).

The uterus of a queen is bicornuate, with each horn measuring 7 – 10 cm (depending on several factors, like number of previous litters, age, cycle phase and pregnancy status) and a uterine body connected to the vagina by the cervix. The uterine wall consists of three layers, the endometrium, the myometrium and the perimetrium. (Eurell & Frappier, 2006; Johnson, 2022; Johnston *et al.*, 2001)

The superficial layer of the endometrium is the functional zone and degenerates partially or fully after pregnancy or estrus. The height and structure of the epithelial cells are related to the secretion of ovarian hormones throughout the cycle. The intensity of the expression of estrogen and progesterone receptors in the uterus has been shown to differ during the different phases of the estrous cycle (Nascimento *et al.*, 2023). The myometrium consists of a thick inner layer, which is mostly circular muscle, and an outer longitudinal layer of smooth muscle cells that increase in number and size during pregnancy. The perimetrium consists of loose connective tissue (Eurell & Frappier, 2006).

The cervix is a thick-walled muscular structure, rich in elastic fibers, caudal to the uterine body and cranial to the vagina, in which it protrudes as a papilla directed ventrocaudally. Increasing quantities of mucus are secreted during estrus, and most passes to the vagina as the cervical canal is open only during this phase (Eurell & Frappier, 2006).

The vaginal fornix is a 2 cm long muscular tube, extending caudally from the cervix and cranial to the external urethral orifice. Immediately caudal to the cervix is a flat longitudinal mucosal-submucosal fold, named the dorsal medial fold. This fold is more prominent in estrus and leads to decreased vaginal diameter and increased fornix length. This increase in the dorsal medial fold is

attributed to the proliferative effects of estrogen on the vaginal tissues, resulting in increased tissue mass and folding. The hormonal variation of the reproductive cycle also causes epithelial height and structure variation (Zambelli & Cunto, 2005).

The vaginal mucosa is lined by a cyclic-dependent epithelium, as in immature or anestrus cats is low cuboidal with a superficial layer of flattened cells, during proestrus, becomes stratified squamous and at the end of estrus, gradually decreases in height changing to low stratified squamous. After estrus, the epithelium becomes columnar and polymorphonuclear leukocytes may be observed between epithelial cells at its lumen (Eurell & Frappier, 2006; Johnson, 2022; Johnston *et al.*, 2001).

The vestibule extends from the external urethral orifice to the vulva, with a total 2 cm in length and 4 mm in diameter (much greater than the fornix). The wall of the vestibule is similar to that of the caudal portion of the vagina. Vestibule's vessels become congested during estrus (Eurell & Frappier, 2006).

The vulva is located just below the anus and is formed by the *labia vulvae*, two small, round, lateral structures, which unite at dorsal and ventral commissures and are covered by skin. The labia may become slightly congested during estrus (Eurell & Frappier, 2006; Johnson, 2022; Johnston *et al.*, 2001).

The reproductive cycle of the queen is regulated by a complex interplay of hormones that coordinate ovarian activity, reproductive behaviour and physiological changes within the reproductive tract (Reece, 2015).

The principal function of the estrogens, a steroid sex hormone, is to cause cellular proliferation and growth of the tissues related to reproduction. Estrogen induces the growth and development of the female reproductive tract and estrus behavior. Estrogen's epitheliotropic function manifests at estrus when the epithelium in the vagina proliferates and cornification is more prevalent. The most commonly found estrogen in non-pregnant queens is estradiol-17 β (Reece, 2015; Johnston *et al.*, 2001).

Progesterone is another steroid sex hormone, produced by the CL of the ovary, placenta, and adrenal cortex. The functions associated with progesterone include the promotion of endometrial gland growth, stimulation of secretory activity of the oviduct and endometrial glands to provide nutrients for the developing embryo before implantation, promotion of lobuloalveolar growth in the mammary gland, prevention of contractility of the uterus, as well as, prevention of follicular maturation and estrus during pregnancy, promotion of behavior appropriate to pregnancy and regulation of secretion of gonadotropins (Reece, 2015; Johnston *et al.*, 2001).

Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are collectively referred to as the gonadotropins. The main function of FSH in the queen is promotion of the growth of follicles and

LH is important for the ovulatory process and the luteinization of the granulosa, an essential aspect of CL formation (Reece, 2015).

FSH and LH concentrations exist in the plasma at a basal level controlled by negative feedback, being increased by estrogen and decreased by progesterone. The release of FSH and LH from the anterior pituitary is controlled by a releasing hormone from the hypothalamus – the gonadotropin-releasing hormone (GnRH) – secreted in response to low levels of LH or FSH. An increasing concentration of estrogen causes an increase in sensitivity of the anterior pituitary to GnRH, and results in increased release of gonadotropins. Progesterone decreases sensitivity of the anterior pituitary gland to GnRH, and LH and FSH concentrations decrease (Reece, 2015).

The progression from secondary to tertiary follicles is regulated by gonadotropins, particularly LH that stimulates androgen production by the theca interna and FSH that promotes their conversion into estrogens by the granulosa cells. The resulting rise in estrogen supports follicular growth and the acquisition of LH receptors by granulosa cells (Eurell & Frappier, 2006; Reece, 2015).

Ovulation happens when the follicles are fully developed and as estrogen concentrations continue to increase. A preovulatory surge of LH occurs and following this surge, estrogen production decreases and the follicular cells begin the process of luteinization (Eurell & Frappier, 2006; Reece, 2015).

Luteinization is the process by which the granulosa and theca cells transform into luteal cells and includes hypertrophy and hyperplasia of both cell types. The first sign of luteal regression is seen in late diestrus. The connective tissue scar remaining after luteal regression is called the *corpus albicans*. In older ovaries, there may be an abundance of such scars (Eurell & Frappier, 2006; Reece, 2015).

The queen is a seasonal polyestrous animal, with several consecutive reproductive cycles followed by an anestrus period, and whose seasonality is determined by the photoperiod and resultant melatonin concentration (Schäfer-Somi, 2017).

An increased photoperiod is associated with a reduced melatonin release by the pineal gland. Lower melatonin concentrations increase the release of GnRH by the hypothalamus, which in turn, increases the secretion of gonadotropins and, consequently, of the sex hormones (Schäfer-Somi, 2017).

In the northern hemisphere, and depending on latitude, cyclic ovarian activity – as opposed to anoestrus – is expected between January/February until late September/mid-October. Due to artificial lighting, some seasonality changes can be expected in indoor cats, with year-round cyclicity being possible. In some breeds, such as Orientals, lower sensitivity to the photoperiod can be noted (Johnson, 2022; Pereira *et al.*, 2024).

Puberty is defined as the beginning of reproductive life, more specifically, in the female, the beginning of ovarian activity. Kittens born in the spring/summer months reach puberty in the next breeding season, at about 6–8 months of age. Kittens born in the fall/early winter only reach puberty in the next breeding season (Reece, 2015). Oriental breed kittens can show early-onset puberty whereas the opposite can be noted in long-hair breeds and Manx kittens (Johnston *et al.*, 2001).

The stages of the feline reproductive cycle are proestrus, estrus, interestrus, diestrus and anestrus (Johnston *et al.*, 2001).

Proestrus is a short (typically less than 24 hours) and behaviourally ambiguous stage, which leads to observational difficulty. In a study from (Shille *et al.*, 1979), proestrus was only observed in 27 of the 168 analysed feline estrous cycles.

Proestrus is the phase of follicular maturation and endometrial proliferation following regression of the corpus luteum of the previous cycle. In this phase, progesterone level falls, allowing the release of FSH. Follicular size increases, the vaginal epithelium thickens and estrogen concentrations rise, leading to estrus (Eurell & Frappier, 2006; Johnson, 2022; Reece, 2015; Johnston *et al.*, 2001)..

Estrus is the phase of sexual receptiveness, lasting for 5-7 days and presenting characteristic behaviours such as, vocalization, rubbing of the face/neck on walls and furniture, lordosis and treading with the hind feet with the rear quarters elevated. The presence of this typical behaviour often lags behind the actual onset of the estrus (Johnson, 2022; Reece, 2015; Johnston *et al.*, 2001).

During this phase, follicles (3 to 6 in each follicular wave) reach maturity, with estradiol increasing (>20 pg/ml), leading to ovulation. Ovulation is preceded by a surge of LH (Eurell & Frappier, 2006; Johnson, 2022; Reece, 2015; Johnston *et al.*, 2001).

Increased serum concentrations of estrogen, produced by developing follicles on the ovarian tissue stimulate proliferation of stratified squamous epithelium in the vagina (Weedon & Kustritz, 2019). At the end of estrus, estrogen levels decline (Reece, 2015).

If ovulation does not occur, the queen enters interestrus. Some authors use the term postestrus/postoestrus to designate this phase (Kanca *et al.*, 2014; Lofstedt, 1982). The term metestrus has also been used in some works, like in the study of Toniollo *et al.* 1995, yet this term means time of corpus luteum development which, while following estrus in some species like the cow, does not occur in the queen. In queens that ovulate, morphological evidence of CL formation becomes apparent following estrus. Therefore, the subsequent reproductive phase should be designated as diestrus, not metestrus (Eurell & Frappier, 2006; Johnston *et al.*, 2001).

Interestrus has a variable duration, averaging 8-9 days. During this time, the dominant follicles undergo atresia and estradiol decreases, while a new wave of follicular development is already under way (Eurell & Frappier, 2006; Johnson, 2022; Reece, 2015; Johnston *et al.*, 2001).

If ovulation does occur, the queen shall enter diestrus, a progesterone-dominated luteal phase, that is confirmed by the presence of one or more CL on the ovary (Eurell & Frappier, 2006; Johnson, 2022; Reece, 2015; Johnston *et al.*, 2001).

The progesterone, secreted by the CL, begins to rise on day four after the first mating occurs, initiating the luteal phase. Endometrial glandular hyperplasia and secretion are maximal during diestrus (Eurell & Frappier, 2006; Reece, 2015; Johnston *et al.*, 2001).

Toward the end of diestrus, the corpus luteum regresses (luteolysis) and endometrial involution sets in. Diestrus can be prolonged into pseudopregnancy or gestational/lactational diestrus, lasting approximately 40 days in the pseudopregnant queen and approximately 60 days in the pregnant queen. Estrus resumes about 7 to 10 days following luteolysis in both the pregnant and the pseudopregnant queen. However, lactation and suckling of kittens, may cause lactational anestrus that may persist until 2 or 3 weeks after weaning (Eurell & Frappier, 2006; Johnson, 2022; Reece, 2015; Johnston *et al.*, 2001).

Anestrus is the prolonged period of sexual inactivity. Plasma estradiol and progesterone concentrations are at baseline levels during this phase (Eurell & Frappier, 2006; Johnson, 2022; Reece, 2015; Johnston *et al.*, 2001).

Although the queen was considered a strict induced ovulator, Lawler *et al.* 1993 identified spontaneous ovulators – as ovulation in some queens happened without intromission or other vaginal *stimuli*. Studies have been conducted and the percent of spontaneous ovulators found ranges from 31% (Pereira *et al.*, 2024) to 87% (Gudermuth *et al.*, 1997).

The duration of CL functions is 40 to 45 days in non-pregnant queens (Paape *et al.*, 1975) – as opposed to 60 to 63 days in pregnant queens (Verstegen *et al.*, 1993) – and the steroidogenic capacity of the CL does not differ from both cases, based on its steroid contents and steroidogenic enzymes' production (Zschockelt *et al.*, 2014).

Similar to the pathogenesis of pyometra in their canine counterparts, the cumulative prolonged effects of progesterone in the endometrium have been suggested as a major predisposing factor for cystic endometrial hyperplasia (CEH) in the queen, which in turn renders the uterus more susceptible to secondary ascending bacterial infection (Binder *et al.*, 2019; Hollinshead & Krekeler, 2016).

The same study from Binder *et al.* 2019 analysed 89 healthy post-pubertal queens, of which 40% were considered spontaneous ovulators, and presented at least one CL. This study found an increased prevalence of CL with weight, indicating that the proportion of adipose tissue and body energy storage may have positive effects on spontaneous ovulation, but no association to age. However, the prevalence of CEH increased in older cats, without a difference regarding the presence or absence of CL in the ovaries (Binder *et al.*, 2019). In a more recent study from Okur *et al.* 2026, with

50 cats, more than half of the queens presenting with CL also presented with CEH, while the ratio in queens with CEH but no CL was lower than 20%.

2.2 Reproductive Control In Queens

The need to control fertility in queens arises from both animal welfare and population management considerations. Due to their high reproductive potential and the frequent occurrence of unplanned matings, domestic cats can rapidly contribute to the growth of free-roaming cat populations (Little, 2012; Welsh *et al.*, 2014). This population expansion is associated with numerous challenges, including shelter overcrowding, abandonment, the spread of infectious diseases, predation on wildlife, and compromised animal welfare (Clyde *et al.*, 2022; McDonald *et al.*, 2025; Tan *et al.*, 2020). Consequently, effective reproductive control has become a fundamental component of modern feline medicine (Johnson & Cicirelli, 2014; Levy *et al.*, 2014).

Reproductive control in the queen can be accomplished through a number of methods, either surgical or non-surgical.

2.2.1 Surgical Methods

Surgical sterilization is one of the most performed procedures in small animal practice. In Europe and United Kingdom, the proportion of neutered private-owned cats ranges from 61% to 93% (Murray *et al.*, 2015).

Queens can be sterilised by removing the ovaries alone (ovariectomy), or with concomitant partial or complete removal of the uterus (ovariohysterectomy). Surgical sterilization with preservation of gonadal hormones (hysterectomy) is also possible, but rarely recommended to client-owned cats due to behaviour issues associated with frequent hormonal cycling and high incidence of malignant mammary gland tumours (Gogua, 2025; Overley *et al.*, 2005; Romagnoli *et al.*, 2024).

The risks of hemorrhage, vaginal bleeding, inadvertent ligation of the ureter, and formation of stump granulomas are less likely in ovariectomy (OVE). Regarding ORS, this approach may allow better visualization of the ovary, since incisions are made more cranially. Incisions were shorter for OVE than OVH but there was no difference in pain scores between the two groups and the surgical time was not significantly different either. There are no differences in long term complications between OVE and OVH (Smith & Séguin, 2012).

A potential disadvantage of OVE is that, since the uterus is not removed, there's a risk of development of uterine tumors (Smith & Séguin, 2012).

Particularly regarding OVH, the most common reason to perform it is to prevent estrus and its associated behaviours, and unwanted litters (Binder *et al.*, 2021). Other reasons for OVH include prevention of mammary tumors or congenital anomalies, prevention and treatment of pyometra, metritis, reproductive tract neoplasia, cysts, trauma, uterine torsion, uterine prolapse, subinvolution of placental sites, vaginal prolapse, pseudopregnancy and vaginal hyperplasia and even control of some endocrine and dermatological abnormalities. Many technical variations of OVH have been described, including open techniques, with midline or flank approaches and laparoscopy/lapascopy assisted techniques (Case *et al.*, 2015; Fossum, 2019; Howe, 2006; Munif *et al.*, 2022).

Queens subjected to OVH should undergo a pre-operative physical exam, including palpation of the abdomen, vulva and mammary glands. Abdominal palpation may reveal an enlarged uterus, mass, visceral displacement and/or pain. Abnormal vulvar skin folds, conformation, discharge, or enlargement may also be noted. Mammary glands may present alterations in symmetry, texture, size, mobility, discharge and the presence of masses (Fossum, 2019).

Some postoperative complications associated with this surgery have been described, such as, hemorrhage, suture dehiscence, ORS, uterine stump pyometra, granuloma, urethral trauma, vagino-peritoneal fistula, urinary incontinence, diarrhea, vomiting, hematochezia, obstipation, colon stenosis, corneal ulceration and discoloration or darkening of the fur after tricotomy in oriental breeds (Adin, 2011; Coe *et al.*, 2006; Smith & Davies, 1996; Xie *et al.*, 2024).

OVH can be performed using different surgical approaches, primarily midline and flank. Several studies have compared these two techniques. For healthy queens with normal reproductive anatomy, subjected to elective OVH, the preferred approach may vary with geographical region/country or the veterinary surgeon's personal preference, since both approaches have advantages and disadvantages. For queen presenting with pyometra, pregnancy, ORS, tumours and other reproductive alterations, the midline approach is preferred, while for queens with enlarged mammary glands, due to, for example, mammary hyperplasia, the flank approach is preferable. The latter is also preferred for wound observation and management in feral or aggressive cats and is associated with a reduced risk of evisceration (Bilen *et al.*, 2023; Burrow *et al.*, 2006; Carbonari *et al.*, 2025; Coe *et al.*, 2006; Del Romero *et al.*, 2020; Grint *et al.*, 2006; Howe, 2006; Lochi *et al.*, 2014; McGrath *et al.*, 2004; Merritt & Collinson, 2018; Munif *et al.*, 2022; Murugesan *et al.*, 2020; Nazari *et al.*, 2025; Rani *et al.*, 2024; Swaffield *et al.*, 2020).

In a 2006 study, Coe *et al.* found to significant difference in the time required to perform each approach, yet, in the flank approach, it took longer to enter the peritoneal cavity and, in the midline approach, it took longer to identify the uterine horn. The same study found that owners' perception of pain (using visual analogic scale scores) was the same for both approaches and overall owners either didn't have a preferred approach or were happy with the one chosen by the surgeon (Coe *et al.*, 2006).

Nazari *et al.*, 2025 showed no significant differences between the two approaches regarding oxidative stress markers. According to Bilen *et al.* (2023), the flank approach resulted in the isolation of less resistant bacteria when compared to the midline approach. However, there is no scientific consensus on the best OVH approach.

2.2.2 Non-Surgical Methods

A number of non-surgical (some of which are temporary) contraceptive methods are available for queens, including products with regulatory approval, as well as some used “off-label”. Non-surgical methods remove the risk of complications related to surgery and offer potential to treat more animals in less time and at lower cost. These methods also appeal to cat breeders seeking temporary/reversible contraception (Murray *et al.*, 2015).

Despite promising developments, concerns regarding efficacy, target-species safety, duration of effect, and potential adverse effects remain, and research is ongoing to develop new effective non-surgical methods of fertility control in cats (Murray *et al.*, 2015).

Deslorelin acetate is a synthetic GnRH agonist. Initially, it stimulates LH and FSH, but then the activation of GnRH receptors suppresses LH and FSH synthesis and release (Romagnoli *et al.*, 2024). The use of deslorelin in queens in Portugal is “off-label”, although labelled application varies by country (Virbac, 2012). Administration of a deslorelin implant can last for over three years and after implant duration or removal, queens returned to normal cyclicity and fertility (Goericke-Pesch *et al.*, 2013; Toydemir *et al.*, 2012).

Megestrol acetate is a synthetic progestogen with a high affinity for the progesterone receptor (Jänne *et al.*, 1978; Schindler *et al.*, 2003). Severe, long-lasting metabolic effects occur when higher dosages are given. Queens have developed mammary gland lesions, such as mammary hyperplasia, benign and malignant tumours, cutaneous xanthomatosis, blindness (due to opacity of the anterior chamber caused by hyperlipidaemia) (Ghaffari, 2008), uterine lesions, such as CEH, pyometra and adenomyosis (Bulman-Fleming, 2008), diabetes and adrenocortical suppression (Hayden *et al.*, 1989; Hinton & Gaskell, 1977; Hollinshead & Krekeler, 2016; MacDougall, 2003; Miller *et al.*, 2003; Pukay & Stevenson, 1983; Watson *et al.*, 1989).

Medroxyprogesterone acetate was the first synthetic progestogen commercially available for veterinary use. It is longer-acting than MA, has an affinity higher than endogenous progesterone for the progesterone receptor and has an affinity lower than MA for the androgen and glucocorticoid receptor (Loretti *et al.*, 2005; Romagnoli, 2015).

Mibolerone is a synthetic androgen that was commercially available in North America and withdrawn from the market due to its potential for human abuse. Serious side effects like hepatic dysfunction have been reported and did not resolve after withdrawal and mortality ensued at higher doses. For these reasons, mibolerone is not recommended in queens (Burke, 1982).

Administration of melatonin mimics the decreasing of the photoperiod, resulting in the suppression of the estrous cycle (Kutzler, 2015). Melatonin implants and oral tablets are commercially available (Romagnoli *et al.*, 2024). Melatonin implant administration resulted in rapid and prolonged estrus postponement (Gimenez *et al.*, 2009; Schäfer-Somi, 2017; Okur *et al.*, 2025). Queens remain fertile after treatment and can produce healthy litters (Schäfer-Somi, 2017). In Portugal, the use of melatonin implants in queens is “off-label” (Ceva, 2020).

Antibodies against GnRH suppress reproductive behaviour as they suppress the synthesis and secretion of LH, FSH and estrogen (Ladd *et al.*, 1994). Currently, there are two GnRH vaccines commercially available in the USA for some wild life species (Romagnoli *et al.*, 2024).

2.3 Vaginal Cytology

Vaginal epithelium changes throughout the feline estrus cycle have been described establishing the biological foundation for the use of vaginal cytology in queens (Mills *et al.*, 1979; Shille *et al.*, 1979). Estrogens induce the thickening of the vaginal epithelium causing epithelial cells to move away from blood supply, degenerating and increasing their size (Kanca *et al.*, 2014).

Clearing – the absence of debris of non-cellular origin and of mucus, as well as a lack of “coalescence of cells into sheetlike aggregates” – appears to be a good indicator, in the feline vaginal cytology, of estrogen activity (Shille *et al.*, 1979)

While vaginal cytology is a tool widely used in the reproductive management of the bitch, its use in the queen still lacks research. Besides this lack of research, there is still a “disputable possibility” of inducing ovulation, that leads to clinics avoiding the use of this technique (Pereira *et al.*, 2025). A study from Kanca *et al.* 2014 swabbed 15 queens in estrus, not inducing ovulation in any of them, suggesting that this concern may be less justified than previously assumed.

Feline vaginal cytology may help with the reproductive management of queens, particularly spontaneous ovulating queens, as well as help identify silent or continuous heat (Pereira *et al.*, 2025).

Vaginal cytology is also used as a tool, in a multimodal approach to diagnostics in cases of infertility, allowing a deeper understanding of the reproductive cycle (Axnér *et al.*, 2008; Little, 2012). In the reproductive management of the queen, specially if infertility is suspected, vaginal cytology may be an useful tool to guarantee that physiological estrus is occurring when the queen present typical estrus behaviour (Fontbonne, 2022).

Vaginal cytology can be used to diagnose ORS, by detecting estrus post-gonadectomy (Ball *et al.*, 2010; Weedon & Kustritz, 2019). In ORS, even a small fragment of ovarian tissue is sufficient to cornify the vaginal epithelium as shown by Moore *et al.* 2026, supporting the use of vaginal cytology as a diagnostic tool for ORS.

Ovarian neoplasia and cysts are rare in queens, becoming increasingly common with old age. Prolonged estrus can be a sign of these sorts of pathologies, making vaginal cytology a possible complementary diagnostic tool, although not the most reliable one. Finding of neoplastic cells on vaginal smears is uncommon (Kustritz, 2020).

Pyometra occurs during or after diestrus. In queens with open-cervix pyometra, vulvar discharge is purulent and foul smelling. A vaginal cytology can be performed and the microscopy fields should be filled with degenerate neutrophils, as this is an inflammatory discharge. All vaginal epithelial cells should be non-cornified (Kustritz, 2020).

A study from Hidalgo *et al.*, (2020), compared four different vaginal cytology techniques in cats, the swab, the gynecological brush, the interdental brush and the Pasteur pipette. Although, the swab technique is easy, relatively atraumatic and the most commonly used in veterinary medicine, this study found that it's associated with lower cellularity and cellular distortion due to adherence to the swab's cotton fibers. While the gynecological brush appears to solve these problems, it's associated with a greater discomfort to the cat, making the smaller interdental brush a similar but more suitable option. At last, the Pasteur pipette technique resulted in good cellular recovery but the procedure (contention, insertion of liquid in the vagina and even the liquid's temperature) may cause discomfort and be difficult to tolerate in cats (Hidalgo *et al.*, 2020).

2.3.1 Interpretation

Four types of vaginal epithelial cells can be identified on vaginal cytology. Parabasal cells are small and round with a prominent nucleus and low cytoplasm to nucleus ratio. Intermediate cells are similar to the parabasal cells, being slightly larger and having a higher cytoplasm/nucleus ratio. These two cell types can be referred to as non-cornified cells (Kustritz, 2020).

Superficial cells result from parabasal cells that induced to divide by the increase in serum estrogen concentration. Superficial cells are large and irregular in shape, with a high cytoplasm/nucleus ratio. Even larger and more irregular cells, with no visible nucleus, are designated anucleate cells. These two cell types can be referred to as cornified cells (Kustritz, 2020).

Other cell types commonly found in vaginal cytology are neutrophils, erythrocytes and bacteria (Kustritz, 2020).

Proestrus is identified by <50% cornified cells and mainly small to large intermediate cells (Johnson, 2022). In cytology, proestrus (a very brief phase) and estrus are difficult to differentiate, which has led some authors (Shille *et al.*, 1979) to consider them as the same reproductive phase.

Estrus is identified by >75% cornified cells, with possible intermediate cells present. Cornification may not reach 100% (as opposed to the bitch). The cell's nucleus becomes pyknotic but usually remains visible – a process designated as parakeratinization (Brady, 2004; Regli & Kress, 2002). Erythrocytes are atypical and may be caused by mucosal irritation (associated to the swab) or by a pathology. Clearing has been consistently noted in vaginal cytologies obtained from queens in estrus. Neutrophils are rare but might be present in all reproductive stages, with no clinical significance (Pereira *et al.*, 2025).

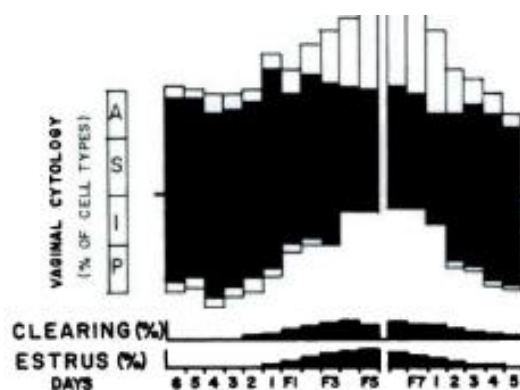


Figure 7. Types of epithelial cells and percentage of clearing during follicular phase (days F1 to F7), from Shille *et al.* (1979).

In interestrus, the cytology will contain mostly parabasal and intermediate cells, with cornified cells decreasing to <50% (Johnson, 2022).

As of diestrus, the cytology will contain mostly small intermediate and parabasal cells (Johnson, 2022). As described by Pereira *et al.*, (2025), in cytology, interestrus and diestrus are phases difficult to distinguish in cats. This is likely because differences in serum P4 concentrations do not result in significant changes in vaginal cell distribution (Termelioğlu *et al.*, 2022). For this reason, the assessment of serum P4 becomes an important aid to vaginal cytology evaluation and identification of ovulation.

During anestrus, the vaginal cytology will contain mostly parabasal cells and some small scattered intermediate cells (Johnson, 2022; Pereira *et al.*, 2025; Toniollo *et al.*, 1995).

2.3.2 Inter-Observer Variability

Inter-observer variability is a commonly encountered challenge in diagnostics, in both human and veterinary medicine, as many diagnostic tools depend on the researcher's subjectivity (McHugh, 2012).

Inter-observer variability in the classification of vaginal cytology has been assessed for the bitch (Arlt, 2018; Moxon *et al.*, 2010; Reckers *et al.*, 2022).

Moxon *et al.*, (2010) acknowledged the potential for variation between researchers' interpretations of the cellular morphology of vaginal epithelial cells of canine origin. Cellular morphology assessment may have an inherently subjective nature, as cells undergo subtle cytological changes throughout the reproductive cycle. For example, parabasal cells might be easily confused with small intermediate cells by observers, especially the less experienced ones. In this study, there were a large number of misinterpretations of the reproductive cycle from all technicians, including the most experienced one. The combination of other techniques, like P4 measurement, was recommended to reduce the risk of phase misinterpretation (Moxon *et al.*, 2010). Ultrasound was also suggested as an additional diagnostic tool, as the sonographic aspect of the ovaries changes during the different phases of the estrous cycle (Pertiwi *et al.*, 2018)

Arlt, (2018), used an online survey to evaluate reproductive medicine clinicians' (63 responders) opinion on the reliability of vaginal cytology as a sole method to determine ovulation timing in the bitch. Afterwards, EVSSAR 2018 congress attendees had the opportunity to blindly classify several random vaginal cytologies. The online survey concluded that the large majority of the surveyed veterinarians did not consider vaginal cytology a reliable tool, when used alone. Blind classifications at the EVSSAR congress supported this opinion, as classifications presented high heterogeneity. It was not possible to test the inter-observer variability in this study (Arlt, 2018).

Reckers *et al.* 2022, further tested the inter-observer variability by requesting 16 participants (worldwide veterinary clinicians interested in reproductive medicine) to classify eight epithelial cells obtained from vaginal cytologies performed in the bitch. The agreement between the participants was considered poor ($\kappa = 0,412$), but after a standardized tutorial provided to five raters, the evaluation of 100 random vaginal cytologies resulted in a strong agreement ($\kappa = 0,858$). The observed improvement in agreement strength suggests that, although vaginal cytology interpretation remains inherently subjective, this subjectivity may be reduced through the implementation of standardized classification criteria (Reckers *et al.* 2022). There are no known studies on the inter-observer variability in the queen's vaginal cytology classification.

3. OBJECTIVE(S)

This study hypothesized that healthy queens, at different reproductive cycle stages, exhibit discernible vaginal cytological patterns, allowing some degree of cytological differentiation. In addition, it was hypothesized that trained researchers would demonstrate a measurable degree of agreement when classifying vaginal cytologies according to reproductive stage, and that this degree of agreement would improve when weighted agreement methods accounting for phase proximity were applied.

The aim of this study was to characterize vaginal cytological patterns in clinically healthy queens undergoing elective ovariohysterectomy and to evaluate inter-observer agreement, diagnostic performance, and potential classification bias in the identification of reproductive stages. By assessing the accuracy and reproducibility of vaginal cytology, this study sought to contribute to the standardization and clinical interpretation of feline vaginal cytology as a practical diagnostic tool in reproductive medicine. These objectives were addressed using Cohen's Kappa and Weighted Kappa analyses, as well as calculations of sensitivity, specificity, and paired McNemar test for the classification of diestrus versus non-diestrus stages.

4. MATERIALS AND METHODS

4.1 Study Design and Ethical Approval

This prospective observational study was conducted between 5th January and 5th March 2026 at the Department of Obstetrics and Gynecology, Department of Reproduction and Artificial Insemination and Department of Histology of the Veterinary Medicine Faculty of the Ankara University. The study protocol was approved by the Local Animal Ethics Committee (number 430/3642) and informed owner consent was obtained for all cats included in the study.

4.2 Animals

A total of 32 client-owned domestic female cats presented for elective ovariohysterectomy (OVH) were enrolled. Sample size was based on convenience sampling of cats presented for elective OVH during the study period. Cats were clinically healthy and had no history of reproductive tract pathology. Detailed demographic information (age, breed and body weight) was not available for all enrolled queens and was therefore not included in the analyses.

One queen ("ID23") was excluded from the study due to extremely low cellularity of the vaginal cytology sample.

4.2.1 Ovariohysterectomy

All cats underwent routine ovariohysterectomy under general anesthesia using a combination of propofol (IV) for induction and isoflurane (INH) for maintenance. A standard left flank approach was performed as described in previous studies (Babu *et al.* 2018; Howe, 2006; Miller *et al.*, 2016).

The cat was placed on right lateral recumbency with the hind limbs secured in caudal extension. A 2 – 3 cm dorsoventral incision was placed caudally to the midpoint between the last rib and the iliac crest, through the skin, the subcutaneous fat, the external aponeurosis, internal and transversal abdominal obliques muscles and peritoneum. The abdominal wall was entered using a blunt dissection. The ovaries and uterus were identified and the ovarian pedicle was isolated, double clamped (with Halstead "mosquito" forceps) and ligated immediately below, with absorbable sutures. The broad ligament was torn, the pedicle was checked for hemorrhage and the uterine horn was followed to the uterine bifurcation, leading to the right ovary. The isolation and ligation of the right ovarian pedicle was made and the broad ligament was torn. Afterwards, traction on both horns was applied, exposing the uterine body and its ligation site. After its ligation and in the absence of

hemorrhage, the abdominal wall was closed in a single layer, in a simple continuous pattern, and a further intradermal continuous suture was used to appose the skin. Each cat also received a prophylactic SC injection of amoxicillin and a SC injection of meloxicam for pain management.

After the ligation and removal of the uterine horns with the respective ovaries, the presence/absence of functional CL in the ovaries was noted, whenever possible. For this effect, a functional CL was considered a macroscopically well-differentiated CL, with orange-yellow pigmentation, and around 3-6mm in diameter (Eurell & Frappier, 2006).

4.2.2 Progesterone Determination

Blood samples for serum progesterone (P4) determination were collected from a subset of 19 queens when considered clinically appropriate, taking into account intraoperative blood loss, duration of anaesthesia and body temperature, as well as, the time available to the clinicians for performing the blood collection between consecutive surgical procedures.

For P4 measurement in feline serum, blood samples were collected into plain tubes, allowed to clot, centrifuged, and the clear serum was transferred into clean tubes. Samples were stored in aliquots at -20°C to avoid repeated freeze-thaw cycles. Commercial enzyme-linked immunosorbent assay (ELISA) kits, supplied by BT LAB®, were used according to the manufacturer's instructions.

Ovulation was considered to have occurred when serum P4 concentrations were equal or higher than 2ng/ml (Pereira *et al.*, 2024).

4.3 Vaginal Cytology Technique

Vaginal smears were obtained before or immediately after surgery using a sterile cotton swab moistened with physiological saline (0.9% NaCl). The vulvar labia were separated with the thumb and index finger and the swab was gently introduced 1-2 cm into the cranial vagina, rotated two to three times, and withdrawn. The collected cellular material was transferred onto a clean glass microscope slide by gently rolling the swab across the slide surface. Smears were then air-dried and stained using the Diff-Quick staining method (Hidalgo *et al.*, 2020).

Slides were examined independently by two trained researchers under light microscopy (×100 and ×400 magnification). The researchers were blinded to serum progesterone concentrations, ovarian findings observed during the surgery, and each other's classification. Epithelial cells were morphologically classified as parabasal, intermediate, superficial, or anucleate according to established descriptions of feline vaginal cytology reported in the literature (Johnson, 2022; Kustritz, 2020; Pereira *et al.*, 2025).

Figure 8 shows representative photomicrographs of the four epithelial cell types observed in feline vaginal cytology: parabasal, intermediate, superficial and anucleate cells.

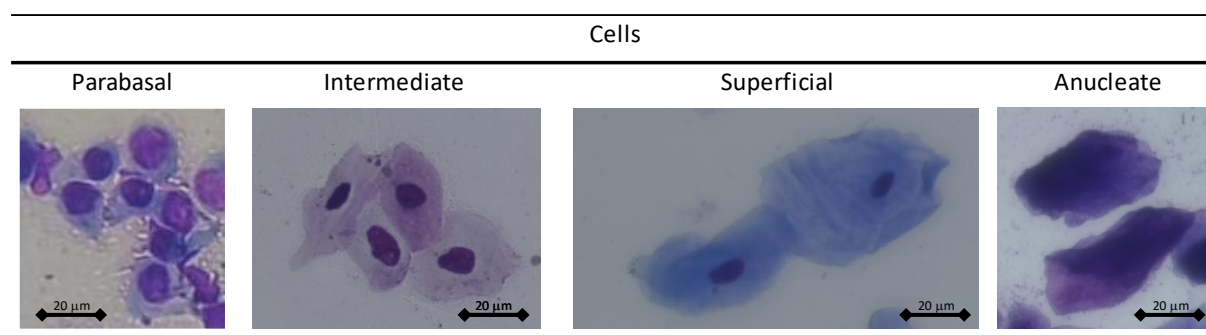


Figure 8. Types of epithelial cells found on the vaginal cytologies.

For each slide, the proportion of each cell type was evaluated across 10 fields at 400× magnification, each containing at least 10 epithelial cells. A semi-quantitative scoring system was developed for this study to characterize the predominance of each epithelial cell type within individual microscope fields. Scoring was performed as follow for each microscopic field, the relative abundance of each epithelial cell type (parabasal (PB), intermediate (I), superficial (S) and anucleate(A)) was scored independently using a semi-quantitative scale from 0 to 4. A score of 4 was assigned when a cell type was the only epithelial cell type present in the field, with all other cell types receiving a score of 0. A score of 3 was assigned when a cell type was clearly predominant over all others; in such cases, the remaining cell types received scores of 0 or 1. A score of 2 was assigned when a cell type was present in proportions similar to one or more other cell types, indicating the absence of a clearly predominant population; the other cell types in the field could therefore receive scores of 2, 1 or 0. A score of 1 was assigned when a cell type was present in low numbers but clearly outnumbered by one or more other cell types. A score of 0 indicated that the cell type was absent from the field. This information is summarized in table 24. The presence of mucus, erythrocytes and neutrophils was also noted for each field.

Table 24. The semi-quantitative scoring system used to characterize the predominance of each epithelial cell type within individual microscope fields.

Score	Interpretation
4	Cell type exclusively present in the field; all other epithelial cell types absent (0).
3	Cell type clearly predominant; all other cell types present only in low numbers (1) or absent (0).
2	Cell type present in proportions similar to one or more other cell types; no clearly predominant cell population.
1	Cell type present but clearly outnumbered by one or more other cell types.
0	Cell type absent.

The scores obtained from the 10 microscopic fields were summarized, for each slide, by determine the modal value for each epithelial cell type (PB, I, S, A). The use of modal value was intended to represent the predominant cytological pattern observed across slide while minimizing the influence of occasional atypical fields. When the modal value was 0 but a cell type was present in at least two microscopic fields, the score was adjusted to 1. In cases where two values occurred with equal frequency, both values were reported (e.g., 2–3). When no clear mode could be identified, the score was assigned as the whole number nearest to the arithmetic mean of the observed values.

The resulting modal values, for each slide, were then interpreted independently by each researcher to assign the reproductive cycle phase. Classification was performed by comparing the observed cytological profile with the expected patterns described in the literature (table 25), based on the work of Feldman & Nelson, (2004); Johnson, (2022); Pereira *et al.* (2025); Shille *et al.*, (1979); Johnston *et al.*, (2001).

Table 25. Expected modal values of vaginal cytology cell types for each estrous cycle phase, compiled from and adapted based on Feldman & Nelson, (2004); Johnson, (2022); Pereira *et al.* (2025); Shille *et al.*, (1979); Johnston *et al.*, (2001).

Estrous Phase	Cell Types			
	Parabasal	Intermediate	Superficial	Anucleate
Anestrus	3 - 4	1	2	1
Proestrus	0	2 - 3	2 - 4	2 – 3
Estrus	0	1	3 - 4	2 – 3
Interestrus	1	2	2	1
Diestrus	1 - 2	3	2	1

4.4 Statistical Analysis

The agreement between the two researchers regarding reproductive cycle phase classification was evaluated using Cohen's Kappa coefficient (McHugh, 2012). In addition, a Weighted Kappa coefficient with quadratic weights was calculated to account for the ordinal nature of the reproductive stages and the fact that disagreements between adjacent stages are biologically less relevant than disagreements between stages representing markedly different hormonal and cytological conditions (Hwang *et al.*, 2020). An alphabetical ordering was considered, as follows: A = estrus; B = proestrus; C = interestrus; D = diestrus; E = anestrus. In this arrangement, proestrus is placed after estrus to better reflect the cyclical nature of the feline estrous cycle. Since the estrous cycle is continuous, proestrus and interestrus were considered adjacent phases, as they are cytologically closer to each other than estrus is to interestrus. This ordering provides a more biologically coherent representation of phase proximity. Furthermore, anestrus is positioned as the "terminal" phase, increasing its distance from estrus while maintaining a relatively closer relationship to the other non-follicular phases (diestrus and interestrus).

The significance of each kappa (κ) value can be found in the table below.

Table 26. Significance of kappa (κ) values (McHugh, 2012)

Value of Kappa	Level of Agreement
0 - 0,20	None
0,21 - 0,39	Minimal
0,40 - 0,59	Weak
0,60 - 0,79	Moderate
0,80 - 0,90	Strong
> 0,90	Almost Perfect

For queens with either serum progesterone measurements or direct observation of corpora lutea (CL) during ovariohysterectomy, the accuracy of cytological classification for diestrus versus non-diestrus was evaluated. A queen was considered to be in diestrus when either corpora lutea were observed or serum progesterone concentrations were ≥ 2 ng/ml. Sensitivity, specificity and their respective 95% confidence intervals (CI) were calculated for each researcher. Confidence intervals were calculated using Wald's method, with Wilson score intervals applied when appropriate for small sample sizes or extreme proportions.

The proportions of correct and incorrect diestrus classifications between researchers were subsequently compared using the paired McNemar test. Statistical significance was established at $P < 0,05$.

Statistical analyses were performed using:

IBM Corp. (2026). IBM SPSS Statistics for Windows (Version 31.0). Armonk, NY: IBM Corp.

Microsoft Corporation. (2025). Excel (Microsoft 365 Subscription). Available at:
<https://www.microsoft.com/en-us/microsoft-365/excel>.

Sergeant, ESG. (2018). Epitools Epidemiological Calculators. Ausvet. Available at:
<http://epitools.ausvet.com.au>.

5. RESULTS

5.1 Vaginal Cytology Classification and Inter-Observer Agreement

Representative microscopic images illustrating the range of vaginal cytological appearances observed in this study are presented in Figures 9–11.

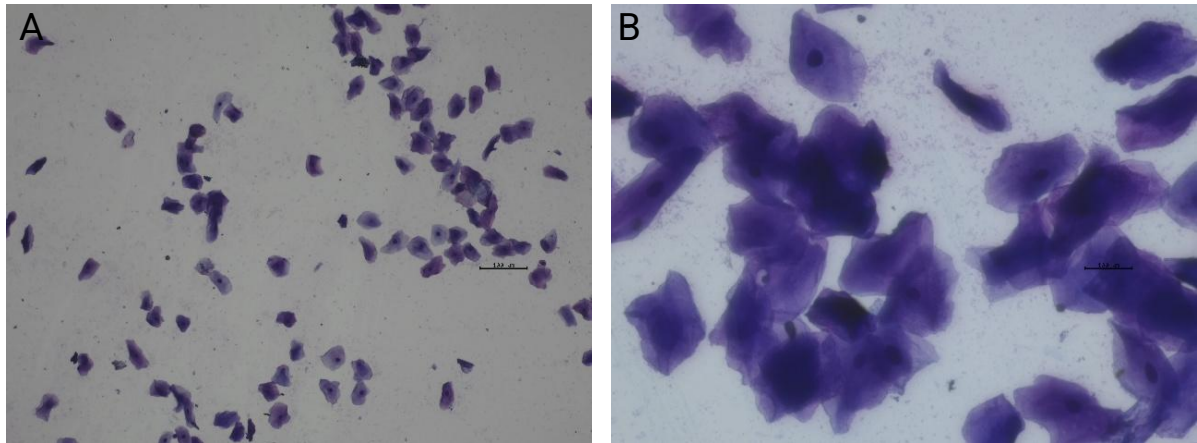


Figure 9. Vaginal cytology of a queen classified as being in proestrus or estrus (A – 100x magnification, B – 400x magnification)

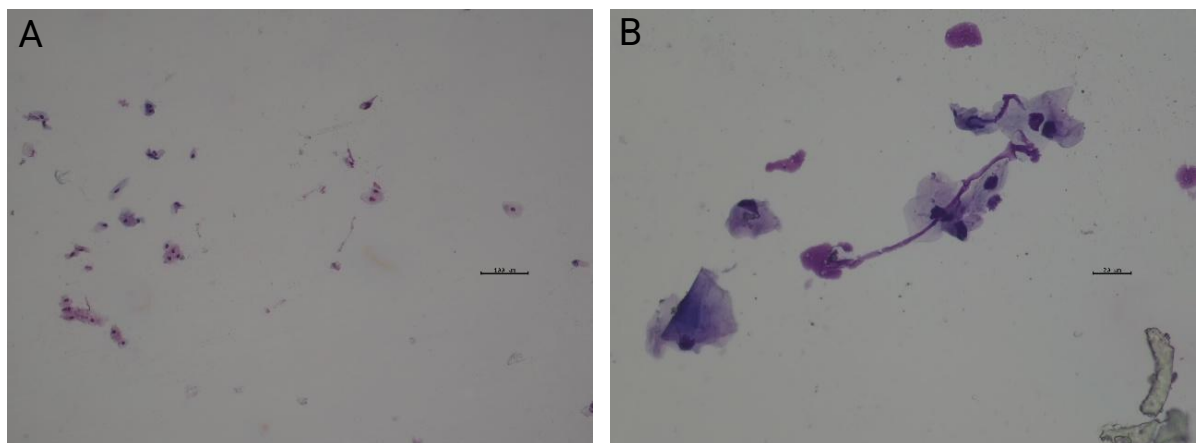


Figure 10. Vaginal cytology of a queen classified as being in diestrus (A – 100x magnification, B – 400x magnification)

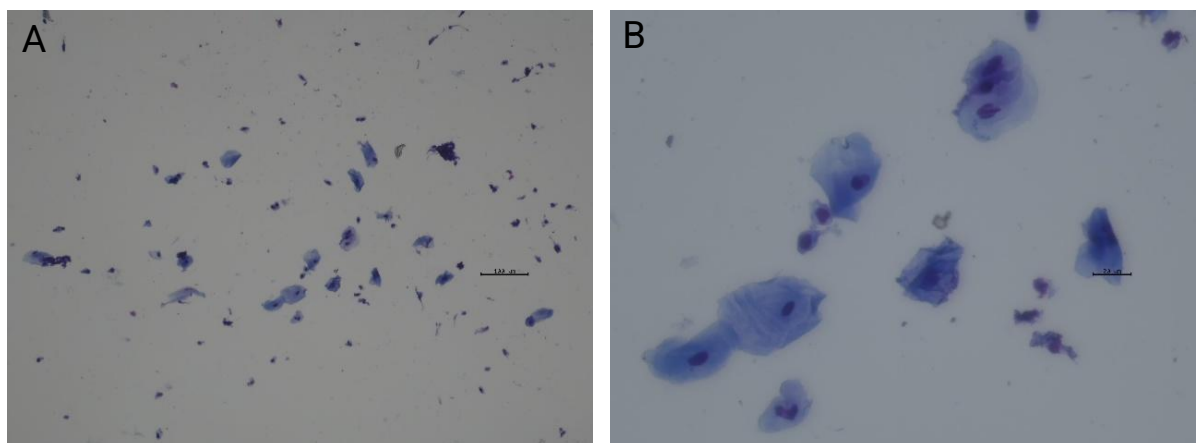


Figure 11. Vaginal cytology of a queen classified as being in interestrus or anaestrus (A – 100x magnification, B – 400x magnification)

Overall, erythrocytes, neutrophils and mucus were uncommon findings in the vaginal cytology samples. Only one sample contained macroscopically visible blood in the swab and a large number of erythrocytes on the cytology, whereas the remaining positive samples showed only small numbers of erythrocytes. Erythrocytes were observed in 5 of 31 samples ($\approx 16,1\%$). Neutrophils were found in moderate amounts in 1 of 31 samples ($\approx 3,2\%$). Mucus was identified in 7 of 31 samples ($\approx 22,3\%$).

The estrous cycle phase assigned to each vaginal cytology sample by each of the two researchers is presented in Table 27. Researcher 1 classified 8/31 samples (25,8%) as anestrus, 8/31 (25,8%) as estrus, 7/31 (22,6%) as diestrus, 5/31 (16,1%) as interestrus and 3/31 (9,7%) as proestrus. Researcher 2 classified 8/31 samples (25,8%) as estrus, 7/31 (22,6%) as diestrus, 7/31 (22,6%) as proestrus, 6/31 (19,4%) as anestrus and 3/31 (9,7%) as interestrus.

Table 27. Vaginal cytology classification by both researchers and presence of neutrophils, erythrocytes and mucus.

ID	Researcher 1	Researcher 2
1	Anestrus	Anestrus
2	Diestrus	Diestrus
3	Proestrus	Proestrus
4	Anestrus	Anestrus
5	Anestrus	Anestrus
6	Anestrus	Anestrus
7	Estrus	Estrus
8	Estrus	Estrus
9	Estrus	Estrus
10	Interestrus	Diestrus
11	Estrus	Estrus
12	Interestrus	Proestrus
13	Estrus	Proestrus
14	Estrus	Interestrus
15	Diestrus	Interestrus
16	Diestrus	Interestrus
17	Anestrus	Anestrus
18	Estrus	Estrus
19	Proestrus	Estrus
20	Anestrus	Diestrus
21	Estrus	Estrus
22	Anestrus	Diestrus
24	Interestrus	Proestrus
25	Anestrus	Anestrus
26	Interestrus	Proestrus
27	Interestrus	Diestrus
28	Diestrus	Diestrus
29	Diestrus	Proestrus
30	Diestrus	Proestrus
31	Proestrus	Estrus
32	Diestrus	Diestrus

Table 28 presents the cross-tabulation of vaginal cytology classifications assigned by both researchers. Agreement on the reproductive cycle phase was observed for 16 of 31 samples (51,6%), and among the 15 discordant classifications, the most frequent disagreement occurred between diestrus and interestrus (4/15 disagreements, 26,7%). Concordance was highest for anestrus, with perfect agreement observed, whereas proestrus and interestrus showed lower agreement. Interestrus showed no exact correspondence between observers, indicating reduced reproducibility based on cytological criteria.

Table 28. Cross-tabulation of vaginal cytological classification between both researchers. Percentages indicating agreement of Researcher 2 within each Researcher 1 category.

		Researcher 2										Total	
		Anestrus		Diestrus		Estrus		Interestrus		Proestrus			
		N	%	N	%	N	%	N	%	N	%	N	%
Researcher 1	Anestrus	6	100,0%	2	28,6%	0	0,0%	0	0,0%	0	0,0%	8	25,8%
	Diestrus	0	0,0%	3	42,9%	0	0,0%	2	66,7%	2	28,6%	7	22,6%
	Estrus	0	0,0%	0	0,0%	6	75,0%	1	33,3%	1	14,3%	8	25,8%
	Interestrus	0	0,0%	2	28,6%	0	0,0%	0	0,0%	3	42,9%	5	16,1%
	Proestrus	0	0,0%	0	0,0%	2	25,0%	0	0,0%	1	14,3%	3	9,7%
Total		6	19,6%	7	22,6%	8	25,8%	3	9,7%	7	22,6%	31	100,0%

Cohen's Kappa test was used to determine interobserver agreement in the classification of the 31 vaginal cytology samples. The Cohen's kappa indicated a minimal level of agreement between observers ($\kappa = 0,391$; 95% CI: 0,286 – 0,496), $p < 0,001$.

Weighted Kappa test with quadratic weights was additionally calculated. This analysis demonstrated a substantially higher agreement between observers ($\kappa = 0,835$; 95% CI: 0,727 – 0,942, $p < 0,001$) indicating that most discrepancies occurred between adjacent reproductive stages rather than between clearly distinct categories, indicating that disagreement was predominantly restricted to adjacent reproductive stages within the cytological classification system.

5.2 Diestrus Classification Accuracy

Out of the 31 queens included in the study, ovulatory status (diestrus or non-diestrus) could be definitively established in 21 animals. The remaining 10 queens were excluded from this analysis due to the absence of either serum progesterone measurements or intraoperative confirmation of corpus luteum presence. From the 19 blood samples collected and processed, 8 ($\approx 42\%$) had serum P4 concentrations equal or superior to 2 ng/ml, the threshold for which the occurrence of ovulation was considered. Overall, serum P4 values ranged from 0,443 ng/ml to 10,8 ng/ml.

For 21 queens, reproductive status was classified as diestrus (N=10) when serum P4 concentrations were ≥ 2 ng/ml or when developed CL were observed during OVH, and as non-diestrus (N=11) when $P4 < 2$ ng/ml or absence of CL in the ovaries. These criteria defined the "Reference" classification, which, together with the blinded cytological assessments of Researchers 1 and 2, serum progesterone values, and corpus luteum observations, is presented in Table 29. The corresponding cross-tabulation table for each researcher is shown in Table 30.

Table 29. Classification of each vaginal cytology into diestrus/non-diestrus classification, according to P4 values, CL observation and blinded researchers' classification. (ND-Non diestrus phase and D-Diestrus phase)

ID	Researcher 1	Researcher 2	Reference	P4 / (ng/ml)	CL
1	ND	ND	ND	--	Absent
2	D	D	D	--	Present
3	ND	ND	ND	1,09	--
4	ND	ND	D	2,23	--
5	ND	ND	ND	1,38	--
8	ND	ND	ND	1,18	--
9	ND	ND	ND	0,443	--
10	ND	D	D	--	Present
11	ND	ND	ND	1,27	--
12	ND	ND	ND	0,787	--
13	ND	ND	ND	1,28	--
14	ND	ND	D	4,17	--
17	ND	ND	D	10,1	--
18	ND	ND	ND	1,03	--
19	ND	ND	ND	1,1	--
22	ND	D	D	10,8	--
24	ND	ND	D	2,92	--
26	ND	ND	D	2,03	--
27	ND	D	ND	0,984	--
28	D	D	D	6,21	--
29	D	ND	D	2	--

Table 30. Cross-tabulation table for each researcher's accuracy in diagnosing diestrus.

	True Diestrus		True Non-Diestrus	
	Researcher 1	Researcher 2	Researcher 1	Researcher 2
Cytology Diestrus	3	4	0	1
Cytology Non-Diestrus	7	6	11	10

For Researcher 1, vaginal cytology correctly identified 3 of 10 diestrus cases, resulting in a sensitivity of 30% (95% CI: 1,6% - 58,4%), while correctly identifying all non-diestrus cases, yielding a specificity of 100% (95% CI: 74,1% - 100%). For Researcher 2, 4 of 10 diestrus cases were correctly classified, corresponding to a sensitivity of 40% (95% CI: 9,6% - 70,4%), while specificity was ≈91% (95% CI: 62,3% - 98,4%)

The paired McNemar test was performed to compare the proportion of correct and incorrect diagnoses between both researchers. No significant difference was detected ($p = 1$, 95% CI: 0,072 – 13,796).

5.3 Spontaneous Ovulation

Ovulatory status could thus be determined in 21 of the 31 queens based on the availability of serum progesterone measurements and/or intraoperative assessment of CL presence/absence. Within this evaluated subset, 10 queens were classified as diestrus, corresponding to an estimated ovulation frequency of 47,6%, while 11 queens were classified as non-diestrus. This proportion reflects the frequency of ovulation within the subset of animals for which complete endocrine and/or ovarian data were available.

6. DISCUSSION

The presence of small amounts of erythrocytes is very likely related to mucosal irritation due to swab insertion (Johnson, 2022) and, for that reason, is not necessarily indicative of pathology. Erythrocytes are not physiologically observed in the queen, as opposed to the bitch, in which their presence during proestrus is considered normal – as estrogen activity increases vascular permeability. A short (<24h) proestrus phase in the queen might justify the absence of erythrocytes in the smear, although the exact mechanisms of this absence remain unclear (Mills *et al.*, 1979; Pereira *et al.*, 2025).

The high number of erythrocytes observed in one queen was most likely an artefact related to the sample collection timing, as the vaginal cytology was obtained immediately after OVH and sanguineous vaginal discharge was present. Therefore, the blood contamination was most likely secondary to the surgical procedure rather than associated with the reproductive phase itself.

In this study, only one smear (classified as diestrus, with P4 = 2 ng/ml) presented neutrophils. The presence of neutrophils in any feline estrous cycle phase is considered normal and non-pathological in the absence of clinical signs (Pereira *et al.*, 2025). While in the vaginal cytology of the bitch the presence of neutrophils is a sign of a shift in reproductive stage (Groppetti *et al.*, 2012), the same cannot be said for the queen (Pereira *et al.*, 2025).

In this study, mucus was found in seven smears. All positive samples were consistently classified as being in non-follicular phases. Although clearing was not assessed in this study, due to the extended interval between sample collection and analysis, which may have compromised its reliable interpretation, the absence of mucus in all follicular-phase cytologies appears to be consistent with the published literature (Pereira *et al.*, 2025; Shille *et al.*, 1979).

Although cellularity was not formally quantified in this study, most samples had apparently poor cellularity, as reported in other studies (Kustritz, 2020; Pereira *et al.*, 2025). A subjective tendency toward increased vaginal cellularity was noted in samples classified as being in the follicular phase.

To the best of the author's knowledge, this is the first inter-observer reliability study on feline vaginal cytology. However, studies on canine vaginal cytology inter-observer reliability do exist. The existence of inter-rated variability was first acknowledged by Moxon *et al.*, (2010). Another study (Arlt, 2018), although not reporting statistical testing, reported weak inter-observer reliability. Later, Reckers *et al.*, (2022), tested the inter-observer reliability on the classification of single epithelial cells, using Fleiss Kappa test. This study obtained a weak kappa value ($\kappa = 0,412$).

The Fleiss' Kappa test is used to assess inter-observer agreement between three or more raters, whereas the Cohen's Kappa test is used when there are only two raters. The obtained Kappa

values from both tests vary from -1 to 1 and have the same significance (table 25) (Landis & Koch, 1977; McHugh, 2012).

This study ran a Cohen's Kappa test, obtaining a κ value of 0,391, lower than the reported values for the canine vaginal cytology. According to McHugh, (2012), this corresponds to a minimal agreement, while the results of Reckers *et al.*, (2022) correspond to a weak (but slightly stronger) agreement. However, the κ values in both studies are very close (difference of 0,021), suggesting a generally limited inter-observer agreement in both canine and feline vaginal cytology.

The estrous cycle is continuous, with hormones such as estrogen and progesterone varying gradually over time, which makes adjacent cycle phases more difficult to distinguish. This limitation has already been reported in the literature. It is particularly evident between follicular phases (proestrus and estrus) and non-follicular phases (diestrus, interestrus and anestrus). Misclassifications between distant phases (e.g. estrus vs anestrus) are considered more severe than disagreements between adjacent phases (e.g. proestrus vs estrus). However, neither Cohen's Kappa nor Fleiss' Kappa account for the magnitude of disagreement. To address this, a Weighted Kappa test was performed to incorporate the degree of disagreement between classifications. No previous studies using Weighted Kappa to assess inter-observer variability on veterinary vaginal cytologies were found.

The Weighted Kappa analysis demonstrated substantially higher agreement estimates when accounting for the ordinal structure of the cycle phases ($\kappa = 0,835$). This supports the idea that most disagreements between the two researchers were between cycle adjacent phases, rather than between distant phases. In other words, the observed disagreements were generally minor and likely reflects the gradual hormonal transitions of the estrous cycle and gradual cytological profile modifications

This study highlighted the need for standardization of vaginal cytology evaluation in queens, in order to reduce inter-observer variability.

The usefulness of weighted/pondered approaches in assessing clinically meaningful agreement in cytological classification systems where categories follow a natural continuum, like the feline estrous cycle phases, was also demonstrated.

The only phase that could be accurately confirmed was diestrus a clear criterion is defined: $P4 \geq 2$ ng/ml (Pereira *et al.*, 2025) or the presence of functional CL in the ovary (Binder *et al.*, 2019).

Assessing the accuracy of diestrus versus non-diestrus classification allows inference regarding the overall accuracy of the researchers' classifications, although it may not fully reflect overall classification accuracy across all reproductive stages.

Sensitivity was relatively low for both researchers (30% and 40%), whereas specificity was high (100% and approximately 91%), demonstrating a tendency to under-diagnose diestrus. Since the

paired McNemar test's p-value was $> 0,05$, no significant differences between the observers ability to correctly classify diestrus could be found.

The high specificity indicates that when diestrus was diagnosed it was rarely incorrect, while the low sensitivity reflects a substantial proportion of false negatives, likely due to the overlapping cytological features between diestrus and the other non-follicular phases (interestrus and anestrus). Furthermore, the absence of significant differences between the researchers further supports that the limitation lies more in the diagnostic criteria and biological proximity of the reproductive cycle phases than in the observers abilities. This reinforces the need to use complementary diagnostic tools, such as serum P4 quantification, when assessing vaginal cytologies to achieve accurate classifications.

None of the queens included in this study had prior access to an intact male. The ovulatory status could be assessed in 21 queens, of which 10 were classified as being in diestrus, corresponding to an estimated ovulation frequency of 47.6%, while the remaining 11 were classified as non-diestrus. All observed ovulations were considered spontaneous, as no ovulation-inducing stimulus was provided. This frequency falls within the range of spontaneous ovulation rates reported in the literature, which vary from 31% (Pereira *et al.*, 2024) to 87% (Gudermuth *et al.*, 1997).

This study had several limitations, including a relatively small sample size, the absence of behavioral anamnesis, the inability to collect blood samples from all cats (which could have allowed serum progesterone and estrogen analysis), and the lack of histological examination of both ovaries in all queens. The latter would have enabled the identification of functional corpora lutea and follicles at different developmental stages. In addition, a previous study in queens (Hidalgo *et al.*, 2020) suggests that the vaginal cytology technique used in this study (swab sampling), although commonly applied, may yield a lower number of cells, which in turn could affected microscopic interpretation.

7. CONCLUSION

In conclusion, feline vaginal cytology provides valuable information regarding broad reproductive status, particularly when distinguishing follicular from non-follicular phases. However, substantial overlap exists between adjacent estrous cycle stages, resulting in limited diagnostic precision when cytology is used in isolation.

The marked improvement observed with weighted agreement analysis suggests that most observer disagreement reflects biologically plausible uncertainty between neighbouring phases rather than major diagnostic error. Consequently, vaginal cytology should be regarded as a useful component of a multimodal reproductive assessment rather than a definitive method for precise cycle staging.

Future studies combining standardized cytological criteria with hormonal and histological validation may further improve the reliability and clinical applicability of vaginal cytology in queens.

This study allowed the creation of a brief informative guide – that can be found in Appendix A – that may contribute to the standardization of vaginal cytology in queens.

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APPENDIX A

A Brief Guide to Vaginal Cytology in the Queen

Breve Guia da Citologia Vaginal na Gata



CITOLOGIA VAGINAL em gatas



É uma ferramenta simples, rápida e de baixo custo para o manejo reprodutivo de gatas, especialmente em casos de ovulação espontânea,aios silenciosos ou contínuos e Síndrome do Ovário Remanescente. É também um método complementar de diagnóstico em caso de piometra.



Rodar a zaragatoa 2-3x e remover.

Inserir a zaragatoa na vagina cranial (1-2cm).

*imagem gerada por IA

Colheita da amostra

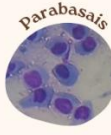
Insere-se na vagina cranial da gata, uma zaragatoa estéril humedecida com solução salina, rodando 2-3x antes de remover. Espalha-se o material colhido numa lâmina, deixa-se secar ao ar e cora-se utilizando o método Diff-Quick. Observa-se no microscópio ótico nas objetivas de 10x e 40x.

Interpretação

Na citologia vaginal podem ser distinguidas células epiteliais parabasais, intermédias, superficiais e anucleadas. Também podem ser detetados neutrófilos, eritrócitos e muco.

No proestro <50% das células são cornificadas com células intermédias pequenas e grandes. No estro >75% das células são cornificadas. No interestro e diestro predominam células intermédias e parabasais, sendo estas mais pequenas no diestro. No anestro, predominam células parabasais.

Por si só, a citologia vaginal não é suficiente para classificar a fase do ciclo reprodutivo devido às grandes semelhanças entre estas.



Parabasais



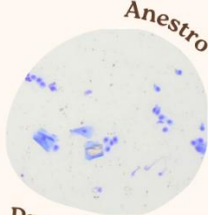
Intermédias



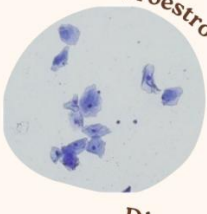
Superficiais



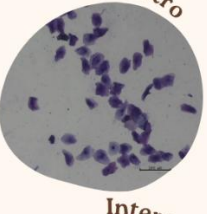
Anucleadas



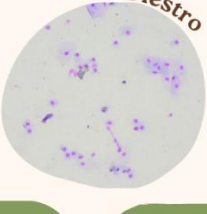
Anestro



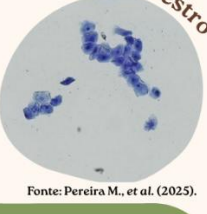
Proestro



Estro



Diestro



Interestro

Fonte: Portela, S., (2026).

Fonte: Pereira M., et al. (2025).

Anamnese

É extremamente importante conhecer o historial reprodutivo da gata, assim como datas/duração deaios anteriores, possibilidade de cópula e presença de comportamentos de cio, tais como, vocalização frequente, lordose e atrito corporal constante.

Progesterona (P4)

Valores de P4>2ng/ml são sugestivos da ocorrência de ovulação. A sua medição permite distinguir fases reprodutivas não foliculares, citologicamente semelhantes.

Presença de Corpo Lúteo (CL)

Quando possível, a visualização direta ou indireta de CL (funcional) no ovário, permite concluir que se deu ovulação, auxiliando na interpretação citológica.

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**Evaluation of Vaginal Cytology for Estrous Cycle Classification in the Queen (*Felis catus*):
Interobserver Agreement and Validation**

**Avaliação da Citologia Vaginal para a Classificação das Fases do Ciclo Éstrico na Gata (*Felis catus*):
Concordância Interobservador e Validação**

Sofia Helena Portela

ICBAS

