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Stress-related cortisol and progesterone concentrations influence the success of ovulation induction in estrous cats

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ABSTRACT

Queens are induced ovulators, and ovulatory success varies with hormonal status and stress. This study aimed to investigate the stress and hormone related factors influencing ovulatory success in 78 queens induced to ovulate by vaginal stimulation (VS), GnRH (50 μg gonadorelin), and human chorionic gonadotropin (hCG, 250 IU) in domestic cats. Before ovulation induction, stress scores, serum anti-Müllerian hormone (AMH) concentrations, age, body weight, and estrus day were recorded. Serum luteinizing hormone (LH), cortisol, and progesterone were determined to characterize hourly from 0 to 4 h post-induction, and estradiol and progesterone were measured once daily for six days. Ovulation was histologically confirmed and progesterone concentrations were ≥1 ng/mL were classified as ovulated. The proportion of ovulated queens differed ($p < 0.05$) among treatments (VS; 46.2 %, GnRH; 73.1 %, hCG; 100 %). There was a positive correlation between the stress score and both serum cortisol and progesterone concentrations. A significant difference in LH concentrations was observed between ovulated and non-ovulated queens ($p < 0.05$). Ovulatory response in VS and GnRH groups of queens was influenced by several physiological factors, with individuals at more advanced estrus days and those exhibiting greater estradiol and lower AMH concentrations associated with a greater likelihood of ovulation ($p < 0.05$), whereas increasing stress score, advancing age, elevated cortisol and progesterone concentrations were associated with reduced ovulation rates ($p < 0.05$). Additionally, a marked post-induction increase in estradiol concentrations in hCG-treated queens ($p < 0.05$). Overall, ovulatory success in queens was influenced by induction method, estrus stage, stres, estradiol and AMH concentrations. Stress-associated adrenal activation may impair LH dynamics and reduce the likelihood of ovulation, whereas hCG maintained consistent efficacy even under heightened stress conditions.

1. Introduction

Felids are induced ovulators, and ovulation generally occurs following mating-induced stimulation of the vagina and cervix, which triggers a surge of luteinizing hormone (LH) from the anterior pituitary [1,2]. Pharmacological induction of ovulation is commonly used in feline reproduction to confirm ovarian remnant syndrome [3,4], terminate estrus in cats for which ovariectomy is not an immediate option [5], manage certain infertility cases [6], or synchronize ovulation for assisted reproductive technologies [7–9]. Ovulation can be induced mechanically, via vaginal stimulation (VS) [10], or pharmacologically through administration of gonadotropin-releasing hormone (GnRH) analogues [5] or human chorionic gonadotropin (hCG) [7].

Multiple factors influence the ovulatory response in cats. The day of estrus [11,12], number of copulations [12,13], and the presence of other cats in the environment have all been reported to affect ovulatory success. Moreover, spontaneous ovulation has been documented in some individuals [14,15]. In addition, stress-related modulation of the hypothalamic–pituitary–adrenal (HPA) axis may also influence ovulation. In pigs [16], rats [17], sheep [18], and humans [19], stress-induced activation of the HPA axis has been reported to blunt or delay the preovulatory LH surge. Supporting this hypothesis, a previous study [20] demonstrated that adrenocorticotropic hormone (ACTH) administration increased cortisol and extragonadal progesterone secretion to a similar extent in both ovariectomized and intact queens, and the authors suggested that such progesterone elevation may be associated with

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reproductive failure.

Cats are inherently sensitive to stress, and a phenomenon analogous to the "white coat effect" described in human medicine [21] has also been reported in felines [22,23]. Queens undergoing ovulation induction in a hospital setting may be particularly susceptible to procedure-related stress, which could further compromise their physiological capacity to mount an adequate LH surge or response. The present study aimed to determine whether stress influences ovulatory outcomes in cats in natural estrus (cats in spontaneous estrus without exposure to males or exogenous hormonal stimulation) administered three different ovulation induction methods. We hypothesized that elevated levels of stress would be associated with a lower likelihood of ovulation in estrous cats undergoing ovulation induction.

2. Materials and methods

All procedures involving animals were conducted in accordance with ethical standards and were approved by the Balıkesir University Animal Experiments Ethics Committee (Approval Date: 01.11.2024; Reference No: 2024/10-9). The study was designed as a prospective, randomized, non-controlled experimental clinical investigation with stratification and was performed on client-owned queens.

2.1. Animals and inclusion criteria

Seventy-eight female domestic cats *(Felis catus)* aged 6–60 months and presented to the Balıkesir University Animal Hospital for elective ovariohysterectomy were enrolled in the study. Queens included in the study were those whose owners reported behavioral signs of estrus and who had no contact with males, ensuring the exclusion of male-induced ovulatory stimuli. Only queens confirmed to be clinically healthy and not receiving corticosteroids or exogenous reproductive hormones were included in the study [24]. Health status was verified through a comprehensive physical examination evaluating body condition, rectal temperature, respiratory and cardiovascular parameters, mucous membrane color, hydration status, and the absence of systemic illness. During the general physical examination, cats that required restraint by more than two veterinarians due to excessive behavioral reactions were excluded from the study to ensure animal welfare and minimize stress-related confounding effects. Reproductive soundness was further assessed by transabdominal ultrasonography, which confirmed normal ovarian morphology, the absence of follicular or luteal cysts, and no evidence of uterine pathology such as fluid accumulation, endometrial thickening, or structural abnormalities. Only queens meeting all of these clinical and ultrasonographic criteria were enrolled [25]. Initially, 109 queens were screened for inclusion in the study. Of these, 31 queens were excluded based on predefined criteria. Exclusion reasons included inability to be safely handled during physical examination (n = 7), body weight <2 kg (n = 2), owner-induced vaginal manipulation prior to presentation (n = 3), cohabitation with a male cat (even if neutered; n = 3), insufficient estrus confirmation by vaginal cytology (<80 % superficial cells; n = 11), intercurrent calicivirus infection after initial examination (n = 2), age <6 months (n = 1), and age >5 years (n = 2). The study sample (n = 78) included 34 Domestic Shorthairs, 28 British Shorthairs, 9 Scottish Folds, 4 British Longhairs, 2 Turkish Angoras, and 1 Sphynx breeds.

2.2. Confirmation of estrus

Vaginal cytology was performed only once and used to confirm estrus status and ensure accurate classification of the estrous phase [26]. Vaginal smear samples were collected using sterile cotton swabs moistened with saline and inserted approximately 1 cm into the vagina, where they were gently rotated against the vaginal floor and lateral walls. The collected material was rolled onto glass slides, air-dried, stained with Diff-Quik, and evaluated under light microscopy at 10 × magnification. Estrus was defined by the presence of ≥80 % superficial and keratinized cells [26]. The estrus day was recorded according to the owner's anamnesis based on behavioral estrus signs, including rubbing of the head and neck against the ground or surrounding objects, rolling in dorsal decubitus, lordosis with tail deviation, increased vocalization [27]. The average behavioral estrus day of the cats participating in the study ranged from 2 to 6 days, and the distribution was equal among the groups (Table 1).

2.3. Behavioral stress assessment

Behavioral stress was assessed using a standardized 7-point scale based on body posture, facial expression, activity, and vocalization [28–30]. Scores range from 1 (fully relaxed) to 7 (terrified), with increasing values indicating greater levels of behavioral stress. No photographic or video recordings were used for scoring. All behavioral assessments were conducted by the same one experienced veterinarian.

Cats were housed individually in a quiet clinical environment and allowed a standardized 1-h acclimation period in the same room where ovulation induction procedures and blood sampling were subsequently performed. No dogs, cats or other animal species were present in the same room or adjacent areas during the acclimation period, ovulation induction procedures, or blood sampling. Stress scoring was performed at the end of the standardized 1-h acclimation period, immediately prior to ovulation induction and any experimental handling. The same housing and acclimation procedures were applied consistently to all animals. During all procedures, the owner remained present alongside the cat to help reduce anxiety associated with the clinical setting. Repeated blood sampling was limited to the minimum number required to address the study objectives and was performed by experienced veterinarians using gentle restraint techniques to minimize discomfort. All cats were handled by the same two veterinarians, and all blood samples were collected by a single experienced veterinarian to ensure procedural consistency. Even when a cat appeared calm enough to be restrained by one person, restraint was still performed by the same two individuals to ensure uniformity of handling across all animals and to minimize variability in stress-related responses attributable to differences in restraint technique. Animals were continuously monitored throughout the study, and no cat exhibited clinical signs requiring premature withdrawal from the study.

2.4. Ovulation induction protocols

Queens were assigned to one of three induction treatments: VS (n = 26), GnRH (n = 26), or hCG (n = 26). Ovulation was induced in the VS group using a 6-mm cotton sterile swab rotated 6–8 times every 30 min for a total of five stimulations [10]. Queens in the GnRH group received a single intramuscular injection of 50 μg gonadorelin (Ovarelin, CEVA

Table 1
Baseline characteristics of queens in the VS, GnRH, and hCG treatment groups.

Variable	VS (n = 26)	GnRH (n = 26)	hCG (n = 26)	p-value
Age (months)	20.8 (8–50)	18.0 (6–49)	19.0 (7–60)	>0.05
Body weight (kg)	2.9 (2.3–4.1)	3.1 (2.6–3.6)	3.2 (2.2–4.2)	>0.05
Estrus day	3.9 (2–6)	3.8 (2–6)	3.2 (2–5)	>0.05
Stress score	4.5 (1–7)	4.1 (1–6)	4.5 (1–7)	>0.05
Superficial cell rate (%)	89.3 (81–100)	91.9 (81–100)	89.9 (80–99)	>0.05
Estradiol (pg/mL)	33.1 (12.9–87.1)	36.2 (12.7–90.8)	32.1 (11.7–87.6)	>0.05
Progesterone (ng/mL)	0.7 (0.2–1.8)	0.6 (0.2–1.6)	0.7 (0.17–2.3)	>0.05
Cortisol	2.7 (0.8–5.3)	3.2 (0.8–7.1)	2.9 (0.4–4.8)	>0.05
AMH (ng/mL)	2.7 (1.1–6.1)	3.2 (0.8–7.3)	3.3 (1.1–8.2)	>0.05

Data are expressed as means with ranges (min–max). Comparisons among groups revealed no significant differences (p > 0.05).

Animal Health, France) [5] and queens in the hCG group were administered a single intramuscular injection of 250 IU human chorionic gonadotropin (Chorulon, Intervet, Germany) intramuscularly [31].

2.5. Blood sample collection

Prior to treatment administration, a single blood sample was collected from each queen (n = 78) to determine baseline reproductive hormone concentrations (anti-Müllerian hormone [AMH], 17β-estradiol, cortisol, and progesterone).

Following treatment allocation, queens were further categorized according to blood sampling frequency as either repeatedly sampled or non-sampled, resulting in a 3 × 2 study design (induction method × sampling status). A subset of 39 queens (13 per treatment group) underwent repeated blood sampling for hourly and daily hormonal analyses after informed owner consent was obtained. Among queens with owner consent, allocation to the repeatedly sampled or non-sampled subgroup was additionally guided by baseline behavioral stress scores to ensure a balanced distribution of stress between sampling groups. The remaining 39 queens (13 per treatment group) were not subjected to repeated sampling and contributed only to baseline hormone measurements and ovulation outcome assessment.

In repeatedly sampled queens, blood samples were collected before treatment (0 h) and at 1, 2, 3, and 4 h thereafter at the same time (±30-min) of day to assess serum LH [12], progesterone, and cortisol concentrations [20]. These samples were used to evaluate the temporal relationship between handling-related stress responses, endocrine dynamics, and ovulatory outcome.

To monitor endocrine changes associated with estrus termination and luteal development, daily blood samples were collected for six days following induction to measure 17β-estradiol and progesterone concentrations. The daily blood sampling period was conducted at the cats' home environment. Blood samples were collected approximately every 24 h (±1 h) for six consecutive days at the same time of day for each cat, in order to minimize the potential effects of circadian variation on hormone concentrations.

All samples were obtained in the presence of the owner by the same experienced veterinarians who performed the initial procedures. No general anesthesia or sedation was used for blood collection; however, a topical local anesthetic ointment (Anestol® %5 Merhem; lidocaine; Sandoz, Turkiye) was applied to the venipuncture site prior to sampling. Blood samples were collected using standardized procedures, with approximately 1.5–3.0 mL of whole blood obtained per sampling time point, depending on the number of hormonal analyses required. Samples were collected via venipuncture or through an over-the-needle intravenous catheter (24 G) placed in the cephalic or saphenous vein. Blood was collected into plain tubes without anticoagulant, allowed to clot at room temperature for approximately 10–20 min, and centrifuged at 3500 rpm for 5 min. The separated serum was aliquoted into labeled microtubes and stored at −20 °C until hormonal analyses were performed.

2.6. Hormonal analysis

2.6.1. Serum estradiol assay

Serum 17β-oestradiol concentrations were measured using the (Cobas® e601) analyser (Hitachi Ltd., Tokyo, Japan) and a commercially available ECLIA (Elecsys® Estradiol III; Roche Diagnostics GmbH, Mannheim, Germany) previously used in feline serum [32]. The assay has a measuring range of 5–3000 pg/mL. Values below the limit of quantification (5 pg/mL) were assigned a value of 2.5 pg/mL (LOQ/2) for statistical analysis.

Reported cross-reactivities included 74.1 % for 6-α-hydroxy-oestradiol and <1 % for all other tested steroids. Intra-assay coefficients of variation ranged from 1.1 % to 8.5 %, depending on concentration level.

2.6.2. Serum progesterone assay

Serum progesterone concentrations were measured using the (Cobas® e601) analyser (Hitachi Ltd., Tokyo, Japan) and a commercially available ECLIA (Elecsys® Progesterone III; Roche Diagnostics GmbH, Mannheim, Germany) previously used in feline serum [32]. The assay has a measuring range of 0.05–60 ng/mL.

Reported cross-reactivities with structurally related steroids included 3.92 % for 11-deoxycorticosterone, while all other tested steroids reported to have <1 % cross-reactivity. Several substances, including DHEA-S, estradiol, and dihydrotestosterone, exhibited no detectable cross-reactivity. Intra-assay coefficients of variation ranged from 1.1 % to 4.2 %, depending on concentration level.

2.6.3. Serum cortisol assay

Serum cortisol concentrations were measured using the Cobas® e601 analyser (Hitachi Ltd., Tokyo, Japan) and a commercially available ECLIA (Elecsys® Cortisol II Roche Diagnostics GmbH, Mannheim, Germany) previously used in feline serum [33]. The assay has a measuring range of 0.054–63.4 μg/dL.

Reported cross-reactivities with structurally related steroids were 6.58 % for cortisone, 4.90 % for 11-deoxycortisol, 2.48 % for corticosterone, and 2.23 % for prednisone. Prednisolone demonstrated a cross-reactivity of 7.98 % at 0.1 μg/mL, while dexamethasone exhibited no detectable cross-reactivity. Intra-assay coefficients of variation ranged from 1.5 % to 7.1 %.

2.6.4. Serum AMH analysis

Serum anti-Müllerian hormone (AMH) concentrations were measured using the Cobas® e601 analyser (Hitachi Ltd., Tokyo, Japan) and a commercially available ECLIA, (Elecsys® AMH; Roche Diagnostics GmbH, Mannheim, Germany) previously used in feline serum [34]. The assay has a measuring range of 0.03–23 ng/mL, defined by the limit of quantitation and upper master curve range. Values below the limit of quantitation (0.03 ng/mL) were reported as 0 ng/mL.

Reported cross-reactivities with structurally related glycoproteins were not detectable for inhibin A, activin A, LH, and FSH. Intra-assay coefficients of variation ranged from 1.3 % to 2.1 %, and intermediate precision ranged from 2.8 % to 3.8 %, depending on concentration level.

2.6.5. Serum LH analysis

Serum LH concentrations were quantified using a sandwich ELISA specifically developed for feline samples (Cat Luteinizing Hormone ELISA Kit, Cat. No. E0118Cat; BT-Laboratory, China). According to the manufacturer, the analytical measurement range of the assay is 0.5–32 ng/mL, with a sensitivity (limit of detection) of 0.24 ng/mL. Assay precision is reported as <8 % intra-assay CV and <10 % inter-assay CV. Standards and samples were assayed in accordance with the manufacturer's guidelines.

2.7. Ovariohysterectomy and ovary examination

To definitively confirm ovulation, all queens underwent ovariohysterectomy six days after induction. Ovariohysterectomy was performed using a left flank approach in all queens. Premedication consisted of medetomidine (30 μg/kg, Domitor®, Orion Pharma, Finland), butorphanol (0.3 mg/kg, Butamidor®, VetViva Richter GmbH, Austria), and midazolam (0.25 mg/kg, Dilemy®, Saba, Turkiye), followed by induction with ketamine (5 mg/kg, Ketasol, VetViva Richter GmbH, Austria).

Both ovaries were exteriorized and examined macroscopically during surgery. The presence of follicles, corpora lutea, and corpora hemorrhagica was recorded [5]. The number of corpora lutea/corpora hemorrhagica (CLs) and their distribution between the left and right ovaries were recorded for each animal by the same investigator. After removal, ovarian tissues were fixed and processed for histological examination to confirm the presence or absence of CLs. Macroscopic and

histological findings [5] and serum progesterone concentration more than 1 ng/mL [27,35] were used as the definitive criteria for the determination of ovulation. Queens in which ovulation was histologically confirmed and progesterone concentrations were ≥1 ng/mL were classified as ovulated, whereas animals lacking either of these two criteria were classified as non-ovulated.

2.8. Statistical analysis

An a priori power analysis was conducted using G*Power (v3.1.9.7). For the primary categorical comparison, a χ^2 test for 3 × 2 contingency tables (df = 2) was planned. Assuming a medium-to-large effect size (Cohen's w = 0.35), α = 0.05, and 80 % power, the required total sample size was 79 queens. For multivariable prediction of ovulation using logistic regression, power analysis was performed assuming a two-tailed test (α = 0.05, power = 0.80), a baseline ovulation probability of 0.50, an anticipated odds ratio of 2.2, and limited shared variance with other predictors (R^2 = 0.05). Under these assumptions, a total sample size of 70 animals was sufficient. For repeated-measures ANOVA (within–between interaction), assuming an effect size of f = 0.25, α = 0.05, power = 0.80, three treatment groups, five repeated measurements, a correlation among repeated measures of 0.50, and ε = 1.0, the required sample size was 12 animals. As the chi-square analysis required the largest sample size among the planned statistical tests, the total number of queens was set to 78 (approximately 26 per treatment group) to satisfy this most conservative requirement. For the 3 × 2 chi-square comparisons, each treatment group was further subdivided according to repeated blood sampling status, resulting in approximately 13 queens per subgroup while preserving the overall sample size requirement.

Ovulation status (ovulated vs. non-ovulated) was analyzed as a binary categorical variable. The association between treatment groups (VS, GnRH, hCG) and ovulation outcome was evaluated using chi-square test. When the overall chi-square test indicated a significant association, post-hoc pairwise chi-square comparisons were performed between treatment groups.

Binary logistic regression was used to identify predictors associated with the likelihood of ovulation. The initial full model included treatment group, age, estrus day (was defined as the number of days since the onset of behavioral estrus based on owner-reported history), body weight, repeated blood sampling (yes/no), stress score, and serum concentrations of estradiol, cortisol, progesterone, and AMH. Model selection used manual backward stepwise (Wald) elimination, retaining predictors with $p < 0.05$ and removing those with $p > 0.10$. Variables with intermediate significance were kept temporarily to avoid premature exclusion. Multicollinearity was assessed using Pearson correlation coefficients, and all pairwise values remained below r = 0.30, indicating no collinearity concerns. Stress score was initially modelled as a categorical variable; however, sparse distribution across categories produced unstable estimates. To improve model performance, stress score was re-specified as an ordinal numeric variable reflecting graded stress intensity. The hCG group was excluded from multivariable modeling because all queens in this group ovulated (100 %), resulting in complete separation. This condition prevents estimation of regression coefficients and inflates standard errors; therefore, logistic regression models were restricted to the VS and GnRH groups. Odds ratios (ORs), 95 % confidence intervals (CI) were reported for all predictors remaining in the final model.

Pearson's correlation coefficient (r) was used to assess linear associations among stress score, serum cortisol concentration, and serum progesterone concentration. Repeated-measures correlation (rmcorr) was conducted to quantify the within-subject association between serum progesterone and cortisol concentrations across repeated time points. Correlation strength was interpreted according to conventional thresholds: weak (r = 0.10–0.29), moderate (0.30–0.49), and strong (r ≥ 0.50).

Serum LH, cortisol, and progesterone concentrations obtained at hourly intervals from 0 to 4 h after treatment were analyzed using two-way repeated-measures analysis of variance. Estradiol and progesterone levels were similarly analyzed in daily samples collected from Day 0 to Day 6. Separate models were fitted for each treatment group. Time was the within-subject factor and ovulation status the between-subject factor. Time effects, ovulation effects, and time × ovulation interactions were tested. When significant between-subject effects or interactions were detected, Tukey-adjusted post-hoc comparisons were performed to identify differences between ovulated and non-ovulated queens. In addition, to compare temporal hormone profiles between induction methods irrespective of ovulation status, treatment group (VS, GnRH, hCG) was included as a between-subject factor in a repeated-measures ANOVA, with time as the within-subject factor, allowing testing of time, treatment, and time × treatment effects.

All descriptive data are presented as mean ± standard error of the mean (SEM). Statistical significance was defined as $p < 0.05$, whereas a statistical tendency was considered for P values between 0.05 and 0.10. Repeated-measures correlation analysis was performed using Jamovi (Version 2.7.9), and all other statistical analyses were conducted using IBM SPSS Statistics (Version 27.0).

3. Results

Baseline characteristics were comparable among the VS, GnRH, and hCG groups, with no significant differences ($p > 0.05$) detected for age, body weight, estrus day, stress score, vaginal superficial cell percentage, or serum hormone concentrations (Table 1).

3.1. Ovulation rate of different induction protocols

A total of 78 queens were evaluated to determine the effect of stimulation method on ovulation. Ovulation occurred in 57 of 78 animals (73.1 %), while 21 of 78 (26.9 %) did not ovulate. Ovulation rates differed markedly among treatment groups (Table 2). Among the 21 queens in which ovulation was histologically excluded, 10 animals (47.6 %) exhibited serum progesterone concentrations exceeding 1 ng/mL at one or more sampling time points. Histological evidence of ovulation was not observed in any queens with progesterone concentrations below 1 ng/mL. Animals exhibiting progesterone concentrations above 1 ng/mL prior to ovulation and on consecutive days, in the absence of macroscopic and histological evidence of corpora lutea or corpora hemorrhagica, were classified as non-ovulated, and the progesterone elevation was considered stress-related. In the vaginal stimulation group, 12 of 26 queens (46.2 %) ovulated, whereas 19 of 26 (73.1 %) in the GnRH group and all 26 queens (100 %) in the hCG group exhibited ovulation. Post-hoc pairwise chi-square comparisons revealed that hCG induced greater ovulation rates than both vaginal stimulation ($p < 0.001$) and GnRH administration ($p < 0.01$). The ovulation rate in the GnRH group was also greater than that in the vaginal stimulation group ($p < 0.05$). Moreover, the mean (±SEM) numbers of CLs were as follows: left ovary 1.5 ± 0.3 (VS), 2.2 ± 0.3 (GnRH), and 2.1 ± 0.2 (hCG); right ovary 1.8 ± 0.2 (VS), 2.0 ± 0.3 (GnRH), and 2.3 ± 0.3

Table 2
Ovulation rates according to ovulation induction method and blood sampling status in queens.

Group	Repeated Blood Sampling (Yes)	Repeated Blood Sampling (No)	Total Ovulation Rate
VS (%, n/n)	23.1 % (3/13)^{aA}	69.2 % (9/13)^{aB}	46.2 % (12/26)^{a}
GnRH (%, n/n)	61.5 % (8/13)^{bA}	84.6 % (11/13)^{aA}	73.1 % (19/26)^{b}
hCG (%, n/n)	100 % (13/13)^{bA}	100 % (13/13)^{aA}	100 % (26/26)^{c}

Different letters (a–c) indicate significant differences between induction methods within the same column ($p < 0.05$).
Different capital letters (A–B) indicate significant differences between blood sampling statuses within the same row ($p < 0.05$).

(hCG). The corresponding total CL numbers were 3.3 ± 0.4, 4.2 ± 0.3, and 4.4 ± 0.3, respectively. No significant differences were detected in left–right ovarian distribution among groups, and the total number of CLs did not differ significantly between the VS and hCG groups ($p > 0.05$), with all other pairwise comparisons also remaining non-significant ($p > 0.05$).

When ovulation rates were stratified according to repeated blood sampling status, distinct patterns emerged between treatment groups (Table 2). In the vaginal stimulation group, ovulation occurred in 3 of 13 repeatedly sampled queens (23.1 %), compared with 9 of 13 non-sampled queens (69.2 %), indicating lower ovulation rate in repeatedly sampled queens ($p < 0.05$). In the GnRH group, ovulation was observed in 8 of 13 repeatedly sampled queens (61.5 %) and 11 of 13 non-sampled queens (84.6 %), with no significant difference between sampling status ($p > 0.05$). In contrast, in the hCG group, all queens ovulated regardless of blood sampling status, with ovulation observed in 13 of 13 repeatedly sampled queens (100 %) and 13 of 13 non-sampled queens (100 %).

In the VS (Fig. 1a) and GnRH (Fig. 1b) groups, all queens that ovulated displayed only corpora lutea on the ovaries, whereas in the hCG group (Fig. 1c), multiple enlarged follicles were observed together with corpora lutea or corpora hemorrhagica in all animals.

3.2. Factors effecting ovulation rates

Human chorionic gonadotropin-treated queens were excluded from multivariable modeling due to 100 % ovulation, preventing reliable odds ratio estimation. Estrus day, stress score, estradiol, cortisol, progesterone and AMH concentration were shown to be significant predictors of ovulation, while age showed a statictical tendency (Table 3). A significant association was observed between estrus day and ovulatory response ($p < 0.05$, OR = 1.352, 95 % CI: 1.048–1.742), indicating a greater likelihood of ovulation in queens at more advanced stages of estrus. A significant association was also observed between increasing stress score and reduced ovulation ($p < 0.05$, OR = 0.534, 95 % CI: 0.303–0.942. Greater estradiol concentrations were associated with an increased likelihood of ovulation ($p < 0.05$, OR = 1.244, 95 % CI: 1.000–1.090). In contrast, greater cortisol ($p < 0.05$, OR = 0.785, 95 % CI: 0.664–0.925), progesterone ($p < 0.05$, OR = 0.814, 95 % CI: 0.692–0.960) and AMH ($p < 0.05$, OR = 0.409, 95 % CI: 0.173–0.964)

Table 3
Logistic regression analysis of predictors of ovulation in queens treated with VS or GnRH.

Variable	p-value	OR	95 % CI
Age	0.07	0.937	0.874–1.005
Body weight	0.93	1.000	0.998–1.002
Stress score	0.04	0.534	0.303–0.942
Repeated blood sampling	0.09	0.545	0.268–1.157
Estrus day	0.04	1.352	1.048–1.742
Estradiol level	0.05	1.244	1.000–1.090
Cortisol level	0.01	0.785	0.664–0.925
Progesterone level	0.02	0.814	0.692–0.960
AMH level	0.04	0.409	0.173–0.964

OR, odds ratio; CI, confidence interval; VS, vaginal stimulation; GnRH, gonadotropin-releasing hormone; AMH, anti-mullerian hormone.
The hCG group was excluded from multivariable modeling because complete separation occurred, as all queens in this group ovulated (100 %), precluding reliable estimation of regression coefficients. Therefore, analyses were restricted to the VS and GnRH groups.

concentrations were associated with a decreased likelihood of ovulation. Age ($p = 0.073$, OR = 0.937, 95 % CI: 0.874–1.005) and repeated blood sampling ($p = 0.096$, OR = 0.545, 95 %Cl: 0.268–1.157) demonstrated a tendency toward decreased ovulatory response. Body weight ($p > 0.05$, OR = 1.00, 95 % Cl: 0.998–1.002) was not associated with ovulation.

3.3. Correlations among stress score, cortisol, and progesterone

Pearson's correlation analysis revealed that stress score was positively correlated with serum cortisol concentration ($r = 0.36$, $p < 0.05$). The association between stress score and progesterone concentration was statistically significant ($r = 0.32$, $p < 0.05$). Repeated-measures correlation analysis demonstrated a moderate and statistically significant positive association between serum progesterone and cortisol levels within individuals (Fig. 2), with an overall repeated-measures correlation coefficient of $r\_rm = 0.540$ ($p < 0.001$, CI: 0.418–0.644).

3.4. Hourly LH, cortisol and progesterone levels

A significant main effect of time was detected ($p < 0.05$), together with significant time × treatment and time × ovulation status

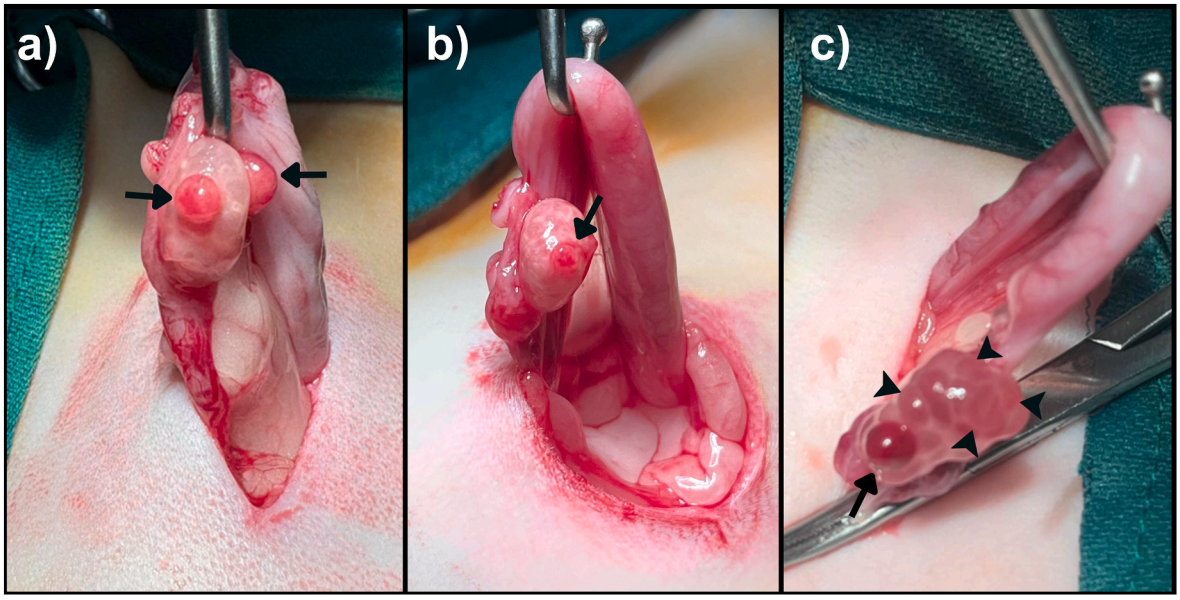

Fig. 1. Ovarian morphology following different ovulation induction methods.
a) VS group; **b)** GnRH group; **c)** hCG group. Arrows indicate corpora lutea and/or corpora hemorrhagica. Arrowheads indicate enlarged follicles.

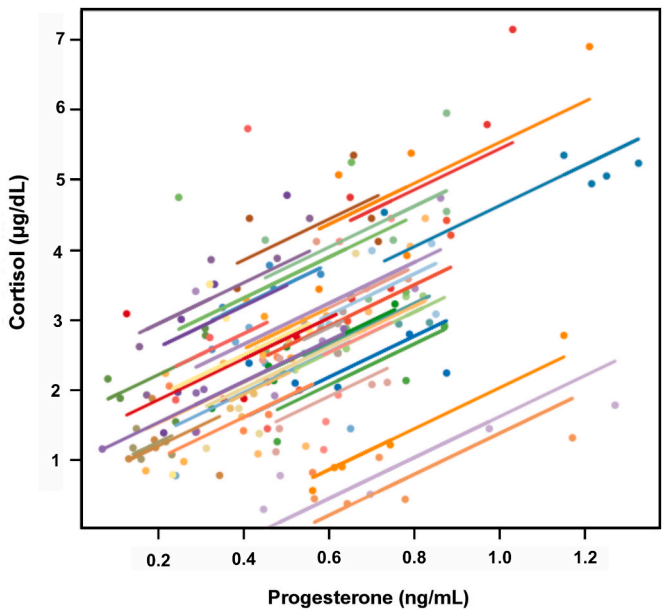

Fig. 2. Repeated-measures correlation graphic between serum progesterone and cortisol concentrations in queens.
Points represent individual measurements obtained from each queen. Lines indicate fitted values derived from the repeated-measures correlation model, which estimates a common within-subject slope while allowing subject-specific intercepts. Thus, lines are parallel and reflect differences in baseline cortisol concentrations among individuals rather than individual-specific regression slopes.

interactions ($p < 0.01$), indicating that LH concentrations changed over time and that these temporal profiles differed between induction methods and ovulatory outcomes.

In the vaginal stimulation (VS) group (Fig. 3a), ovulated queens exhibited a significant increase in serum LH from 0 h to 2 h ($p < 0.05$), reaching their maximal LH concentrations at 2 h. In contrast, non-ovulated queens exhibited only a transient increase from 0 h to 1 h ($p < 0.05$), followed by a decline toward 2 h ($p < 0.05$). At 2 h, LH concentrations were greater in ovulated queens than in non-ovulated queens (16.43 ± 1.92 vs. 8.30 ± 1.02 ng/mL, $p < 0.05$). In the GnRH group (Fig. 3b), ovulated queens demonstrated a marked rise in LH from 0 h to 2 h ($p < 0.001$), followed by a gradual decline thereafter ($p < 0.01$). Non-ovulated queens showed an increase from 0 h to 1 h ($p < 0.05$), followed by a decrease from 1 h to 2 h ($p < 0.05$). As in the VS group, LH concentrations differed between ovulated and non-ovulated queens at 2 h ($p < 0.05$), with greater values observed in ovulated animals. In the hCG group (Fig. 3c), all queens ovulated, and serum LH concentrations declined gradually over time ($p > 0.05$). Because ovulation occurred in 100 % of hCG-treated queens, no ovulation-based subgroup comparisons were applicable in this group. When LH concentrations were compared across induction methods, no significant differences were observed between the VS and GnRH groups ($p > 0.05$). In contrast, LH concentrations in the hCG group were lower than those measured in both VS- and GnRH-treated queens across the sampling period ($p < 0.05$).

No significant effects of time, group, or time × group interaction on cortisol concentrations were detected when ovulatory outcome was not considered ($p > 0.05$ for all). Accordingly, no significant differences in cortisol concentrations were observed among treatment groups over the 4-h sampling period. However, a significant time × ovulation status interaction was detected within treatment groups ($p < 0.05$), indicating that cortisol dynamics differed between ovulated and non-ovulated queens. In the VS group (Fig. 3d), ovulated queens (2.11 ± 0.28 ng/mL) had lower cortisol than non-ovulated queens (4.10 ± 0.49 ng/mL) at 0 h ($p < 0.05$). In the GnRH group (Fig. 3e), a significant difference occurred at 2 h, where cortisol was lower in ovulated queens (1.82 ± 0.32 ng/mL) compared to non-ovulated queens (3.75 ± 0.40 ng/mL; $p < 0.05$). In the hCG group, all queens ovulated; therefore, comparisons based on ovulatory outcome were not applicable (Fig. 3f).

No significant effects of time, group, or time × group interaction on progesterone concentrations were detected when ovulatory outcome was not considered ($p > 0.05$ for all). Accordingly, no significant differences in progesterone concentrations were observed among treatment groups over the sampling period. However, a significant time × ovulation status interaction was detected within treatment groups ($p < 0.05$), indicating that progesterone profiles differed between ovulated and non-ovulated queens. In the VS group (Fig. 3g), non-ovulated queens had greater progesterone at 0 h (ovulated: 0.46 ± 0.07 ng/mL; non-ovulated: 0.87 ± 0.10 ng/mL; $p < 0.05$) and 2 h (ovulated: 0.50 ± 0.08 ng/mL; non-ovulated: 0.97 ± 0.12 ng/mL; $p < 0.05$). In the GnRH group (Fig. 3h), non-ovulated queens exhibited greater progesterone at 0 h (ovulated: 0.41 ± 0.08 ng/mL; non-ovulated: 0.76 ± 0.07 ng/mL; $p < 0.05$) and 1 h (ovulated: 0.40 ± 0.06 ng/mL; non-ovulated: 0.81 ± 0.06 ng/mL; $p < 0.05$). No differences were present at later time points ($p > 0.05$). In the hCG group, all queens ovulated; therefore, comparisons based on ovulatory outcome were not applicable (Fig. 3i).

3.5. Daily estradiol and progesterone levels

A significant main effect of time and a significant time × treatment and time × ovulation interaction were detected for estradiol concentrations ($p < 0.05$). In queens that ovulated following VS treatment (Fig. 4a), estradiol declined markedly from Day 0 = 27.61 ± 5.73 pg/mL to Day 6 = 3.21 ± 3.21 pg/mL. A similar decline was observed in ovulated queens treated with GnRH (Fig. 4b), decreasing from 40.82 ± 9.18 pg/mL on Day 0–14.29 ± 6.21 pg/mL on Day 6. In contrast, hCG-treated queens (Fig. 4c) showed a significant ($p < 0.001$) rise in estradiol concentrations, increasing from 33.91 ± 8.20 pg/mL on Day 0–200.94 ± 30.06 pg/mL on Day 6. Greater ($p < 0.05$) estradiol concentrations were observed in the hCG group compared with the VS and GnRH groups on Days 3, 4, 5, and 6. In non-ovulated queens (Fig. 4a and b), estradiol concentrations did not show significant temporal changes across the sampling period ($p > 0.05$). In the VS group estradiol concentrations were lower in ovulated queens compared with non-ovulated queens on Days 3, 4, and 5 ($p < 0.05$ for all; Fig. 4a). No such differences were detected between ovulated and non-ovulated queens in the GnRH group at any time point ($p > 0.05$; Fig. 4b). Because all hCG-treated queens ovulated, non-ovulated comparisons were not applicable in this group.

A significant main effect of time was detected for progesterone concentrations ($p < 0.05$), and a significant time × ovulation status interaction was observed ($p < 0.05$), whereas no significant time × treatment interaction was found ($p > 0.05$). Across all treatment groups, progesterone concentrations increased markedly only in queens that ovulated ($p < 0.05$). In the VS group (Fig. 4d), progesterone rose from Day 0 = 0.46 ± 0.07 ng/mL to Day 6 = 17.56 ± 6.16 ng/mL. A comparable increase occurred in GnRH-treated queens (Fig. 4e), from 0.43 ± 0.05 ng/mL on Day 0–18.13 ± 2.45 ng/mL on Day 6, and hCG-treated queens (Fig. 4f) demonstrated a similar pattern, with progesterone increasing from 0.42 ± 0.09 ng/mL to 20.21 ± 2.14 ng/mL by Day 6. Progesterone exceeded 1 ng/mL earlier in the GnRH and hCG groups (on Day 2), whereas this threshold was reached one day later (on Day 3) in the VS group. No significant differences in progesterone concentrations were observed among ovulated cats in the VS, GnRH, and hCG groups on Day 6. In all non-ovulated queens (Fig. 4d and e), progesterone remained below 1 ng/mL without significant change ($p > 0.05$). A significant effect of ovulation status was detected in both the VS and GnRH groups ($p < 0.05$), indicating that progesterone concentrations were overall greater in ovulated than in non-ovulated queens across the daily sampling period (Fig. 4d and e). In non-ovulated queens, progesterone remained low and did not change over time ($p > 0.05$).

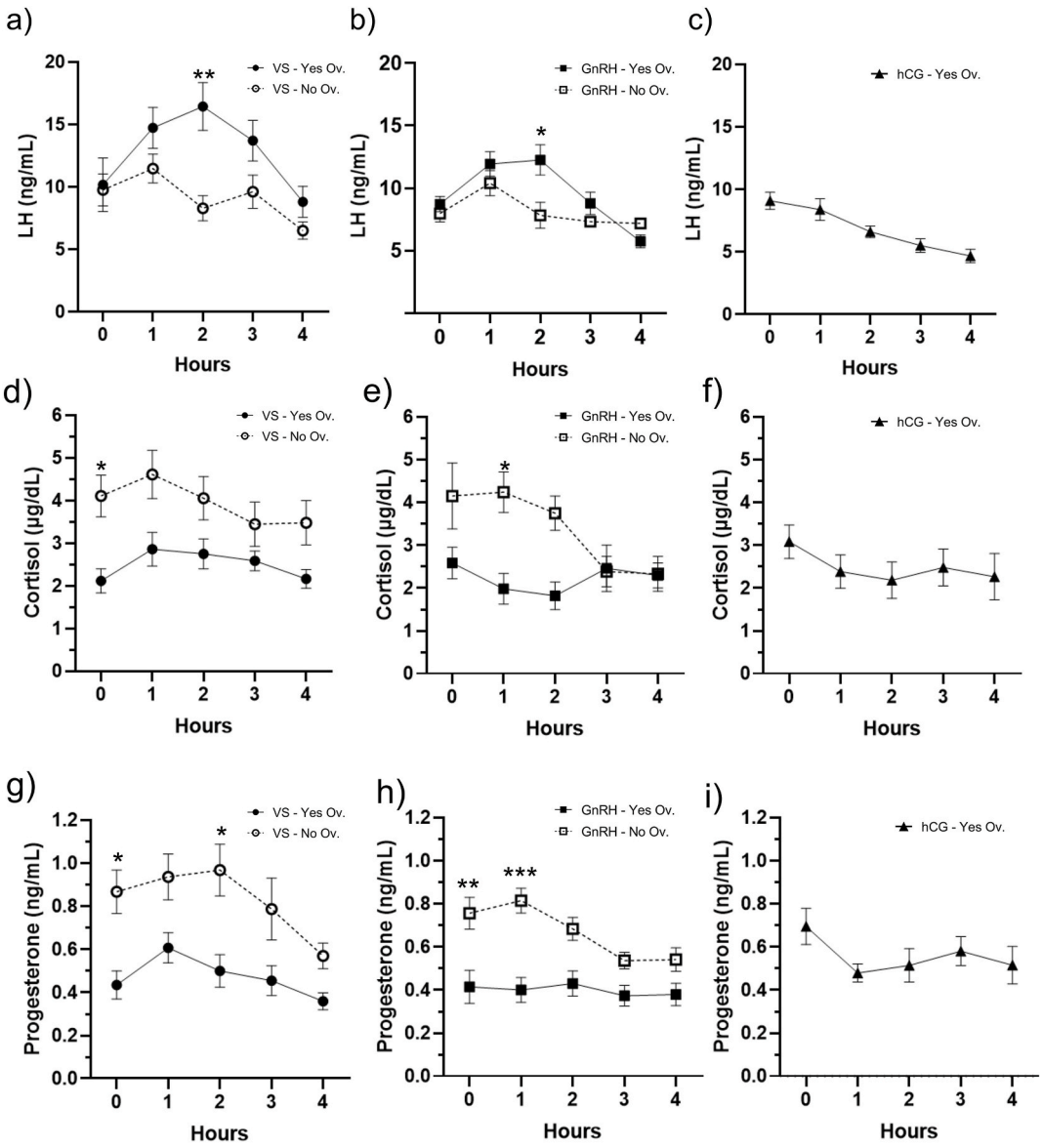

Fig. 3. Temporal profiles of LH, cortisol, and progesterone levels (mean ± SEM) after ovulation induction protocols.
Panels (a–c) present LH levels; (d–f) show cortisol levels; and (g–i) illustrate progesterone levels measured over the 4-h sampling period following treatment. (a, d, g) VS group, showing hormone levels in queens that ovulated (black circles) and those that did not ovulate (white circles). (b, e, h) GnRH-treated queens that ovulated (black squares) and those that did not ovulate (white squares). (c, f, i) hCG-treated queens (all ovulated), represented with black triangles. Significant differences between ovulated and non-ovulated animals at individual time points are indicated (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

4. Discussion

The present study provides ovulatory success in natural estrous queens by considering ovulation induction methods together with stress-related physiological and hormonal variables. Queens treated with hCG consistently ovulated, consistent with its pharmacological action as a direct LH receptor agonist that bypasses the hypothalamic–pituitary axis and is therefore less susceptible to stress-related neuroendocrine suppression [36]. In contrast, ovulatory responses following GnRH administration or vaginal stimulation were more variable. Both of these approaches rely on intact neural and endocrine pathways: GnRH requires adequate pituitary responsiveness, whereas vaginal stimulation depends on the integrity of the afferent–efferent neuroendocrine reflex arc. Because these pathways can be influenced by biological variability or environmental factors, their outcomes tend to be more variable [37,38]. Findings from previous studies reflect this variability. Reported ovulation rates following GnRH administration range from 40 % after a single 25 µg dose [39] to 100 % in previous studies [40]. Administering 25 µg twice at 12-h intervals resulted in approximately 80 % ovulation [41]. A higher single dose of 50 µg GnRH, which matches the dose used in the present study, induced ovulation in 84 % of queens [5]. For hCG, Wildt & Seager (1978) [42] reported a 66.6 % ovulation rate with 250 IU, whereas Tsutsui et al. (2000) [31] observed an 82.4 % rate with 100 IU. In contrast, the present study achieved 100 % ovulation with 250 IU. Such differences may reflect variation in hormonal sensitivity, follicular status at the time of treatment, and methodological differences among studies.Estrus day and circulating estradiol concentrations were positively associated with ovulation in the present study. This finding is consistent with previous reports showing that queens mated on days 3–5 of estrus achieve greater ovulation and pregnancy rates compared with those bred earlier in the cycle [11,12]. These observations align with evidence indicating that maximum follicular diameter is typically reached at approximately 3.8 ± 0.3 days of estrus [27]. Collectively, these results suggest that follicular maturity and sufficient estrogenic

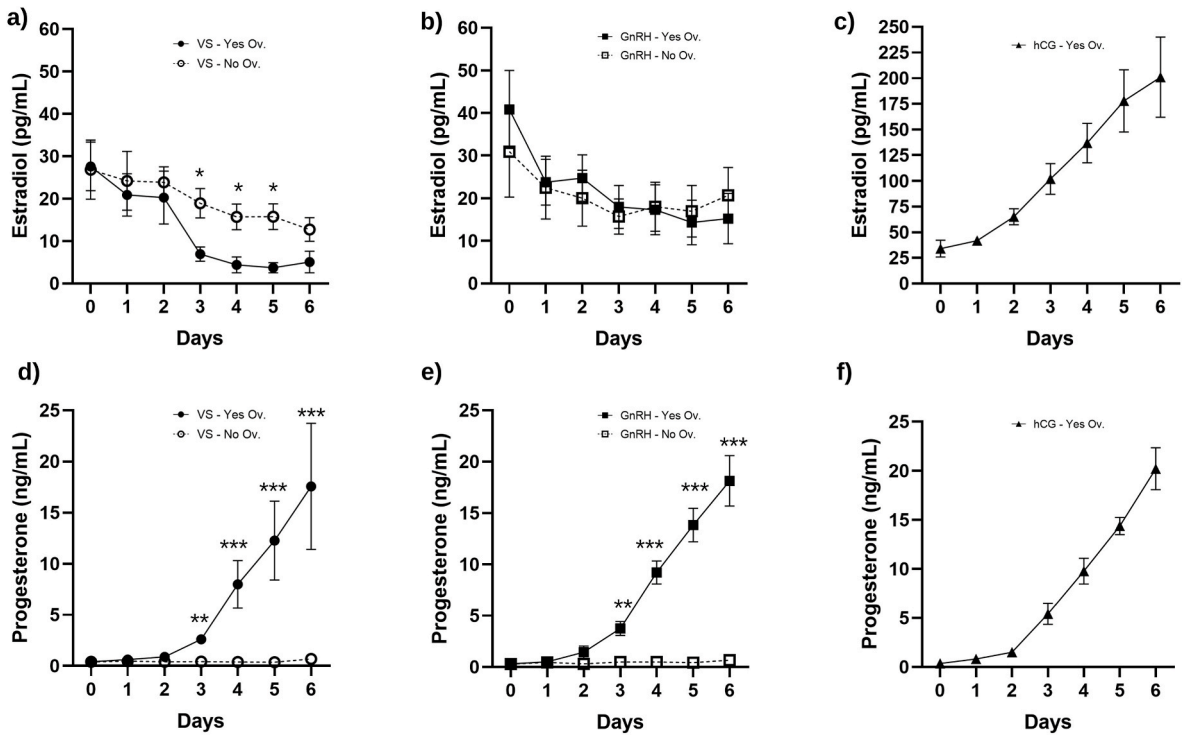

Fig. 4. Estradiol and progesterone levels (mean ± SEM) during the 6 days following ovulation induction protocols.
Panels (a–c) show estradiol levels, and panels (d–f) show progesterone levels. (a, d) Vaginal stimulation (VS) group, illustrating hormone levels in queens that ovulated (black circles) and those that did not ovulate (white circles). (b, e) GnRH-treated queens that ovulated (black squares) and those that did not ovulate (white squares). (c, f) hCG-treated queens (all ovulated), represented with black triangles. Significant differences between ovulated and non-ovulated animals at individual time points are indicated (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

priming may enhance pituitary responsiveness to GnRH, thereby increasing the likelihood of generating an appropriate LH surge [43]. Thus, queens in more advanced stages of estrus may be physiologically better positioned to mount a successful ovulatory response.

The present study revealed an inverse relationship between serum AMH concentrations and ovulatory outcome. similar pattern was reported by Chumsrui et al. (2024) [44] in clouded leopards, where individuals that failed to ovulate after eCG + LH treatment had markedly greater AMH concentrations than those that ovulated. In other mammalian species, including humans, elevated or persistently high AMH levels have been associated with reduced follicular responsiveness to FSH and altered granulosa cell differentiation, which may compromise the final stages of follicular maturation and ovulation [45,46]. In domestic cats, AMH concentrations normally decline during estrus and into metestrus as follicles progress toward advanced maturation stages [47]. Therefore, elevated AMH concentrations observed in some estrous queens may reflect an atypical follicular endocrine profile, potentially characterized by the persistence of smaller AMH-secreting follicles alongside larger preovulatory follicles. While such a profile could be associated with reduced ovulatory efficiency, direct evidence linking AMH to impaired ovulation in queens is currently lacking. Further studies integrating follicular morphology, gonadotropin responsiveness, and AMH signaling dynamics are needed to clarify these relationships.

The findings of the present study suggest that stress may contribute to reduced ovulatory success in queens. Higher stress scores were associated with a lower likelihood of ovulation, supporting the hypothesis that stress-related neuroendocrine disruption may impair preovulatory signaling [48]. Notably, within the VS group, queens subjected to repeated blood sampling exhibited substantially reduced ovulatory success, suggesting that restraint and venipuncture may have acted as acute stressors. Repeated venipuncture should be regarded as an uncontrolled stress variable, especially in the VS group, in which repeated sampling was associated with reduced ovulation. Handling and restraint are known to elicit rapid cortisol responses in cats [30]. This interpretation is further supported by the elevated cortisol concentrations detected at 3 and 4 h in the VS group compared with the GnRH and hCG groups, likely reflecting the greater degree of handling inherent to the VS protocol, which involved five episodes of vaginal stimulation administered at 30-min intervals. However, cortisol should not be interpreted solely as a marker of pathological stress, as activation of the hypothalamic–pituitary–adrenal axis also occurs in response to novelty, social interactions, and mating-related stimuli. Natural mating itself may therefore be associated with cortisol release, indicating that cortisol elevations are not unique to clinical handling or blood sampling. In this study, methodological stress from repeated venipuncture may have contributed to reduced ovulation through cortisol-related stress responses. Future studies should consider less invasive sampling methods or cortisol-corrected analytical models.

Positive correlations among stress score, cortisol, and progesterone indicate a coordinated adrenal response to stress. Several queens displayed progesterone concentrations >1 ng/mL prior to induction, a value typically interpreted as indicative of luteal activity [27,35]. Notably, in the present study, some queens in which ovulation was histologically excluded had progesterone concentrations exceeding 1 ng/mL at one or more time points, despite the complete absence of corpora lutea or corpora hemorrhagica. This finding provides direct evidence that progesterone concentrations above 1 ng/mL can occur in the absence of ovulation in this population. When progesterone concentrations are interpreted in conjunction with cortisol and estradiol profiles, vaginal cytology, and behavioral indicators of estrus, the observed progesterone levels are unlikely to represent functional luteal activity. This finding aligns with previous reports demonstrating that ACTH stimulation induces simultaneous adrenal release of cortisol and progesterone in both intact and ovariectomized cats [20]. Similar adrenal-derived progesterone responses have been reported in several species [16–19], in which stress-related progesterone has been

associated with reduced GnRH pulsatility and attenuation of the LH surge. Previous study [32] reported that queens exposed to chronic glucocorticoid treatment still ovulated when induced using an eCG + hCG protocol. A similar pattern was observed in the present study. All queens treated with hCG ovulated regardless of variation in stress scores or cortisol concentrations. This observation may be related to the mechanism of action of hCG, which does not depend on endogenous LH release but instead acts through direct activation of ovarian LH receptors [36].

The hormonal patterns observed in the present study further support a stress-sensitive disruption of the ovulatory cascade. In both the VS and GnRH groups, queens that ovulated exhibited a clear early rise in LH, with maximal concentrations occurring approximately 2 h after stimulation—an LH profile consistent with previous reports following mating-induced stimulation [12,13] or GnRH administration [49,50]. In contrast, non-ovulated queens demonstrated only a transient increase in LH at 1 h followed by an early decline, insufficient to initiate ovulation. Cortisol and progesterone profiles closely mirrored LH patterns, demonstrating a clear positive association among the three hormones. Queens that failed to ovulate in both the VS and GnRH groups exhibited greater early cortisol and progesterone concentrations. Elevated cortisol and progesterone may contribute to decreased ovulatory LH secretion [51]. supporting the hypothesis that stress-induced adrenal steroidogenesis interferes with the hypothalamic–pituitary signaling required for a sustained periovulatory LH rise.

Vaginal cytology was performed only once and used to confirm estrus status and ensure accurate classification of the estrous phase. The mechanical stimulation associated with swab insertion raises the question of whether limited activation of the vaginal–cervical–LH reflex could have occurred [5,15]. No published evidence indicates that a single, brief cytology swab is sufficient to induce measurable LH release in queens, and this possibility should therefore be interpreted with caution. Nonetheless, the unexpectedly elevated baseline LH concentrations observed in the hCG group, followed by a gradual decline, may be compatible with a limited neuroendocrine response triggered by handling or minor mechanical stimulation. Such an effect, if present, could contribute to baseline variability in LH measurements, particularly in studies conducted under field or clinical conditions.

The estrogen and progesterone profiles observed in ovulated queens align with previous report [52] showing that ovulation occurs roughly 24 h after the LH surge and that luteal progesterone becomes detectable as early as days 2–3. The widely accepted threshold of 1 ng/mL for confirming luteal activity [1,52] was also supported in the present study. Progesterone exceeded 1 ng/mL on days 2–3 in queens that ovulated in the VS, GnRH, and hCG groups, whereas non-ovulating queens remained within low ranges throughout the monitoring period. In the VS and GnRH groups, the decline in estradiol preceding the rise in progesterone is consistent with follicular rupture and transition to luteal activity. The observation that ovulation precedes the progesterone increase by 1–2 days [1,35] further supports this periovulatory estradiol decline. In contrast, hCG-treated queens exhibited a marked increase in estradiol beginning on Day 2, which may reflect the prolonged LH/FSH-like activity of hCG, capable of stimulating both luteal and residual follicular steroidogenesis [36]. These results are consistent with earlier report [53] showing that hCG can create an abnormal endocrine milieu and prolonged estradiol elevations in domestic cats. Although the present findings demonstrate that hCG is highly effective for inducing ovulation even in queens experiencing elevated stress, it should be used with caution in situations where subsequent fertility is desired or when ovulation is induced solely to manage persistent estrous behavior.

The present study has several limitations that should be considered when interpreting the findings. First, although the study incorporated a randomized controlled design, it was conducted under field–clinical conditions in privately owned queens maintained in diverse husbandry environments and exposed to varying degrees of environmental and stress-related factors. This heterogeneity may have contributed to the inter-individual variability observed in cortisol, progesterone, and LH profiles. This variability reflects the conditions under which ovulation induction protocols are applied in routine clinical practice and therefore provides a realistic representation of how queens respond outside of tightly controlled experimental settings. Additionally, a potential limitation of the present study relates to the hormone assays used. Although the immunoassays applied for cortisol, progesterone, estradiol, and AMH measurements have been previously used in feline and other felid studies, they have not been formally validated for domestic cats. Each subsequent assay run with felid serum adds to their practical credibility; however, the absence of species-specific validation studies should be acknowledged when interpreting the endocrine results. Future studies employing fully validated feline-specific assays would further strengthen the interpretation of hormonal dynamics associated with ovulation induction. The absence of a non-treated or placebo control group represents an additional limitation, as such a group would have allowed estimation of spontaneous ovulation rates and provided a baseline against which the endocrine and stress effects of vaginal stimulation and hormonal induction could be compared. Although both repeatedly sampled and non-sampled queens were included, the lack of a true non-intervention control limits separation of spontaneous ovulation from procedure-related effects.

Repeated venipuncture should be regarded as an uncontrolled stress variable in this study, particularly in the VS group where repeated sampling was associated with reduced ovulation. This procedural stress may have influenced cortisol secretion and downstream reproductive endocrine responses. Future studies should therefore consider less invasive sampling or analytical approaches that account for cortisol as a confounder. Additionally, natural breeding in felids may similarly elicit cortisol release, and future studies comparing naturally bred and clinically induced queens may help clarify whether a stress threshold exists that compromises ovulation.

5. Conclusion

This study demonstrated that ovulation success was influenced by the method of ovulation induction, estrus day, estradiol concentrations, AMH levels, and stress in domestic queens. hCG proved to be the most effective treatment inducing ovulation in all queens regardless of stress status. The hormonal patterns characterized in this study indicate that stress-related elevations in progesterone and cortisol levels may affect LH secretion and thereby reduce ovulatory success. Given the evident interaction between stress physiology and reproductive function, further studies are warranted to clarify the mechanisms by which stress affects ovulation in felids and to refine reproductive management strategies that account for stress susceptibility.

CRediT authorship contribution statement

Buse Ozturk: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Yusuf Bilal Cetinkaya:** Investigation. **Aslihan Ayalp-Erkan:** Investigation, Formal analysis. **Tunahan Ozturk:** Investigation. **Baris Guner:** Writing – review & editing, Resources, Project administration, Methodology, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dr. Baris GUNER reports financial support was provided by Balıkesir

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2026.117849.

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