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# Changing the route of GnRH administration according to the initial cyclic phase of the Ovsynch protocol may optimize reproductive outcomes in dairy cows

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## Abstract

**Background** This study aimed to investigate the effects and interactions of gonadotropin-releasing hormone (GnRH) analogues (lecirelin and buserelin), administration routes (intramuscular and epidural), and initial estrus cycle phases (follicular and luteal) on fertility outcomes in cyclic dairy cows subjected to the Ovsynch protocol.

**Results** A total of 406 multiparous Holstein cows were randomly assigned to four groups: intramuscular lecirelin ($L_{im}$, $n=102$), intramuscular buserelin ($B_{im}$, $n=102$), epidural lecirelin ($L_{epi}$, $n=101$), and epidural buserelin ($B_{epi}$, $n=101$). During the Ovsynch protocol, the first GnRH treatment was administered on D0, followed by $PGF_2\alpha$ on D7, the second GnRH treatment on D9, and fixed-time artificial insemination (FTAI) 20 h later on D10. At each GnRH administration, cows received either 62.5 µg lecirelin acetate or 10 µg buserelin acetate by the intramuscular or epidural route. Blood samples for serum progesterone ($P_4$) determination were collected on D0, D7, D9, D10, and D13. The initial cyclic phase was determined on D0 by ovarian ultrasonography and serum $P_4$ concentration. Pregnancy status was assessed on D30 after FTAI, and pregnancy losses on D60. No significant differences in pregnancy or pregnancy-loss rates were detected between GnRH analogues or routes of administration, irrespective of the initial cyclic phase ($P>0.05$). Likewise, within each initial cyclic phase, the route of administration did not significantly affect pregnancy rate ($P>0.05$), although opposite numerical trends were observed. Pregnancy rates were numerically higher after intramuscular administration in cows starting the protocol during the luteal phase (44.31% vs. 31.95%, $P=0.083$), but numerically higher after epidural administration in those starting during the follicular phase (46.66% vs. 34.48%, $P=0.083$). Epidural GnRH administration was also associated with higher $P_4$ concentrations on D13 ($P<0.001$).

**Conclusion** Consequently, although changing the GnRH analogue and administration route did not significantly affect overall fertility outcomes, the findings suggest a possible phase-dependent relationship between administration route and pregnancy outcome. However, this trend requires confirmation in further studies.

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**Keywords** Dairy cow, Epidural, GnRH analogues, Intramuscular, Ovsynch, Reproductive performance

## Background

The Ovsynch protocol has become one of the most widely used synchronisation strategies to overcome the limitations of estrus detection [1]. This protocol, which consists of sequential injections of gonadotropin-releasing hormone (GnRH) and prostaglandin $F_2\alpha$ ($PGF_2\alpha$), enables fixed-time artificial insemination (FTAI) without the need for estrus detection, thereby facilitating reproductive management, particularly in large herds [2]. Nevertheless, the fertility outcomes achieved with Ovsynch remain suboptimal, with only 30–50% of cows reported to be pregnant 30–40 days after FTAI [3–7].

One of the most important biological constraints contributing to the limited success of Ovsynch is insufficient synchronisation [3, 8]. Ovulation of the targeted dominant follicle and initiation of a new follicular wave, particularly with the first GnRH injection administered at the beginning of the protocol, occurs in only 66–85% of cows [1]. In non-responding cows, the persistence of an old dominant follicle or a lack of ovarian response leads to a mismatch between the timing of ovulation and insemination [9, 10]. Indeed, cows that ovulate in response to the first GnRH injection have a 15–20% higher fertility rate compared to non-responders [11].

The response to the first GnRH injection in the Ovsynch protocol is closely related to the phase of the estrus cycle at the beginning of the protocol. Previous studies have shown that initiating Ovsynch between days 5 and 9 of the estrus cycle is key to successfully synchronising ovulation [8, 12]. This is likely due to the presence of a functional dominant follicle capable of ovulation in response to the luteinizing hormone (LH) surge induced by the first GnRH injection of Ovsynch [8]. The progesterone ($P_4$) concentration in the plasma of cows lacking a mature corpus luteum (CL) at the beginning of the synchronisation protocol is not sufficient to support proper growth of the ovulatory follicle, which is considered a significant obstacle to fertility in high-yielding dairy cows [9, 13, 14]. Indeed, several studies have reported a significantly reduced chance of pregnancy in cows starting the Ovsynch protocol in the follicular phase (absence of an active CL and low $P_4$ levels) compared to those starting in the luteal phase [1, 2, 13, 15, 16]. Various modifications have been proposed to increase protocol success at this stage. For example, attempts have been made to initiate the protocol in the luteal phase by administering Double-Ovsynch before the protocol [17]. Similarly, the addition of exogenous $P_4$ devices (PRID, CIDR, etc.) has been attempted to offset the negative effects of low initial $P_4$ levels [13]. Furthermore, alternative hormonal approaches, including the administration of equine chorionic gonadotropin or human chorionic gonadotropin [3, 18], as well as increasing the GnRH dose [16, 19], have been investigated to improve the ovulatory response. Although varying degrees of success have been reported with these approaches, most involve modifications such as prolonging the protocol and/or adding supplementary hormonal treatments, including exogenous $P_4$, which increase the overall intervention burden. Therefore, it remains relevant to investigate whether reproductive outcomes can be improved within the conventional Ovsynch schedule, without extending the protocol or introducing additional hormonal treatments, by modifying the route of GnRH administration.

The type of GnRH analogue used represents an important variable in the Ovsynch protocol. GnRH agonists such as buserelin, lecirelin, and gonadorelin are commonly used in synchronisation programs [20, 21]. Although these compounds differ structurally and pharmacologically [22], previous studies have generally reported comparable LH release, ovulation, and fertility outcomes among commonly used GnRH analogues under standard conditions [20, 23]. Thus, any potential differences between analogues are more likely to emerge under specific treatment conditions than as a consistent general effect [24].

Traditionally, GnRH is administered intramuscularly, allowing systemic absorption and pituitary stimulation. By contrast, epidural administration has been proposed as an alternative route that may alter drug disposition and tissue exposure [25, 26]. This approach is supported by the presence of GnRH receptors not only in the pituitary but also in ovarian tissues and along the spinal cord [27]. The efficacy of drugs administered into the epidural space depends on several factors, including pharmacokinetics, injection volume and rate, lipophilicity, and molecular weight [28–30]. Previous studies in cows with follicular cysts or anoestrus have suggested that epidural administration of GnRH analogues may influence reproductive responses, including estrus induction and corpus luteum formation [25–27]. However, evidence remains limited, particularly in cyclic dairy cows managed under the Ovsynch protocol.

In this context, it remains unclear whether the reproductive response to GnRH treatment during Ovsynch varies with the analogue used, the route of administration, and the ovarian status at the start of the protocol. Therefore, the present study was designed to compare two GnRH analogues (lecirelin and buserelin) administered by two different routes (intramuscular vs. epidural) in cyclic dairy cows subjected to the Ovsynch protocol, while also evaluating outcomes according to the initial

cyclic phase (follicular vs. luteal). The aim was to determine whether modifying the GnRH treatment strategy within the conventional Ovsynch schedule could influence fertility outcomes and $P_4$ dynamics.

## Methods

### Ethical approval and location

The experiment was conducted from June to December at Plato Integrated Livestock and Agriculture Industry and Trade Inc., a private livestock enterprise located in the Bünyan District of Kayseri Province, Middle Anatolia, Türkiye (38°52′04.2″N, 35°52′13.4″E; altitude: 1,337 m). Informed consent for the use of animals in this study was obtained from the owner of the livestock before study initiation. The study protocol was reviewed and approved by the Erciyes University Animal Experiments Local Ethics Committee, Kayseri, Türkiye (Approval No: 12/47, 2012).

### Animals management

A total of 406 multiparous, clinically healthy, 3–4 years old, and 50 days postpartum Holstein cows were enrolled in the study. All cows received uniform care and management, were milked three times daily, and fed a total mixed ration (TMR) consisting of corn silage, alfalfa, and barley, supplemented with a soybean meal-based concentrate twice daily. The TMR was formulated to meet the minimum nutritional requirements for lactating dairy cattle [31], and water was provided ad libitum. To ensure accurate reproductive status, each cow underwent systematic postpartum gynaecological examinations, including rectal palpation and transrectal ultrasonography with a multi-frequency linear transducer (7.5 MHz, HS-2100, Honda Electronics, Japan) performed at two-week intervals starting from the third week postpartum until the beginning of the study. These evaluations aimed to detect uterine involution, presence of a functional CL, ovarian pathologies (e.g., follicular/luteal cysts, ovarian atrophy), and to record any reproductive abnormalities [32].

Serum $P_4$ concentrations were measured concurrently as an additional criterion to verify ovarian cyclicity. In accordance with previously described criteria [14], cows in which no CL was identified on consecutive examinations and whose serum $P_4$ concentration was ≤ 1 ng/mL were classified as anovular and excluded from the study. Consequently, only cows with evidence of normal cyclic ovarian activity were enrolled in the experimental groups.

### Establishment of experimental groups and application of the Ovsynch protocol

In this study, lactating Holstein cows ($n$ = 406) were randomly assigned by simple randomisation to two main groups according to the GnRH analogue administered: Group L ($n$ = 203) (62.5 µg lecirelin acetate, Dalmarelin®, Vetaş, Türkiye) and Group B ($n$ = 203) (10 µg buserelin acetate, Receptal®, Intervet, Türkiye). Each main group was further subdivided by route of administration: intramuscular ($_{im}$) or epidural ($_{epi}$). Intramuscular injections were performed in the area between the semitendinosus and semimembranosus muscles. In contrast, epidural administrations were carried out in the sacrococcygeal intervertebral space (between S5 and Co1) using a sterile 18 G (1.20 × 38 mm, Sterile Needle, Ayset Medical Products Inc., Türkiye, Cat No: 70504) needle under strict aseptic and antiseptic precautions [33].

Accordingly, Group $L_{im}$ ($n$ = 102) received lecirelin acetate intramuscularly, while Group $L_{epi}$ ($n$ = 101) was treated via the epidural route. Similarly, Group $B_{im}$ ($n$ = 102) received buserelin acetate intramuscularly, whereas Group $B_{epi}$ ($n$ = 101) received epidural buserelin acetate. In accordance with the GnRH analogues and administration routes defined for each experimental group, the Ovsynch protocol was applied as follows: the first GnRH injection was administered on Day 0 (D0), followed 7 days later (D7) by an intramuscular injection of 25 mg dinoprost tromethamine (Dinolytic®, Zoetis, USA). Subsequently, the second GnRH treatment was administered 48 h (h) later (D9), and fixed-time artificial insemination (FTAI) was performed approximately 20 h after the second GnRH injection (D10). All cows in the experimental groups were inseminated using high-quality, qualified imported semen by an experienced technician (Fig. 1). In addition, estrus signs at the time of FTAI (D10) were evaluated through the observation of behavioural and physical indicators. Animals showing typical estrus signs (vulvar oedema, mucus discharge, restlessness, mounting other cows, or standing to be mounted) [34] were recorded, and the estrus rate was determined accordingly.

### Ultrasonographic ovarian and pregnancy examination

Ovarian examination was performed transrectally using a real-time B-mode ultrasound scanner (HS-2100, Honda Electronics, Japan) equipped with a 7.5 MHz linear-array transducer. On D0, immediately before the first GnRH administration, transrectal ultrasonographic examination of the ovaries was performed to identify the presence of follicles and luteal structures. Cows without a functional CL and with serum $P_4$ concentrations ≤ 0.5 ng/mL were classified as being in the follicular phase, whereas cows with at least one active CL and serum $P_4$ concentrations above ≥ 1 ng/mL were classified as being in the luteal phase [14]. A limited number of cows ($n$ = 24) had intermediate serum $P_4$ concentrations of 0.5-1.0 ng/mL. These animals were classified according to ultrasonographic CL status: cows with a functional CL were assigned to

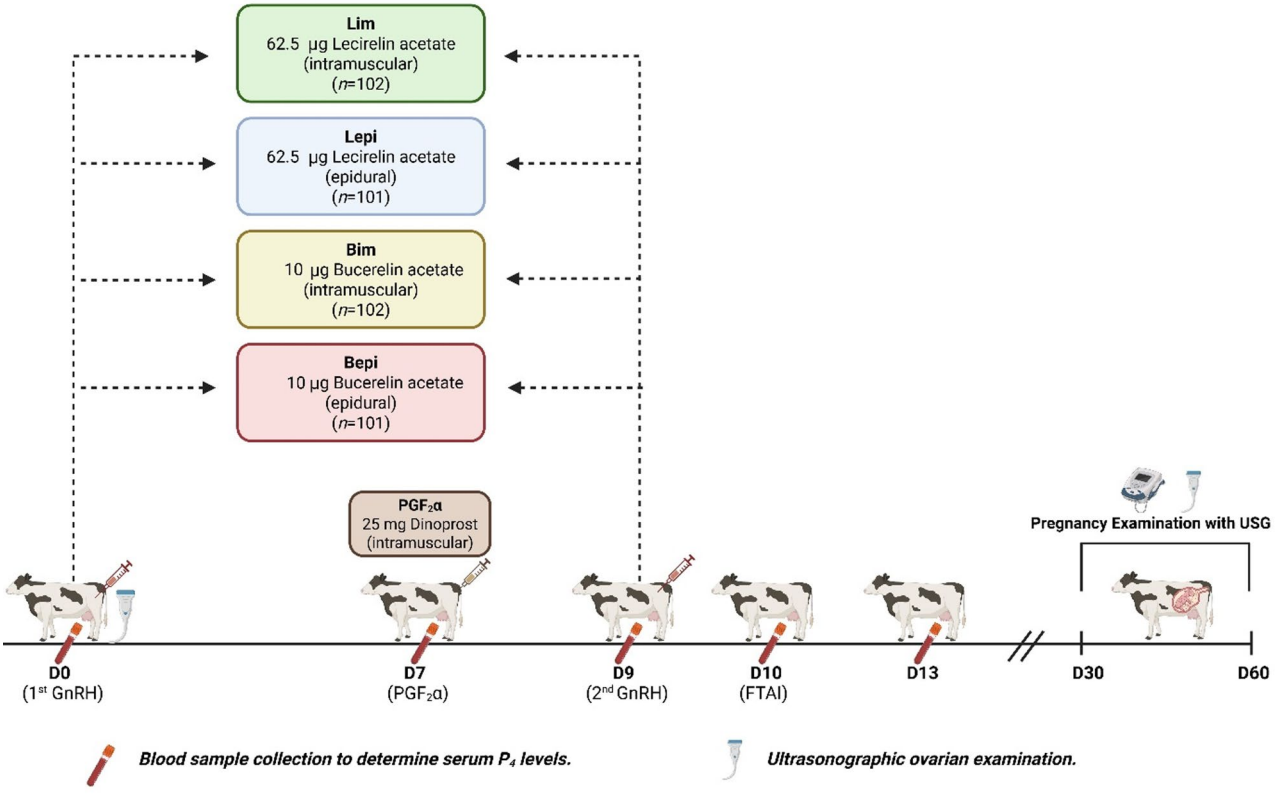



**Fig. 1** Schematic representation of the experimental design used in the Ovsynch protocol. Cows ($n=406$) were allocated into four groups according to GnRH analogue (lecirelin [L] or buserelin [B]) and route of administration (intramuscular [$_{im}$] or epidural [$_{epi}$]). Blood samples for serum progesterone ($P_4$) determination and ovarian ultrasonography (USG) were performed on D0, D7, D9, D10 and D13. Pregnancy examinations were performed by USG on days 30 and 60 (D30 and D60) after fixed-time artificial insemination (FTAI)

the luteal phase, and those without a functional CL were assigned to the follicular phase.

Pregnancy diagnosis was performed by transrectal ultrasonography at 30 (D30) and 60 (D60) days following FTAI. The detection of a fluid-filled uterine horn containing embryonic vesicles together with a functional CL at D30, or the visualisation of a viable fetus at D60, was considered a positive criterion for pregnancy. Pregnancy loss rates were calculated based on cows diagnosed pregnant at the first examination (D30) but found non-pregnant at the second examination (D60) (Fig. 1).

### Blood collection and hormone assessment

For serum $P_4$ level (ng/ml) determination, approximately 8 mL of blood samples were collected from the coccygeal vein or artery (D0, D7, D9, D10 and D13) by puncturing them with a 21 G (0.8 × 38 mm, Blood Collection Needle, Ayset Medical Products Inc., Türkiye, Cat No: 70524) cannula into vacuum tubes (Vacusera®, Disera, Türkiye, Cat. No: 436308) containing clot activator gel (Fig. 1). After collection, blood samples were stored at +4 °C in the farm refrigerator once sampling of each treatment group had been completed. Subsequently, all samples from that group were transported together to the laboratory under cold-chain conditions within 2 h, after which serum was separated by centrifugation at 3000 rpm for 10 min. Serum samples were stored at -20 °C until the day of analysis. Serum $P_4$ concentration was measured by enzyme immune assay (EIA) (DRG Aurica ELISA Progesterone Kit, DRG Intl. Inc., Germany, Cat. No: EIA-1561) with a sensitivity ≤ 0.1 ng/ml, a specificity of 97.8% and intra-assay oath inter-assay coefficients of variation (CV) of 5.40–6.99% and 4.34–9.96% for samples between 0 and 40 ng/ml.

### Statistical analysis

A post hoc power analysis was performed using G*Power (version 3.1) based on the available sample size ($n=406$). Accordingly, the power of the test was calculated as 0.994 when the effect size was accepted as 0.30 and the significance level as 0.05 for comparisons of fertility parameters among groups, and as 0.997 when the effect size was accepted as 0.025 and the significance level as 0.05 for comparisons of serum $P_4$ concentrations between groups and across days. These calculations were based on the final sample size and were included to provide additional statistical context for the analyses, rather than as an a priori justification of the sample size.

**Table 1** Baseline days in milk (DIM), body condition score (BCS), and milk yield (kg/day) of cows in the experimental groups at the initiation of the Ovsynch protocol (D0). Data are presented as mean ± standard deviation (SD)

| Groups | DIM Mean ± SD (min-max) | BCS Mean ± SD (min-max) | Milk yield Mean ± SD (min-max) |
|---|---|---|---|
| $L_{im}$ | 86.4 ± 26.3 (51–154)[a] | 2.6 ± 0.3 (2.00–3.00)[a] | 23.7 ± 4.1 (13.10-33.05)[a] |
| $B_{im}$ | 83.8 ± 20.9 (50–135)[a] | 2.7 ± 0.3 (2.00–3.00)[a] | 24.3 ± 4.0 (15.10–34.10)[a] |
| $L_{epi}$ | 79.9 ± 18.9 (59–164)[a] | 2.6 ± 0.4 (2.00–3.00)[a] | 32.5 ± 6.6 (13.60–45.10)[c] |
| $B_{epi}$ | 69.5 ± 14.8 (51–136)[b] | 2.5 ± 0.3 (2.00-3.50)[b] | 27.8 ± 5.5 (15.40–44.80)[b] |
| *P*-value | < 0.001 | 0.023 | < 0.001 |

*Different superscripts (a, b, c) in the same row indicate significant differences among the groups ($P < 0.05$)

Descriptive statistics were calculated and presented as mean ± standard error. Data distribution was assessed for normality using the Shapiro-Wilk test. Homogeneity of variances was evaluated using Levene's test, which was preferred because it is a widely used and relatively robust method for assessing variance equality across groups. The sphericity assumption was evaluated using Mauchly's test. The main effects and interactions of group ($L_{im}$, $L_{epi}$, $B_{im}$, and $B_{epi}$), initial cyclic phase (follicular and luteal), and time (D0, D7, D9, D10, and D13) were analysed using repeated-measures analysis of variance (ANOVA). When the sphericity assumption was violated, the Greenhouse-Geisser correction was applied. Multiple comparisons were performed using the Bonferroni test. Differences between groups and cyclic phases in estrus, pregnancy, and pregnancy loss rates were evaluated using Pearson's chi-square test or the Fisher-Freeman-Halton exact test.

In addition, a multivariable binary logistic regression analysis was performed to identify factors associated with pregnancy status at D30. In this model, pregnancy outcome (pregnant/non-pregnant) was the dependent variable, and days in milk (DIM), body condition score (BCS), milk yield, initial cyclic phase, GnRH analogue, route of administration, and estrus expression were the independent variables. Categorical variables were coded using appropriate reference categories. Overall model significance was assessed using the Omnibus test, model fit was evaluated using the Hosmer-Lemeshow goodness-of-fit test, and explanatory power was assessed using the Nagelkerke $R^2$ coefficient. The effects of independent variables on the probability of pregnancy were reported as odds ratios (OR; Exp(B)) with 95% confidence intervals (CI).

In all analyses, $P < 0.05$ was considered statistically significant, whereas $0.05 \leq P \leq 0.10$ was interpreted as a numerical tendency. All analyses were performed using IBM SPSS Statistics 30.0 (IBM Corp., Armonk, NY, USA).

**Table 2** Combined effects of GnRH administration route (intramuscular vs. epidural), analogue (lecirelin vs. buserelin), and initial cyclic phase (follicular vs. luteal) on the reproductive outcomes of the Ovsynch protocol in dairy cows

| | | Route | Fertility Parameters % (*n*) | | |
|---|---|---|---|---|---|
| | | | Estrus | Pregnancy | Pregnancy loss |
| Analogue | **Lecirelin** | **Intramuscular** | 93.27 (94/102)[a] | 42.15 (43/102) | 6.97 (3/43) |
| | | **Epidural** | 75.24 (76/101)[b] | 39.60 (40/101) | 7.50 (3/40) |
| | **Buserelin** | **Intramuscular** | 87.25 (89/102)[a] | 35.29 (36/102) | 5.55 (2/36) |
| | | **Epidural** | 87.12 (88/101)[a] | 39.60 (40/101) | 7.50 (3/40) |
| P-value | | | < 0.001* | 0.821 | NS |
| Cyclic Phase | **Follicular** | **Intramuscular** | 85.34 (99/116)[b] | 34.48 (40/116)[c] | 5.00 (2/40) |
| | | **Epidural** | 82.85 (87/105)[b] | 46.66 (49/105)[d] | 12.24 (6/49) |
| | **Luteal** | **Intramuscular** | 95.45 (84/88)[a] | 44.31 (39/88)[d] | 7.69 (3/39) |
| | | **Epidural** | 79.38 (77/97)[b] | 31.95 (31/97)[c] | 0 (0/31) |
| P-value | | | < 0.001* | 0.083** | 0.249 |

*Different superscripts (a, b) in the same column indicate significant differences among the groups ($P < 0.05$)

**Different superscripts (c, d) in the same column indicate tendency differences ($0.05 \leq P \leq 0.1$) among the groups

## Results

At the initiation of the Ovsynch protocol (D0), significant between-group differences were detected for DIM ($P < 0.001$), BCS ($P = 0.023$), and milk yield ($P < 0.001$). Specifically, DIM was lower in the $B_{epi}$ group than in the other groups, milk yield was highest in the $L_{epi}$ group and intermediate in the $B_{epi}$ group, and BCS was lower in the Bepi group than in the remaining groups (Table 1).

### Fertility outcomes

The fertility parameters of the experimental groups are presented in Table 2. Overall, estrus rates were significantly higher in $L_{im}$ (93.27%), $B_{im}$ (87.25%), and $B_{epi}$ (87.12%) than in $L_{epi}$ (75.24%) ($P < 0.001$). However, there were no significant differences among the groups in pregnancy or pregnancy loss ($P > 0.05$). When the effect of the administration route was evaluated according to the GnRH analogue, intramuscular administration of lecirelin (93.27%) resulted in significantly higher estrus expression compared to epidural treatment (75.24%) ($P < 0.001$). However, no differences were detected in other parameters between GnRH analogues and administration routes ($P > 0.05$).

Evaluation of the administration route irrespective of the GnRH analogue showed that intramuscular treatment

in the luteal phase produced significantly higher estrus expression (95.45%) than epidural treatment (79.38%) ($P < 0.001$). Although not statistically significant, pregnancy rates tended to be higher with intramuscular administration in the luteal phase (44.31%) compared to epidural administration (31.95%) ($P = 0.083$). Conversely, when the protocol was initiated in the follicular phase, the trend was reversed, with higher pregnancy rates in the epidural group (46.66%) than in the intramuscular group (34.48%) ($P = 0.083$).

A multivariable binary logistic regression analysis was also performed to evaluate factors associated with pregnancy at D30, including DIM, BCS, milk yield, initial cyclic phase, GnRH analogue, route of administration, and estrus expression (Table 3). The model was not statistically significant (Omnibus test: $\chi^2 = 2.721$, df = 8, $P = 0.951$), although it showed acceptable fit according to the Hosmer-Lemeshow test ($\chi^2 = 10.883$, df = 8, $P = 0.208$). The model's explanatory power was low (Nagelkerke $R^2 = 0.009$), and none of the included variables was significantly associated with pregnancy outcome ($P > 0.05$).

### Serum progesterone concentrations

Significant main effects were found for group (G: $P < 0.001$), cyclic phase (C: $P < 0.001$), and time (T: $P < 0.001$), indicating that measured values differed significantly among treatment groups, between follicular and luteal phases, and across measurement time points. Significant two-way interactions were observed between group and cyclic phase (G × C: $P = 0.021$), group and time (G × T: $P < 0.001$), and cyclic phase and time (C × T: $P < 0.001$). These interactions indicate that the effect of the cyclic phase varied across groups, that group differences changed over time, and that the cyclic-phase effect was time-dependent. The three-way interaction among group, cyclic phase, and time was not statistically significant (G × C × T: $P = 0.452$), suggesting that the pattern of group-cyclic phase interactions remained consistent across all measurement time points.

Within each treatment group, $P_4$ concentrations did not differ significantly between cows that entered the protocol in the follicular or luteal phase, except on D0. The difference observed on D0 reflected the physiologically higher $P_4$ levels in cows at the luteal stage compared with those at the follicular stage, which was considered an expected physiological finding and therefore disregarded in the analysis (Fig. 2a-d).

When the Ovsynch protocol was initiated during the luteal phase, a significant difference was observed among the groups. On D7, the $L_{im}$ had a mean $P_4$ concentration of 3.31 ± 0.28 ng/mL, which was significantly higher than $L_{epi}$ (2.45 ± 0.21 ng/mL), $B_{im}$ (2.06 ± 0.15 ng/mL), and $B_{epi}$ (2.00 ± 0.19 ng/mL) ($P < 0.001$) (Fig. 2f). In both the follicular and luteal phases, irrespective of the GnRH analogue used, epidural administration resulted in higher $P_4$ concentrations on D13 ($P < 0.001$) (Fig. 2e-f).

When the results were evaluated regardless of the initial cyclic phase, the $L_{im}$ showed the highest $P_4$ concentration on D7 (2.92 ± 0.14 ng/mL) compared with $L_{epi}$ (2.27 ± 0.13 ng/mL), $B_{im}$ (1.94 ± 0.10 ng/mL), and $B_{epi}$

**Table 3** Multivariable binary logistic regression analysis for factors associated with pregnancy at D30

| Variables | *β* | *SE (β)* | Wald | df | *P* | Exp(B) | %95 CI of Exp(B) |
|---|---|---|---|---|---|---|---|
| DIM (day) | −0.002 | 0.005 | 0.166 | 1 | 0.684 | 0.998 | 0.988–1.008 |
| BCS | | | 1.587 | 2 | 0.452 | | |
| 2 (ref.) | | | | | | | |
| 2.5 | −0.122 | 0.332 | 0.134 | 1 | 0.714 | 0.885 | 0.462–1.697 |
| 3 | 0.168 | 0.350 | 0.229 | 1 | 0.632 | 1.183 | 0.595–2.349 |
| Milk Yield (kg/day) | −0.004 | 0.020 | 0.042 | 1 | 0.837 | 0.996 | 0.958–1.035 |
| Initial Cycle | | | | | | | |
| Follicular (ref.) | | | | | | | |
| Luteal | −0.004 | 0.216 | 0.000 | 1 | 0.983 | 0.996 | 0.652–1.520 |
| Analog | | | | | | | |
| Buserelin (ref.) | | | | | | | |
| Lecirelin | 0.107 | 0.217 | 0.242 | 1 | 0.623 | 1.113 | 0.727–1.703 |
| Route | | | | | | | |
| Intramuscular (ref.) | | | | | | | |
| Epidural | −0.094 | 0.247 | 0.146 | 1 | 0.702 | 0.910 | 0.561–1.476 |
| Estrus | | | | | | | |
| Negative (ref.) | | | | | | | |
| Positive | 0.158 | 0.303 | 0.273 | 1 | 0.601 | 1.171 | 0.647–2.119 |

Methods: Enter. Omnibus testi: $\chi^2 = 2.721$; sd = 8; $p = 0.951$; −2 Log likelihood = 529.936; Cox & Snell $R^2 = 0.007$; Nagelkerke $R^2 = 0.009$; Hosmer–Lemeshow testi: $\chi^2 = 10.883$; sd = 8; $p = 0.208$

*β:* Estimated slope coefficient, *SE (β):* Standard error of the estimated slope coefficient, *Wald: Wald statistic*, which tests whether the slope coefficients for the model are equal to zero, *df:* Degrees of Freedom, *P: P* value of Wald statistics, *Exp(B):* Estimated odds ratio, *%95 CI of Exp(B):* confidence interval values are given

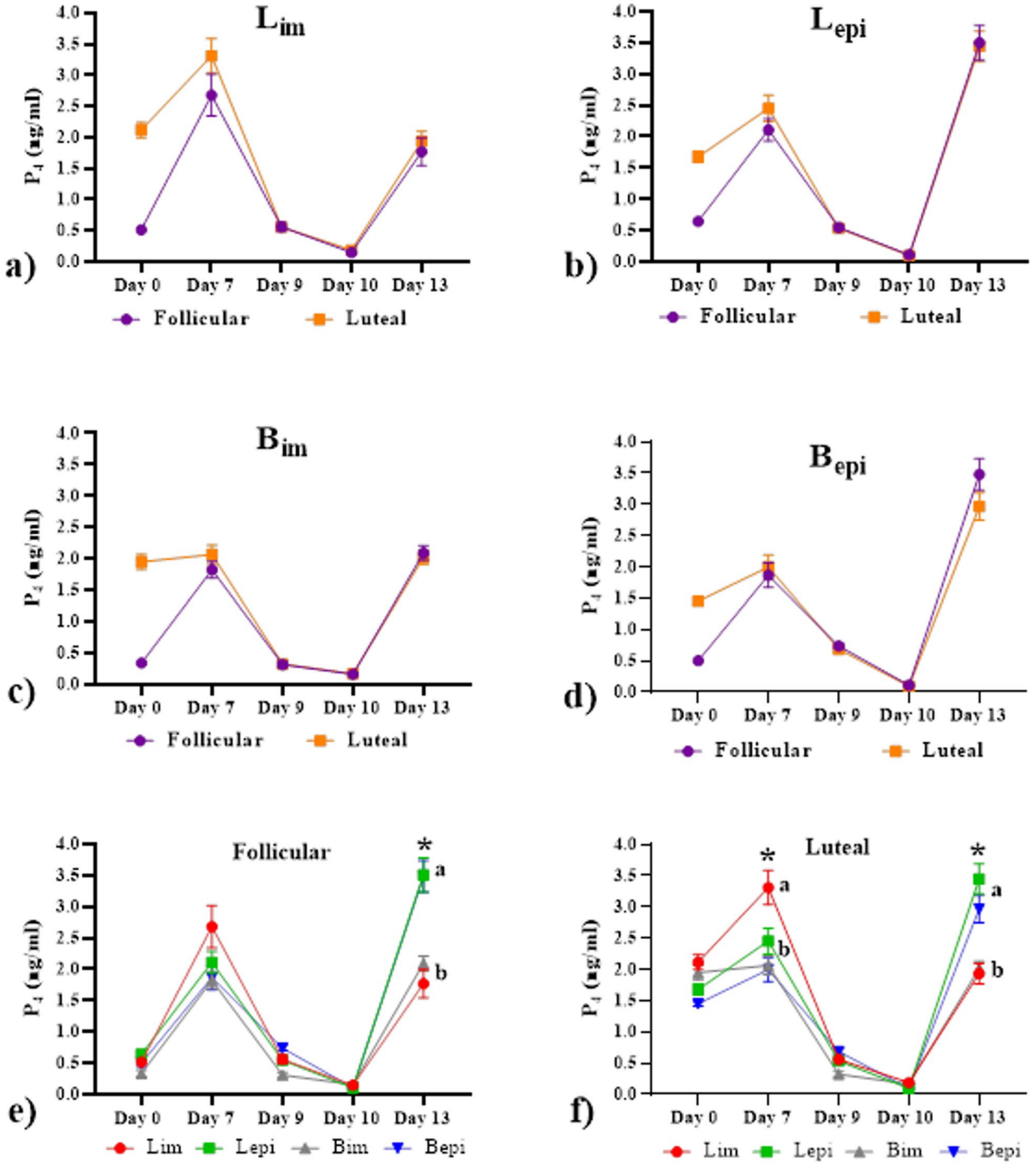



**Fig. 2** Serum progesterone ($P_4$) concentrations (mean ± SEM, ng/mL) during the Ovsynch protocol in relation to GnRH analogue, route of administration, and initial cyclic phase. (**a**) $L_{im}$ (lecirelin, intramuscular); (**b**) $L_{epi}$ (lecirelin, epidural); (**c**) $B_{im}$ (buserelin, intramuscular); (**d**) $B_{epi}$ (buserelin, epidural); (**e**) comparison of all groups in the follicular phase; (**f**) comparison of all groups in the luteal phase. *Means with different superscripts (a, b) at the same time point are different ($P < 0.05$)

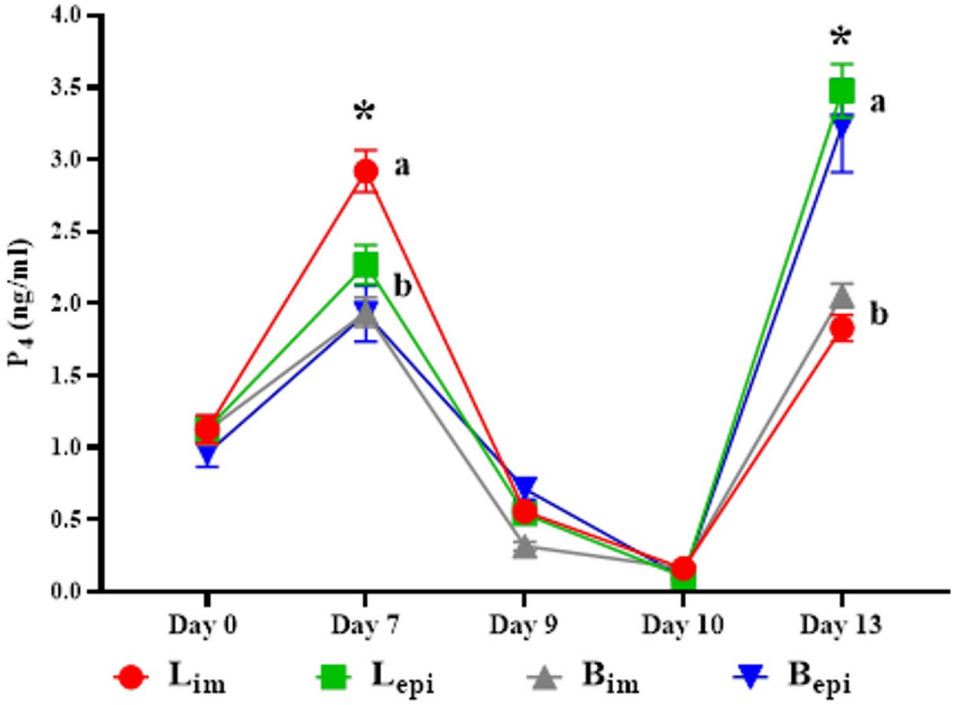



**Fig. 3** Serum progesterone ($P_4$) concentrations (mean ± SEM, ng/mL) measured on days 0, 7, 9, 10, and 13 of the Ovsynch protocol in dairy cows, regardless of initial cyclic phase, treated with different GnRH analogues and routes of administration. Groups: $L_{im}$ (62.5 µg lecirelin acetate, intramuscular; $n = 102$), $L_{epi}$ (62.5 µg lecirelin acetate, epidural; $n = 101$), $B_{im}$ (10 µg buserelin acetate, intramuscular; $n = 102$), and $B_{epi}$ (10 µg buserelin acetate, epidural; $n = 101$). *Means with different superscripts (a, b) at the same time point are different ($P < 0.05$)

(1.92 ± 0.19 ng/mL) ($P < 0.001$) (Fig. 3). On D13, epidural groups maintained significantly higher $P_4$ concentrations ($L_{epi}$: 3.47 ± 0.18 ng/mL, $B_{epi}$: 3.23 ± 0.32 ng/mL) than intramuscular groups ($L_{im}$: 1.83 ± 0.09 ng/mL, $B_{im}$: 2.05 ± 0.08 ng/mL) ($P < 0.001$) (Fig. 3).

## Discussion

In the present study, we examined the combined effects of the initial cyclic phase, the type of GnRH analogue used, and the route of administration on fertility outcomes in dairy cows treated with the Ovsynch protocol. Contrary to our initial hypothesis, changing the route of GnRH administration did not significantly affect overall fertility. The only difference between the groups was observed in estrus rates, but this was inconsistent. Cows in the $L_{epi}$ group exhibited the fewest signs of estrus at the time of FTAI. However, this did not result in a decrease in pregnancy rates. Within the Ovsynch protocol, pregnancy outcome is primarily determined by the synchronisation of ovulation induced by timed GnRH and $PGF_2\alpha$ administration rather than by the behavioural expression of estrus [1]. Consistent with this, previous studies have shown that estrus expression is not always closely associated with pregnancy rate in synchronised cows [13, 35, 36]. Therefore, it was not unexpected that pregnancy rates were similar despite lower estrus expression in the $L_{epi}$ group. On the other hand, pregnancy and pregnancy-loss rates were similar across all groups ($P > 0.05$). In parallel with our results, Salar et al. [37] investigated the effects of epidural or intramuscular administration of a GnRH analogue on the Ovsynch protocol and found no significant difference between pregnancy rates (44.26% vs. 36.49%, respectively) ($P > 0.05$). In addition, changing the GnRH analogue did not significantly affect fertility. Indeed, consistent with our results, Baruselli et al. [23] reported that the efficacy of buserelin and lecirelin was similar. Picard-Hagen et al. [20] investigated the efficacy of different GnRH analogues on LH secretion and reported no difference in efficacy between lecirelin and buserelin. Consistent with the literature, the interaction between analogue and route of administration was not significant for pregnancy rate ($P > 0.05$).

At baseline, DIM, BCS, and milk yield differed significantly among the groups. In particular, DIM was lower in the $B_{epi}$ group, whereas milk yield was higher in the $L_{epi}$ group (Table 1). However, these initial differences did not translate into differences in fertility outcomes. Despite the lower DIM in the $B_{epi}$ group, the synchronisation protocol appeared to function effectively across all groups, resulting in comparable pregnancy rates. This is consistent with previous reports indicating that timed insemination protocols, such as Ovsynch, can be successfully applied after 50 days postpartum and may reduce the impact of postpartum interval on reproductive

performance [1, 2]. Similarly, although BCS differed statistically among groups, the biological variation was limited, with values remaining within a relatively narrow range (2.5–2.7). This may explain why BCS did not appear to influence fertility outcomes in the present study, since the effect of body condition on reproduction is generally more evident at more extreme values [38]. Milk yield was significantly higher in the $L_{epi}$ group. Although high milk production has been associated with increased metabolic load, reduced estrus expression, and impaired fertility in some studies [39, 40], this pattern was not evident here, as pregnancy rates did not differ significantly among groups. This interpretation was further supported by the multivariable logistic regression analysis, which showed that DIM, BCS, and milk yield were not significantly associated with pregnancy at D30 (Table 3). Taken together, these findings suggest that, under the conditions of the present study, the baseline differences observed among groups were not of sufficient magnitude to affect pregnancy outcome.

However, the results suggested a possible interaction. This interaction was between the route of administration and the cyclic phase at the beginning of the protocol. More specifically, opposite numerical pregnancy patterns were observed according to the initial cyclic phase. It has previously been reported that cows initiating the Ovsynch protocol during the anovulatory or follicular phase of estrus have reduced pregnancy rates compared to cows initiating the protocol during the luteal phase [2, 12–14, 41, 42]. Consistent with these results, pregnancy rates in cows starting the Ovsynch protocol in the luteal phase (with a $P_4$ level ≥ 1 ng/mL and functional CL) tended to be higher in the intramuscular administration groups compared to cows starting in the follicular phase ($P$ = 0.083). These findings are consistent with reports suggesting that the physiological environment at the beginning of the protocol plays a critical role in determining the efficacy of GnRH treatment [1, 7, 13, 43]. Interestingly, however, the opposite trend was observed with epidural administration of GnRH. Cows starting the protocol in the follicular phase ($P_4$ level ≤ 0.5 ng/mL and having no functional CL) tended to have a higher pregnancy rate than those starting in the luteal phase ($P$ = 0.083). Although these numerical differences may indicate a phase-related pattern, they did not reach statistical significance and therefore should be interpreted with caution. The multivariable logistic regression analysis also supports this more cautious interpretation. When pregnancy at D30 was evaluated in relation to DIM, BCS, milk yield, initial cyclic phase, GnRH analogue, route of administration, and estrus expression, none of these variables showed a statistically significant independent association with pregnancy outcome, and the model's explanatory power was low. Accordingly, the opposite numerical tendencies observed between luteal- and follicular-phase cows should be regarded as preliminary rather than definitive evidence of a phase-dependent effect of administration route.

Although further studies are needed to elucidate the mechanism underlying this difference, one possible explanation may be that the LH release pattern varies depending on the administration route. The effects of intramuscular GnRH analogues on LH secretion have been extensively investigated in the literature. In cattle, intramuscular GnRH administration has been reported to cause acute increases in LH and follicle-stimulating hormone (FSH), the magnitude of which depends on the phase of the follicular wave [44] and circulating steroid hormone concentrations [45, 46]. Common findings suggest that intramuscular administration results in high amplitude but short-lived LH surges [21, 22, 47–49]. This appears to be necessary, particularly during the luteal phase, to overcome the strong negative feedback of high $P_4$ levels on the hypothalamic-pituitary axis to trigger ovulation [3, 16, 50].

Studies directly examining the LH pattern after epidural GnRH injection are limited. Amin et al. [25] reported that epidural administration of 20 µg buserelin in anovulatory cows significantly increased FSH and LH levels, thus having the potential to induce the estrus response and pregnancy frequency in treated cows. Rizzo et al. [26] also reported that epidural administration of 50 µg lecirelin in cows with follicular cysts increased FSH and LH levels, resulting in significantly higher estrus induction (75.0% vs. 57.0%) and pregnancy rate (71.0% vs. 53.0%) compared to intramuscular administration. Similarly, Rizzo et al. [27] reported that epidural administration of 50 µg lecirelin in cows was a highly effective treatment for follicular cysts, with regression occurring 8 ± 2 days after treatment in 82% of treated cows and increased ovarian vascularisation.

Although potential additional benefits of epidural GnRH administration have been reported, insufficient evidence exists regarding the amplitude and duration of LH release. However, other drug models have shown that epidural injection provides a longer duration of action compared to intramuscular administration. For example, epidural morphine has been reported to produce a slower onset but a more prolonged effect than intramuscular administration, supporting the distinct pharmacokinetic characteristics of the neuraxial route [51]. Similarly, in Holstein cows, epidural xylazine produced more potent and prolonged perineal analgesia than intramuscular xylazine; onset, magnitude, and duration metrics confirmed this difference [52]. These findings suggest that epidural administration may result in lower peak plasma concentrations but a longer duration of effect than intramuscular administration, due to factors such as drug delivery

to cerebrospinal fluid/spinal cord receptors, a reservoir effect in epidural tissues, and slower systemic absorption [53]. In light of these data, we predict that epidural GnRH administration may have similarly provided lower-amplitude but longer-lasting LH stimulation. Such a pattern may be insufficient to overcome the high $P_4$ pressure during the luteal phase. In contrast, during the follicular phase, it may have resulted in a stronger ovulatory response and higher pregnancy rates through prolonged LH signalling and possible extra-pituitary effects [54].

The situation is quite different in the follicular phase, where $P_4$ is low, and there is no significant negative feedback [3]. Under these circumstances, prolonged LH support generated by epidural GnRH may be advantageous. Prolonged LH stimulation may enhance oocyte maturation, optimise the timing of ovulation, and improve synchrony with endometrial priming [55]. This may explain the tendency for epidural GnRH administration during the follicular phase to improve pregnancy outcomes in our study. Mechanistically, GnRH receptors are present not only in the pituitary but also in the ovary and neural tissues [27, 54, 56]. Due to their lipophilic structure, epidural GnRH analogues can cross the meninges, reach the cerebrospinal fluid, and activate spinal GnRH receptors [26, 57]. Stimulation of these receptors increases sympathetic outflow, which is known to influence follicular maturation, steroid secretion, and ovulation [22, 58]. Therefore, epidural GnRH may have not only endocrine but also extra pituitary effects, increasing reproductive success, particularly in the hormonal milieu of the follicular phase. However, further studies directly examining GnRH/LH release patterns after epidural administration are needed to confirm this conclusion.

The $P_4$ profiles between the groups also support these interpretations. Higher $P_4$ values on D7 in the Lim group suggest acute luteinisation triggered by a strong LH surge. In contrast, significantly higher $P_4$ levels observed in the epidural groups on the third day (D13) after FTAI reflect longer-lasting luteal activity (Fig. 3). This could indicate either stronger luteal support or the formation of accessory CLs [15]. It is particularly noteworthy that this finding occurs independently of the cyclic phase or the GnRH analogue used. These higher $P_4$ levels obtained on D13 following epidural administration are consistent with the prolonged LH release pattern that develops following ovulation triggered by the second GnRH administration. In fact, this also supports our previous prediction. The possibly longer-lasting but lower-amplitude LH stimulation generated by epidural GnRH may not only initiate ovulation but also increase the functional capacity of the luteal tissue by maintaining its luteotrophic activity and increasing $P_4$ levels [10].

The physiological basis for this lies in the differential sensitivity of cell populations in the early luteal formation. While small luteal cells directly undergo steroidogenesis dependent on LH, large luteal cells have higher basal $P_4$ secretion and a limited response to LH [59]. Therefore, prolonged LH stimulation provided by epidural GnRH may increase total $P_4$ production by promoting steroidogenesis activity, particularly in small luteal cells [40]. Adequate luteal $P_4$ support in the period after FTAI is critical for embryo survival and maternal recognition of pregnancy. In this context, the ability of epidural GnRH to maintain higher $P_4$ levels at D13 can be considered a significant practical advantage that contributes to embryo implantation and maintenance of pregnancy [60]. Overall, the results suggest that GnRH effectiveness in the Ovsynch protocol is conditional. In this context, while intramuscular administration is advantageous in the $P_4$-dominant (luteal) setting due to the strong LH surge, epidural administration in the $P_4$-low (follicular) setting may yield better results due to its prolonged stimulation. This dual effect is consistent with previous studies reporting better outcomes with epidural GnRH in pathological or anoestrus cases [24–26].

The limitations of our study should also be considered. Although the total number of animals was substantial, subdivision according to analogue, route, and cyclic phase reduced the effective sample size for some comparisons. This may be considered an important limitation, particularly in multivariable models and interaction analyses, where the effective sample size can be further reduced, thereby limiting statistical power for some comparisons. In addition, the multivariable logistic regression model showed low explanatory power and failed to identify any independent predictors of pregnancy outcome, further indicating that the observed subgroup patterns should not be overinterpreted. Furthermore, LH patterns and ovulatory responses of GnRH analogues could not be assessed based on the route of administration. Therefore, while the present findings highlight a possible phase-dependent pattern, confirmation requires more comprehensive studies specifically designed to test interaction effects and underlying endocrine mechanisms.

## Conclusion

In conclusion, opposite numerical pregnancy patterns were observed according to the initial cyclic phase, with intramuscular treatment tending to perform better in cows starting the protocol during the luteal phase and epidural treatment tending to perform better in cows starting during the follicular phase. However, because these differences were not statistically significant and were not confirmed as independent predictors in the multivariable logistic regression analysis, they should be interpreted with caution. Therefore, the main contribution of this study is to highlight a possible phase-dependent pattern in the reproductive response to GnRH

treatment within the Ovsynch protocol, which requires confirmation in more comprehensive studies specifically designed to evaluate interaction effects and underlying endocrine mechanisms.

**Abbreviations**

| | |
|---|---|
| BCS | Body Condition Score |
| CL | Corpus luteum |
| CV | Coefficients of variation |
| DIM | Days in milk |
| EIA | Enzyme Immune Assay |
| ($_{epi}$) | Epidural |
| FSH | Follicle-stimulating hormone |
| FTAI | Fixed-time artificial insemination |
| GnRH | Gonadotropin-releasing hormone |
| ($_{im}$) | Intramuscular |
| LH | Luteinizing hormone |
| P4 | Progesterone |
| $PGF_2\alpha$ | Prostaglandin $F_2\alpha$ |
| TMR | Total mixed ration |

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**Authors' contributions**
Murat Abay : Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Validation, Visualization, Writing–original draft, Writing–review and editing. Gökhan Uyanık: Validation, Visualization, Writing–original draft, Writing–review and editing. Aytaç Akçay : Conceptualization, Data curation, Formal analysis, Methodology, Writing–review and editing. Esra Canooğlu : Writing–review and editing. Kutlay Gürbulak : Writing–review and editing. Tayfur Bekyürek : Conceptualization, Investigation, Methodology, Supervision, Writing–review and editing. Ömer Orkun Demiral : Methodology, Supervision, Writing–review and editing.

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**Data availability**
The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Declarations

**Ethics approval and consent to participate**
Plato Integrated Livestock and Agriculture Industry and Trade Inc., a private livestock enterprise, owned the animals used in this study. Informed consent for the use of animals in this study was obtained from the owner of the livestock before study initiation. All procedures were performed in accordance with relevant guidelines and regulations, and the study protocol was reviewed and approved by the Erciyes University Animal Experiments Local Ethics Committee, Kayseri, Türkiye (Approval No: 12/47, 2012).

**Competing interests**
The authors declare no competing interests.

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