












# EFFICIENCY OF HORMONAL BIOMARKERS IN PREDICTING THE REPRODUCTIVE POTENTIAL OF DONOR COWS

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**ABSTRACT:** The critical decline in the cattle population in Ukraine necessitates the implementation of effective biotechnologies, particularly embryo transfer. The successful application of such programs largely depends on the accurate selection of high-producing donor cows. Therefore, the aim of this study was to evaluate the predictive value of estradiol and anti-Müllerian hormone as biomarkers for assessing the reproductive potential of donor cows. Blood samples were collected daily from 30 cows during days 3–10 of the estrous cycle to determine the concentrations of estradiol and anti-Müllerian hormone using chemiluminescent immunoassay (CLIA). Simultaneously, transrectal ultrasonography was performed to count the number of antral follicles measuring 2–10 mm in diameter. Statistical analysis included one-way ANOVA followed by Tukey's post hoc test. The results showed that the concentration of anti-Müllerian hormone remained relatively stable throughout the study period, whereas estradiol levels exhibited dynamic fluctuations with a peak on day 7. The highest mean number of antral follicles ( $11.14 \pm 2.41$ ) was observed in animals with high anti-Müllerian hormone concentrations ( $> 0.3 \text{ ng/cm}^3$ ), which was significantly higher compared to groups with low and medium hormone levels. For estradiol, the highest number of antral follicles was recorded in the group with medium hormone levels ( $15\text{--}25 \text{ pg/cm}^3$ ), with values also significantly higher than those observed in the low and high concentration groups. Thus, the concentrations of anti-Müllerian hormone and estradiol can be considered reliable predictive biomarkers for assessing the reproductive potential of donor cows. It was established that the highest number of antral follicles is associated with high anti-Müllerian hormone concentrations and medium estradiol levels, which enables the optimization of selecting animals with high ovarian reserve for biotechnological reproduction programs.

**Keywords:** anti-Müllerian hormone (AMH), estradiol (E2), antral follicle count (AFC), ovarian reserve, biomarkers, reproductive potential, reproductive biotechnologies, fertility

## INTRODUCTION

Cattle breeding is a strategic sector of the agricultural economy of Ukraine, as it ensures food security and contributes to the development of rural areas through job creation, infrastructure development, and improved community welfare (Lomovskykh et al., 2025). However, a critical decline in livestock numbers has been observed against the backdrop of ongoing military actions and economic crisis. Thus, at the beginning of 2000, the cattle population amounted to 10.6 million head, decreasing to 4.8 million in 2010, 2.6 million in 2022, and 2.0 million as of January 1, 2025; the number of cows declined from 5.4 million in 2000 to 2.7 million in 2010, 1.5 million in 2022, and less than 1.2 million in 2025 (Dukhnytskyi & Dukhnytskyi, 2025). Under such conditions, the further development of cattle breeding in Ukraine is only possible through the implementation of innovative technologies, automation of production processes, and improved resource management, which can ensure profitability and competitiveness of the sector (Lomovskykh et al., 2025).

Embryo transfer is an advanced technology widely used in cattle breeding in Europe, Canada, and the United States for genetic improvement and accelerated reproduction of high-value animals (Dochi, 2019; Kovpak et al., 2022a; Valchuk et al., 2023). Since 2016, ovum pick-up (OPU) followed by in vitro embryo production (IVEP) has become the leading reproductive technology in cattle breeding (Viana, 2020). This is associated with significant technological progress, allowing for the production of a greater number of embryos and pregnancies per unit time, a broader range of potential donor females from which oocytes can be obtained (including prepubertal heifers and cows in early pregnancy) (Ferré et al., 2020), the possibility of fertilizing oocytes with semen from multiple bulls (Bo et al., 2019), and obtaining offspring from cows with reduced fertility due to reproductive pathologies, impaired sperm transport, or terminal diseases (Salek et al., 2025).

Despite the annual increase in global embryo production (Viana, 2024), the use of embryo transfer technology in cattle breeding in Ukraine has declined to critically low levels (Burkat et al., 2005). This is largely due to certain biases among domestic livestock owners, who perceive the technology as costly and economically inefficient. Such perceptions are largely associated with reliance on outdated and expensive in vivo embryo production methods, as well as insufficient understanding of the mechanisms of hormonal regulation of folliculogenesis and oogenesis.

Despite the fact that modern biotechnological approaches significantly enhance embryo production efficiency (Crowe et al., 2021; Kovpak et al., 2022b; Hansen, 2024), the number of embryos obtained per donor cow remains limited (Bó & Mapletoft, 2014). This underscores the need to develop scientifically grounded approaches for evaluating the suitability of individual animals as donors. In this context, studies have already investigated the effects of genetic profile (Hirayama et al.,

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2019), antral follicle count (Zangirolamo et al., 2018), anti-Müllerian hormone (AMH) levels (Fushimi et al., 2020; Kovpak et al., 2024), and other indicators on the performance of superovulation programs. However, the effective implementation of advanced methodologies requires a comprehensive approach that integrates the analysis of hormonal status with the assessment of ovarian reserve characteristics. Therefore, the aim of this study was to determine the predictive value of AMH and estradiol (E2) levels as biomarkers for selecting donor cows for oocyte retrieval under field conditions.

The efficiency of both ovum pick-up (OPU) and multiple ovulation protocols is directly dependent on the number of antral follicles in the donor cow. However, the introduction of new donor selection protocols into practice requires, first and foremost, a clear understanding of the patterns of follicular development, as the follicle represents the fundamental functional unit of the ovary in which oocyte growth and maturation occur.

Folliculogenesis takes place within a highly dynamic ovarian environment. As a result of interactions among hormonal, paracrine, and autocrine factors, key processes occur, including cyclic follicular recruitment, neoangiogenesis, spatial redistribution, follicular atresia, and ovulation (Pakarainen et al., 2005; Fiorentino et al., 2023). It should be noted that approximately two-thirds of the initial population of germ cells in the fetus undergo degeneration before birth (Britt, 2008). Cattle are born with a finite number of follicles and oocytes in the ovaries, which varies considerably among individuals (ranging from 10,000 to 350,000) (Aerts & Bols, 2010). Nevertheless, this reserve is sufficient to support accelerated genetic progress and improve fertility in cattle populations (de Lima et al., 2020).

According to Fair (2003), a primordial follicle in cattle requires approximately 4–6 months to reach ovulation, and each stage of folliculogenesis is tightly regulated, beginning with the activation of primary follicles from a quiescent state. Follicular activation occurs gradually and in waves; therefore, during the estrous cycle, the ovary contains primary, secondary, and tertiary follicles simultaneously (Shimizu, 2016). Morphologically, activated primary follicles are identified by the transformation of granulosa cells from a flattened to a cuboidal shape. However, the mechanisms governing follicular activation and recruitment at any given cycle remain incompletely understood (Morton et al., 2023).

Following recruitment, follicles enter the growing pool; however, the majority do not complete maturation and instead undergo atresia. Fiorentino et al. (2023) report that gonadotropin receptors are not yet present in primary follicles, suggesting that their initial activation is likely independent of pituitary control and regulated by biomechanical, autocrine, and paracrine pathways. In contrast, Candelaria et al. (2020) demonstrated that follicle-stimulating hormone (FSH) is already expressed in growing preantral follicles at the primary stage in cattle and that these follicles respond to FSH by enhancing specific cellular functions and signaling pathways. Furthermore, data obtained by Cossigny et al. (2012) from *in vitro* culture of whole rat ovaries indicate that the addition of FSH to the culture medium reduces apoptosis within the pool of primary follicles compared to hormone-free conditions. Candelaria et al. (2020) also confirmed the proliferative effects of FSH in preantral follicles through the upregulation of genes involved in energy metabolism and MAPK (mitogen-activated protein kinase) signaling pathways. Meanwhile, Abel et al. (2000) emphasize that preantral follicles do not have an absolute requirement for this hormone, as evidenced by their normal growth up to the late secondary stage in FSH receptor knockout mice. Thus, although FSH is not essential for preantral follicle development, it may play a physiological role in the early stages of folliculogenesis (Oliynyk et al., 2005).

In cattle, the transition of follicles to gonadotropin dependence occurs at the stage of antral follicles measuring 1–3 mm, after which their further growth and maturation depend on adequate support from circulating follicle-stimulating hormone (FSH) (Jaiswal et al., 2004; Clark et al., 2022). Therefore, the development of antral follicles is of particular interest for biotechnological reproduction methods, as this stage can be effectively modulated. This process is typically divided into two phases. The first, “slow” growth phase ensures final oocyte development and lasts more than 30 days, from the formation of an antral follicle measuring approximately 0.3 mm to the “small” follicle stage of 3–5 mm in diameter. The second, “rapid” growth phase involves the recruitment of small follicles ( $\geq 3$  mm), selection of the dominant follicle, and its subsequent growth; this stage lasts only 5–7 days (Mihm et al., 2002) and is entirely dependent on elevated FSH concentrations and appropriate luteinizing hormone (LH) pulsatility (Clark et al., 2022).

Given that antral follicles contain a fluid-filled cavity detectable by ultrasonography, it has been established that they grow in an organized pattern known as follicular waves (Rajamahendran & Taylor, 1990; Motta et al., 2025), which are already evident in calves as early as 2 weeks of age (Krause et al., 2022). Cattle are a mono-ovulatory species in which two (approximately 65% frequency) or three major follicular waves develop during the interovulatory interval (Ginther, 2018). Moreover, Jaiswal et al. (2009) reported a high repeatability of the wave pattern within individuals. The emergence of a follicular wave in cattle is characterized by the rapid (within 2–3 days) synchronous growth of a cohort of small follicles (Ginther et al., 1989). Evidence indicates that the peak number of antral follicles  $\leq 3$  mm per wave is highly variable among individuals (range 8–54; mean 24) (Rajamahendran & Taylor, 1990; Mossa & Evans, 2023), but shows high repeatability within the same animal across consecutive estrous cycles (Burns et al., 2005). Notably, both ovaries function largely as a single unit, meaning that each follicular wave includes follicles from both ovaries responding in a coordinated manner (Adams et al., 2008). Although the processes controlling antral folliculogenesis have been extensively studied, significant knowledge gaps remain, particularly for domestic species such as cattle, as most detailed investigations have been conducted in rodents (Morton et al., 2023).

Morphological changes in the ovary correlate with wave-like fluctuations in plasma FSH concentrations, as follicles measuring 1–3 mm actively respond to increases in this hormone (Jaiswal et al., 2004). Each major follicular wave is preceded by a transient rise in FSH, which begins 2–4 days before ultrasonographic detection of the wave (follicles of 4–5 mm), reaches its peak within 1–2 days, and declines as follicles diverge into a dominant follicle and subordinate follicles (6–7 mm) (Adams et al., 1992). The growth rates of follicles within a wave are similar for approximately 4 days, after which one follicle is selected to continue development (the dominant follicle), while the others (subordinate follicles) undergo atresia and regression (Jaiswal et al., 2009). Once the largest follicle reaches a critical diameter of  $\geq 8.5$  mm, FSH concentrations decline below the threshold

required to sustain smaller follicles, yet remain sufficient to support the dominant follicle (Adams et al., 1993; Ginther et al., 1999).

The largest follicle becomes dominant and, following selection, continues to grow from approximately 9 to 15 mm in diameter over a period of about 4 days (Price & Estienne, 2018). During this growth phase, the dominant follicle is characterized by high estrogen production. Follicular enlargement is driven by granulosa cell proliferation, initially under the influence of FSH, and subsequently maintained by LH and insulin-like growth factor 1 (IGF-1) as plasma FSH concentrations decline (Shimizu, 2016). As the follicle approaches the end of the growth phase, intrafollicular estradiol concentrations decrease (Price et al., 1995). The dominant follicle then enters a static phase lasting 4–5 days, during which minimal further growth occurs (Price & Estienne, 2018). Each follicular wave produces a dominant follicle exceeding 10 mm in diameter. In a two-wave interovulatory interval, the first wave is anovulatory and the second is ovulatory; in a three-wave pattern, the first and second waves are anovulatory, while the third is ovulatory (Ginther et al., 2013). Early static dominant follicles remain estrogen-active, and approximately half are morphologically healthy based on light microscopy (Price et al., 1995; Clark et al., 2004), often responding to induced luteolysis by ovulation (Ali et al., 2001). In contrast, late static dominant follicles are estrogen-inactive, do not ovulate following luteolysis, and instead undergo atresia (Ali et al., 2001; Price & Estienne, 2018). Because the dominant follicle is functionally as well as morphologically dominant, its ablation—depending on timing—can initiate a new wave of follicular recruitment or rescue a subordinate follicle from regression (Ko et al., 1991; Ginther et al., 1999; Siddiqui et al., 2015).

Despite extensive and well-structured research on the bovine estrous cycle, the limited practical implementation of these findings necessitates further investigation. Accordingly, the aim of this study was to determine the predictive value of estradiol and anti-Müllerian hormone as biomarkers of the follicular pool status in cattle, with the ultimate goal of optimizing biotechnological reproduction methods.

## MATERIALS AND METHODS

### Study period and experimental site

The study was conducted from March to July 2025. The experimental base was the farm of the Educational and Production Center of the National University of Life and Environmental Sciences of Ukraine “Agronomical Research Station” (Pshenychna village, Bila Tserkva district, Kyiv region, Ukraine).

### Experimental animals and estrous cycle stage selection

The experiment was performed on 30 Ukrainian Black-and-White dairy cows in their first or second lactation during days 3–10 of the estrous cycle. The selection of this period was based on the findings of Marion & Gier (1971), who reported an increased number of follicles larger than 8 mm in diameter in bovine ovaries during this stage compared with other phases of the cycle.

### Ethical approval and regulatory compliance

Ethical and legal requirements for animal use were fully complied with in accordance with the Law of Ukraine “On Protection of Animals from Cruel Treatment” (2006, Article 230), the General Ethical Principles of Animal Experiments, and the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986).

### Animal housing and welfare conditions

Animal housing conditions complied with the requirements of the Order of the Ministry for Development of Economy, Trade and Agriculture of Ukraine “On Approval of Requirements for the Welfare of Farm Animals During Their Keeping” (No. 224, 2021). Ethical approval for the use of experimental animals was obtained from the Bioethics Commission of the National University of Life and Environmental Sciences of Ukraine prior to the study (application No. 031 dated 17.03.2025).

### Veterinary monitoring and animal welfare

Daily veterinary monitoring of the clinical condition of the experimental animals was carried out throughout the study period. In case of any pathological signs, cows received immediate qualified veterinary assistance to prevent pain and suffering. All procedures were performed according to humane principles, minimizing stress and pain and ensuring animal welfare.

### Determination of anti-Müllerian hormone and estradiol concentrations in bovine serum

Blood samples were collected daily from the coccygeal vein of cows with simultaneous recording of the estrous cycle day. A total of 4 cm<sup>3</sup> of blood was collected after disinfection of the puncture site using sterile vacuum tubes “Vacutainer PREMIUM” with silica activator (Greiner Bio-One, Austria). Samples were transported to the laboratory at +4 °C and analyzed within 2 hours after collection, maintaining the cold chain. Serum samples were processed in the Medical Laboratory of the Diagnostic Center “CSL LAB” LLC (Kyiv, Ukraine, 45 Vasylkivska Street), where hormone concentrations were determined using a chemiluminescent immunoassay (CLIA).

Based on serum anti-Müllerian hormone (AMH) concentrations, animals were divided into three groups:

Group 1 (low level): < 0.1 ng/cm<sup>3</sup>

Group 2 (medium level): 0.1–0.3 ng/cm<sup>3</sup>

Group 3 (high level): > 0.3 ng/cm<sup>3</sup>

A similar classification was applied according to estradiol concentrations:

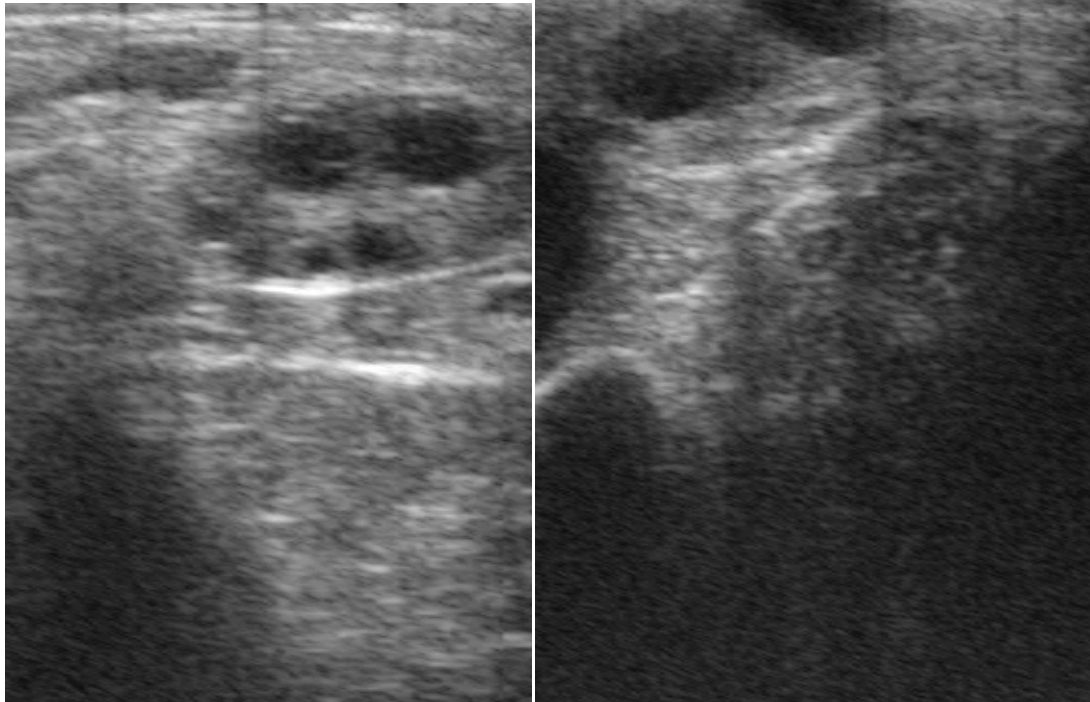
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Group 1 (low level): < 15 pg/cm<sup>3</sup> Group 2 (medium level): 15–25 pg/cm<sup>3</sup>

Group 3 (high level): > 25 pg/cm<sup>3</sup>

#### Antral follicle count (AFC) assessment

The number of antral follicles (AFC) in donor cows was determined using clinical examination and transrectal ultrasonography. Ovarian structures were evaluated in B-mode using an ultrasound scanner (Kaixin KX5200, China). Follicles with a diameter of 2–10 mm were counted in both ovaries (Figure 1). The examination was performed by systematically adjusting the position of the probe to ensure complete visualization of ovarian structures. Animals with non-ovulatory follicles or ovarian cysts were excluded from the analysis.



**Figure 1-** Ultrasound image of bovine ovaries on day 5 of the estrous cycle

Note: a — antral follicles (AFC); b — ovarian tissue.

#### Statistical analysis

The results are presented as mean  $\pm$  standard deviation ( $\bar{x} \pm SD$ ). Differences were considered statistically significant at  $p < 0.05$ . One-way analysis of variance (ANOVA) was used to compare values between groups, followed by Tukey's honestly significant difference (HSD) post hoc test for multiple comparisons.

## RESULTS AND DISCUSSION

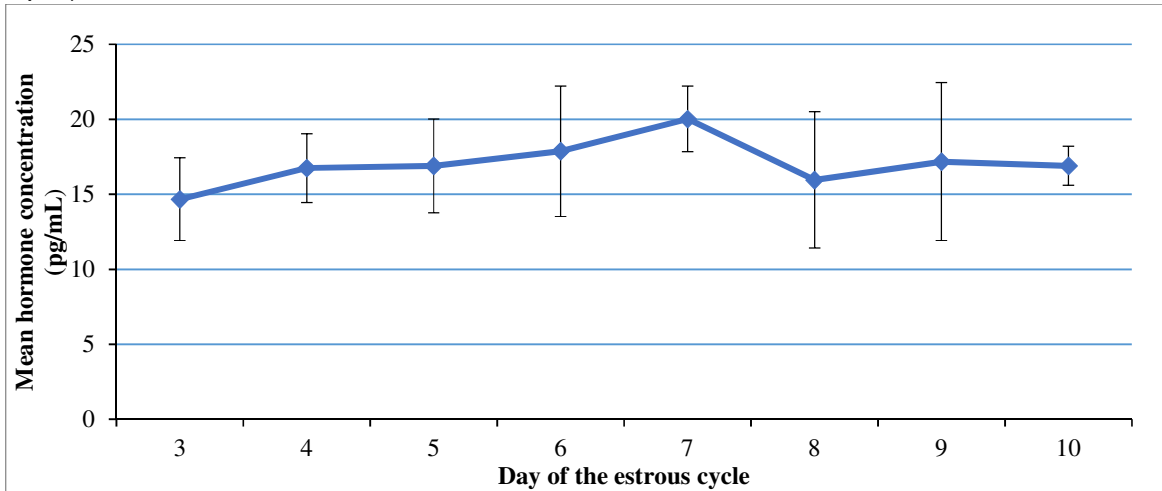
For successful oocyte formation in the ovaries, normal follicular development is critically important. Hormones play a key role in this process by regulating various aspects of folliculogenesis, including granulosa cell growth, follicular fluid formation, cell proliferation, apoptosis, angiogenesis, and other related processes (Chou & Chen, 2018). In this context, the present study aimed to analyze changes in hormone levels from days 3 to 10 of the bovine estrous cycle and to determine how their concentrations influence the number of antral follicles.

One of the most important hormones in the female reproductive system is estrogen, a steroid hormone that regulates fertility, particularly through the control of folliculogenesis. In animals, three forms of estrogen are produced: estradiol-17 $\beta$  (E2), estrone (E1), and estriol (E3). Among them, estradiol (E2) is the most physiologically active and predominant circulating estrogen in reproductive-age females and is synthesized in ovarian granulosa and theca cells (Çiftci, 2013; Mauvais-Jarvis & Lindsey, 2024). At the stage of antral cavity formation, granulosa cells of early antral follicles secrete E2, which is essential for oocyte growth and maturation in cattle (Endo et al., 2013). Its role in follicular development has also been confirmed in hamsters (Wang & Roy, 2007) and baboons (Zachos et al., 2002).

However, both excessive and insufficient estrogen levels may have adverse effects. Elevated concentrations of estrogen or estrogen-like compounds can disrupt oogenesis and lead to the formation of multi-oocyte follicles (MOFs) in mice (Chen et al., 2009). Conversely, low estradiol levels may impair the maturation of developing follicles and promote the accumulation of small antral follicles (Yu et al., 2019). Estradiol also contributes to the initial formation and activation of primary follicles and regulates oocyte size (Britt et al., 2004). Its concentration is considered a key factor in follicular maturation and an important marker of oocyte quality (Chiu et al., 2002).

Sakaguchi et al. (2017) demonstrated that E2 production is higher in oocyte-cumulus-granulosa complexes (OCGCs) that yield mature oocytes after in vitro maturation (IVM) compared with OCGCs that produce immature oocytes. Furthermore, Sakaguchi et al. (2018) reported higher E2 production by granulosa cells and greater oocyte developmental competence in ovaries with a high antral follicle count compared with those with a low count. Collectively, these findings indicate that estradiol production is not only an indicator of follicular health but also a predictive marker of oocyte quality and developmental competence. At the same time, its most well-documented role is the selection of dominant preovulatory follicles and regulation of their subsequent development (Chauvin et al., 2022).

The dynamics of estradiol (E2) production play a crucial role in bovine reproductive cycles. Therefore, to further investigate changes in estradiol concentration during the estrous cycle in cattle, serum levels were monitored throughout the study period (Graph 1).



Graph - 1 Mean serum estradiol concentrations in cattle at different days of the estrous cycle ( $\bar{x} \pm SD$ )

A general increasing trend in E2 levels was observed from the initial values on day 3 ( $14.67 \pm 4.95$  pg/mL) to a peak value on day 7 ( $20.03 \pm 2.19$  pg/mL). Notably, the lowest standard deviation was recorded on day 7, indicating low variability among animals during this period. It should be noted that both Smith et al. (1975) and Ahmad et al. (1996) reported a similar estradiol peak during the luteal phase, well before the onset of estrus (days 6–8 of the cycle). These results can be explained by the fact that the dominant follicle of the first follicular wave reaches its maximum development and secretory activity during this period, after which it undergoes atresia. Following the peak on day 7, a gradual decrease in E2 concentration was recorded, reaching  $16.90 \pm 1.29$  pg/mL on day 10. Despite observed fluctuations in mean hormone levels during the study period, no statistically significant differences were found between the examined days of the estrous cycle.

The absence of statistically significant differences in estradiol dynamics from day 3 to day 10 may be explained by the physiological characteristics of the bovine estrous cycle (Figure 2). In cows exhibiting two or three follicular waves per cycle, a pronounced preovulatory estradiol peak typically occurs only at the end of the growth phase of the dominant follicle, immediately prior to ovulation (Mekonnin et al., 2017). The period from day 3 to day 10 encompasses the growth phases of the first and second follicular waves. During this interval, estradiol levels, although fluctuating, remain within the physiological range. While variations in E2 concentrations were observed, they did not reach statistical significance compared with preovulatory peak levels, which exceed 40 pg/mL according to Setyorini et al. (2023). Nevertheless, the recorded E2 values were higher than basal levels in cattle (1–10 pg/mL) (Mekonnin et al., 2017), indicating active growth of antral follicles.

Therefore, determination of E2 levels during the early stages of a follicular wave may serve as an informative indicator for predicting the number of follicles within that wave.

In this context, the next stage of the study involved assessing the relationship between antral follicle count (AFC) and serum estradiol concentration in cattle. For this purpose, data were analyzed over the entire study period without stratification by specific cycle day (Table 1).

Estradiol concentration, pg/cm <sup>3</sup>	Number of observations per group	Mean antral follicle count, n
<15 (low level)	11	7.27±2.05 <sup>a</sup>
Between 15 and 25 (medium level)	12	10.67±2.67 <sup>b</sup>
> 25 (high level)	6	8.00±3.29 <sup>a</sup>

Notes: Different superscript letters indicate statistically significant differences between groups ( $p < 0.05$ ) according to Tukey's HSD post hoc test.

A statistically significant difference in AFC was observed among groups differing in E2 concentration. This indicates that estradiol is not a constant factor of the follicular pool; rather, it correlates with dynamic changes in antral follicle

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development. The highest mean AFC ( $10.67 \pm 2.67$ ) was recorded in the group of animals with medium E2 levels ( $15\text{--}25 \text{ pg/cm}^3$ ). This value was significantly higher ( $p < 0.05$ ) than in both the low ( $7.27 \pm 2.05$ ) and high ( $8.00 \pm 3.29$ ) E2 groups. No statistically significant differences in AFC were found between the low- and high-estradiol groups.

Thus, the highest AFC is associated with an intermediate range of estradiol concentration ( $15\text{--}25 \text{ pg/cm}^3$ ). This finding suggests the existence of an optimal physiological level of E2 that is most favorable for follicular growth and survival. Our results are consistent with Sakaguchi et al. (2019), who also reported higher estradiol concentrations in cows with a greater number of antral follicles compared with animals with a low AFC. The authors attributed this to reduced E2 production by granulosa cells in low-AFC cows, resulting in decreased systemic estradiol levels. This highlights a direct relationship between follicular functional activity and the systemic hormonal profile, supporting the concept that a certain estradiol threshold is critical for maintaining an optimal follicular pool.

The observed reduction in AFC at high estradiol concentrations has no direct analogues in the existing literature. Although this finding may appear contradictory, as increased follicular number is generally associated with higher estradiol production, it can be physiologically explained. In conditions of reduced ovarian reserve, remaining follicles may exhibit compensatory hyperfunction and secrete excessive amounts of E2 (Mutlu & Erdem, 2012; Chauvin et al., 2022). In addition, such a profile may reflect endocrine dysregulation affecting folliculogenesis or abnormal follicular development leading to premature atresia despite active steroidogenesis. It should also be noted that granulosa cells from atretic follicles may remain steroidogenically active (Henderson et al., 1987), which can contribute to elevated estradiol levels. A shortened follicular phase may represent another contributing factor (Chauvin et al., 2022). Furthermore, the negative feedback mechanism, whereby high estradiol levels suppress GnRH and subsequently FSH secretion, may lead to increased atresia of smaller follicles while allowing the dominant follicle to persist (Shaw et al., 2010). Additionally, persistently elevated E2 levels may paradoxically impair the recruitment of new follicles (McEvoy et al., 2022). This complex interaction likely explains the pronounced variability in AFC ( $8.00 \pm 3.29$ ) observed in the high-estradiol group, reflecting heterogeneity in reproductive dynamics within this subgroup.

Although estradiol (E2) measurement is not routinely used in cattle production, its role in monitoring follicular development is well established in human reproductive medicine. In controlled ovarian stimulation protocols, E2 monitoring is crucial. Poor ovarian responders typically exhibit low systemic estradiol levels, which correlate with a reduced number of retrieved oocytes and lower fertilization rates (Grin et al., 2020). In contrast, high estradiol concentrations are associated with ovarian hyperresponse and an increased risk of ovarian hyperstimulation syndrome (Corbett et al., 2014; Li et al., 2021). These clinical observations support the notion that estradiol levels reflect the number of actively developing follicles. Moreover, an excessive rise in E2 per retrieved oocyte may indicate an imbalance between stimulation intensity and ovarian response, serving as a prognostic indicator of reduced oocyte quality and fewer embryos available for cryopreservation. Kumari et al. (2021) demonstrated a relationship between AFC, serum estradiol levels, and the number of mature follicles in women undergoing IVF. Similarly, Melo et al. (2009) reported lower estradiol levels and fewer mature oocytes in donors with AFC below 10. These cross-species parallels highlight the universal regulatory role of estradiol in reproductive physiology and demonstrate the potential value of E2 monitoring in veterinary reproduction for assessing follicular dynamics and predicting reproductive outcomes.

The next stage of the study involved determining serum anti-Müllerian hormone (AMH) levels in cattle during days 3–10 of the estrous cycle.

AMH, also known as Müllerian inhibiting substance (MIS), is a glycoprotein hormone that selectively influences the reproductive system and plays a key role in regulating reproductive function (La Marca & Volpe, 2006; Hugon et al., 2010). In cattle, AMH expression is cell-specific, being restricted to granulosa cells of growing follicles (Rico et al., 2011). AMH plays a crucial role in the formation of the primordial follicle pool, as it modulates oocyte apoptosis during early folliculogenesis (Nilsson et al., 2011). At later stages, AMH produced by small antral follicles acts as a paracrine inhibitor, preventing excessive activation of primordial follicles, maintaining them in a quiescent state, and thereby regulating the rate of follicular recruitment (Durlinger et al., 2002; Gigli et al., 2005; Visser & Themmen, 2005). AMH also exerts autocrine effects by modulating follicular sensitivity to FSH (Zhou et al., 2022). In vitro and in vivo studies have confirmed that follicles become more sensitive to FSH in the absence of AMH (Durlinger et al., 2001), which is associated with reduced granulosa cell proliferation.

Although AMH is generally considered a stable biomarker of ovarian reserve independent of the estrous cycle phase, some studies suggest minor fluctuations in its concentration (Pfeiffer et al., 2014; Koca et al., 2023). Rico et al. (2011) reported a dynamic AMH profile during the estrous cycle that is independent of follicular waves. Therefore, to confirm its stability in our experimental population, serum AMH levels were monitored from day 3 to day 10 of the estrous cycle.

The results demonstrated low intra-individual variability in AMH concentrations, while considerable inter-individual variation was observed. Importantly, these differences were independent of the estrous cycle day.

Based on the observed stability of AMH, the next stage of the study focused on evaluating the relationship between AMH concentration and antral follicle count (AFC). For this purpose, data were analyzed over the entire observation period without stratification by specific cycle day.

**Table 2. Relationship between anti-Müllerian hormone concentration and antral follicle count in cattle ( $\bar{x} \pm SD$ )**

Anti-Müllerian hormone concentration, ng/cm <sup>3</sup>	Number of observations per group	Mean antral follicle count, n
< 0.1 (low level)	4	3.75±0.50 <sup>a</sup>
Between 0.1 ≥ and 0.3 (medium level)	5	7.20±1.48 <sup>b</sup>
> 0.3 (high level)	7	11.14±2.54 <sup>c</sup>

Notes: Different superscript letters indicate statistically significant differences between groups ( $p < 0.05$ ) according to Tukey's HSD post hoc test.

Table 2 demonstrates a direct and statistically significant relationship between serum anti-Müllerian hormone (AMH) concentration and mean antral follicle count (AFC) in cattle. This correlation indicates that AMH is not a random indicator but is closely associated with changes in follicular pool dynamics. The analysis revealed that the highest mean AFC ( $11.14 \pm 2.41$ ) was observed in animals with high AMH levels ( $> 0.3 \text{ ng/cm}^3$ ). This value was significantly higher ( $p < 0.05$ ) compared with both the low ( $3.75 \pm 0.50$ ) and medium ( $7.20 \pm 1.48$ ) AMH groups. Importantly, statistically significant differences in AFC were detected among all three groups (low, medium, and high hormone concentrations). These results emphasize the high informative value of AMH as an indicator of the follicular pool in cattle.

Our findings are consistent with previous studies and confirm that AMH concentration is a reliable predictor of the efficiency of reproductive biotechnologies. Rico et al. (2009) reported that plasma AMH levels are positively correlated with the number of small antral follicles and superovulation efficiency. This conclusion is supported by other studies demonstrating a direct relationship between high AMH concentrations and improved outcomes in donor cow superovulation programs. In particular, Monniaux et al. (2010) and Kovpak et al. (2024) reported a positive correlation between AMH levels and the number of high-quality embryos obtained. Hirayama et al. (2017) and Koca et al. (2024) showed that cows with higher AMH concentrations yield significantly more oocytes and embryos. Meanwhile, Mossa & Ireland (2019) explained the physiological mechanism underlying this relationship: low AMH levels reduce follicular cell sensitivity to follicle-stimulating hormone (FSH), thereby resulting in a diminished ovarian response to stimulation.

Thus, the present study confirms that AMH is a reliable and stable biomarker for assessing ovarian reserve in cattle. A direct and statistically significant correlation between AMH levels and AFC was established.

## CONCLUSION

The present study demonstrated that a combined assessment of estradiol and anti-Müllerian hormone (AMH) levels is appropriate for predicting the efficiency of reproductive biotechnologies based on evaluation of the follicular pool. AMH is a stable biomarker, and its high concentration is directly correlated with a greater number of antral follicles. This makes it a reliable indicator for rapid and accurate assessment of the overall ovarian reserve in cattle. Estradiol, although not a reliable long-term marker, serves as an important indicator of current follicular dynamics. Its concentration above basal levels during days 3 to 10 of the estrous cycle reflects active follicular growth at the time of sampling, which is critical for justified selection of donor cows. Thus, the combined use of AMH and estradiol measurements provides a comprehensive assessment of the reproductive status of animals, enabling more efficient donor selection and optimization of hormonal stimulation protocols.

## DECLARATIONS

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### Authors' contribution

Maksym Salizhenko, Yurii Zhuk, Vitalii Kovpak contribute to the research, data analysis, and manuscript writing.

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### Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request

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### Conflict of interests

The authors have not declared any conflict of interests.

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