



Estrus Signs and Estrogen Levels in Brahman Cross and First-Generation (F1) Crossbred Cattle: Implications for Pregnancy and Repeat Mating

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Abstract | Brahman Cross cattle are grown extensively in Indonesia due to their outperformance compared to local cattle in reproduction and daily weight increase. Although it may result in reproductive problems, crossbreeding with *Bos taurus* breeds, such as Belgian Blue cattle, increases growth potential. In Brahman Cross and first-generation F1 Crossbred cattle, estrus signs and estrogen levels are recommended to be examined during artificial insemination (AI) and 30 days after AI. Twenty blood samples consisting of ten samples of F1 crossbred cattle with Belgian Blue bulls and Brahman Cross dams, and ten samples of Brahman Cross cattle grown at PT. Widodo Makmur Perkasa were taken. Estrogen levels and visual indicators of estrus were tracked both during and 30 days after AI. Both Brahman Cross and F1 Crossbred cattle showed strong estrus signals during AI, with estrus signs of 125.50 ± 5.64 for Brahman Cross and 128.30 ± 5.41 for F1 Crossbred, decreasing to 120.80 ± 6.05 after 30 days. Estrogen levels were 463.60 ± 76.98 for Brahman Cross and 475.94 ± 38.92 for F1 Crossbred during AI, dropping to 276.44 ± 119.04 and 326.64 ± 147.42 , respectively, after 30 days. After AI, F1 Crossbred cattle in particular exhibit the lowest levels of estrogen, which is correlated with conception. On the other hand, non-pregnant Brahman Cross cattle exhibit lower levels of estrogen and estrus, probably as a result of repeated mating. The significance of keeping an eye on estrogen levels and estrus signs in cattle breeding programs is highlighted in these findings.

Keywords | Brahman cross, Estrogen levels, Estrus signs, F1 Crossbreeding, Pregnancy, Repeat mating

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In Indonesia, a tropical country, Brahman Cross cattle are widely bred because Brahman Cross (*Bos indicus*) shows better reproductive conditions and daily weight gain than local cattle (Sutarno and Setyawan, 2015). Crossbreeding between Brahman and *Bos taurus*, such as Belgian Blue which has a large body size and grows at a moderate to fast rate (Panjono *et al.*, 2022), is conducted to increase their weight (Sigala *et al.*, 2022). *Bos taurus* is known for its high fertility and short gestation period, contrasting with *Bos indicus* which tends to have lower fertility and a longer gestation period (Leite *et al.*, 2021). However, *Bos indicus* is renowned for its docile estrus behavior, making it difficult to determine when cows are in estrus (Cooke *et al.*, 2020).

Reproductive efficiency is a crucial determinant of profitability (Budisatria *et al.*, 2021). Accurate monitoring of estrogen levels and successful estrus diagnosis are essential components of artificial insemination (AI) (Hubner *et al.*, 2022). Cows in estrus can be visually identified by their enlarged vulva (Xu *et al.*, 2023) and standing to be mounted (Endo, 2022). Other behavioral indications include vulvar mucus secretion, vulvar redness (Atmoko *et al.*, 2020), sniffing and chin resting (Dobson *et al.*, 2017a), and groaning and restlessness (Kaas *et al.*, 2023). Reproductive hormones like estrogen, which influence estrus behavior, are responsible for these estrus signs (Reith and Hoy, 2018). Cows typically go through a 21-day estrus cycle, with an average estrus duration of 15 hours (Morrel, 2011). Low levels of estrogen in cattle are functionally recognized as a sign of pregnancy, as estrogen plays a crucial role in regulating reproductive processes, indicating that the cow is pregnant (Molefe and Mwanza, 2020). At 30 days post-artificial insemination (AI), signs of estrus and estrogen levels can provide crucial information regarding the pregnancy status and reproductive health of cattle. After artificial insemination, estrogen levels typically decrease if pregnancy is successful, as this hormone is involved in maintaining the estrus cycle and is not needed to support pregnancy (Yamamoto *et al.*, 2018). A decrease in estrogen levels at 30 days post-AI often indicates that pregnancy may have occurred, as the hormone levels are no longer high enough to trigger estrus signs. Conversely, estrogen levels remain high and estrus signs such as an enlarged vulva, vulvar mucus secretion, and standing to be mounted are still observed. In that case, it may suggest that the cow is not pregnant and could be experiencing failure of the AI process or other reproductive issues (Madureira *et al.*, 2019).

Rehash breeding could be a major issue in dairy bovines. Rehash breeders are bovines without any anatomical or irresistible anomalies that don't ended up pregnant after three or more breeding endeavors or various manufactured inseminations (AI) (Tanimura *et al.*, 2022). The estrus cycle

in cows is largely regulated by hormones (Liu *et al.*, 2023). Therefore, it is important to note that this study does not use synchronization protocols, and new regulations have been implemented prohibiting the use of hormones for this purpose in the future (Bo and Mechaca, 2023). Thus, this study aims to understand estrus signs and estrogen levels in Brahman Cross and F1 crossbred cattle both during artificial insemination and 30 days post artificial insemination. The uniqueness of this study lies in determining estrus signs and estrogen levels as indicators of pregnancy or repeat mating consequences.

MATERIALS AND METHODS

ETHICAL COMMITTEE

All strategies including creatures in this ponder were authorized by the Animal Ethics Commission of the Faculty of Veterinary Medication at Universitas Gadjah Mada in Yogyakarta, Indonesia, under ethical number 012/EC-FKH/Eks/2023, the permission was given.

MATERIALS

A total of 20 blood samples were taken, comprising samples from first-generation (F1) crossbred Belgian Blue bulls and Brahman Cross cows and samples from Brahman Cross heifers. Ten Brahman Cross samples and ten F1 Crossbred samples were present, and they were all reared at PT. Widodo Makmur Perkasa. A 2x2 tren design was used for statistical analysis to test the effects of cattle breeds, specifically Brahman Cross and F1 Crossbred, as independent variables, and estrus phase on estrus hormone levels and signs of estrus before and after artificial insemination within each breed as dependent variables.

METHODS

ESTRUS DETECTION BEFORE TO ARTIFICIAL INSEMINATION:

Estrus detection is performed before artificial insemination. If the cow exhibits multiple signs of estrus, artificial insemination is then carried out. As stated in the study by Gatius (2022), scientists observe cows to identify estrus, or the heat phase, at particular times: 6:00 AM, 9:00 AM, 12:00 PM, 4:00 PM, and 9:00 PM. The criteria set by Eerdenburg *et al.* (1996) are used to evaluate estrus signs. These criteria consider several factors, including behavior, physical activity, and particular physical traits that indicate the estrus condition in female cows. Table 1 presents these assessment criteria specifics.

ESTROGEN HORMONE OBSERVATION DURING ARTIFICIAL INSEMINATION:

Blood was drawn from the cow's coccygeal vein, which had been cleansed by wiping the region with 70% alcohol. Afterward, a venoject fitted with a venoject holder was inserted into the coccygeal vein to draw blood, about 3 ml per head. The blood then flowed out from the needle. After the suction, the blood was put into sterile tubes

treated with anticoagulants ethylenediaminetetraacetic acid (EDTA). The samples were thoroughly mixed with EDTA to guarantee that all of the blood was mixed, labeled or coded, and then centrifuged (3000xg) for 5 minutes to extract the plasma. The plasma was then refrigerated at -4°C for additional analysis (Cadamuro *et al.*, 2017).

Table 1: Criteria and scores for estrus behavior by van Eerdenburg *et al.*, (1996).

No.	Signs	Score
1.	Vulva wet, swollen, and red, (this sign indicates physical changes in the cow's vulva, which are associated with estrus. It reflects hormonal changes preparing the cow for mating).	3
2.	Groaning, (groaning is a vocalization that can be associated with discomfort or signaling readiness for mating. It suggests physiological changes linked to estrus).	3
3.	Restlessness, (restlessness refers to the cow's agitation or inability to settle down, often pacing or moving about more frequently. It's a behavioral indicator of estrus).	5
4.	Sniffing, (sniffing involves cows investigating or smelling the genital area of other cows, which increases during estrus due to hormonal changes and the release of pheromones).	10
5.	Chin resting, (chin resting occurs when a cow places its chin on another cow's back or flank, which is more frequent during estrus as a form of mounting behavior).	15
6.	Standing heat, (Standing heat is when a cow stands still and allows mounting by other cows or attempts to mount other cows itself. It's the most reliable indicator of estrus).	100

ARTIFICIAL INSEMINATION

To prepare the frozen semen insemination, 37°C water was used for 15 seconds to thaw frozen semen. The frozen semen should be thawed before the sperm could be inserted into the female cow's reproductive system using an apparatus named an insemination gun . In this study, artificial insemination was performed at 6 a.m. According to Gatius (2022), AI is typically conducted 12-24 hours after the onset of standing estrus (when the cow is receptive to being mounted) to enhance the chances of ovum fertilization. This study does not use synchronization protocols, and new regulations have been implemented prohibiting the use of hormones for this purpose in the future (Bo and Mechaca, 2023).

ESTRUS DETECTION AT 30 DAYS POST ARTIFICIAL INSEMINATION: A comprehensive visual examination was conducted 30 days after artificial insemination (AI), the cattle had a comprehensive visual examination to look for signs of estrus . Observing 30 days after artificial

insemination (AI) is crucial because it aligns with the time needed to confirm pregnancy status. By this period, if pregnancy has occurred, estrogen levels typically decrease, which helps to verify the success of AI. Checking one or two estrus cycles (approximately 42-60 days) could delay the detection of pregnancy or reproductive issues and is less efficient for confirming early pregnancy outcomes (Szenci, 2021). The surveillance covered a variety of markers akin to those previously mentioned. This entailed, among other things, closely examining behaviors including mounting, restlessness, increased vocalization, and vulvar enlargement.

ESTROGEN HORMONE OBSERVATION AT 30 DAYS POST ARTIFICIAL INSEMINATION: Subsequently 30 days following AI, blood samples were taken from the cow. The process for separating plasma and storing samples was the same as that aforementioned. The levels of the hormone estrogen were measured in the laboratory using the plasma samples.

RECTAL PALPATION

Pregnancy was identified on the 60th day following artificial insemination by placing the hand inside the rectum and feeling the uterine and rectal walls to find the fetus. Rectal palpation in this study was performed by a trained veterinary technician with specialized skills. The procedure begins with the technician carefully inserting a gloved hand into the cow's rectum, usually conducted in a calm environment to minimize stress on the animal. The technician then palpates reproductive organs such as the uterus and ovaries (Bond *et al.*, 2019). In pregnant cows, the uterus will feel enlarged and heavier compared to the uterus in non-pregnant cows. The technician will look for the fetal sac, which may be felt as a small lump within the uterus. Additionally, pregnancy detection involves examining changes in the ovaries, such as the presence of the corpus luteum, a structure formed after ovulation that indicates pregnancy. Other features examined include a decrease in uterine consistency and changes in tissue texture (Romano *et al.*, 2020).

ESTROGEN LEVEL CHECK

The collected serum underwent an examination using the enzyme-linked immunosorbent assay (ELISA) method (Aydin, 2015), with the Fine Test ELISA Kit (Wuhan, China). The ELISA kit, reagents, and samples were prepared at room temperature. Standard arrangements were arranged employing a standard dissolvable with concentrations of 1000; 500; 250; 125; 62.5; 31.25; 15.625; and pg/ml. The plate was washed twice with 1x wash buffer, and then standards, blanks, and undiluted 50 µl samples were added to the plate. A 100x diluted biotin-labeled antibody solution was added at 50 µl to all wells. The plate was fixed with a plastic sealer and brooded for 45 minutes at 37°C. After hatching, the plate was washed three times with 1x wash buffer, enduring 1-2 minutes. Following, 100 µl of SABC working arrangement was included to all wells and

brooded for 30 minutes at 37°C. The plate was washed 5 times with 1x wash buffer, enduring 1-2 minutes. Substrate arrangement (90 µl) was included to all wells and brooded for 10-20 minutes at 37°C within the dim. A short time later, 50 µl of halt arrangement was included to all wells, changing color from blue to yellow/orange. Optical Thickness (OD) readings were taken employing a Microplate Peruser at a wavelength of 450 nm.

STATISTICAL ANALYSIS

An independent t-test was utilized to compare differences between breeds, as this test evaluates the means of two independent groups. A paired t-test was employed to observe changes in the estrus phase within each breed, suitable for comparing measurements taken at different times within the same group. Every data point is displayed as mean ± SD.

RESULTS AND DISCUSSION

Based on the findings of this study, Brahman Cross and F1 Crossbred cattle both exhibit strong visual estrus markers during artificial insemination (AI), with Brahman Cross cattle displaying the strongest indicators. However, after 30 days post artificial insemination (AI), estrus signals in these cattle typically diminish and, in some cases, completely disappear. This suggests the presence of pregnancy and the absence of estrus. Although estrogen levels decreased significantly in both breeds, all Brahman Cross cattle were not pregnant, while two F1 Crossbred cattle were pregnant. This indicates that while decreased estrogen levels are associated with reduced estrus activity, other factors beyond estrogen levels might influence pregnancy success (Table 3). Specifically, the average dropped from 123.30 to 121.30 for Brahman Cross, and from 128.50 to 121.60 for F1 Belgian Blue (Figure 1). Signs of estrus that have been noticed include enlarged, red, and mucus-producing vulva, as well as groaning, restlessness, sniffing, resting the chin, and standing heat. This supports Reith et al. (2014) findings that cows, both Simmental and Frisian Holstein, become restless and more active by an average of 38.7% during estrus. There is a typical pattern of restlessness in cows during specific times during the estrus cycle, as evidenced by the fact that cows in estrus tend to be more restless between 02:00 and 08:00 (Gaillard et al., 2016). Between 2.3 and 6 times during their estrus cycle, Holstein cows become restless (Silper et al., 2015).

Estrus signs in cattle during estrus have an average score of 125.90 with a standard deviation of ±1.09. Meanwhile, during the phase 30 days after AI, the estrus signs score decreases to 121.45. Statistical analysis shows a significant difference between the estrus phase and 30 days after AI in both types of cattle ($P < 0.05$) (Table 2). Dobson et al. (2018) also report research on estrus indicators and find that standing heat lasts 4.68 ± 1.49 hours, ranging from

0.25 to 18.25 hours. Smelling at another cow's vulva takes an average of 5.5 ± 1.3 hours (range = 0.25–18.25 hours) to occur. According to research by Yizengaw (2017), some animals in estrus will have clear mucus secreted from the vagina, swelling, and redness of the vulva, and cows in estrus may also exhibit vocalization behavior during long-distance travel to find bulls, possibly mounting or being mounted by other animals. Taurine cows exhibit licking and rubbing behaviors with one another, while zebu cows rest their chins (Layek et al., 2011). Cheong and Gilbert (2014), also examined estrus, particularly vulva swelling, and discovered that during estrus, vulva swelling increased from a width of 30 cm at registration to 40 cm just before calving. When pressure is applied, the swelling depresses and feels cold to the touch.

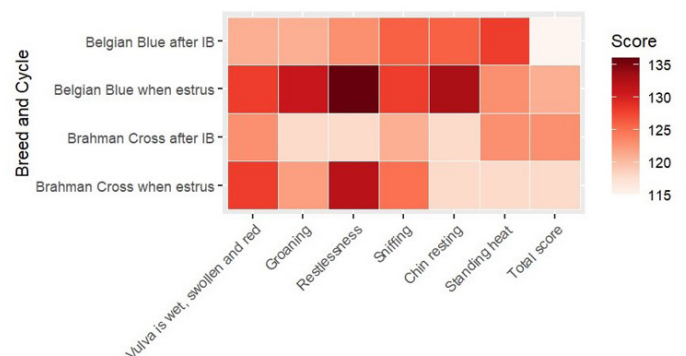


Figure 1: the sign of estrus during estrus and 30 days post artificial insemination in brahman cross and F1 crossbred cattle.

Table 2: The significance in signs estrus between the two types of cattle, both during estrus and 30 days after artificial insemination (AI) (mean±SD).

Phase	Brahman Cross	F1 Crossbred
Estrus	125.50 ^b ±5.64	128.30 ^b ±5.41
30 days after AI	120.80 ^a ±6.05	120.80 ^a ±6.05

^{ab}Different superscripts on the same column indicate significant differences ($P < 0.05$). No significant differences were found among values within the same row.

The study indisputably demonstrates that artificial insemination raises estrogen levels; the highest levels were observed in cows that were Brahman Cross (581.641 pg/ml) and F1 Crossbred (573.191 pg/ml). However, the estrogen levels of both types of cattle decreased 30 days after artificial insemination; the lowest levels were observed in Brahman Cross cattle at 173.880 pg/ml and in F1 Crossbred cattle at 132.082 pg/ml. The lower estrogen levels in these cattle indicate that they are either not in estrus or pregnant (Table 3). A common time to confirm pregnancy in cattle is 30 days after artificial insemination (AI), as this period allows for reliable detection of pregnancy through hormone testing or physical exams such as rectal palpation. At this stage, a drop in estrogen levels typically signifies that estrus

Table 3: The estrogen levels during estrus and 30 days post artificial insemination in Brahman Cross and F1 Crossbred (Belgian Blue) cattle.

No	Breed	Artificial Insemination Concentration pg/ml	30 Days Post Artificial Insemination Concentration pg/ml	Rectal Palpation	Number of Artificial Insemination
1.	BX (1)	511.110	173.880	Not Pregnant	3
2.	BX (2)	405.402	168.893	Not Pregnant	3
3.	BX (3)	452.430	405.402	Not Pregnant	3
4.	BX (4)	386.100	156.000	Not Pregnant	3
5.	BX (5)	547.235	168.893	Not Pregnant	3
6.	BX (6)	384.222	330.300	Not Pregnant	3
7.	BX (7)	581.641	422.564	Not Pregnant	3
8.	BX (8)	435.115	435.115	Not Pregnant	3
9.	BX (9)	384.222	173.910	Not Pregnant	3
10.	BX (10)	548.571	329.495	Not Pregnant	3
11.	F1 (1)	465.121	395.115	Not Pregnant	2
12.	F1 (2)	459.100	367.718	Not Pregnant	2
13.	F1 (3)	460.221	329.495	Not Pregnant	3
14.	F1 (4)	465.888	165.237	Not Pregnant	3
15.	F1 (5)	509.865	454.643	Not Pregnant	3
16.	F1 (6)	435.115	553.949	Not Pregnant	3
17.	F1 (7)	573.191	146.821	Pregnant	2
18.	F1 (8)	460.221	254.430	Not Pregnant	3
19.	F1 (9)	473.890	467.006	Not Pregnant	3
20.	F1 (10)	456.866	132.082	Pregnant	2

BX: Brahman Cross; **F1:** F1 Crossbred.

has concluded and that pregnancy needs to be verified. Estrogen levels are generally elevated during estrus, but if the female is not pregnant, these levels will decrease once estrus ends (Scully *et al.*, 2014). Consequently, assessing estrogen levels 30 days post-AI can help detect changes that align with pregnancy status. Estrogen levels remain high, and estrus signs such as an enlarged vulva, vulvar mucus secretion, and standing to be mounted are still observed. This could indicate that the cow is not pregnant and may be experiencing failure of the AI process or other reproductive issues (Hayati *et al.*, 2021). This is consistent with the findings of Schubach *et al.* (2017) study, which found that rising levels of circulating estrogen hormone trigger the hypothalamic initiation process, therefore initiating estrus expression in Holstein heifers. According to other research findings, estrus was associated with greater estrogen levels in crossbred cattle when compared to other reproductive stages. Estradiol levels increased significantly ($P<0.05$) in estrus cows relative to heifers (Mekonnin *et al.*, 2017).

The analysis results show that there is no significant difference in estrogen levels (E2) between the two types of cattle, both during estrus and 30 days after AI ($P>0.05$). Nevertheless, there is a significant difference in estrogen levels between the estrus phase and 30 days after AI in each

type of cattle ($P<0.05$) (Table 4). The research data indicates that lowered estrogen levels and estrus signs in F1 crossbred cattle are caused by pregnancy, as proven by rectal palpation (Table 3). This is consistent with the idea that variations in the majority of estrus indicators are linked to peaks in estrogen (Sumiyoshi *et al.*, 2014). Estradiol levels have a direct impact on the strength of estrus, which is equally significant. Within a herd, identifying estrus has a direct impact on the success rates of pregnancies (Nogueira *et al.*, 2019).

Table 4: The significance in estrogen levels (E2) between the two types of cattle, both during estrus and 30 days after artificial insemination (AI) (mean $\pm$ SD).

Phase	Brahman Cross	F1 Crossbred
Estrus	463.60 ^b $\pm$ 76.98	475.94 ^b $\pm$ 38.92
30 days after AI	276.44 ^a $\pm$ 119.04	326.64 ^a $\pm$ 147.42

^{ab}Different superscripts on the same column indicate significant differences ($P<0.05$). No significant differences were found among values within the same row.

According to the research findings, 30 days after artificial insemination (AI), Brahman Cross cattle no longer exhibit estrus signs or elevated estrogen levels. Rectal examination

indicates that these cattle are not pregnant, possibly due to frequent mating, as evidenced by three instances of artificial insemination (Table 3). Repeat Breeder Cow (RBC) often leads to a significant decrease in serum estrogen levels. Lower estrogen levels can disrupt normal estrus cycles and ovulation, making it difficult for cows to conceive despite multiple inseminations (Yaginuma *et al.*, 2019). This decrease in estrogen is associated with frequent repeat mating or excessive artificial insemination, which can result in delayed or absent ovulation (Maulana *et al.*, 2022). Repeat Breeder Cow (RBC) syndrome refers to cows that have failed to conceive three or more times (Marin and Quintela, 2023). According to Sood *et al.* (2015), who discovered significantly decreased serum estrogen levels in cows undergoing repeat mating ($P < 0.05$), this could potentially be the result of repeat mating. Furthermore, Ahmmed *et al.* (2018), found that these cows had considerably decreased estrogen levels ($P < 0.01$). Friesian Holstein Cross Breed cows that are repeat breeding have lower levels of all estradiol parameters, according to Darmawan *et al.* (2020) study. If a cow has normal estrus, estrus cycles, and reproductive tracts but has mated with a fertile bull or semen at least three times without becoming pregnant, then the cow is classified as a recurrent breeder (Marin and Quintela, 2023). Repeat breeding has a complex and multifaceted set of reasons. About 40.1% of the reasons for repeat breeding in Jersey cows are related to hormone imbalances and disorders (Tiwari *et al.*, 2019). Heat stress causes low-intensity estrus in zebu cows, and these cows frequently show moderate signs of estrus with few external markers (Stevenson *et al.*, 2004). A broad analysis of zebu cow estrus suggests that 30–40% of the activity takes place at night.

CONCLUSIONS AND RECOMMENDATIONS

According to the study findings, visual estrus signs and estrogen levels in Brahman Cross and F1 Crossbred cattle both decline 30 days following artificial insemination (AI). This decline in F1 Crossbred cattle, where two out of the total were pregnant, indicates successful conception. In contrast, Brahman Cross cattle that were not pregnant after AI showed lower estrogen levels and fewer estrus signs, consistent with the impacts of repeated mating and potential issues such as Repeat Breeder Cow (RBC) syndrome.

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This study found that a decrease in estrogen levels in F1 Crossbred cattle after AI correlates with successful pregnancy, while non-pregnant Brahman Cross cattle showed lower estrogen and estrus signs, possibly linked to Repeat Breeder Cow (RBC) syndrome. These findings provide new insights into monitoring reproductive outcomes and identifying breeding issues.

AUTHOR'S CONTRIBUTIONS

Devi Ermawati: Conceptualization, visualization, writing-original draft, data curation, formal analysis, investigation.

Tety Hartatik: Conceptualization, supervision, funding acquisition, project administration.

Panjono: Validation, investigation.

Sigit Bintara: Conceptualization, methodology, investigation.

Bertha Yudistyra: Resources, data curation.

Rifai Mustofa: Resources, data curation.

Ali Agus: Validation.

Budi Prasetyo Widyobroto: Validation.

CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest.

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