

# Uterine inflammation is dynamic during a timed AI protocol, and its resolution before AI is associated with pregnancy in young primiparous *Bos indicus* beef cows

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## Research Article

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# Abstract

Uterine PMN proportion is dynamic during a timed AI (TAI) protocol, and its resolution by AI may be associated with restore fertility in postpartum beef cows. Our objective was to characterize changes in uterine polymorphonuclear neutrophils (PMN) proportion between D0 and D8 of a TAI protocol and to determine the association between uterine status and fertility in young primiparous (YP) *Bos indicus* beef cows. The YP cows (~ 26 mo; n = 107) underwent a TAI protocol, with uterine cytobrush samples collected on D0 and D8. Cows were classified by the proportion of uterine PMN on D8 as Low (L; PMN  $\leq$  5%; n = 77) or High (H; PMN > 5%; n = 30), and by their D0-to-D8 transition: HH (n = 8), HL (n = 11), LL (n = 66), and LH (n = 22). Uterine status changed between D0 and D8 in 33 of 107 cows (30.8%; HL, n = 11; LH, n = 22). Pregnancy per AI (P/AI) was higher in cows classified as L PMN than in cows classified as H PMN on D8 (50.7% vs. 30.0%; p = 0.02). Among transition groups, P/AI was 51.5%, 45.5%, 31.8%, and 25.0% for LL, HL, LH, and HH cows, respectively. Cows that resolved inflammation during the protocol (HL) attained pregnancy rates similar to cows that remained with Low (L/L), whereas cows classified as H PMN on D8 (LH or HH) had lower fertility. Uterine status at the end of the TAI protocol rather than at protocol onset was associated with pregnancy.

## Introduction

Postpartum uterine health is a critical determinant of reproductive performance in beef cattle (Oliveira Filho *et al.* 2022; Sheldon and Dobson 2004; Sheldon *et al.* 2006, 2008). Optimal reproductive performance is closely associated to postpartum uterine health (Gilbert *et al.* 2005; LeBlanc *et al.* 2002; Pfeifer *et al.* 2018), particularly the effective resolution of the marked polymorphonuclear neutrophil (PMN) influx that occurs during the first week after calving (Gilbert and Santos 2016; Sheldon *et al.* 2009). Evidence suggests that successful restoration of uterine function is characterized by a progressive decline in the proportion of uterine PMN to < 5% by 21–35 days postpartum (DPP), followed by a reduction to minimal levels (0–2%) by approximately 45 DPP in mature beef *Bos indicus* cows (Andrade *et al.* 2023). These low PMN proportions are subsequently maintained through the time of insemination and are associated with improved fertility outcomes (Bogado Pascottini *et al.* 2017; Drillich *et al.* 2012; Pfeifer *et al.* 2018).

Subclinical endometritis, diagnosed by uterine cytology, has been consistently associated with reduced pregnancy per artificial insemination (P/AI) in *Bos indicus* cows (Oliveira Filho *et al.* 2022). Studies have shown that a high proportion of uterine PMN at the initiation of a timed artificial insemination (TAI) protocol is associated with impaired fertility and reduced P/AI, indicating that persistent uterine inflammation may serve as a marker of reproductive performance (Andrade *et al.* 2021; Pfeifer *et al.* 2018; Ricci *et al.* 2015). In that regard, a proportion of PMN cells > 5% in uterine samples has been validated as a practical threshold to identify postpartum beef cows at risk of impaired fertility (Pfeifer *et al.* 2018). This concern is particularly relevant in young primiparous (YP) *Bos indicus* cows included in TAI programs early in the postpartum period (Pfeifer *et al.* 2026), in which uterine involution may be incomplete by 35 DPP (Andrade *et al.* 2023). However, recently, Pfeifer *et al.* (2026) observed that despite

these clear indicators of uterine inflammation, the P/AI did not differ significantly between healthy YP cows and those with endometritis at the onset of the TAI program. Most studies have evaluated uterine health at a single time point relative to AI, providing a static assessment of what is likely a dynamic process. Therefore, the persistence or resolution of the uterine inflammation by the end of the TAI protocol, rather than its status at protocol onset, could be a more proximate determinant of pregnancy success.

Estradiol-progesterone Timed AI (TAI) based protocols expose the uterus to a defined sequence of hormonal signals, including progesterone, estradiol, prostaglandin F2 $\alpha$ , and equine chorionic gonadotropin (eCG), over approximately one week (Baruselli *et al.* 2004; Bo *et al.* 1995; Meneghetti *et al.* 2009), which may influence the uterine environment and the dynamics of uterine inflammation. Whether uterine inflammation resolves, persists, or develops during a TAI protocol, and how these transitions relate to pregnancy, remains largely unexplored in *Bos indicus* cows. The objective of the present study was to characterize changes in the proportion of uterine PMN cells between the onset (D0) and the end (D8) of a TAI protocol, and to determine the association between uterine health status at the time of AI and pregnancy in YP *Bos indicus* beef cows.

## Materials and Methods

### Animals

All experimental procedures were approved by the Animal Care and Use Committee of the Brazilian Agricultural Research Corporation (Embrapa Rondônia; Protocol No. 01/2024). The experiment was conducted on a commercial farm in Ariquemes, Rondônia (RO), Brazil. A total of 107 YP Nelore cows (*Bos indicus*), aged  $26 \pm 1.8$  months, body condition score (BCS) between 2.75 and 3.5 (1–5 scale), and at  $43.8 \pm 1.2$  (mean  $\pm$  SE) days postpartum (DPP), were enrolled in the study. Cows were maintained on *Brachiaria brizantha* pasture with *ad libitum* access to water and mineral supplementation.

### Gynecological evaluation and hormonal treatments

Endometrial tissue was collected from all cows on D0 and D8 using the cytobrush technique, as described by Cardoso *et al.* (2017). After tissue collection, the brush was gently rolled onto a glass slide, which was stored for subsequent analysis. In the laboratory, slides were stained using a commercial kit (Quick Panoptic®, Laborclin, Pinhais, Brazil). A total of 200 cells per slide were counted under a light microscope (1000 $\times$  magnification), including polymorphonuclear (PMN), mononuclear, and epithelial cells, to determine the PMN proportion.

The timed artificial insemination (TAI) hormonal protocol consisted of intramuscular (i.m.) administration of 2 mg of estradiol benzoate (Gonadiol®, Zoetis, São Paulo, Brazil) and insertion of an intravaginal progesterone releasing device (CIDR®, Zoetis, São Paulo, Brazil) on D0. On D8, the CIDR® was removed and cows received 526  $\mu$ g of cloprostenol i.m. (Ciclase® DL, Zoetis, Argentina), 0.6 mg of

estradiol cypionate i.m. (Cipiotec®, Agener União, São Paulo, Brazil), and 300 IU i.m. of equine chorionic gonadotropin (eCG; Novormon®, Zoetis). On D10, all cows were artificially inseminated by the same technician using semen from two Nelore bulls. Pregnancy diagnosis was performed 30 days after TAI by ultrasonography (Mindray®).

## Definitions and experimental groups

The experimental design is shown in Fig. 1. Cows were classified using a 5% PMN cut-off (Low, L: PMN  $\leq$  5%; High, H: PMN > 5%; Adapted from Pfeifer *et al.* 2026). Cows were classified according to uterine condition (UC) on D8 (UCD8, end of the TAI protocol) as Low PMN (n = 77) or High PMN (n = 30). To characterize the dynamics of uterine health during the protocol, cows were additionally classified according to the transition in PMN proportion between D0 and D8: HH (High on D0 and D8; n = 8), HL (High on D0 and Low on D8; n = 11), LL (Low on D0 and D8; n = 66), and LH (Low on D0 and High on D8; n = 22; Fig. 1).

## Statistical analyses

All analyses were performed using GraphPad Prism version 10.2 (GraphPad Software, San Diego, CA, USA) and SAS software (SAS Institute Inc., Cary, NC, USA). Results are expressed as means  $\pm$  SEM or as percentages.

P/AI was analyzed by logistic regression. In preliminary analyses, the association between uterine condition and P/AI was evaluated on D0 (UCD0) and UCD8 (low vs. high PMN), with models including sire, calving to TAI (C-TAI) interval (early,  $\leq$  35 DPP; middle, > 35 to  $\leq$  45 DPP; late, > 45 DPP), replicate, and BCS. UCD0 was not associated with P/AI ( $p < 0.05$ ), whereas UCD8 was associated with P/AI ( $p < 0.05$ ). Accordingly, all subsequent analyses were performed using the UCD8 classification. The variables sire, replicate and BCS had no significant effect on the P/AI and were, therefore, excluded from the model. Finally, the P/AI was analyzed by UCD8, C-TAI interval, and its interaction (UCD8  $\times$  C-TAI interval). The proportion of cows classified as High PMN was calculated on D0 and D8, and the two proportions were compared using a chi-square test. The complementary analysis evaluated P/AI among transition groups (HH, HL, LL, LH), with a specific contrast between HL and LL cows. Among transition groups (HH, HL, LL, LH), P/AI was analyzed by chi-square test followed by pairwise comparisons. Fisher's exact test was used when expected cell frequencies were < 5. As PMN data were not normally distributed (Shapiro–Wilk test), the effects of uterine condition on D8 (UCD8), calving-to-TAI (C-TAI) interval, and their interaction on uterine PMN proportion were evaluated using a two-way ANOVA on ranked data, followed by Dunn's test for multiple comparisons when appropriate. Within-group changes in uterine PMN proportion between D0 and D8 were analyzed using the Wilcoxon signed-rank test.

In all analyses, differences were considered significant when  $P \leq 0.05$ , and a probability between 0.051 and 0.1 was considered a trend.

## Results

The uterine PMN proportion on D0 and D8 in YP Nelore beef cows classified according to uterine condition on D8 and calving-to-TAI interval (Early, Middle, Late) is shown in Table 1. On D0, PMN proportion was higher ( $p = 0.007$ ) in cows subsequently classified as H PMN on D8 than in cows that remained with L PMN in the uterus. The PMN proportion on D0 also differed among calving to TAI intervals ( $p < 0.01$ ), and a significant group  $\times$  interval interaction was detected ( $p < 0.001$ ), indicating that the association between calving to TAI interval and PMN on D0 differed between cows classified as L PMN or cows classified as H PMN on D8.

Table 1

Pregnancy per AI and uterine polymorphonuclear (PMN) cell proportion on D0 and D8 in young primiparous Nelore (*Bos indicus*) beef cows ( $n = 107$ ) classified according to uterine health status on D8 (Low, PMN  $\leq 5\%$ , vs. High, PMN  $> 5\%$ ) and calving-to-TAI interval (Early, Middle, Late).

|                                                                                                                                                                                                                                                 | Uterine condition on D8 |                | Calving – TAI intervals |                 |                 | P-Value |                |            |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------|----------------|-------------------------|-----------------|-----------------|---------|----------------|------------|
|                                                                                                                                                                                                                                                 | Low PMN                 | High PMN       | Early                   | Middle          | Late            | UCD8    | C-TAI interval | UCD8*C-TAI |
| PMN on D0                                                                                                                                                                                                                                       | 4.1 $\pm$ 1.6           | 7.3 $\pm$ 2.3  | 5.8 $\pm$ 3.1           | 3.6 $\pm$ 1.5   | 8.6 $\pm$ 2.3   | 0.007   | < 0.01         | < 0.001    |
| PMN on D8                                                                                                                                                                                                                                       | 1.4 $\pm$ 1.9           | 15.8 $\pm$ 2.7 | 6.9 $\pm$ 3.7           | 6.1 $\pm$ 1.8   | 7.6 $\pm$ 2.8   | < 0.001 | 0.36           | 0.31       |
| Pregnancy per AI. %                                                                                                                                                                                                                             | 50.7<br>(39/77)         | 30.0<br>(9/30) | 43.7<br>(7/16)          | 43.3<br>(26/60) | 48.4<br>(15/31) | 0.02    | 0.53           | 0.23       |
| Abbreviations: PMN, polymorphonuclear; TAI, timed artificial insemination; UCD8, uterine condition on D8. P values refer to the effects of uterine status (UCD8), calving-to-TAI interval (C-TAI), and their interaction (UCD8 $\times$ C-TAI). |                         |                |                         |                 |                 |         |                |            |

On D8, PMN proportion was higher in cows classified as H PMN than in cows classified as L PMN in the uterus ( $p = 0.001$ ), as expected from the classification criteria. PMN proportion on D8 was not affected by calving to TAI interval ( $p = 0.36$ ) or by the group  $\times$  interval interaction ( $p = 0.31$ ).

The proportion of cows classified as H PMN increased ( $p = 0.02$ ) from 17.8% (19/107) on D0 to 28.0% (30/107) on D8 (Fig. 1), and 33 of 107 cows (30.8%) changed uterine status during the protocol. Of the cows classified as H PMN on D0, 11 of 19 (57.9%) had resolved the uterine condition by D8 (HL), whereas 22 of 88 cows (25.0%) classified as L PMN on D0 developed H PMN by D8 (LH).

Paired comparison of uterine PMN proportion between D0 and D8 in cows classified as L PMN or H PMN on D8 is shown in Fig. 2. The proportion of PMN tended ( $p = 0.07$ ) to change from D0 to D8 in cows classified as L PMN on D8 ( $n = 77$ ). In contrast, PMN proportion increased ( $p < 0.001$ ) from D0 to D8 in cows classified as H PMN on D8 ( $n = 30$ ).

The P/AI in YP cows classified according to uterine condition on D8 and calving-to-TAI interval is shown in Table 1. The P/AI was higher ( $p = 0.02$ ) in cows classified as L PMN on D8 than in cows classified as H PMN on D8. Calving to TAI interval did not affect P/AI ( $p = 0.53$ ), and no group  $\times$  interval interaction was detected ( $p = 0.23$ ).

Among transition groups, P/AI was lowest in HH cows (25.0%) and highest in LL cows (51.5%), which differed from each other ( $p < 0.05$ ), whereas HL (45.5%) and LH (31.8%) cows were intermediate and did not differ ( $p < 0.05$ ) from the other groups (Fig. 3). A tendency for lower P/AI was observed in LH compared with LL cows ( $p < 0.10$ ). Cows that resolved uterine inflammation during the protocol (HL) attained P/AI similar to those of cows that remained with L (LL), whereas cows that developed (LH) or maintained (HH) High PMN had lower fertility.

## Discussion

To our knowledge, this is the first study to describe the dynamics of uterine inflammation during an estradiol-progesterone based TAI protocol in beef cows. Approximately one-third of cows changed uterine status between D0 and D8, and more than half (11/19) of the cows with high PMN on D0 had resolved the condition by D8. Notably, cows that resolved inflammation during the protocol (HL) attained pregnancy rates similar to those of cows that remained with L PMN (LL), whereas cows that developed (LH) or maintained (HH) H PMN had markedly lower fertility. Consistent with this, uterine status at the end of the TAI protocol was closely associated with pregnancy, indicating that the uterine environment near the time of insemination is associated with conception.

The paired analysis of uterine PMN proportion between D0 and D8 is consistent with the dynamic nature of uterine inflammation during the TAI protocol. We observed that the proportion of endometrial PMN increased from D0 to D8 in cows classified as H PMN on D8, whereas it did not change in cows classified as L PMN on D8. These uterine dynamics indicate that a single cytological evaluation at protocol onset may not accurately predict uterine health near insemination. In the present study, 57.9% of cows that presented H PMN on D0 showed a reduction in PMN proportion on D8. This result is consistent with the progressive reduction in uterine inflammation observed during the postpartum period by Andrade *et al.* (2021). Therefore, the reduction in PMN proportion observed during the protocol may represent the continuation of the resolution of uterine inflammation already present at the onset of TAI. Conversely, 25% of cows classified as L PMN on D0 showed an increase in PMN proportion on D8. This result indicates that the absence of detectable inflammation at the onset of the TAI protocol did not guarantee that this condition would be maintained until AI. Therefore, the uterine response during the TAI period was not characterized by a uniform change in PMN proportion, but by different trajectories according to the initial condition. A possible explanation for this differential response is the alteration of the endocrine environment during the protocol. Sugiura *et al.* (2018) demonstrated that endometrial thickness in cows is related to plasma concentrations of progesterone and estradiol, both during natural estrus and after hormonal induction. These results demonstrate that the endometrium responds to changes in the steroidal environment during the estrous cycle and after hormonal manipulation. In the

present study, therefore, the change in PMN proportion between D0 and D8 occurred during a period of programmed hormonal changes, which may have contributed to the modulation of uterine condition. Progesterone also exerts anti-inflammatory effects directly on the bovine endometrium (Cui *et al.* 2020). The authors demonstrated that progesterone reduced the inflammatory response of bovine endometrial epithelial cells stimulated with *E. coli* or LPS, involving modulation of the NF- $\kappa$ B and MAPK pathways. Subsequently, Cui *et al.* (2022) demonstrated a similar effect in bovine endometrial stromal cells, showing that progesterone reduced the inflammatory response through mechanisms related to the same pathways. These results support the possibility that changes in progesterone exposure during the protocol may contribute to changes in the uterine inflammatory response.

Pfeifer *et al.* (2018) observed a higher PMN proportion at the onset of TAI in cows submitted to the protocol between 20 and 30 days postpartum (9.0%) than in those submitted between 31–45 (3.8%) and 46–60 days postpartum (2.2%). These animals also showed higher expression of *IL1* and *IL8* and lower P/AI. Similarly, Andrade *et al.* (2021) observed a reduction in PMN proportion with increasing postpartum interval in Nelore cows submitted to TAI. These studies indicate that uterine condition at the onset of the protocol is strongly influenced by the stage of postpartum recovery. However, in the present study, YP cows included late in TAI were the ones with greater proportion of PMN in the uterus. Within the range of intervals evaluated, C-TAI interval did not modulate P/AI or uterine PMN at the end of the TAI protocol, indicating that the association between uterine status on D8 and pregnancy was independent of the interval between calving and timed AI. The greater inflammatory activity in YP cows late postpartum period was also described by Andrade *et al.* (2023), who observed a higher PMN proportion and higher *IL1B* and *IL8* expression in YP cows compared with conventional primiparous and multiparous cows. The authors suggested that differences between categories could be related to the process of uterine recovery after calving. Therefore, in YP cows, the uterine condition observed at the onset of TAI may reflect different stages of resolution of postpartum inflammation, contributing to the heterogeneity of the response observed between D0 and D8.

The dynamics of uterine inflammation during hormonal protocols for timed AI (TAI) in early postpartum beef cows remain poorly understood. Lietaer *et al.* (2022) observed that, in healthy early postpartum cows, endometrial PMN showed a decreasing proportion of apoptotic cells, an increasing proportion of necrotic cells, and a decreasing proportion of cells actively engaged in phagocytosis over time. A lower proportion of apoptotic PMN in the endometrium may indicate resolution of the inflammatory process. In addition to a quantitative reduction in the uterine PMN proportion in cows with greater P/AI, the functional status of these cells may also be modulated by the hormonal changes imposed by the TAI protocol. In the present study, it is not possible to directly attribute the reduction or increase in PMN proportion to the hormonal environment promoted by the protocol. The absence of a control group not exposed to the protocol precludes determining whether the change observed between D0 and D8 was caused by the hormonal treatment or resulted from the natural dynamics of postpartum inflammation. Thus, the results suggest a possible role of the hormonal environment in modulating the uterine response. Further research on the viability of endometrial PMN may help clarify how the hormonal

environment of TAI protocols affects uterine inflammation and the functional capacity of PMN involved in postpartum uterine health.

The P/AI was associated with uterine status at the end of the TAI protocol rather than at protocol onset. Cows classified as H PMN on D8 had lower P/AI than cows classified as L PMN, whereas cows that resolved uterine inflammation during the protocol achieved P/AI comparable to those of cows that remained with L PMN (45.5% vs. 51.5%). Calving to TAI interval did not affect P/AI, indicating that this association was independent of the interval between calving and timed AI. These findings indicate that a single cytological evaluation at protocol onset has limited predictive value for uterine health near the insemination time. This result contrasts with that observed by Pfeifer *et al.* (2026), who specifically evaluated YP *Bos indicus* cows and did not observe difference in P/AI between healthy and cows with endometritis on D0 of TAI. In that study, P/AI was 58.2% and 58.3% for healthy and cows with endometritis, respectively. The absence of an association between PMN on D0 and P/AI observed by Pfeifer *et al.* (2026), together with the association between uterine condition on D8 and P/AI observed here, suggests that the relationship between uterine inflammation and fertility may depend on the time of evaluation. The pregnancy outcome also differed from that described by Pfeifer *et al.* (2018), who observed lower P/AI in cows with a higher PMN proportion at the onset of TAI. This difference may be related to animal category and postpartum timing. Pfeifer *et al.* (2018) evaluated beef cows submitted to TAI at different postpartum times, whereas Pfeifer *et al.* (2026) and the present study specifically evaluated YP *Bos indicus* cows. Therefore, the available results suggest that the association between uterine inflammation and fertility is not uniform and may depend on animal category, postpartum interval, and the time of uterine evaluation.

Because of the small number of cows in the HH (n = 8) and HL (n = 11) groups, the statistical power to detect differences among transition groups was limited; therefore, the lack of significant differences involving these groups should be interpreted with caution, and these comparisons should be considered exploratory.

In conclusion, the present study demonstrates that uterine inflammation is a dynamic condition during a timed AI protocol in YP *Bos indicus* beef cows. The proportion of cows classified as H PMN increased from 17.8% on D0 to 28.0% on D8, and 30.8% of cows changed uterine status during the protocol; notably, more than half of the cows classified as H PMN on D0 (57.9%) had resolved the condition by D8. Paired analysis confirmed that PMN proportion is maintained from D0 to D8 in cows classified as L PMN on D8 and increasing in cows classified as H PMN on D8. From a practical standpoint, cytological evidence of H PMN at protocol onset does not preclude pregnancy. Our results support maintaining YP *Bos indicus* cows with early inflammation in the breeding program and prioritizing cytological evaluation near the time of AI as a more informative tool for fertility prognosis in extensive beef systems.

## Declarations

**Author contributions** IPB: investigation, data curation, formal analysis, writing – original draft, writing – review and editing. DACG: investigation, methodology, and data curation. JSA: investigation, data curation, writing – review and editing. RRS: investigation, methodology, and validation. ÉN: conceptualization, methodology, supervision, resources, writing – review and editing. LCLF: conceptualization, methodology, formal analysis, and investigation. SAS: investigation, data curation, writing – review and editing. LFMP: conceptualization, methodology, supervision, project administration, funding acquisition, writing – original draft, writing – review and editing.

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**Data availability** The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

**Code availability** Not applicable.

**Ethical approval:** All experimental procedures were approved by the Animal Care and Use Committee of the Brazilian Agricultural Research Corporation (Embrapa Rondônia; Protocol No. 01/2024).

**Consent for publication** The author allows the publication of the manuscript.

**Consent to participate** The author agreed to participate in the article.

**Conflict of interest** The authors declare no competing interests.

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## Figures

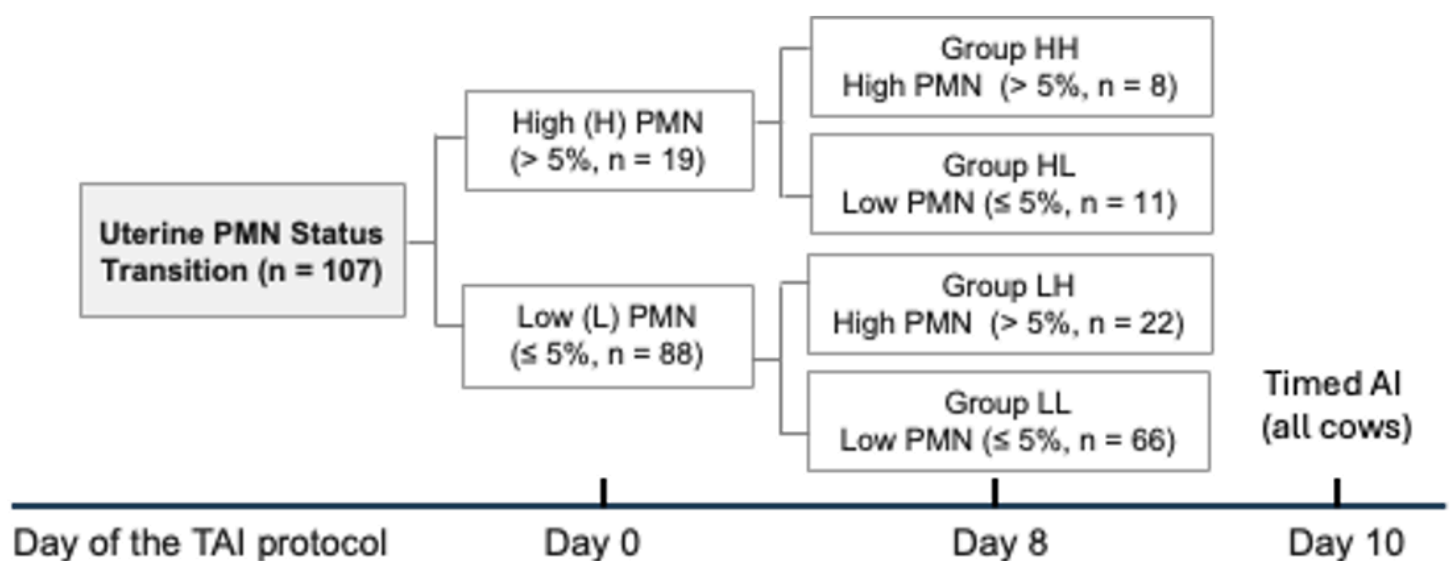


Figure 1

Transitions in uterine PMN proportion between D0 (onset) and D8 (end) of a timed AI protocol in young primiparous *Bos indicus* beef cows. Cows were classified as Low (PMN ≤ 5%) or High (PMN > 5%) at each day; group names reflect D0–D8 status (H = High, L = Low).

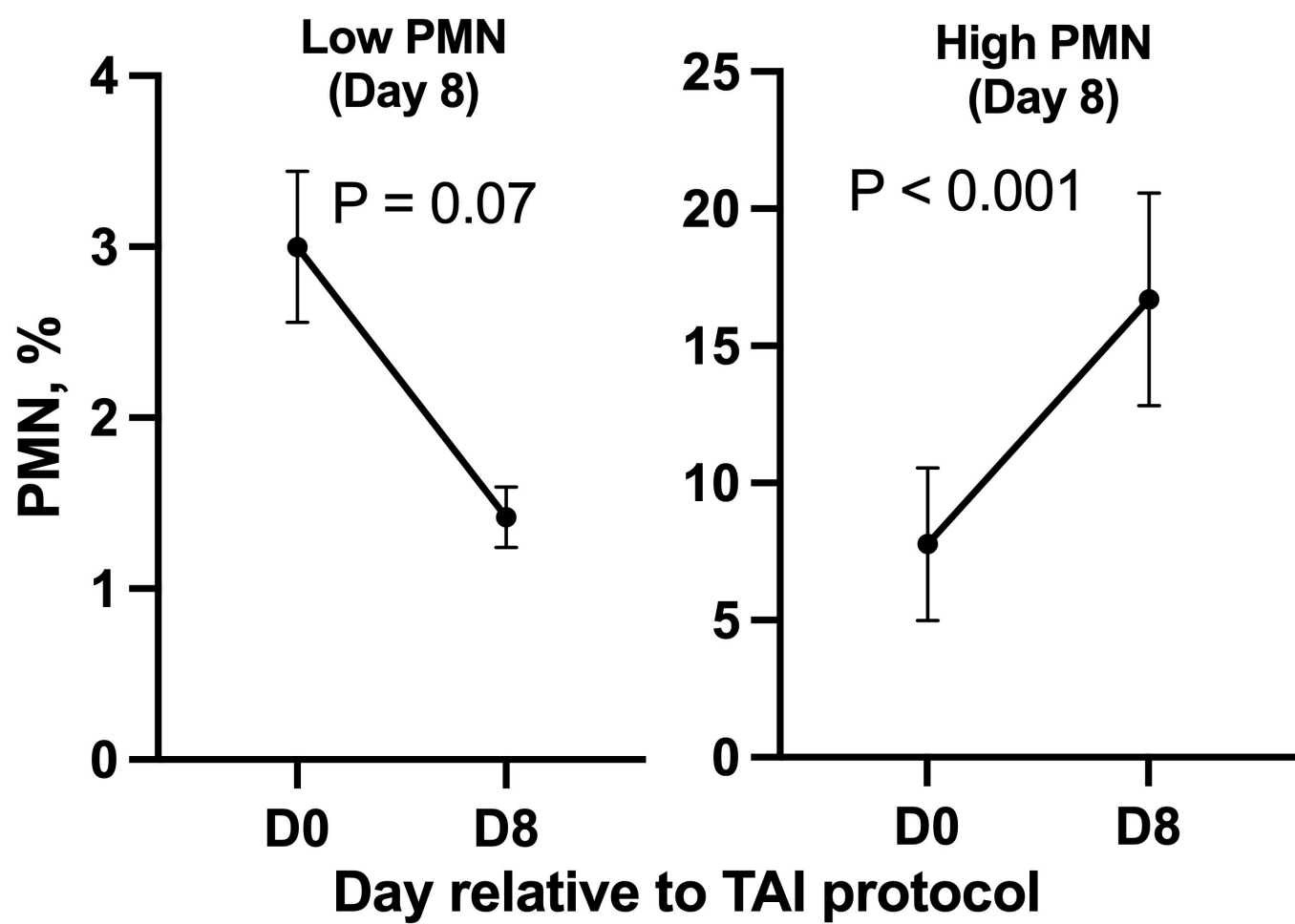


**Figure 2**

Paired comparison of uterine PMN proportion between D0 and D8 in young primiparous Nelore (*Bos indicus*) beef cows with Low PMN (PMN ≤ 5%; n = 77) or with High PMN (PMN > 5%; n = 30) on D8.

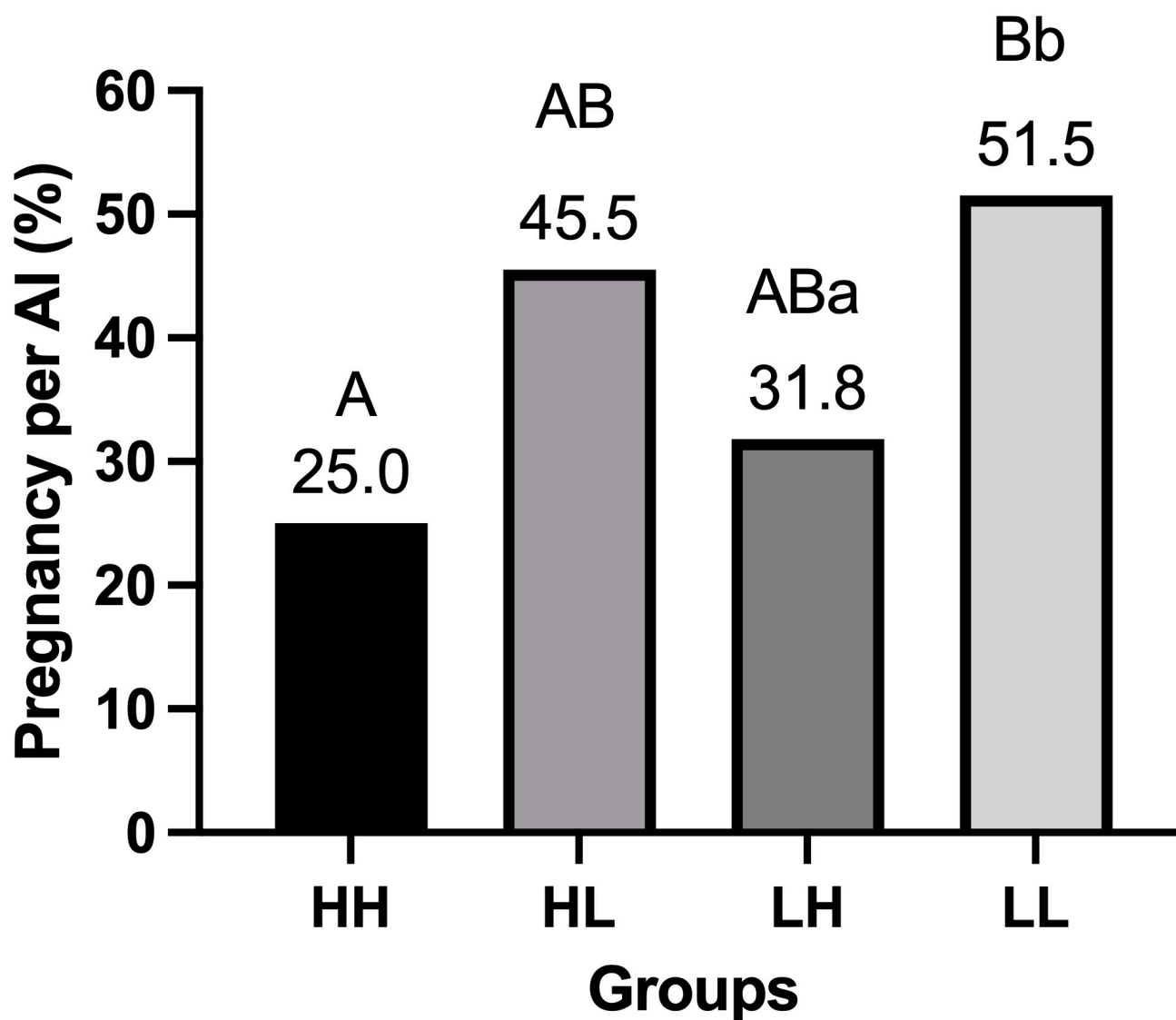


**Figure 3**

Pregnancy per AI (%) in young primiparous Nelore (*Bos indicus*) beef cows (n = 107) classified according to the transition in uterine PMN proportion between D0 and D8 of a timed AI protocol. Cows were classified as H (High, PMN > 5%) or L (Low, PMN ≤ 5%) at each day; group names reflect the D0–D8 status: HH (n = 8), HL (n = 11), LL (n = 66), and LH (n = 22).

AB Different letters indicate differences among groups ( $p < 0.05$ ).

ab Different letters indicate tendency among groups ( $p < 0.1$ ).