PEER REVIEW, Vol. 6, Nº 11, 2024
DOI: 10.53660/PRW-2238-4127
ISSN: 1541-1389

Effect of estrus behavior on the paint removal score of the sacral region of nulliparous Bos indicus

Efeito do comportamento de estro sobre o escore de remoção de tinta da região sacral de nulíparas *Bos indicus*

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RESUMO

O objetivo deste estudo foi correlacionar o comportamento do estro com o escore de remoção de tinta da região sacral (ERT) e avaliar a associação do ERT com o número de montas e comportamentos secundários do estro em fêmeas nulíparas de *Bos indicus*. Vinte e quatro protocolos de inseminação artificial em tempo fixo (IATF) de três manejos foram implementados para investigação do objetivo do estudo. A comparação entre ERT 3 e ERT 2 revelou diferenças significativas nas variáveis monta recebidas, execução de monta, descanso de queixo, cheirar ou ser cheirada, lamber e ser lambida, favorecendo o grupo ERT 3 ($P < 0,05$), indicando estro. Entretanto, a comparação entre ERT 2 e ERT 1 não resultou em diferenças significativas nas características citadas, exceto no ser cheirada ($P < 0,05$), indicando ausência de estro para esses dois escores. O número de montas recebidas em ERT 2 e 1 foi insuficiente para apagar as marcações. Conclui-se que o ERT se mostrou um indicador eficiente da manifestação do estro. Os comportamentos de estro das vacas nulíparas na ERT 3 foram mais evidentes que os da ERT 1 e 2, e os comportamentos manifestados na ERT 1 foram semelhantes aos da ERT 2, indicando ausência de comportamento de estro. Assim, sugere-se que no futuro o ERT seja apenas dividido em duas classes.

Palavras-chave: Bastão marcador; Expressão de estro; IATF; Manifestação de estro; Monta.

Recebido: 18/04/2024 | Aceito: 21/05/2024 | Publicado: 24/05/2024

ABSTRACT

The objective of this study was assess the effects of estrus behavior on the tail paint score (PRS) and evaluate the association of PRS with mounting events and secondary estrous behaviors in nulliparous *Bos indicus* females. Twenty-four fixed-time artificial insemination (FTAI) protocols across three management strategies were implemented to investigate the study's objectives. Comparisons between PRS 3 and PRS 2 revealed significant differences in received mounts, mounting behavior, chin resting, sniffing or being sniffed, licking and being licked, favoring the PRS 3 group ($P < 0.05$), indicating estrus. However, comparisons between PRS 2 and PRS 1 did not result in significant differences in the mentioned characteristics, except for being sniffed ($P < 0.05$), indicating absence of estrus. The number of mounts received in PRS 2 and 1 was insufficient to remove the paint marking. It is concluded that PRS proved to be an efficient indicator of estrus manifestation. Estrus behaviors in nulliparous cows in PRS 3 were more evident than those in PRS 1 and 2, and behaviors exhibited in PRS 1 were similar to PRS 2, indicating absence of estrus behavior. Future studies can apply the PRS using only two classifications.

Keywords: Marker stick; Estrus expression; FTAI; Estrus manifestation; Mount.

INTRODUCTION

Behavioral estrus expression in beef cattle at FTAI programs serves as a strategy to identify females with a higher likelihood of pregnancy (Sá Filho et al., 2011; Bonato et al., 2021). Among the various techniques for assessing estrus expression, the paint removal score (PRS) is widely utilized (Nogueira et al., 2019). This technique involves marking females with a paint stick on the sacral-caudal region on the day of progesterone (P4) removal. On the FTAI day, the painted area is evaluated, establishing a correlation with estrus sign expression. Classification is based on the extent of paint removal, with scores of 1 (little or no paint removal = no estrus expression), 2 (removal ≤ 75% = low estrus expression), and 3 (removal > 75% = high estrus expression) (Nogueira et al., 2019).

Although the PRS is commonly used during mating of females in estrus, no study has validated this technique for assessing behavioral estrus signs associated with PRS and their variations. Bovine females in estrus typically exhibit behaviors such as standing still when mounted (primary estrous behavior), mounting, sniffing and licking the genital area, and chin resting (secondary estrous behaviors, Sverbeg et al., 2013). The manifestation of estrus behaviors in females undergoing timed artificial insemination (FTAI) protocols may impact PRS, validating this behavior as an indicator of heat expression. The objective of this study was to measure the association between estrus behavior with the tail PRS.

MATERIAL AND METHOD

This study received approval from the Committee for the Ethical Use of Animals (Integrated University Center, Protocol n.16707/2021, Campo Mourão, Paraná, Brazil). The research was conducted at the Experimental Farm of the Integrated University Campus, situated at latitude 24º2´46´´S; Longitude 52º23´2´´W. Six nulliparous Nellore breed females, aged 24 months (23 - 25), with a body condition score (BCS) ranging between 3.5 and 4.0 (1-thin to 5=obese; Houghton et al., 1990) were included in the study. They fed on a diet consisting of pasture (Cynodon niemfuensis) supplemented with protein mineral salt (400g/day, Miner Plus Proteinado®, Prado, Paraná, Brazil), and 12 kg of crushed sugar cane (Saccharum officinarum) per animal/day.

Each heifer underwent the FTAI protocol, for 4 consecutive times at 16-day intervals but was not inseminated. At the study's conclusion, a total of 24 FTAI protocols were executed, and behavioral assessments, were performed.

The FTAI protocol was administered as follows: on day zero (D0), the heifers received 2 mg of estradiol benzoate (im) (Fertilcare synchronization®, MSD, São Paulo, Brazil) + an intravaginal device with 0.6 g of P4 (Fertilcare implant 600®, MSD, São Paulo, Brazil). On D8, 1 mg of estradiol cypionate (im) (Fertilcare ovulação®, MSD, São Paulo, Brazil) + 250 **µ**g of cloprostenol (im) (Ciosin®, MSD, São Paulo, Brazil), + 300IU equine chorionic gonadotropin (im) (Folligon®, MSD, São Paulo, Brazil), + P4 removal (Figure 1) + paint marking with a stick in the sacral region (Raidex®, GmbH, Dettingen/Erms, Germany). From 6 am on D10, the animals were continuously observed to detect signs of estrus for 30 to 48 h after removing the P4 device. The same protocol was repeated 16 days later.

For behavioral data evaluation, an ethogram was constructed, recording behaviors in the perineal region, including standing still when mounted, mounting, accepting a chin rest, sniffing, and licking (Table 1, adapted from Sverbeg et al., 2011).

The observation period for estrus behaviors (Table 1) occurred between 30 and 48 hours after P4 removal on day 8 of the protocol. Three evaluators continuously recorded estrus signs during this period. To assess estrus expression by PRS, heifers were marked with a marker stick (Raider®, GmbH, Dettingen/Erms, Germany) in the sacro-caudal region, between the sacral tuberosity and the first coccygeal vertebra. The stick was

rubbed four to five times consecutively, resulting in a total paint width of approximately 5 cm on the coat (Figure 1).

Table 1. Description of estrus behaviors recorded between 30 and 48 hours after progesterone device removal.

Behavior	Description of behavior
Receive stationary mounts	The female remains stationary when mounted; the cow makes no effort to escape when ridden by other cows.
Mounting	Mounts another animal supporting both thoracic limbs on the back of mounted animal.
Accept chin rest	Another female rests her chin on the rump of the evaluated female.
Smell/sniff	Smell/sniff the anogenital region of another animal.
Being sniffed	Another female smells the anogenital region of the evaluated female.
Licking	Lick the anogenital region.
Being licked	Another female licks the anogenital region of the evaluated female.

Figure 1 - Nulliparous Nellore breed subjected to paint marking with a marker stick for reading of the paint removal score on D10 of the protocol.

The heifers were categorized into scores 1 to 3: PRS 1(removal < 25% = without expression of estrus), PRS 2 (removal between 25 and 75% = low estrus expression) and PRS 3 (removal > 75% = high estrus expression) (modified from Nogueira et al., 2019).

To assess estrus and ovulation synchronization, ovarian scans were conducted on D8 and D10 to measure the diameter of the dominant follicle (DF), and on D21 to check the CL presence and diameter (Gastal et al., 2008). A veterinary ultrasound transrectal linear transducer (7.5 MHz, DP-2200 Vet®, Mindray, China) was utilized. Ovulation status was confirmed when the CL was detected in the same ovary where the DF was identified on D10.

Statistical analyses were performed using R software (R Core Team, 2020) and the RStudio interface (RStudio, 2020). Linear mixed models were employed to determine the average number of behavioral events in each PRS, with individuals included in all models to account for repeated measures. Means were estimated using the least squares method. To detect the difference between the number of primary and secondary behaviors between the different estrus expressions by PRS, four observation moments were established: the first one occurred between 30 and 34.5 h, the second between 34, 5 and 39, third between 39 and 43.5 and fourth between 43.5 and 48 h. At the end of each moment, behavioral data were accumulated, and the PRS was identified. Thus, the number of primary and secondary behaviors was associated with the PRS four times for each heifer per FTAI protocol. The PRS was checked at the beginning of the observation period (30 h). Females with a PRS 3 at the begging of the observational period were excluded from the study. The dimensions of the detected follicles (US examinations), such as the average dimensions of the DF (D8 and D10) and CL (D21) of the PRS (1, 2, and 3), were compared using Tukey's test. Females lacking DF or CL on D8 or D10 were excluded from calculations. Fischer's test was employed to calculate differences in ovulation rates among PRS 1, 2, and 3. Values were considered to have significant differences when the value was $P < 0.05$.

RESULTS

Table 2 details the modes of estrus behaviors (receiving mounting while still, carrying out mounting, accepting chin rest, sniffing, sniffing, licking, and licking),

corresponding to PRS 1, 2, and 3. Females with PRS 3 exhibited higher behavioral averages, followed by PRS 2 and 1 (Table 2).

Table 2. Minimum, maximum and average numbers of primary and secondary behaviors presented by paint removal scores 1, 2 and 3, during the TAI protocols.

Paint removal score	1			2			3		
Behaviors (n)	Min.	Average	Max.	Min.	Average	Max.	Min.	Average	Max.
Receive stationary mounts	0	0.8	3	0	4.4	13	0	14.0	32
Mounting	0	7.3	23	0	12.0	32	0	13.4	36
Accept chin rest	0	0.7	2	0	0.7	3	0	2.0	8
Smell/sniff	1	20.6	56	1	18.5	35	7	28.2	63
Being sniffed	3	16.4	34	3	18.2	36	5	24.3	49
Licking	0	7.1	34	0	9.2	61	1	9.5	81
Being licked	0	1.9	9	0	3.0	10	0	7.2	23

Legend: 1 (paint removal < 25% = no expression of estrus); 2 (dye removal 25 to 75%=low expression of estrus); 3 (dye removal >75%=high expression of estrus).

Table 3 presents estimated differences and degrees of significance between PRS behaviors. All PRS 3 behaviors differed significantly ($P < 0.05$) from both PRS 2 and 1. No differences were observed in behaviors between PRS 2 and 1, except for licking in PRS 2 ($P < 0.05$) (Table 3). Out of the 24 FTAI protocols, 16 protocols began the evaluation of estrous behavior (30 h after the removal of P4) in PRS 1, six with PRS 2, and two with PRS 3. At the end of the 48 h observation period, four FTAI protocols presented PRS 1 (16.67%), five protocols had PRS 2 (20.83%), and PRS 3 was observed in 15 (62.5%) (Table 4).

Table 3. Estimated difference and degree of significance between primary and secondary paint removal scores (PRS) behaviors.

Escore Remoção Tinta*	PRS 3 x PRS2		PRS 2 x PRS 1		PRS 3 x PRS 1	
X Comportamentos	ED±SE	P value	ED±SE	P value	ED±SE	P value
Receive stationary mounts	12.82±1.89	<0.01	2.11±1.76	0.47	14.93±2.27	<0.01
Make mounts	7.92±1.87	<0.01	3.97±1.74	0.10	11.89±2.26	<0.01
Accept chin rest	2.50±0.63	<0.01	0.35±0.60	0.82	2.14±0.74	<0.05
To smell	15.49±3.56	<0.01	3.41±3.30	0.57	18.90±4.31	<0.01
Being sniffed	10.61±2.87	<0.01	8.74±2.67	<0.05	19.35±3.47	<0.01
To lick	2.74±0.40	<0.01	0.12±0.41	0.95	2.86±0.53	<0.01
Being licked	5.05±1.50	<0.05	0.94±1.41	0.78	6.00±1.82	<0.05

* – Key: 1 (paint removal < 25% = no expression of estrus); 2 (dye removal 25 to 75%=low expression of estrus); 3 (dye removal >75%=high expression of estrus). ED: expected difference. SE: standard error

Table 4 displays the diameter of the dominant follicle (DF) on days 8 and 10, its association with PRS, and the overall ovulation rate scores. No differences were noted between PRS 1, 2, and 3 regarding DF dimensions on D8 and D10. The ovulation rate was 100% for PRS 3 and 2, and 50% for PRS 1 ($P > 0.05$).

Table 4. Dimensions of the diameter of the dominant follicle on day 8 and 10 of Bos taurus heifers and crossbreeds submitted to TAI protocols (03 managements) with a view to their association with estrus behavior.

Nulliparous PRS	FD dimension in D8 (mm) (mean±SD)	FD dimension in D10 (mm) (mean±SD)	CL dimension in D21 (mm) (mean±SD)	Ovulation rate (general) % (n)
1	6,72±0,70[a]	9,33±1,51[a]	17,90±1,27[a]	50(2/4)[a]
2	8,82±2,13[a]	12,60±1,72[a]	19,00±2,77[a]	100(5/5)[a]
3	10,10±1,93[a]	13,30±1,98[a]	20,83±2,41[a]	100(15/15)[a]

Identical superscript letters in the columns indicate there is no difference between them.

DISCUSSION

Behavioral studies have garnered significant attention due to their direct correlation with reproductive performance (Brandão and Cooke, 2021). However, few studies have explored the combined effects of estrus behavior and the sacral region paint removal score (PRS) as an indicator of estrus manifestation in beef cattle. This study is the first to validate this technique regarding the behaviors associated to each level of PRS.

In this investigation, six out of 24 protocols applied to the heifers resulted in estrous behaviour commencing the observation period with PRS2, and two under PRS 3, indicating earlier estrus in these animals compared to those under PRS 1. These results were expected, as heifers subjected to the protocols, FTAIs manifested estrus with ovulation before the group's average (Carvalho et al., 2016). In this study, 62.5% of the animals completed the observation period (48 h after P4 removal) under a PRS of 3, a percentage consistent with reports by Nogueira et al. (2019), Rodrigues et al. (2019), and Bonato et al. (2021). This result supports previous findings that lactating Bos indicus cows in the postpartum period exhibit a PRS 3 of 53–70% in response to TAI protocols.

A notable percentage of females under PRS 3 displayed estrus signs, as evidenced by the number of mounts in the "acceptance of mounts standing" behavior, comparable to zebu females showing natural estrus (Zavaleta et al., 1991) or those induced to estrus

by cloprostenol administration (Orihuela et al., 1983). In this study, the difference in the number of "accepting the mount standing still" was significant ($P<0.05$) when comparing PRS 3 with PRS 1 (Table 2). "Accepting mount while standing still" is a primary indicator of estrus in Bos indicus animals, occurring almost exclusively in females during estrus (Orihuela, 2000).

Although the evaluated secondary estrus behaviors do not influence the ERT, the increase in these signs in PRS3 along with the behavior of accepting mounting while standing still are indicators of estrus (Kerbrat and Disenhaus, 2004).

Despite estrus behaviors were numerically higher in ERT2 compared to ERT1 (Table 2), suggesting that ERT2 represents a less intense form of estrus (Nogueira et al.,2019), these numerical differences were so small that they were not significant except for the characteristic "being sniffed" (Table 3).

Regarding the behavior of females classified in ERT 1, these did not show estrus and were similar to females in ERT 2, suggesting they could be discussed as a single group. Although pregnancy rates of these females were not evaluated, studies have shown that physiologically, heifers in ERT 1 and 2 have the potential to become pregnant (Nogueira et al., 2019). This is possible because dominant animals initiate mounting in 60% of cases, which can inhibit mounting activity in subordinate animals (Orihuela, 2000). Therefore, some females in ERT 1 and 2 may be in physiological estrus but are not mounted due to social interactions, resulting in the persistence of paint in the sacral region of the animal.

Due to the methodology employed in this study, it was possible to identify that complete paint removal (100%) occurred on average with 14 mounts. However, it can be stated that the number of mounts experienced by females not truly in estrus was insufficient to achieve 100% paint removal.

Data from the present study concerning ovarian ultrasound examinations align with studies comparing pregnancy rates with PRS (Nogueira et al., 2019). Females in PRS 3 exhibited greater dimensions in DF (absolute values) on D8 and D10, as well as in Cl (D21), than females under PRS 2 and 1 (Table 4). Such data could potentially lead to higher pregnancy rates in PRS 3 compared to the other three PRS (Nogueira et al., 2019). Females classified as PRS 1 might have a lower fertility rate, as some of these heifers did not respond to estrus synchronization, delaying ovulation or not ovulating at all, as observed in two animals.

CONCLUSION

It is concluded that PRS proved to be an efficient indicator of estrus manifestation. Estrus behaviors in nulliparous cows in PRS 3 were more evident than those in PRS 1 and 2, and behaviors exhibited in PRS 1 were similar to PRS 2, indicating absence of estrus behavior. Future studies can apply the PRS using only two classifications.

ACKNOWLEDGEMENT

To the Integrated Institute of Science and Technology (IN2) for financing the project. To the Coordination for the Improvement of Higher Education Personnel – Brazil (CAPES), for the scholarship granted.

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