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Effect of bull biostimulation on the oestrous behaviour of pubertal Sahiwal (*Bos indicus*) heifers

Sanjay Choudhary*, Madan Lal Kamboj

Livestock Production Management Section, ICAR-National Dairy Research Institute, Karnal, Haryana, 132001, India



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ABSTRACT

The study was conducted to determine effects of biostimulation of Sahiwal heifers through fenceline bull contact and fenceline combined with direct bull contact on oestrous behaviour when there was ovulation occurring in the absence of behavioural oestrus ("silent oestrus - SE) and overt behavioural oestrus (OBE). Prepubertal Sahiwal heifers were allotted to three treatments (T₀, T₁, T₂, n = 8 heifers/treatment). In the T₀ group, there was no bull exposure; in T₁, exposure to a bull through fenceline contact for 24 h and in T₂, exposure to a bull as in T₁ along with direct bull contact with another bull for a 6-h period daily. The oestrous behaviours were recorded on day (d) -3, -2 and -1 (prior to oestrus), d 0 (day of oestrus) and d +3, +2 and +1 (post-oestrus). With both SE and OBE, the mean frequency of sniffing, micturition, chin resting and standing to be mounted differed ($P < 0.05$) from d -2 to d +1 among treatment groups. The mean times devoted to eating, ruminating and lying during SE and OBE were less ($P < 0.05$) on d-1 and d 0 in heifers of all three groups than the respective values on reference days in both T₁ and T₂ groups. Biostimulation of Sahiwal heifers with bull exposure, therefore, resulted in greater expression of oestrous behaviour than in non-exposed heifers during the periods around when there was SE and OBE.

1. Introduction

Oestrous behaviour is important for oestrous detection and determining the timing of artificial insemination in dairy animals. Shorter durations and less intense oestrous behaviour can lead to the excessive occurrence of "silent" oestrus (ovulation when there is an absence of easily detectable behavioural symptoms of oestrus) in *Bos indicus* breeds of cattle and are major impediments in achieving greater accuracy and efficiency of oestrous detection (Walker et al., 2008; Layak et al., 2011; Choudhary et al., 2019). Detection of oestrus in *Bos indicus* females is a challenging task as these animals fail to express overt symptoms of behavioural oestrus even when there are typical physiological functions that are associated with oestrus occurring (e.g., ovulation; Vaca et al., 1985). There are frequent inaccuracies or inconsistencies in human observation approaches and interpretations of observations during periods when personnel are observing the females in an attempt to detect oestrus. These observational problems are probably due to the behavioural differences between *Bos indicus* and *Bos taurus* breeds of cattle (Chenoweth, 1994). Besides breed, environmental, management and social cues are also important for the expression of oestrous behavioural symptoms in dairy cattle (Orihuela, 2000). In last 50 years, the duration of oestrus in dairy cattle has been reported to have decreased from 18 to 8 h (Allrich, 1994; Dolecheck et al., 2015) and the occurrence of oestrous behaviour when associated with the time of ovulation in Holstein dairy cows has been reduced from 80% to 50% (Dobson et al., 2008). This decrease in expression and intensity of oestrous behaviour has been

* Corresponding author.

E-mail addresses: rajsaya07@gmail.com (S. Choudhary), kamboj66@rediffmail.com (M. Lal Kamboj).<https://doi.org/10.1016/j.anireprosci.2019.106149>

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hypothesized to have occurred mainly due to modern intensive management and lack of social enrichment. Generally, oestrous detection is based on behaviours such as standing to be mounted, mounting other cows, chin resting, vulval discharge and some other general behaviours (Van Vliet and van Eerdenburg, 1996; López-Gatius et al., 2005; Yániz et al., 2006). The behavioural symptom that is most frequently considered to be reliable in detecting oestrus is standing to be mounted by other animals is not observed due to a lack of overt symptoms of oestrus (Lyimo et al., 2000). The provision of some social cues in the form of biostimulation has been suggested to be an effective suitable approach to enhance the expression of oestrous behaviour and reproductive efficiency in dairy cows (Mat, 2014). Biostimulation is the stimulatory effect of the male by means of pheromonal stimulation and external visual, olfactory, tactile and auditory cues that elicit both behavioural and endocrine responses in conspecifics (Chenoweth, 1983). Previously, it has been reported that direct exposure to a male improves the expression and intensity of oestrous behaviour in pigs (Langendijk et al., 2000), sheep (Rekwot et al., 2001) and post-partum beef cows (Mat, 2014). Fenceline bull exposure does not necessarily affect behavioural expression of oestrus in dairy cows (Roelofs et al., 2008).

Studies on the expression of cardinal signs of oestrus during the pubertal oestrus (referred to as “silent” oestrus) and subsequent pro-oestrous, oestrous and post-oestrous phases of the oestrous cycle in the presence of a bull as a biostimulant in dairy cattle heifers have not been attempted. It is hypothesized that expression of oestrous behaviour in pubertal dairy heifers may be improved with the cumulative effects of direct bull exposure along with fenceline bull exposure. The aim of the present study, therefore, was to compare the effects in Sahiwal heifers of biostimulation with the provision of fenceline bull contact and with the combination of fenceline plus direct bull contact on oestrous behaviour when there was a SE or an overt behavioural oestrus (OBE).

2. Material and methods

The experiment was approved by the Institutional Animal Ethics Committee (IAEC, registration no. 41-IAEC-18-20) and conducted in ways that were consistent with the IAEC established standard as per Article Number 13 of the committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) policies of the Government of India.

2.1. Experimental approaches

The experiment was conducted at the Livestock Research Center, National Dairy Research Institute, Karnal, Haryana, India which is situated at 29°42' 20' N latitude and 76°58' 52.5'E longitude, at an altitude of 227 m above the mean sea level. The maximum ambient temperature ranges from 40 to 43 °C in summer and 2 to 15 °C in winter. The experiment was conducted from September 2017 to April 2018. At the Livestock Research Center, National Dairy Research Institute, a total of about 342 Sahiwal cattle (152 mature cows) are maintained. Of these cows, about 109 cows are in milk throughout the year with about 6 to 7 calving's occurring every month on an average.

The experimental animals were housed in a loose housing system with a covered shed and an adjoining open paddock with a total floor space of 8 m² per animal with a shared feeder through a fenceline feed barrier and a shared drinker. The allocation of floor space and feeding space was consistent with the standards of the Bureau of Indian Standards for housing of cattle in loose housing systems (BIS: 1223–1987). The bulls were housed in between the sheds in which the heifers of the T₁ and T₂ groups were housed separated by 5-foot-high fence made of galvanized iron pipe railings of 1.5 in. diameter. The distance between the pipe railings from ground level was 24, 18 and 18 in. which allowed sufficient visual, olfactory and a limited tactile interaction between bull and the heifers through the fence. The floors of animal houses were made of cement concrete with grooves under the covered area and paved with brick-on-edge in adjacent open paddocks. All heifers were fed similar rations based on the feeding standards provided by the ICAR (2013) for feeding of dairy cattle and buffalo.

In the present study, the female calves born during the period from April to October 2016 were selected on the basis reproductive soundness (assessed through ultrasonography), age and body weight. A total of 24 Sahiwal heifers were selected and randomly allotted to three treatment groups of equal number (eight in each group) of heifers (T₁, T₂ and T₀) based on age (14.44 ± 0.28 months) and body weight (152 ± 6.12 kg). In the T₁ group, heifers were exposed to two intact Sahiwal bulls (aged 28 and 31 months) for 24-h continuously via fenceline contact. The fenceline exposure occurred along the complete length of the covered area as well as in the open paddock of a shed used to house heifers. In the T₂ group, heifers were exposed to a different bull for 24-h via continuous fenceline contact similar to that for the T₁ group but in addition there was direct contact with one of the two bulls used for fenceline contact for 6 h daily during two periods of 3 h each (from 07.00 to 10.00 and 16.00 to 19.00). During the period the bull was in the pen with the heifers, there was placement of an apron on the bull to inhibit intromission during mounting of the heifers. The heifers of the T₀ group were housed in a similar shed at a distance of about 0.5 km from the locations of heifers in the T₁ and T₂ groups to minimize effects of airborne bull pheromones. The bulls were selected on the basis of age, sexual maturity and sexual preferences toward the oestrous heifers (which was assessed through visual observations).

2.2. Recording of the oestrous behaviour

The frequency of different oestrous behaviours (number of times particular behaviour was observed in 24 h) were recorded for 24 h for 3 days before the onset of oestrus (d -3, -2, -1), on the day of oestrus (d 0) and 3 days after oestrus (d +3, +2, +1) using digital video recording by installing nine closed circuit television (CCTV) outdoor cameras (CP Plus, Noida, Uttar Pradesh, India). The cameras had 8x digital zoom capacity for closer viewing and were enabled with array infrared technology for night vision and were situated to allow for obtaining video recordings of the whole shed area. The images and videos were stored in a 16-channel digital

Table 1
Scoring scale for observed signs of oestrus (Van Eerdenburg et al., 2000).

Oestrous symptoms	Score
Flehmen	3
Mucous discharge from vulva	3
Cow restlessness	5
Sniffing the vulva of another cow	10
Mounting but not standing	10
Resting the chin on the back of another cow	15
Mounting or attempt to mount other cows	35
Standing oestrus	100

video recorder (DVR) having a hard disk of 1 TB space. Additionally, duration of eating, ruminating and lying was observed in hours per day for 24 h throughout periods when there was SE and OBE. The reference values of duration of eating, rumination and lying were recorded as the mean of these variables recorded consecutively for 7 days before the onset of proestrus. Heifers were numbered on the flank and back region with black or white water-paints twice a week to facilitate individual identification. The oestrous symptoms were scored (Table 1) using the procedures described by van Eerdenburg et al. (2000). Each time an animal displayed behavioural signs of oestrus, the assigned number of points were recorded. If the sum of points during the observation periods exceeded 100, the animal was considered to be in OBE. The midpoint in time between the first observation where the animal expressed signs of oestrus and the previous observation without any behavioural signs was considered as the time of onset of oestrus. Similarly, the end of oestrus was considered as the midpoint between the last observation where the animal expressed signs of oestrus and the following observation where the animal was no longer expressing signs of oestrus.

2.3. Progesterone assay

Blood samples (5 ml) were collected to quantify progesterone concentrations at 10 days post-oestrus. After collection, blood samples were immediately placed on ice and serum was separated by centrifugation at 3000 RPM for 15 min. Blood serum samples were stored at -20°C until concentrations of progesterone (P_4) were quantified using the Bovine Pg. (Progesterone) ELISA Kit (Wuhan Fine Biological Technology Co., Ltd., Wuhan, Hubei, China). The detection range and sensitivity of the kit was 0.313–20 ng/ml and < 0.188 ng/ml and the intra-assay CV was $< 8\%$.

2.4. Definition of OBE and SE

At each time there was detection of oestrus using the P_4 profile, there was classification of the oestrus as an OBE or SE. A cow was defined as having an OBE when the sum of the points scored for oestrous behaviour, as observed on the video recording, exceeded 100 (Van Eerdenburg et al., 2002). In this previous study, a cow was considered to have been in SE when there was not detection of any behavioural signs of oestrus or the oestrus score was < 100 points at or around the day when ovulation occurred based on milk P_4 profile for a cow (Van Eerdenburg et al., 2002). These same variables and methods were used to indicate that there was an SE in the present study.

2.5. Statistical analyses

Statistical analyses were performed using the general linear model in the statistical package for the social science (SPSS, computer software version 21, Armonk, New York, United States) and data graphs were prepared using GraphPad Prism version 5.0 San Diego California USA. The data on mean frequencies of the various oestrous behaviours were compared between treatments on 3 days before oestrus (d -3, -2, -1), the day of oestrus (d 0) and 3 days after oestrus (d +1, +2, +3), using Duncan's Multiple Range test and a one-way ANOVA for assessing both SE and OBE. Similarly, a one-way ANOVA was used to compare the data for mean duration of time devoted to eating, ruminating and lying on the 3 days before oestrus (d -3, -2, -1), day of oestrus (d 0) and 3 days after oestrus (d +1, +2, +3) relative to the values on respective reference days when there was a SE and an OBE, where there was no interaction between groups. The duration of SE, OBE and standing oestrus among the three groups of heifers was compared using a one-way ANOVA. Differences were reported as significant at $P < 0.05$. The following formula was used for these assessments.

$$\text{General linear model: } Y_{ij} = \mu + T_i + e_{ij}$$

Where:

Y = Observation of frequency of oestrous behaviours j^{th} heifer in the i^{th} treatment

μ = Overall mean, T_i = Effect of the i^{th} treatment, e_{ij} = Error

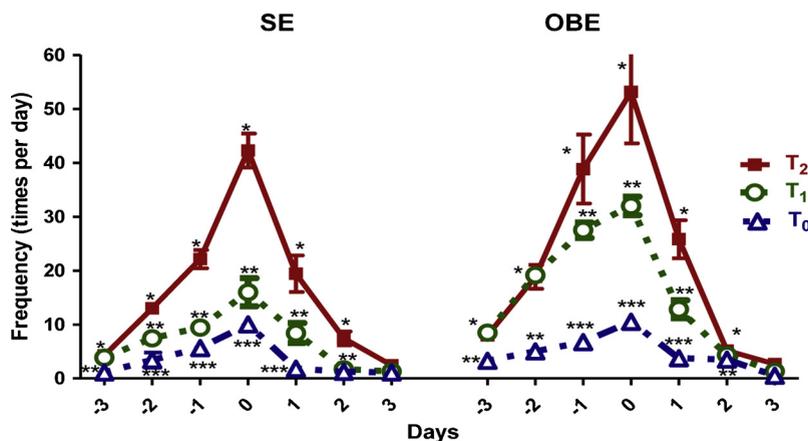


Fig. 1. Frequency of sniffing/licking by bull or other pen-mates in Sahiwal heifers without bull exposure (T_0), with fenceline bull exposure (T_1), or fenceline and direct bull exposure (T_2) 3 days before (d -3, -2, -1), day of (d 0) and 3 days after oestrus (d +1, +2, +3) when there was a silent oestrus (SE, $n = 18$) or an overt behavioural oestrus (OBE, $n = 21$); Where *, **, *** indicates differences between the mean values of different groups ($P < 0.05$).

3. Results

3.1. Oestrous behaviour

The data for the different oestrous behaviours (sniffing/licking, tail raising, micturition, chin resting, allowing mounting attempts and standing to be mounted) are presented in the sequential pattern of expression of these behaviours in cattle (Roelofs et al., 2005).

3.1.1. Sniffing/licking

The average frequency of sniffing/licking of heifers by a bull or other pen-mates during SE varied ($P < 0.05$) among the three treatment groups on d -2, d -1, d 0 and d +1. There was the greatest frequency in heifers of the T_2 (13.00 ± 1.02 , 22.14 ± 1.69 , 42.28 ± 3.14 and 19.42 ± 3.38), followed by T_1 (7.42 ± 0.71 , 9.42 ± 0.57 , 16.00 ± 2.61 and 8.42 ± 1.84) and T_0 (3.40 ± 1.40 , 5.60 ± 0.60 , 10.00 ± 0.54 and 1.80 ± 0.20) groups. Similarly, the mean frequency of sniffing/licking during OBE differed ($P < 0.05$) among heifers of all three groups with values on d -1 to d +1, being the greatest in T_2 (38.83 ± 6.39 , 53.16 ± 9.55 and 25.83 ± 3.52) followed by the T_1 (27.50 ± 1.43 , 32.00 ± 1.71 and 12.83 ± 1.66) and T_0 (6.75 ± 1.03 , 10.50 ± 1.19 and 3.75 ± 0.47) groups. Mean frequencies on d -3 and d -2, however, were greater ($P < 0.05$) in both the T_1 and T_2 than T_0 group. The frequency of sniffing/licking was markedly greater (1.5 to > 2 times) during OBE in heifers of the T_1 and T_2 groups in comparison to heifers where there was a SE, whereas in heifers of the T_0 group these frequencies were similar of heifers with an OBE and a SE (Fig. 1).

3.1.2. Tail raising

During SE, the average frequency of tail raising in response to sniffing/licking by a bull or other pen-mates in heifers of the T_1 and T_2 groups was greater ($P < 0.05$) from d -2 to d -1 of the period preceding oestrus than in heifers of the T_0 group. Furthermore, the frequency of tail raising on d 0 and d +1 was greater ($P < 0.05$) in the heifers of the T_2 (33.71 ± 3.47), followed by the heifers of the T_1 (12.42 ± 1.54) and T_0 (4.00 ± 0.91) groups. The average frequency of tail raising during OBE was greater ($P < 0.05$) in heifers of both T_1 and T_2 as compared with the T_0 group on d -3 (6.67 ± 0.42 , 7.67 ± 0.34 and 0.00 ± 0.00), d -2 (14.17 ± 0.70 , 13.50 ± 0.17 and 4.25 ± 0.25), d -1 (24.84 ± 1.81 , 24.00 ± 1.77 and 6.75 ± 0.75) and d 0 (48.33 ± 2.31 , 52.67 ± 9.57 and 15.75 ± 0.62). The frequencies of tail raising were almost 1.5 to 3.0 times greater in heifers of all three groups during OBE than the respective values during SE (Fig. 2).

3.1.3. Micturition

The frequency of micturition during SE was greater ($P < 0.05$) in heifers on d -1, d 0 and d +1 in the T_2 (13.67 ± 0.66 , 25.67 ± 1.68 and 13.83 ± 1.62) than in the T_1 (8.83 ± 0.30 , 14.34 ± 1.72 and 8.34 ± 0.49) and T_0 (9.25 ± 0.94 , 11.50 ± 0.95 and 7.75 ± 0.47) groups, respectively. During OBE, however, the frequency of micturition on d -1 was greater ($P < 0.05$) in heifers of the T_1 (18.83 ± 0.54) and T_2 (18.50 ± 0.71) than those in the T_0 (9.75 ± 1.03) group. Furthermore, on d 0, the frequency of micturition varied ($P < 0.05$) among treatment groups with the greatest frequency in the T_2 (39.50 ± 1.02), followed by the T_1 (27.50 ± 2.14) and T_0 (16.75 ± 1.30) group. Notably, the frequency of micturition on the day of oestrus when there was an OBE was 91.7% (T_1), 53.7% (T_2) and 45.6% (T_0) greater compared to the frequency observed during SE (Fig. 3).

3.1.4. Chin resting

The willingness of heifers to allow chin resting by a bull or other pen-mates on the tail head region during SE was first observed on

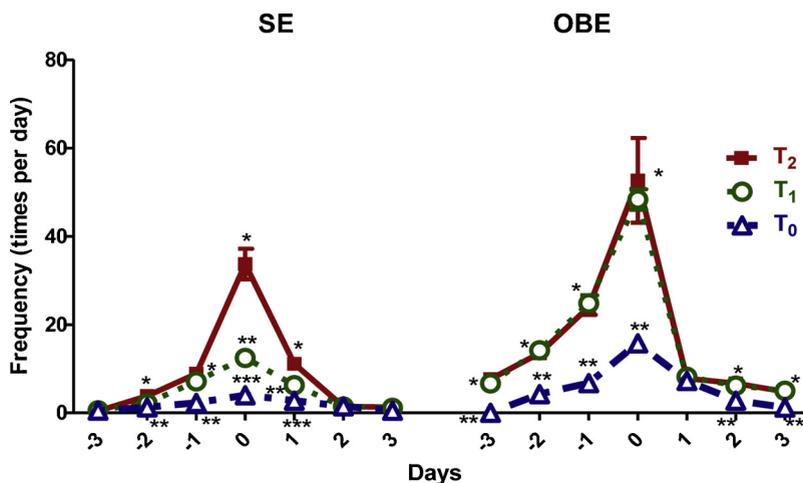


Fig. 2. Frequency of tail raising in response to sniffing/licking by bull or other pen-mates in Sahiwal heifers without bull exposure (T_0), with fenceline bull exposure (T_1), or fenceline and direct bull exposure (T_2) 3 days before (d -3, -2, -1), day of (d 0) and 3 days after oestrus (d +1, +2, +3) when there was a silent oestrus (SE, $n = 18$) or an overt behavioural oestrus (OBE, $n = 21$); Where there are *, **, *** indicates differences between the mean values of different groups ($P < 0.05$).

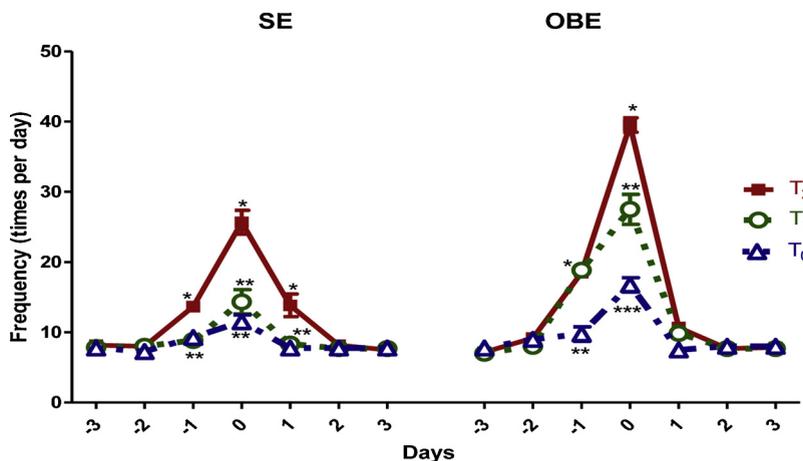


Fig. 3. Frequency of micturition by the bull or other pen-mates in Sahiwal heifers without bull exposure (T_0), with fenceline bull exposure (T_1), or fenceline and direct bull exposure (T_2) on the 3 days before (d -3, -2, -1), day of (d 0) and 3 days after oestrus (d +1, +2, +3) when there was a silent oestrus (SE, $n = 18$) or an overt behavioural oestrus (OBE, $n = 21$); Where there is *, **, *** indicates differences between the mean values of different groups ($P < 0.05$).

d -2 and extended until d +2 in all treatments. The mean frequency of allowing chin resting on d 0 and d +1 when there was a SE was greater in the heifers of the T_2 (23.85 ± 2.77 and 7.00 ± 0.30), followed by T_1 (10.42 ± 1.26 and 5.00 ± 0.84), and T_0 (5.00 ± 0.31 and 3.00 ± 0.31) ($P < 0.05$) group, respectively, whereas on d -2, d -1 and d +2, the frequency of chin resting was greater ($P < 0.05$) in the T_1 (3.28 ± 0.64 , 4.14 ± 0.60 and 4.14 ± 0.40) and T_2 (3.85 ± 0.50 , 5.57 ± 0.78 and 4.28 ± 0.28) groups as compared to the T_0 (1.20 ± 0.20 , 2.20 ± 0.20 and 1.20 ± 0.20) group, but there were no differences between heifers of the T_1 and T_2 groups. The frequency of chin resting in heifers with an OBE was similar to that observed in heifers with a SE, albeit, on d -2, d -1 and d 0 the mean values were more than twice that of all three groups as compared to the respective values when there was a SE (Fig. 4).

3.1.5. Mounting attempts

The onset of heifers soliciting mounting attempts by the bull or other pen-mates was discernible only from d -2 and extended until d +2 when there was a SE as well as when there was an OBE. The mean frequency of allowing mounting was greater ($P < 0.05$) on d -2, d -1, d 0 and d +1 in heifers of both the T_1 (3.85 ± 0.50 , 5.28 ± 0.71 , 16.42 ± 2.72 and 5.00 ± 0.21) and T_2 (4.42 ± 0.78 , 7.71 ± 0.83 , 21.28 ± 1.30 and 5.00 ± 0.21) compared with those in the T_0 (0.00 ± 0.00 , 1.80 ± 0.20 , 5.00 ± 0.31 and 2.20 ± 0.20) group when there was a SE and when there was an OBE with mounting attempts being greater ($P < 0.05$) on d -2, d -1 and d 0 in heifers of the T_1 (4.83 ± 1.01 , 10.33 ± 0.84 and 42.33 ± 2.84) and T_2 (6.16 ± 0.54 , 12.16 ± 1.16 and 48.33 ± 3.34) then T_0 (1.25 ± 0.25 , 5.00 ± 0.40 and 12.00 ± 0.70) group. The frequency of mounting attempts when there was an OBE on d 0

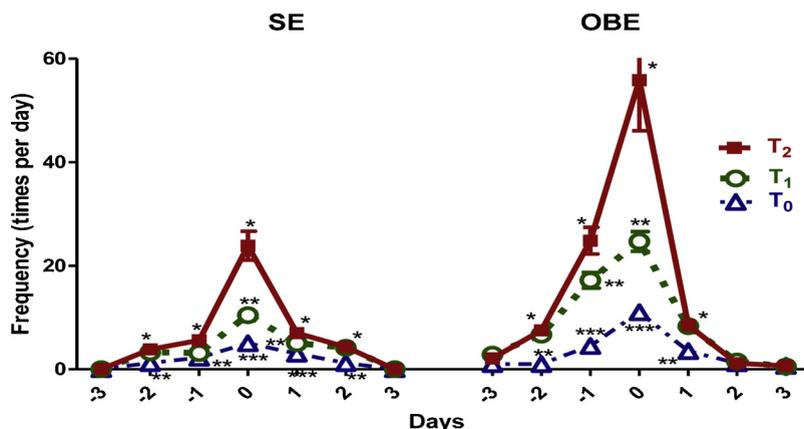


Fig. 4. Frequency of chin resting by the bull or other pen-mates in Sahiwal heifers without bull exposure (T_0), with fenceline bull exposure (T_1), or fenceline and direct bull exposure (T_2) on the 3 days before (d -3, -2, -1), day of (d 0) and 3 days after oestrus (d +1, +2, +3) when there was a silent oestrus (SE, $n = 18$) or an overt behavioural oestrus (OBE, $n = 21$); Where there is *, **, *** indicates difference between the mean values of different groups ($P < 0.05$).

was 2.0 to 2.5 times greater in heifers of the T_1 , T_2 and T_0 than the respective values for heifers with a SE (Fig. 5).

3.1.6. Standing to be mounted

None of the heifers were standing to be mounted by a bull or other pen-mates during SE. In heifers with an OBE, this behaviour was only detected on the day of oestrus and was greater ($P < 0.05$) in heifers of the T_2 (37.2 ± 8.21) followed by T_1 (10.4 ± 0.60) and T_0 (5.5 ± 0.50) group, respectively.

3.1.7. Eating time and rumination time

There was a lesser ($P < 0.05$) mean time devoted to eating (ranging from 78 to 87 min.) on the day of oestrus when there was a SE in heifers of the T_1 and T_2 groups as compared with values on the reference days for these groups (Table 2A). Notably, there was a greater time devoted to rumination on d +2 and d +3 (ranging from 45 to 55 min) compared to the values on reference days for these groups. There was no difference in the time devoted to eating on d 0 for heifers of all treatment groups. When there was an OBE, there was a lesser ($P < 0.05$) mean time devoted to eating (123 min) on d 0 compared to the values on reference days for in the heifers of the T_0 group (Table 2B) and again there was an increase on d +2 (18.65%) and d +3 (19.08%) as compared to values on the reference days for these groups. In the heifers of the T_1 and T_2 groups, the decrease (14.70% and 22.85%) was already evident on d -1 ($P < 0.05$) and continued on d 0, to thereafter increase on d +2 (14.41% and 16.59%) and d +3 (12.57 and 17.32%) compared to the respective reference values. The time devoted to eating on d 0 was less in heifers of the T_1 as well as T_2 (42.50 and 43.23%) than in the T_0 (29.41%) group (Table 2A, B).

When there was a SE and an OBE, the mean time devoted to ruminating (Table 3A, B) on d -1, d 0 and d +1 was less ($P < 0.05$)

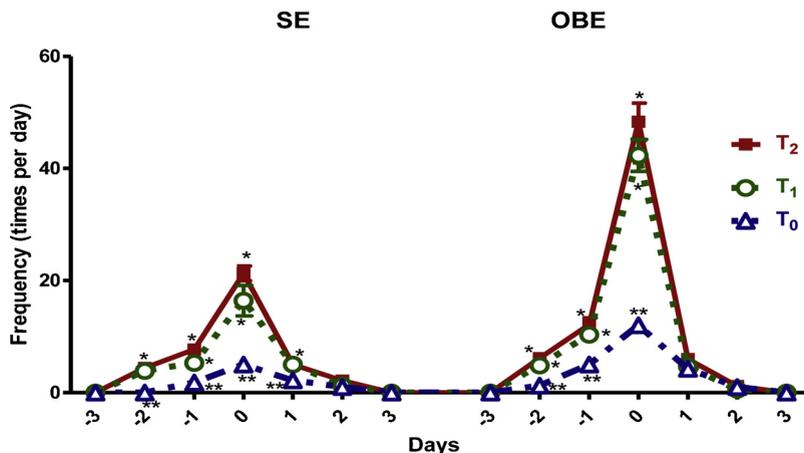


Fig. 5. Frequency of mounting attempts of heifers by the bull or other pen-mates in Sahiwal heifers without bull exposure (T_0), with fenceline bull exposure (T_1), or fenceline and direct bull exposure (T_2) on the 3 days before (d -3, -2, -1), day of (d 0) and 3 days after oestrus (d +1, +2, +3) when there is a silent oestrus (SE, $n = 18$) or an overt behavioural oestrus (OBE, $n = 21$) Where there are *, **, *** indicates differences between the mean values of different groups ($P < 0.05$).

Table 2

Mean duration of time devoted to eating (h/day) of Sahiwal heifers without bull exposure (T_0), with fenceline bull exposure (T_1), or fenceline and direct bull exposure (T_2) on the 3 days before (d -3, -2, -1), day of (d 0) and 3 days after oestrus (d +1, +2, +3) when there is a silent oestrous (SE, $n = 18$) (A) or an overt behavioural oestrus (OBE, $n = 21$) (B).

(A)									
Treatments	SE								
	Ref days	Days before oestrus			On the day of oestrus	Days after oestrus			
		-3	-2	-1		0	+1	+2	+3
T_0	6.72 ^a ± 0.14	6.84 ± 0.19	6.98 ± 0.11	6.32 ± 0.19	6.18 ± 0.21	6.86 ± 0.40	7.41 ^b ± 0.61	7.58 ^b ± 0.12	
T_1	6.65 ^a ± 0.20	6.75 ± 0.14	6.94 ± 0.78	6.31 ± 0.17	5.34 ^b ± 0.17	6.81 ± 0.29	7.31 ^b ± 0.11	7.45 ^b ± 0.11	
T_2	6.70 ^a ± 0.16	6.72 ± 0.15	6.92 ± 0.10	6.20 ± 0.15	5.25 ^b ± 0.14	6.64 ± 0.30	7.47 ^b ± 0.10	7.45 ^b ± 0.11	

(B)									
Treatments	OBE								
	Ref days	Days before oestrus			On the day of oestrus	Days after oestrus			
		-3	-2	-1		0	+1	+2	+3
T_0	6.97 ^a ± 0.07	6.92 ± 0.04	6.72 ± 0.05	6.57 ± 0.07	4.92 ^b ± 0.14	6.17 ± 0.02	8.27 ^b ± 0.17	8.30 ^b ± 0.12	
T_1	6.87 ^a ± 0.06	6.88 ± 0.04	6.70 ± 0.07	5.86 ^b ± 0.33	3.95 ^b ± 0.04	6.65 ± 0.33	7.86 ^b ± 0.12	7.73 ^b ± 0.21	
T_2	6.87 ^a ± 0.06	6.76 ± 0.03	6.71 ± 0.04	5.30 ^b ± 0.14	3.90 ^b ± 0.10	6.06 ± 0.08	8.01 ^b ± 0.13	8.06 ^b ± 0.12	

Values with different superscripts in the same row are different ($P < 0.05$).

Values with different superscripts in the same row are different ($P < 0.05$).

Table 3

Mean duration of time devoted to ruminating (h/day) of Sahiwal heifers without bull exposure (T_0), with fenceline bull exposure (T_1), or fenceline and direct bull exposure (T_2) on the 3 days before (d -3, -2, -1), day of (d 0) and 3 days after oestrus (d +1, +2, +3) when there was a silent oestrus (SE, $n = 18$) (A) or an overt behavioural oestrus (OBE, $n = 21$) (B).

(A)									
Treatments	SE								
	Ref days	Days before oestrus			On the day of oestrus	Days after oestrus			
		-3	-2	-1		0	+1	+2	+3
T_0	9.45 ^a ± 0.11	9.68 ± 0.38	8.40 ± 0.13	8.32 ± 0.11	7.60 ^b ± 0.18	8.26 ± 0.10	8.44 ± 0.12	10.34 ± 0.26	
T_1	9.38 ^a ± 0.15	9.08 ± 0.13	8.28 ± 0.11	7.57 ^b ± 0.12	5.67 ^b ± 0.14	7.14 ^b ± 0.11	8.67 ± 0.11	10.04 ± 0.16	
T_2	9.37 ^a ± 0.14	9.10 ± 0.12	8.32 ± 0.11	7.61 ^b ± 0.04	5.38 ^b ± 0.11	6.30 ^b ± 0.12	8.71 ± 0.10	10.04 ± 0.16	

(B)									
Treatments	OBE								
	Ref days	Days before oestrus			On the day of oestrus	Days after oestrus			
		-3	-2	-1		0	+1	+2	+3
T_0	9.45 ^a ± 0.11	9.43 ± 0.03	8.83 ± 0.13	8.53 ± 0.17	6.20 ^b ± 0.10	8.40 ± 0.11	9.23 ± 0.03	9.40 ± 0.10	
T_1	9.70 ^a ± 0.65	9.18 ± 0.10	8.46 ± 0.10	7.15 ^b ± 0.14	5.23 ^c ± 0.16	7.68 ^b ± 0.24	8.93 ± 0.11	9.55 ± 0.15	
T_2	9.38 ^a ± 0.15	9.05 ± 0.03	8.51 ± 0.01	7.43 ^b ± 0.03	5.10 ^b ± 0.11	7.28 ^b ± 0.03	9.00 ± 0.10	9.71 ± 0.01	

Values with different superscripts in the same row are different ($P < 0.05$).

Values with different superscripts in the same column are different ($P < 0.05$).

in heifers of the T_1 and T_2 groups compared to the values on the respective reference days for these groups. In heifers of the T_0 group this value was less ($P < 0.05$) only on d 0 when there was a SE (7.60 ± 0.18 h) as well as when there was an OBE (6.20 ± 0.10 h) as compared to the reference value (9.45 ± 0.11 h Table 3A, B).

3.1.8. Lying time

There was a lesser ($P < 0.05$) mean amount of time devoted to lying on d -1 (8.34 ± 0.12, 7.85 ± 0.58 and 7.61 ± 0.11 h) and d 0 (6.87 ± 0.24, 5.17 ± 0.50 and 5.78 ± 0.11 h) when there was a SE in heifers of the T_0 , T_1 and T_2 groups compared to the values on the reference day for these groups (10.25 ± 0.25, 10.50 ± 0.24 and 9.88 ± 0.10) (Table 4A). When there was an OBE, however,

Table 4

Mean duration of time spent lying (h/day) of Sahiwal heifers without bull exposure (T_0), with fenceline bull exposure (T_1), or fenceline and direct bull exposure (T_2) on the 3 days before (d -3, -2, -1), day of (d 0) and 3 days after oestrus (d +1, +2, +3) when there was a silent oestrous (SE, $n = 18$) (A) or an overt behavioural oestrous (OBE, $n = 21$) (B).

(A)									
Treatments	SE								
	Ref days	Days before oestrus			On the day of oestrus	Days after oestrus			
		-3	-2	-1		0	+1	+2	+3
T_0	10.25 ^a ± 0.25	10.17 ± 0.44	9.43 ± 0.12	8.34 ^b ± 0.12	6.87 ^b ± 0.24	9.74 ± 0.12	9.93 ± 0.15	10.70 ± 0.25	
T_1	10.50 ^a ± 0.24	9.61 ± 0.49	9.10 ± 0.49	7.85 ^b ± 0.58	5.17 ^b ± 0.50	9.18 ± 0.65	9.87 ± 0.58	10.17 ± 0.50	
T_2	9.88 ^a ± 0.10	9.10 ± 0.10	8.32 ± 0.10	7.61 ^b ± 0.11	5.78 ^b ± 0.11	8.91 ± 0.11	9.71 ± 0.10	10.04 ± 0.16	

(B)									
Treatments	OBE								
	Ref days	Days before oestrus			On the day of oestrus	Days after oestrus			
		-3	-2	-1		0	+1	+2	+3
T_0	10.25 ^a ± 0.25	10.15 ± 0.20	9.95 ± 0.10	7.30 ^b ± 0.20	6.25 ^b ± 0.10	9.80 ± 0.20	10.25 ± 0.25	11.10 ^b ± 0.10	
T_1	10.80 ^a ± 0.24	10.18 ± 0.26	8.46 ^b ± 0.18	7.94 ^b ± 0.34	5.62 ^b ± 0.13	9.65 ± 0.62	11.80 ^b ± 0.24	11.34 ^b ± 0.21	
T_2	9.88 ^a ± 0.10	9.72 ± 0.10	8.08 ^b ± 0.18	7.50 ^b ± 0.10	5.52 ^b ± 0.17	9.74 ± 0.35	11.88 ^b ± 0.10	11.18 ^b ± 0.18	

Values with different superscripts in the same row are different ($P < 0.05$).

Values with different superscripts in the same row are different ($P < 0.05$).

the mean lying time was less ($P < 0.05$) on d -2, d -1, and d 0 in heifers of the T_1 and T_2 , and d -1 and d 0 in the T_0 group compared to values on the reference day for these groups. Furthermore, there was a greater ($P < 0.05$) amount of mean lying time on d +3 in heifers of the T_0 and on d +2 and d +3 in heifers of the T_1 and T_2 groups as compared with the values on the reference days for these groups (Table 4B).

3.2. Diurnal pattern of onset of oestrus

Of a total 21 heifers recorded to be in oestrus, 47.62% commenced oestrus during evenings (1500–2100 h) followed by 38.10% during early mornings (0300–0900 h). Conversely, the percentage of heifers commencing oestrus during night (2100–0300 h) and day time (0900–1500 h) was 9.52% and 4.76%, respectively.

3.3. Duration of oestrus

The duration of oestrus ranged from 4 to 10 h for heifers with a SE and from 9 to 21 h for heifers with an OBE in all treatment groups. The mean duration of oestrus did not differ ($P > 0.05$) for heifers of the T_0 , T_1 and T_2 groups with a SE (6.12 ± 0.65 h, 7.41 ± 0.40 h and 7.98 ± 0.47 h for the T_0 , T_1 and T_2 groups, respectively) or an OBE (15.70 ± 0.80 h, 15.95 ± 0.66 h and 16.16 ± 0.75 h for T_0 , T_1 and T_2 groups, respectively). Standing oestrus was not observed in any of the heifers with a SE. The mean duration of standing oestrus in heifers with an OBE was 10.11 ± 0.50 h, 10.81 ± 0.61 h and 11.78 ± 0.58 h for the T_0 , T_1 and T_2 groups, respectively, and did not differ among treatment groups ($P > 0.05$).

4. Discussion

4.1. Oestrous behaviour

The oestrous behaviours of sniffing/licking, tail raising, micturition, chin resting and soliciting mounting attempts by heifers were first observed on d -3 or d -2 before oestrus and then the frequencies increased consistently until the time standing oestrus was detected. Thereafter, these frequencies gradually decreased and ceased completely by d +2 to d +3.

4.1.1. Sniffing/licking

Normally the behaviour of sniffing the genitalia and licking the vulva of other cows occurs much more frequently when there is an OBE during pro-oestrus. When there was a SE, the frequencies of this behaviour remained basal because of a lack of oestradiol at concentrations sufficient to induce behavioural symptoms of oestrus (Sveberg et al., 2011). In the present study, however, the average for frequencies of sniffing/licking when there was a SE was greater from d -2 to d +2 in heifers of the T_2 than T_1 and T_0 group. This may be attributed to the release of olfactory molecules by oestrous females responsible for attracting the bull and subsequently

stimulating bull sexual behaviour. The bull in the T₂ treatment group had a greater opportunity of sniffing/licking as it was physically present within the group, while there were less opportunities for the bull to sniff/lick heifers through a fenceline barrier in the T₁ group. Lopez-Gatius et al. and Vyas et al. also reported an increased physical activity of females during direct bull exposure, which could explain the greater amount of activity of heifers in the T₂ compared to T₁ groups. If there are more oestrous females in a group, oestrous behaviours will be expressed to a greater extent (e.g., if two or more cows are approaching the time of sexual receptivity at the same time, nudging, sniffing and licking are frequently observed; Orihuela et al., 1983; Kerbrat and Disenhaus, 2004). In the present study, frequencies of sniffing/licking were less in heifers of the T₀ group when there was a SE and an OBE, probably due to the fact that only one female was in oestrus at any time in the group. When there was an OBE, the average frequency of sniffing/licking in heifers of the T₁ and T₂ group was greater as oestrous females approached the bulls via fenceline contact more often or were in direct bull contact. The findings in present study are consistent with those in previous studies of Mondal et al. (2006) and Roelofs et al. (2008) in dairy cows, and Ahmed et al. (2006) in Mithun cows. A substantial increase (almost 1.5 to 2.0 times) in the frequency of this behaviour when there is an OBE as compared to the respective value when there is a SE may have occurred due to a greater concentration of oestradiol during oestrus. The sniffing/licking by bulls on d -3, d -2 and d +2, d +3 may be attributed to the investigative behaviour of the bull because this behaviour is also expressed during non-oestrus periods (Phillips and Schofield, 1990).

4.1.2. Tail raising

Generally, the tail raising behaviour is expressed in response to sniffing of the genitalia by males and other socially active pen-mates and occurs more frequently if the cow being investigated is in oestrus or as a result of previous exposure to progesterone (Silper, 2010). Greater frequencies of tail raising when there is a SE or an OBE, in both bull exposed groups of heifers before and during oestrus, may be attributed to the greater frequency of sniffing/licking by the bull. Importantly, when there is a SE, on d 0 the frequency of tail raising was greater in heifers of the T₂ as compared with the T₁ group. This may have resulted from greater opportunity for the bull to sniff due to direct contact, in addition to the sniffing by the bull housed in fenceline contact. The heifers of the T₁ group were attracted towards the bull housed across the fenceline barrier, but the frequency of tail raising was less, indicating the importance of direct bull contact for the expression of this behaviour. These results are similar to those reported by Lopez-Gatius et al. and Roelofs et al. (2008). The frequency of tail raising was markedly less on d +1 in all treatment groups when there was a SE and an OBE, likely due to a similar reduction in frequency of sniffing/licking after the day of oestrus.

4.1.3. Micturition

In accordance with the pattern of tail raising behaviour, micturition is also shown in response to sniffing/licking and, therefore, similar patterns for these two variables were observed. The frequency of micturition when there was a SE or an OBE was greater in heifers of the T₂ than T₁ group which may be due to the cumulative effect of direct combined with fenceline bull exposure on sniffing/licking behaviour. The results of the present study are consistent with findings of Houpt et al. (1989) where there was a greater micturition in response to greater sniffing and licking by a bull during oestrus. The frequency of micturition was less in heifers of the T₀ group which might be due to the absence of a sexually active group. The frequency of micturition was less in the present study than values reported by Mangal (2009) which could be due to differences in age and parity of the animals.

4.1.4. Chin resting

Prior to mounting, bull/cows often rest or rub their chin on the rump or back of the cow when there is a mounting attempt. The chin pressuring is used as a signal to the bull regarding the receptivity of the cow to be mounted. Findings in the present study regarding this behaviour both when there was a SE or an OBE are consistent with those of Roelofs et al. (2005) and Sveberg et al. (2011).

4.1.5. Mounting attempts by a bull or other pen-mates

There were greater frequencies of bull mounting attempts in the present study in heifers of both groups where there was exposure to a bull can be attributed to the fact that bulls may have been attracted by pheromones of oestrous females that stimulated the bull to assess female receptivity by making sham mounting attempts. Results of the present study are consistent with findings by Orihuela et al. (1983) where it was observed that there was a greater frequency of mounting attempts when cows were directly exposed to a bull compared to when there was no bull exposure. On the contrary, Roelofs et al. (2008) reported that fenceline bull exposure did not result in an increased number of mounting attempts in dairy cows exposed to bulls, possibly because the cows had to walk through one of the entrances to be able to see and interact with the bull. In the present study, however, the bull was housed across a fenceline barrier through which the bull was able to attempt to mount the females resulting in an increased frequency of mounting attempts. The frequency of mounting attempts in heifers of the T₀ group was less when there was a SE or an OBE because the mounting attempts were made by other heifers in the group which were not in oestrus. These findings are supported by results from the study of Esslemont and Bryant (1976) and Hurnik (1987) where it was concluded that mounting episodes are longer when two cows are in oestrus than when only one animal is receptive. The frequency of mounting attempts was two times greater on d 0 when there was an OBE compared with a SE which can be explained by the longer duration of oestrus-related sexual behaviours in heifers with an OBE compared with those with a SE which increases the chances of mounting occurring. Hernandez et al. (2002) also observed that there was a greater number of mounts in cows where there was expression of behavioural oestrus as compared with cows where there was a SE.

4.1.6. Standing to be mounted

Absence of expression of standing to be mounted behaviour by heifers when there was a SE in the present study may be attributed to the absence of progesterone priming prior to the first ovulation, which is likely to cause a lesser concentration of oestradiol, resulting in the absence of an immobile female stimulus to a bull for mounting. Findings in the present study are similar to those reported by Senger (1994) and Allrich (1994) in which there was no observed standing to be mounted behaviour in the absence of a bull in post-partum dairy cows when there was a SE. The greatest frequency of standing to be mounted observed in heifers of the T₂ group when there was an OBE may have occurred due to a greater concentration of oestradiol inducing heifers to take an immobile stance near the bull in the pen which in turn induced mounting by the bull. The finding in the present study is partially consistent with the findings of Orihuela et al. (1988) where there were similar frequencies of standing to be mounted in post-partum zebu cows when there was synchronization of oestrus imposed using PGF2 α before bull exposure. Mat (2014) also observed that there was a greater frequency of standing to be mounted when there was direct bull contact in post-partum Holstein Friesian cows. The lesser frequency of standing to be mounted in heifers of the T₁ compared to T₂ group in current study can be explained by the difficulty for the bull to complete mounting due to the presence of the fenceline barrier, and also due to the fact that oestrus females devoted most of the time to standing close to the fenceline barrier opposite the bull, resulting in less opportunities for female-female mounting. Orihuela et al. (1988) and Hernández et al. (2006) observed that homosexual mounting activity was inhibited to some extent in Indo-Brazil and Angus cows whenever bulls were present in cow sheds.

4.1.7. Time devoted to eating and rumination

In the present study, there was a reduction in the mean time devoted to eating on d 0 when there was a SE in heifers of the T₁ (19.67%) and T₂ (21.64%) groups. This may be attributed to the presence of bulls and release of pheromones leading to the release of oestradiol which is reported to reduce appetite (Lopez et al., 2004). Furthermore, the presence of bulls stimulated sniffing/licking, chin resting and mounting behaviours in heifers that probably resulted in a decrease in feeding duration. Ingvarstsen and Andersen (2000) and Zebari et al. (2018) reported that there was an increase in oestradiol concentration when there was a SE that might lead to inhibition of feed intake but not sufficiently adequate to increase oestrous activity, lending credence to the current findings. When there was an OBE, there was a markedly greater reduction in time devoted to eating in both bull exposed groups (T₁: 42.5% and T₂: 43.2%) and heifers of the T₀ group (29.4%) on d 0. This might be due to the increase in physical activity of heifers of all treatment groups and the involvement of heifers in mounting and other oestrous behaviours on account of the presence of a bull in both bull exposed groups. Previously, it has been suggested that the expression of behavioural oestrus was strongly correlated with oestradiol reaching peak concentration on the day before oestrus, resulting in increased activity and reduced appetite and feed consumption (Lyimo et al., 2000; Diskin and Sreenan, 2000; Lopez et al., 2004; Pahl et al., 2015 and Zebari et al., 2018). In the present study, there was a reduction in rumination time in heifers of the T₀ group only, on d 0 when there was a SE or an OBE, which might have resulted from the increased activity of heifers on the day of oestrus resulting in reduced time devoted to eating. Reith et al. and Pahl et al. (2015) also reported similar findings in Holstein Friesian dairy cows. Findings in the present study with respect to eating and rumination during oestrus are consistent with those of Zebari et al. (2018) where results indicated that heifers with a SE may have changes in their behavioural symptoms that are not apparent using routine oestrous detection approaches.

4.1.8. Time devoted to lying

In dairy cattle, restlessness is one of the most important secondary indicators of oestrus (Firk et al., 2002). In the current study, lying time decreased in heifers of all treatment groups on d -1 and d 0 when there was a SE. The magnitude of decrease in time devoted to lying when there was an SE on d -1 was 18.63%, 25.23% and 22.97% respectively, for heifers in the T₁, T₂, and T₃ groups and there was a further decrease on d 0 with values being 32.97%, 50.76% and 41.49%, respectively, as compared to the reference values for the three groups of heifers. Zebari et al. (2018) reported that there was no difference in lying time on d 0 from 3 day before and 3 day after oestrus when there was a SE. This variation might be due to differences in age, breed, season, presence of pheromonal cues and oestrous detection regimen. The frequency and duration are mostly dependent on hormone secretion by the ovaries and is also affected by the presence of bulls and other cows in oestrus (Mat, 2014). Thus, changes in hormone secretion resulting from bull exposure may promote greater behavioural expression in oestrus heifers. In the present study, heifers in the T₁ and T₂ groups devoted less time to lying from d -2 to d 0 when there was an OBE compared to the time devoted to lying on the reference days. The most probable reason for a decreased resting time during oestrus is the increased activity in response to increased release of oestrogen commencing during the pre-oestrus period. Furthermore, bulls engaged oestrous heifers in sniffing, chin resting and mounting thereby reducing the resting time for heifers. The findings in the present study are consistent with those of Roelofs et al. (2007) and Mat (2014). The results in the current study with the heifers of the T₀ group are consistent with those of Kerbrat and Disenhaus (2004); Dolecheck et al. (2015) and Zebari et al. (2018).

4.2. Duration of sexual behaviours as related to heifers with a SE and an OBE

Generally, duration of oestrus depends on the age, parity and milk production of the animal, as well as season, number of oestrous females and presence of bulls (Orihuela, 2000; Kerbrat and Disenhaus, 2004). There were shorter durations of sexual-related behaviours when there was a SE in the present study and this may be attributed to the absence of progesterone priming prior to the first ovulation with no standing oestrus (Allrich, 1994). These findings are consistent with those of Floyd et al. (2001) and Hernández et al. (2006). Furthermore, in the present study there was not any effect of bull exposure on the duration sexual behaviours associated with heifers having a SE or an OBE. The shorter OBE duration might have resulted due to the capacity of the bull to precisely detect

the onset of standing oestrus which facilitated the artificial insemination of these heifers at mid-oestrus. Natural mating or artificial insemination is known to shorten the duration of oestrus in cows (Orihuela, 2000). The mean oestrus duration in present study was 2–3 h shorter than that reported by Layek et al. for multiparous Sahiwal cows when there was no bull exposure. The mean duration of standing oestrus when there was an OBE observed in the present study was similar to that reported by At-Taras and Spahr (2001); Lopez et al. (2004) and Sveberg et al. (2011) in lactating Holstein cows. Khanh et al. however, reported a longer duration of oestrus in bull-exposed than in non-exposed multiparous dairy cows.

4.3. Diurnal pattern of oestrus

The onset of more than 85% of the periods of oestrus occur during morning and evening hours and less than 15% are initiated during the day time or late-night hours. The most probable reason for this markedly greater percentage of heifers initiating oestrous behaviour during the early morning and evening hours is the lack of activity on the farm premises meaning the animals were free to express these behaviours without much external distraction. In contrast, routine farm operations were conducted during day time with the presence of a large number of farm workers and operations of farm machinery. Furthermore, during the largest part of the day the animals remained engaged in maintenance activities such as feeding, drinking, rumination, grooming and socialization. Orihuela et al. (1983) also reported the greatest number of Holstein heifers in oestrus is between 0600 h and 0800 h and with a greater frequency of oestrous onset from 1800 h to 0000 h. Similarly, Pinheiro et al. (1998) observed that in the majority of Nellore cows (53.8%) oestrus began during the night (between 1801 and 0600 h), and that in 34.6% of cows the initial symptoms of oestrus were detected and there was cessation of these behaviours during the night when there was a prostaglandin-induced oestrus. Negussie et al. (2002) and Venkata Naidu and Babu Rao (2006), however, reported that the onset of oestrus occurred most frequently between 1200 to 1800 h in primiparous Fogera (Zebu) and Ongole cows.

5. Conclusion

It is concluded that the biostimulation of Sahiwal heifers by bull exposure elicited a greater expression of oestrous behaviour than that of non-exposed heifers when there was a SE or an OBE. The direct bull exposure in conjunction with fenceline bull exposure appeared to have a greater effect than only fenceline exposure in improving the oestrous behaviour expression in pubertal heifers.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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