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Effects of Biostimulation on Oestrus Behaviour, Ovulation Time and Conception Rate in Primiparous and Multiparous Beef Cows

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Abstract: The objective of this experiment is to describe the influences of biostimulation on oestrus behaviour, ovulation time, progesterone levels and conception rate in beef cows. Three groups of cattle were investigated; CNB, PB and MB which comprised 17 multiparous, 12 primiparous and 13 multiparous cows, respectively. On the 3rd day of CIDR insert, three bulls were introduced into the MB and PB groups for biostimulation and breeding. CNB cows were AI 12 h after standing heat. Blood samples were collected every 3 days from CIDR insertion to end of experiment. Ovulation time and pregnancy were determined by ultrasound twice daily for 4 consecutive days and on day 35 after CIDR removal, respectively. Overall, mean percent of cows in oestrus was 90.52%. PB and MB scored higher than CNB for oestrus intensity. Mounting another cow being mounted but not standing and standing oestrus were commonly exhibited during oestrus. Oestrus duration was significantly longest in PB. Conception rate in PB and MB were three times higher than CNB. Only primiparous cows displayed longer, more intense oestrus and longer ovulation time. Conception rate is higher by natural mating than AI.

Key words: Biostimulation, oestrus behavior, ovulation, pregnancy rate, natural mating, Malaysia

INTRODUCTION

For successful fertilization, determination of time from insemination to ovulation is indispensable and observation of behavioral oestrus is pertinent. Signs of oestrus include standing to be mounted, sniffing the vagina of another cow and resting of chin and mounting behaviour (Roelofs *et al*., 2005). Detection of oestrus behavioral signs must be done accurately and efficiently and these depend on certain factors such as frequency as well as duration of oestrus and timing of observable oestrus periods (Galina and Orihuela, 2007). Ultrasound is another method that has been successfully applied to determine the correlation between oestrus signs and ovulation time. As is generally known, not all cows show oestrus behavioral signs; cows with silent oestrus could be checked to determine ovulation time by ultrasonography. This will contribute to the success of determining the optimal time for artificial insemination (Hanzen *et al*., 2000).

Biostimulation, a stimulatory technique that uses stud bulls to enhance oestrus and ovulation through genital or pheromonal stimulation can contribute to the successful detection of oestrus behavioral signs and eventually improve the success rate of AI and eventually reproduction. Close physical contact of bulls with anoestrus and postpartum nursing cows have been proven to accelerate resumption of ovarian cyclicity (Custer *et al*., 1990; Rekwot *et al*., 2000; Berardinelli and Joshi, 2005a). When beef cows were exposed to bulls, the postpartum anovulatory interval was reduced (Berardinelli and Joshi, 2005a). Berardinelli and Joshi (2005a) have shown that pheromones present in bulls' excretory products were the mediating factors that hastened postpartum resumption of luteal function.

There is a correlation between oestrus behavioral signs and time of ovulation in cattle without biostimulation (Roelofs *et al*., 2005). The stimulatory effect of bulls on oestrus manifestation and ovulation in cows is not well understood. Failure to detect oestrus that leads to mistimed AI in breeding cows has become an increasing problem to farmers causing huge economic loss. Therefore, the aim of the present study was to compare oestrus behavioral signs, ovulation time and pregnancy rate between biostimulated and non-biostimulated beef cows.

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MATERIALS AND METHODS

Animals and management: The experiment was conducted from August to December 2010. At the start of the study, 34 multiparous and 17 primiparous cows were selected from a herd of 105 KK cows. No cows selected via palpation per rectum were pregnant. Then, the 34 multiparous cows were equally divided into two groups. The 1st group (CNB) consisted of 17 multiparous cows without bulls whilst the 2nd group (MB) were with bulls. The 3rd group (PB) comprised 17 primiparous cows with bulls. The KK cows were between 2 and 6 years old, weighed from 150-225 kg and with Body Condition Score (BCS) of 5-7 (Scale from 1 = Emaciated to 9 = Obese) (Eversole *et al*., 2009). Three 4-5 years old KK bulls with body weight from 280-350 kg were used to biostimulate the cows. The semen quality of the three bulls, evaluated prior to the experiment met the standards for breeder bulls. All the animals were managed under the same environmental condition and followed the same vaccination programme. They were allowed to graze freely on pasture and water was given *ad libitum*. The cows were supplemented with a commercial concentrate (crude protein≤16%, crude fibre≥22.5%, calcium≤1.5%, crude fat≥2.6%, moisture≤10.6%, phosphorous 0.49%) at 2 kg/cow.

Oestrus synchronization: The experiment was carried out in two batches. The 1st batch involved cows in the MB and CNB groups. In this batch, oestrus was synchronized by CIDR® (Controlled Intravaginal Drug-releasing Device; Pharmacia Limited Company, Auckland, New Zealand; 1.38 g progesterone) and each cow was injected with 2 mg oestradiol benzoate (Cidirol®; Victoria, Australia) intramuscularly on the 1st day of the experiment. On day 10, CIDR was withdrawn and the cows were injected with 250 ug cloprostenol (Estrumate®; Schering-Plough, Canada). On day 11, the cows received an additional 1 mg oestradiol benzoate. Oestrus signs were observed as well as dominant follicles were scanned twice a day for 4 days. After the oestrus synchronization is completed for the first batch, we proceeded with the 2nd batch that involved the primiparous cows (PB). The same oestrus synchronization protocol used in the 1st batch was followed.

Biostimulation of cows: The three bulls were first introduced into the MB group on the 3rd day of oestrus synchronization for 12 days. The MB cows were housed in a paddock that was 1 km away from the other 2 groups of cows. After biostimulation of MB cows has completed, the three bulls were rested for 10 days. Then, the three bulls were introduced into the PB group on the 3rd day of oestrus synchronization for the same length of time as the MB cows. The PB cows also were housed 1 km away from the paddocks of MB and CNB cows. Thus, it is unlikely that the bulls were seen, heard or protected by olfaction of the cows in the CNB groups.

Table 1: Scoring scale for oestrus signs observed

Oestrus signs	Points
Vulva mucus discharge	3
Flehmen reflex	3
Restlessness	5
Sniffing the vulva of another cow	10
Mounted but not standing	10
Resting with chin on the back of another cow	15
Mounting other cows (attempt)	35
Mounting head side of other cows (attempt)	45
Standing heat	100

Restlessness can be recorded only once during an observation period (Van Eerdenburg *et al.*, 2002)

Detection of oestrus: Cows in each group were observed for oestrus signs twice a day after the removal of CIDR at early morning (6:00-9:00a.m.) and late evening (18:00-21:00p.m.). Oestrus behavioral signs were observed, scored and recorded according to Van Eerdenburg *et al.* (2002) scoring points as shown in Table 1. Observation was done at a suitable location, without animals being disturbed. A cow was considered in oestrus if the sum of the points of oestrus signs was ≥100 (Roelofs *et al.*, 2005).

Ultrasonography of the ovaries: After CIDR removal, the ovaries of each cow were examined twice a day (morning and at night following oestrus) for 4 consecutive days beginning on day 10 via an ultrasound scanner (Aloka SSD-500, Japan) per rectum. The scanner was equipped with a 5 MHz linear rectal transducer. Ovulation was determined by the disappearance of the largest follicle (>7 mm) between two consecutive scannings.

Artificial insemination and pregnancy diagnosis: The CNB cows were artificially inseminated with frozen-thawed KK semen, 12 h after the cows displayed standing heat. For CNB cows that did not express any oestrus behaviours or standing heat, AI was done at 50 h after CIDR removal (Bremer *et al*., 2004). Pregnancy status of all the cows in the 3 groups was diagnosed via ultrasonography 35 days after CIDR removal.

Blood sampling and progesterone assay: Blood samples were collected every 3 days from the beginning of treatment until the day of CIDR removal (day 1, 4, 7, 9) and a day after CIDR removal (day 10), on the day of ovulation (12 days) and twice time a week after ovulation (15, 18, 21, 24, 27, 30, 34, 38, 42 and 45 days). About 5 mL blood was collected from the coccygeal vein and placed into heparinised tubes (Vacutainer 20 IU heparin/mL). After collection, the samples were labeled, placed on ice and centrifuged at 1340×g for 15 min. Plasma was harvested and stored at -20°C pending assay for plasma progesterone.

Plasma progesterone level was estimated by using a sensitive Radioimmunoassay (RIA) kit (Immunotech, a Beckman Coulter Company, France). The standards are 0, 0.12, 0.55, 2.0, 8.7 and 49 ng mL^{-1}. The standards and quality control were tested in duplicates. The analytical sensitivity for progesterone is 0.05 ng mL^{-1}. The Non-specific Binding (NSB) was 1.4%. The regression for assays (r^2) was 0.997%. Intra and inter-assay coefficient of variation of progesterone is around 6.5 and 9.0%, respectively.

Statistical analysis: Duration of oestrus intensity of oestrus and ovulation time and pregnancy rate were analyzed by one-way Analysis of Variance (ANOVA) and Chi-square (χ^2) using statistical software SPSS Version 17. Duncan's multiple range test system was used to compare differences between and among treatments. Values were presented as the mean±Standard Error of the Mean (SEM). Differences were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

During CIDR treatment, 5 and 4 cows from the PB and MB groups had run away and mixed with other cowherds. Therefore, the results of the study were based on only 42 cows; about 17 cows from the CNB group, 12 and 13 cows in the PB and MB groups, respectively.

Duration and intensity of oestrus: Results of the present study showed a high proportion (80-100%) of cows in oestrus from all the 3 groups investigated (Table 2). However, there was no statistical difference ($p>0.05$) in the proportion of cows that showed oestrus between groups, although MB group had all its cows in oestrus. In addition, biostimulation did not influence the duration of oestrus in MB compared with CNB but PB cows (23.64±4.15 h) showed significantly ($p<0.05$) longer duration of oestrus than MB (12.69±2.36 h) and CNB (13.40±1.50 h). For total points scored, PB cows scored the highest points among the three groups but not statistically different compared with MB.

Distribution of individual oestrus behavioral signs: In this study, not all oestrus signs were expressed by the cows during oestrus period. Most of the cows showed mucus discharge, signs of being mounted but not standing, mounting other cows and standing heat (Table 3). However, there was significant difference on the effect of biostimulation on the oestrus sign being mounted but not standing. The proportion of oestrus cows that expressed sign of being mounted but not standing in PB (100%) and MB (92.31%) was significantly higher than CNB (52.94%).

Table 4 shows the total points scored by cows that expressed common individual oestrus signs. Flehmen and mounting other cows were signs that were significantly ($p<0.05$) higher in PB compared with MB and CNB. However, sign of being mounted but not standing was displayed more frequent and more intensive in both PB and MB than in CNB. However, frequencies of standing heat and mucus discharge were not different in the three groups.

Plasma progesterone concentration: Progesterone profiles of PB, MB and CNB cows are shown in Fig. 1. The mean plasma progesterone concentration on the day of CIDR insertion was 4.53±0.87, 7.62±1.62 and 1.66±0.34 ng mL^{-1} in PB, MB and CNB cows, respectively.

Table 2: Duration of oestrus and total points scored for oestrus signs observed among cows in each group

Groups	No. of cows	No. of cows in oestrus after treatment	Duration of oestrus (h)	Total points
PB	12	10 (83.33%)^a	23.64±4.15^a	662.40±67.08^a
MB	13	13 (100%)^a	12.69±2.36^b	547.38±78.80^{a,b}
CNB	17	15 (88.24%)^a	13.40±1.50^b	432.40±54.11^b

Table 3: Percentage of cows displayed individual oestrus signs

	Individual signs of oestrus (%)		
Oestrus signs	PB	MB	CNB
Vulva mucus discharge	75.00^a	92.31^a	64.71^a
Flehmen reflex	66.67^a	61.54^a	29.41^a
Restlessness	33.33^{a,b}	53.85^a	5.88^b
Sniffing vulva of other cows	50.00^a	30.77^a	0.00^b
Mounted but not standing	100.00^a	92.31^a	52.94^b
Chin resting on the back of other cows	8.33^a	15.38^a	5.88^a
Mounting other cows	91.67^a	100.00^a	88.24^a
Mounting head side of other cows	8.33^a	15.38^a	11.76^a
Standing heat	83.33^a	84.62^a	82.35^a

Table 4: Total points of common individual signs of oestrus expressed by cows in the three groups

	Oestrus points		
Oestrus signs	PB	MB	CNB
Vulva mucus discharge	6.67±1.20^a	4.25±0.45^a	6.00±1.21^a
Flehmen	5.62±0.68^b	3.75±0.49^a	3.60±0.60^a
Mounted but not standing	35.83±8.30^a	40.00±5.43^a	13.33±1.67^b
Mounting other cows	302.27±42.81^b	158.85±14.08^a	128.33±20.68^a
Standing heat	270.00±59.72^a	381.82±69.83^a	314.29±47.87^a

Values with different superscripts in the same row are significantly different ($p<0.05$)

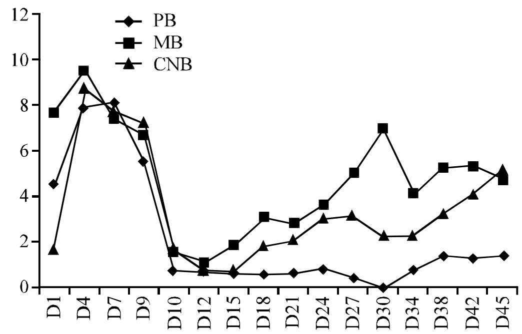

Fig. 1: Mean plasma progesterone in PB, MB and CNB cows from the day of CIDR insertion to the end of experiment

Progesterone levels of the PB and CNB groups rapidly increased 2- and 4-fold, respectively, on day 4 of treatment and slightly increased in MB cows (from around 7.5-9.5 ng mL^{-1}). Then, it gradually decreased until day 9 in the 3 groups. After CIDR removal and $PGF_{2\alpha}$ injection, the progesterone level markedly dropped and maintained low level on day 10 until day 12 in the 3 groups. However for MB cows, there was a gradual increase of progesterone level until day 18 and the progesterone levels fluctuated between 4 and 4.5 ng mL^{-1} from day 19 until day 45. Meanwhile for PB group, the progesterone level was still at low level until day 27 and dropped almost 0 ng mL^{-1} on day 30. Then, it lightly rose on later days. For the CNB, the progesterone level slowly increased from days 15-27. Then, it decreased on day 30 and rose again on later days and reached almost 5.5 ng mL^{-1} on day 45.

Ovulation time and conception rate: Ovulation was defined as the disappearance of a large follicle (>0.7 cm) that was previously recorded (Fig. 2 and 3). Table 5 shows that ovulation from onset of mounting and from onset of being mounted in PB was longer than CNB ($p<0.05$). However, ovulation from onset of standing heat was not different between each group. In PB and MB intervals from onset of being mounted to and onset of standing heat to ovulation were not statistically significant. The conception rate of PB and MB cows was significantly higher than artificially inseminated CNB cows. However, there was no significant difference in conception rate between PB and MB groups (Table 6).

Berardinelli and Joshi (2005b) have proven that the effect of bull biostimulation was mediated by pheromones. Understanding the role of pheromones can be of potential economic importance to solve some of the problems associated with animal production. Some reports on the

Fig. 2: The left ovary of primiparous cow contains a dominant follicle with 1 cm in diameter (arrow)

Fig. 3: The left ovary with 1.6 cm in diameter (big arrow) of primiparous cows after ovulation has 2 small follicles with 0.5 cm in diameter (small arrows)

Table 5: Ovulation time of oestrus cows in PB, MB and CNB groups

	Ovulation time (h)		
Parameters	PB	MB	CNB
Ovulation from onset of being mounted	54.80±3.86[a]	51.91±2.60[a]	29.67±3.07[b]
Ovulation from onset of mounting	50.70±4.09[a]	37.17±1.87[b]	37.40±2.99[b]
Ovulation from onset of standing heat	26.27±2.49[a]	26.17±2.12[a]	23.44±2.11[a]

Table 6: Comparison of conception rate between natural mating (PB and MB) and AI (CNB)

Groups	No. of cows examined	No. of pregnant cows	Pregnancy (%)
PB	12	8	66.67[a]
MB	13	9	69.23[a]
CNB	17	4	23.53[b]

Values with different superscripts in the same column are significantly different ($p<0.05$)

significant roles of biostimulation in reproduction include hastened sexual maturity induction of ovulation, reduction of postpartum anoestrus in many mammalian species (Burns and Spitzer, 1992), resumption of ovarian activity (Rekwot *et al*., 2000) and enhancement of heat and ovulation through genital stimulation (Rekwot *et al*., 2001). With this in mind, a question arises if there are any effects of biostimulation on oestrus behaviour, duration and intensity of oestrus, ovulation time and conception rate in primiparous cows.

In the present study, the proportion of cows in oestrus detected was quite high but not significantly different among the groups. Compared with a previous study (Hussein, 2008) that was conducted using the same protocol but without bulls, the percentage of MB cows in the present study detected in oestrus was higher (100% vs. 91.7%, respectively). However in another study by Alberio *et al*. (1987), they reported a significant higher proportion ($p<0.01$) of cows in oestrus when exposed to bulls (67.9%) compared with when unexposed to bulls (32.7%). The difference observed between the present study and Alberio's study can be explained by the use of vasectomised bulls for biostimulation then, there was no scoring system of Van Eerdenburg *et al*. (2002) for oestrus, beef breeds studied were Aberdeen Angus and its crossbreed and the study was carried out under different environmental condition and temperature.

The duration of oestrus was longer in PB cows than in MB and CNB cows. This probably implies the presence of a relationship between parity and exposure to bulls for biostimulation. The exposure of primiparous cows to bulls appeared to have prolonged the duration of oestrus but not for multiparous cows when compared with CNB cows. This is in agreement with two previous studies of Roelofs *et al*. (2005) with 13.6 h in primiparous cows and 10.8 h in multiparous cows and Mondal *et al*. (2008) with 14.6 h in primiparous cows and 7.7 h in multiparous cows in duration of oestrus. However, these two studies were conducted in dairy cows and without biostimulation. In the present findings the duration of oestrus was longer in PB than in MB compared with previous findings whereby, the duration of oestrus was either about the same between primiparous and multiparous cows (Lyimo *et al*., 2000) or shorter in primiparous cows (7.4 h) compared with multiparous cows (13.6 h) (Walker *et al*., 1996).

In addition, the duration of estrus (12.69 h) in the present study is longer compared with the report of Hernandez *et al*. (2006) which was 7.9 h. The current study also is in agreement with Hernandez *et al*. (2006) that biostimulation did not affect the duration of oestrus in multiparous cows. In another earlier study by Hernandez *et al*. (2004), the duration of oestrus was reported as 9.1 h in Senepol cows and 22.1 h in Brahman cows that were not biostimulated. The difference in oestrus duration observed in the present study compared with Hernandez *et al*. (2004, 2006) findings might be attributed to the differences in how oestrus was observed, types of housing and frequency of handling (Orihuela, 2000), season of the year, humidity as well as genetic factors (Hernandez *et al*., 2002). Cows in heat with high total points scored based on the scoring system of Van Eerdenburg *et al*. (1996) reflected a high frequency of oestrus signs displayed as well as strong intensity of oestrus. When an oestrus sign is intensely exhibited during oestrus, the chance of detecting cows in oestrus is higher (Van Eerdenburg *et al*., 1996; Van Vliet and Van Eerdenburg, 1996; Roelofs *et al*., 2005). Unlike primiparous cows, the influence of biostimulation was not appreciated in multiparous cows.

In the present study, the total points scored for oestrus was higher in primiparous cows (662.40±67.08) than multiparous cows (432.40±54.11). However, Mondal *et al*. (2006a) who carried out the experiment without biostimulation showed that total points of oestrus was not statistically significant between primiparous and multiparous cows (1236±125 vs. 1152±103). However, the total points scored for oestrus by Mondal *et al*. (2006a) was higher than in the present study. The reason for the higher total points scored by Mondal *et al*. (2006a) was that oestrus observation in their research was carried out at every 2 h interval. The prolonged duration of oestrus recorded in PB cows compared with CNB group was likely associated with the strong intensity of oestrus manifested. Generally, biostimulation in the present study has shown its impact on intensity of oestrus and prolonging oestrus duration in primiparous cows but not in multiparous cows.

Thus, this would give a better opportunity to detect oestrus and to carefully time artificial insemination which would result in high breeding success. A cow in oestrus usually expresses common oestrus signs such as vulva mucus discharge, flehmen reflex, chin resting, restlessness, mounting other cows, being mounted and standing heat. In the present study, not all signs were displayed simultaneously during oestrus; the frequency of expressing each oestrus sign was also different. These results are in agreement with previous results of Roelofs *et al*. (2005) and Hernandez *et al*. (2006). Irrespective of parity, the most frequent oestrus signs observed in the present study were being mounted, mounting other cows and standing heat with less frequent signs observed for chin resting, flehmen reflex and mounting head side. This present findings are in agreement with the study of Mondal *et al*. (2006a) whereby cows exposed to bulls showed more intense oestrus signs of restlessness, sniffing of vulva being mounted by bulls and other cows than cows without exposure to bulls. Frequencies of expressing mounting other cows and standing to be mounted were also very strong but not significantly different among the three groups. Among the oestrus signs expressed by biostimulated cows, the sign of being mounted but not standing accounted for the highest frequency. Hence, this sign may be used as a landmark to predict ovulation time. However, individual signs of oestrus cannot be used alone for oestrus detection because of its low frequency of occurrence and low reliability for confirmation of estrus. Using either the scoring system of Van Eerdenburg *et al*. (2002) or the method of visual detection by Mondal *et al*. (2008) is suggested as a method to determine cows in oestrus due to their higher efficiency and accuracy.

The progesterone concentration was significantly lower ($p<0.05$) in the PB cows compared with MB and CNB cows. This can be explained because the primiparous cows were younger and smaller in body size than the multiparous cows (Macmillan *et al*., 1991). On day 12, the progesterone concentration was very low 0.76±0.19 ng mL^{-1} in PB cows, 1.08±1.10 ng mL^{-1} in MB cows and 0.78±0.07 ng mL^{-1} in CNB cows and there was no significant different between each group.

In the present study, the cows also expressed oestrus behaviours the most frequent and intensed on 12 days. This is in agreement with many previous reports (Siah, 2002; Hussein, 2008; Mondal *et al*. 2006b). On day 15, the progesterone concentration still remained low level in PB and CNB cows while it slightly increased to 2 ng mL^{-1} in MB cows. The highest progesterone concentration on 15 days in the MB cows illustrates that MB had shortest duration of oestrus.

In addition, the lowest progesterone concentration maintained too long in PB cows proved that PB have spent oestrus phase quite long compared with the other two groups as well as longer duration of oestrus than the MB and CNB groups. Determining the time of ovulation plays an important role to the success of AI (Rorie *et al*., 1999). In general, the present study reveals that ovulation time in primiparous cows is longer than in multiparous cows. However, ovulation from standing heat was not statistically significant between primiparous and multiparous cows. When compared with previous reports, ovulation time in the present study was longer than what was reported previously in cows without biostimulation (Roelofs *et al*., 2005; Mondal *et al*., 2006a). Mondal *et al*. (2006a) reported the ovulation time in primiparous cow (25.3 h from mounting activity and 23.1 h from standing heat) was significantly longer ($p<0.05$) compared with multiparous cows (21.7 h from mounting activity and 20.4 h from standing heat). Although the actual cause of longer ovulation time is not clear, the stronger intensity and the longer duration of oestrus observed in primiparous cows in the present study may account for the longer ovulation time compared with multiparous cows and with those previously reported (Roelofs *et al*., 2005; Mondal *et al*., 2006a). Moreover, the observed difference in length of ovulation time might be attributed to the variations in the methods of oestrus observation, determination of ovulation time, breed, nutrition and environment (Hunter *et al*., 2004).

In the present study, higher conception rate was observed in biostimulated cows than non-biostimulated cows. This result is in accordance with a previous study (Berardinelli *et al*., 2007). The quality of frozen semen combined with stress of restraining and handling the KK cows, a very aggressive breed during AI process and ultrasonography examination (8 times) may have caused lower pregnant proportion in AI compared with natural mating (Siah, 2002). In addition, according to Mann (2001) stress was one of the factors poor pregnancy rate. Despite the difference in parity, the conception rates in primiparous cows (65%) and multiparous cows (70%) were not different. This is in agreement with a report by Lamb *et al*. (2008).

CONCLUSION

In this study, primiparous cows exposed to bulls displayed oestrus signs of being mounted and mounting activities for a longer duration and more intense than multiparous cows. Three parameters; mounting other cows, being mounted and standing heat appeared to be useful to predict ovulation time. Number of cows in oestrus and oestrus sign of standing heat were not affected by biostimulation. Conception rate in natural mating was higher than AI with no significant effect of parity on conception rate of biostimulated cows.

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