EFFECT OF BULL BIOSTIMULATION ON ANESTRUS IN MURRAH BUFFALO HEIFERS

THESIS SUBMITTED TO THE
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF

MASTER OF VETERINARY SCIENCE
IN
LIVESTOCK PRODUCTION MANAGEMENT
BY

Dr. ADITYA CHANDRAKAR
(B.V.Sc. & A.H.)

DIVISION OF LIVESTOCK PRODUCTION MANAGEMENT
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE,
(DEEMED UNIVERSITY)
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EXTERNAL EXAMINER

Dr. M.L. Kamboj
MAJOR ADVISOR AND CHAIRMAN

Members of the Advisory Committee

1. **Dr. Pawan Singh**
   (Principal Scientist, LPM Division)

2. **Dr. Ashutosh** 17/9/22
   (Principal Scientist, Animal Physiology)

3. **Dr. Nishant Kumar** Nishant Kumar 17.09.2022
   (Senior Scientist, LPM Division)

4. **Dr. Vikas Vohra** On deputation
   (Principal Scientist, Animal Genetics and Breeding)
   (Jt. Director's Nominee)

LIVESTOCK PRODUCTION MANAGEMENT DIVISION
NATIONAL DAIRY RESEARCH INSTITUTE
(DEEMED UNIVERSITY)
(Indian Council of Agricultural Research)
KARNAL – 132001 (HARYANA), INDIA

Dr. M.L. Kamboj
Principal Scientist (LPM)

CERTIFICATE

This is you certify that the thesis entitled, **"EFFECT OF BULL BIOSTIMULATION ON ANESTRUS IN MURRAH BUFFALO HEIFERS"** submitted by **ADITYA CHANDRAKAR** towards the partial fulfilment of the requirements for the award of the degree of **MASTER OF VETERINARY SCIENCE IN LIVESTOCK PRODUCTION MANAGEMENT** of the **ICAR- NATIONAL DAIRY RESEARCH INSTITUTE (DEEMED UNIVERSITY)**, Karnal (Haryana), India, is a bonafide research work carried out by him under my supervision and guidance, and no part of the thesis has been submitted for any other degree or diploma.

Dated September 17, 2022

Dr. M.L. Kamboj
Major Advisor

Dedicated

to Almighty God

and

Beloved Family

कोई फर्क़ नहीं सब कुछ जीत लेने में

और अंत तक हिम्मत न हारने में

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I apologize for the faux pass of the persons who have extended the help in a way or other & eventually I desire to convey my sincere thanks to such kind of hearts who helped me directly or indirectly in implementation of this voluminous work.

Where emotions are involved, words cease to come! No lexicon could have the word to express the feelings and it is like a tiny drop in the ocean of words that can reach its mark to acknowledge infinite love, affection, emotional support, constant guidance, encouragement & everlasting blessings of my beloved parents for giving shape to my personality and career.

I apologize for the faux pass of the persons who have extended the help in a way or other & eventually I desire to convey my sincere thanks to such kind of hearts who helped me directly or indirectly in implementation of this voluminous work.

Date: **ADITYA CHANDRAKAR**

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LIST OF ABBREVIATIONS

AI	Artificial insemination
AM	Antemeridian
avg.	Average
CL	Corpus luteum
cm	Centimeter
CNS	Central nervous system
CR	Conception rate
D	Day
d.f	Degrees of freedom
DMI	Dry matter intake
et al.	Co-workers
Exp.	Experiment
Fig.	Figure
FSH	Follicle Stimulating Hormone
h	Hour
hrs.	Hours
HF	Holstein Friesian
i. e.	That is
gm	Gram
kg	Kilogram
LH	Luteinizing hormone
ml	Milliliter
Mo	Month
mins.	Minutes
mg	Milligram
ng	Nanogram
N	Number of observations
No.	Number
PG	Prostaglandin
PM	Postmeridian
SE	Standard error

Sl. No.	Serial number
SS	Sum of Square
viz.	Namely
vs.	Against
VNO	Vomeronasal organ

Abstract

Anestrus is one of the most common reproductive disorders in buffalo. The aim of the present study was to investigate the effect of biostimulation on onset of estrus, estrus behaviour and reproductive performance of Murrah buffalo heifers. For this study, 24 heifers were allotted to 2 groups: T_0 (control) and T_1 (treatment) of 12 each for a period of 5 months from December 2021 to April 2022. In T_0 the heifers were not exposed to the bull and in T_1 group, the heifers were exposed to the bull directly for 6 hours daily (3hr in morning and 3hr in evening). Both groups of heifers were housed separately at a distance of about 0.5 km from each other. All the experimental animals were housed under a loose housing system and floor space (i.e., 4 m^2 covered area, 8 m^2 open area). The feeding of both groups of heifers were similar comprising of *ad libitum* feeding of seasonal green fodders and the concentrate mixture feeding. Data were analysed using SPSS software (Version 21.0) using t-test: two sample assuming equal variance. The average age and body weight of the T_0 and T_1 group before the start of the experiment was (25.85±0.32 months, 333.5±6.29 kg) and (25.94±0.48 months, 332.83±7.51 kg). The mean dry matter intake 10.43±0.24 and 10.72±0.28 kg per day in T_0 and T_1 respectively did not differ significantly between the 2 groups of heifers. The mean daily gain 507±28.1 gm/day and 605±19.34 gm/day in T_0 and T_1 respectively which was significantly ($p<0.05$) higher in T_1 group. The mean body weight 362.47±7.32 and 369.07±8.2 kg in T_0 and T_1 respectively did not differ significantly between the 2 groups of heifers. The average age at first estrus in T_0 and T_1 was 28.04±0.01 and 27.58±0.33 months. The average age at first service in the T_1 group was 28.18±0.57 months and in T_0 none of the heifers was inseminated. The conception rate in T_1 group was 57.14%. The average frequencies of estrus behaviour including sniffing and licking on the day of estrus are (12.00±3.00 vs. 26.50±3.42), tail raising (9.00±3.00 vs. 20.12±2.64), chin resting (4.00±1.00 vs. 12.00±1.34), stand to be mounted (3.00±1.00 vs. 9.75±1.16) in T_0 and T_1 respectively. which was higher in T_1 group. The mean frequency of standing in T_0 and T_1 was 678.25±4.43 and 722.57±4.93 minutes/day which was significantly ($p<0.05$) higher in T_1 group. The mean daily times spent on lying in T_0 and T_1 was 728.06±4.01 and 689.59±3.87 minutes/day which was significantly ($p<0.05$) higher in T_0 group. The mean frequency of eating and rumination in T_0 and T_1 was (249.04±2.27 and 260.89±2.14) and (375.80±5.36 and 396.22±5.25) minutes/day which was significantly ($p<0.05$) higher in T_1 group. The mean drinking frequency in T_0 and T_1 was 3.76±0.39 and 4.36±0.56 which did not differ significantly among the 2 groups of heifers. The mean allogrooming frequency in T_0 and T_1 were 9.94±2.49and 20.09±2.26 which was significantly ($p<0.05$) higher in T_1 group. It was concluded that the biostimulation of anestrus buffalo heifers through direct bull exposure was effective in bringing the most of the heifers into estrus with normal expression of estrus behaviour and better reproductive performance.

सारांश

एनेस्ट्रस भैंस में सबसे आम प्रजनन विकारों में से एक है। वर्तमान अध्ययन का उद्देश्य एस्ट्रस की शुरुआत एस्ट्रस व्यवहार और मुर्रा भैंस बछिया के प्रजनन प्रदर्शन पर बायोस्टिम्यूलेशन के प्रभाव की जांच करना था। इस अध्ययन के लिए दिसंबर 2021 से अप्रैल 2022 तक 5 महीने की अवधि के लिए 2 समूहों: टी 0 (नियंत्रण) और 12 प्रत्येक के टी 1 (उपचार) के लिए 24 बछिया आवंटित किए गए थे। टी 0 में गायों को साँड के संपर्क में नहीं लाया गया था और टी 1 में समूह में गायों को प्रतिदिन 6 घंटे (सुबह में 3 घंटे और शाम को 3 घंटे) सीधे साँड के संपर्क में रखा जाता था। बछिया के दोनों समूहों को एक दूसरे से लगभग 0.5 किमी की दूरी पर अलग-अलग रखा गया था। सभी प्रायोगिक जानवरों को एक ढीले आवास प्रणाली और फर्श की जगह (यानी 4 एम^2 कवर क्षेत्र 8 एम^2 खुला क्षेत्र) के तहत रखा गया। गायों के दोनों समूहों का आहार एक समान था जिसमें मौसमी हरा चारा और सांद्र मिश्रण खिलाना शामिल है। डेटा का विश्लेषण SPSS सॉफ़्टवेयर (संस्करण 21.0) का उपयोग करके परीक्षण का उपयोग करके किया गया: दो नमूने समान विचरण मानते हुए। प्रयोग शुरू होने से पहले टी 0 और टी 1 समूह की औसत आयु और शरीर का वजन (25.85±0.32 महीने, 333.5±6.29 किलो) और (25.94±0.48 महीने, 332.83±7.51 किलो) था। टी 0 और टी 1 में क्रमशः शुष्क पदार्थ का सेवन 10.43 ± 0.24 और 10.72±0.28 प्रति दिन बछिया के 2 समूहों के बीच महत्वपूर्ण रूप से भिन्न नहीं था। औसत दैनिक लाभ क्रमशः टी 0 और टी 1 में 507±28.1 ग्राम / दिन और 605±19.34 ग्राम / दिन है जो टी 1 समूह में काफी (पी<0.05) अधिक था। टी 0 और टी 1 में औसत शरीर का वजन 362.47±7.32 और 369.07±8.2 किलोग्राम क्रमशः बछिया के 2 समूहों के बीच महत्वपूर्ण रूप से भिन्न नहीं था। टी 0 और टी 1 में यौवन की औसत आयु 28.04±0.01 और 27.58±0.33 महीने थी। टी 1 समूह में पहली सेवा में औसत आयु 28.18±0.57 महीने थी और टी 0 में किसी भी बछिया का गर्भाधान नहीं हुआ था। टी 1 समूह में गर्भाधान दर 57.14% थी। एस्ट्रस के दिन सूँघने और चाटने सहित एस्ट्रस व्यवहार की औसत आवृत्तियाँ हैं (12.00±3.00 बनाम 26.50±3.42), पूंछ उठाना (9.00±3.00 बनाम 20.12±2.64), ठुड्डी पर आराम (4.00±1.00 बनाम 12.00±1.34), माउंट किए जाने के लिए स्टैंड (3.00±1.00 बनाम 9.75±1.16)। जो टी 1 समूह में अधिक था। टी 0 और टी 1 में खड़े होने की औसत आवृत्ति 678.25±4.43 और 722.57±4.93 मिनट/दिन थी जो कि टी 1 समूह में काफी (पी<0.05) अधिक थी। टी 0 और टी 1 में लेटने का औसत दैनिक समय 728.06±4.01 और 689.59±3.87 मिनट / दिन था जो टी 0 समूह में काफी (पी<0.05) अधिक था। टी 0 और टी 1 में खाने और जुगाली करने की औसत आवृत्ति (249.04±2.27 और 260.89±2.14) और (375.80±5.36 और 396.22±5.25) मिनट/दिन थी जो कि टी 1 समूह में काफी (पी<0.05) अधिक थी। टी 0 और टी 1 में औसत पीने की आवृत्ति 3.76±0.39 और 4.36±0.56 थी जो बछिया के 2 समूहों के बीच महत्वपूर्ण रूप से भिन्न नहीं थी। टी 0 और टी 1 में माध्य आवंटन आवृत्ति 9.94±2.49 और 20.09±2.26 थी जो कि टी 1 समूह में काफी (पी<0.05) अधिक थी। यह निष्कर्ष निकाला गया कि प्रत्यक्ष बैल जोखिम के माध्यम से एनेस्ट्रस भैंस बछिया का बायोस्टिम्यूलेशन एस्ट्रस व्यवहार की सामान्य अभिव्यक्ति और बेहतर प्रजनन प्रदर्शन के साथ अधिकांश बछिया को एस्ट्रस में लाने में प्रभावी था।

CHAPTER –1

Introduction

INTRODUCTION

Heifers are the future cows/buffaloes of the herd but unfortunately, heifers are the most neglected stock because of poor feeding, care and management. Buffalo is regarded as a sluggish breeder because of certain characteristics such as late maturity, poor expression of estrus signs, especially during the summer, irregular oestrous cycle, silent heat, seasonality in breeding, poor conception rate and a long inter-calving interval which reduce the reproductive efficiency (Madan,1990). Buffalo is also a difficult breeder because of its natural susceptibility to environmental stress, which promotes anestrous and repeats breeding. These two factors are to blame for the buffaloes protracted inter-calving time, which results in significant financial losses (Rangamma *et al*., 2016). The traditional goal is to produce healthy calves each year. This can only be achieved by improving the fertility of the animal. Therefore, reproductive efficiency is one of the most important factors in a viable dairy system. Delayed puberty in dairy heifer's costs farmers and the country a lot of money because it reduces the number of calves produced and milk yield, as well as increases the cost of feeding before they start producing.

Buffalo farming can be made profitable with frequent breeding and calving at the appropriate times. Oestrous cycle length in buffaloes ranges from 17 to 26 days with a mean of around 21 days (Jainudeen and Hafez, 1993). Anestrus is one of the most common reproductive disorders in buffalo. Buffalo anestrus is observed in India at a rate ranging from 25 to 67% (Singh *et al*., 2003; Pandit, 2004). In India, a clinical assessment indicated that buffaloes have a higher rate due of anestrus and inactive ovaries than cows (Nagaraju *et al*., 1991). Rural areas have a higher percentage of anestrus, mainly to hunger and poor management practices (Kumar and Sharma, 1991). In buffaloes, oestrous behaviour is critical for detecting oestrus and deciding the timing of artificial insemination. Shorter oestrous durations and less intense oestrous behaviour might lead to an increase in "silent" oestrus (ovulation without easily detectable oestrus behavioural symptoms), which is a major hurdle to improving oestrous detection accuracy and efficiency (Walker *et al*., 2008; Layek *et al*., 2011; Choudhary *et al*., 2019).

Heifers must be produced to replace the farm's older and less productive females through voluntary culling. Heifer production is the most costly segment of dairy farming (Heinrichs, *et al*.,1993). It requires more input over the long term and has no concrete

benefit. Buffalo's reproductive performance is significantly influenced by age at puberty, especially in systems with seasonal breeding (Ferrell,1982). Early puberty is necessary to reduce the age at first conception and first calving, and to achieve optimal lifetime productivity (Lesmeister *et al.*,1973). Therefore, one of the primary goals in dairy farming is to shorten anestrus periods in order to achieve a more sustainable level of output. In buffalo, this means (a) lowering the age of the first service (Patterson *et al*., 2003) and (b) shortening the cyclic activity during the postpartum period (Peter *et al*., 2009).

Under Indian conditions the age at first calving in buffaloes ranges from 40 to 45 months, but it can be reduced to as little as 30-32 months with better management. So far, major research efforts have been concentrated on improving feeding by supplemental feeding of energy and protein or growth promoters, feed additives, vitamins and minerals, which has given mixed results. Another approach has been tried by the use of hormones but these are costly and unwarranted use of hormones may disturb the normal endocrine physiology of the animal. Priming of the peripubertal dairy heifers by the provision of social cues such as through exposure to adult males (biostimulation) has given encouraging results in indigenous breeds of cattle (Choudhary and Kamboj, 2019) and in buffaloes (Dutt, 2020). Biostimulation (male/bull effect) refers to the stimulatory effect of a male on estrus and ovulation through genital stimulation, olfactory pheromones or other less well-defined external cues such as tactile, visual and auditory **(**Fiol and Ungerfeld, 2010). The contact of a bull with a cow influences reproductive activity via olfactory cues via pheromone which plays a very important role in biostimulation.

Pheromones are chemical signals that are utilised by animals to communicate with one another (Wyatt, 2010). Pheromones are a primary biostimulatory factor that can work in two ways: as a signalling pheromone that induces an immediate behavioural response, or as a priming pheromone that affects physiological processes by inhibiting or stimulating the neuroendocrine system, influencing reproduction. Pheromones in mammals communicate species-specific information by activating the vomeronasal organ, which comprises bipolar, sensory neurons. These neurons provide chemical signals to the olfactory bulb, which projects to a vomeronasal nucleus in the amygdala, causing the hypothalamus to release luteinizing hormone-releasing hormone in response (LHRH). LHRH's extra pituitary activity stimulates the secretion of LH, which aids in the regulation of reproductive behaviour and puberty induction. (Meredith,1991). Independent of cognitive recognition, these specialised neurons are likely to trigger a cascade of neuro-

endocrine reactions (Patra *et al.*, 2012).

Any of the following strategies can be used to achieve biostimulation: I) Direct bull contact; ii) Bull contact along the fenceline; iii) Bull urine contact iv) exposure to testosterone-treated steers or females, or a combination of the previous methods. Biostimulation has been introduced to increase the percentage of cows in oestrus while also shortening the post-partum anestrus period (Zalesky *et al*., 1984). Biostimulation has been found to have a positive effect on uterine involution and the duration of the first postpartum estrous cycle (Landaeta, 2004). Several studies have found that having a male present not only helps to detect estrus more accurately, but also promotes estrus behaviour and ovarian activity (Mat *et al*., 2014).

By enhancing postpartum ovarian activity and promoting estrus expression, it has been discovered that the presence of a sexually mature male animal during the mating season has a positive impact on the onset of estrus in sheep and goats as well as raising the expression of estrus in sows. (Langendijk *et al*., 2000; Rekwot *et al*., 2001). In this backdrop, it was hypothesized that biostimulation can be employed to reduce the economic losses due to delayed and prolonged anestrous in heifers. The study was therefore, undertaken with the following specific objectives:

1. **To study the effect of direct bull exposure on the onset of estrus and estrus behaviour in anestrus buffalo heifers.**
2. **To study the reproductive performance of buffalo heifers exposed to a bull for overcoming anestrus.**

CHAPTER –2

Review of Literature

REVIEW OF LITERATURE

1.1 Anestrus in buffaloes

One of the most essential dairy animals is the buffalo, which is primarily found in tropical and subtropical regions of the world. One of the most common reproductive issues in buffaloes is anestrus. Anestrus is defined as the absence of the regular signs of estrus with absence of physiological sign of estrus associated with corpus luteum or absence of palpable follicular or luteal structure. One of the most significant factors contributing to anestrus in buffaloes is an inactive or non-functional ovary. Buffalo's poor reproductive performance is still a major global economic issue. Reduced reproductive performance in buffaloes can be attributed to a variety of factors, including delayed sexual maturity, postpartum anestrus, silent estrus, and the seasonal breeding schedule. Anestrus causes financial losses due to an increase in inter-calving period, poor net calf crops, productivity losses, medical costs, and the cost of replacing mature animals with first-calving heifers (Kumar *et al*., 2014). According to Kumar *et al*. (2013), anestrus in a buffalo causes an estimated loss of Indian Rs. 372.90 each day. Anestrus in buffalo is observed in post-pubertal heifers, as well as during pregnancy, lactation, the early postpartum phase, and the non-breeding season. Incidence of anestrus in buffaloes in India state wise is presented in Table 2.1

Table 2.1 Incidence of anestrus in buffaloes

State	Incidence (%)	References
Andhra Pradesh	30.76–50	Rao and Sreemannarayanan, 1982; Naidu, 1981
Gujarat	20.84 – 45.97	Kulkarni, 2002; Modi *et al*., 2011
Madhya Pradesh	29.12 – 60. 83	Pandit, 2004; Kumar *et al*., 2013
Maharashtra	29.5–41.40	Bharkad and Markandeya, 2003;
Tamilnadu	9.09	Selvaraju *et al*., 2005
Punjab	38.98–55.5	Singh *et al*., 2006
Uttar Pradesh	14.69–45.20	Luktuke *et al*., 1973
Karnataka	56.0	Hussain, 1984

The classification of anestrus defined for cattle by Roberts (1971) can also be applied to buffalo. Roberts classified the two varieties of anestrus class I, which refers to females with a normal functioning corpus luteum, and class II, which refers to females without a functional corpus luteum. Class I anestrus includes anestrus due to pregnancy, due to persistent corpus luteum (CL), due to 'weak' or 'silent' estrus and due to unobserved estrus. Buffaloes naturally exhibit anestrus due to weak estrus, often known as "silent estrus" or "silent ovulation," which is one of the biggest problems with buffalo reproduction (Zicarelli *et al*., 1997). Class II anestrus include anestrus due to small or inactive ovaries with no functional corpus luteum. These are non-cyclic animals and termed as 'true anestrus'.

The multiple causes of anestrus in buffalo include inadequate nutrition, stress from the environment, uterine pathology and poor management techniques. The frequency of postpartum anestrus was found to be influenced by nutrition, calving season, parity, management techniques and abnormal parturition in buffaloes (Reviewed by ElWishy, 2007).

(a) **Genotype**: Under similar management conditions, the duration of postpartum anestrus is often longer in buffalo than in cattle (Dobson and Kamonpatana, 1986; Jainudeen and Hafez, 1993). Buffaloes differ from cows in that they have fewer preantral and antral follicles, smaller pre-ovulatory follicles and a greater tendency for follicular atresia (Danell, 1987; Baruselli *et al*., 1997; Samad and Nasseri, 1979) It could be the cause of the increased rate of anestrus in buffaloes.

(b) **Nutrition**: Animal nutrition has an effect on follicular development, maturation and ovulation (Diskin *et al*., 2003). One of the most common causes of anestrus in heifers is undernutrition. Reduced feed intake in the late stages of pregnancy or the early postpartum period or negative energy balance (NEB) caused by a very high metabolic load after delivery, especially in high yielders, delays the restoration of LH pulsatility and prolongs the postpartum anestrus (Wiltbank *et al*., 1962; Rutter and Randel, 1984; Connor et al., 1990; Hegazi *et al*., 1994). Anestrus is also linked with a shortage in minerals like calcium (Ca), phosphorus (P), copper (Cu), zinc (Zn), and manganese (Mn) (Hidiroglou, 1979; Campbell *et al*., 1999). It is well known that minerals act as an intermediary between hormones and enzymes at the

cellular level and their deficiency ultimately has an impact on how effectively females reproduce (Bearden *et al*., 2004).

(c) **Season/Environment**: According to several authors (Shah *et al*., 1989; Singh and Lal, 1994; Zicarelli, 1997), buffalo cows display a significant seasonal fluctuation in estrus, rate of conception and calving rate. Even though buffalo are effectively used in humid and hot climates. The heat stress significantly decreases ovarian activity, and expressed in the form of anestrus (Singh *et al*., 2000).

2.2 Male effects on the female

To boost buffalo reproductive performance, a variety of approaches and strategies have been developed, although the majority of them require the use of several hormones as therapies. Nonetheless, there is a growing public awareness and demand for a "clean, green, and ethical" production process (Martin *et al*., 2004). As a result, other more natural ways to farm management, such as biostimulation, have been promoted to limit the use of hormone treatments (Fiol *et al*., 2010). The stimulatory pathway of biostimulation is shown in Fig 2.1.

It has been discovered that the presence of a sexually mature male animal during breeding season causes early onset of oestrus in sheep and goats as well as greater oestrus expression in sows because it stimulates postpartum ovarian activity (Langendijk *et al*., 2000). The oestrus cows will generally move closer to the bull if a bull is kept in a corral near the cows (Gordon, 1996).

Izard and Vandenbergh (1982) added to the evidence that social interaction between bulls and prepubescent heifers can lead to a younger puberty age because LH release was enhanced immediately after exposure to the bull. Custer *et al*. (1990) speculated that the effect of bull stimulation on the restart of ovarian cyclicity could be mediated through the central nervous system. The specific method of biostimulation and the transmission of cues from bull to cow remain unknown.

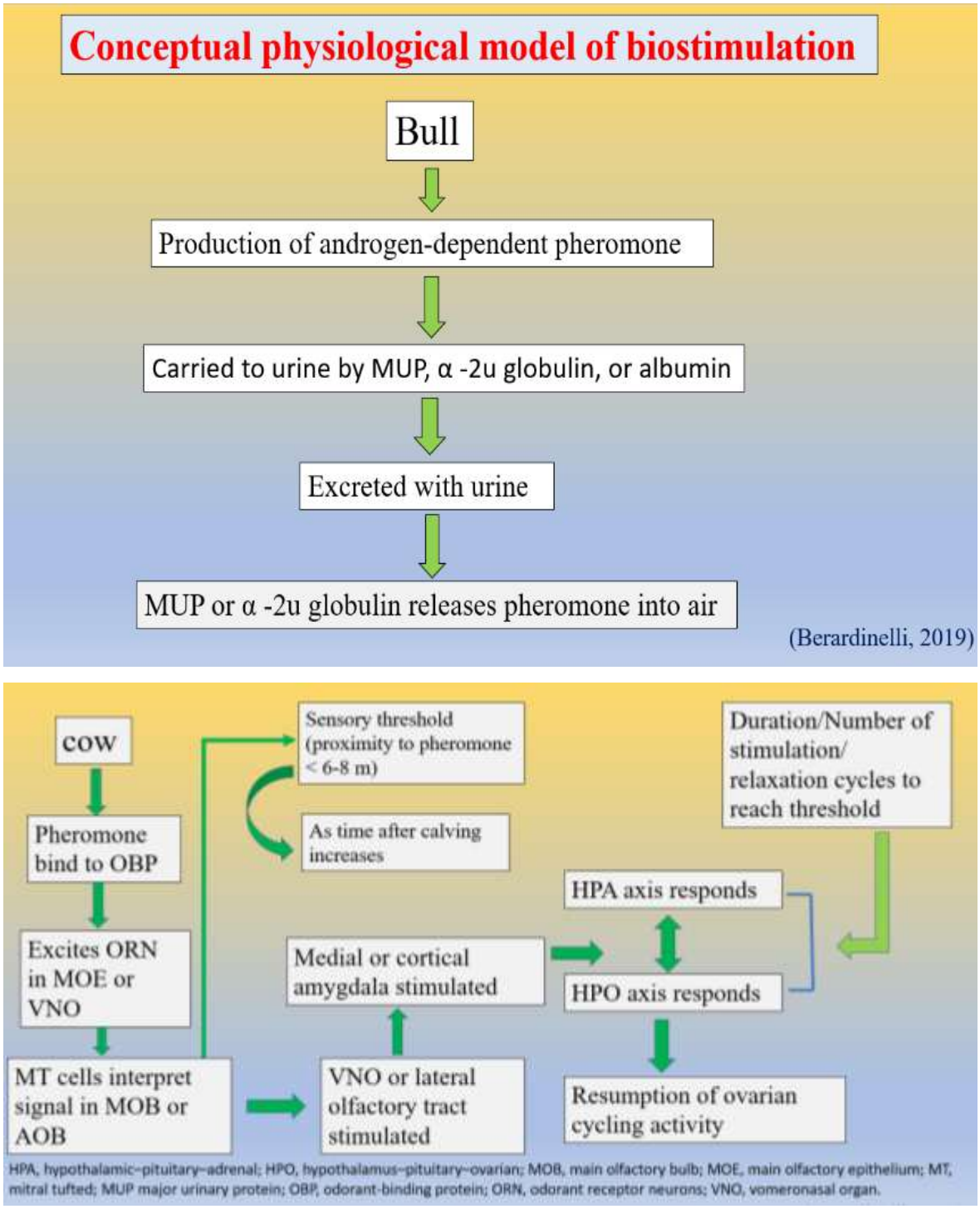


Fig 2.1 Stimulatory pathway of biostimulation

2.2.1. Biostimulatory effect of bull exposure on the onset of puberty

A heifer's puberty is determined by the time she has her first estrus. Many physiological events involving the brain, pituitary gland and ovaries lead to it. Many factors determine the age of puberty in heifers, including the dam's age and breed, the sire's

breed, environmental temperature, nutrition, body weight, growth rate, and social environment (Schillo *et al*., 1992). When a female hits puberty, she begins to cycle on a regular basis. Delays in puberty will result in a delay in the age of first breeding and the age of calving, increasing the cost of producing heifers.

The use of a biostimulation approach to expedite puberty in heifers could be a beneficial and practical tool. The presence of a male can hasten the development of puberty in rodents (Vandenbergh, 1974). In pigs, Mavrogenis and Robinson (1976) discovered that gilts who were exposed to a boar achieved puberty at a younger age than gilts who were not exposed to a boar. As a result, biostimulation by exposing female animals to males has been shown to assist speed puberty in other species. Heifers who were exposed to bulls reached puberty earlier than those who had not been exposed (Zelensky *et al*., 1984).

Izard and Vandenbergh (1982) found that 67 percent of heifers exposed to urine and 32 percent of heifers subjected to simply water reached puberty, supporting the concept that bull urine includes a priming pheromone that accelerates heifer puberty.

At the Montana State University Livestock Center in Bozeman, Roberson *et al.* (1991) conducted two experiments to test the following hypotheses: 1) Sterile bull exposure increases the proportion of beef heifers reaching puberty by 14 months of age, and 2) the rate of growth interacts with bull exposure to influence age at puberty in beef heifers demonstrated utilizing stimulated heifers from 11.5 to 14 months of age. Heifers were randomly assigned to one of two treatments in Exp. I: 1) bull exposure (BE; roughly 70 days of exposure) or 2) heifer isolation from bulls (NE) and served as controls. Heifers were fed to gain at a moderate (MG: 600 gm/d) or high (HG: 800 gm/d) growth rate in Exp. II. They were assigned to either BE or NE treatments (175 days of bull exposure) and were fed to gain at a moderate (MG: 600 gm/d) or high (HG: 800 gm/d) growth rate. Progesterone concentrations indicative of the onset of corpus luteum function and puberty were measured twice weekly in blood samples. By 14 months of age, a higher proportion of heifers getting the BE treatment than those receiving the NE treatment had begun corpus luteum function. In Exp. II, there was an interaction between bull exposure and growth rate, with the effect of bull exposure being stronger in the HG groups than in the MG groups. Finally, they showed that post-weaning gain and bull exposure have a significant relationship, with heifers exposed to bulls reaching puberty at a younger age (BE 61.8 percent vs. 45.4 percent NE). They concluded that biostimulation method presents a potentially beneficial and practicable method for enhancing reproductive efficiency in

livestock species in tropical regions.

Rekwot *et al*. (2000) studied the effect of bull exposure on 97 prepubertal heifers of Bunaji and Friesian breeds heifers, aged 13-15 months. They randomly assigned to two treatment groups: (1) heifers exposed to mature teaser bulls (MBE; n=48) and (2) heifers isolated from bulls (NBE; n=49) for a period of 15 months while working at National Animal Production Research Institute, Shika, Ahmadu Bello University Zaria Guinea. Heifers were scored for body condition and live weight at 28-day intervals and were judged to have reached puberty if they displayed oestrus and had palpable CL with a P4 concentration of more than 1ng/ml. They found that the start of puberty in MBE heifers (23.1±0.4 months) was considerably sooner than in NBE heifers (26.4 ± 0.4 months) at the end of the experiment. More MBE heifers (70.8%) reached puberty between the ages of 17 and 24 months than NBE heifers (18.3%). It was concluded that bull-cow biostimulation affects the cow's oestrus, perhaps by olfactory signals, which alters reproductive activity (pheromones).

Oliveira *et al.* (2009) conducted research to determine the effect of bull exposure on age at puberty in Nellore heifers on a farm situated in middle-west Brazil. He selected heifers (n=392) who were 360±10 days of age, weighing 180±10 kg (initial body weight, IBW) and were randomly assigned to one of four treatment groups (n=98/group) as follows 1) Heifers in the bull exposed (BE) group were maintained on pasture while being kept in the presence of bulls.; 2) the animals in the bull exposed+supplemented (S) group were kept in the presence of bulls while being managed on pasture and were fed a diet with greater energy and protein content to produce 0.49 kg of BW gain/day; 3) the animals in the control group (no bull exposure) were kept away from bulls and under pasture conditions; 4) the animals in the no bull exposed+supplemented group was kept away from bulls, were maintained on pasture, and were fed the same diet as the BE+S group. Animals were weighed monthly for weight gain and weekly ultrasound scanning to detect the CL. The first pubertal animals were identified at 480 days of age which means 120 days after the beginning of the experimental phase I in all groups. A significant BE×BE+S interaction was detected for BW at 480 days of age. At this age, the number of pubertal heifers differs between BE+S (5 out of 33), BE (5/33) vs NBE (7/33), and NBE+S (2/33). At 510 days exposure period, the number of pubertal heifers differs between BE+S (12/33), BE (8/33) then NBE (8/33), and NBE+S (12/33) and at 540 days exposure period the number of pubertal heifers was increasing BE+S (19/33), BE (19/33) vs NBE (11/33) and NBE+S

(15/33). A significant effect of biostimulation on age at puberty ($p<0.05$) was found at the end of the experiment. They concluded that differences between animal groups were primarily due to effects of early exposure on adult males. This control could represent an alternative practice in large-scale beef cattle production systems, especially in areas where climatic conditions limit the availability of feed for replacement heifers.

Fiol *et al*. (2010) reported that exposing peripubertal beef heifers to androgenized steers for 35 days advanced puberty in heavier heifers while conducting their research in the experimental unit "Palo a Pique" INIA Treinta y Tres, Uruguay with 131 Hereford and Aberdeen Angus heifers that were 12 months old and were divided into two treatment groups. 1) Exposed group (E, n=66) was exposed to androgenized steers from days 0 to 35. 2) control group (C, n=65) was isolated from the steer. Estrus behaviour detection (twice daily) and weekly ultrasound scanning to detect the CL were used to determine the cyclic activity. As of day 21, exposed heifers had a higher cumulative proportion of cyclicity than control heifers. By the end of the exposure period, more exposed heifers (16/66 vs. 2/65; $P<0.001$) had reached puberty than control heifers. It was concluded that puberty was accelerated in heavier beef heifers after being exposed to androgenized steers for 35 days; this response was shown earlier in heifers who were closer to the androgenized steers.

2.2.3. Biostimulatory effect of a bull on postpartum ovarian cyclicity

The first evidence of a bull effect on postpartum ovarian activity in cows was provided by Skinner and Bonsma (1964). They discovered that introducing a vasectomized bull to a breeding herd of females 30 days before breeding season increased postpartum ovarian activity.

Gokuldas *et al*. (2010) examined the impact of bull exposure on the restoration of ovarian cyclicity and reproductive response in postpartum buffaloes raised under typical farm conditions. A total of 24 Murrah buffaloes were assigned randomly to one of the following treatments: (1) bull-exposed (BE, n = 11) and (2) non-exposed (NE, n = 13). Animals were exposed to a vasectomized bull from the 40th to the 90th day postpartum. The restart of ovarian cyclicity was assessed using changes in progesterone levels. Bull-exposed animals had a shorter postpartum gap to resumption of ovarian cyclicity (47±2.58 days vs. 56±2.37 days, $p<0.05$) and behavioural estrus (573.61 days vs. 715.13 days, $p<0.05$) than control animals. Similarly, BE animals exhibited a significantly shorter

postpartum ovulation interval (48.69 days vs. 57.23 days, $p<0.05$). When compared to the NE group, the BE group had a lower rate of silent ovulation (18.18 percent vs. 50 percent). By 60 days postpartum, more than half of the animals in the BE group had conceived, compared to a very low number of animals in the NE group (54 percent vs. 15%, $p<0.05$). Furthermore, BE animals had a higher percentage of first service conception than NE animals (100 percent vs. 37.50 percent, $p<0.05$). Finally, continual bull exposure to buffaloes during the later postpartum period speeds up the restoration of ovarian cyclicity, reduces the incidence of silent ovulation and improves the first service conception rate. These findings suggest that introducing males to a buffalo herd could be a sound management option for minimizing postpartum anestrus and improving buffalo reproductive performance.

The effect of bull exposure on ovarian cyclicity in postpartum Murrah buffaloes was investigated by Barman *et al*. (2011). Twenty-one buffaloes, ranging in age from the first to the fifth lactation and calving between September and early February, were randomly assigned to one of two treatments:(1) a vasectomized bull (bull exposed, BE) and (2) isolation from a vasectomized bull (non-exposed, NE). From the 3rd to the 60th day postpartum, the buffaloes in the BE group (n=11) were continually exposed to a vasectomized bull, which was tethered in a semi-open shed, whereas the buffaloes in the NE group (n=10) were housed in another semi-open shed, about 0.13 km away. Following the completion of the treatment period, all BE and NE animals were mixed together and their cyclicity was observed for another 30 days. Blood samples were taken from each animal at weekly intervals from day 3 to day 30 following delivery, and then at 4-day intervals until day 90 after delivery. The criterion for the restart of ovulatory cycles was a rise in progesterone concentration of 1.0 ng/ml for at least two consecutive samples. The animals in each group were tested for signs of estrus twice a day, at 7:00 a.m. and 17:00 p.m., by parading a teaser bull. Animals in standing estrus were confirmed by rectal examination before insemination with 0.5 ml of frozen-thawed viable bull semen at 12 and 24 hours following commencement of standing estrus. At 60 days after insemination, a rectal examination was used to confirm pregnancy. When the buffalo cows were exposed to the vasectomized bull, neither (1) the postpartum interval to uterine involution, nor the incidence of initial behavioural estrus and ovulation, nor (2) the proportion of cows who resumed cyclic activity changed ($p>0.05$). When compared to the NE group, the incidence of silent ovulation was considerably ($p>0.05$) lower in the BE group (57.14 percent) (85.71

percent). In addition, the BE animals had a greater conception rate ($p>0.05$) than the NE animals (81.82 percent versus 40.0 percent). They concluded that continuous exposure of postpartum buffalo cows to a vasectomized bull curtails the incidence of silent ovulation and enhances the conception rate.

Akhtar *et al*. (2018) investigated the influence of bull exposure after calving on the postpartum interval to estrus in Nili-Ravi buffaloes at the Bahauddin Zakariya University in Multan, Pakistan. In this study, 48 buffaloes of the Nili-Ravi breed were used. A fully randomised plan utilising a 4x1 factorial design was used to assign all buffaloes to one of the four treatments. There were four groups of buffaloes:1) exposed continuously (the bull was present in the herd at all times) to the physical presence of a bull (BEC; n=12); 2) exposed intermittently (the bull was introduced for 2 h daily) to the physical presence of a bull (BEI; n=12); 3) exposed to bull discharge waste (urine and faeces) for 13.5 hrs daily (EPB; n=12); and 4) not exposed to a bull or bull discharge waste (NE; Throughout the investigation, the buffalo-to-bull ratio (12:1) was maintained. During the research, estrus was identified twice a day (07.00 a.m. and 18.00 p.m.). A buffalo stood for mounting considered to be in estrus. Buffaloes were exposed on day 5 after parturition. For each therapy, day 5 postpartum was used as d 0. The BEC therapy had a shorter postpartum gap to the first behavioural estrus than the BEI, EPB, and NE treatments. The mean serum progesterone concentration was not substantially different between BEC and the other treatments (BEI, EPB, and NE). It was concluded that buffalo that were regularly exposed to bulls had shorter postpartum intervals between estrus. Bull presence improves Nili Ravi buffalo by shortening the calving interval. It was clearly shown that having a bull around tends to help the buffalo reproduce more effectively.

During the low breeding season, Zaidi and Anwar (2018) investigated the effect of biostimulation by bull exposure on estrus expression, ovarian activity, and fertility in anestrus, pluriparous buffaloes (Bubalus bubalis) from May to July 2012. Bull exposed (24 h exposure per day, N=20, BE), bull partly exposed (1 h per day, N=20, BP), and bull non-exposed (no bull exposure, N=20, BN) groups were formed from non-pregnant, lactating Nili-Ravi buffaloes with no corpus luteum (CL) on ovaries as palpated per rectum twice at 11-day intervals. Over the course of 60 days, estrus expression and the time between services were documented (June and July). On day 60 after service, the pregnancy was evaluated. The number of animals in the BE (60%) and BP (40%) groups that displayed behavioural estrus was substantially larger ($p<0.05$) than in the BN (5%) group,

although the BE and BP groups did not differ significantly. The progesterone profile in five animals per group (blood samples taken once a week) revealed ovarian activity that matched the visually observed estrus expression. BE had a shorter time to service from the start of the experiment ($p<0.05$) than the BP and BN groups (26.3±4.9, 37.0±3.7, and 40.0±0.4 days) respectively. The difference between the BP and BN groups is not significant. The pregnancy rate was considerably greater in the BE (40%) and BP (20%) groups than in the BN (0%) group ($p<0.05$), with the difference between the BE and BP groups being non-significant. It was concluded that during the low breeding season, biostimulation for 24 hours per day was found to restore ovarian cyclicity in 60% of anestrus buffaloes.

Berardinelli *et al*. (2007) also tested postpartum resumption of ovulatory cycles in primiparous, suckled beef cows exposed to close physical touch or limited, fence line interaction with mature bulls in two tests. Experiments (Exps.) 1 and 2 included 53 and 54 spring-calving, primiparous Angus×Hereford cows, respectively. Experiments were conducted 12 months apart at the Bozeman Livestock Teaching and Research Center at Montana State University. Cows were assigned randomly to be exposed to close physical contact experiment 1 (Exp.1), BE; (n=14); limited, fence line contact (Exp. 2, BEFL; n = 22); or not exposed (Exp.1 and 2, NE; n = 37) to mature bulls beginning 59 days (Exp.1) and 68 days (Exp.2) after calving and exposed for 35 and 42 days. The percentage of cows that resumed luteal activity by the end of the Exp.1 was greater ($p<0.05$) for BE cows (100%) than for NE cows (68.8%). 2. Interval from the start of the exposure period to the resumption of luteal activity was shorter ($P<0.05$) for BE (12.2 days) and BEFL (18.1 days) cows than for NE cows (28.1) in Exps. 1 and 2, respectively. It was concluded that degree of the exposure (frequency, duration, and quantity of stimuli) to the pheromonal stimuli produced by bulls may affect how anovular, primiparous cows react to their biostimulatory effects.

Miller and Ungerfeld (2008) reported that exposing postpartum anestrus suckled beef cows to weekly bull exchanges speeds up the resumption of cyclicity when compared to continuous exposure to the same bulls and that the conception rate was higher (56.2%) in bull exchanged animals than in non-exchanged animals (35.6%).

At Montana State University in Bozeman, Tauck *et al*. (2010) observed early resumption of ovulatory activity (OA) and the percentage of cows that restarted ovarian activity in two-year-old Angus×Hereford primiparous, suckled beef cows (n = 39), which

were randomly assigned after calving to bulls exposed for 12 hours (BE12; n=15), 6 hours (BE 6; n=14), or not exposed to bulls (NE; n=10) for 45 days beginning at 51 days after calving. The interval from calving or from D 0 to the resumption of OA was shorter for BE12 (87.7 days), BE6 (90.0 days) vs NE (101.2days) ($p<0.05$), and the proportion of cows that resumed OA during the experiment was greater ($p<0.05$) for BE12 (60.0%), BE6 (64.3%) than for NE cows NE (10.0%)**.** An increase in progesterone concentration above the average progesterone baseline of individual cows in three consecutive samples that exceeded 1 ng/mL was used to determine the occurrence of resumption of OA. Blood samples were collected every other day from D 0 to the end of the experiment (D 44) from each cow by jugular venepuncture. It was concluded that compared to continuous exposure to a single pair of bulls, weekly interchange of two pairs of bulls decreased postpartum anestrus in suckled multiparous cows.

During the spring season at the University of Florida's Santa Fe Beef Research Unit, Hernández *et al*. (2013) investigate the effect of social structure and biostimulation generated by bull exposure on the restart of ovarian activity. Thirty Angus cows were divided into three groups (10 each) based on parity; two groups were exposed to bulls, while a third group was not exposed to bulls and acted as a control. Biostimulation ($p<0.002$) and dominance order ($p<0.004$) impacted the time between calving and resumption of ovarian cyclicity (ICR). As dominance order dropped, the ICR increased (D=34.5±6 days; I=45.0±6 days; S=53.1±4 days; $p< 0.01$). When comparing cows from different social groups, ICR was lower in the bull-exposed group (D=26.3±8.2 days; I=42.0±6.4 days; S=46.1±4.1 days) than in the non-exposed group (D 43.0±8.2 days; I=48.0±10.1 days; S=60.2±6.4 days). They came to the conclusion that biostimulation and social dominance had an impact on the ICR.

2.2.4. Effects of biostimulation on estrous behaviour and hormone secretion

Zicarelli *et al*. (1997), investigate the effect of biostimulation on estrus behaviour. They show the effects of the presence or absence of vasectomized male buffaloes on the reproductive efficiency of Italian buffalo cows (n=396) were tested on six farms owned and maintained by a single consortium. On each farm, lactating cows were divided into two groups of varying sizes and housed in semi-range circumstances. One group had vasectomized bulls with a bull/empty-cow ratio of 1:30. In the other group, there were no bulls. Over a 3.5-month period, reproductive efficiency was compared and evaluated between the two groups based on: 1) the number of spontaneous overt estruses associated

with feeble or intense signs of estrous behaviour; 2) the number of functional estrous cycles, i.e., estrous cycles with luteal phases defined as normal, based on specified progesterone concentrations in milk or blood plasma 8-10 days after estrus; and 3) the number of consecutive functional estrous cycles. The reproductive effectiveness of groups with bulls was substantially higher than that of those without them. There was a higher incidence of spontaneous estrus (92% versus 69%; $p<0.01$); spontaneous estrus of high intensity (62.2% versus 31.1%; $p<0.01$); and functional estrous cycles following both spontaneous (65.8% versus 57.1%; $p<0.05$) and induced (77.0% versus 59.5%; $p<0.05$) estrus. The pregnancy rate in cows inseminated at spontaneous (42.5 versus 18.9%; $p<0.01$) or induced (51.1 against 33.3%; $p<0.05$) estrus was considerably higher after exposure to vasectomized bulls (90.5 versus 68.1%; $p<0.01$. In the absence of bulls, the pregnancy rate per AI was higher in cows inseminated at induced than in cows inseminated at spontaneous estrus (33.3 versus 18.9%).

Roelofs *et al*. (2008) examined the behaviour of cows exposed to the bull at the Wageningen University and Research Center in the Netherlands. The control (no bull on the farm), the bull treatment (a bull housed behind the fence), and the no bull treatment (a bull present on the farm but not housed behind the fence). Cows were fitted with pedometers and signs of oestrus were observed every 4 hours for 30 minutes. On the day of oestrus, cows were more frequent in the contact area during the bull treatment (12.0±9.8 times) and the no bull treatment (13.9±10.2 times) than during the control treatment (2.6±2.5 times) on the day of oestrus. The number of visits to the contact region was modest (2.2±1.9 times) and did not differ between treatments on other days. On the day of oestrus (71.4%), more cows had direct contact with the bull than on days outside of oestrus (21.4-30%). Based on their findings, they concluded that dairy cows in oestrus appear to be attracted by the presence of a fence line housed bull, but fence line bull exposure does not affect oestrus behavioural expression.

The biostimulatory effect on circulating LH concentration is minimal, and it may not be enough to stimulate estradiol secretion, which would influence oestrus behaviour modifications. Cows that were continually exposed to bulls and cows who were exposed to bulls on a regular basis had higher mean LH concentrations and more LH pulses than cows who were not exposed to bulls (Fernandez *et al*., 1996). Roelofs *et al*. (2005) found that fence line bull exposure to anoestrus dairy cows during the early postpartum period had an effect on LH release, with an increase in basal and average LH concentrations as

well as the frequency of LH pulses.

The different researcher has reported the effect of biostimulation on the estrus behavior of cows as given below in table 2.2

Table 2.2: Occurrence of different estrous signs and symptoms

Estrous symptom	**Khanh *et al.* (2012)**	**Roelofs *et al.* (2005a/b) in H.F. cows**	**Mat *et al.* (2014)**
Vulva mucus discharge	75 %	--	16.7%
Flehmen reflex	66.67 %	29 %	--
Restlessness	33.33 %	--	--
Sniffing the vulva of another cow	50 %	100 %	--
Mounted but not standing	100 %	--	58.3%
Resting with chin on the back of another cow	8.33 %	100 %	41.7%
Mounting other cows(attempt)	91.67 %	--	66.7%
Mounting head side of other cows (attempt)	8.33 %	12 %	--
Standing heat	83.33	47 %	--

2.2.5. Effects of biostimulation on conception rate

By exposing buffalo cows to the bull and accelerating the restoration of ovarian cyclicity from biostimulation, the conception rate in buffaloes could be boosted. Continuous bull exposure of buffaloes during the later postpartum period (40 days post-calving) accelerates postpartum resumption of ovarian cyclic activity, reduces the incidence of silent ovulation, and improves the first service conception rate, according to Gokuldas *et al*. (2010). Biostimulation using bull stimuli is an effective approach for increasing the number of pregnant animals during the early days after calving.

Barman *et al*. (2011) discovered that exposing postpartum buffalo cows to a vasectomized bull increases the conception rate. According to Ebert *et al*. (1972), the conception rate to the first service was greater in cows exposed to the bull (68 percent),

compared to (48 percent) in cows not exposed to the bull. In a similar experiment conducted by Izard (1982), postpartum beef heifers exposed to a vasectomized bull for 3 to 4 hours, two times a day, conceived to a viable mating earlier than cows not exposed to the bull.

While conducting research at Government Farm, Pantai Timor, Kelantan, Malaysia, Khanh *et al*. (2012) found that spontaneous mating has a greater conception rate than AI. They studied three groups of cattle: 1) Controlled non-exposed multiparous (CNB; n=17) 2) Bull primiparous (PB; n=12) 3) MB (n=13) is a multiparous cow with a bull. Three bulls were put into the MB and PB groups for biostimulation on the third day of the CIDR insert. From the time the CIDR was implanted until the end of the experiment, blood samples were taken every three days. The conception rate (pregnancy rate) in PB (66.67%) and MB (69.23%) was three times higher than in CNB (23.53 percent).

2.3. Stimulation methods used in exposing the heifers to a bull

Various methodologies were used in past studies on the biostimulation of heifers and postpartum cows by bull exposure in order to define the mechanism of biostimulation.

2.3.1. Continuous or direct contact bull exposure

Previous research on beef heifers indicated that long-term continuous exposure of heifers to a mature bull had no effect on age at puberty (Roberson *et al*., 1987) and that short-term exposure of heifers to a mature bull had no effect on age at puberty (Berardinelli *et al*., 1978).

2.3.2. Fenceline bull exposure

In a study by Fike *et al.* (1996), 30 days postpartum, crossbred primiparous and multiparous beef cows from the University of Nebraska Agricultural Research and Development Center beef physiology herd were randomly allocated to one of two treatments: fence line exposure to bulls or isolation from bulls. Blood samples were taken twice weekly for 13 weeks, beginning 1 to 4 weeks after delivery, in each experiment. These samples were utilised to measure progesterone levels and establish when ovarian luteal function began after calving ($p<0.05$); however, there was no difference between the treatments in the multiparous group. Primiparous cows with fence line exposure had a shorter duration of postpartum anoestrus (BE=78 days) compared to heifers without bull exposure (NE=92 days).

In other investigations, in primiparous, postpartum, and anestrus cows, fence line exposure to bulls accelerated the restart of cyclic activity, although not as successfully as direct male-female contact. (Berardinelli *et al*., 2007).

2.4. Application of the biostimulation technique on dairy farms

On most dairy farms in India, the use of a bull is currently restricted to breeding purposes. However, exposing dairy cows to a bull and employing biostimulation technology to boost their reproductive condition has a lot of potentials. As a result, the purpose of this research is to explore if the biostimulation effect can help buffaloes reduce their anestrus period and boost their reproductive performance, in the hopes of enhancing lifetime performance and extending individual buffaloes' production lives.

2.5 Biostimualtory effect on age at puberty and reproductive performance

Choudhary *et al.* (2019) investigated the effect of biostimulation on the age of puberty and reproductive performance of Sahiwal heifers. They selected 24 pre-pubertal heifers and divided them into three groups based on age and body weight: non-bull exposed, fenceline bull exposed, and fenceline bull exposed+direct bull exposed. Heifers in the non-bull exposed group were not exposed to bulls, while those in the fenceline bull exposed group had 24 hours of fenceline interaction and those in the direct bull exposed group had 6 hours of direct bull contact. The average age and body weight at puberty in fenceline bull exposed groups (19.33±0.36 months and 226.20±6.35 kg), fenceline bull exposed + direct bull exposed (19.11±0.58 months and 224.19±4.54 kg) heifers were almost similar but significantly lower than non-bull exposed group heifers (24.13±0.16 months and 262.50±8.50 kg). The average age at first service and first calving was similar in fenceline bull exposed groups (20.41±0.45 and 30.20±0.73 months) and fenceline bull exposed + direct bull exposed (20.78±0.36 and 29.90±0.44 months) heifers but significantly lower than non-bull exposed group heifers (25.15±0.14 and 34.29±0.53 months). They concluded that biostimulation of heifers by fenceline contact with the bull is as effective in hastening puberty and enhancing reproductive performance as fenceline bull exposure + 6 h of daily direct bull contact.

2.6 Biostimualtory effect on puberty and estrus behaviour

Sahu (2022) observed that late puberty and poor estrus detection are severe reproductive difficulties in water buffaloes. Biostimulation has been demonstrated to hasten puberty and aid in the accurate detection of estrus in cattle, sheep, goats, and pigs.

In this study 24 pre-pubertal Murrah heifers were allotted to 3 groups of 8 each on the basis of age (15.08±0.16 months) and body weight (199.5±0.21 kg). In the no bull exposure group, heifers were not exposed to a bull; in fenceline bull exposure, heifers were exposed to a bull through a fenceline contact round-the-clock, and in direct bull exposure, heifers were exposed to a bull through direct contact twice daily for 6 hours. Estrus behaviours were recorded 3 days prior to estrus, d 0 (on the day of estrus), and on 3 days post estrus using 24 hours CCTV cameras. The number of heifers detected in estrus in fenceline bull exposure, direct bull exposure and non-bull exposure were 5, 8, and 5; age at first estrus was 23.55±0.85, 21.50±0.44 and 25.61±0.70 months with a body weight of 342±13.46, 348±8.53 and 330±12.45 kg respectively after 10 months of the experiment. Frequencies of estrus behaviours sniffing/licking, tail raising, micturition, chin resting, and mounting attempts on the day of the first estrus were in DBE (89.00±0.63, 14.25±0.65, 23.00±0.80, 55.13±0.13, and 23.63±0.94 respectively), in FBE (64.00±0.63, 11.20±0.74, 19.20±0.80, 32.60±1.08 and 13.00±0.71 respectively) and in NBE (35.80±0.66, 5.80±0.58, 16.40±0.51, 10.20±0.66 and 6.60±0.40 respectively). DBE has the highest incidence of these frequencies, followed by FBE and NBE. Most estrus activities began on d-3 and increased in frequency until d-0 when they began to drop on d+1 and nearly disappeared on d+3. They concluded that direct bull contact or fenceline bull contact hastened puberty and increased the expression of estrus behaviours in buffalo heifers.

2.7 Biostimualtory effect on estrus behaviour and reproductive performance

Rajput (2019) investigate the effect of biostimulation on behaviour and energy balance of Sahiwal cows during peri-estrus. The prolonged inter-calving interval caused by a delay in the commencement of post-partum ovarian function and poor estrus detection is one of the most serious reproductive difficulties, especially in native breeds of cattle in India. Biostimulation has been found to improve both estrus detection and reproductive performance in various beef breeds of cattle during the postpartum period. In this study 24 postpartum cows were divided into three groups (T_0, T_1, and T_2) of eight each based on their yield in the previous lactation in pleuriparous cows and estimated producing capacities of primiparous cows. T_0 cows were not exposed to the bull; T_1 cows were introduced to the bull through round-the-clock fenceline interaction 15-30 days after calving. The cows in T_2 were exposed to direct bull interaction for 12 hours per day. The estrus behaviours were captured by 24-hour CCTV camera recording on 3 days prior to estrus, d 0 (on the day of estrus), and 3 days after estrus. During first estrus, mean

frequencies of different estrus behaviours viz., sniffing/licking (8.75±0.52, 16.00±1.22 and 26.88±0.78), tail raising (5.88±0.61, 14.50±0.63 and 23.63±1.36), micturition (10.13±0.80, 15.50±0.48 and 18.88±0.71), chin resting (8.38±0.65, 18.62±0.77and 25.25±0.86), the total number of steps (3053.88±55.86, 3767.75±81.39 and 4106.50±87.69), mounting attempts (8.37± 0.41, 15.50±1.01 and 23.62±1.48), flehmens response (0, 6.38±0.88 and 10.88±0.97) and cow-bull agonistic interactions (6.25±0.32, 13.88±0.47 and 19.50±0.66) were significantly ($p<0.05$) higher in T_2 than in T_1 and T_0 on the d 0. In all three groups of cows, the frequency of estrus behaviours increased in the second estrus compared to the first estrus. The average days to first estrus post-partum in T_1 and T_2 were significantly lower than in T_0. They concluded that the biostimulation of sahiwal cows by exposure to bull contact from 15 to 30 days postpartum enhanced the expression as well as the intensity of estrus symptoms and decreased the days of the first estrus in postpartum when compared to non-bull exposed cows. Direct contact with a bull was found to have a greater effect on increasing the expression and intensity of estrus than contact with a bull from the fenceline.

2.8 Effect of bull biostimulation on growth performance, ingestive and resting behaviour

Dutt (2020) investigated the effect of biostimulation on growth performance of buffalo heifers. On the basis of age (16.09±0.17 months) and body weight (210.88± 2.68 kg), twenty-four prepubertal heifers were divided into three groups of eight each. Heifers in the no bull exposure group were not exposed to a bull; heifers in the fenceline bull exposure group were exposed to a bull 24 hours a day through fenceline contact, and heifers in the direct bull exposure group were exposed to a bull twice daily for 6 hours. Mean dry matter intake (kg/day) and mean dry matter intake (kg/100 kg body weight) differed significantly ($p<0.05$) among the 3 groups; being highest in DBE (8.52±0.55 and 2.73±0.15 kg) followed by FBE (7.76±0.44 and 2.67±0.14 kg) and NBE (7.14±0.36 and 2.62±0.15 kg). Feeding time, watering time, and rumination time (min/day) were lower ($p<0.05$) in NBE (261.35±3.95, 14.36±0.77, and 344.76±6.75 respectively) than in DBE (282.67±4.46, 16.86±0.90 and 368.79±6.30) and FBE (273.10±3.02, 16.32±0.94 and 361.61±5.58 respectively). The feed conversion ratio was higher ($p<0.01$) in DBE and FBE than in NBE. There were no differences in feeding frequency, water drinking frequency, rumination frequency, idle laying time, or idle standing time among the three groups. Total standing time (min/day) in DBE (692.42±14.70), FBE (709.87±14.04), and

NBE (774.21±9.97) and lying time (min/day) in FBE (724.62±13.51), DBE (744.56±15.37) and in NBE (665.79±9.97) were different ($p<0.05$) among 3 groups. Final weights and daily weight gain after 8 months differed ($p<0.01$) among 3 groups; being highest in DBE (310.34±8.02 kg; 0.764±0.02 gm) followed by FBE (289.33±5.19 kg; 0.678±0.02 gm) and NBE (271.07±4.49 kg; 0.545±0.01 gm). They concluded that biostimulation of buffalo heifers, either directly or through fenceline bull interaction, tended to improve feed intake, resting and improve the growth of buffalo heifers.

CHAPTER –3

Materials & Methods

MATERIALS AND METHODS

The present work entitled “Effect of bull biostimulation on anestrus in Murrah buffalo heifers” was carried out on Murrah buffaloe heifers maintained at Livestock Research Center (LRC), National Dairy Research Institute, Karnal. In order to achieve the specific objectives of the study the following materials and methods were used:

3.1 Location of experiment and climatic conditions

The experiment was conducted at the LRC, ICAR, National Dairy Research Institute, Karnal, which is situated at 29°43" N Latitude and 77° 2" Longitude, at an altitude of 227 m above mean sea level respectively. The maximum ambient temperature in summer goes up to 45°C and the minimum temperature in winter comes down to 4°C with a diurnal variation in the order of 15-20 °C. The annual rainfall is 70 cm, most of which is received from July to mid-September.

3.2 Experimental study

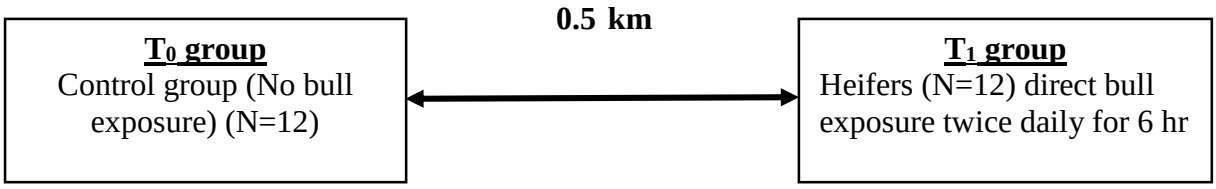
For this study, 24 Murrah heifers was selected from the LRC. These heifers were above 24 months of age and had a body weight >300 kg. Buffalo heifer divided into two groups (control and treatment) based on their body weight and age. The average age and body weight of heifers at the start of the experiment were (25.85±0.32 months, 333.5±6.29 kg) in control (T_0) group and in treatment group (25.94±0.48 months, 332.83±7.51 kg). The selected animals were confirmed as being healthy and in the anestrus condition respectively. The list of animals with their age and body weight at the start of the experiment is presented in Table 3.1.

3.2.1 Duration of experiment

The experiment was started in the month of December 2021 and lasted up to April 2022. An adjustment period of 15 days was given to the animals before the actual start of the experiment.

3.3 Experimental treatments

A total of 24 Murrah buffalo heifers selected from the herd as described in previous sections were randomly allocated to one of the following 2 treatments with 12 animals each.



3.3.1 Treatment 1 (T_1): Direct bull contact

The heifers were exposed directly to an apronized intact bull by letting in the heifer shed for a total duration of 6 hours daily (3 hours in the morning from 07.00 to 10. 00 am and again for 3 hours in the evening from 4.00 to 7.00 pm) in the heifer shed. The bull used for direct contact in this group of heifers was weekly replaced with another bull.

Plate 3.1 Heifers of treatment T_1 group (Direct bull contact group)

3.3.2 Control (T_0): No bull contact

The heifers in this group were not exposed to the bull and were housed at a distance of about 0.5 km from the sheds used for housing treatment groups of heifers.

Plate 3.2 Heifers of T_0 group (Control group)

Table 3.1: List of animals with their age and body weight at the start of the experiment

Sr. No.	Animal No.	Control (T_0) group	
		Body weight (kg)	Age (months)
1	7912	360	24.4
2	7864	359	26.22
3	7855	352	26.29
4	7871	348	26.9
5	7836	336	27.15
6	7862	324	26.24
7	7851	312	26.3
8	7866	302	26.19
9	7830	305	27.28
10	7820	320	24.9
11	7913	325	24.23
12	7923	360	24.1
Mean		**333.5±6.29**	**25.85±0.32**

Sr. No.	Animal No.	Treatment (T_1) group	
		Body weight (kg)	Age (months)
1	7832	380	27.18
2	7828	376	28.1
3	7826	338	28.6
4	7916	345	24.18
5	7842	334	26.12
6	7841	332	27.12
7	7902	305	24.25
8	7844	303	27.1
9	7863	309	26.26
10	7812	304	24.23
11	7965	326	24.13
12	7924	342	24.1
Mean		**332.83±7.51**	**25.94±0.48**

3.4 Bulls used for biostimulation

Two adult Murrah buffalo bulls aged 50 to 54 months were selected for exposure to treatment group of heifers. The bulls were intact and bull apron was tied around the animal body during exposure to heifers in order to prevent mating while mounting on heifers

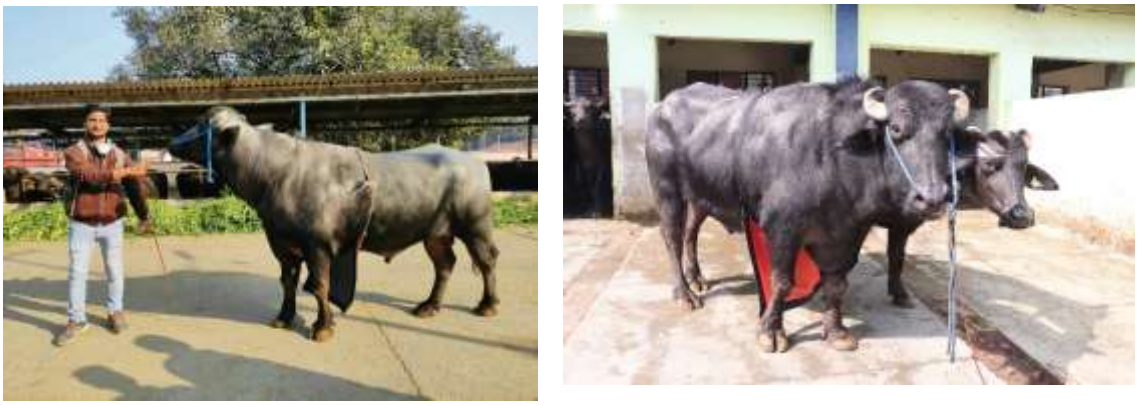


Plate 3.3 Bulls used for biostimulation

3.5 Housing management

All the experimental animals were housed under a loose housing system and floor space (i.e., 4 m^2 covered area, 8 m^2 open area), and other housing conditions were provided according to BIS standard recommendations. The feeding of all 2 groups of heifers was similar comprising of feeding of seasonal green fodders and the concentrate mixture feeding. In order to ward off the effect of airborne pheromones; the two groups of animals housed at an approximate distance of 0.5 Km from each other. Experimental sheds were equipped with CCTV cameras 24x7 hours.

3.6 Feeding management

Both the experimental groups of heifers were fed at a similar ration as per ICAR (2013) feeding standards for buffaloes. The animals were provided *ad libitum* all seasonal farm-grown green fodder, dry roughage and concentrate mixture. Green fodders available for feeding during different months of the experimental period are presented in Table 3.2

Table 3.2 Green fodder availability in LRC

Sr. No.	Month	Name of fodder crop fed to experimental heifers
1	November	Mustard and jowar
2	December	Berseem, mustard and jowar
3	January	Berseem, oats, mustard
4	February	Berseem, oats, wheat bhusa
5	March	Berseem, oats, wheat bhusa
6	April	Berseem, oats, wheat bhusa

The available concentrate mixture with 16-18 % DCP and 70 % TDN with the ingredients as mentioned in Table 3.3 was offered to animals at the rate of 1 kg per 100 kg body weight per day (around 3.0 kg to 3.5 kg/day).

Table 3.3: Ingredient composition (%) of concentrate

Ingredient	Part of total (%)
Maize grain	25.00
Barley grain/ bajra grain	10.00
Soyabean meal	12.00
Groundnut cake	6.00
Mustard oil cake	13.00
Cotton seed cake	5.00
Gram chuni	10.85
Wheat bran	10.00
Deoiled rice bran	5.00
Toxin binder	0.15
Mineral mixture	2.00
Commen salt	1.00

Fresh water was available for drinking to all the animals round the clock. Animal sheds and animals were cleaned daily and good hygienic conditions were maintained throughout the experimental period. Animals were monitored daily for the early detection of any health problem. The feeding managers were cleaned once a day in the morning at 6:00 AM, the feces were collected twice daily. The water troughs were disinfected with a fresh coat of lime at monthly intervals.

3.7 Parameters recorded

3.7.1 Recording of growth performance

3.7.1.1 Dry matter

Total dry matter intake (DMI) was calculated at fortnightly intervals on two consecutive days by tying the heifers individually by taking into account of the daily average amount of roughage and concentrate mixture consumed. The amount of concentrate mixture, green fodder, and roughage offered daily was weighed out, and the residues that remained weighed the next morning (i.e., 24 h later). A sample of all offered and remaining feed was taken for the dry matter (DM) analysis, so the DM consumed could be calculated.

$$\text{DM (\%)} = \frac{\text{(Wt. of aluminium tray + sample after drying – Wt. of aluminium tray)}}{\text{Weight of fresh sample}} \times 100$$

DMI of each heifer was calculated using the following formula: DMI = DM of the feed offered – DM of residual feed

Plate 3.4 Weighing of green fodder and drying of feed

3.7.1.2 Body weight

The body weight of the experimental animals was recorded initially at the start of the experiment and then on a fortnightly basis till the end of the experimental period. The body weight of each animal was measured early in the morning before providing the animals any feed or water, using an electronic weighing scale. The change of body weight between key periods was assessed by deducting the values of body weight at the first record in time from the second record in time.

Plate 3.5 Recording of body weight of heifers

3.7.2 Recording of heifer behaviour

The general heifer behavior and various estrus behaviour were observed through direct visual observation and video cameras were installed to record the activity of cows 24 hours per day. Two video cameras were attached to view the area of cows in the control group (cows without any bull contact), and another three were placed to view the area of cows in the treatment groups (cows with bull exposure). The video observations were analyzed for changes in behaviour of contacting the bull on the day of estrus.

All the behaviour parameters were recorded by digital video recording done by five CCTV outdoor cameras (CP Plus). The camera had 8x digital zoom for closer viewing. The cameras were enabled with an array of infrared technology for the best night vision. Cameras were installed at different places and at different angles in the experimental shed so that the whole shed can be covered from a viewing angle. The images and video were

stored in 16 channel digital video recorder (DVR) with a hard disk of 1 TB space. All the parameters were recorded in hours: minutes format initially which were later changed to hours according to the needs of the parameters.

3.7.3 General behaviour

1. Eating time

The average eating time was recorded at the fortnightly interval by video recording of 24 h. It is the total time the animal has taken for feeding all feedstuffs including green and dry fodders and concentrate mixture.

2. **Rumination time**

Rumination time was recorded at the fortnightly interval as the total time spent by an animal to ruminate the previously ingested food during the day. It is represented in minutes per day.

3. **Lying time**

It is the time when the animal was sitting calmly in the shed. It is represented in minutes per day. It was also recorded fortnightly.

4. **Standing time**

It is the total amount of time spent by the animal in standing posture. It is represented in minutes per day. It was also recorded fortnightly.

3.7.4 Estrus behaviours

Expression of the estrus behaviour defined as "stand to be mounted" was considered as the primary sign of estrus, while signs including sniffing/licking of the vulva by a bull and other herd mates, tail raising in response to sniffing/licking of the vulva by a bull and other herd mates, chin resting, vulval discharge and mounting other heifers were considered as the secondary signs of estrus.

For this purpose, the following signs of estrus were recorded

- Stand to be mounted
- Mounting other heifers
- Chin resting
- Mucus discharge
- Tail raising

- Sniffing/licking

A brief description of estrus behaviour recorded is given as below:

3.7.4.1 Stand to be mounted

The score for the "stand to be mounted" sign was derived by observing CCTV camera recording for 24 h observations during that cycle on the day of estrus. The designated points were given for each time the cows showed "stand to be mounted" behaviour. If a particular heifers stood to be mounted and then walked away before standing to be mounted again, this was counted as two occasions, and points were given again.

3.7.4.2 Mounting other heifers

The scores were based on the CCTV camera photoaged and points were given for each occasion in that estrus cycle a heifers was detected mounting another heifers on the day of estrus. If she walked away and then repeated the behaviour, this was counted as two occasions and points were given again.

3.7.4.3 Chin resting

The same scoring procedure as for mounting other heifers was used for assigning points for chin resting behaviour.

3.7.4.4 Vulval discharge

For vulva discharge, points were only assigned one time for any estrus cycle.

3.7.4.5 Cervical mucus characteristics

(a) Appearance

Classification of appearance was done as described by Deo and Roy (1971):

1. Clean- Like the white of an egg
2. Turbid - Cloudy in appearance, not homogenous in look, and
3. Dirty – Colour like yellowish, grey, red etc.

(b) **Consistency**

It was noted as described by Deo and Roy (1971):

1. Thin - Freely flowing mucus on the 45 inclined glass slides
2. Medium - Mucus moves slowly

3. Thick - Mucus is sticky and moves in lumps

(c) **Arborisation**

It was classified as follows as described by Luktuke and Roy (1967):

1. Typical, fern pattern with primary, secondary and tertiary branches.
2. Atypical, fern pattern with primary and secondary branches.
3. Nil, fern pattern with no primary, secondary and tertiary branches.

(d) Spinnbarkeit value of mucus

Few drops of cervical mucus were taken on a grease-free glass slide; then another grease-free glass slide was placed over it; then the upper glass slide was slowly moved away from the first slide; the mucus was stretched between two slides; the slide was moved until the mucus breaks and the distance between the two slides was measured just before the breakage of the mucus string through a scale (cm scale) mounted on the wall (Panigrahi, 1964). The obtained Spinnbarkeit values were grouped into three groups:

1. 0-8 cm
2. 8-16 cm
3. 16-24 cm

(e) pH

The pH of cervical mucus was measured using a pH-Conductivity Benchtop (Orion 4 star, Thermo Electron Corporation, USA). The observed pH was categorised into three categories:

1. 7-7.5
2. 7.5-8
3. Above 8

3.7.5 Ovarian cyclicity using ultrasonography (USG)

The ovarian structure of all the heifers in each treatment and control group was observed ultrasonographically using a real-time, B-mode, diagnostic scanner equipped with a transrectal 7.5 MHz, linear array transducer (**Aloka, Prosound 2 Japan).** Ultrasound examination were performed fortnightly of all the animals. On each examination the diameter of the follicle in each ovary was measured.

Plate 3.6 USG machine

3.7.6 Collection of blood samples

The blood samples were collected from all animals in treatment and control groups in vacutainer tubes by jugular venipuncture using 20G sterile disposable needles at fortnight intervals. The plasma was harvested from blood samples using a refrigerated centrifuge machine (REMI) at 3000 rpm for 15 minutes and was stored in different aliquots at -20°C till hormonal analysis (Progesterone hormone).

3.7.6.1 Hormone assay

3.7.6.2 Bovine progesterone ELISA Kit

A. Description of the assay kit

This sandwich kit was for the accurate quantitative detection of Bovine Progesterone (also known as PROG) in serum, plasma, cell culture supernates, cell lysates, and tissue homogenates.

B. Detection range

Range: 0.5-100 ng/ml

Sensitivity: 0.22 ng/ml

C. Test principle

This kit was an Enzyme-Linked Immunosorbent Assay (ELISA). The plate had been pre-coated with Bovine PROG antibody. PROG present in the sample was added and binds to antibodies coated on the wells. And then biotinylated Bovine PROG Antibody was added and binds to PROG in the sample. Then Streptavidin-HRP was added and binds to the Biotinylated PROG antibody. After incubation unbound Streptavidin-HRP has

washed away during a washing step. Substrate solution was then added and color develops in proportion to the amount of Bovine PROG. The reaction is terminated by the addition of acidic stop solution and absorbance was measured at 450 nm.

D. Reagent preparation and storage

All reagents were brought to room temperature before use.

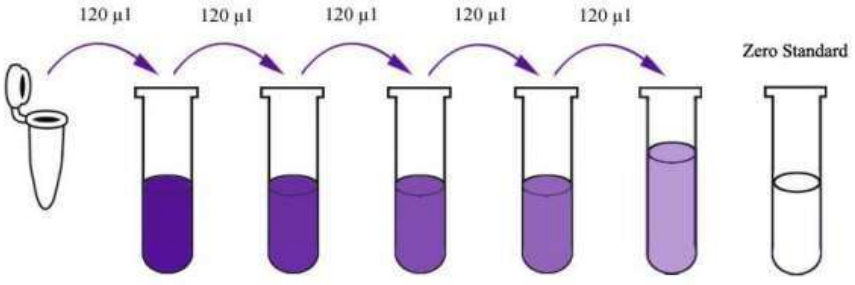
1. Wash Buffer:

Diluted 20 ml of wash buffer concentrate 30x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

2. Standard dilution:

Standard reconstituted the 120 µl of the standard (128 ng/ml) with 120 µl of standard diluent to generate a 64 ng/ml standard stock solution. Allowed the standard to sit for 15 mins with gentle agitation prior to making dilutions. Prepared duplicate standard points by serially diluting the standard stock solution (64 ng/ml) 1:2 with standard diluent to produce 32 ng/ml, 16 ng/ml, 8 ng/ml, and 4 ng/ml solutions. Standard diluent serves as the zero standard (0 ng/ml). Any remaining solution were frozen at -20°C and used within one month. Dilution of standard solutions suggested were as follows:

64ng/ml	Standard No.5	120µl Original Standard + 120µl Standard Diluent
32ng/ml	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
16ng/ml	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
8ng/ml	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
4ng/ml	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
128ng/ml	64ng/ml	32ng/ml	16ng/ml	8ng/ml	4ng/ml

E. Assay procedure

1. Prepared all reagents, standard solutions, and samples as instructed. Brought all reagents to room temperature before use. The assay was performed at room temperature.
2. Determined the number of strips required for the assay. Inserted the strips in the frames for use. The unused strips were stored at 2-8°C.
3. Added 50 µl standard to standard well. Note: Didn't add antibody to standard well because the standard solution contains biotinylated antibody.
4. Added 40 µl sample to sample wells and then added 10 µl anti-PROG antibody to sample wells, then added 50 µl streptavidin-HRP to sample wells and standard wells (Not blank control well). Mixed well. Covered the plate with a sealer. Incubated for 60 minutes at 37°C.
5. Removed the sealer and wash the plate 5 times with wash buffer. Soaked wells with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirate all wells and wash 5 times with wash buffer, overfilling wells with wash buffer. Blotted the plate onto paper towels or other absorbent material.
6. Added 50 µl substrate solution A to each well and then add 50 µl substrate solution B to each well. Incubated plate covered with a new sealer for 10 minutes at 37°C in the dark.
7. Added 50 µl stop solution to each well, the blue colour will change into yellow immediately.
8. Determined the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Plate 3.7 Blood collection and pipetting are done for progesterone estimation

3.8 Statistical analysis of data

The data generated were subjected to the following test statistics so as to arrive at meaningful inferences:

(a) Mean and their standard errors

(b) Frequencies

(c) Percentages

T-test assuming equal variance

The significance of differences between the mean values of two groups of experimental animals was analysed by subjecting the data to T-test assuming equal variance using SPSS computer software version 21.

CHAPTER –4

Results and Discussion

RESULTS AND DISCUSSION

Anestrus is one of the most common reproductive disorders in buffalo. Anestrus in buffalo is observed in India at a rate ranging from 25 to 67%. Shorter oestrous durations and less intense oestrous behaviour might increase "silent" oestrus, which is a major hurdle to improving estrus detection accuracy and efficiency. Buffalo is also a difficult breeder because of its natural susceptibility to environmental stress, which promotes anestrus and repeats breeding. These two factors are to blame for the dairy protracted inter-calving time in buffaloes, which results in significant financial losses. The goal of the current study was to address the issue of anestrus by using a unique stimulus known as biostimulation. The data were analysed using standard statistical techniques as outlined in the previous chapter. The results are presented and discussed in this chapter as under:

4.1 Growth performance

4.1.1 Dry matter intake (DMI)

The data on average dry matter intake (DMI) of two groups measured during experimental period at fortnight intervals are presented in table 4.1 and graphically represented in fig 4.1. In T_0 and T_1, the average DMI was 10.43±0.24 and 10.72±0.28 and DMI/100 kg body weight was 2.87±0.011 and 2.9±0.014 kg, respectively. There was no significant difference in DMI in between the two groups of buffalo heifers. The average DMI/100 kg of body weight in both groups of buffalo heifers was within the acceptable range (2.5 to 3 percent) for feeding of buffalo heifers as per ICAR (2013) feeding standards. This is in agreement with the findings of Dutt (2020) who reported that DMI was higher in bull exposure group of growing buffalo heifers as compared to no bull exposed heifers.

4.1.2 Body weight and average daily gain (ADG)

The average body weight of both groups of heifers, recorded at the beginning and then at fortnightly intervals, is shown in table 4.3 and graphically represented in fig 4.2. In T_0 and T_1, the final body weight was 362.47±7.32 and 369.07±8.2 kg respectively which did not differ significantly from each other. The data on the fortnightly average daily gain in T_0 and T_1 is presented in table 4.4 and graphically represented in fig 4.3. The overall daily average gain in body weight in T_1 was 605±19.34 gm/day which was significantly ($P<0.05$)

higher than T_0 (507±28.1 gm/day). Significantly greater amounts of leptin hormone and growth hormone may be responsible for the significantly higher ADG in the treatment groups of heifers.

Dutt (2020) reported that the higher growth in bull exposed heifers at about 15 months of age was mainly due to higher level of growth hormone. Choudhary *et al.* (2019) found that overall average daily body weight gain was significantly ($p<0.01$) higher in direct bull exposure+fenceline bull exposure group (508.11±14.57gm) and fenceline bull exposure group (501.6±4.3gm) in comparison with no bull exposed group (441.8±2.9gm) in Sahiwal heifers. Earlier Harder *et al.* (2003) and Garratt *et al.* (2016) had reported that female mice exposed to male pheromones grew at a faster rate than non-exposed females. The increased growth rate observed in female mice exposed to male pheromones was attributed by both of these researchers to the activation of the hypothalamic-pituitary-gonadal axis, which led to higher levels of the growth hormone (HPG).

Table 4.1 Mean DMI at a fortnightly interval (kg/head/day) of two groups of heifers

Day	T_0 (No bull exposure)	T_1 (Direct bull exposure)
0	9.38±0.21	9.52±0.24
15	9.93±0.34	9.86±0.28
30	9.83±0.27	10.28±0.31
45	10.39±0.25	10.57±0.35
60	10.52±0.33	11.02±0.30
75	10.92±0.29	11.30±0.31
90	11.10±0.27	11.43±0.34
120	11.40±0.29	11.78±0.32
Overall	**10.43±0.24**	**10.72±0.28**

Table 4.2 Mean DMI (kg/day/100 kg body weight) of two groups of heifers

Day	T_0 (No bull exposure)	T_1 (Direct bull exposure)
0	2.82±0.05	2.84±0.04
15	2.85±0.04	2.85±0.05
30	2.86±0.05	2.89±0.04
45	2.90±0.04	2.89±0.04
60	2.87±0.04	2.95±0.04
75	2.92±0.04	2.95±0.05
90	2.90±0.04	2.91±0.04
120	2.89±0.04	2.92±0.05
Overall	**2.87±0.011**	**2.90±0.014**

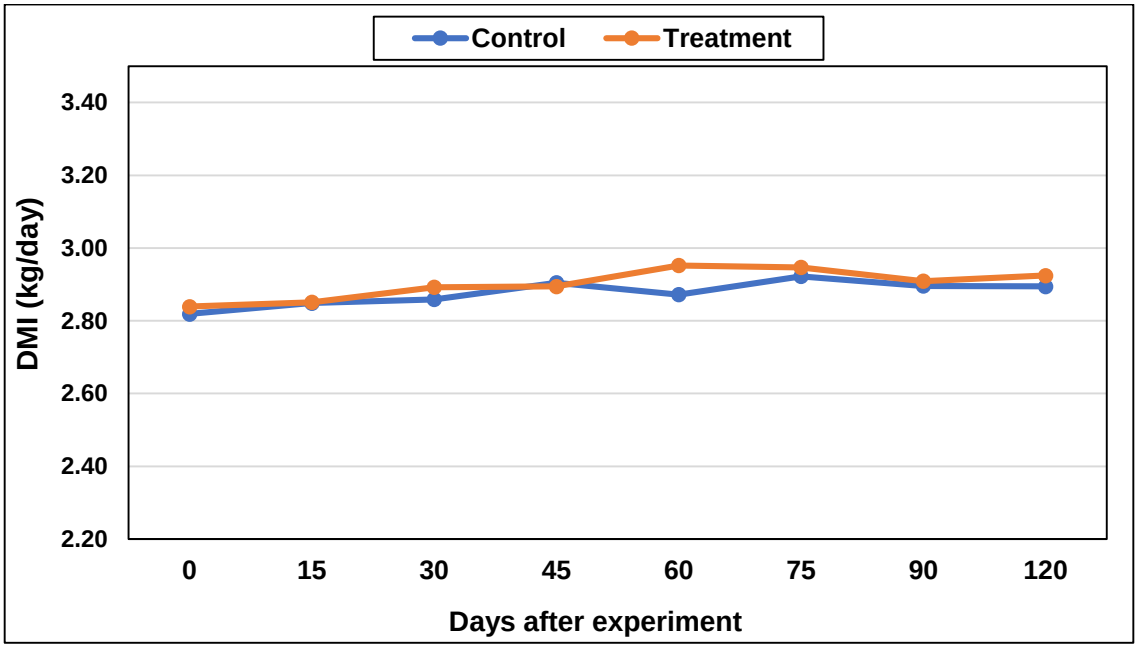


Fig 4.1 Total dry matter intake per 100 kg body weight (kg/day/animal) of heifers at fortnightly intervals

Table 4.3 Mean body weight (kg) of two groups of heifers at fortnightly intervals

Day	T_0 (No bull exposure)	T_1 (Direct bull exposure)
0	333.11±7.7	335.78±9.44
15	341.44±7.97	345.33±9.12
30	350.44±8.04	354.89±8.94
45	358.22±8.29	364.78±8.89
60	366.11±8.82	373.56±8.86
75	373.78±8.79	383.22±8.64
90	383.11±8.25	392.56±8.64
120	393.56±7.02	402.44±8.08
Overall	**362.47±7.32**	**369.07±8.21**

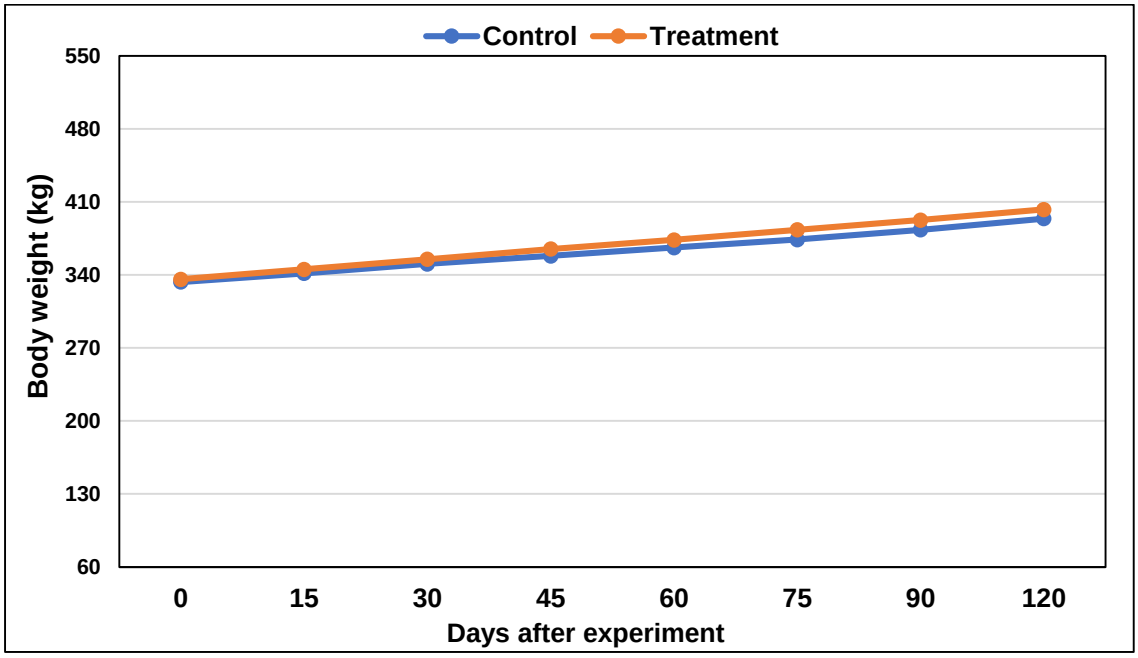


Fig 4.2 Average body weight (kg) of two groups of heifers at fortnightly intervals

Table 4.4 Average daily body weight gain (kg/day) of two groups of heifers

Day	T_0 (No bull exposure)	T_1 (Direct bull exposure)
15	0.56±0.04	0.64±0.05
30	0.6±0.02	0.64±0.06
45	0.52±0.04	0.66±0.05
60	0.53±0.05	0.59±0.02
75	0.51±0.04	0.64±0.06
90	0.42±0.03	0.58±0.06
105	0.47±0.07	0.6±0.07
120	0.45±0.06	0.49±0.05
Overall	**0.507^{a}±0.028**	**0.605^{b}±0.019**

Mean bearing different superscripts in a row differ significantly (P<0.05)

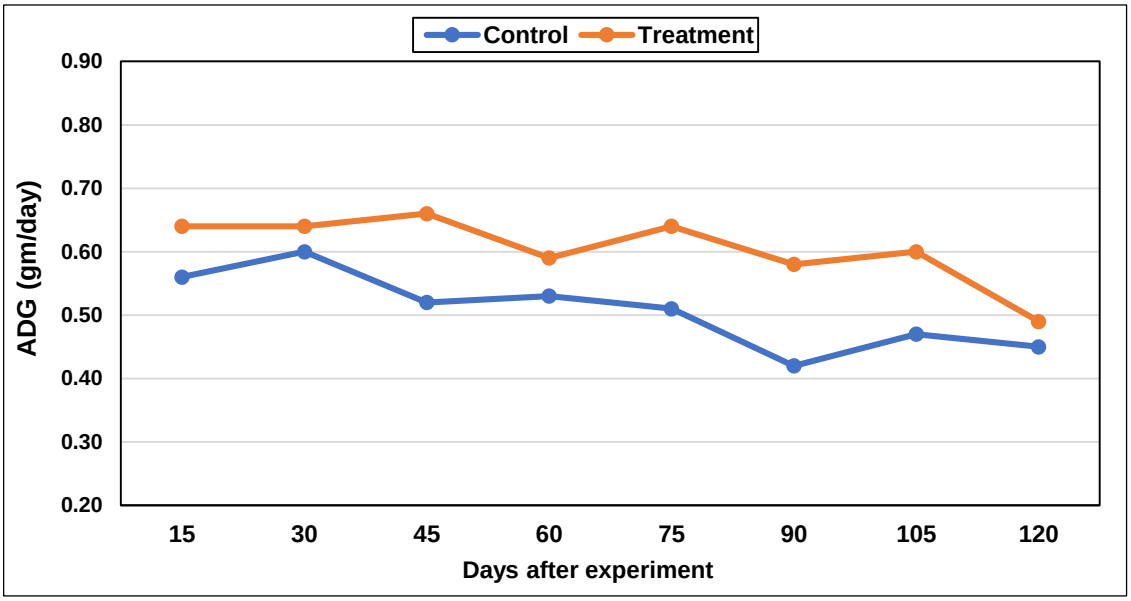


Fig 4.3 Average daily body weight gain (kg/day) of two groups of heifers at fortnightly intervals

4.2 Reproductive performance

4.2.1 Ovarian activity through ultrasonography (USG)

Before the start of the experiment i.e., on d 0 the heifers of two groups were examined through ultrasound scanning. The average size of a follicle in both groups ranged from 2-6 mm. It was therefore, confirmed that all animals were in an anestrus condition. The data on follicle diameter of two groups of heifers is presented in table 4.5.

According to Sahu (2002) and Adams (1999), anestrus is characterised by the presence of anovulatory follicular waves. Instead of lacking GnRH responsive follicles, these heifers' small size follicles may be the result of insufficient hypothalamic GnRH impulses to induce the anterior pituitary release of gonadotropins (Peters and Lamming, 1984).

Table 4.5 Follicles diameter of two groups of heifers

Sr. No.	Control		Treatment	
	Animal number	Follicular diameter (mm)	Animal number	Follicular diameter (mm)
1	7855	5	7828	3.3
2	7836	5.6	7826	5.5
3	7851	5.9	7832	5.1
4	7830	2.0	7841	4.9
5	7871	3.5	7863	5.4
6	7820	4.2	7916	2.8
7	7913	4.4	7902	5.2
8	7823	3.8	7965	3.7

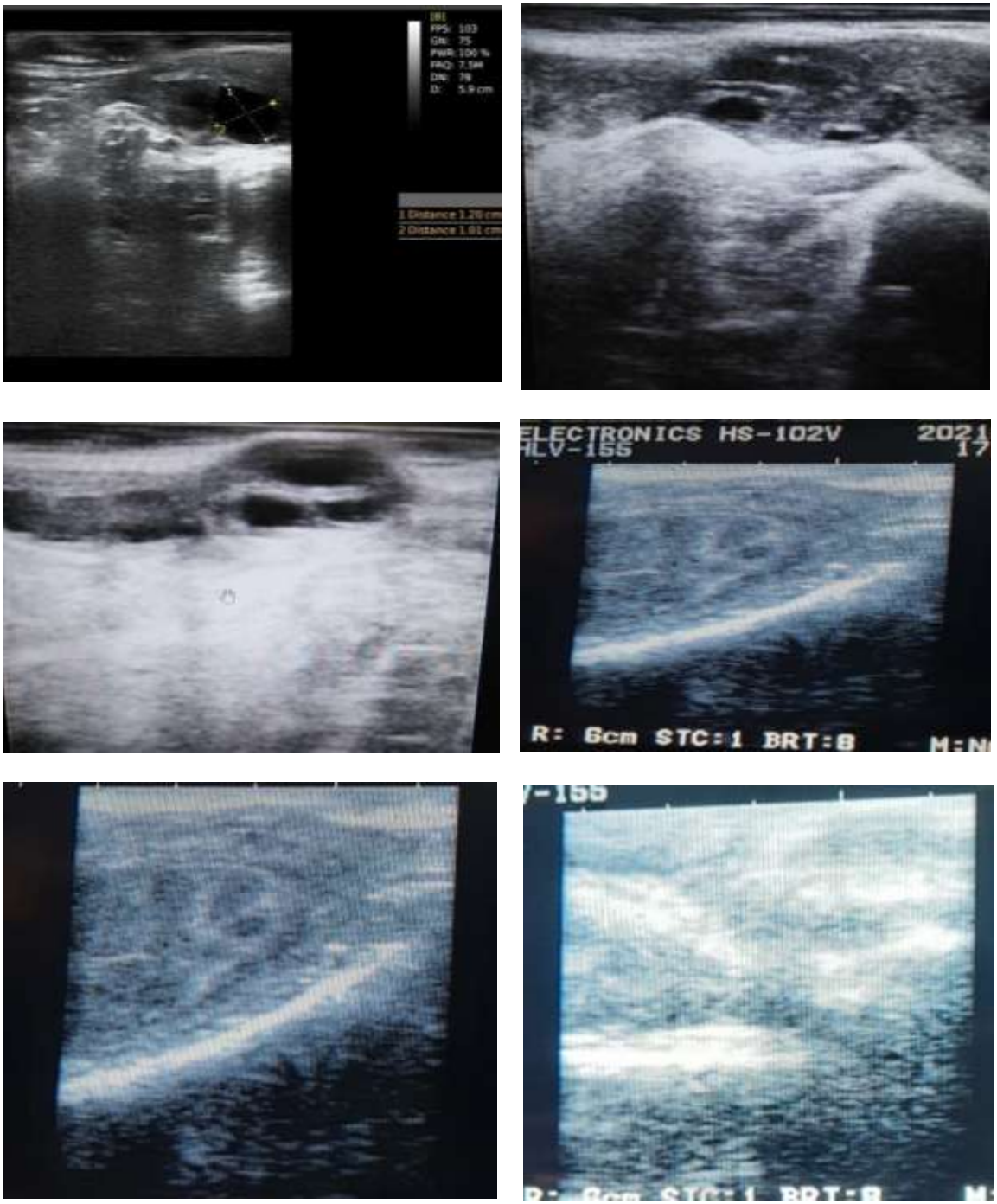


Plate 4.1 Ultrasound images depicting follicle size in two groups of heifers

4.2.2 Age at first estrus

The average age and body weight of the T_0 and T_1 group before the start of the experiment was (25.85±0.32 months, 333.5±6.29 kg) and (25.94±0.48 months, 332.83±7.51 kg). In the control group out of 12 animals, only 2 animals came into heat, and in the treatment group out of 12 animals, 10 came into heat. The average age at first estrus in T_0 and T_1 was 27.89 and 28.29 months (table 4.6).

Heifers in treatment groups may have come into estrus because the presence of a bull through visual, tactile, olfactory, and vocal signals primed their neuroendocrine response. In domestic animal priming pheromones from male have been reported (Izard, 1983) to influence on termination of anestrus and shortening of post-partum anestrus. The male pheromones may have triggered the hypothalamus and there is release of LHRH (luteinizing hormone releasing hormone). LHRH acts on pituitary and controls the pulsatile release of luteinizing hormone (LH). The increase in LH secretion is supposed to probably stimulate the ovarian secretion of estrogen that via positive feedback effect at the hypothalamic level induces the preovulatory LH surge and hence ovulation occurs in heifers.

Earlier Gokuldas *et al*. (2010) reported that exposing post-partum Murrah buffaloes to buffalo bull (kept tied inside buffalo shed) considerably shortened postpartum intervals to the resumption of ovarian cyclicity as compared to non-exposed buffaloes. Further, Akhtar *et al*. (2018) found that post-partum interval to first behavioural estrus was shorter in (BEC) buffalo exposed than (BEI) buffalo exposed intermittently, EPB (buffalo exposed to excretory product of the bull) and NE (non-exposed) treatments. In contrast to the non-exposed group, there was a lower incidence of silent ovulation in the bull exposed group. Barman *et al*. (2011) found that bull exposed buffaloes had a significantly lower incidence of silent ovulation than the NE group.

Sahu (2022) reported that growing buffalo heifers came into estrus as early as 21.50±0.44 months in direct bull exposure group as compared to 25.61±0.70 months in no bull contact. Choudhary *et al.* (2019) reported first estrus at 19.11±0.58, 24.00±0.12 months in bull exposed group than no exposed group in Sahiwal heifers. Miller and Ungerfeld (2008) reported that weekly exchange of two pairs of bulls shortened postpartum anestrus in beef cows as compared to continuous exposure to a single pair of bulls. Roberson *et al*. (1991) observed that those heifers exposed to bulls attained puberty at younger ages 61.8 % than non-exposed 45.4%. Although at lower body weights of the heifers during the bull exposure phase, Berardinelli *et al*. (1978) and Macmillian *et al*. (1979) claimed that the presence of the bull did not have an impact on accelerating onset of estrus in the heifers. Previous reports on Angus heifers by Roberson *et al*. (1991), Angus heifers by Izard and Vandenbergh (1982), Angus heifers by Rekwot *et al*. (2000), Nelore heifers by Oliveira *et al*. (2009) and Hereford, as well as Angus, crossbred heifers by Fiol and Ungerfeld (2016) all, support the findings of the current study. They concluded that exposing the heifers to a bull lowered the age at first estrus in heifers.

4.2.3 Age at first service

The total number of 7 heifers out of 10 which were seen in estrus in the treatment group were inseminated while the other three were not inseminated as they were in weak estrus. In control group the two heifers observed in estrus were also not inseminated because they also had weak symptoms. The average age at first service in the treatment group was 28.18±0.57 months.

Sahu (2022) found that age at first service in buffalo heifers was 22.28±0.50 months in bull exposed group as compared to 26.29±0.70 months in no bull exposed group. Choudhary *et al.* (2019) reported 24.25±0.25, 20.37±0.54, 20.78±0.52 months in no bull exposed, fenceline+direct and direct exposed group of Sahiwal heifers. Earlier studies of Izard and Vandenbergh (1982) in Holstein heifers; Rekwot *et al*. (2000) in Bunaji heifers; Oliveira *et al*. (2009) in Nelore heifers; Fiol and Ungerfeld (2016) in Hereford and Angus crossbred heifers reported the reduction of age at first service in bull exposed animals.

The reduction in age at first service in the treatment groups of heifers might be attributable to the priming of these heifers by the presence of bull via visual, tactile, olfactory, and auditory cues, which could have resulted in the activation of the neuro-endocrine response. Fiol *et al*. (2010) had reported that distance between androgenised steers and females was positively related to the likelihood of ovulating in prepubertal heifers, indicating that physical closeness modulates the response. As a result, cues related to courting (tactile, physical touch) are crucial and stimulation is not only dependent on chemical signals but also another possibility may be due to the duration of exposure time.

4.2.4 Conception rate

The conception rate of the treatment group was 57.14% This might be explained by the bull recognising estrus early and the A.I. of identified heifers at the appropriate time and the average service per conception was 1.75. Sahu (2022) reported higher conception rate in bull exposure group as compared to no bull contact in Murrah buffalo heifers. Gokuldas *et al*. (2010) reported first service conception rate in bull exposed group was 100% as compared to 37.50% in non-exposed group of Murrah buffalos. Barman *et al*. (2011) found that bull exposed group had higher conception rate than non-exposed group. Miller and Ungerfeld (2008) reported that there is higher conception rate 30 days after the end of bull exposure. These results concur with those of Ungerfeld (2009), Khanh *et al*. (2012), Filho *et al*. (2015), and Choudhary *et al.* (2019) who found that bull exposed heifers and cows had a higher conception rate than non-exposed counterparts.

It might be connected to precise time of detection of onset of estrus by the bull and insemination of the heifers at right time during standing estrus. It has been reported that a higher first-service conception rate was strongly correlated with insemination during clean cervical mucus discharge (Loeffler *et al*. 1999).

4.2.5 Numbers of heifers come into heat at different months

The data on a number of heifers that come into heat at different months are presented in table 4.7. Most of the heifers in T_1 (50%) come into heat within the first month due to the stimulatory effect of a male. This indicated the stimulatory response of the presence of bull of the heifers was stronger in the beginning of exposure during the first month.

Table 4.6 Reproductive performance of two groups of heifers

Parameter	T_0 (Control) (n=12)	T_1 (Treatment) (n=12)
Average body weight at the start of the experiment (kg)	333.5±6.29	332.83±7.51
Average age at the start of the experiment (months)	25.85±0.32	25.94±0.48
No. of heifers come into heat	2 (16.7 %)	10 (83 %)
No. of heifers inseminated	0	7 (70 %)
Average age at first estrus (months)	28.04±0.01	27.58±0.33
Average age at first service (months)	-	28.18±0.57
Conception rate (%)	-	57.14
Number of services per conception	-	1.75

Table 4.7 Total number of animals came into heat in different months

Group of heifers	Month after start of experiment			
	0-1	1-2	2-3	3-4
Control	-	-	1	1
Treatment	5	2	2	1

4.3 ESTRUS CHARACTERISTICS

4.3.1 Estrus behaviour

The suggested order for the various behaviours associated with the onset of estrus in cattle is sniffing, tail raising, micturition, chin resting, mounting attempts, and finally standing to be mounted (Roelofs *et al.*, 2005). The different symptoms were recorded through CCTV camera in the proforma as described in materials and methods. The data on the average frequency of various estrus behaviours were recorded on the day of estrus in two groups of Murrah heifers and are presented and discussed here as:

Table 4.8 Average frequency of estrus behaviour expressed by heifers on the day of estrus

Average frequency	Control	Treatment
Sniffing and licking	12.00±3.00	26.50±3.42
Tail raising	9.00±3.00	20.12±2.64
Chin resting	4.00±1.00	12.00±1.34
Stand to be mounted	3.00±1.00	9.75±1.16

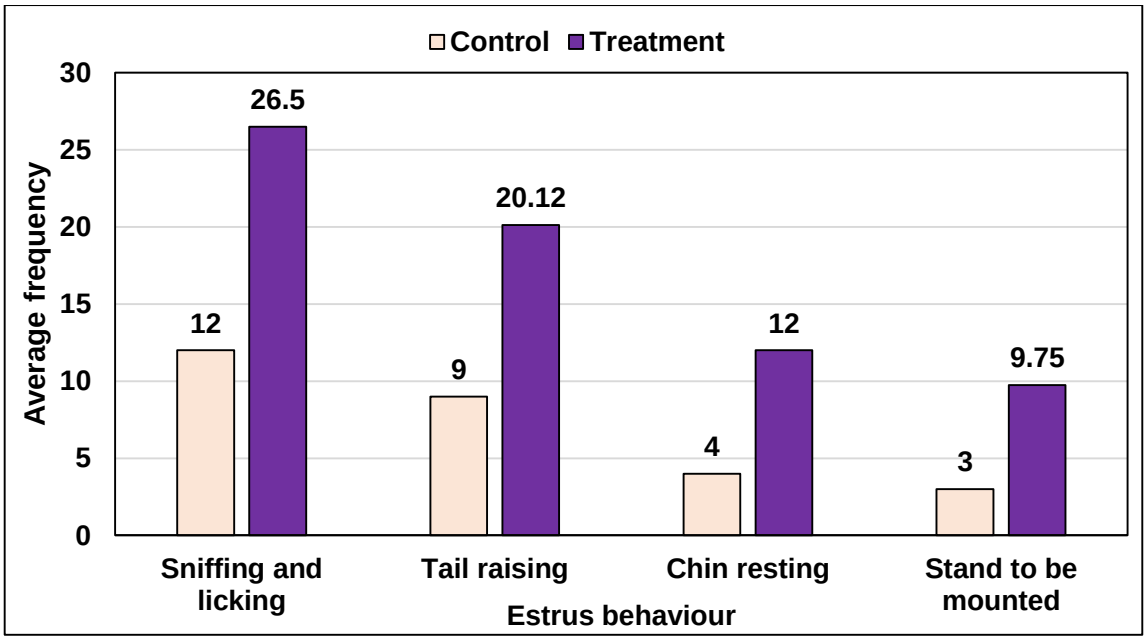


Fig 4.4 Average frequency of estrus behaviour of heifers on the day of estrus

4.3.1.1 Sniffing and licking by a bull and other herd mates

The data on average frequency of sniffing and licking by a bull and other herd mates during estrus is presented in table 4.8 and graphically represented in fig 4.3**.** The average frequency of sniffing and licking by a bull and other herd mates during estrus in T_1 group heifers was higher (26.50±3.42 vs. 12.00±3.00) than in T_0 group heifers. The sniffing/licking of external genitalia of treatment group of heifers was observed to a greater extent since the bull had a greater chance to do the same as it was directly present in the same shed with heifers as compared to the control group in which no bull was present.

Sahu (2022) reported sniffing/licking was higher in bull exposed group as compare to fenceline + direct and no bull exposed group in Murrah buffalo heifers on the day of estrus. Due to an inadequate concentration of the hormone estrogen during the first estrus, the frequency of this behaviour remained low (Sveberg *et al*., 2011). It's possible that direct bull exposure increased females' physical activity because T_1 heifers were more active and allowed more sniffing and licking than T_0 heifers. Previously, when a teaser bull was placed into a herd of buffaloes for 20 to 30 minutes each time to detect estrus, a bull's sniffing or licking of an estrus female's external genitalia was seen to a great extent. The current study's findings support those of Mondal *et al*. (2006), Roelofs *et al*. (2008), and Choudhary and Kamboj (2019), who reported that bull exposed cows showed a higher frequency of sniffing and licking than non-exposed cows/ buffaloes.

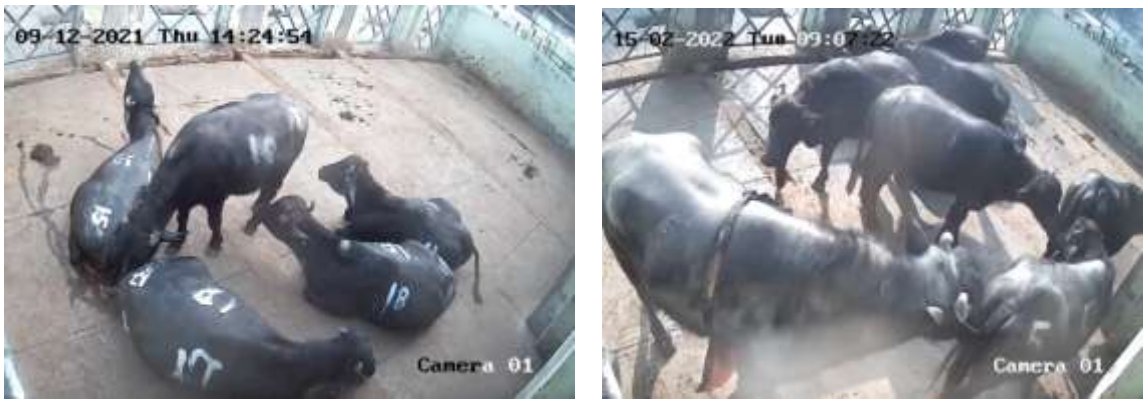


Plate 4.2 Sniffing and licking by a bull and other herd mates

4.3.1.2 Tail raising

The values of average frequencies of tail raising in response to sniffing and licking by a bull and other herd mates during estrus is presented in Table 4.8 and graphically represented in fig 4.3. The average frequency of tail raising in response to sniffing and

licking by a bull and other herd mates during estrus in T_1 group heifers was higher (20.12±2.64 vs. 9.00±3.00) than in T_0 group heifers.

Sahu (2022) observed tail raising was higher in bull exposed group, than fenceline + direct and direct bull exposure in Murrah buffalo heifers on the day of estrus. The results of the current study are similar to those of Silper (2010), who showed that as the day of estrus approaches, the behaviour of the bull and other herd members to sniffing and licking increases. Verma *et al*. (2014) also noted that on the day of estrus, the degree of tail-raising by buffalo heifers was greatly augmented by the teaser male's sniffing or licking.

Plate 4.3 Tail raising by estrus buffalo heifers

4.3.1.3 Chin resting

The average frequency of chin resting by a bull and other herd mates during estrus is presented in Table 4.8 and graphically represented in fig 4.3. The average frequency of chin resting by a bull and other herd mates during estrus in T_1 group heifers was higher (12.00±1.34 vs. 4.00±1.00) than in T_0 group heifers.

Prior to mounting, bulls, and cows frequently rub or rest their chins on the rump or back of the cow. The cow's or buffalo's reaction to chin pressing serves as a cue to the bull about the animal's receptivity and preparedness for mounting. In the current investigation, it was observed that the bull's chin-resting behaviour was an investigative strategy for determining whether an estrus heifer was ready to accept the mount.

Sahu (2022) reported higher chin resting frequency in bull exposed group than fenceline + direct and no bull exposed group in Murrah buffalo heifers on the day of estrus. Kumar *et al*. (2007), Joshi (2016) and Verma *et al*. (2014) observed that teaser bulls paraded in buffalo enclosures for estrous detection allowed chin resting in the majority of buffaloes in estrus. The results of this study are in agreement to those of Roelofs *et al*. (2005) and Sveberg *et al*. (2011) who found that cows in estrus were more likely to

favorably respond to chin rest when a bull and other cows were present than when there were no cows in estrus.

Plate 4.4 Chin resting by a bull during direct contact

4.3.1.4 Stand to be mounted

The average frequency of stand to be mounted by a bull and other herd mates during estrus is presented in Table 4.8 and graphically represented in fig 4.3. The average frequency of stand to be mounted by a bull and other herd mates during estrus in T_1 group heifers was higher (9.75±1.16 vs. 3.00±1.00) than in T_0 group heifers. In the current research, it was revealed that the mean frequencies of stand to be mounted showed the similar pattern with previous findings of Hernandez *et al*. (2002) who reported that more mounts were obtained by cows with longer estrus duration than during silent estrus.

Esslemont and Bryant (1976) and Hurnik *et al*. (1987), mounting episodes last longer when two cows are in estrus than when just one animal is receptive. The current study's findings that mounting episodes last longer when two cows are in estrus are confirmed by the lower frequency of mounting attempts in the control group.

Plate 4.5 Stand to be mounted by estrus heifers

4.4 General behaviour

4.4.1 Standing time

The data on average standing time of two groups of Murrah heifers at fortnightly intervals is presented in table 4.9 and graphically represented in fig 4.4. The mean ± SE of standing time between treatment (T_1) and control group (T_0) of heifers was 722.57±4.93, 678.25±4.43 minutes per day respectively.

The standing time was significantly higher ($p<0.05$) in the treatment group of heifers when compared to no bull exposed. The higher standing time in bull exposed heifers was attributed to more activity and more expression of estrus behaviour which kept the heifers engaged and standing.

Dutt (2020) recorded a standing time of 692.42±14.70 minutes/day in direct bull contact and 774.21±9.97 minutes/day in no bull exposure in Murrah buffalo heifers. Choudhary (2021) observed 652.75±9.58, 686.42±7.54, 689.48±11.174 minutes/day in fenceline contact, restricted contact and no bull contact in Murrah buffaloes. Singh *et al*. (1985) recorded a daily standing time of 758 minutes. This study's findings of standing time are similar to those reported by Singh *et al.* (1985) and Choudhary (2021).

Table 4.9 Average time spent on the standing (minutes/day) of two groups of heifers

Days after the start of the experiment	T_0 (no bull exposure)	T_1 (bull exposure)
0	696.33±12.35 (11.60)	707.44±13.01 (11.79)
15	676.78^{a}±14.37(11.27)	724.56^{b}±12.81 (12.07)
30	685.89±13.23 (11.43)	699.44±16.37 (11.65)
45	681.78±11.20 (11.36)	718.67±15.35 (11.97)
60	657.89^{a}±20.63 (10.96)	723.11^{b}±18.57 (12.05)
75	681.00^{a}±13.58 (11.35)	722.33^{b}±13.58 (12.03)
90	673.56^{a}±14.49 (11.22)	741.33^{b}±18.64 (12.35)
120	688.22^{a}±10.2 (11.47)	734.67^{b}±18.22 (12.24)
Overall	**678.25^{a}±4.43 (11.30)**	**722.57^{b}±4.93 (12.04)**

Means bearing different superscripts in a row differ significantly (P<0.05)
Figures in parenthesis represent the standing time in hours

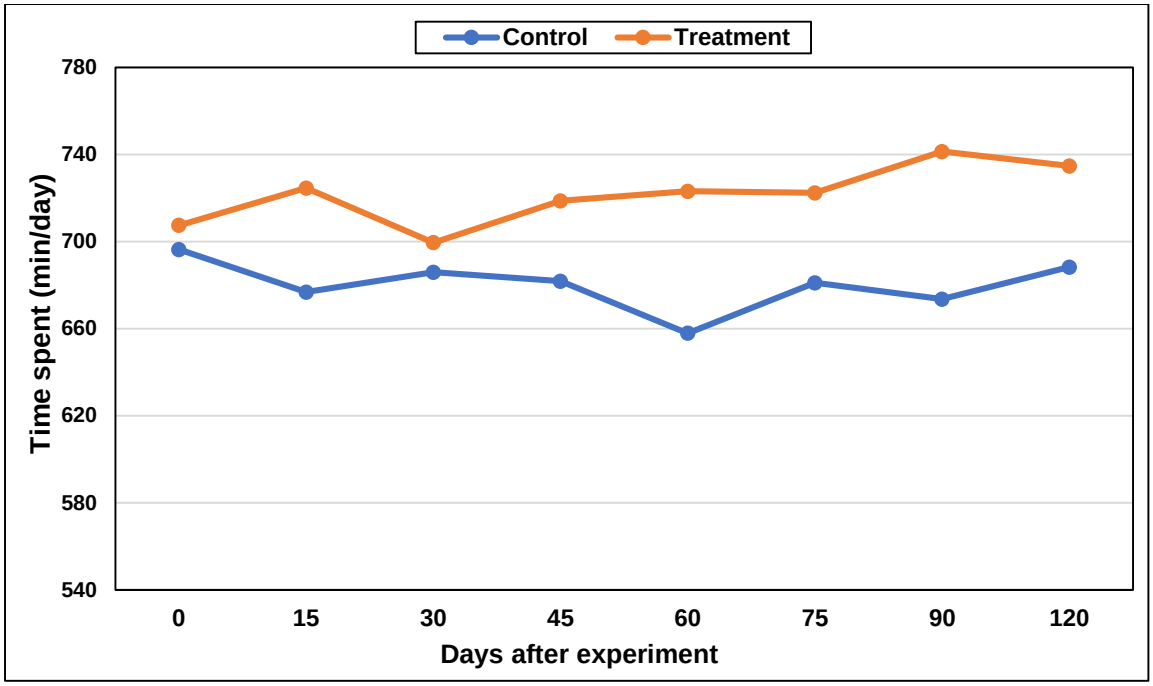


Fig 4.5 Average standing time of two groups of heifers

Plate 4.6 Standing animal of two groups of heifers

4.4.2 Lying time

The data on average lying time of two groups of Murrah heifers observed at fortnightly intervals is presented in table 4.10 and graphically represented in fig 4.5. The mean ± SE of lying time between treatment (T_1) and control group (T_0) were 689.59±3.87, 728.06±4.01 minutes per day respectively.

The lying time was significantly higher ($p<0.05$) in the control group as compared to the treatment group. The lying time was more in the control group of heifers as the heifers were not disturbed by the presence of bull and further the estrus symptoms may also be weak and duration of estrus lower in these heifers and therefore these heifers rested more.

Dutt (2020) recorded a lying time 744.56±15.37 minutes/day in direct bull contact and 665.79±9.97 minutes/day in no bull exposure in buffalo therefore at lower age, Singh *et al*. (1985) found lying times of 594 minutes in the summer and 679 minutes per day in the winter. In dairy animals, restlessness is one of the most significant secondary oestrus signs (Firk *et al*., 2002). Greater physical restlessness in response to an increase in estrogen secretion that started at the pro-oestrus phase and more engagement in mounting and other estrous behaviours of heifers as a result of the presence of a bull may likely be attributed to the greater reduction in resting time in bull exposed heifers.

The results of the current study are consistent with those of Singh *et al*. (1985), Kerbrat and Disenhaus (2004) and Dolecheck (2015), who reported a decrease in the amount of time spent resting each day during estrus.

Table 4.10 Average time spent on lying (minutes/day) of two groups of heifers

Days after the start of the experiment	**T_0 (no bull exposure)**	**T_1 (bull exposure)**
0	717.56^{a}±12.73 (11.95)	674.33^{b}±13.17 (11.23)
15	736.11^{a}±15.63 (12.26)	681.56^{b}±13.15 (11.35)
30	723.33^{a}±9.12 (12.05)	686.56^{b}±11.15 (11.44)
45	727.11±10.82 (12.11)	707.78±13.09 (11.79)
60	731.33^{a}±10.81 (12.18)	685.33^{b}±15.08 (11.42)
75	716.11±8.17 (11.93)	684.33±13.18 (11.40)
90	722.22±11.41 (12.03)	699.00±10.86 (11.65)
120	750.78^{a}±14.78 (12.51)	697.89^{b}±16.38 (11.63)
Overall	**728.06^{a}±4.01 (12.13)**	**689.59^{b}±3.87 (11.49)**

Means bearing different superscripts in a row differ significantly (P<0.05)

Figures in parenthesis represent the lying time in hours

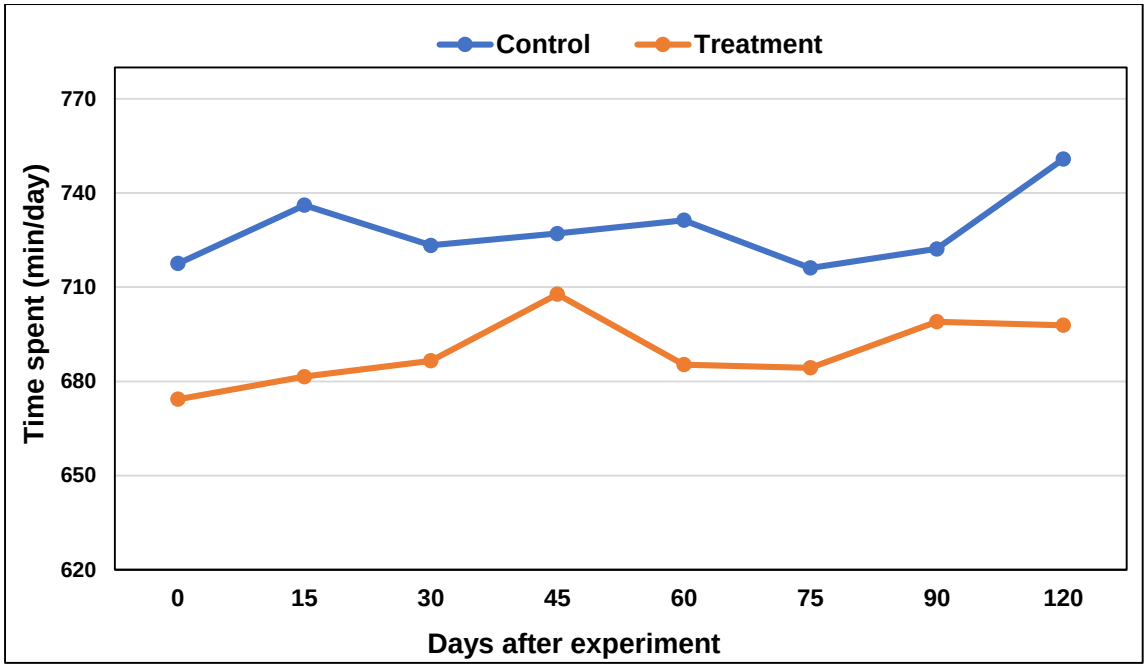


Fig 4.6 Average lying time of two groups of heifers

Plate 4.7 Lying behaviour of two groups of heifers

4.4.3 Eating time

The mean ± SE of eating time in treatment (T_1) and control group (T_0) was 260.89±2.14, 249.04±2.27 minutes per day respectively. The eating time was significantly higher ($p<0.05$) in the treatment group of heifers as compared to control group of heifers.

Dutt (2020) recorded an eating time of 282.67±4.46 minutes/day in direct bull contact and 261.35±3.95 minutes/day in no bull exposure of buffalo heifers. Different researchers have reported feeding times of 3-5 hours per day in cattle (Grant and Albright 2000), daily meal time of 272.18±82.14 minutes/day Dado and Allen (1994). The results of the current study are consistent with those of Dutt (2020) in bull exposed buffalo heifers and Rajput (2019) in Sahiwal heifers.

Table 4.11 Average time spent on eating (minutes/day) two groups of heifers

Days after the start of the experiment	T_0(no bull exposure)	T_1 (bull exposure)
0	247.67±6.72 (4.12)	267.67±7.4 (4.46)
15	246.22±6.16 (4.10)	253.89±8.91 (4.23)
30	243.11±6.13 (4.05)	263.33±7.61 (4.38)
45	252.22±5.33 (4.20)	267.00±5.98 (4.45)
60	254.44±6.74 (4.24)	263.78±8.91 (4.39)
75	257.22±4.84 (4.28)	257.56±7.81 (4.29)
90	253.33±5.98 (4.22)	251.11±7.78 (4.18)
120	238.11^{a}±6.64 (3.96)	262.78^{b}±8.65 (4.37)
Overall	**249.04^{a}±2.27 (4.15)**	**260.89^{b}±2.14 (4.34)**

Means bearing different superscripts in a row differ significantly (P<0.05)

Figures in parenthesis represent the eating time in hours

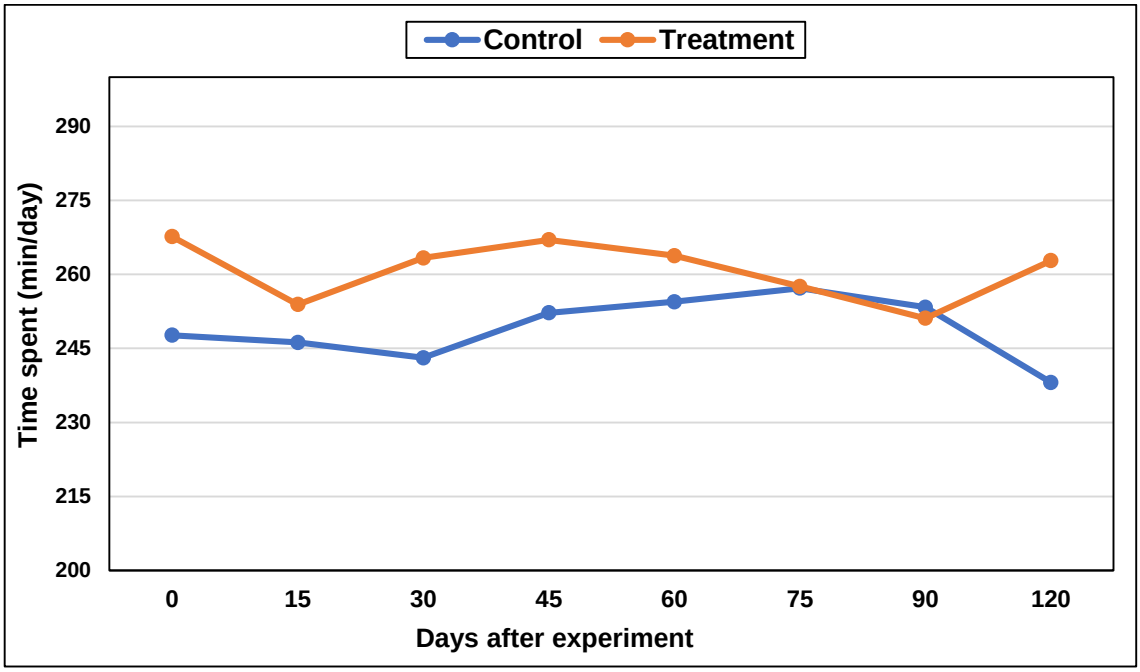


Fig 4.7 Average eating time of two groups of heifers at fortnightly interval

Plate 4.8 Eating behaviour of two groups of buffalo heifers

4.4.4 Rumination time

The mean ± SE of rumination time in treatment (T_1) and control group (T_0) was 396.22±5.25, 375.80±5.36 minutes per day respectively (Table 4.12). The rumination time was significantly higher ($p<0.05$) in the treatment group of heifers as compared to control group of heifers.

Table 4.12 Average time spent on rumination (minutes/day) of two groups of heifers

Days after the start of the experiment	T_0 (no bull exposure)	T_1 (bull exposure)
0	379.78±16.23 (6.32)	389.89±8.09 (6.49)
15	373.44±15.79 (6.22)	386.67±11.42 (6.44)
30	397.56±17.45 (6.62)	393.56±7.59 (6.55)
45	358.89^{a}±13.23 (5.98)	415.67^{b}±14.40 (6.92)
60	396.67±13.21 (6.61)	392.89±12.54 (6.54)
75	372.33^{a}±10.10 (6.20)	421.89^{b}±20.36 (7.03)
90	370.89±11.31 (6.18)	378.00±13.07 (6.3)
120	356.89^{a}±10.88 (5.94)	391.22^{b}±10.31 (6.52)
Overall	**375.80^{a}±5.36 (6.26)**	**396.22^{b}±5.25 (6.60)**

Means bearing different superscripts in a row differ significantly ($P<0.05$)
Figures in parenthesis represent the rumination time in hours

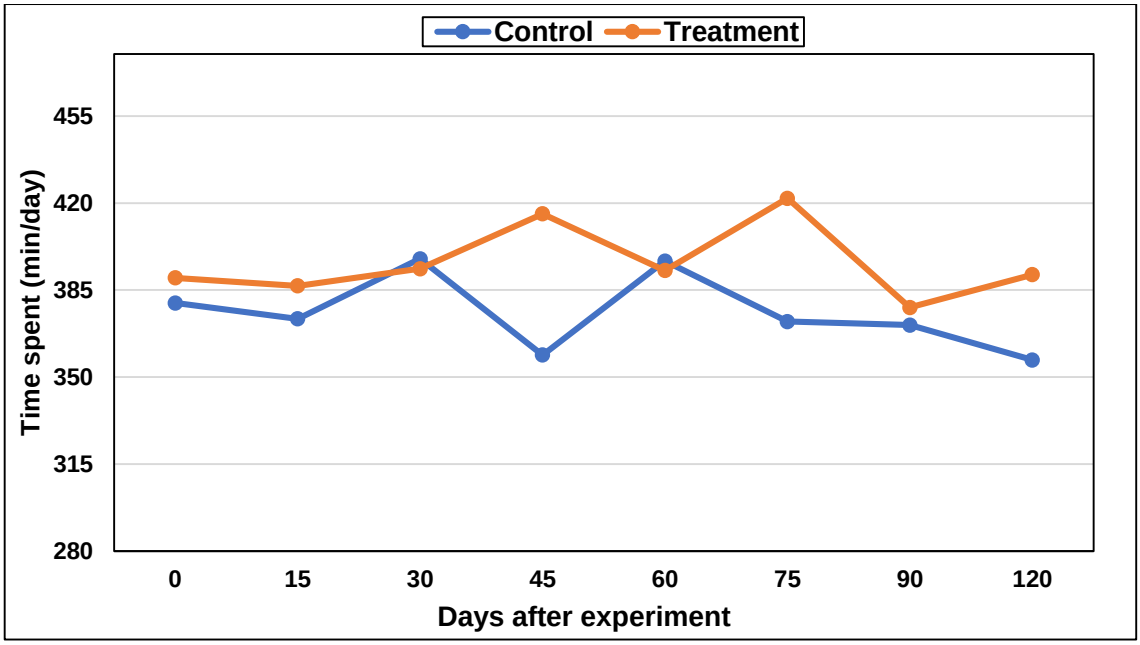


Fig 4.8 Average rumination time of two groups of heifers

Plate 4.9 Rumination behaviour of two groups of heifers

Dutt (2020) reported a rumination time of 368.79±6.30 minutes in direct bull contact and 344.76±6.76 in no bull exposed buffalo heifers. Rumination time was estimated to be 475.57 minutes per day for Murrah buffalo heifers living in loose housing by Singh *et al*. (1985). Similar findings were also reported by Choudhary and Kamboj, (2019) in fenceline bull exposed Sahiwal heifers and Rajput and Kamboj (2019) in bull exposed Sahiwal cows.

4.4.5 Drinking frequency

The data on average frequency of drinking observed for heifers is presented in table 4.13 and graphically represented in fig 4.7. The average frequency of drinking in the T_1 and T_0 group of heifers was 4.36±0.56 and 3.76±0.39. The drinking frequency was not statistically different between the two groups of buffalo heifers.

Dutt (2020) recorded drinking frequency 10.81±0.68, 12.24±0.80, 11.65±0.73 in control, fenceline and direct contact in Murrah buffalo heifers. The drinking frequency of dairy cattle has been reported by various workers as 5.2/day (Jago *et al*., 2005), 9.4/day (Huzzey *et al*., 2005), 7.3/day (Cardot *et al*., 2008), 8.1 drinking bouts/day (Gavojdian *at al*., 2008). The observations of drinking frequency in this study are lower than reported. It might be due to the experiment being conducted in the winter months.

Table 4.13 Average drinking frequency (times/day) of two groups of heifers

Days after the start of the experiment	T_0 (no bull exposure)	T_1 (bull exposure)
0	3.44±0.67	3.67±0.29
15	3.00±0.33	3.56±0.18
30	2.78^{a}±0.32	3.89^{b}±0.35
45	3.44^{a}±0.29	2.33^{b}±0.17
60	3.22^{a}±0.36	4.22^{b}±0.22
75	3.22±0.55	3.78±0.43
90	5.22±0.89	6.00±0.78
120	5.78±0.91	7.44±1.19
Overall	**3.76±0.39**	**4.36±0.56**

Means bearing different superscripts in a row differ significantly (P<0.05)

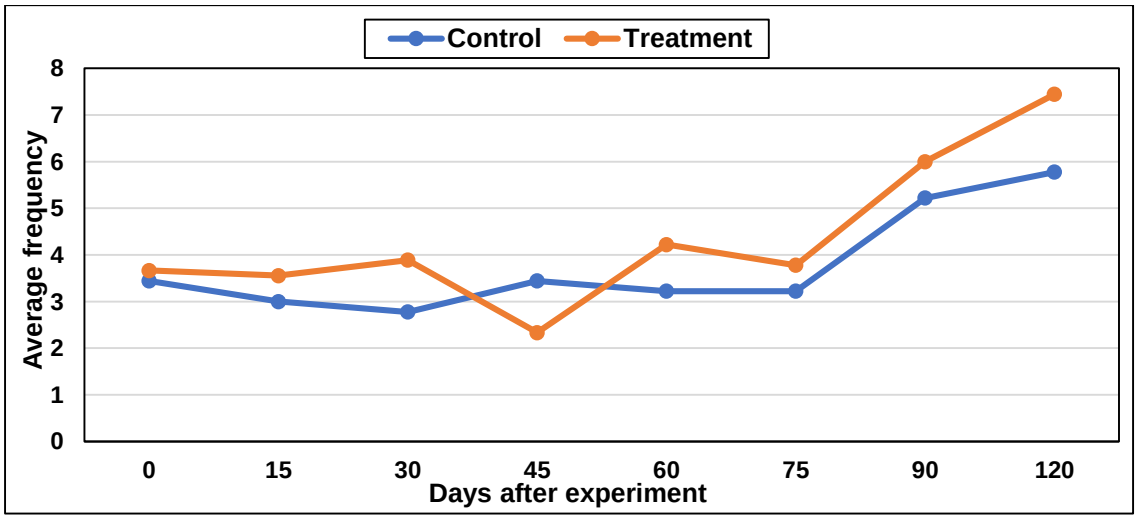


Fig 4.9 Average drinking frequency of two groups of heifers recorded at fortnightly intervals

Plate 4.10 Drinking behaviour of two groups of heifers

4.4.6 Allogrooming frequency

The average frequency of allogrooming observed for heifers is presented in table 4.14 and graphically represented in fig 4.8. The overall average frequency of allogrooming in the T_1 and T_0 group of heifers were 20.09±2.26 and 9.94±2.49. The allogrooming frequency was significantly higher ($p<0.05$) in the treatment group of heifers as compared to control group. During first month of experiment most of the animals in treatment group came into estrus under the influence of high estrogen in these heifers. The allogrooming frequency (licking and grooming) was observed to be more in treatment group.

Table 4.14 Average allogrooming frequency (times/day) of **two groups of heifers**

Days after the start of the experiment	T_0 (no bull exposure)	T_1 (bull exposure)
0	4.56^{a}±1.53	19.67^{b}±5.39
15	14.67^{a}±3.84	29.89^{b}±5.83
30	02.11±1.63	11.11±6.09
45	21.56±4.84	23.11±6.78
60	10.89±4.10	15.11±7.87
75	3.00^{a}±1.84	16.00^{b}±4.51
90	16.22±5.90	18.33±6.66
120	6.56^{a}±4.07	27.56^{b}±5.21
Overall	**9.94^{a}±2.49**	**20.09^{b}±2.26**

Means bearing different superscripts in a row differ significantly ($P<0.05$)

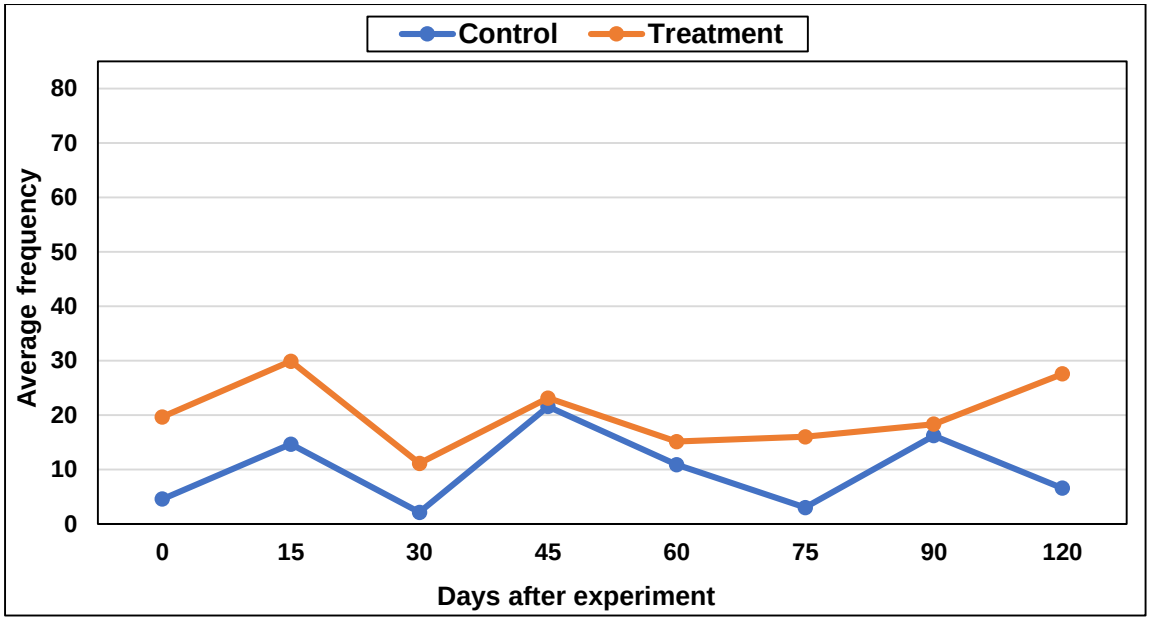


Fig 4.10 Average allogrooming frequency of two groups of heifers

Plate 4.11 Allogrooming behaviour of two groups of heifers

Although there haven't been any published scientific studies on buffalo allogrooming. Winckler *et al*. (2002) and Wasilewski (2003) reported that social licking in cattle can be a stress-relieving behavior that also deepens and stabilises social ties. Wood (1977) and Sato (1984) reported a positive link between being licked and weight gain and milk production in this species, respectively.

4.5 Cervical mucus characteristics

4.5.1 Physical properties of cervical mucus

The frequency distribution of some physical properties of cervical mucus viz. quantity (amount), appearance (colour), consistency, spinnbarkeit value and arborisation pattern (fern pattern) are presented in table: 4.15.

Table 4.15 Physical properties of cervical mucus of heifers

Sr. No.	Parameter	Percent of total heifers	
		Control (n=2)	**Treatment (n=10)**
1	**Quantity**		
	Copious	-	20 (2)
	Moderate	50 (1)	70 (7)
	Scanty	50 (1)	10 (1)
2	**Appearance**		
	Clear	50 (1)	80 (8)
	Cloudy	50 (1)	20 (2)
	Dirty	-	-
3	**Consistency**		
	Thin	-	20 (2)
	Moderate	100 (2)	80 (8)
	Thick	-	-
4	**Spinnbarkeit Value**		
	0-8 cm	50 (1)	30 (3)
	8-16 cm	50 (1)	50 (5)
	16-24 cm	-	20 (2)
5	**Arborization pattern**		
	Typical	50 (1)	60 (6)
	Atypical	50 (1)	30 (3)
	Nil	-	10 (1)

(a) Quantity

The majority of estrus episodes (70%) in the treatment group were connected to moderate mucus flow during AI (Table 4.15). For the characteristic changes in the cervical mucus during the estrus threshold estrogen level is required which may be the reason for more copious discharge in cows.

Verma (2012) reported 53.84% moderate, 30.78% copious, 15.38% scanty. Layek (2011), claimed that 70.17 % of estruses were accompanied by profuse mucus discharge. The results found in the current study are higher than those reported by Verma (2012).

(b) Appearance

In the majority of the heifers in treatment group cervical mucus was clear in appearance and in the rest, mucus was cloudy in appearance (table 4.15). No animal in the present study was observed with dirty discharge.

Sahu (2022) reported 60% clear and 40% cloudy appearance in Murrah buffalo heifers. Verma (2012) observed 76.92% clear and 23.08% cloudy appearance in Murrah buffaloes. Similar to the current study, Gill *et al*. (1974) observed 82.42 % clean mucus discharge in Murrah buffaloes. Gunasekaran *et al*. (2007) reported a lower proportion of 67.9 and 62.86 % respectively of clean discharge in buffaloes.

(c) Consistency

A higher percentage of buffalo heifers displayed cervical discharges with moderate consistency. In the treatment group 80% (n=10) and in the control group 100% (n=2) of heifers showed moderate consistency of mucus (table 4.15).

Sahu (2022) reported 40% thin, 40% medium and 20% thick consistency in bull exposed buffalo heifers. Verma (2012) observed 15.38% thin, 61.54% medium and 23.08% thick consistency in buffaloes. Gunasekaran *et al*. (2007) reported more findings in Murrah buffaloes (68.57 %). Deo and Roy (1971) and Agarwal and Purbey (1983), reported the presence of a larger percentage of thin mucus was present (61.8 and 54.17% respectively). The consistency of mucus mostly depends on the estrus stage at the time the sample is taken, and this may account for the variations in results among studies since the animals used for insemination may not always be in the same estrus stage. Consistency is a subjective quality, and how it is defined will depend on the observer or researcher, which could be a contributing factor to the difference between studies. In the present study, most

of the buffalo heifers are associated with moderate consistency of cervical mucus which implies that the majority of the animals were in mid-estrus during the time of insemination.

(d) Spinnbarkeit value

Cervical mucus of 50 % of estruses was in the 8-16 cm range whereas 16.7 % of estruses were in the 16-24 cm range and 33.3 % of estruses were in the 0-8 cm range (Table 4.13) with respect to Spinnbarkeit value.

Verma (2012) reported 30.77% (0-8cm), 46.15% (8-16cm), 23.08% (16-24cm) in Murrah buffalo heifers. The present Spinnbarkeit values of which are almost similar to the results in present study. The higher values i.e., 19 cm. were observed by Pattabiraman *et al*. (1967) in crossbred cows whereas the lower values were reported by Rao and Rao (1982) in crossbred heifers and Tsiligianni *et al*. (2001) in Friesian heifers (7.25 cm and 7.6 cm., respectively).

Plate 4.12 Spinnbarkeit value of cervical mucus

(e) Arborisation pattern

Cervical mucus in a higher proportion of estruses (58.3%) (n=7) had shown the typical fern pattern predominantly with primary, secondary and tertiary venation. Among the remaining estruses, 33.3% represented the atypical and 0.083 % represent the nil fern pattern (Fig. 4.13).

Sahu (2022) also reported higher percentage (75) of bull exposed buffalo heifers showing typical fern pattern of mucus whereas Verma (2012) reported lower (38.4%) number of buffaloes showing typical fern pattern. The present study finding is slightly lower than the findings observed by Kumar (1989) in buffaloes (60.29%) and Rangnekar *et al*. (2002) in HF cows (60%) whereas the typical fern pattern value of the present finding

is higher than the values reported by Deo and Roy (1971) and Agarwal and Purbey (1983) in buffaloes (42.1 and 25%, respectively).

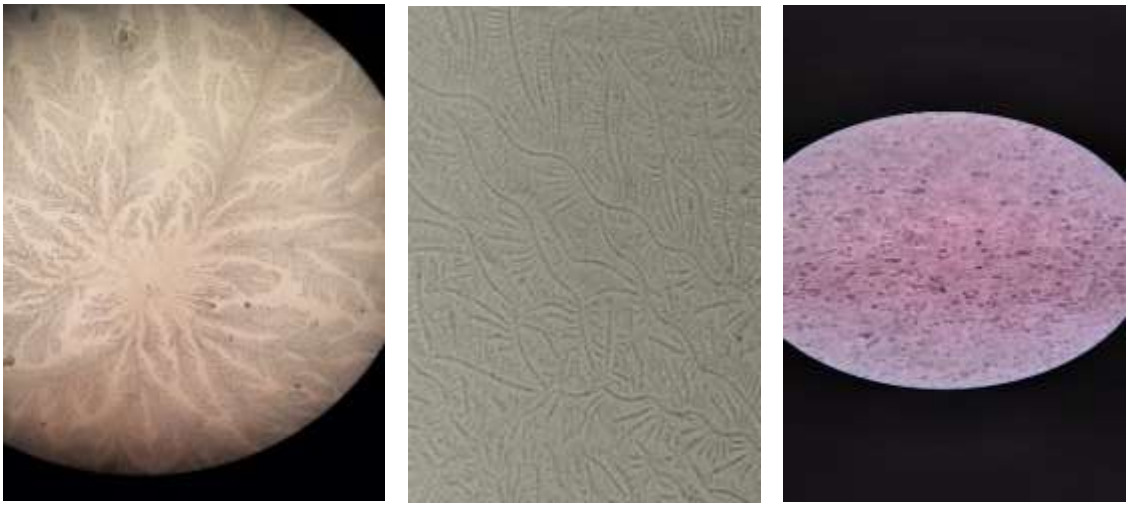


Plate 4.13 Typical, Atypical and nil fern pattern of cervical mucus

4.5.2 Chemical properties of cervical mucus

(a) pH

In the present study in the majority of the animals, the average pH value was 7.5 - 8.0. The pH value in our study is lower than that found by Chhatry *et al*. (1999) in Murrah buffaloes (8.18). Cervical mucus of estruses (41.6%) was within pH range 7.5- 8.0 while 0.16% and 41.6% of cervical mucus were in pH range >8 and 7.0-7.5 respectively (table 4.16).

Verma (2012) and Joshi (2016) also reported that cervical mucus during estrus in most of buffaloes had a pH range of 7.5-8.0.

Table 4.16 Number of heifers with different cervical mucus pH

Group	pH		
	7.0-7.5	**7.5-8.0**	**Above 8.0**
Control	2 (100)	-	-
Treatment	3 (30)	5 (50)	2 (20)

Figures in parenthesis represent the percentage of animals

4.6 Hormone assay

4.6.1 Plasma progesterone concentration

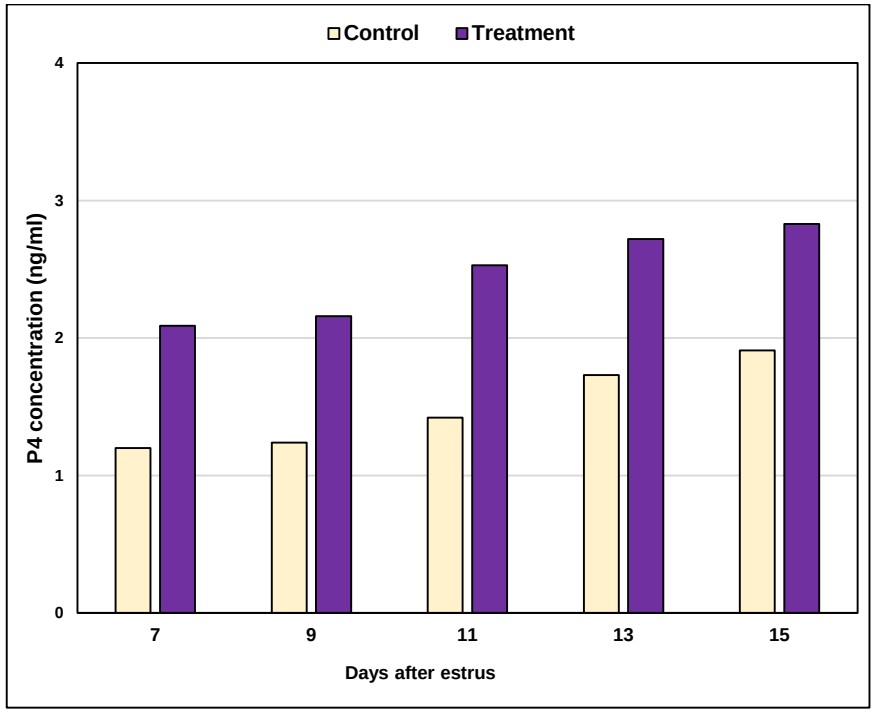
The data on average progesterone concentration (ng/ml) on alternate days between the 7^{th} to 15^{th} days of the estrous cycle during both the first and second estrous cycle are presented in table 4.17 and graphically presented in fig 4.9. The reason for selecting the days ranging from the 6th to 16th days of the estrus cycle was that the active corpus luteum is present during this period of the estrous cycle. The pattern of P4 concentration had shown variations between the above-mentioned period of the oestrus cycle but the peak concentration of P4 was seen from the 11th day of the oestrous cycle during both the oestrus cycles in two groups. It was also observed that the amount of progesterone hormone was significantly ($P<0.05$) higher in the bull exposed groups as compared to the no bull exposed group which might be due to the presence of a bigger size of corpus luteum in the bull exposed groups. The amount of progesterone hormone in the body is more than 1 ng/ml of blood between the 7th to 15th days of the oestrous cycle proving that all the heifers were in estrus in the recent period.

These results are consistent with those of Fike *et al*. (1996b), who found that cows exposed to a bull elevated progesterone concentration quicker than cows who were not exposed to the bull. Patra (2006), on the other hand, found no difference in progesterone levels between bull-exposed and no bull exposed postpartum cows.

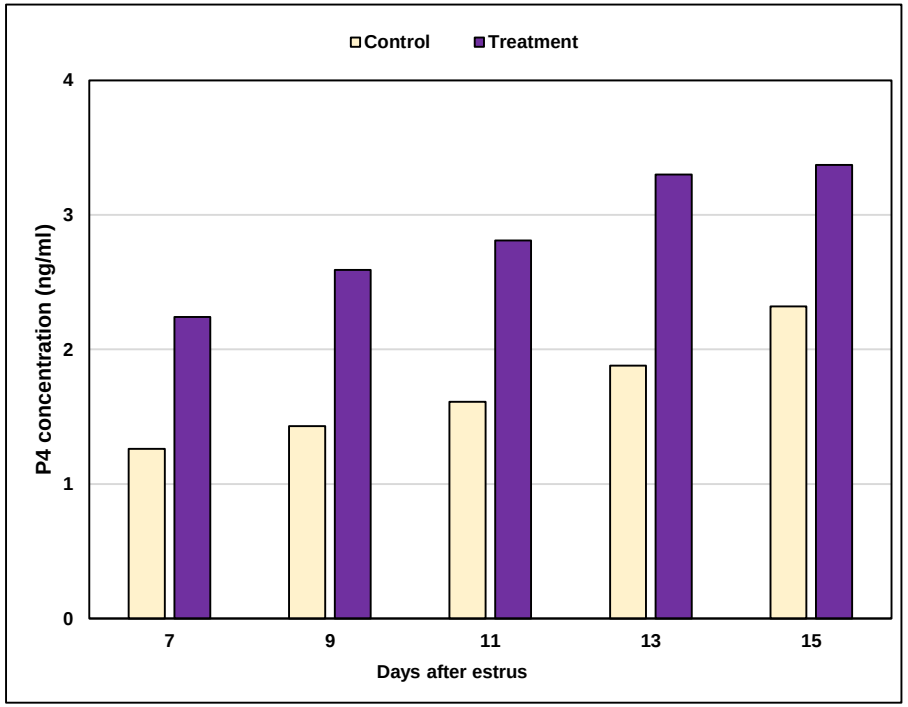
Table 4.17 Average progesterone (P4) hormone concentration (ng/ml) in blood plasma in two groups of heifers

Group	Days after 1st estrus					Days after 2nd estrus				
	7	**9**	**11**	**13**	**15**	**7**	**9**	**11**	**13**	**15**
T_0	1.20^{a}±0.06	1.24^{a}±0.12	1.34^{a}±0.14	1.73^{a}±0.19	1.91^{a}±0.24	1.26^{a}±0.05	1.36^{a}±0.2	1.61^{a}±0.21	1.88^{a}±0.22	2.32^{a}±0.18
T_1	2.09^{b}±0.20	2.16^{b}±0.17	2.53^{b}±0.13	2.72^{b}±0.22	2.83^{b}±0.12	2.24^{b}±0.21	2.59^{b}±0.20	2.81^{b}±0.27	3.30^{b}±0.20	3.37^{b}±0.21

Means bearing different superscripts differ significantly (P<0.05)



First estrus



Second estrus

Fig 4.11 Average progesterone (P4) hormone concentration (ng/ml) of heifers in first and second estrus.

CHAPTER –5

Summary and Conclusions

SUMMARY AND CONCLUSIONS

Buffalo is regarded as a sluggish breeder because of certain characteristics such as late maturity, poor expression of estrus signs, especially during the summer, irregular oestrous cycle, silent heat, seasonality in breeding, poor conception rate and a long inter-calving interval which reduce the reproductive efficiency. Buffalo is also a difficult breeder because of its natural susceptibility to environmental stress, which promotes anestrous and repeats breeding. These two factors are to blame for the buffaloes protracted inter-calving time, which results in significant financial losses. Delayed puberty in dairy heifer's costs farmers and the country a lot of money because it reduces the number of calves produced and milk yield, as well as increases the cost of feeding before they start producing. Anestrus is one of the most common reproductive disorders in buffalo. Priming of the peripubertal dairy heifers by the provision of social cues such as through exposure to adult males (biostimulation) has given encouraging results in indigenous breeds of cattle and in buffaloes. However, there is a great potential for application of the biostimulation technique by exposure to a bull for an early onset of estrus. For this reason, this study has been planned to explore the possible potential of the biostimulation effect to improve their reproductive performance, with the intention that this will improve lifetime performance and longer production life for individual buffalo heifers.

Experimental methodology

The experiment was conducted on Murrah buffalo heifers to study the effect of biostimulation on onset of estrus, estrus behaviour and reproductive performance. For this study, 24 heifers were allotted to 2 groups: T_0 (control) and T_1 (treatment) of 12 each for a period of 5 months from December 2021 to April 2022. An adjustment period of 15 days was given to the animals before the actual start of experiment. In T_0 the cows were not exposed to the bull and in T_1 group, the cows were exposed to the bull directly for 6 hours daily (3hr in morning and 3hr in evening). Both groups of heifers were housed separately at a distance of about 0.5 km from each other. All the experimental animals were housed under a loose housing system and floor space (i.e., 4 m^2 covered area, 8 m^2 open area). The feeding of both groups of cows was similar comprising of ad libitum feeding of seasonal green fodders and the concentrate mixture feeding. Apart from concentrate mixture, all experimental heifers were fed twice a day on a ration consisting of roughages

as per the availability in the farm at 9 A.M. and 3.30 P.M. respectively. Fresh water was available for drinking to all the heifers round the clock inside the shed. At fortnightly intervals, the body weights were taken. A CCTV camera was used to track the heifers' estrus behavior and general behavior around the clock. The blood was collected on 7^{th}, 9^{th}, 11^{th}, 13^{th} and 15^{th} day of estrous cycle for estimation of level of progesterone. Data were analysed using SPSS software (Version 21.0) using t-test: two sample assuming equal variance.

Salient findings

- The final body weight was in T_0 (362.47±7.32) and T_1 (369.07±8.2) kg respectively which did not differ significantly from each other.
- The overall daily average daily gain in body weight in T_1 was 605±19.34 gm/day which was significantly ($P<0.05$) higher than T_0 (507±28.1 gm/day).
- The mean dry matter intake (DMI) of both groups was not significantly different in the two groups in T_1 (10.72±0.28 kg/head) and in T_0 (10.43±0.24 kg/head)
- A total of 10 anestrus heifers out of 12 in bull exposed group and only 2 in control group were observed in estrus. Seven heifers in treatment group were inseminated and 4 were conceived whereas none of the T_0 heifers was inseminated.
- The average age at first estrus in T_0 and T_1 was 28.04±0.01 and 27.58±0.33 months. The average age at first service in the T_1 group was 28.18±0.57 months.
- The conception rate in T_1 group was 57.14% and number of services per conception in T_1 was 1.75.
- The average frequencies of estrus behaviour including sniffing and licking on the day of estrus were (12±3.00 vs. 26.5±3.42), tail raising (9±3.00 vs. 20.12±2.64), chin resting (4±1.00 vs. 12±1.34), stand to be mounted (3±1.00 vs. 9.75±1.16) in T_0 and T_1 respectively. which were higher in T_1 group as compared to T_0 group of heifers.
- The mean standing time in treatment (T_1) and control group (T_0) was 722.57±4.93, 678.25±4.43 minutes per day respectively. which was significantly higher ($p<0.05$) in bull exposed heifers as compared to no bull exposed heifers.

- The average lying time in treatment (T_1) and control group (T_0) of heifers was 689.59±3.87, 728.06±4.01 minutes per day respectively. This was significantly higher ($p<0.05$) in the control group as compared to treatment group of heifers.
- The mean eating time in treatment (T_1) and control group (T_0) was 260.89±2.14, 249.04±2.27 minutes per day respectively. The eating time was significantly higher ($p<0.05$) in the treatment group as compared to control group of heifers.
- The average rumination time in treatment (T_1) and control group (T_0) were 396.22±5.25, 375.80±5.36 minutes per day respectively. The rumination time was significantly higher ($p<0.05$) in the treatment group as compared to control group of heifers.
- The average frequency of drinking in the T_1 and T_0 group of heifers was 4.36±0.56 and 3.76±0.39. The drinking frequency was not statistical different in the two groups of buffalo heifers.
- The average frequency of allogrooming in the T_1 and T_0 group of heifers was 20.09±2.26 and 9.94±2.49 respectively. The allogrooming frequency was significantly higher ($p<0.05$) in the treatment group as compared to control group of heifers.

Conclusions

1. The biostimulation of anestrus buffalo heifers through direct bull exposure was effective in bringing most of the heifers into estrus with normal expression of estrus behaviour.
2. The reproductive performance of bull exposure anestrus heifers was better in comparison to non-bull exposure heifers.

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