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# Temporal Changes in Endogenous Estrogens and Expression of Behaviors Associated with Estrus during the Periovulatory Period in Doublesynch Treated Murrah Buffaloes (*Bubalus bubalis*)

Research Article

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## ABSTRACT

The aims of this study were (a) to establish the occurrence, duration and intensity of behavioral estrus symptoms, and (b) to determine the relationship between behavioral estrus symptoms with the total estrogen profiles in cycling Murrah buffaloes (n=10) subjected to Doublesynch protocol (0 $PGF_{2\alpha}$, 2 GnRH, 9 $PGF_{2\alpha}$ and 11 GnRH). After both GnRH injections, estrus and its behavioral signs were detected at hourly intervals using visual observations, transrectal examination of genitalia, and bull parading twice a day for 30 min each. Frequent urination (100%) and excitement (100%) were the best indicators of estrus followed by swollen vulva (90%) and bellowing (85%). The longest duration of estrus signs viz. swollen vulva, was observed up to 8.9 ± 0.5 h and 11.1 ± 0.3 post first and second GnRH administrations, respectively. The mean intervals between the onset of estrus symptoms appearance and ovulation were 13.8 ± 0.9 h and 13.4 ± 1.0 h post first and second GnRH inactions, respectively. The mean total estrogen concentrations at the time of both GnRH injections were above the basal level (8.4±1.9 pg/mL; range 2.9-18.4 and 7.9±1.9 pg/mL; range 2.9-17.6 at the time of the first and second GnRH injections, respectively), temporarily increased post GnRH treatments and then gradually declined to its basal level during ovulation time window. Frequent urination, excitement and swollen vulva appeared to be the most notable estrus symptoms after GnRH injections. Our findings also suggest that the AI can be done at a fixed time (once 20 to 24 h posts second GnRH) in buffaloes treated with Doublesynch protocol.

KEY WORDS Doublesynch, estrogen, estrus behavior, Murrah buffalo.

## INTRODUCTION

In many south and south-east Asian countries, and in particular in India, the riverine buffaloes are the mainstay of milk and meat production system. Despite their low reproductive efficiency, they can utilize the native roughage of low quality. Poor estrus expressivity and silent estrus are perhaps the major limitations which contribute to the low reproductive efficiency of this species, particularly during hot seasons.

It has been reported that a high number of buffaloes (30-40%) experience a prolonged period of anestrus in India which can incur an estimated loss of 19-20 million tons of milk per year (Madan and Prakash, 2007). To maximize the chance of successful artificial insemination (AI) practices, proper estrus detection is essential. In Murrah buffaloes, because of less obvious estrus symptoms, the accuracy of estrus detection is one of the major problems limiting the use of AI. Therefore, the use of estrus synchronization protocols can be highly beneficial because they allow for timed

artificial insemination (TAI) of buffaloes without the need for estrus detection. Very recently, we have performed a newly-developed estrus-synchronization protocol named Doublesynch (d 0 $PGF_{2\alpha}$, d 2 GnRH, d 9 $PGF_{2\alpha}$, d 11 GnRH, TAI 16 and 24 h) in Murrah buffaloes (Mirmahmoudi and Prakash, 2012). Nonetheless, understanding of the expression of estrus behaviors and their relationship with the hormonal injections and associated endocrine changes that control estrus are also fundamental for the construction of strategies aimed at improving the detection of estrus and fertility improvement. Estrogen acts as a key regulator of the endocrine and behavioral events associated with the natural estrous cycle and induces estrus behavior, the release of GnRH, an LH surge, and finally ovulation (Hafez and Hafez, 2000). Several studies have reported the expression of various estrus symptoms in hormonally-induced estrus as well as spontaneous detected estrus in buffaloes (Singh *et al*. 1984; Paul 2003; Mohan and Prakash, 2010; Mohan *et al*. 2010), however, there are no reports on the occurrence, duration and intensity of estrus signs in relation to the preovulatory estrogen changes and ovulation in Murrah buffalos subjected to the Doublesynch protocol. This study was therefore undertaken to determine the duration and intensity of various estrus symptoms associated with the periovulatory total estrogen release and timing of ovulations after the first and second GnRH injections in cycling Murrah buffaloes treated with the Doublesynch protocol during the low breeding season.

## MATERIALS AND METHODS

### Animals and management

This study was conducted on 10 cycling Murrah buffaloes (3 to 4 parity) during the period extending from the first week of April to the end of May (low breeding season) when the humidity was 75-85% and the ambient temperature was 30-39 ˚C. The maximum incidence of silent heat in Murrah buffaloes has already been reported for this particular time period of the year (Prakash *et al*. 2005). The cyclicity of the buffaloes was confirmed by progesterone analysis from blood plasma samples that were collected twice weekly over a 21-day period (Kamboj and Prakash, 1993) prior to treatment. All of the animals were selected from the herd maintained at the National Dairy Research Institute, Karnal, India. The animals were fed a diet consisting of a concentrated mixture of maize grain, groundnut cake, mustard cake, wheat bran, mineral mixture, salt and roughage (either berseem, maize or oat fodder based on availability) following the standard feeding practices employed at the NDRI farm. Ad libitum fresh drinking water was available throughout the day and night to all of the animals. The Animal Ethics Committee of the Institute approved these experiments.

### Doublesynch treatment

Estrus was synchronized by administering 25 mg of $PGF_{2\alpha}$ (dinoprost tromethamine; Lutalyse™, Novartis India Limited, Maharashtra, India) without regard to the estrous cycle stage (day of first $PGF_{2\alpha}$ treatment=day 0), followed by 10 µg of a GnRH analog (Buserelin Acetate, Receptal® VET, Intervet India Private Ltd., Pune, Maharashtra, India) on day 2, a second $PGF_{2\alpha}$ dose (25 mg) on day 9, and a second GnRH dose (10 µg) 48 h after the second $PGF_{2\alpha}$ dose (day 11). All injections were given i.m.

### Monitoring of estrus behaviors, detection of ovulation, and timing of events

Occurrence and intensity of estrus in the animals were monitored by hourly observation of various behavioral estrus signs and by vasectomized bull (teaser) parading twice daily for a 30 min and further confirmed by observing uterine tone on rectal palpation. The value for intensity has been given according to visual appraisal on a 3-point scale (+ low; ++ medium; +++ high). The behavioral signs monitored were excitement, bellowing, frequent urination, swollen vulva, uterine tone, tail raising, mucus discharge, chasing by bull, bull mounting and chin resting on other animal. In addition, ovulation was detected by transrectal palpation of the ovaries conducted every 2 h from both GnRH treatments until detection of ovulation (or up to 96 h after the GnRH treatment if ovulation was not detected). Ovulation was confirmed by the change of ovarian surface from turgid to flaccid (Moioli *et al*. 1998).

### Collection of blood samples and hormone assay

After both the first and second GnRH treatments, blood samples were collected via an indwelling jugular catheter every 30 min for 8 h and then at 2-h intervals until 2 h after ovulation was confirmed. The samples were maintained at 4 °C and transported to the laboratory within 1 h of collection. The samples were then centrifuged, and the plasma was stored at -20 ˚C until hormone analysis. Before catheterization, local anesthesia (Lidocaine Hydrochloride, Xylocaine® Astra Zeneca Pharma India Ltd., Bangalore, India) was administered, and after removal of the catheter, the animals were given an antibiotic treatment (Terramycin, Oxytetracycline®, Pfizer Ltd., Aana Salai, Chennai, India) for the next 3 days. The total estrogen concentration was measured using a sensitive heterologous enzyme immunoassay previously developed in the laboratory (Mondal *et al*. 2006). This procedure utilized 50 µl of extracted and reconstituted plasma.

The sensitivity of this assay was 0.4 pg/50 µL/well, which corresponded to a plasma concentration of 2.9 pg/mL. The inter- and intra-assay coefficients of variation were 12.2 and 6.1%, respectively.

**Data analysis**

The data for estrus symptoms and changes in the plasma total estrogen concentrations after both of the GnRH treatments were analyzed by repeated measures ANOVA. We used a t-test to determine significant differences between the means. All of the statistical analyses were conducted using SAS (1996). The means and standard errors of the mean are quoted in the text.

## RESULTS AND DISCUSSION

**Estrus behavior**

The incidence of various behavioral estrus symptoms after the first and second GnRH administrations of the Doublesynch protocol are presented in Table 1. Frequent urination and excitement were the prominent estrus signs and were observed in all buffaloes after both the first and second GnRH treatments; swollen vulva and bellowing were the next two most exhibited symptoms. Other behavioral estrus signs exhibited in buffaloes treated with the Doublesynch protocol were uterine tone, bull mounting, tail raising, mucus discharge, chasing by bull, and chin resting on other animal.

No significant difference (P>0.05) was found between the total numbers of estrus symptoms expressed per buffalo after the first (6.7) and second (6.9) GnRH injection of the Doublesynch protocol (Table 1). Also, similar intensities of estrus signs post first (1.6) and second (1.9) GnRH treatments were found (P>0.05).

**Duration of behavioral estrus symptoms**

The onset, end and duration of various behavioral estrus signs exhibited after both the first and second GnRH administrations for estrus synchronization with the Doublesynch protocol in 10 cycling Murrah buffaloes are presented in Table 2.

Swollen vulva and bellowing were the first estrus signs which appeared after both the first and second GnRH treatments. Six of a total of 10 observed estrus behaviors, viz. frequent urination, mucus discharge, uterine tone, bull mounting, chasing by bull, and chin resting on other animal appeared earlier after the first GnRH rather than the second GnRH injection (Table 2). This made a significantly (P<0.05) shorter average time between the first GnRH injection and appearance of estrus behaviors (8.4±0.4 h) compared with that after the second GnRH treatment (9.8±0.4 h).

In addition, the appearance 6 of a total of 10 observed estrus behaviors, viz. swollen vulva, uterine tone, tail raising, bull mounting, chasing by bull, and chin resting on other animal lasted longer after the first GnRH injection than after the second GnRH injection (Table 2). Nonetheless, such differences in the appearance and disappearance of estrus signs did not result in significant changes between the overall duration of the behavioral estrus symptoms after the first (6.2±0.4 h; range 3.0-12.0) and second (6.1±0.4 h; range 2.0-12.0) GnRH administrations (Table 2).

Ovulation occurred in 9 / 10 buffaloes (90%) at 22.2 ± 1.2 h (Mean±SEM; range 18.0-26.0 h) and 10 / 10 buffaloes (100%) at 23.2 ± 1.0 h (Mean±SEM; range 20.0-28.0) after the first and second GnRH treatments, respectively. The intervals between the onset of estrus symptoms and ovulation were 13.8 ± 0.9 h and 13.4 ± 1.0 h (P>0.05) post first and second GnRH inactions, respectively. Likewise, the intervals between the disappearance of estrus symptoms and ovulation recorded post-first 7.6 ± 0.8 h and second 7.3 ± 0.9 h GnRH administrations of Doublesynch protocol were similar (P>0.05).

**Periovulatory plasma total estrogen profile after the first and second GnRH treatments in Doublesynch protocol**

The temporal periovulatory changes in plasma total estrogen after both GnRH injections in Doublesynch treated cycling buffaloes are presented in (Figs. 1 and 2). The mean total estrogen concentrations at the time of both GnRH injections were above the basal level (8.4±1.9 pg/mL; range 2.9-18.4, and 7.9±1.9 pg/mL; range 2.9-17.6 at the time of the first and second GnRH injections, respectively). Individual variation in the temporal changes of total estrogen after both GnRH injections were observed in the buffaloes. Except in a non-responder buffalo (with ovulation) post first GnRH injection, the remaining of buffaloes exhibited a temporal increase (with great variability), and then a gradual decline in total estrogen concentration until ovulation (Figures 1 and 2). To the best of our knowledge, this is the first study reporting the expression and duration of various behavioral estrus symptoms associated with GnRH injections in cycling Murrah buffaloes subjected to the Doublesynch protocol during the low breeding season. The behavioral estrus signs expressed after the first and second GnRH treatments of the Doublesynch program in cycling Murrah buffaloes were frequent urination, excitement, swollen vulva, bellowing, presence of uterine tone, mucus discharge, bull mounting, tail raising, chasing by bull, and chin resting on other animal (Table 1). The four most important symptoms were frequent urination, excitement, swollen vulva, and bellowing (with an average number of responders after both GnRH injections of 9.4±0.3).

**Table 1** Incidence of various behavioral estrus signs exhibited by buffaloes (n=10) after first and second GnRH injections in Doublesynch protocol

| Parameters | First GnRH | | Second GnRH | |
|---|---|---|---|---|
| | No. of responders | Intensity (Mean) | No. of responders | Intensity (Mean) |
| **Excitement** | 10 | + | 10 | ++ |
| **Bellowing** | 8 | ++ | 9 | ++ |
| **Frequent urination** | 10 | +++ | 10 | ++ |
| **Swollen vulva** | 8 | +++ | 10 | +++ |
| **Mucus discharge** | 6 | ++ | 5 | +++ |
| **Uterine tone** | 6 | + | 5 | ++ |
| **Tail raising** | 5 | + | 6 | + |
| **Bull mounting** | 5 | + | 5 | + |
| **Chasing by bull** | 6 | + | 6 | ++ |
| **Chin resting on other animal** | 3 | + | 3 | + |
| **Number of symptoms and average intensity of estrus signs observed/animal** | 6.7 | 1.6 | 6.9 | 1.9 |

On visual appraisal on a 3-point scale (+ low; ++ medium; +++ high).

**Table 2** Duration of estrus signs (Mean±SEM) after the first and second GnRH injections in buffaloes (n=10) treated with the Doublesynch protocol

| Estrus signs | First GnRH | | | | Second GnRH | | | |
|---|---|---|---|---|---|---|---|---|
| | Mean±SEM (h) | | | | Mean±SEM (h) | | | |
| | Onset (h) | End (h) | Duration (h) (Mean±SEM) | Range (h) | Onset (h) | End (h) | Duration (h) (Mean±SEM) | Range (h) |
| **Excitement** | 7.1±0.6 | 13.7±0.4 | 6.6±0.3$^{a}$ | 5.0-7.0 | 7.6±0.5 | 13.3±0.4 | 5.7±0.3$^{b}$ | 4.0-7.0 |
| **Bellowing** | 6.6±0.5 | 13.2±0.3$^{a}$ | 6.6±0.4$^{a}$ | 5.0-7.0 | 7.2±0.4 | 12.1±0.5$^{b}$ | 4.9±0.3$^{b}$ | 4.0-6.0 |
| **Frequent urination** | 6.8±0.3$^{b}$ | 14.5±0.5 | 7.7±0.6$^{a}$ | 4.0-9.0 | 8.3±0.6$^{a}$ | 14.1±0.5 | 5.9±0.5$^{b}$ | 4.0-8.0 |
| **Swollen vulva** | 6.6±0.3 | 15.5±0.6$^{b}$ | 8.9±0.5$^{b}$ | 6.0-10.0 | 7.2±0.3 | 18.3±0.4$^{a}$ | 11.1±0.3$^{a}$ | 9.0-12.0 |
| **Mucus discharge** | 10.2±0.5$^{b}$ | 16.8±0.4 | 6.6±0.3 | 6.0-8.0 | 11.7±0.6$^{a}$ | 17.8±0.6 | 6.1±0.4 | 5.0-9.0 |
| **Uterine tone** | 9.9±0.4$^{b}$ | 13.1±0.2$^{b}$ | 3.2±0.5 | 3.0-5.0 | 11.6±0.4$^{a}$ | 14.6±0.3$^{a}$ | 3.0±0.5 | 2.0-4.0 |
| **Tail raising** | 10.2±0.2$^{a}$ | 14.9±0.2$^{b}$ | 4.7±0.3$^{b}$ | 4.0-5.0 | 9.3±0.1$^{b}$ | 15.8±0.2$^{a}$ | 6.5±0.3$^{a}$ | 5.0-7.0 |
| **Bull mounting** | 10.4±0.4$^{b}$ | 16.6±0.5$^{b}$ | 6.2±0.6 | 6.0-8.0 | 12.5±0.5$^{a}$ | 18.3±0.6$^{a}$ | 5.8±0.7 | 5.0-8.0 |
| **Chasing by bull** | 7.3±0.5$^{b}$ | 14.8±0.6$^{b}$ | 7.5±0.4 | 6.0-12.0 | 11.8±0.3$^{a}$ | 18.9±0.4$^{a}$ | 7.1±0.3 | 6.0-9.0 |
| **Chin resting on other animal** | 8.8±0.2$^{b}$ | 13.2±0.2$^{b}$ | 4.4±0.3 | 4.0-5.0 | 10.9±0.3$^{a}$ | 15.6±0.2$^{a}$ | 4.7±0.3 | 4.0-5.0 |
| **Mean** | 8.4±0.4$^{b}$ | 14.6±0.4$^{b}$ | 6.2±0.4 | 3.0-12.0 | 9.8±0.4$^{a}$ | 15.9±0.4$^{a}$ | 6.1±0.4 | 2.0-12 |

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

The minor behavioral estrus signs after both GnRH injections were presence of uterine tone, mucus discharge, bull mounting, tail raising, chasing by bull, and chin resting on other animal (expressed by almost 50% of buffaloes post first and second GnRH injections; the average numbers of responders was 5.1±0.3). Previous studies reported the expression of various behavioral estrus symptoms during spontaneous or induced estrus in buffaloes (Singh *et al.* 1984; Gunasekaran, 1998; Mohan and Prakash, 2010; Mohan *et al.* 2010). Behavioral estrus signs like bellowing, frequent urination, restlessness, swollen vulva, and mucus discharge were observed by Singh *et al.* (1984) during estrus in buffaloes. Gunasekaran (1998) suggested that uterine tone and turgid or coiled cornuae on rectal palpation can be used for confirmation of estrus in Murrah buffaloes. In addition, other studies suggested that discharge of cervical mucus is one of the most important estrus symptoms in buffaloes (Singh *et al.* 1984; Danell *et al.* 1984). However, in the current study, the mucus discharge and uterine tone were observed only in 55% of the buffaloes after both first and second GnRH administrations.

This is in agreement with the previous findings in our laboratory with cycling Murrah buffaloes during spontaneous estrus (50%) reported by Mohan *et al.* (2010) as well as using Heatsynch (60%) protocol reported by Mohan and Prakash (2010). Janakiraman (1978) considered frequent urination at the onset of estrus as a sure constant sign of estrus in Surti buffalo heifers. Similarly, in the present study, this sign has been observed as a major symptom in all cycling Murrah buffaloes. In the previous studies, it was not seen in all cycling Murrah buffaloes (Mohan and Prakash, 2010; Mohan *et al.* 2010) however, it was recognized as a major symptom. The discrepancy between results obtained about estrus symptoms in different studies may be attributed to different feeding, management, environmental conditions, degree of cyclicity, breed of buffaloes, and the type of administrated hormone and stage of estrous cycle at the time of hormonal treatment (if estrus is induced). The overall average number of estrus signs exhibited per buffalo after both GnRH injections in Doublesynch protocol (6.8) was higher than (5.7) reported by Paul (2003) and (6.0) reported by Mohan *et al.* (2010) in Murrah buffaloes during

spontaneous estrus. However, it is lower than (7.1) reported by Mohan *et al*. (2010) in Heatsynch treated Murrah buffaloes.

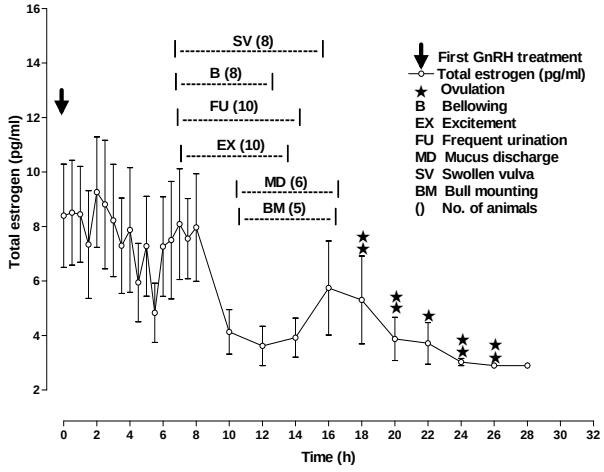


**Figure 1** Changes in the plasma total estrogen profile (Mean±SEM) and mean duration of estrus signs after administration of first GnRH in buffaloes (n=10) treated with the Doublesynch protocol

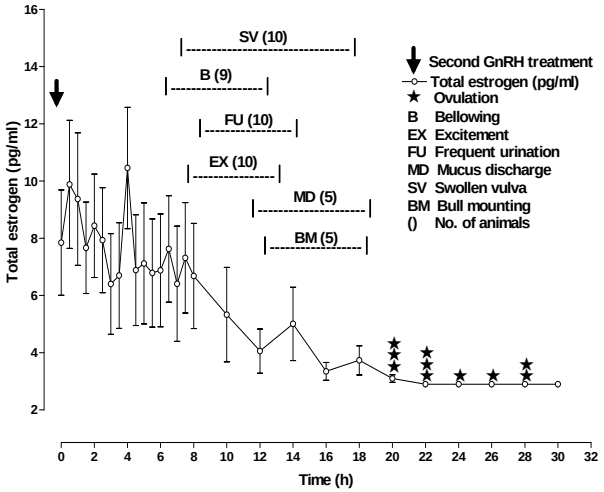


**Figure 2** Changes in the plasma total estrogen profile (Mean±SEM) and mean duration of estrus signs after administration of second GnRH in buffaloes (n=10) treated with the Doublesynch protocol

It can be due to external application of estrogen in Heatsynch protocol which has been recognized as the key regulator of the endocrine and behavioral events associated with estrous cycle (Hafez and Hafez, 2000).

The total estrogen concentration profile in the cycling Murrah buffaloes treated with both first and second GnRH in the present study is lower than those found earlier after spontaneous detected estrus (Mohan *et al*. 2010). It may be because of differences in the stage of estrous cycle of the individual buffaloes used in the present study rather than the experiment done by Mohan *et al*. (2010) in which all buffaloes were in their natural detected estrus. Further, it can be due to the fact that the animals used in the present study are less cycling since this experiment has been conducted during the low breeding season (70% silent heat has already been reported at this particular time of the year by Prakash *et al*. 2005). In addition, Kobayashi (1995) showed that administration of GnRH after $PGF_{2\alpha}$ caused a cessation of estrogen secretion by the preovulatory follicle as evidenced by a decline in blood concentrations of estrogen.

Our observations also indicate that all the estrus symptoms were recorded while the total estrogen concentration is declining, and the basal estrogen levels were recorded at the time of ovulation (Figure 1). Similar patterns were found with buffaloes during spontaneous detected estrus and as well as buffaloes subjected to Heatsynch protocol (Mohan *el al*. 2009; Mohan and Prakash, 2010; Mohan *et al*. 2010). Nonetheless, in the present study, despite with the lower recorded estrogen level and lower cyclicity of animals used than those in the work done by Mohan *et al*. (2010), a higher number of responder buffaloes with estrus and intensity of estrus symptoms were recorded. These can be due to the use of GnRH itself because, that apart from the gonadotropin releasing effects of GnRH analogous and they are able to directly cause the sexual behavior through affect on the limbic structures (Schneider *et al*. 2006).

## CONCLUSION

In conclusion, among the estrus signs, frequent urination, excitement, and swollen vulva appeared to be the three most notable estrus symptoms. From a practical viewpoint, our findings suggest that the AI of cycling Murrah buffalo can be done once 20 to 24 h post second GnRH injection as ovulations were recorded 23.2 h post second GnRH administration in Doublesynch protocol.

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## REFERENCES

Danell B., Gopakumar N., Nair M.C.S. and Rajgopalan K. (1984). Heat detection symptoms in Surti buffalo breeds. *Indian J. Anim. Reprod*. **5,** 1-7.

Gunasekaran M. (1998). Estrous behaviour and fertility in crossbred cattle and Murrah buffaloes. MS Thesis. Deemed Univ., Karnal, India.

Hafez E.S.E. and Hafez B. (2000). Reproduction in Farm Animals. Wiley.

Janakiraman K. (1978). Control of optimizing of reproductive cycle in buffalo. Pp. 220-225 in Proc. Semina. buffalo Reprod. Artif. Insemin, NDRI, Karnal, India.

Kamboj M. and Prakash B.S. (1993). Relationship of progesterone in plasma and whole milk of buffaloes during cyclicity and early pregnancy. *Trop. Anim. Health Prod*. **25,** 185-192.

Kobayashi Y. (1995). Gonadotropin-releasing hormone at estrous in the bovine: changes in characteristics of the preovulatory follicle and corpus luteum and *in vitro* production of progesterone and oxytocin. MS Thesis. Kansas State Univ., Manhattan, New York.

Madan M.L. and Prakash B.S. (2007). Reproductive endocrinology and biotechnology applications among buffaloes. *Soc. Reprod. Fertil*. **64,** 261-281.

Mirmahmoudi R. and Prakash B.S. (2012). The endocrine changes, timing of ovulation and efficacy of the Doublesynch protocol in the Murrah buffaloes (*Bubalus bubalis*). *Gen. Comp. Endocrinol*. **177,** 153-159.

Mohan K., Kumar V., Sarkar M. and Prakash B.S. (2010). Temporal changes in endogenous estrogens and expression of behaviors associated with estrus during the periovulaory period in Murrah buffaloes (*Bubalus bubalis*). *Trop. Anim. Health Prod*. **42,** 21-26.

Mohan K. and Prakash B.S. (2010). Changes in endogenous estrogens and expression of behaviors associated with estrus during the periovulatory period in Heatsynch treated Murrah buffaloes (*Bubalus bubalis*). *Trop. Anim. Health Prod*. **42,** 947-952.

Mohan K., Sarkar M. and Prakash B.S. (2009). Efficiency of Heatsynch protocol in estrus synchronization, ovulation and conception of dairy buffaloes (*Bubalus bubalis*). *Asian-Australas J. Anim*. **22,** 774-780.

Moioli B.M., Napolitano F., Puppo S., Braile V.L., Terzano G.M. and Malfatti A. (1998). Patterns of oestrus, time of LH release and ovulation and effects of time of artificial insemination in Mediterranean buffalo cows. *Anim. Sci*. **66,** 87-91.

Mondal M., Rajkhowa C. and Prakash B.S. (2006). Relationship of plasma estradiol-17-β, total estrogen and progesterone to estrus behavior in mithun (*Bos frontalis*) cows. *Horm. Behav*. **49,** 626-633.

Paul V. (2003). Studies on estrus synchronization methods, timing of ovulation and endocrine profile in buffaloes. Ph D. Thesis. National Dairy Research Institute, Karnal, Haryana, India.

Prakash B.S., Sarkar M., Paul V., Mishra D.P., Mishra A. and Meyer H.H.D. (2005). Postpartum endocrinology and prospects for fertility improvement in the lactating riverine buffalo (*Bubalus bubalis*) and yak (*Poephagus grunniens*). *Livest. Prod. Sci*. **98,** 13-23.

SAS Institute. (1996). SAS®/STAT Software, Release 6.11. SAS Institute, Inc., Cary, NC.

Schneider F., Tomek W. and Gründker C. (2006). Gonadotropin-releasing hormone (GnRH) and its natural analogues: a review. *Theriogenology*. **66,** 691-709.

Singh G., Singh G.B., Sharma S.S. and Sharma R.D. (1984). Studies on oestrus symptoms of buffalo heifers. *Theriogenology*. **21,** 849-858.