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# RIASSUNTO

La precoce ripresa della ciclicità ovarica nel periodo post-partum è di fondamentale importanza per le produzioni di latte. L'anaestro post-partum nelle bufale è la maggior causa del lungo interparto, della bassa fertilità e determina una perdita economica da parte dell'allevatore. Per un'ottimale gestione della riproduzione è necessario identificare il metodo migliore di sincronizzazione dell'estro per ottenere un buon tasso di gravidanza dopo inseminazione artificiale con seme congelato. Con questo obiettivo, sono stati condotti due esperimenti per valutare l'efficacia del protocollo Ovsynch (GnRH + $PGF_{2\alpha}$ + GnRH) per la sincronizzazione degli estri e la fertilità successiva all'inseminazione artificiale in bufale di due nazioni (Italia e Bangladesh).

Lo scopo del primo esperimento è stato quello di valutare l'efficacia di un protocollo Ovsynch utilizzando due analoghi del GnRH (Buserelina e Gonadorelina), a due orari di somministrazione (mattutino e pomeridiano), per la sincronizzazione dell'ovulazione e per la fertilità successiva ad inseminazione con seme sessato in bufale mediterranee allevate in Italia. Sono state utilizzate trentadue bufale di razza Mediterranea Italiana. Tutti i soggetti hanno risposto positivamente alla seconda somministrazione di GnRH del protocollo Ovsynch presentando l'estro e sono state inseminate artificialmente con 2.5 milioni di spermatozoi sessati (Spermatozoi X), vivi allo scongelamento (4 milioni in totale), entro le 18-21 ore dopo la seconda somministrazione dell'analogo del GnRH. L'impedenza elettrica del muco vaginale, la temperatura vulvare e quella perivulvare sono state misurate in diversi stadi della sincronizzazione per monitorare l'induzione dell'estro. La gravidanza è stata accertata tramite ecografia trans-rettale a 42 giorni dall'inseminazione. Il tasso di gravidanza è stato del 37.5 % quando è stata utilizzata la Buserelina ed ha raggiunto il 50 % quando il trattamento ormonale è stato mattutino. I valori d'impedenza elettrica sono diminuiti e la temperatura vulvare è aumentata al tempo di somministrazione della seconda dose di GnRH, quando le bufale hanno avuto l'estro

indotto. I risultati del primo esperimento forniscono le basi per futuri studi sull'ottimizzazione della cronologia e della tipologia di trattamento per la sincronizzazione dell'estro e dell'ovulazione con analoghi del GnRH. L'ottimizzazione porterebbe ad un sensibile aumento del tasso di gravidanza così come delle capacità produttive delle bufale Mediterranea italiana.

Nel secondo esperimento, sono state utilizzate 114 bufale indigene del Bangladesh per studiare l'efficienza del protocollo Ovsynch per la sincronizzazione dell'ovulazione e la fertilità post inseminazione con seme congelato di razza Bufala Mediterranea Italiana. È stato valutato anche l'effetto di parametri come il numero di follicoli, la presenza di corpi lutei e i giorni di lattazione al momento del reclutamento dei soggetti. Le bufale sono state selezionate in due aree del Bangladesh e sono stati suddivisi in tre gruppi in dipendenza del tipo di protocollo. Ogni gruppo è stato suddiviso in AM e PM in dipendenza dell'orario di somministrazione (mattutino o pomeridiano) degli ormoni nel protocollo di sincronizzazione. Tra i tre gruppi, il più alto tasso di gravidanza (78% in AM) è stato osservato nel gruppo di trattamento 3 che era caratterizzato da un'ulteriore somministrazione di GnRH al momento dell'inseminazione. Simile a quanto osservato nell'esperimento 1, si è osservato un più alto tasso di gravidanza quando il trattamento è stato mattutino (74%) rispetto al trattamento pomeridiano (63%). Per ciò che riguarda il periodo post-partum, si è osservato un più alto tasso di gravidanza nelle bufale che si trovavano in un periodo tra il 90 e i 180 giorni (77%) rispetto a quelle che si trovavano a più di 180 giorni di lattazione (66 %). Non sono state riscontrate differenze significative nel tasso di gravidanza tra bufale con (59%) o senza (57,1%) corpo luteo al momento dell'inizio del protocollo Inoltre, non sono state evidenziate significati effetti del numero di follicoli e di corpi lutei. Lo studio indica che la cronologia del trattamento dovrebbe essere preso in considerazione per i protocolli Ovsynch. I risultati suggeriscono che i protocolli ormonali possono migliorare I programmi di selezione delle bufale indigene e

che la terza somministrazione di GnRH al momento dell'inseminazione può aumentare il tasso di gravidanza quando si usa seme congelato. Il miglioramento genetico risultante dall'applicazione dell'inseminazione artificiale con seme congelato in bufale sincronizzate potrebbe apportare un significativo guadagno economico  degli allevatori del settore lattiero.

PAROLE CHIAVE: Bufalo, analoghi del GnRH, protocolli di sincronizzazione dell'ovulazione, inseminazione artificiale.

# ABSTRACT

The achievement of early cyclicity after calving is of vital importance in maintaining a dairy industry. Post-partum anoestrous in buffalo is a major cause of long calving interval and infertility resulting in economic loss to buffalo breeders. For sustainable breeding program it is necessary to identify an oestrous synchronization treatment that is more reliable for successful pregnancy after artificial insemination (AI) with frozen semen. Two experiments were carried out to evaluate the efficacy of an Ovsynch protocol (GnRH + $PGF_{2\alpha}$ + GnRH) for oestrous synchronization and subsequent AI fertility in water buffaloes of two countries (Italy and Bangladesh). The aim of the first experiment was to evaluate the efficacy of an Ovsynch protocol with two GnRH analogues (Buserelin and Gonadorelin acetate) and at two times of administration (AM and PM) for synchronization of ovulation and fixed–timed AI using sexed frozen semen in Italian Mediterranean buffalo cows. Thirty-two Mediterranean postpartum buffaloes cows were used for this study. All animals were inseminated by AI with 2.5 million live (4million total) sex-sorted frozen thawed spermatozoa (X-chromosome bearing spermatozoa) within 18 to 21 hours after using of 2nd GnRH. Vaginal electrical impedance (VEI), vulvar and perivulvar temperatures were recorded at different stages of synchronization protocol to asses oestrous. Pregnancy was confirmed by transrectal ultrasonography after 42 days of AI. The pregnancy rate was 37.50 % when Buserelin was used for synchronization, whereas the pregnancy rate was 50% when hormones were administered at morning. Vaginal Electric Impedance decreased and vulvar temperature, recorded with thermocamera, increased during administration of second dose of GnRH, when buffaloes were supposed to be in oestrous. However, the first study served as a basis for further study on time and type of treatment with GnRH on synchronization of estrus and ovulation to improve pregnancy rate as well as reproductive quality in Mediterranean buffalo cows.

In second experiment, a total of 114 lactating Bangladeshi Indigenous buffalo cows were used to study the efficiency of Ovsynch protocol for oestrous and fertility after AI with frozen semen of Italian Mediterranean buffaloes. The effect of some factors such as follicles and corpus luteum numbers, postpartum period on the fertility of synchronized Bangladeshi water buffaloes was also studied. Buffaloes were selected from two different areas in Bangladesh and were divided into three groups. Buffaloes of each group were subdivided as AM and PM according to time of administration of hormones of synchronization protocols. Among three groups, higher pregnancy rate (78% in AM) was observed in buffaloes of Group-3 where Ovsynch protocol was combined with a third GnRH injection at the time of AI. Similar to first experiment, we observed higher pregnancy rate (74 %) in buffaloes received induction treatment and insemination at AM time than that of PM counterpart (63%). Regarding the post–partum days, pregnancy rate was higher (77%) in buffaloes received induction treatment at 90 to 180 days between calving to ovulation induction than that of more than 180 days (66%). We did not observe any significant difference in pregnancy rate in buffaloes with (59%) or without (57.1%) CL at the time of synchronization. In addition, this study did not show any significant in regards to follicles and CL numbers in both pregnant and non-pregnant buffaloes. This study results that time of day should considered during administration of hormones for Ovsynch protocols. The findings of the study suggests that Ovsynch protocol can be successfully used for selective breeding program of Bangladeshi Water buffaloes and a third injection of GnRH during AI could help to improve pregnancy rate of water buffaloes when inseminated with frozen-thawed semen. The genetic improvement resulting from AI with frozen-thawed semen in synchronized buffaloes could bring a significant economic gain for farmers of dairy industry.

Key words: buffalo, GnRH analogues, ovulation synchronization protocol, artificial insemination

# CHAPTER 1. INTRODUCTION

Buffaloes are known to be seasonally polyestrous animal. The breeding frequency in buffaloes is highest during the winter, decreased in autumn and spring, and is lowest in the summer due to increasing daylight and heat (Shah, 1988). Irrespective of breeding or non-breeding season, buffaloes show less intense expression of oestrous, mounting behavior (Roy and Prakash, 2009) compared with cattle (Ohashi, 1994). The duration of the oestrous cycle in buffalo ranges from 17 to 26 days with a mean of around 21 days (Jainudeen and Hafez, 1993). However, there is greater variability of oestrous cycle length in buffalo, with a greater incidence of both abnormally short and long oestrous cycles, attributed to various factors including adverse environmental conditions, nutrition and irregularities in secretion of ovarian steroid hormones (Kaur and Arora, 1982; Nanda *et al.,* 2003). The basic pattern of changes in hormone profiles of buffaloes during the estrous cycle closely resembles that of cattle. Post-partum anoestrous in buffalo is responsible for a long calving interval (Borghese *et al*., 1993; Campanile *et al*., 1993) and is a major cause of infertility resulting in economic loss to buffalo breeders in many countries (El-Wishy, 2007). The period of postpartum anoestrous or anoestrus is usually longer in buffalo than in cattle under comparative management conditions (Jainudeen and Hafez, 1993). Under optimal conditions buffalo resume ovarian cycle by 30–90 days, but factors such as poor nutrition and body condition (Baruselli *et al*., 2001), suckling management (Usmani *et al*., 1990) and climate (Nanda *et al*., 2003), can delay this considerably. The achievement of early cyclicity after calving is of vital importance in maintaining a dairy industry (Darwash *et al.,* 1997). These considerations indicated a need for oestrous synchronization using fixed-timed AI for the implementation of breeding programs in buffalo (Presicce *et al*., 2004; Ali and Fahmy, 2007). Efforts have been made to breed buffaloes throughout the year by using different hormones (Rajamahendran and Thamotharam, 1983; Singh, 2003), but with limited success. Fertility improvements of buffalo cows require an adequate knowledge of reproductive behavior (Ireland *et al.*, 2000). It has been documented that a precise manipulation of follicular development is needed to achieve better synchrony of ovulation followed by improved fertility in buffaloes. Ovarian activity is manipulated by (1) controlling the luteal phase of the cycle through the administration of prostaglandins or progesterone analogues between reproductive activity and ecological conditions is related to the amplitude and consistency in timing of seasonal fluctuations (Colwell, 1974); (2) controlling follicle development and ovulation using different combinations of prostaglandins, progesterone, GnRH, hCG, eCG and estradiol. Understanding of the hormonal interaction is essential for relieving reproductive problems of endocrine origin, a considerable attention has been focused in the last two decades on utilizing reproductive endocrinology as a means to identify problems specific to this species and to devise means for improving reproductive performance. Various synchronization method used in buffalo are mainly based on those developed for cattle. Previously, oestrous synchronization was performed either by inducing premature luteolysis using prostaglandins or prolonging the luteal phase using progestagens (Perera, 1987). Recent knowledge on the ovarian follicular wave dynamics have prompted studies aimed at manipulating follicular development to achieve greater oestrous synchrony and improved fertility (De Rensis and López-Gatius, 2007).

Moreover, it is necessary to identify an oestrous synchronization treatment that is more reliable for successful pregnancy after AI with frozen semen in water buffaloes. Among these, synchronization with GnRH + PGF + GnRH (called Ovsynch protocol) has been extensively practiced in dairy industry. The hypothalamic area that controls reproduction is represented by scattered neurons producing gonadotropin releasing hormone (GnRH), a decapeptide hormone considered as the master molecule for the reproductive control in mammals. GnRH controls gonadal activity by regulating the production and release of pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH).

GnRH pulse generator drives endocrine signals that allow the onset of reproductive activity, either by the transition from a pre-pubertal state to puberty, or the transition from seasonal anestrous to a full reproductive activity, as well as the maintenance of proper reproductive activity in adult  (Víctor *et al.,* 2012). This protocol promotes ovulation of dominant follicle (DF) by GnRH administration, corpus luteum regression by prostaglandin administration 7 days later, and thereafter, the control of ovulation of the new DF by a second injection of GnRH (Qin *et al*., 2009). In most studies the success rate was lower when treatment was done during the periods of low breeding activity or during seasonal anoestrous, and various modified protocols have been tried to overcome these problems.

It is well accepted that an injection of a GnRH agonist at any stage of the estrous cycle in buffalo 1) increases the number of medium-sized follicles within 3 days of treatment, 2) eliminates the large follicles by ovulation or atresia and 3) induces the emergence of a new follicular wave within 2 to 3 days of treatment (Jazayeri *et al*., 2010). The ovsynch protocol for synchronization of the ovulation in buffaloes has been tested during different seasons elsewhere (Paul and Prakash, 2005; Carvalho *et al*., 2007; Warriach *et al.,* 2008) and dramatically decreased conception rate was reported during non-breeding season (Baruselli, 2001).

The production potential of livestock can be increased by genetic improvement using artificial insemination (AI) in order to obtain faster diffusion of individuals of higher genetic merits for improvement of production traits. AI with frozen-thawed semen has been used potentially in cattle breeding strategies. Though AI has been adopted in buffaloes, it remains unpopular. Covert signs of oestrous, the wide variation in duration of oestrous (4-64 h) and difficulty in predicting the time of ovulation in female buffaloes (Baruselli, 2001) are major constraints to the broader adoption of AI for genetic improvement in buffaloes.

Studies done to date have not been designed to study the time of day on synchronization protocols. Furthermore, a satisfactory conception rate can be achieved only if the insemination is performed at the correct time relative to ovulation. Therefore, it is very important to monitor reproductive system during oestrous synchronization to detect oestrous properly. Recent efforts to overcome the problem of oestrous detection in the water buffalo include use of a vasectomized teaser, androgenized females (Drost *et al.,* 1985) plasma and milk progesterone, laparoscopy, use of vaginal electrical resistance (VER) (Gupta and Purohit, 2001) and ultrasonography (Manik *et al.*, 1992).

Previously, rectal palpation of ovary was performed to determine ovulation in buffalo (Kanai and Shimizu, 1983). A number of noninvasive *in vivo* methods have been used to study reproductive events in female mammals (Bollwein *et al.,* 2000; Singh *et al.,* 2003). A better understanding of follicular wave dynamics could facilitate the development of a methodology for influencing ovarian function and oestrous in both cyclic and non-cyclic animals. Protruded mature follicles from the surface of the ovary (Jainudeen et al., 1983) and deeply embedded CL in ovarian stroma make accurate identification of ovarian structures by rectal palpation in buffalo more difficult than in cattle (Perera et al., 1987; El- Wishy, 2007). The use of real-time ultrasound imaging has greatly improved our knowledge of follicular dynamics in cattle and buffaloes (Vassena *et al.* 2003). With the advent of real-time ultrasonography, it becomes possible to visualize the various reproductive events in large animal species over a prolonged period of time and without any interruption of normal physiological events. Determining the state of oestrous with the help of ultrasonography along with measuring vaginal electrical impedance and vulvar temperature in buffaloes undergoing Ovsynch protocol has not yet been reported.

The river buffalo is a triple purpose species contributing milk, draft-work and meat, therefore, plays a prominent role in rural economy particularly in Asia. In Bangladesh, buffalo also serves as a capital asset to protect against economic risks such as crop failure, and features in religious and cultural events in some communities. The production systems are mostly extensive or semi-intensive, with free or tethered grazing in home gardens, fallow fields and communal lands. Application of ovulation synchronization programs in Bangladeshi buffaloes has not been widely applied. Despite its importance as a tool for economic growth and poverty alleviation in rural areas of Bangladesh, they are much neglected till now. The true scenario of buffalo production includes no selective breeding program and no AI. As a result, buffalo number is increasing naturally without any significant growth rate, even without any increase in its milk production. As management-related defect, errors in heat detection and suckling may prolong the interval between parturition and onset of oestrous which may ultimately affect the pregnancy rate in oestrous induced buffaloes. To the best of my knowledge, previously only one study on application of oestrous synchronization protocols and FTAI in Bangladeshi river type buffaloes has been conducted with few animals (Hoque, 2009). However, no study has been reported in Bangladeshi river type buffaloes on effects of time of administration of hormone for synchronization protocol, and successive timed AI with frozen semen of Italian Mediterranean buffaloes.

The first experiment was designed (i) to determine the effect of two GnRH analogues administered at two different times of a day for oestrous synchronization, (ii) to study relation of vaginal electrical impedence and vulvar and perivulvar temperature recorded with Infra-red thermography with pregnancy rates in Italian Mediterranean buffaloes inseminated with sexed frozen semen. Second experiment was carried out (i) to study the time effect on efficiency of Ovsynch protocol in Bangladeshi Indigenous water buffaloes and fertility after AI with frozen semen of Italian Mediterranean buffaloes. Simultaneously, the effect of some factors such as follicles and corpus luteum numbers, postpartum period on the fertility of Bangladeshi water buffaloes inseminated with frozen-thawed semen was also studied.

# CHAPTER 2. REVIEW OF LITERATURE

## 2.1. Reproductive Physiology in Buffaloes

The water buffalo is characterized by ineffectively reproductive performance. Late puberty reduces the duration of fertile life of this animal while silent heat and variable time of ovulation cause the difficulty to the oestrous detection which results in either missing oestrous unnoticed or obtaining low conception rate. Due to the low level of blood oestradiol-17 beta, the expression of oestrous in buffaloes is very poor (Nam, 2010). Buffalo heifers usually attain puberty when they reach about 55–60% of their adult body weight, but the age at which they attain puberty can be highly variable, ranging from 18 to 46 months (Jainudeen and Hafez, 1993). The factors that influence this are genotype, nutrition, management, social environment, climate, year or season of birth and diseases.

The duration of oestrous is similar in river and swamp buffalo, varying between 5 and 27 h, and ovulation occurs about 24–48 h (mean 34 h) after onset of oestrous, or 6–21 h (mean 14 h) after the end of oestrous (Kanai *et al*., 1990; Perera, 1999). Externally detectable physical changes around the time of oestrous include swelling of the vulva and reddening of the vestibular mucosa (Danell, 1987; Kanai *et al*., 1990). Mucus secreted from the cervix during oestrous is less copious than in cattle, does not usually hang as strands from the vulva, and be discharged either when the animal is lying down (Perera *et al.,* 1977) or with the urine (Kanai *et al.,* 1990). Srivastava and Sahni (2003) reported that buffaloes showed more oestrous activity in the morning (06:00–07:30 hour) than in the afternoon (14:00– 15:30 hour) or during the night (22:00–23:30 hour).

The buffalo ovary is elongated and considerably smaller than that of cattle. The average dimensions of the buffalo ovary vary between 22–26 X 11–18 X 11–14 mm$^3$ (Fadle *et al.*, 1974) with a maximum and minimum weight of 6.1 and 2.9 g, respectively (El-Wishy *et al.*, 1971). The ovaries of post-pubertal buffalo heifers have a reservoir of only 10,000–20,000 primordial follicles (Danell, 1987). When palpated per rectum, mature follicles in  tend to protrude from the surface of the ovary and can be mistaken as an early developing corpus luteum (Jainudeen *et al.*, 1983). The corpus luteum CL of the buffalo is often deeply embedded in ovarian stroma and is generally smaller than that of cattle. The corpus luteum does not protrude markedly from the surface of the ovary and sometimes lacks a clear crown. These characteristics make accurate identification of ovarian structures by rectal palpation in buffalo more difficult than in cattle (Perera *et al*., 1987; El- Wishy, 2007).

The phases of reproductive cycle are regulated by several sequential events and interactions between hypothalamic releasing hormones, hormones secreted from the pituitary and sex steroids secreted by the ovary. Lack of integration or synchronization or endocrine imbalances at any phase of the sequence may result in reproductive failure. Therefore, understanding of the hormonal interaction is essential for relieving reproductive problems of endocrine origin and a considerable attention has been focused on utilizing reproductive endocrinology to devise means for improving reproductive performance.  External and internal conditions are perceived by way of specialized neural functions that influence reproduction through the hypothalamic-pituitary-gonadal (HPG) axis. The hypothalamic area that controls reproduction is represented by scattered neurons producing gonadotropin releasing hormone (GnRH), a decapeptide hormone considered as the master molecule for the reproductive control in mammals. GnRH controls gonadal activity by regulating the production and release of pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). It is well known that pulsatile GnRH secretion induces an identical pattern of LH secretion and increases the FSH synthesis at the pituitary level.  Pulsatile GnRH secretion controls gonadal development and function, including steroidogenesis in both females and males, while surge GnRH secretion drives ovulation in the females. The GnRH pulse generator drives endocrine signals that allow the onset of reproductive activity, either by the transition from a pre-pubertal state to puberty, or the transition from seasonal anestrous to a full reproductive activity, as well as the maintenance of proper reproductive activity in adult. Later, gonadal steroid or peptide hormones regulate the hypothalamic/pituitary function by feedback mechanisms, completing the loop of endogenous control of reproductive activity. In general, reproductive function is influenced by the animal's nutritional condition, particularly the energy status (Imakawa *et al.,* 1986; Schillo, 1993), which includes amount of body energy stores and energy obtained from food consumption on a daily basis.

The basic pattern of changes in hormone profiles of buffaloes during the estrous cycle closely resembles that of cattle. The average length of the estrous cycle has been reported to be 20–22 days for river buffaloes (Singh *et al*., 2000). The average duration of oestrous appears to be slightly longer in river buffalo 23.8 ± 6.2 h (Danell, 1987) than in swamp buffalo 19.9 ± 4.4 h (Shimizu, 1987). A seasonal variation was reported in one study wherein the duration of oestrous was estimated to be 14, 18, and 8–10 h in the monsoon, winter, and summer seasons, respectively, in river buffaloes (Janakiraman, 1978).

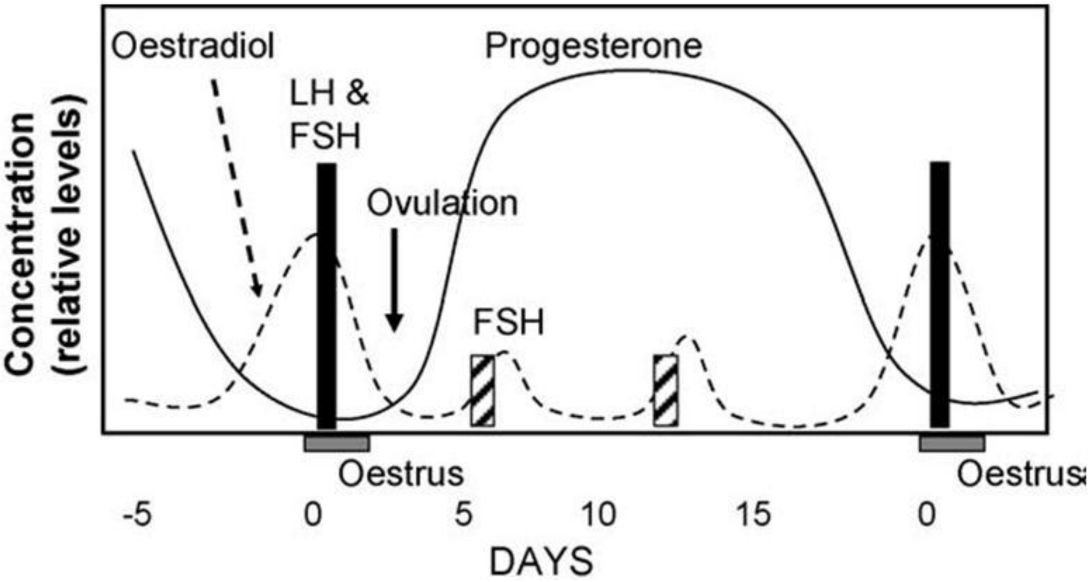
Though the changes in the peripheral ovarian steroids and gonadotropins profiles during the cyclic ovarian activity in buffalo cows are very similar to that in cattle (Jainudeen and Hafez, 2000), lower levels of several sexual hormones and some differences in the oestrous behavior and in other reproductive aspects in buffalo species in comparison to that of bovine, have also been reported (Perera, 2008). The temporal patterns of both LH and FSH are basically similar to those in cattle (Kanai *et al*., 1990), with a preovulatory LH surge occurring on the day of oestrous and lasting 7–12 h. The concentration of oestradiol-17 in blood during the follicular phase of the oestrous cycle also appears to be relatively less than that in cattle (Avenell et al., 1985; Kanai *et al*., 1990; Roy and Prakash, 2009) and this has been suggested as a possible reason for the lesser intensity of oestrous exhibited by buffaloes. Peak progesterone values of 4.0–5.1 ng/ml (Arora and Pandey, 1982; Takkar *et al*., 1983) have been recorded about 15 days after oestrous. Circulating estradiol concentrations remain low during the luteal phase with minor fluctuations 10–20 pg/ml around Days 4 and 10 of the oestrous cycle in river buffalo (Batra and Pandey, 1982; Samad *et al.,* 1988) . Peak concentrations of estradiol 30–35 pg/ml were detected on the day of oestrous or one day before ( Batra and Pandey, 1982), followed by a decline to 5–10 pg/ml within two days. The duration of the LH surge has been estimated to be 7–12 h (Batra and Pandey, 1982; Kanai and Shimizu, 1986). Peak LH concentrations were estimated to occur about 14.8 h after the peak in estradiol concentration (Batra and Pandey, 1982). Higher FSH levels 57–65 ng/ml have been observed during the beginning of oestrous cycle than during the luteal phase 10–17 ng/ml ( Razdan *et al.,* 1982).

Ovarian follicular growth occurs in waves. Follicular dynamics is defined as the process of continual growth and regression of antral follicles that leads to development of the preovulatory follicle (Lucy *et al*., 1992). Studies on Italian buffalo indicate differences in ovarian follicular dynamics between heifers and parity cows (Presicce, 2004). A wave of follicular growth is defined as the simultaneous initiation of developing a group (5–7) of follicles in ovaries (Mapletoft *et al.,* 1994). During each wave, small follicles (4 mm in diameter) enter a common growth phase, one follicle of the cohort grows rapidly to attain ovulatory diameter (dominant follicle) (Ginther *et al*., 2003), suppressing the growth of other follicles (subordinate follicles) and preventing emergences of a new follicular wave (Armstrong and Webb 1997). Each antral follicular wave is stimulated by an increase in FSH secretion (Adams *et al*., 1992) caused by the regression of the dominant follicle of the previous wave (Ginther *et al.,* 2003). The morphologically and functionally dominant follicle (the largest follicle of the final wave, i.e. ovulatory follicle) appears to ovulate under appropriate hormonal conditions (Fortune, 1993). The dominant follicle subsequently acquires

LH dependency for its own continued growth and suppresses FSH secretion, starving subordinate follicles of sufficient FSH support (Austin *et al.,* 2003).

The dynamics of follicular turnover, including the length of the inter-ovulatory interval (Knopf *et al*., 1989; Savio *et al*., 1990), emergence of waves, number of follicles having 3 mm size at emergence of waves (Knopf *et al.,* 1989), persistence and maximum diameter of the dominant anovulatory and/or dominant ovulatory follicles (Bo *et al*., 1995; Fortune *et al*., 1988; Ginther *et al.,* 1989a) were studied and similarities were found between buffaloes and cattle. The development of one and two ovarian follicular waves in buffaloes (Presicce *et al.,* 2005; Warriach and Ahmad 2007) is comparable with follicular wave phenomenon described in cattle. Primordial follicles smaller than 4 mm in diameter are highly increased at the beginning of the oestrous cycle, might be resulted from the pulsatile release of FSH (Ginther *et al.,* 2003). Later, number of primordial follicles decrease in other stages of the oestrous cycle might be due to the suppressive effect of DF that is usually dominant on day, 5–21 of the oestrous cycle (Mihm *et al.,* 2002). Following the gonadotropin surge and almost coincide with ovulation secondary or peri-ovulatory transient FSH rise occurs which stimulate up to 24 small antral follicles (the cohort) to grow beyond 4 mm in diameter (Mihm *et al.,* 2002). During declining FSH, fewer follicles from the original cohort continue growing until only one single follicle (DF) is selected, and the remaining cohort members become static and undergo atresia via apoptosis (Austin *et al.,* 2001). The first wave commenced on day 1 (day0 = ovulation) in all categories of animals, while the second wave emerged on days 10.8 and 9.3 for the animals with 2 and 3 wave cycles, respectively, and the third wave emerged on day 16.8 in the latter group (Perera, 2011).

Figure: 2.1. Schematic representation of the hormonal changes occurring in blood of buffalo during the oestrous cycle (Source-Perera, 2011).



Buffaloes have oestrous cycles with 1, 2 or 3 follicular waves, 2-wave cycles are the most common and the number of waves in a cycle is associated with the luteal phase and with oestrous cycle length (Baruselli *et al*., 1997). Awasthi *et al.,* (2006) have stated that one follicular wave patterns are quite usual in the oestrous cycles of the river buffalo. According to a study in the Egyptian river buffalo, there were two patterns of follicular waves (Barkawi *et al.,* 2009). In contrast, the one wave oestrous cycles in the Thai swamp buffalo were depicted to be *22.7%* while the two wave- patterns were *77.3%* (Promdireg *et al.,* 2004). In one wave model, the ovulatory follicles persisted much longer than those in the 2 wave-pattern, and at the middle of the cycle there is a regression before a resurrection. The growth rate of ovulatory follicles is slower than that in the 2 wave patterns. Therefore, the small size and slow growth rate of ovulatory follicles in the one follicular wave oestrous result in the low concentration of estrogen which is one of the causes of the silent heat in the river buffalo (Awasthi *et al.,* 2007). Furthermore, the oocytes ovulated from the small sized follicles seem to have low quality which might be a cause of the low fertility and conception rate. Beside the follicular model, the partial luteolysis is also pointed out as a probable reason of the silent heat observed in the studies of using PGF$_2$α to induce the luteolysis in buffaloes, (Dhaliwal *et al.,* 1988, El-Belely *et al.,* 1995).

## 2.2. Postpartum period in buffaloes

The postpartum period is characterized by involution of the uterus and re-establishment of ovarian function, intended to prepare the animal for a new pregnancy and uterine involution results from three overlapping processes: contraction, loss of tissue and tissue repair (Kindahl *et al.*, 1999; Yavas and Walton, 2000). The postpartum period in the buffalo like the cow starts with parturition and ends with complete uterine involution and resumption of cyclic ovarian activity and normal estrous expression. Hormonal changes during the peri-parturient period besides regulating lactogenesis and parturition have their impact on postpartum reproductive activity. El-Wishy (2007) have reviewed the hormonal changes during late gestation and postpartum period, initiation of follicular activity after calving and factors which influence uterine involution period. Pituitary release of LH and FSH in response to exogenous GnRH declined progressively with the advancement of pregnancy and low response is observed after parturition regardless of the stable LH and FSH contents in the pituitary, and this could be one of the reasons of long postpartum anestrus in the buffalo (Palta and Madan, 1996). No significant variations observed in basal level of FSH between Days 2, 20 and 35 postpartum (Palta and Madan, 1995). Similarly, no significant variations were observed in the basal LH levels between Days 3 through 90 postpartum or between milked (0.9 ± 0.2 to 1.3 ± 0.2 ng/ml) and suckled (0.9 ± 0.1 to 1.5 ± 0.2 ng/ml) anestrous Murrah buffaloes (Arya and Madan, 2001a). Batra and Pandey (1983) reported that basal plasma LH concentration was significantly higher in buffaloes showing oestrous than in anestrous animals. Basal values of estradiol-17 were reported between Days 2 and 7 after calving (Eissa *et al.,* 1995; Arya and Madan, 2001b) with minor fluctuations thereafter (11 ± 3 to 18 ± 3 pg/ml) until day 45 postpartum (Madan *et al.,* 1984). Fluctuations of total estrogens between 38 ± 10 and 61 ± 5 pg/ml were observed during the first 75 days postpartum in acyclic buffaloes (Soliman *et al.,* 1981) probably reflects waves of follicular growth and atresia.

As in cattle, uterine involution in buffalo is usually completed in 25–35 days after calving (Jainudeen and Hafez, 1993; Perera *et al.,* 1987). The stimulus of suckling shortens involution time (Usmani *et al.,* 1990). To maintain a calving interval of 13–14 months in buffaloes, successful breeding must take place within 85–115 days after calving. Disturbances during this period due to delay of uterine involution or resumption of estrous activity are likely to prolong the calving interval and reduce the lifetime reproductive and productive efficiency (EI-Wishy, 2007). Uterine involution is also a key factor that impacts the time of postpartum anestrus in the water buffaloes. The long postpartum anestrus in the buffalo depends on several factors including: season of calving, uterine involution, suckling, milk yield, nutrition and body condition score at calving (Nam, 2010).

Perera (2011) have stated that the first postpartum ovulation is frequently followed by one or more short oestrous cycles (<18 days) and cessation of oestrous cyclicity occurred after the first or second ovulation in about 25% of animals due to ovulatory failure or prolonged luteal activity.

The response of LH secretion to exogenous GnRH at 25–35 days postpartum, is greater in non-suckled than suckled buffalo (Singh *et al.,* 2006). These considerations indicated a need for oestrous synchronization using fixed-time insemination for the implementation of breeding programs in buffalo (Presicce *et al*., 2004; Ali and Fahmy, 2007). Ovulation and oestrous activity after calving are delayed when the positive feed back effects of estradiol on release of LH from the pituitary are reduced due to various factors. Therefore, treatment includes hormonal and management strategies (Rhodes *et al.,* 2003).

Qureshi *et al*., (2002) reported that BCS and postpartum ovulation interval were correlated with ME intake BCS was higher in oestrous buffaloes than anestrous ones. (P<0.01). Higher and lower metabolizable energy (ME) intakes are associated with anoestrus, while a moderate energy intake is associated with a postpartum estrus interval (PEI) of less than 75 days (Qureshi *et al*., 2002). Very recently, Qureshi (2012) has stated that excess intake of crude protein, associated with higher serum urea levels and low energy intake, associated with poor body condition, are the key factors for low reproductive efficiency.

Some previous studies showed that high milk yield (>8kg of milk per day) (El-Fadaly *et al.,* 1980, El-Azab *et al.,* 1984) and suckling prolonged time of postpartum anestrus. Arya and Madan, (2001) observed that milked buffaloes had longer acyclic interval of 72 ± 11 days compared with 44 ± 9 days in those who were weaned. Free suckled buffaloes had longest postpartum anestrus interval (82 ± 11 days) in comparison to restricted suckled (69 ± 10 days) and early-weaned buffaloes (50 ± 7 days) (Nordin and Jainudeen, 1991).

Nutrition plays a considerably important role in reproduction of water buffaloes. Shorter postpartum anestrus period achieved in those buffaloes who were fed high-energy prepartum (Hegazy *et al.,* 1994a). In the buffaloes whose body condition score are low, the body reserves are used for the production of milk and other daily activities then they do not have enough energy stored for the next oestrous cycle. Therefore, the thin buffaloes may experience a longer time of acyclic postpartum than those have moderate body condition score; 63 days compared with 47 days (Hegazy *et al.,* 1994b). Baruselli *et al*., (2001) also observed that buffaloes those had higher body condition were found to have shorter postpartum anestrus interval than those with lower body condition score.

Data from Egypt, India and Pakistan indicated that only 34–49% of buffaloes showed oestrous during the first 90 days after calving and 31–42% remained anoestrous for more than 150 days (reviewed by El Wishy, 2007). Anovulatory follicles are reported in postpartum anestrous dairy ( El-Wishy, 1979; Abul-Ela *et al.,* 1988; Suthar and Kavani, 1992) and swamp buffaloes (Jainudeen *et al.,* 1983). Restoration of pulsatile LH secretion could be a limiting factor for development and maturation of dominant follicles (Manik *et al.,* 2002) in postpartum buffalo cows. Early studies demonstrated that suckling (Honnapagol et al., 1993; Tiwari and Pathak, 1995), level of milk production (Singh *et al.,* 1979) and prepartum nutrition (Usmani *et al.,* 1990) did not influence the interval to initiation of follicular activity. The interval to first postpartum oestrous is also significantly affected by the season of calving (Madan, 1988; Singh, 1993; Singh and Nanda, 1993). Buffaloes calving in late winter and early summer have lower reproductive efficiency compared to those calving during other periods. The resumption of ovarian activity after calving was significantly delayed in buffaloes that calved from February to May (116–148 days compared to the rest of the year 38–64 days) (Singh and Nanda, 1993).

Under-nutrition and environmental stress have been recognized as causes of long anoestrous periods in buffaloes (Kaur and Arora, 1984). It is suggested that delay in post-partum ovarian activity in ruminants is related to lower level of minerals in blood (Krop, 1993; Manspeaker and Robl, 1993). Deficiency of mineral elements like phosphorus (P), copper (Cu) and zinc (Zn) are associated with subnormal fertility and anoestrous conditions in cows (Campbell *et al.,* 1999). Moreover, Ca, P, Zn and manganese (Mn) have been found affecting post partum reproduction in cattle (Hidiroglou, 1979).

The achievement of early cyclicity after calving is of vital importance in maintaining a dairy industry (Darwash *et al.,* 1997). During pregnancy, high concentrations of circulating progesterone are associated with a marked reduction of LH content at the anterior pituitary and this is one of the initial limitations on the resumption of normal ovarian activity in post-partum cows (Peters *et al.,* 1981). It is hypothesized that the capability of the pituitary gland to respond to exogenous GnRH is restored by Day 20 postpartum in dairy buffaloes (Palta and Madan, 1995). Therefore, studies on ultrasonographic assessment of ovarian follicular dynamics together with hormonal profiles (progesterone and LH) are needed to define the circumstances under which early return of cyclic activity can be achieved in the postpartum buffalo (El-Wishy, 2007).

### 2.3. Anoestrous in buffaloes

Silent ovulation (lack of overt signs of oestrous) and unobserved oestrous (poor oestrous detection efficiency) can greatly increase the incidence of anoestrous. It is important to distinguish between these conditions (physiology vs. management). Anoestrous is broadly classified into physiologic and pathologic (clinical) types,

In type I anoestrous, there is growth of follicles to emergence without further deviation or establishment of a dominant follicle. The pathophysiology of this condition is not well understood, but it is presumed to be due to extreme under nutrition. In that regard, under nutrition and severe energy deficit may cause this condition through a lack of essential LH support to sustain follicular growth and dominance (Sangsritavong *et al*., 2002).

In type II anoestrous, there is deviation and growth, followed by either atresia or regression. In certain cases, the regression or atresia occurs only after a follicle has reached a dominant status. Regression of this follicle results in the emergence of a new follicular wave 2 to 3 days later. In these cases, there are sequential follicular waves prior to first ovulation, which may be delayed for a prolonged interval. Some follicles grow further and regress prior to ovulation (Wiltbank *et al.,* 2002).

In type III anoestrous, there is deviation, growth and establishment of dominant follicle, but it fails to ovulate and becomes a persistent follicular structure. A single follicular structure >8 mm in diameter was observed in the absence of a CL or cyst in two ultrasonographic examinations 7 days apart (Sangsritavong *et al.,* 2002). This may be due to insensitivity of the hypothalamus to the positive feedback effect of estradiol or to altered follicular responsiveness to gonadotrophic support, mediated via metabolic hormones (e.g., insulin-like growth factor and insulin). Persistent follicular structures may become follicular cysts or they may luteinize (luteal cysts). The latter occurs in 10% to 13% of the cases (Ambrose *et al.,* 2007).

Type IV anoestrous, is due to a prolonged luteal phase. These cows have normal oestrous, ovulation and formation of a CL, with prolonged luteal function due to a lack of luteal regression. A contributing factor may be the lack of an estrogenic dominant follicle at the expected time of luteal regression. In that regard, estradiol from a dominant follicle is believed to induce the formation of uterine oxytocin receptors, leading to pulsatile release of PGF2α (Mwaanga ES, 2000).

Post-partum anoestrous in buffalo is responsible for a long calving interval (Borghese *et al*., 1993; Campanile *et al*., 1993) and is a major cause of infertility resulting in economic loss to buffalo breeders in many countries (El-Wishy, 2007). The period of postpartum anoestrous or anoestrous is usually longer in buffalo than in cattle under comparative management conditions (Jainudeen and Hafez, 1993). Under optimal conditions buffalo resume anoestrous by 30–90 days, but factors such as poor nutrition and body condition (Baruselli *et al.,* 2001), suckling management (Usmani *et al.,* 1990) and climate (Nanda *et al.,* 2003), which also influence nutrition through feed quality and availability, can delay this considerably.

Dairy animals in the tropics face numerous challenges under tropical environments. Rasby *et al*., (1992) reported that nutrition restriction has a negative influence on LH release. Suboptimal nutrition coupled with stress due to high environmental temperatures may be responsible for long anoestrous in buffaloes (Terzano *et al.,* 2012).

Animals in anoestrous showed decrease in diameter of the dominant follicle and in ovulation rate to the GnRH treatment. Nutritional status of an animal is reflected by the BCS. Several studies also demonstrate the negative effect of low BCS on ovarian cyclicity and pregnancy rates in beef cows (Viscarra *et al*., 1998).

Furthermore, investigations on postpartum reproduction indicate that BCS is a useful indicator of energy status and rebreeding potential (DeRouen *et al.*, 1994). It was suggested that buffaloes have to present BCS ≥ 3.5 for a satisfactory response to the treatment with GnRH and prostaglandins for fixed time artificial insemination (FTAI).

Research in cattle shoed that, ovulation is delayed by inhibition of LH pulse frequency and by suppressing blood concentrations of glucose, insulin and insulin-like growth factor- 1 (IGF-1) which reduce estrogen production by dominant follicles (Butler *et al.,* 2000).

In addition to energy deficits, increased feed intake also suppresses reproduction by promoting steroid hormone metabolism. Increased feed intake enhances hepatic perfusion, thereby promoting clearance of estradiol and progesterone, which can contribute to anovulation (Sangsritavong *et al.,* 2002), ovulation of larger dominant follicles, multiple ovulations (Sartori *et al*., 2004), poor luteal function and delayed luteal regression, perhaps due to development of estrogen-inactive dominant follicles, resulting in inadequate endometrial PGF2α production. Furthermore, diets high in crude protein, typically in excess of 16% to 17%, support high milk yield but may be detrimental to reproductive performance (Tamminga *et al.,* 2006) due to elevated blood urea concentrations (Butler, 2000).

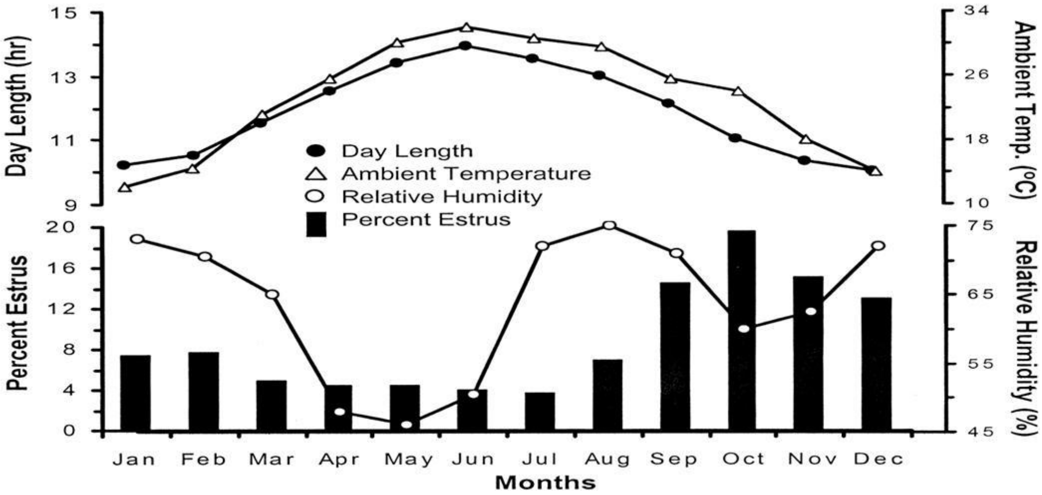
### 2.4. Seasonality in buffalo reproduction

Buffalo cows exhibit a distinct seasonal change in displaying oestrous, conception rate and calving rate (Tailor *et al.,* 1990; Barile, 2005). In hot climates duration of oestrus tends to be shorter and signs of oestrus may be exhibited only during the night or early morning. Oestrous often passes unoticedly, especially in the hot and dry seasons when grass, wallowing pools and shades are in deficiency which made the expression more Dubious (Nam, 2010).

The buffaloes calved in the spring seemed to have longer postpartum anestrus period than those calved during August to November. Longer acyclic postpartum period in the low breeding season than that in the breeding season was also described by Qureshi *et al.,* (1998) and Perera *et al.,* (1987).

Seasonal anestrus has been associated to biometeorological factors such as day length, ambient temperature, relative humidity, rainfall. Reduced sexual activity in the buffalo coincides with both an increase in ambient temperature (Singh *et al.,* 1989) and increasing day length.

Figure 2.2. Percent distribution of display of first oestrous after calving by buffaloes during a 1-year interval in relation to day length, ambient temperature and relative humidity in Punjab, India (Singh, 1993; Singh and Nanda, 1993).



This reproductive seasonality is considered to be dependent on climate and particularly on photoperiod. It is widely accepted that environmental photoperiod cues are translated into endogenous signals, where the hormone melatonin plays a central role for this (Di Palo *et al.,* 1997; Zicarelli, 1997). Photoperiodism can be defined as the physiologic response of animals and plants to the variations of light and darkness. Seasonal changes in the photoperiod are a major determinant of reproductive activity, considered the most precise cue involved in the shift of the behavior in the animals. This reproductive pattern depends on the duration and the intensity of the light source that is captured by the retina (retinal photoneurons) and subsequently passes through different neural connections: first, it is developed by the suprachiasmatic nucleus (biological clock which regulates the endogenous circadian rhythm). From this level, the information reaches the superior cervical ganglion and then the pineal gland. Therefore, the pineal gland is the main regulatory organ in the seasonality of breeding: it has no efferent projections, and therefore it affects neuroendocrine function by humoral means (Cardinali, 1984) producing indoleamins, of which melatonin is the most important. Melatonin is synthesized and secreted mainly by the pineal gland, under a neuronal control system in which the perception of light in the retina blocks the synthesis and secretion of pineal melatonin. Thus, the concentration of melatonin during daylight hours is very low, increasing dramatically during the night-time darkness. Because the almost perfect correlation between the hours of darkness and the presence of high concentrations of melatonin in the blood, the daily pattern of the hormone becomes an annual pattern, in which short photoperiod seasons are consistent with a higher proportion of hours a day with high concentrations of melatonin, in comparison with that observed in long photoperiod seasons. It has been proposed that melatonin may act by means several neural pathway including catecholaminergic, serotoninergic, opioidergic (Vector *et al.,* 2012). Melatonin is produced and secreted during the night (dark). As days become shorter, the exposure to melatonin increases; this hormone, through a complex action on the hypothalamus-pituitary- gonads axis, simulates the condition of the beginning of oestrous via exerting a stimulating effect on GnRH secretion by the hypothalamus in short-day breeders. On the contrary, in long-day breeders, such as the horse, increased melatonin exposure has the opposite effect, inhibiting GnRH release by the hypothalamus.

Generally, photoperiod, which entrains the endogenous circannual rhythms of reproduction, exerts its action through two different but complementary and dependent pathways, by adjusting the phases of gonadal development with external natural conditions and by synchronizing the reproductive period between individuals of the same species (Chemineau *et al.,* 2008). Buffaloes are more affected by reproductive seasonality with distance from the equator (Zicarelli *et al.,* 1997a); in particular, females that calve during the non-breeding season have an extended postpartum anoestrous period with a proportion not resuming ovulation until the following breeding season (Zicarelli, 1997c). Borghese *et al.*, 1995) reported that the melatonin trend shows remarkable differences between seasons. The lowest values and less persistence of melatonin peak were found because of the shortest night in June, while the highest values were noted particularly in September. The more seasonal buffalo showed a melatonin profile below 20 pg / ml during the hours of light and systematic peaks after sunset (with an average of 60 pg / ml), while, less seasonal buffalo showed high concentrations of melatonin frequently during daylight hours (30-40 pg / ml), with a lack of a clear increase in melatonin during the night (Terzano *et al.,* 2012).

The degree of correlation between reproductive activity and ecological conditions is related to the amplitude and consistency in timing of seasonal fluctuations (Colwell, 1974). Qureshi *et al.,* (1999a) reported shortest postpartum ovulation interval and lowest incidence of silent ovulations during autumn (August to October). He also stated that it coincided with the minimum intake of crude protein (CPI) and maximum intake of metabolizable energy (MEI), and it was also associated with higher calcium and zinc intake and lower phosphorus, copper and magnesium intake. Qureshi, 2012 summarizes that seasonality is associated with reproductive activities through changing daylight length, availability of fodders mass and changes in ambient temperatures.

The nutrition and heat stress measured throughout temperature/humidity indexes (THI) play an important role in the reproductive functions of buffaloes (Vale, 2007). The increase of day-length determines a seasonal decline in reproductive activity, which is manifested by a reduced incidence of oestrous behavior, a decrease in the proportion of females that undergo regular oestrous cycles and generally lower conception rates (Qureshi, 2012).

It has been observed that embryonic loss in animals inseminated artificially is 20-40% during seasons characterized by high number of light hours (Campanile *et al*., 2005), whereas, embryo loss is around 7% recorded in Brazil during decreasing light days (Baruselli *et al*., 1997).

Campanile and Neglia, (2007) found a higher incidence ($P<0.01$) of fetal mortality during a period of increasing daylight length (transitional period: December- March) compared to the April-July period. The water buffalo is heat intolerant by nature (Chiu, 2003), they need shade and water or mud to get rid of the heat from the environment. In the summer, while the temperature is high, pools of water disappear and grass is also scarce; those factors contribute to a decrease in activities of buffalo that results in weak libido in the male and poor reproductive performance in the female. In the low breeding season, the female river buffalo features a high concentration of prolactin and low concentration of progesterone and oestradiol-17beta (Roy and Prakash, 2007). This endocrine pattern may also be partially responsible for the low sexual activities and low fertility in the buffalo in low breeding season. The survival of embryo in the uterus is impaired due to the deficiency of progesterone in the hot season (Bahga and Gangwar, 1988). Some authors suggest that luteal function is adversely affected during the summer, higher concentrations of progesterone were recorded during summer than during fall and winter (Singh and Chaudhary, 1992). Lower circulating concentrations of FSH measured during oestrous and during the luteal phase were detected during summer than winter months (Janakiraman *et al.,* 1980; Razdan *et al.,* 1982). Similarly, the amplitude and frequency of LH secretion during the follicular phase were lower during the summer than during the winter (Aboul-Ela and Barkawi, 1988). Plasma E2 concentrations are also affected by weather. Rao and Pandey, (1983) found E2 concentrations to be lower in summer compared to cooler months. Lower peak values of E2 around oestrous coupled with decreased P4 concentrations was attributed to be the major reason responsible for a higher incidence of silent oestrous during summer (Rao and Pandey, 1982).

P4 concentrations have also been found to vary with seasons (Srivastava *et al.*, 1999) and the nutritional status ( Ronchi *et al.,* 2001). Lower P4 levels at oestrous as well at midluteal phase in hotter (0.14 ± 0.05 and 2.05 ± 1.16 ng/ml, respectively) than in cooler months (0.49 ± 0.06 and 3.11 ± 0.20 ng/ml, respectively) are believed to be responsible for the poor expression of oestrous and low conception rate during summer season (Rao and Pandey, 1982). In contrast, Mondal *et al.,* (2004) have observed P4 concentrations to be significantly higher during summer compared to those in winter season. A significant increase in peripheral plasma P4 concentrations during prolonged heat exposure could be from a stress-induced rise in P4 from adrenal cortex (Abilay *et al.,* 1975).

The incidence of true anoestrous was significantly correlated with mean maximum and minimum air temperature and with mean relative humidity (Singh *et al.,* 1985). The proportion of buffaloes exhibiting oestrous during the period of short day length was significantly greater than during the period of long day length 74% versus 26%, respectively (Tailor *et al.,* 1990). Decreasing day length may be a stronger determinant of the onset of postpartum ovarian activity, whereas ambient temperature and relative humidity may have relatively lesser influence (Singh *et al.,* 2000). Tailor *et al*., (1990) reported that 60.7% of the buffaloes showed oestrous activity in winter, 17.6% in the rainy season, 17.3% in spring and 4.2% in summer.

However, evidence suggests a strong influence of biometeorological factors on the endocrine system of buffaloes i.e., day length, ambient temperature, relative humidity and rain fall (Shah, 1988). The seasonal pattern may also be attributed to be a consequence of, or adjustment to, meager availability of green fodder during the summer months.

### 2.5. Synchronization protocols used in buffalo reproduction

Ovarian activity may be manipulated and the time of ovulation may be predicted.

This is achieved by

(1) controlling the luteal phase of the cycle through the administration of prostaglandins or progesterone analogues

(2) controlling follicle development and ovulation using different combinations of prostaglandins, progesterone, GnRH, hCG, eCG and estradiol.

1. ***Control of the luteal phase***

Figure 2.3. Control of the luteal phase with single PGF2α treatment (De Rensis and Lo´pez-Gatius, 2007)

Figure 2.4. Control of the luteal phase with double PGF2α plus GnRH protocol (De Rensis and Lo´pez-Gatius, 2007)

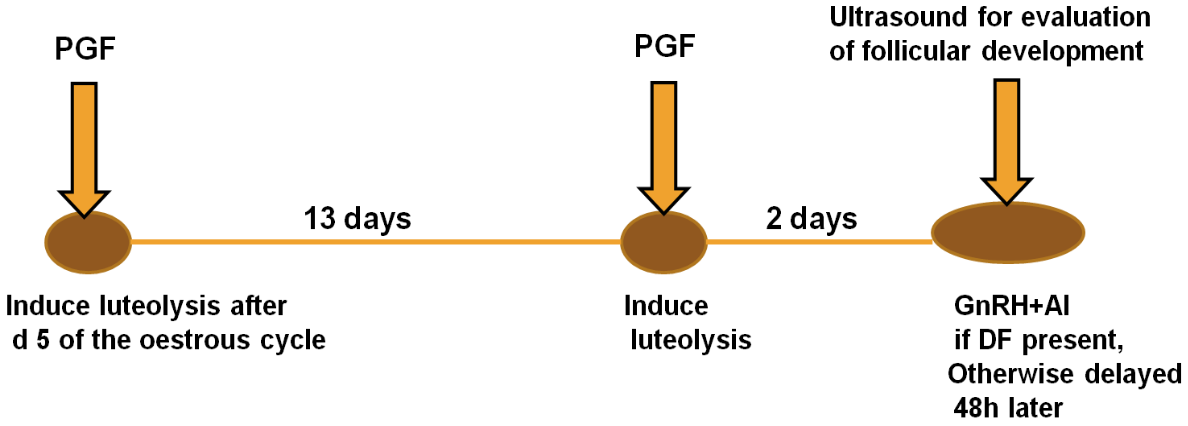
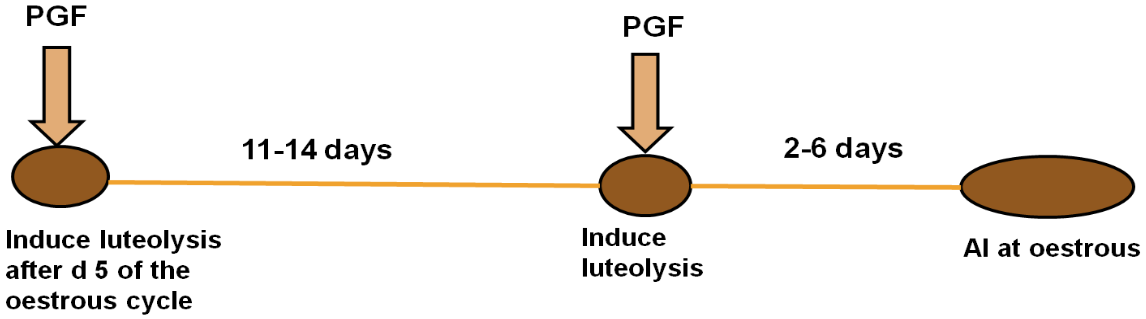


Figure 2.5. Control of the luteal phase with double PGF2α treatment (De Rensis and Lo´pez-Gatius, 2007)



Efficiency in synchronizing oestrous with PGF depends on the presence of CL. If animals treated when follicles are in the pre-dominance stage of development display oestrous 4–6 days later, whereas animals treated in the presence of a dominant follicle (DF) display oestrous 2– 3 days after PGF2α administration. Since the intervals between treatment, oestrous, and ovulation vary after PGF2α administration, a timed artificial insemination protocol cannot be applied. Regimens involving the use of progesterone or progestagen to synchronize oestrous and ovulation in buffalo are limited, compared to protocols based on prostaglandins.

Treatment includes the administration of estradiol valerate (or benzoate) at the time of progestagen application, and/or PGF2α the day before progestagen removal.

Ovulation occurs 40–96 h following progestagen withdrawal and oestrous has been observed in 80–93% of treated animals. The interval from progesterone device removal to oestrous may varies from 43 to 117 h. Pregnancy rate following treatment varies from 20 to 50%.

### 2. *Control of follicular development and ovulation*

Figure 2.6. Control of follicular development and ovulation with the ovsynch protocol (De Rensis and Lo´pez-Gatius, 2007)

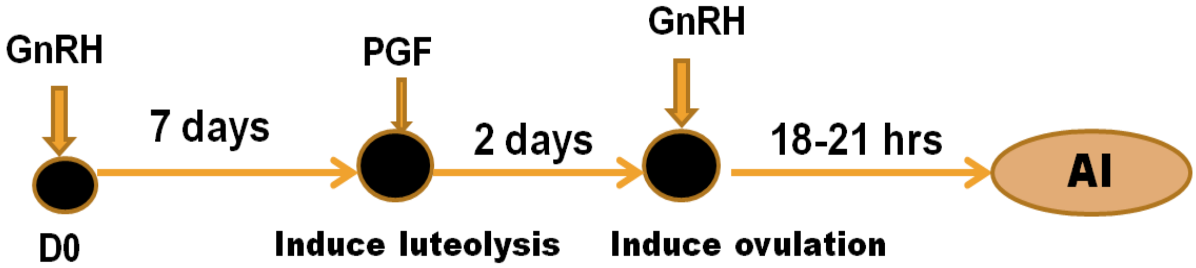
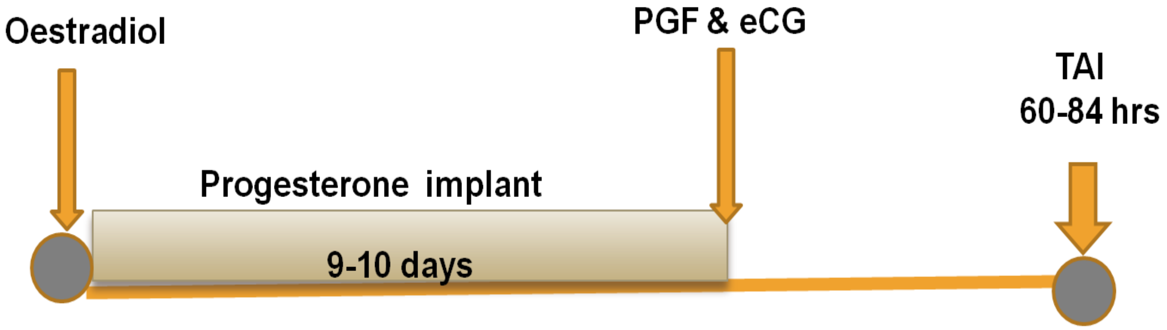


Figure 2.7. Progesterone based estrus synchronization protocol (De Rensis and Lo´pez-Gatius, 2007)



Efforts, however, have been made to breed the buffaloes throughout the year by using different hormones (Rajamahendran and Thamotharam, 1983; Singh, 2003), but with limited success. It is well accepted that an injection of a GnRH agonist at any stage of the oestrous cycle in buffalo 1) increases the number of medium-sized follicles within 3 days of treatment, 2) eliminates the large follicles by ovulation or atresia and 3) induces the emergence of a new follicular wave within 2 to 3 days of treatment (Jazayeri *et al*., 2010). Several reports have showed that the first GnRH injection can successfully synchronize a new follicular wave 1-3 days after treatment (Neglia *et al*., 2003; Ali and Fahmy, 2007) and this wave results in the development of a new dominant follicle, which may be attributed to the subnormal P4 concentrations. Sub-luteal circulating P4 levels have been reported to increase the frequency of LH pulses, and a prolonged growth phase of the dominant follicle (Bridge and Fortune, 2003). The subsequent injection of PGF2α increases the percentage of synchronized animals by lysis of both the cyclic CL and the CL resulting from ovulation of the dominant follicle (Pursley *et al*., 1995). After the PGF2α injection, if the dominant follicle did not ovulate, a new wave of small follicles needs several days to grow and become able to produce estradiol-17β, leading to an induction of the preovulatory LH-surge (Bridge and Fortune, 2003). It has been previously suggested that high P4 levels at the time of PGF2α application may be an important factor to improve conception rates for subsequent inseminations (Hussein, 2003, De Rensis *et al*., 2005). In order to increase synchrony of ovulation, a second dose of GnRH is injected to ovulate the preovulatory follicle at a precise time (Wiltbank, 1998). The second GnRH injection on day 9 of the protocol causes an induced LH surge responsible for ovulation of the dominant follicle and formation of a new CL (Senger, 2003).

The time to ovulation after GnRH injection depends mainly on the diameter of the largest follicle at the time of injection (Wiltbank, 1998; Hussein *et al*., 2002; Hussein, 2003) and it is a determining factor for the successful synchronization of ovulation and high conception rates (De Rensis *et al.*, 2005). In a recent study, Campanile *et al.* (2008) have demonstrated that follicle size in buffalo that ovulated compared to those that did not ovulate is quite similar. Moreover, the stage of follicular development (growth or regression phases) greatly affects its response to GnRH treatment (Dharani *et al.*, 2010). Conception rate (CR) achieved 33–60% in buffaloes with the ovsynch protocol (Paul and Prakash, 2005).

De Rensis and López-Gatius,(2007) have reported that the Ovsynch protocol appears to be more effective when supplemented by administration of progesterone for 7 days between the first GnRH and prostaglandin treatments. Study comparing the effects of GnRH and LH in the Ovsynch conducted by de Araujo Berber *et al.*, (2002) showed quite high fertility rates of 56.5% and 64.2%. By contrast, a very poor fertility rate (12.5-25%) was reported by Honnappagol and Patil (1991).

Recent studies indicate GnRH induced ovulation of follicles ≤1 mm decreased pregnancy rates and increased the incidence of late embryonic/ fetal mortality (Perry *et al.,* 2005). Consequently, GnRH-induced ovulation of physiologically immature follicles can reduce pregnancy rate and late embryonic/fetal survivability which was associated with decreased serum concentrations of estradiol on the day of insemination (Perry *et al.,* 2005), and estradiol has been associated with the induction of endometrial progesterone receptors (Zelinski *et al.,* 1980). The presence of a follicle with a size >10 mm in ovaries at the time of inclusion of buffaloes in oestrous synchronization programs is essential for obtaining good results (Presicce *et al.*, 2005; De Rensis and Lopez-Gatius, 2007). This is due to the fact that small follicle size during the first GnRH treatment is related to a high probability of preliminary ovulation of the dominant follicle – between the PgF2α analogue injection and the second GnRH administration (Baruselli *et al.,* 2003).

De Rensis *et al*. (2005) observed a relatively higher conception rate in animals with follicle size ≥10 mm on the first day of ovsynch program, compared to buffaloes with follicle size of <10 mm: 44% and 8%, respectively. The ovulation in response to GnRH treatment in postpartum buffaloes was 60% (Baruselli *et al*., 2003). Warriach *et al*. (2008) established different conception rates in buffaloes, insemination after ovulation synchronization: 36.3% during the oestral and 30.4% during the anoestral season.

With respect to season, Baruselli *et al*. (2003) demonstrated a considerable difference in conception rates: 48.8% and 6.9% during the oestral and anoestral seasons, respectively. Similarly, Carvalho *et al.* (2007) reported conception rates of 46.8% after using the Ovsynch protocol during the reproductive season. The efficiency of different ovulation inducers (hCG or GnRH) on follicular dynamics and conception rate were studied by Carvalho *et al*., 2007b. They found ovulation rates of 76.5% and 81.3% and conception rates of 52.3% and 51.8% with hCG and GnRH respectively. GnRH is cheaper than hCG and it would reduce the cost of the synchronization protocol.

Previous studies carried out in postpartum anoestrous cows have demonstrated that progesterone treatment stimulates an increase in LH pulse frequency during and following the treatment period (Rhodes *et al*., 2002). Treatment of anoestrous cows with progesterone results in greater follicular fluid and circulating of estradiol, increased pulsatile release of LH and increased numbers of receptors for LH in granulosa and theca cells in preovulatory follicles, compared with untreated animals (Rhodes *et al*., 2003). Furthermore, a short period of elevated progesterone concentrations during anoestrous period is important for the expression of oestrous as well as subsequently normal luteal function (McDougall *et al*., 1992). Results indicate that the use of intravaginal progesterone device for three times provided satisfactory conception rate in buffalo during the off breeding season and it might reduce the cost of the protocol for fixed time artificial insemination Carvalho *et al*., 2007a.

Pursley *et al*. (1997) reported that P4 supplementation on the day of PGF2 $\alpha$ injection had no effect on the pregnancy rates. Attempts to replace the second GnRH injection with hCG have also been failed to improve conception rates in buffalo after fixed time AI in Brazil (Carvalho *et al*., 2007).

The conception rates achieved with prostaglandins either alone or in combination with GnRH ranged from 7 to 56%, while CR following the use of a progesterone releasing devices either alone or in combination with eCG, and in some cases further supplemented with hCG or GnRH, ranged from 8 to 64% (Perera, 2011). CR of water buffaloes were reported to range from 22.2% to 37.5% when PRID (contained progesterone and estradiol-17beta) and PMSG were used (Zicarelli *et al.,* 1997, Barile *et al.,* 2001, Pacelli *et al*, 2001). The fertility rate of buffaloes induced oestrous by GnRH and PMSG was from 28.2% to 36% (Neglia *et al.,* 2003; Paul and Prakash, 2005; Chaikhun *et al*., 2009).

However, for successful implementation of any protocol selected, the following factors must also be addressed in buffalo (Perera, 2008): (a) selection of animals that are in good body condition score and free from disease; (b) minimize stress during the treatment administration and AI, when animals may be herded together, tethered or moved to other locations; and (c) scheduling treatments for the more favourable periods or during the peak of the breeding season when the majority of animals are likely to be having oestrous cycles.

## 2.6. Artificial Insemination in Buffaloes

AI has made a significant contribution to genetic improvement in cattle and has the potential to improve genetic characteristics in buffalo. However, the widespread use of AI in buffalo is still limited due to a relatively low expression of oestrous behavior (Seren *et al.*, 1993; Ohashi, 1994), highly variable duration of estrus (4-64 h) and difficulty in predicting the time of ovulation in female buffaloes (Baruselli, 2001).

Buffaloes require exogenous hormone treatments that induce elevated P4 concentration throughout the period from initial development to embryonic attachment. The use of pharmacological treatments in order to increase P4 systemic levels between 25 and 40 days post-AI, characterized by a 45% embryo mortality rate in buffalo, play a determinant role in farms with a high incidence of embryonic mortality (Campanile *et al.*, 2005, 2007b). In addition, early application of the program during the post-partum period may be another possible cause of lowering conception rates in buffalo cows (Derar *et al., 2012*).

Embryo mortality in buffalo occurs usually between 25 and 40 days from AI (Campanile *et al.*, 2005). In buffaloes naturally mated, 8.8% showed embryonic mortality between 28-45 days and 13.4% showed fetal mortality between 46-90 days (Vecchio *et al.*, 2007).

In the spontaneous oestrous buffaloes, the conception rate varies depending on the time at which AI is performed. Kumaresan and Ansari, (2001) conducted AI at 6-12h, 12-18h and 18-24h on buffaloes after estrus, and observed 16.67%, 28.99% and 33.33%, pregnancy rates respectively.

Noncyclic buffaloes were announced to achieve lower fertility rate of 4.7%-30% compared with that of 35.7% - 51.5% in cyclic buffaloes (De Rensis *et al.,* 2005).

Low pregnancy rate in buffaloes might be explained by that the embryonic mortality rate between day 25 and day 40 post insemination was reported to be very high of 21%-50% (Campanile *et al.,* 2005, Campanile et al. 2008, Vecchio *et al.*, 2008).

The correct management of estrus detection requires continuous observation of the herd and qualified, responsible and knowledgeable labour. In herds in which estrus detection is inefficient, there is a decrease in the reproductive performance and a consequent increase in the breeding period and in the calving interval, what causes serious economic losses for the breeder. Therefore, the use of management schemes that do not require the identification of estrus contribute for the increase in the use of AI in buffalo herds, mainly because it is easy to perform. The use of estrous synchronization protocols has been collaborated with the widespread of the artificial insemination in buffalo herds, and enables genetic improvement, increasing milk and meat productions.

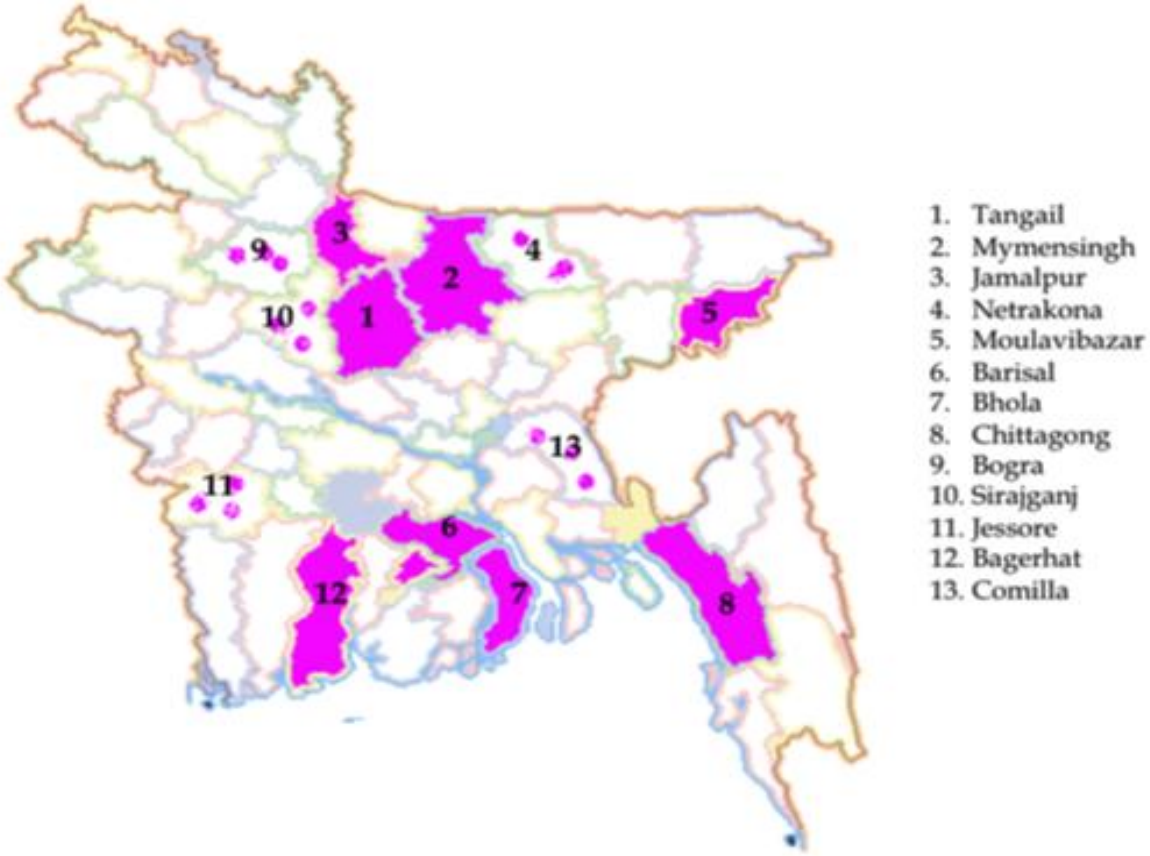
### 2.7. Buffalo Farming System in Bangladesh

Bangladesh lies between 20°34$^{/}$ N and 26°38$^{/}$ N latitude, and with a 724 km long coastline, on the northern coast of the Bay of Bengal. Bangladesh possesses about 1.26 million buffaloes (DLS, 2008), which are of swamp, crossbred and river types. These are found in the Brammaputra- Jamuna flood plain of central Bangladesh and Ganges-Meghna Tidal Flood Plain of Southern Bangladesh. Buffalo forms a part of the property, possession and profession of rural farmers. They are an easily 'convertible currency' and a reliable 'living bank' to serve the immediate needs of the rural masses in several communities.

Table 2.1. Types/breeds of buffaloes found in Bangladesh (Farouque, 2003).

| Type\breed | Location | Phenotype and Genotype |
|---|---|---|
| Indigenous River type | Western and central part of the country | Coat colour-Jet-Black to Black<br>Chromosome no.-50<br>Medium in size |
| Bangladeshi | Central and South West | Coat colour- Light black<br>Chevron and white stocking present.<br>Chromosome no.-50<br>Medium in size |
| Indigenous<br>Swamp type | Eastern part of the country | Grey coat colour, chevron, white stocking and crescent horn are present.<br>Chromosome no 48<br>Small in size |
| Crossbred type (Indigenous X Nili Ravi) | Southern part of the country | Phenotypes combination n of Swamp type and Nili-Ravi<br>Medium in size |
| Nili Ravi | Buffalo breeding farm | - |
| Non-descriptive type | Southwest and Southern part of the country | - |

Figure 2.8. Major Buffalo raising areas in Bangladesh (pink colored areas indicate large number)



Among different types of buffaloes, local non-descript, Surti and Murrah crossbreds are very popular to farmers. The Surti and Murrah crossbreds have the local names “Nepali” and “Gujrati”, respectively. Each breed has advantages for particular traits. The Murrah has the highest milk yield, highest market value, and best temperament. The Surti is the best for meat production. The local buffalo is the most resistant to disease and most adapted to poor quality feeds. The local animal also has the most regular reproduction, but has the poorest temperament.

Husbandry and production systems for buffaloes vary depending on the topography and vegetation patterns of the country. Buffaloes are raised under an extensive system in the coastal and hilly areas where large-scale pastureland and enough green forage are available. Buffaloes are raised under a semi-intensive system on plain land and marshy land where there is limited pastureland. An intensive system for buffalo production is not practiced anywhere in Bangladesh even for institutional or commercial herds. The husbandry and care of the animals differs somewhat in these two systems.  All the swamp buffaloes are rearing extensively. Each farmer keeps one or two buffaloes and maintains them by grazing on roadside grass or on common land. Rice straw and crop residues are supplemented feed. Dairy buffaloes are reared in semi-intensive system, they are raised mainly for milk production and are stall fed. In these farms, forage and grasses are cut, carried and fed at stall. Crops by-products are used to mix with concentrate to feed the animal. Not so often, the buffaloes are supplied by urea molasses block or rice straw urea treatment.

The buffaloes are kept for multi purposes: milk, meat and work. Most of the farmers have one or two animals. Very few herds larger than a dozen are noted in some places. Despite its importance as a tool for economic growth and poverty alleviation in rural areas of Bangladesh, they are much neglected till now. The true scenario of buffalo production includes the absence of defined housing system, artificial insemination system, routine vaccination program and animal identification and record-keeping system. As a result, buffalo number is increasing naturally without any significant growth rate, even without any increase in its milk and milk production. The production of milk and meat from buffaloes in Asian countries over the last decades has shown a varying pattern in countries such as India, Sri Lanka, Pakistan and China, the milk yield per animal has increased by 2.44 %, 1 %, 1.45 % and 1.55 %, respectively, while there has been either no change or only a negligible change in milk production in Bangladesh (Borghese and Mazzi, 2005).

There is a great lack of information on the production and reproduction performance of buffaloes in the farmer herds across the country. One study to compare the performance of river and swamp buffaloes involving milk recording activity in the farmers reared buffaloes has been reported (Khan *et al.,* 2007).

### *Major constrains for sustainable buffalo production*

In the past, all the experiences are drawn to the exploitation of draught power. Little/no attention is given to buffaloes because of the longevity of buffalo cows. The following factors can be considered to understand the reason for a low rate of reproduction:

- Scarcity of breeding males at village level
- Unawareness in using of breeding bulls
- The poor knowledge of farmers about the reproduction of buffaloes has been an obstruction to the development of buffalo herd.
- Absence of AI service (mainly available for cattle only)
- The unfair management of draught power through the hard ploughing seasons, above all in summer. This leads to the disorder of physiological heating that has a negative effect on insemination and abortion problem.
- The poor nutrition management during the hot season.
- The grazing system is not suitable for a reproduction system. All the buffaloes are kept in the farmers' household and graze separately by different grazers.
- Sound national breeding policies have never been implemented
- The neglect of buffalo development at the national level. Almost no resource has been put aside for a national research project on buffalo development.
- Lack of infra-structure and trained personnel
- Existing buffalo production system
- Lack of financial support
- Limited milk collection and processing facilities and low prices at collection points

- Absence of market information

Along with these constrains, reproductive efficiency is the primary factor affecting productivity and is hampered in female buffalo by

a. inherent late maturity

b. poor oestrus expression in summer

c. distinct seasonal reproductive patterns

d. prolonged intercalving intervals (Madan, 1988; Madan and Raina, 1984).

The buffaloes of Bangladesh have been recognized to possess low reproductive performance (Alam and Ghosh, 1991). Production is interlinked with reproductive performances. During breeding season, buffalo farmers borrow a bull from neighbour or leave their buffaloes to be bred in a village field by unknown bulls. The silent heat-the outstanding feature of buffalo is a disadvantage for implementing appropriate technologies for increasing reproduction such as artificial insemination. The low reproductive rate has caused a slow growth of population.

Table 2.2. Some reproductive parameters Bangladeshi buffalo cows (Islam *et al.,* 2005)

| Reproductive parameters | Bangladeshi buffalo breeds | |
|---|---|---|
| | Nili-Ravi | Cross breed |
| Average conception rate (%) | 95.24% | 88.46 |
| Service per conception | 1.05 | 1.13 |
| Gestation period (days) | 301.486±6.72 | 303.200±7.07 |
| Age at first calving (months) | 61.857 ± 3.44 | 63.048 ± 3.89 |
| Birth weight (kg) | 33.266 ± 3.49 | 30.508 ± 3.43 |
| Calving interval (days) | 572.633± 116.54 | 581.481 ± 94.15 |
| Postpartum heat period (days) | 167.800 ± 27.92 | 174.500 ± 41.04 |

The possible cause may be less fodder availability and high environmental stress together with under-nutrition might be responsible for the long periods of seasonal anoestrus in buffaloes (Shah *et al.,* 1989). The seasonality in buffalo could be due more to management factors and unavailability of green fodder rather than to the inability of the species to reproduce throughout the year. Therefore, breeding plans for improving genetic potential and regular production are essential. But they must comply with the animals' adaptation to the local climatic conditions, technical expertise and the needs of the human population.

## 2.8. Practical use of ultrasonography in reproduction

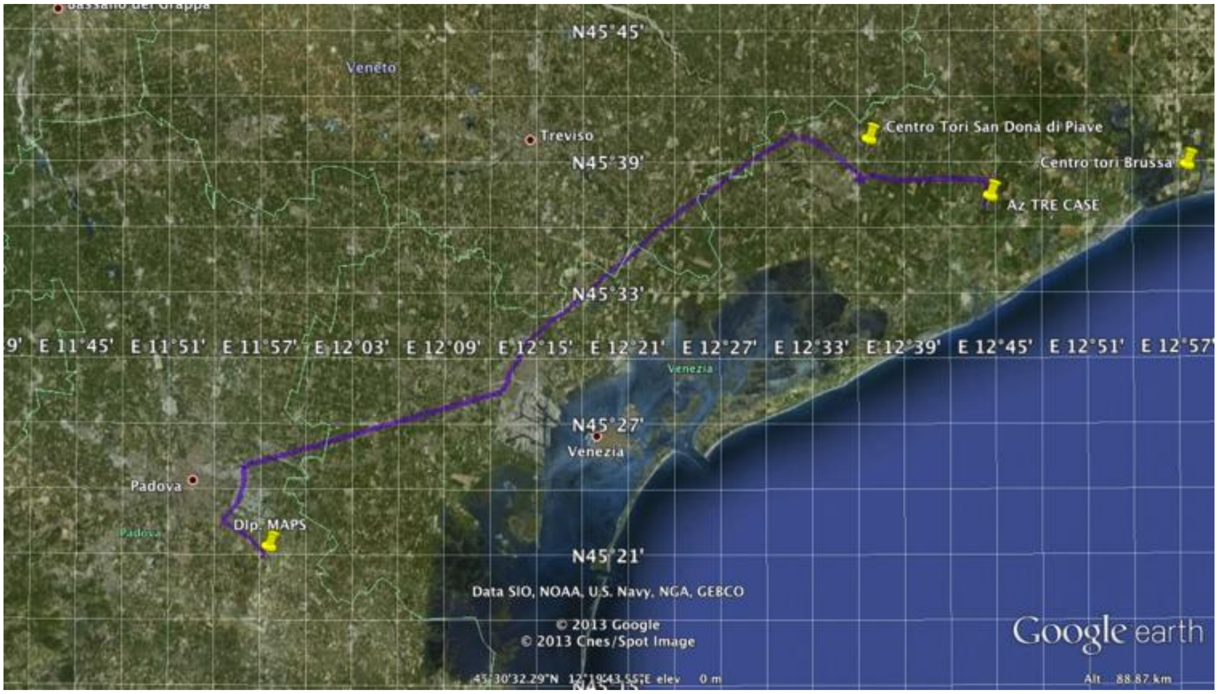
Since the advent of bovine transrectal ultrasound machine in 1984, enormous progress has been made in our understanding of folliculogenesis and the development of the bovine corpus luteum (Pierson and Ginther, 1988b; Sirois and Fortune, 1988; Ginther, 1998; Durocher *et al*., 2005). Ultrasound enables us to describe the dynamics of follicular growth in follicles greater than 1 mm in diameter (Durocher *et al*., 2005). As a diagnostic aid, ultrasonography is well suited for bovine practice, particularly for the examination of reproductive organs (Rajamahendran *et al*., 1994). The pregnancy examination is generally considered essential and profitable for dairy producers (DesCoteaux *et al*., 2002). In routine clinical practice of commercial dairy farms, ultrasound scanning (US) is one of the useful tools to monitor reproductive organs since the US monitoring technique has been reported to be an accurate and reliable method of observing dynamic changes in ovarian structures (Pierson and Ginther, 1984; Rajamahendran *et al.,* 1994) and determining the patterns of postpartum resumption of ovarian activity (Rajamahendran and Taylor, 1990; Savio *et al.,* 1990a, b). Moreover, the US technique can be efficiently utilized in the early detection of silent oestrous, anoestrous and cystic ovarian conditions, and thus, useful in reducing the calving interval (Taylor and Rajamahendran, 1991). Unfortunately, there are few studies on the use of transrectal ultrasonography as a diagnostic tool for reproductive disorders in Bangladesh.

# CHAPTER 3. EXPERIMENT 1: EFFICACY OF OVSYNCH PROTOCOLS WITH TWO DIFFERENT GnRH ANALOGUES FOR SYNCHRONIZATION AND FIXED-TIMED AI WITH SEXED FROZEN SEMEN IN ITALIAN MEDITERRANEAN BUFFALO COWS

## 3.1. MATERIALS AND METHODS

This study was conducted in a private buffalo farm "Azienda Agricola Tre Case Srl" of Caorle in Venezia Province, Italy (Figure 3.1.), during a period extending from August to October 2010, with ambient temperature recorded from 16 to 25°C.

Figure 3.1. Localization of the Italian buffaloes farm used for the experiment 1.



### 3.1.1. Animals

Thirty-two healthy Mediterranean buffaloes with variable post-partum period (16 to 174 days) were used for this a study and they were free from any detectable reproductive problems. The daily feed allocation (Total Mixed Ration) consisted of 5 kg ryegrass Italian hay, 18 kg corn silage (30% dry matter), 2 kg soybean meal (44% crude protein), 4 kg grain mix (22% crude protein), 2 kg corn meal and 0.1 kg hydrolyzed fats. The buffaloes were maintained in good health under hygienic and optimum management condition. They were milked twice per day.

### 3.1.2. Experimental design

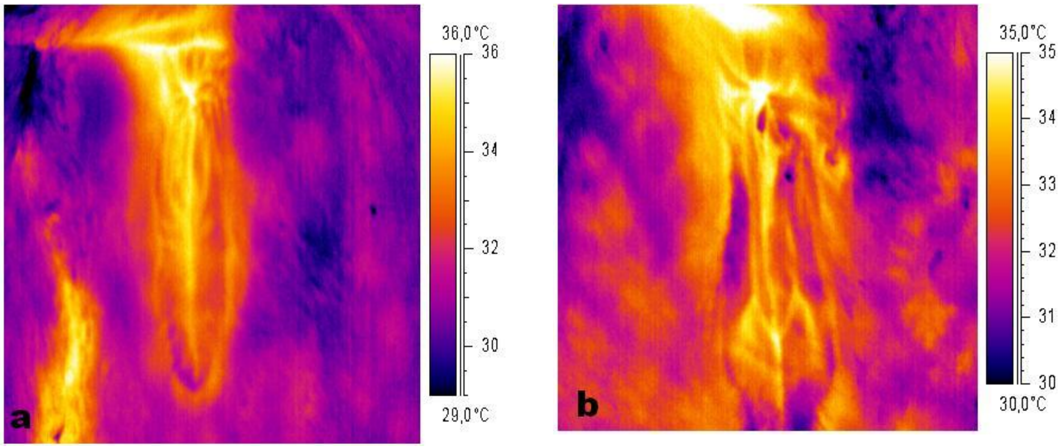
All buffaloes were divided randomly into two groups depending on GnRH analogue, named as group F and R. According to the time of administration of hormones, every group had two subgroups such as AM (hormones administered at 8.00) and PM (hormones administered at 16.00). In group F, buffaloes were synchronized following Ovsynch protocol with Fertagyl ® (Gonadorelin acetate, Intervet, schering-Plough Animal Health) administered at morning (n=8) and at evening (n=8). Similarly, in group R, Receptal® (Buserelin, Intervet, schering-Plough Animal Health) was used for synchronization of sixteen buffaloes, where Ovsynch protocol was started at morning (n=8) and at evening (n=8).

Before starting synchronization protocol, a transrectal ultrasound examination was carried out to identify follicular status in ovaries. Follicles ≥3mm in diameter and the CL were measured and recorded. The Ovsynch protocol (Pursley et al., 1995; De Rensis and López-Gatius, 2007) was started regardless of the stage of estrous cycle in animals. 5ml of Fertagyl or Receptal was injected at gluteal muscle at first day ($D_0$). At day seven ($D_7$) 2ml of Estrumate (Cloprostenol) was injected. At day nine ($D_9$), second dose of Fertagyl or Receptal (2.5) ml was administered. All female buffaloes were inseminated with sexed frozen semen (X-chromosome bearing spermatozoa) from a bull of known high fertility after 18-21 hours of administration of second dose of GnRH.

### 3.1.3. Use of Infra-red thermography, transrectal ultrasonography and vaginal electrical impedance

Infrared thermography of vulvar and perivulvar region were performed using a thermocamera (Thermacam P25, Flir system) at D7 and before AI. Figure 3.2. shows thermographic images taken by thermocamera. Images taken by thermocamera were analyzed with Therma Cam Researcher Basic software 2.8 to obtain temperature (°C) of vulvar and perivulvar region (Figure 3.2 a and b). Moreover, Transrectal ultrasonography was performed to evaluate the condition of ovary at D7. Electrical impedance of vaginal mucus (VEI) was measured with Draminski probe before administration of Estrumate, after administration of second dose of GnRH and before AI for proper detection of oestrous condition in buffaloes. For confirmation of pregnancy, transrectal ultrasonography was performed after 42 days of AI.

Figure 3.2. Thermographic images of vulva and perivulva regions taken during administration of second dose of GnRH (a) and AI (b). The measuring scale indicates the color relating to temperature(°C).



### 3.1.4. Statistical analysis

All data were analyzed in GLM (General Linear Model) in statistical software SIGMASTAT 3.50. Difference in values were measured by two ways ANOVA (Analysis of Variance), where time and treatment for synchronization protocols were considered as independent variable s and values of VEI, temperature of vulvar region and δT (difference between temperatures of vulvar and perivulvar region), pregnancy rate were considered as dependent variables. Differences were considered significant at $P < 0.05$. Values were presented as means and S.E.M.

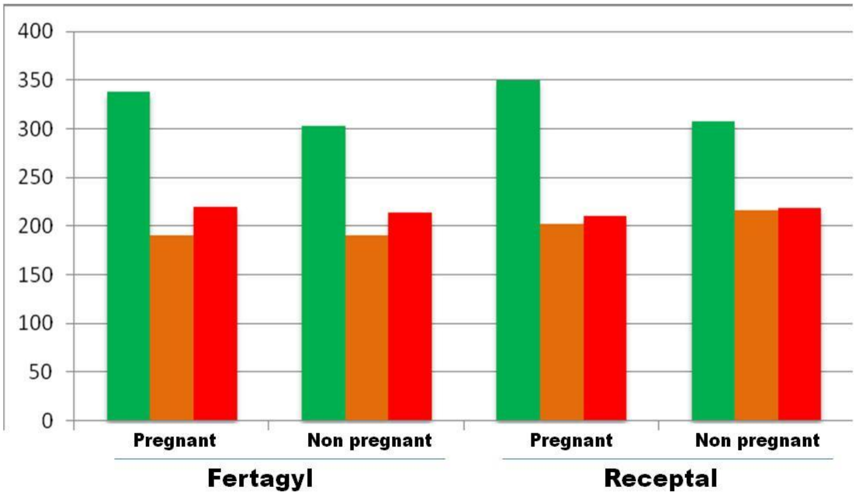
## 3.2. RESULTS

The objective of this study was to compare the effects of Ovsynch protocol using two GnRH analogues on conception rate of Italian Mediterranean buffaloes inseminated with sexed frozen semen. Moreover, this was a preliminary study to observe the time effect on synchronization protocol. In this study, pregnancy rate was 37.5% in R group and 25% in F group. Considering the time of administration of hormones as well as time of insemination, 50% buffaloes were pregnant in morning group, whereas 25% were pregnant in evening group. There were significant differences between pregnancy rates regarding to type and time of treatment ($P<0.05$).

Differences between values of VEI in pregnant (P) and non-pregnant (NP) buffaloes of F and R groups are shown in Figure 3.3. VEI recorded before administration $PGF_2\alpha$ was comparatively higher in pregnant buffaloes (337.5±18.3 and 350±23.6 mmOhm) in comparison to non-pregnant buffaloes (303.1±16.7 and 308±18.3 mmOhm) of F and R groups respectively. After administration of second dose of GnRH, VEI valued significantly ($P < 0.05$) decreased in all buffaloes of F (P: 190 ± 28.9; NP: 190.9 ± 16.7 mmOhm) and R (P: 201.6 ± 23.6; NP: 216.0 ± 18.3

mmOhm) groups. Whereas, VEI values slightly increased at the time of AI in pregnant (220.0 ± 18.3 and 210.0 ± 23.6 mmOhm) and non-pregnant (213.4 ± 16.7 and 219 ± 18.3 mmOhm) buffaloes of F and R groups respectively. There was significant ($P<0.05$) difference in values of VEI of buffaloes in two groups and values were higher in buffaloes of R groups.

Figure 3.3. Values (mean± SEM) of vaginal electrical impedance (VEI-mmOhm) in pregnant and non-pregnant buffaloes treated with Fertagyl and Receptal: 🟩VEI values recorded before administration PGF$_{2\alpha}$, 🟧 VEI values recorded after administration of second dose of GnRH 🟥 VEI values recorded during AI.



When we considered time of treatment (AM and PM), significant ($P<0.05$) difference was observed between VEI values of pregnant and non pregnant buffaloes at different observations (Table 3.1.). VEI values were significantly ($P<0.05$) higher in PM subgroups in both pregnant and non pregnant buffaloes. These data suggested that VEI values decrease during administration of 2$^{nd}$ dose of GnRH when buffaloes are supposed to be in oestrous.

Table 3.1. Values (mean± SEM) of vaginal electrical impedence (VEI-mmOhm) in pregnant and non-pregnant buffaloes where hormones were administered at morning (AM) and evening (PM).

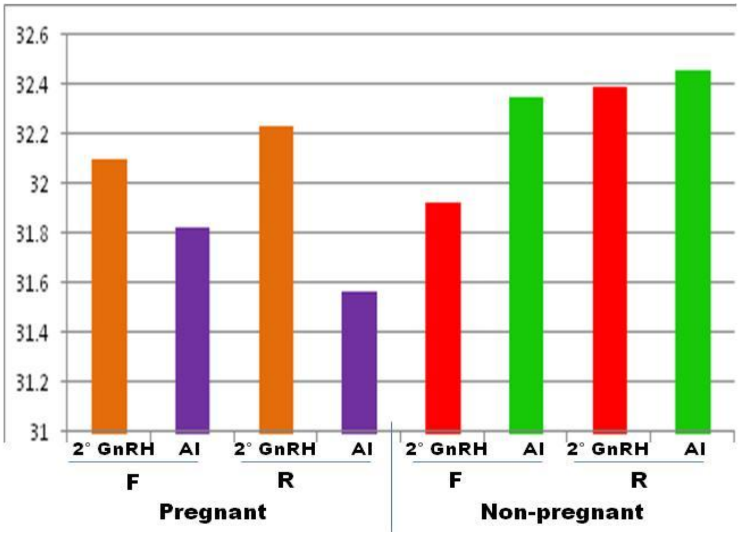
| Time of observation | Pregnant | | Non-pregnant | |
|---|---|---|---|---|
| | AM | PM | AM | PM |
| 1 | $303.3 \pm 22.2^{a*}$ | $407.5 \pm 27.2^{b*}$ | $299.8 \pm 17.2^{a**}$ | $310 \pm 15.7^{b**}$ |
| 2 | $206.6 \pm 22.2^{a*}$ | $182.5 \pm 27.2^{b*}$ | $183.1 \pm 17.2^{a**}$ | $218.3 \pm 15.7^{b**}$ |
| 3 | $223.3 \pm 22.2^{a*}$ | $200 \pm 27.2^{b*}$ | $205.1 \pm 17.2^{a**}$ | 225 ± 15.7 b** |

1- VEI values recorded before administration $PGF_{2\alpha}$, 2- VEI values recorded after administration of second dose of GnRH, 3- VEI values recorded during AI.

*,** indicate significant ($P<0.05$) differences between values of VEI within pregnant and non-pregnant buffaloes, a, b indicate ($P<0.05$) differences between values of AM and PM sub-groups

Thermographic data showed some interesting results (Figure 3.4.). Vulvar temperature recorded at two times; during administration of 2nd GnRH and AI. In Pregnant animals, T values were higher during 2nd GnRH administration in comparison to that recorded during AI in F (32.1 ± 0.5 vs 31.8 ± 0.5°C) and R (32.2 ± 0.4 vs 31.5 ± 0.4°C) groups. In contrast, we observed higher T values in non-pregnant buffaloes during AI than that recorded during GnRH administration in both F (32.5 ± 0.3vs 31.9 ± 0.3°C) and R (32.4 ± 0.3vs 32.4 ± 0.3 °C).

Figure 3.4. Values of vulvar temperature (°C) recorded by Infrared thermography in pregnant and non pregnant animals treated with fertagyl (F) and Receptal (R) during 2nd administration of GnRH and AI.



There was significant difference between T values recorded at two times in response to time of treatment (AM and PM) (Table 3.2.). T values were significantly higher in both pregnant and non/pregnant buffaloes when protocol started at evening (PM).

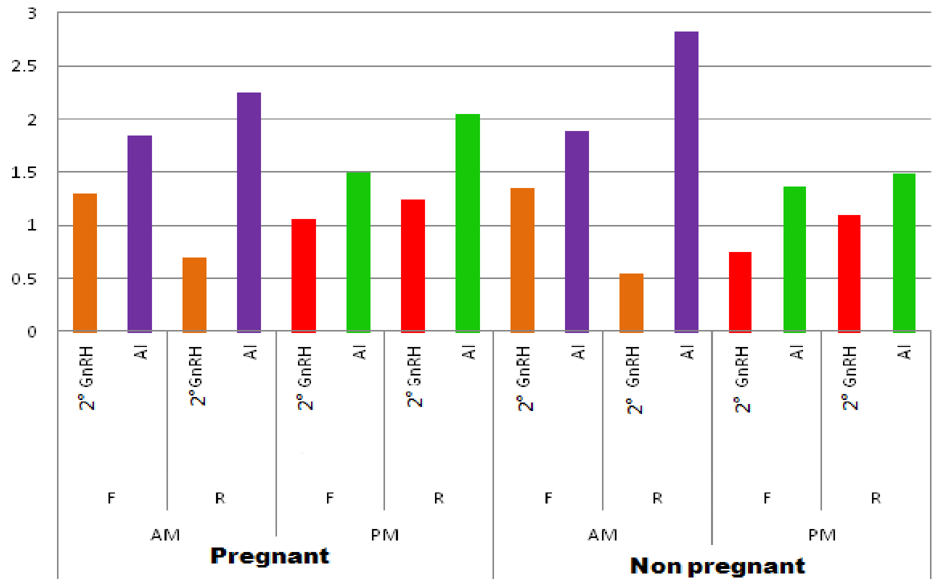
Table 3.2. Values (mean± SEM) of vulvar temperature (T/°C)) recorded by Infra-red thermography in pregnant and non-pregnant buffaloes treated with hormones administered at morning (AM) and evening (PM).

| Time of observation | Pregnant | | Non pregnant | |
|---|---|---|---|---|
| | AM | PM | AM | PM |
| 1 | 31.8 ± 0.3$^a$ | 32.7 ± 0.3$^b$ | 31.7± 0.2$^a$ | 32.4 ± 0.2$^b$ |
| 2 | 30.7 ± 0.3$^a$ | 33.1 ± 0.3$^b$ | 31.6 ± 0.2$^a$ | 33.05 ± 0.2$^b$ |

1- T (°C) values recorded after administration of second dose of GnRH,
2- T (°C) values recorded during AI.
a,b indicate ($P<0.05$) differences between values of AM and PM subgroups.

We also analyzed δT (difference between temperatures of vulvar and perivulvar region) values in both pregnant and non-pregnant buffaloes recorded after administration of second dose of GnRH and during AI, and results are shown in Figure 3.5. There were significant differences in δT values in response to time and type of treatment. δT values were higher in both pregnant and non-pregnant buffaloes when treated with Receptal. Moreover, higher δT values were observed in buffaloes of AM subgroups irrespective of treatment.

Figure 3.5. Values recorded by Infrared thermography in pregnant and non-pregnant animals treated with Fertagyl (F) and Receptal (R) during 2nd administration of GnRH and AI.



In this study, Pearson's correlation test of data obtained during this protocol revealed a significant positive correlation between time of treatment and T value (0,626) and between time of observation and T value (0,617) in both pregnant and non-pregnant buffaloes. We recorded higher T values during second administration of GnRH. Results of this study hypothesize that buffaloes in oestrous during AI were pregnant. On the other hand, non-pregnant buffaloes might be at advanced stage of oestrous (might ovulate) around the time of AI.

## 3.3. DISCUSSION

An improvement in buffalo reproductive efficiency can be obtained by utilizing controlled breeding techniques. It is worthy to point that out-of-season breeding of postpartum buffaloes is practiced in Italy to produce milk throughout the year in order to achieve continuity of market supply (Zicarelli, 1997). Hormonal treatments have been proved useful in reducing the calving interval and increasing fertility in the non-breeding season (Barile, 2005). The study had as objective the evaluation of the Ovsynch protocol application according to type of GnRH analogues and time of treatment. There were significant differences between pregnancy rates regarding to type and time of treatment ($P < 0.05$). Pregnancy rate was higher (37.5%) when Receptal (Buserelin) was used for synchronization. There are some differences in chemistry and diluents among the GnRH products. Differences among products used in this study may be attributed to differences in absorption (Zia *et al*., 1991), in the timing of the GnRH-induced LH surge, its magnitude and ovulatory response (Martínez *et al*.,2003). Research in cattle has shown that the change that is caused by the GnRH in the function of follicle or the corpus luteum is the indirect action of the GnRH on the regulation of the LH and FSH surge, because there is no particular GnRH receptor on the ovary tissue of the cow (Qin *et al.,* 2009). Therefore, different GnRH agonists have been used to increase stability and capacity to bind plasma proteins and GnRH receptors (Thatcher *et al*., 1993) as well as to manipulate ovarian function (Drost and Thatcher, 1992).

The overall conception rate (31.25%) obtained in the present study is higher in comparison to that (6.9%) reported by Baruselli *et al.* (2003) when applied timed-AI after using Ovsynch protocol in non-breeding season. Neglia *et al*., (2003) obtained a conception rate of 36% using the Ovsynch protocol in the period of transition to seasonal anoestrous. De Rensis *et al*. (2005) reported very low conception rate (4.7%) in Italian non-cyclic buffalo cows during unfavorable breeding season. In contrast, some Authors (Baruselli *et al.,* 2003*;* Ali and Fahmy, 2007*)* have reported 42.8–60% conception rate after single timed AI during high breeding season. Considering the time of administration of hormones as well as time of insemination, 50% buffaloes were pregnant in morning group, whereas 25% were pregnant in evening group. High temperatures of summer may be the reason for the lower conception rate obtained in our study. Reduced sexual activity in the buffalo has been associated to biometeorological factors such as day length, ambient temperature, relative humidity and rainfall (Razdan *et al*., 1981; Singh and Nanda, 1993). The heat stress is associated with increased uterine temperature and reduced blood supply to the uterus in cows. These changes inhibit embryonic development and increase embryonic loss (De Rensis and Scaramuzzi, 2003).

The use of AI is limited to just the 10% of the buffaloes enrolled to the Herd Book and AI with sexed frozen semen is very rare in Italy. Because the efficiency of AI is still low and is significantly affected by the seasonality of the species (Campanile *et al*., 2008). Due to the sensitivity of the Mediterranean buffalo breed to photoperiod, there is a decrease in the conception rate during the spring–summer seasons (Barile, 2005). Different studies have summarized that reduced ovarian cyclicity in buffaloes during summer is characterized by suboptimal functioning of the hypothalamus-pituitary-gonadal axis (Rao and Pandey, 1983), low plasma circulating concentrations of pituitary and gonadal hormones specially lower peaks of FSH and LH and variable plasma progesterone plasma levels, high concentrations of prolactin as well as variable duration of oestrous (Razdan *et al.,* 1981; Rao and Pandey,1983; Jainudeen *et al*., 1993). It is important to point that buffaloes used in this study were in variable postpartum period as well as were milked twice per day. Lactation period also influences the responses to synchronization protocol. Females later in lactation will have lower milk production, and this may increase circulating progesterone concentrations (Wiltbank *et al*., 2006) that, in turn, may decrease GnRH-induced LH secretion (Dias, 2008) producing the decreasing ovulation efficiency.

Therefore, greater doses of GnRH might be necessary to maximize ovulation when GnRH is administered during the luteal phase. It is important to mention that we have started synchronization protocol irrespective of stage of oestrous cycle. Previous studies show that for successful synchronization, Ovsynch protocol requires the presence of a DF at the time of the first GnRH treatment (Vasconcelos *et al.,* 1999; De Rensis *et al.,* 2005). Moreover, sexed frozen semen used in this study might also be accounted for reduced pregnancy rate.

Using the semen with a high number of X chromosome cells is of a deep interest for the farmers raising milk cows, due to the fact conception products are mostly females. Though most of the reproductive management techniques in cattle can be successfully applied to water buffalo because of the similarities in the anatomy, physiology, and endocrinology of reproduction (Drost, 2007), the fertility rate in buffaloes inseminated with frozen semen is generally lower than in cows (Andrabi *et al*., 2008). The impairment and cell degradation suffered by spermatozoa during the technologic flow with high physical forces for separation of X and Y chromosome bearing spermatozoa is considered an important cause for the poor fertility (Fetrow, 2007).

Most synchronization programs applied to buffalo allow AI without oestrous detection, because of the relatively low intensity of estrous behavior in buffaloes (Ohashi, 1994). In this study, all buffaloes were examined with trans-rectal ultrasonography (for follicular size and number) along with VEI impedance and infrared thermography of vulvar and perivulvar region (immediately before AI) to assess oestrous status. Moreover, buffaloes with a tonic uterus with the presence of mucous vaginal discharge (when palpated per rectum during AI) were considered as in oestrous. Ultrasonographic imaging has afforded the ability to characterize dynamic physiologic events as they occur and has been intensively applied in the study of ovarian function (Adams and Pierson, 1995). The ideal time of treatment can be established by determining ovarian activity by ultrasound (De-Rensis and Lopez-

Gatius, 2007). Along with transrectal ultrasonography, vaginal electrical resistance (VER) or VEI can be used successfully to predict the stage of oestrous cycle, ovarian status and ovulation (Gupta and Purohit, 2001). We observed that VEI values were significantly ($P<0.05$) higher in PM subgroups in both pregnant and non pregnant buffaloes (Table 3.1.) and VEI values decreased during administration of 2nd dose of GnRH when buffaloes were supposed to be in oestrous (Figure 3.3). Schams *et al.* (1977) also reported that vaginal impedance declined markedly at oestrous in cattle and this could be triggered by estradiol (Heckman *et al.,* 1979). Ovarian follicular growth and corpus luteum formation and regression are associated with histological and histochemical changes in the vagina that are accompanied by alterations in its passive electrical properties (Řezáč, 2008). Study showed that changes in vaginal impedance had a closer relationship to the timing of ovulation than to the timing of oestrous (Smith *et al.,* 1989) and AI at a low VEI distinctly improved the conception rate in buffaloes (Gupta and Purohit, 2001).

Infrared thermography was used to measure the heat emitted from superficial capillaries in vulva and perivulva (referred as vulvar and perivulvar temperatures) in response to hormonal effect during Ovsynch protocol. Thermographic results showed that there were significant differences in T and δT values in response to time and type of treatment. T values were higher during 2nd GnRH administration in pregnant buffaloes in both F and R groups (Figure 3.4.). T values were significantly higher in both pregnant and non-pregnant buffaloes when protocol started at evening (PM) (Table 3.2). Whereas, higher δT values were observed in buffaloes of AM subgroups irrespective of treatment (Figure 3.5.). Unfortunately, we are unaware of data regarding relation of pregnancy rate with T values obtained by thermography. There are very few studies where infrared thermography has been successfully used to monitor oestrous in animals (Stelletta *et al*., 2006; Calabria *et al.,* 2010). Studies in ruminants demonstrate that vulvar temperature reflects circadian variation of body temperature and fluctuation of body temperature might be resulted from different physiological activities of body in response to different hormone's action occurred during oestrous (Piccione *et al.,* 2003).

Results from this study will help to devise better strategies for oestrous synchronization and should lead to optimum timing of insemination and improved fertility in buffaloes, especially when sexed frozen semen is used. Variation in pregnancy rates using to GnRH analogues demands more precise study to know the ultimate effect of different exogenous GnRH on LH surge, ovulation induction in buffaloes. It is necessary to use large number of buffaloes to verify the results obtained from administering hormones at different times of day and temperature variation within a day might be accounted for different hormones.

The efficiency of AI largely depends on the proper detection of oestrous as well as ovulation. For proper monitoring of oestrous and time of ovulation vaginal electrical impedance and vulvar temperature could be recorded. However, study of relationship between values of VEI and T values and pregnancy may assist in detecting oestrous as well ovulation for AI in buffaloes where detection of oestrous behavior is difficult or oestrous signs are inconclusive (Gupta and Purohit, 2001). To improve buffalo production during non-breeding season, it is very important to optimize hormonal treatment. Moreover, the use of sexed spermatozoa at high priority together with more efficient synchronization protocols for AI, could revolutionize the dairy buffalo industry.

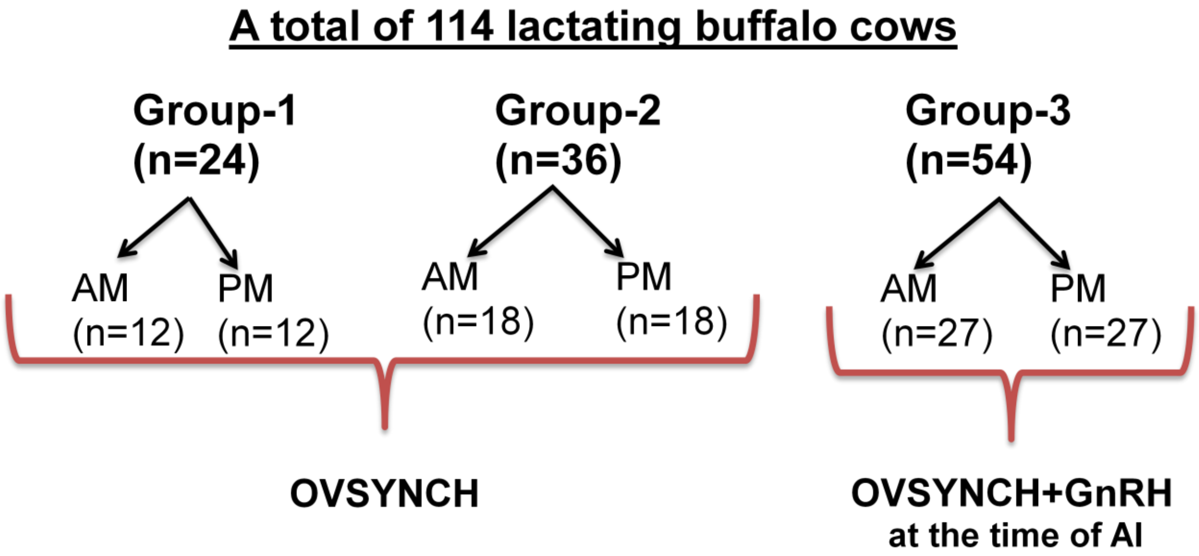
# CHAPTER 4. EXPERIMENT 2: EFFECT OF OVSYNCH PROTOCOL APPLIED IN DIFFERENT TIMES OF DAY ON THE FERTILITY OF INDIGENOUS BANGLADESHI BUFFALOES INSEMINATED WITH FROZEN SEMEN OF ITALIAN MEDITERRANEAN BUFFALOES

## 4.1. MATERIALS AND METHODS

### 4.1.1. Animals selection and management

A total of 114 lactating buffalo cows were used to study the effect of OVSYNCH protocols on the fertility. Buffaloes were selected from two different areas in Bangladesh and were divided into three groups. Buffaloes of each group were subdivided as AM and PM according to time of administration of hormones of synchronization protocols (Figure 4.1). All non-pregnant indigenous river type buffaloes were selected on the basis of presence of two or more follicles of ≥5 mm in diameter examined by ultrasonography. The buffaloes were between 4 and 8 years of age and their postpartum period ranged from 90 to 410 days at the beginning of the experiment. The body condition score (BCS) of the buffalo cows varied from 3.0 to 4.0 (1.0=very thin to 5.0=very fat). The parity of the buffaloes ranged from 1 to 4. They were milked once a day daily with their calves used for stimulating milk let down. Calves survived on residual milk after the hand milking. Weaning was not controlled in the buffaloes. Vitamin and Mineral premixes (Renavit DB Plus, Animal Health Division, Renata Limited, Dhaka, Bangladesh and DB-Vitamin, Square Pharmaceuticals Limited, Agro Vet Division, Dhaka, Bangladesh) were also supplied to the buffaloes with concentrate mixture. All the selected buffaloes were dewormed by using Triclabendazole 900 mg and levamisole 600 mg/tablet (Tablet Renadex, Animal Health Division, Renata Limited, Dhaka, Bangladesh) 7 days before beginning of the experiment. The buffaloes used were free from any apparent anatomical, physiological or reproductive disorders.

Figure 4.1. Buffaloes used for the Experiment 2.



In group-1, 24 buffalo cows were selected from 7 small holding buffalo farms situated in Kanihari village of Trishal upazila, Mymensingh, Bangladesh and synchronized during September 2011 to March 2012 (Figure 4.2.). Management of these animals was nearly similar. The buffaloes were reared under open yard rearing system and were fed on rice straw, cut-and-carry grass with grazing on roadside and community land. The animals were housed in collective stall during the night, and they were released extensively during the day for free grazing in the surrounding backyards (Figure 4.3 a). The animals had free access to water.

Buffaloes in group-2 (n=36) and group-3 (n=54) were selected from a private farm “Lal Teer Animal breeding House”, Lal Teer Livestock Limited situated at Tangail, Bangladesh (Figure 4.2). This is the only private buffalo farm in Bangladesh. The buffaloes were kept under loose housing conditions in clean, hygienic paddocks with brick flooring, asbestos roofing, and sufficient space for the free movement of the animals (Figure 4.3.b). All buffaloes were fed a ration consisting of concentrates (maize grain, mustard cake, molasses), rice straw, a mineral mixture, and salt. Fresh tap water was available ad libitum. The animals were fed according to their body weight and milk production.

Figure 4.2. Localization of the farms used for the Experiment 2.

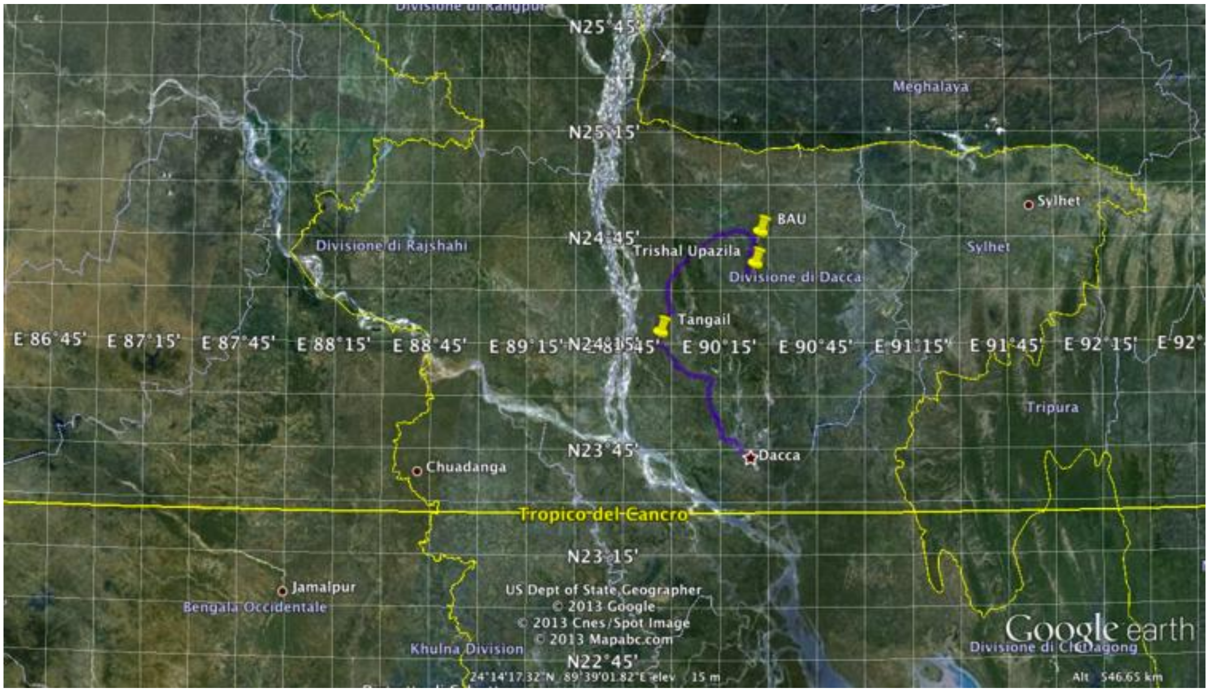
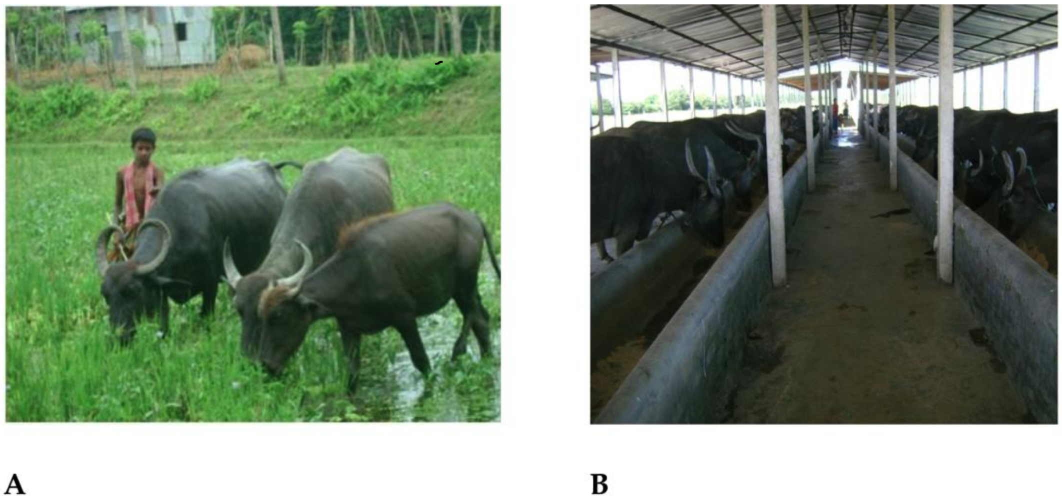


Figure 4.3. Open yard rearing system of buffaloes (A) and a private farm "Lal Teer Animal breeding House", Lal Teer Livestock Limited(B).



### 4.1.2. Synchronizaion and AI

The Ovsynch protocol was administered regardless of the stage of oestrous cycle in animals. For AM subgroup, each animal was treated with hormones at 7.00 AM morning and for PM subgroup, hormone was administered at 5.00 PM afternoon. For oestrous induction, in group-1 (Figure 4.4.); 500mg GnRH analogue-Gonadorelin (5ml Gonadon inj., Dong Bang Co.,Ltd., South Korea ) at $D^0$ , 500µg PGF2α-Cloprostenol (5ml Dinorin inj., Dong Bang Co.,Ltd., South Korea) at $D^7$ , 250mg of 2nd GnRH (2.5ml Gonadon inj.) at $D^9$ , in group-2 (Figure 4.5.); at $D^0$ 500mg GnRH analogue-Gonadorelin (5ml Ovurelin inj., Bomac Laboratories Ltd., New Zealand), 500µg PGF2α-Cloprostenol (2ml Ovuprost inj., Bomac Laboratories Ltd., New Zealand) at $D^7$ , 250mg of 2nd GnRH (2.5ml Ovurelin inj.) at $D^9$ and in group-3 (Figure 4.6); at $D^0$ 500mg GnRH analogue-Gonadorelin (5ml Ovurelin), 500µg PGF2α-Cloprostenol ( 2ml Ovuprost) at $D^7$ , 250mg of 2nd GnRH (2.5ml Ovurelin) at $D^9$ were injected intramuscularly in hip region. At 20-22 hours after second gonadorelin injection, AI of buffaloes was carried out by a trained AI technician using frozen semen from a Italian Mediterranean bull of known high fertility. Buffaloes in Group-3 were administered i.m route in hip region another third dose of GnRH (0.5ml Ovurelin inj.) at the time of AI (Figure 4.5.). The frozen semen was imported from Italy (COFA, Agricultural Cooperative Society, Orezola – 26048, Tidolo sigh of Cremona, Italy). Post-thaw sperm motility was 50-60%. Fixed time AI was done without considering the onset of prominent oestrous signs. However, some oestrous signs were observed such as, reddish vestibules, swollen vulva with marked edema in vulva lips and clear mucus hanging from the vulva (Figure 4.6. A and B).

Figure 4.4. Schedule of synchronization protocols administered at morning (AM) and afternoon (PM), ultrasonogrhy monitoring (US) and AI in buffaloes of group-1 and group-2.

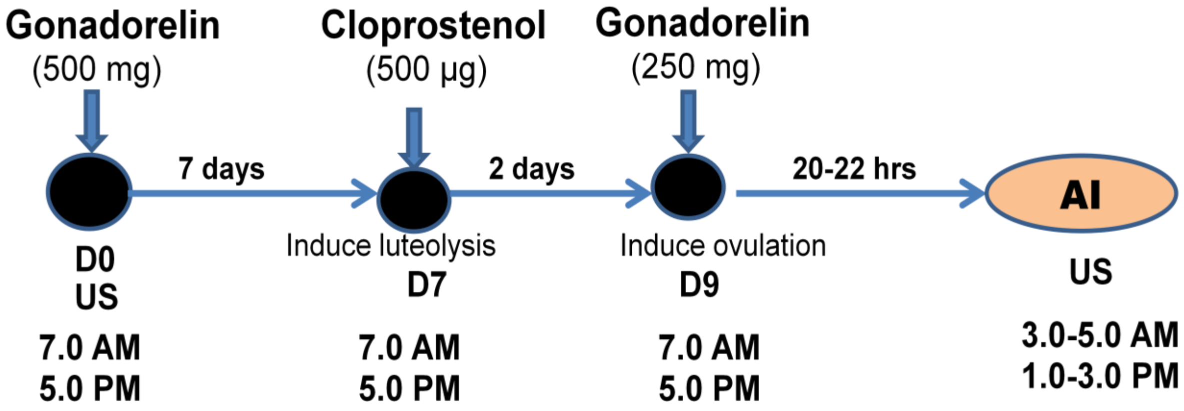


Figure 4.5. Schedule of synchronization protocols administered at morning (AM) and afternoon (PM), ultrasonography monitoring (US) and AI in buffaloes of group-3

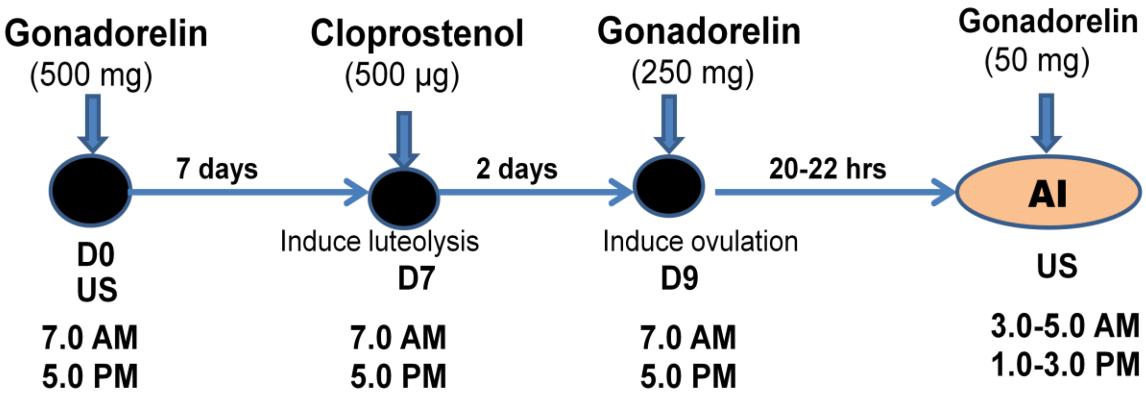


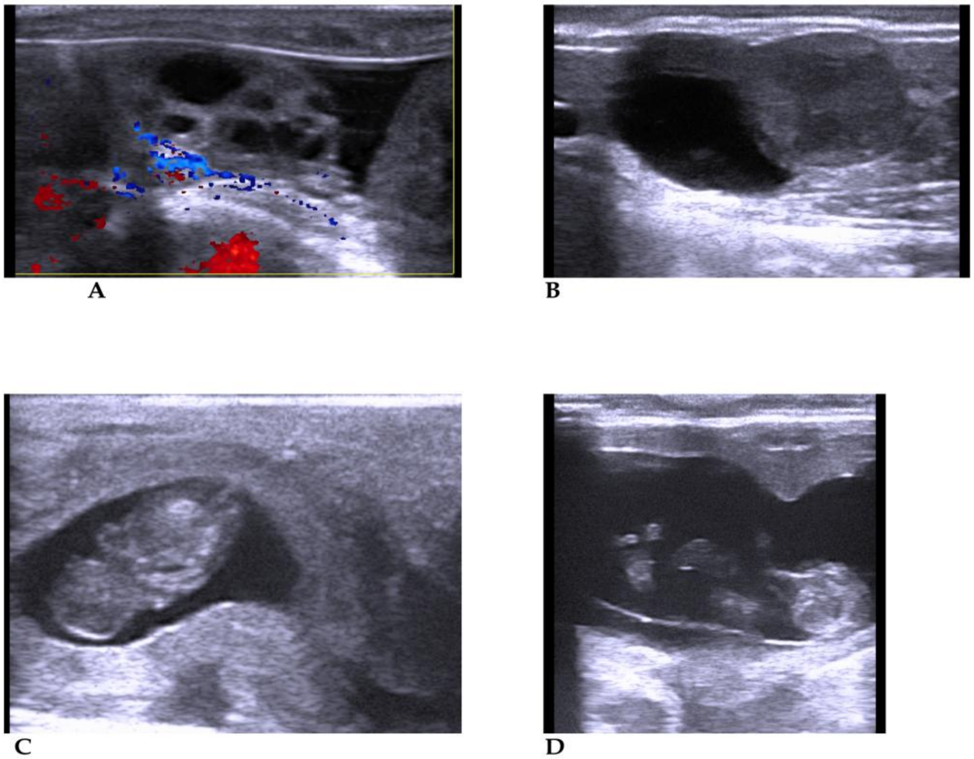
Figure 4.6. Reddish vestibules, swollen vulva with marked edema in vulva lips (A) and clear mucus hanging from the vulva (B).

**A** **B**

### 4.1.3. Ultrasonographic examinations

Real-time B-mode ultrasound (Tringa Linear VET®, Esaote Pie Medical, Genova, Italy) equipped with a 7.5 MHz linear-array rectal transducer was used to perform examination of the ovaries (Follicle and CL size). All animals were examined by the same operator. Number of follicles and corpus luteum were recorded (Figure 4.7. A and B). Ultrasonography was performed during the first administration of GnRH and just before AI. Pregnancy diagnosis were performed by observing embryos with transrectal ultrasonography at Day 42 after insemination (Figure 4.7. C&D)

Figure 4.7. Ultrasonogaphic monitoring presence of small follicles (A), corpus luteum (CL) and dominant follicle (FL) (B) at the time of AI, embryos of 42 days (C & D).



### 4.1.4. Statistical analysis

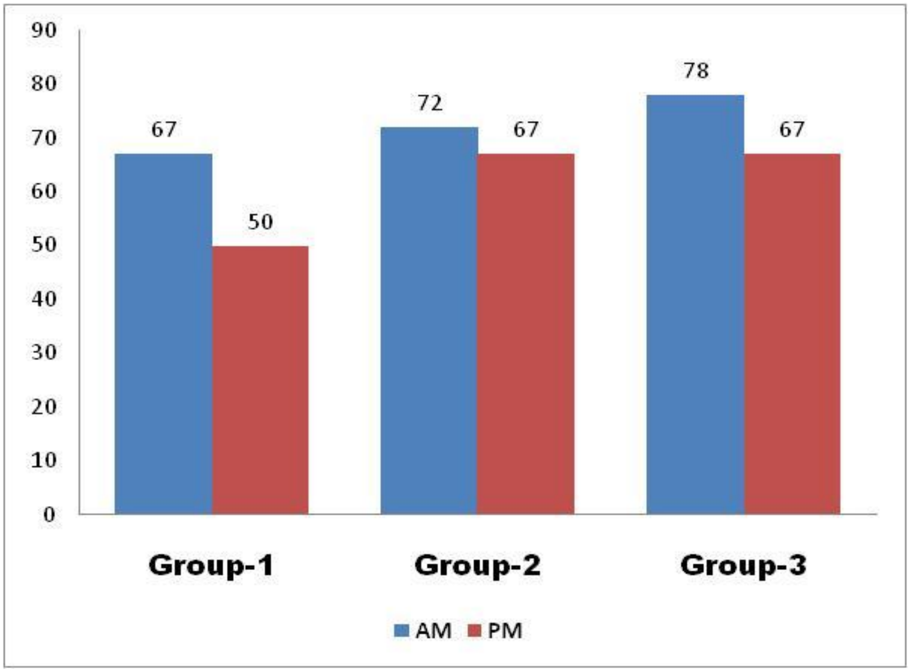
We used Chi square test to analyze binomials variables (farm, time of protocol) and pregnancy rate (Steel and Torrie, 1991). ANOVA was used to evaluate continuos variables (focllicle and corpus luteum number) for repeated measure. All data were analyzed in statistical software SIGMASTAT 3.50. Differences were considered significant at $P < 0.05$.

## 4.2. RESULTS

This study was carried out to study the efficiency of Ovsynch protocol in Bangladeshi Indigenous water buffaloes and fertility after AI with frozen semen of Italian Mediterranean buffaloes

Differences in pregnancy rates in three groups are shown in Figure 4.8. Pregnancy rate was higher (78%) in Group-3 when Ovsynch protocol started at morning and was accompanied with a third GnRH injection at the time AI. This result revealed that a third injection of GnRH during AI could help to improve pregnancy rate of water buffaloes when inseminated with frozen-thawed semen of Italian Mediterranean buffaloes.

Figure 4.8. Effects of time of initiation synchronization protocol on pregnancy rate in buffaloes of different groups.



The effect of some factors such as follicles (FL) and corpus luteum (CL) numbers, postpartum period on the fertility of Bangladeshi water buffaloes inseminated with frozen-thawed semen was also studied and pregnancy rates obtained in response to these factors are given in Table 4.1.

Table 4.1. Effects of time, post-partum days and presence or absence of CL at the beginning of ovulation induction on pregnancy rate in buffaloes.

| Factors | Number of AI | Pregnancy rate (%) |
|---|---|---|
| Time | AM | $74^a$ |
| | PM | $63^b$ |
| Post-Partum days | 90-180 d | $77^a$ |
| | >180d | $66^b$ |
| CL | With CL | 59 |
| | Without CL | 57 |

a.b superscript indicate significant difference (P>0.5) in the factors.

Effects of time of ovulation induction and insemination on pregnancy rate of Bangladeshi river type buffaloes are given in Table 4.3. When compared, pregnancy rate was higher (74 %) in buffaloes received induction treatment and insemination at AM time than that of PM counterpart (63%). Regarding the post–partum days, pregnancy rate was higher (77%) in buffaloes received induction treatment at 90 to 180 days between calving to ovulation induction than that of more than 180 days (66%). We did not observe any significant difference in pregnancy rate in buffaloes having CL (59%) and buffaloes having no CL (57.1%) at the time of synchronization.

We monitored ovarian follicular status, number of follicles (FL) and corpus luteum (CL) with transrectal ultrasonography during first administration of GnRH and during AI. After analyzing data regarding number of FL and CL in all buffaloes (n=114) used in this study, we did not found any significant difference in regards to follicles and CL numbers in both pregnant and non-pregnant buffaloes (Table 4.2.).

Table 4.2. Comparison between follicular and corpus luteum number at the time of selection (Mean±SEM) between pregnant and non-pregnant buffaloes of three groups.

| **Groups** | **Buffalo** | **Left Ovary** | | **Right Ovary** | |
|---|---|---|---|---|---|
| | | Number of Follicle | Number of Corpus luteum | Number of Follicle | Number of Corpus luteum |
| 1 | Pregnant | 3.46±0.37 | 1.00±0.11 | 3.15±0.42 | 1.00±0.13 |
| | Non-pregnant | 3.00±0.43 | 1.25±0.14 | 2.50±0.47 | 1.00±0.16 |
| 2 | Pregnant | 3.38±0.27 | 1.20±0.13 | 2.58±0.31 | 1.09±0.08 |
| | Non-pregnant | 3.00±0.41 | 1.00±0.14 | 2.80±0.47 | 1.00±0.28 |
| 3 | Pregnant | 3.82±0.22 | 1.00±0.17 | 3.95±0.24 | 1.33±0.16 |
| | Non-pregnant | 3.27±0.35 | 1.00±0.20 | 3.33±0.39 | 1.00±0.16 |

However, the time of day should be considered during administration of hormones for Ovsynch protocols. The findings of the study suggest that Ovsynch protocol can be successfully used for selective breeding program of Bangladeshi Water buffaloes.

### 4.3. DISCUSSION

The reproductive function of buffaloes is a major factor determining the economic significance of this animal species (Barile, 2005). It is influenced by the late onset of sexual maturity (Jainudeen & Hafez, 2000; Mondal & Prakash, 2004), high percentage of animals with silent oestrous (Barile, 2005), the longer period between calving and the reduced ovarian activity during the hot months of the year (Singh *et al.*, 2000; De Rensis & Lopez-Gatius, 2007; Dimitrov *et al.*, 2009). The different duration of oestrous (4 to 64 h) impedes the exact detection of ovulation and results in limited application of AI in buffaloes (Neglia *et al.*, 2003). That is why, in buffaloes, oestrous induction and a fixed-timed AI is regarded as more efficient approach (Presicce *et al.*, 2005).

The present study demonstrated that 50-78% of buffaloes become pregnant when oestrous was induced by using Ovsynch protocol and AI using frozen semen. The conception rate obtained in this study is considered high when compared to results of other experiments that ranged from 27.2 to 42.4% (Irikura *et al.*, 2003; Neglia *et al.*, 2003; Paul and Prakash, 2005). However, the obtained pregnancy rate of the present study is consistent with the earlier study conducted by Berber *et al*. (2002) who reported 56.5% pregnancy rate in buffaloes. The reason for difference in pregnancy rates among studies may be due to differences in breed of buffaloes, ovulation induction protocols and agro-climatic conditions of study areas in different studies. Presence or absence of corpus luteum (CL) at the beginning of ovulation induction did not affect the pregnancy rate in Bangladeshi river type buffaloes as indicated by insignificant difference in pregnancy rates between buffaloes received induction treatment in presence or absence of CL.

Diaz *et al.* (1994) stated that cows having palpable CL had higher conception rate (67%) than that of cows having no palpable CL (43%). Higher incidence of anoestrous due to inactive ovaries in buffaloes than in cows has been reported by

Tanwar *et al.* (2003). Sometimes the anoestrous is prolonged due to sudden climatic variation such as a fall in temperature, exposure to cold wind, heavy rain associated with low temperature or hot weather without any possibility of bathing or sheltering from the sun (Zicarelli, 1997). Moreover, it is likely that suckling significantly increases the interval from parturition to first postpartum oestrous in buffaloes. The extension of anoestrous period in buffaloes due to calf suckling is also reported elsewhere (Usmani *et al.,* 1990). Silent oestrous is a common problem in buffaloes even under good management and non-stressful periods of the year (Kamboj and Prakash, 1993; Abdalla, 2003).

In this study, ovsynch treatment induced oestrous symptoms of variable intensity in all buffaloes. We observed higher pregnancy rate (74%) in Bangladeshi river type buffaloes received induction treatment and insemination at morning. We are unaware of data regarding similar to this type of finding. Srivastava and Sahni (2003), who reported that buffaloes showed more oestrous activity in the morning (06:00–07:30 hour) than in the afternoon (14:00– 15:30 hour) or during the night (22:00–23:30 hour).

The ovsynch protocol for synchronization of the ovulation in buffaloes has been tested during different seasons (Paul and Prakash, 2005; Carvalho *et al.*, 2007; Warriach *et al.,* 2008). Different studies indicated 33.3-46.8%, pregnancy rate during breeding season in water buffaloes (Paul and Prakash, 2005; Carvalho *et al.,* 2007). Baruselli *et al*. (2003) reported a considerable difference in pregnancy rates between seasons of oestrous induction in buffaloes (breeding vs. non-breeding season: 48.8% and 6.9%). When compared, Warriach *et al*. (2008) obtained no difference in pregnancy rates in buffaloes during breeding (36.3%) and non-breeding season (30.0%). In the present study, all buffaloes were treated for ovulation induction in the period of transition to breeding season. However, the lower temperature and humidity at morning than that of evening during ovulation induction treatment and insemination might be a favorable condition for obtaining higher pregnancy rate in AM group than that of PM counterpart.

The present study demonstrated that the induction of oestrous at early post partum interval (90 to 180 days) in Bangladeshi river type buffaloes improve the pregnancy rate. The postpartum period is designed to allow involution of the cervix and uterus after calving. The reproductive tract typically returns to normal size by 35 d after calving, while the uterus takes approximately 45 days to return to normal dimension. Harrison *et al*. (1990) have stated that high milk production is antagonistic to the expression of oestrous behavior.

Taylor *et al*. (2004) have stated that negative relationship between milk yield and return to cyclicity was stronger in the multiparous cows than in the primiparous cows. We did not consider parity in our study. Researches in cattle suggest that the burning of fatty acids due to negative energy balance in under-fed or high production animals causes a release of some amount of progesterone, found in fat tissues, which in turn inhibits follicular growth and signs of oestrous. In addition, many cows do not receive their first postpartum Al until after 100 days in milking (DIM) (Fricke, 2005). Several studies show that 20 to 30% of lactating cows do not become cyclic by 60 DIM (Pursley *et al*., 2001; Gumen *et al*., 2003). Early application of the program during the post-partum period may be another possible cause of lowering conception rates in buffalo cows (Derar *et al*., 2012). It is hypothesized that the capability of the pituitary gland to respond to exogenous GnRH is restored by Day 20 postpartum in dairy buffaloes (Palta and Madan, 1995).

Real-time ultrasonography has gained tremendous popularity in recent years as a diagnostic as well as a research tool in veterinary and animal science. As a diagnostic aid, ultrasonography is well suited for bovine practice, particularly for the examination of reproductive organs (Rajamahendran *et al*., 1994). The technique is noninvasive, relatively simple and effective, safe to both the subject and the operator, portable and ultrarapid, and it facilitates immediate interpretation and diagnosis in most circumstances. Accordingly, applications of ultrasound in research on cattle (Akter, 2008; Rahman, 2010) and buffalo reproduction have been documented in Bangladesh (Hoque, 2009).

The time to ovulation after GnRH injection depends mainly on the diameter of the largest follicle at the time of injection (Hussein *et al*., 2002; Hussein, 2003) and it is a determining factor for the successful synchronization of ovulation and high conception rates (De Rensis *et al*., 2005). In a recent study, Campanile *et al*. (2008) have stated similar size of follicles in ovulated and non-ovulated buffaloes. However, the stage of follicular development (growth or regression phase) greatly affects its response to GnRH treatment (Dharani *et al*., 2010). The existence of a wave-pattern of follicular dynamics in buffaloes and understanding the process recruitment, development, atresia and temporal pattern follicle selection, dominance, subordinate follicle suppression, follicle numbers, and, preovulatory changes of follicular dynamics temporal relationships among follicles, all are that have direct impact on the design of, and response to, synchronization and superstimulation protocols (Singh *et al.,* 2000). Therefore, understanding and investigating follicular ''waves'' as a functional unit, rather than the estrous cycle, could help' in the proper synchronization protocol for the improvement of reproductive efficiency in river buffaloes. A better understanding of follicular wave dynamics could facilitate the development of a methodology for influencing ovarian function and oestrous in both cyclic and non-cyclic animals.

For efficient and economic reproductive management constraints that limit the achievement of reproductive goals should be eliminated. Good nutrition management in both pre- and post partum periods, efficient and accurate heat detection, prevention and treatment of reproductive and metabolic diseases would be considered helpful for better reproduction. Recommended AI management should be done accordingly. Hormonal treatments under various protocols will improve the reproductive performances and thus the reproductive goals can be achieved.

# CHAPTER 5. CONCLUSIONS

Reproductive management and selection for fertility have been used less for water buffalo than for cattle. Poor expression of estrous behavior is the primary factor responsible for low reproductive efficiency. Difficulties in oestrous detection can be ameliorated by the use of teaser animals. In addition, artificial control of the oestrous cycle has provided an efficient means of increasing reproductive efficiency.

This study concluded that significant differences ($P < 0.05$) existed between pregnancy rates regarding to type and time of treatment when Ovsynch protocol was performed with two different GnRH analogues. Vaginal electrical impedence (VEI) and vulvar temperature (T) recorded by Infrared-thermography could be used for proper monitoring of oestrous and time of ovulation. VEI values decreased and T values increased during administration of 2nd dose of GnRH when buffaloes were supposed to be in oestrous. Higher pregnancy rate observed river type buffaloes received induction treatment and insemination at morning. Induction of oestrous at early post partum interval improves the pregnancy rate in Bangladeshi river type buffaloes inseminated with frozen-thawed semen.

Results of the study confirm the beneficial effect of Ovsynch treatment. The present study has shown that Ovsynch achieve good synchronization of oestrous in buffaloes. Treatment with Ovsynch is associated with a higher pregnancy rate in both Italian and Bangladeshi buffaloes inseminated with frozen semen. Therefore, this study clearly indicates the opportunity for practical application of the Ovsynch protocol for TAI in buffaloes.

However, further studies are needed in order to better understand the time of ovulation from the second-GnRH for increasing the efficiency of AI in buffaloes. It is suggested that this is an important frontier for discovery that will lead to the next major advance in technology to improve reproductive performance in buffaloes. It will be important to further elucidate the factors that can contribute to increase pregnancy in buffalo cows so that strategies can be developed to optimize fertility to synchronization and AI during periods of reduced reproductive activity.

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# APPENDIX I – LIST OF FIGURES

# APPENDIX II – LIST OF TABLES

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