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XXV CICLO

Timed artificial insemination in the reproductive management of buffalo(Bubalus bubalis) herds.

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1. INTRODUCTION

The Artificial Insemination (AI) can be considered the very first applied reproductive technology, and it was rudimentarily attempted for the first time in 1779 by L. Spallanzani in the dog. That early attempt consisted in the collection of the semen from animals of high genetic merit and its dilution and utilization in more females. The application of this technique is worldwide carried out in several animal species as well as in humans. In particular, in the field of animal science it allows healthy, genetic and economic advantages.

From a sanitary point of view it avoids the transmission of some venereal infectious diseases, such as *vibrio* and *trichomonas*, that are usually spread through natural mating. Furthermore, this technique avoids the transmission and reduces the possibilities of diffusion of other infectious diseases, since the males that are selected for AI undergone severe sanitary controls to verify that they have been in contact with agents, such as brucellosis, leucosis, tuberculosis and IBR. This aspect is particularly important, especially in some Countries were such pathologies are endemic. It is worth pointing out that some pathogens can be transmitted in semen through AI, but the collection process allows for the screening of disease agents.

From a genetic point of view, AI allows for evaluation of sires through progeny testing of daughters in production, whose productive, morphological and reproductive characteristics can be tested. It has been estimated that one bull can mate 20 females in one month by natural insemination and its reproductive activity lasts approximately 12 years: hence, if all the mating yielded in a pregnancy, it has been calculated that one bull can produce less than 3,000 calves in its lifetime. By using AI, one bull can produce up to 200,000 calves, taking into consideration the number of straws that can be obtained from a single ejaculate following dilution and freezing. Further advantages derived from the use of frozen/thawed semen through AI are: i) the availability of semen even after death of proven sires, and ii) the ease of moving semen to promote genetic improvement in Countries where the livestock population is not very productive. Another important aspect related to AI is the customization of the best sire to the recipient characteristics. This allows correction of some morphological and productive defective traits of recipients that will not be carried over to their progeny. Furthermore, AI can be a flexible tool to obtain calves with different characteristics, following insemination of recipients with semen from different sires.

The economic advantage linked to the utilization of AI is mainly due to a better management of the farm. Bulls are expensive to rear, relatively unproductive, vulnerable to disease or accident and may even prove to be infertile. In addition, they are characterized by higher feed requirement and need special housing and handling equipment. By using AI, farmers can save on space otherwise used by bulls, for a better farm management, and erase the possibility of any dangerous situation linked to their aggressiveness.

We have to consider though, some possible disadvantages derived from the application of AI. In fact, a long time is needed in order to prove sires through progeny testing, i.e. testing of their daughters for milk production. In the buffalo species, this process requires at least four to five years, since: i) a little over 10 months is required for pregnancy, ii) at least other 20 months are needed to the progeny to reach puberty, and iii) additional 10 months are required for the subsequent pregnancy of the progeny. Hence, at the end of the first lactation, data will be analyzed, and it is possible that after all this time a bull may show negative effects on milk production and therefore its use would be no longer adopted. Furthermore, the diffusion and use of few proven sires across many farms may cause, in the long run, a considerably increase of the phenomenon of consanguinity and inbreeding. For this reason specific breeding programs must be implemented.

Although buffaloes are probably among the easiest mammals to be trained to serve an artificial vagina, natural mating is still responsible for most of the pregnancies occurring in buffalo herds. In fact, the use of AI in both Swamp and River buffaloes is still marginal for several reasons. The implementation of AI in Swamp buffaloes has its drawbacks in the low number of heads per owner, lack of proper heat detection and the overall poor management. On the contrary, in intensive River buffalo breeding, the use of AI has been kept marginal until a decade ago, due mainly to lack of truly superior progeny tested bulls and to unacceptable low pregnancy rates when using both natural or synchronized oestrus (Zicarelli et al. 1997). Recently though, an improvement of the efficiency in protocols for synchronization of estrus and ovulation has been reported in buffaloes (Neglia et al., 2008), paving the road for a wider implementation of assisted reproduction in this species. Buffalo bulls can breed throughout the year, although seasonal fluctuation in reproductive function is witnessed. In fact, semen quality is also affected together with testosterone blood concentration, being poorer and lower during summer than in winter, and such aspect is reflected on its freezeability, reporting more fragile spermatozoa to withstand freezing protocols in the summer season (Bahga et al., 1991; Sansone et al., 2000). A series of aspects need to be considered and further investigated then, in order to spread the diffusion of AI in the buffalo management worldwide for a faster enhancement of the genetic potential and its distinctive production.

2. BUFFALO INDUSTRY IN ITALY AND IN THE WORLD

The importance and competitiveness of buffalo breeding in Italy, compared with the other more established forms of livestock rearing, is demonstrated by the increase in the National population (Italy). The increasing demand for buffalo milk and the need to cut production costs make planning to improve milk production essential to farming. In this scenario reproductive biotechnologies are extremely important, allowing to plan selective programs and their accomplishment in a shorter time. Furthermore, the use of these technologies is crucially important to provide for genetic requests from developing Countries. These requests are aimed to crossbreed the local working buffalos with the high productive Mediterranean buffalo, in order to increase animal proteins and meet human needs. In this sense the AI represents the simplest reproductive technology that can be improved.

3. BUFFALO FEMALE GENITALIA

The knowledge of differential anatomic features among species is of paramount importance to the employment of reproductive techniques like AI and embryo transfer. A comparison on the biometry of the genital system among species and different breeds has to take into consideration size and body weight (Singh & Singh, 1985), body condition score, physiological status, environmental conditions, season and management practice of the animals subjected to comparison, together with the intrinsic individual variability. Danell (1987) also reported a positive correlation between the weight of the genital organs and the body and weight of the animals themselves. Nevertheless, it can be stated that the morphology of the reproductive tract of the water buffalo (Bubalus bubalis) is quite similar to that of domestic cattle (Bos taurus and Bos indicus). Following is a representation of the various organs comprising the non pregnant reproductive tract of the river buffalo in comparison to swamp buffalo and to cattle breeds.

3.1. Vulva and vagina

The vulva in the buffalo has a similar length to cattle, highly pigmented and characterized by Pacini, Ruffini and Meissner corpuscles together with other featuring fibers firstly described by Cecio & Pelagalli (1958). Within the vestibulum, the position of the urethral opening constitutes an anatomical feature in the buffalo, characterized by a papillary structure of approximately 1 cm in length and situated at the boundary between vagina and vestibulum (Vittoria, 1997). The length of the vagina in the Mediterranean Italian adult buffaloes is similar to Murrah breed (~29 cm) but different from Swamp buffaloes (~ 20 cm).

3.2. Cervix, uterus and oviducts

The cervix has also a different size between Mediterranean and Swamp buffaloes (~ 5-6 cm length and ~3 cm width) and cattle (~8-10 cm length and ~6 cm width). In addition, usually buffalo cervix has at least one cervical ring less (~3) when compared to cattle, which may account for a reduced dilation during parturition and estrus (Asdell, 1955; El-Fouly, 1983; Lohachit et al., 1981). The body of the uterus is also different in length among Indian and Mediterranean buffaloes (~2-4), cattle (~4 cm) and Swamp buffaloes (~1-3 cm). The tubular genitalia in buffaloes, are slightly smaller and lighter, but at the same time more tonic, muscular and firmer, and the uterine horns are more coiled (Sane et al., 1964). In Swamp buffaloes, average length of both horns is around 25 to 28 cm, whereas both Indian and Mediterranean breeds approximate the length in cattle (~34 to 38 cm). Oviducts are shorter in swamp buffaloes (~17-20 cm) when compared to Indian and Mediterranean breeds and to cattle (~25 cm).

3.3. Ovaries

The first available information on prenatal development of the buffalo ovary is by Ghannam & Deeb (1967, 1969), reporting differentiation of the primordial gonads as occurring at a crown-rump length of 7 mm, sex differentiation at 20 mm, and observation of the first primordial follicles at 600 mm and a follow up development until birth. With regard to fully developed gonads and as a general differential aspect, the buffalo ovaries are usually round in shape, whereas cattle ovaries are more elongated. The size and weight of ovaries in Indian and Mediterranean breeds are slighter reduced (~2-3 cm and 3-5 gr, respectively) when compared to cattle (~4 cm and 9 gr, respectively) (Razzaque et al., 2008). Therefore, ovaries in the buffalo species are smaller compared with their bovine counterparts and contain roughly a 10-fold lower number of primordial follicles (Danell, 1987). Due to their smaller size when compared to European cattle, palpation per rectum and the identification of the ovarian structures can be more challenging, especially for corpora lutea which are often found deeply embedded in the ovarian stroma (Vittoria, 1997), mostly as a consequence of a smaller ovarian size and volume. The ovulation papilla is also less prominent in the water buffalo, hence more difficult to palpate per rectum (Drost, 2007).

Figure 1. Corpus luteum of the water buffalo (left) and Bos indicus (right).

The smaller size of the buffalo corpus luteum (CL) when compared to cattle breeds makes its identification through palpation per rectum more difficult (Figure 1). With regard to the size of ovaries and the implementation of reproductive technologies in buffaloes and in particular to MOET programs, the discouraging results following non surgical flushing of uterine horns may be related more to a difficulty to recover all the ovulated oocytes deriving from multiple ovulation, rather than an inadequacy of the buffalo to respond to superovulation protocols (Neglia et al., 2010a). This would occur if the ovaries become too large following superovulation, so that the oviduct’s fimbria cannot receive and hold all the ovulated oocytes (Shea et al.,1983). Furthermore, it has to be underlined that buffalo ovaries are characterized by fewer primordial and antral follicles, compared to cattle (Danell, 1987; Ty et al., 1989). The pool of primordial follicles in buffalo heifers is approximately one fifth (12,636 vs 50,000) compared with cattle (Danell, 1987). Furthermore, buffalo heifers also show a greater rate of follicular atresia (67%; Danell, 1987) relative to cattle (50%; Rajakoski, 1960). As a consequence of the intrinsic differences of the species, the number of follicles recruited into a follicular wave is lower in buffaloes than in cattle (Danell, 1987; Ty et al., 1989). Interestingly, the number of follicles recruited into the follicular waves is relatively constant for individual buffaloes (Baruselli et al., 1997a; Neglia et al., 2007), like in cattle (Boni et al., 1993). Given that the follicular response to ovarian superstimulation treatment appears to be related to the number of follicles available to be recruited at the start of treatment in cattle (Romero et al., 1991), it may be hypothesized that the heritability of non-atretic antral follicles may prove to be an important genetic parameter for selecting donors in assisted reproduction programs. In this regard, the follicular response to ovarian superstimulation remained relatively constant for individual buffaloes (Misra et al., 1991).

4. BUFFALO REPRODUCTIVE CHARACTERISTICS

Wild animals are generally seasonal, although this characteristic was gradually diminishing with domestication. However, it is still present in some genetic types of the bovine (Bos taurus taurus) species bred freely such as the Podolica, the Sarda, the Maremmana and the Highland bovine and in zebù (Bos taurus indicus). As regard the horse, sheep, goat and the buffalo itself reproductive seasonality has only been partially influenced by domestication and the change of breeding area. The selection and improved nutritive requirements have decoupled the dairy cow from strict reproductive seasonality. However, it does not mean that this is or will be just as easily achieved in other species. For example, the egg-laying hen must be conditioned by pre-established light programmes so as to ensure constant egg production throughout the year although there is a shorter interval between the generations - and hence more favourable for fixing traits over a shorter period of time - and that its nutritional requirements are satisfied better than it is currently possible in high-production dairy cow. A comparison between the two species raises serious questions from a physiological, ethological and genetic point of view. The place of origin and the duration of gestation influenced the reproductive seasonality. In fact, the natural necessity to coincide calving and weaning with the most suitable parts of season in order to satisfy the reproduction and nutritive requirements of the offspring through a period in which etiologic agents (infectious and parasites) are less aggressive and/or present (Zicarelli, 1997) represents one of the causes of this 'adaptation' process. Those subjects acquired under the most favourable conditions, have brought about natural selection of subjects endowed with a more ideal reproductive seasonality towards the survival of the species. Their reproductive characteristics have probably been determined by receptive stimulus of the central nervous system (CNS) during gestation or the early days of life.

4.1. Reproductive seasonality

The majority of the authors report that buffalo is characterized by delayed puberty, prolonged post-partum ovarian inactivity, long intercalving intervals and a tendency for seasonality (Madan, 1988; Misra and Tyagy, 2007).

Seasonal breeding is a survival strategy adopted by many wild animals to ensure that their progeny are born at the most favorable period of the year. This biological programming of births or synchronization of reproductive response to appropriate environmental conditions, clearly leads to distinct advantages for the offspring being born at the time of mildest weather and maximal food availability during the early part of offspring’s life (Wood et al., 2006). In tropical zones where the domestic buffalo developed (between 31°N and 2°S) forage availability is usually adequate after the rainy season (July through September). Therefore, in species like the domestic buffalo, with a pregnancy length of 310 to 316 days, the reproductive season takes place between September and January. Around the equatorial belt, where the light/dark ratio varies little throughout the year, the reproductive season is highly conditioned by forage availability (Vale et al. 1990; 1996).

Buffalo species shows a pattern of reproductive efficiency closely related to the environmental and climatic conditions of the area of origin, that is the Indo Valley (currently territory of Pakistan and India). Indeed, the buffalo is a short-day breeder but in equatorial zones can show oestrous cycles throughout the year provided that nutrition is adequate to maintain reproductive function (Seren et al., 1995). Buffaloes become increasingly seasonally polyoestrus with distance from the equator (Zicarelli, 1997; Campanile et al. 2010; Baruselli et al., 2001a) and 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, 2007).

The majority of authors attribute reproductive seasonality to nutritional factors. The breeding period in areas where 97% of the buffalo population is bred takes place in the months of greater forage availability (Roy et al.

1968; Singh, 1988; Singh & Lal 1992; Qureshi et al. 1999). Greater forage availability is found between July and November, months characterized by decreasing daylight length in tropical Countries at North of the equator. In southern Brazil (Da Silva & Grodzki 1991; Baruselli et al. 2001a) and Argentina, the wet season (and consequently, increased pasture availability) starts in October to November and continues until March to April. Pasture scarcity goes from May to June until October to November. The buffalo calving period under these conditions is mainly concentrated from February to May. The breeding period is from April to July and the calf weaning under free range and suckling calf conditions is from September to December. These events permit the coincidence of forage availability within the first 2 to 4 months of lactation and most of the dry period (October to April). The breeding period, however, is mainly concentrated during the pasture scarcity period (May to July).

Authors from India and Pakistan attribute the decline of reproductive activity that is observed in summer to the heat stress (Ahmad et al., 1980; Madan, 1988; Singh & Nanda, 1993). However, in Italy for out-of-season mated buffaloes, the conception rate increases between July and September, i.e. the warmest months of the year, in contrast to Holstein cows that show a low conception rate. In Italy, in order to meet the milk market demand, the out-of-breeding mating strategy (OBMS) is used, that entails the interruption of natural mating or the use of AI between October and late January in adult females and between September and late March in heifers. These months are the most-favorable periods for reproductive activity (Zicarelli 1994b; Zicarelli 1997; Campanile et al. 2009). In Italian herds where the OBMS technique is not used (Zicarelli et al. 1997), as well as 30 to 40 years ago on the majority of farms (Ferrara 1957), the resumption of the reproductive cycle occurs from September (decreasing light period) until January (light increasing period but predominantly dark hours). Sensitivity to the negative photoperiod is also found on farms where a constant balanced diet is provided year-round (Zicarelli 1994a). This type of seasonality, where reproductive events are not synchronized with forage availability, indicates that the buffaloes bred in Italy are not autochthonous in the sense that they will sometimes calve during periods of forage scarceness and low temperature, which in turn hinders the survival of the calf. Italian findings should be sufficient to define the buffalo as a short day breeder. Indeed, a similar seasonality to that found in Italy and Asian tropical areas is also found in Venezuela (Montiel, 2000) and Egypt (Zoheir et al., 2007). Therefore, while the annual cycle in rainfall, with the consequent cycles in food availability, are important variables in tropical regions, the daily photoperiod and the annual cycles in environmental temperature are the most striking examples in temperate regions (Vivien-

Roels and Pévet, 1983). In Italy (latitude between 47°05'29 N and 35°29'24 N), because of the advanced model of farming, in which forage is always available, buffalo is a seasonally polyestrus species, in which the increase of ovarian cyclic activity and, subsequently, fertility coincides with decreasing day-light hours. The fact that the reproductive efficiency improves with negative photoperiod demonstrates that moving into new areas of domestication and breeding has not modified the sensitivity of the hypothalamic hypophyseal axis to a decreasing light/dark ratio. Interestingly, in all Countries where buffalo is bred, despite differences in environmental temperature, rain fall and forage availability, the common feature is that reproductive activity benefits from a negative photoperiod (Zicarelli, 2007). The influence of photoperiod means that, without intervention, buffaloes have seasonal cycles in conception, calving and milk production (Campanile et al., 2010).

The need to make calving and weaning coincide with both the most suitable season, in order to satisfy the heat and nutritive requirements of the offspring, and with the period in which the causal agents of infections and infestations express less pathogenic effect (Zicarelli, 1994a), represents one of the causes which gave rise to this adaptation process. The individuals born in more favourable conditions have given rise to natural selection of subjects with a seasonal reproductive behaviour better suited to the survival of the species, whose reproductive features were probably determined by stimuli incorporated in the central nervous system during gestation or during the first days of life (Zicarelli 1997).

This reproductive pattern depends on the duration and the intensity of the light source, which is captured by the retina (retinal photoneurons) and then 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 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 estrus (Lincoln, 1992) via exerting a stimulating effect on GnRH secretion by the hypothalamus in short-day breeders.

Plasma melatonin concentrations two hours after sunset result higher in buffaloes that are more sensitive to photoperiod (Parmeggiani & Di Palo 1994). Furthermore, this feature does not change by moving these animals in another places, where other females show low plasma melatonin concentrations and less sensitivity towards light stimulation (Di Palo et al. 1997). The importance of this finding is related to the high repeatability (0.733) of plasma melatonin levels (Di Palo et al. 1993), suggesting that it is hereditable and that may be incorporated into genetic selection programmes for buffalo (Zicarelli 1994b). It has been observed that heifer fertility is not compromised by the season (Campanile et al. 1991; Avallone et al.1994). Interestingly, the differences between night and day concentrations of plasma melatonin in March were less marked in heifers (5.0 times) than in adult buffaloes (28.3 times) (Borghese et al. 1995). Both buffaloes that calve in spring – more adaptable to out-of-breeding-mating-strategy (Di Palo et al. 1993) – and the heifers – less sensitive to the photoperiod (Borghese et al. 1995; Zicarelli 1994b) – show the same behavioral pattern. During the summer and when daylight hours are more than dark hours, there is an increase in prolactin in the blood but, contrary to assertions by Madan (1988), buffaloes regularly conceive. It is likely that hyperprolactinemia is a consequence of the hypothyroidism (Campanile et al. 1994) during the warm months (Zicarelli 1994a; 1997).

Recently, it has been highlighted the effect of the season on follicular population and oocyte developmental competence. Although the follicular population and the number of oocytes recovered per ovary is not influenced, a significant increase of both cleavage (71.7 vs 58%) and blastocyst (26.5 vs 18.8%) rates is observed during autumn months compared to spring months, indicating a seasonal effect on oocyte developmental competence, that reflects the in vivo reproductive pattern (Di Francesco et al., 2011). This finding suggests that when planning ovum pick-up trial (Neglia et al., 2003a ; Neglia et al., 2011a) it is better to avoid oocyte collection during months with increasing daily light length to optimize benefits/costs ratio.

It is worth noting that the season may also affect male reproductive activity (see below). The effects are clear when the animals are maintained in free mounting condition, since a higher pregnancy rate is observed between January and April in buffaloes inseminated by AI compared with those naturally mated. This may occur because the AI avoids the negative effect of the bull. In fact, in April, only 23%, 31% and 29% of the bulls show testosterone, diihydrotestosterone and androstenedione values higher than the average value (Pelagalli et al. 2009).

4.2. Characteristics of the estrous cycle

In addition to seasonality, buffalo presents several peculiarities of the estrous cycle. The estrous cycle in buffaloes can vary from 16 to 28 days (Manik et al., 1994; Neglia et al., 2007; Baruselli et al., 1997a) and in buffaloes showing regular estrous cycles, the duration of oestrus is typically

10–20 h if animals are cycling during the breeding season (Gill et al., 1973; Rao et al., 1981; Vale et al., 1984). In females cycling during the non breeding season the duration of estrous is highly variable, from 2 to 72h (Zicarelli et al., 1988a; Baruselli, 2001). Irrespective of breeding or non-breeding season, buffaloes show less intense expression of estrous compared with cattle (Ohashi, 1994) and also less estrous-associated mounting behaviour (Roy & Prakash, 2009). In buffalo, in contrast to what happens in cattle, only 3.44% of the females present homosexual behavior (Baruselli, 1994). The lower intensity of estrous behavior, together with the wide variation in the duration of the estrous in buffalo (6 to 48 hours), make estrus detection more difficult and impair the use of AI in this species. Therefore, other techniques have been developed, such as the use of teaser bulls (Zicarelli et al., 1997), the radiotelemetry (Baruselli, 2001) and the use of pedometers (Di Palo et al., 2001). The radiotelemetry system (Heatwatch®; DDx Inc., Boulder, Colorado, USA) has been recently developed, and successfully used to detect estrus signs (Porto-Filho et al, 1999). The use of pedometers is largely applied in dairy cattle (Lehrer et al., 1992). These instruments are localized at the tether of the animals and are able to record the motory activity of the subjects throughout the day. The rationale of their use is that the animals in estrous status show a higher motory activity than in other stages of the cycle. In buffalo cows the pedometers are able to reveal the etsrous staus in 80% of the animals, with an accuracy of 75%. Furthermore, if a vasectomized bull is present in the herd, the sensibility of the system exceeds 90% (Di Palo et al., 2001).

It has been suggested that the reduced intensity of estrous in buffaloes is associated, at least in part, with relative low circulating concentrations of 17-β-estradiol (estradiol) in comparison with dairy cattle (Seren et al., 1995). The pre-ovulatory follicle in buffaloes is smaller than in dairy cattle (Baruselli et al., 1997a) and this could be associated with lower estradiol levels, although a different estradiol metabolism and clearance from circulation may be present between buffaloes and dairy cattle. The interval between the onset of estrous and the LH surge can vary from 1 to 12 h in buffaloes (Gill et al., 1973; Rao et al., 1981; Vale et al., 1984; Seren et al., 1995) and ovulation occurs between 26 and 33 h after the LH surge (Seren et al., 1995; Porto-Filho et al., 1999).

Another peculiar feature of buffalo reproduction is the high incidence of double ovulations (Zicarelli et al. 1988). Interestingly, it has been reported that only 0.06% of double ovulation leads to twin pregnancies. However, the occurrence of double ovulations reduces the efficiency of AI only in case of spontaneous estrouses, but not in case of induced estrouses (Zicarelli et al. 1997). The hormonal pattern in cyclic buffalo is similar to that described in a cyclic Bos taurus cow (Seren & Parmeggiani, 1997). The main difference between the two species is the percentage of cyclic individuals in the different seasons. Buffaloes show waves of ovarian follicular development during estrous cycles similar to cattle (Baruselli et al., 1997a). The fundamental features of follicle recruitment, selection, dominance and atresia during a follicular wave in buffaloes (Baruselli et al., 1997a) are similar to those described for cattle (Sirois and Fortune, 1988; Ginther et al., 1989; Mapletoft et al., 1994). The number of follicular waves during an estrous cycle can vary from 1 to 3 in buffaloes, with 2 waves being the most common (63%; Baruselli et al., 1997a; Presicce et al., 2004; Neglia et al., 2007). There has been no report of 4 follicular waves within an estrous cycle in buffaloes, although 4 waves do occur in cattle (Rhodes et al., 1995).

After calving, there are a low number of ovarian follicles and follicular waves and few cycles occur. If conception does not take place, an anoestrus condition of variable length begins (Zicarelli et al. 1994). With regard to this topic, the transition period and the postpartum period have a major importance for fertility in buffalo (see below). It is not clear whether the seasonality of the species depends on the reduced follicular population or if this latter effect is the cause of buffalo seasonality. If the calving calendar is not modified, the delayed resumption of cyclic activity after calving can be due to the absence of the bull (bull effect; Zicarelli et al. 1997) and/or to poor nutritional conditions. Unless the feed deficiencies are serious, however, nutritional factors in general do not play a significant role (Zicarelli 1997). The buffalo cannot yet be considered exactly as having a “lactiferous habitus” and therefore a “catabolicus habitus” such as that found in the high milk producing bovine cow which uses its reserves to compensate energy and protein deficiency during early lactation. As a matter of fact, within reasonable limits, the buffalo uses its reserves in its aim to reproduce and to the detriment of her own milk production.

4.3. Seasonal anoestrous

The anoestrous can be defined as a condition during which the female is not able to exhibit regular estrous cycle (Senger, 2005). When an animal is in this condition, the ovaries are moderately inactive and no ovulations or corpora lutea are present. From a hormonal point of view, the anoestrus is probably due to a low GnRH release from the hypothalamus, hence the gonads are not continuously stimulated by gonadotrophins. It can occur in relation to several conditions, such as pregnancy, lactation, presence of offspring, season, nutrition, environmental conditions, stress and pathologies. All these features need to be considered in the analysis of anoestrus.

The “gestational” anoestrus is due to the high progesterone secretion by CL and/or placenta. This hormone is able to block the GnRH secretion by acting on the neurons of hypothalamus through a negative feedback mechanism. For this reason FSH and LH hormones are at basal levels and are not able to stimulate the ovaries, avoiding the ovulation. It is worth pointing out, however, that in rare cases (around 3-5% in sheep and bovine and 1-2% in buffaloes, considering heats detected by using pedometers), estrous behaviour may be present even during pregnancy, although the physiological meaning of this phenomenon is still unclear.

Just before parturition, progesterone rapidly decreases, and estradiol increase after or shortly before parturition, but the animal remains in anoestrous for some days. This post-partum anoestrous allows uterine involution before a new pregnancy can occur. It is likely that other factors are also involved in this process: the mean interval for completing uterine involution in buffaloes is between 19 and 52 days, although the ovarian follicular activity may be resumed earlier, around 15-30 days. In particular, the responsiveness of pituitary gland to exogenous GnRH occurs by day 20 and 30 in dairy (Palta and Madan, 1995) and Swamp buffaloes (Jainudeen et al., 1984), respectively. In any case the first postpartum estrous in buffalo species occurs between 44 and 87 days after delivery (For review see El-Wishy, 2007a). Interestingly, a short luteal phase of 6-13 days, characterized sometimes by low progesterone levels, has been observed at the first estrous in many buffaloes.

The establishment of the seasonal anoestrus is peculiar in buffalo species and similar to that described in sheep. As described above, the presence of few primordial follicles at birth, causes a low number of estrous cycles after calving: if the animal does not conceive in these periods, the phenomenon of anoestrous occurs. In fact, it has been observed that the interruption of cyclic activity is usually preceded by a normal estrous cycle, which is followed by an inadequate luteal secretion. In fact, a typical scenario for buffaloes in the transition to seasonal anoestrous is a progressive decline in CL activity in successive cycles before entering anoestrous (Zicarelli et al., 1988a). The proportion of females in a group that show reduced progesterone in cycles approaching the onset of anoestrus is reduced by the presence of a buffalo bull (Zicarelli et al., 1997). These reproductive disorders may arise into two different conditions: a short luteal phase or a normal luteal phase with low progesterone production (Garverickh et al., 1992).

In both cases three or more estrus cycles lasting 17-22 days can be observed, but progesterone levels are lower than in physiological conditions (Zicarelli et al., 1988a). This phenomenon, defined as acyclic estrus, has been found upon the onset of anoestrus season in sheep, upon the first ovulation postpartum in bovine and during the pre-pubertal phase in both species. No differences have been observed regarding to the length of the acyclic estrus cycles and ovulation rates. In fact, acyclic estrus cycles are similar to normal counterparts, except for progesterone production. The result is the occurrence of a functional and behavioural anoestrus, which lasts variable length. The acyclic heats have been particularly studied during the "transitional period" (Campanile & Neglia, 2007), which corresponds to the midwinter in Italy (transition from periods characterized by decreasing day-length to periods with increasing day-length). It has been observed that the incidence of acyclic heats is similar for both spontaneous and induced heats (Zicarelli et al., 1988a) from December to January, but the phenomenon is more evident in spontaneous heats during the spring period (February-April).

Environmental conditions that are responsible for the anoestrous are accompanied by changes in blood hormones such as prolactin (Razdan et al. 1981), follicle stimulating hormone (Janakiraman et al. 1980), luteinizing hormone (Batra & Pandey 1982; Aboul-Ela et al. 1983), progesterone (Kaur et al. 1983; Qureshi et al. 2000), estradiol (Sheth et al. 1978; Heranjal et al. 1979; Razdan et al. 1981), thyroid hormones (Khurana & Madan 1985; Gupta & Dhoble 1988), and corticosteroids (Madan et al. 1983). Some of these hormones represent the response to stressful factors rather than the cause of the arrest in reproductive activity. Furthermore, the volume of oviductal fluid collected in cyclic buffaloes is definitely higher than that collected from acyclic subjects (Vecchio et al. 2007a). The oviduct is the organ where fertilization and early embryo development occur and hence its secretions, a combination of selective transudate from the blood and secretory products from the epithelial cells (Leese, 1988; Malayer et al., 1988), are particularly important throughout these processes, for the presence of several growth and nutritive factors. Since lower energetic substrates have been dosed in the oviductal fluid of anoestrus buffaloes (Vecchio et al., 2009), it can not be ruled out that this condition may influence oocyte quality and embryonic development, representing one of the causes of embryonic mortality (see later).

The incidence of acyclic subjects is variable in relation to farm, management, climate and, obviously, photoperiod. More than 50% of the animals that calve between the end of the winter and the beginning of spring in Italy, are acyclic within 90 days open, compared to 3% of the buffaloes that calve between the end of summer and the beginning of Autumn (Zicarelli et al., 1985). Furthermore, the phenomenon is more evident in primiparous (80%) than in pluriparous (40%) buffaloes. This type of anoestrus mainly recorded in primiparous animals has also been described in cattle (Senger, 2005). Similarly to primiparous animals, old buffaloes (>

10 years old) frequently show anoestrus condition in periods characterized by increasing daylight length. The reasons for explaining the anoestrus in primiparous and old buffaloes are quite different. In the first case, poor management during their early years, stress of first lactation, gestation and first post-partum period or puerperium are the main factors involved in the occurrence of anoestrus. In fact, it is known that after parturition a negative energy balance condition occurs in all cows, but it is more accentuated in primiparous subjects, due to growth and lactation requirements. Therefore, these animals are more subjected to undergo “nutritional” anoestrus and need more time for resuming ovarian activity compared to adult cows. This would be confirmed by the evidence that a diet characterized by high energy and starch content leads to high fertility rate in primiparous animals, while this rationing schedule negatively affects fertility in pluriparous buffaloes (Vecchio et al., 2007). On the contrary, as mentioned above, old buffaloes are affected by the calving season, since they solve the anoestrus condition when daylight hours decrease (Autumn in Italy). Different techniques regarding weaning, growing subjects together with diet supplied during puerperium could be contributing factors.

According to the calving season, two different “types” of seasonal anoestrus have been described in buffalo species in Italy.

a temporary anoestrus (animals with less than 150 days open), which is observed in buffaloes that calve after the beginning of spring. These animals are characterized by tonic uterus and smooth ovaries with small and medium follicles, that are not able to reach the ovulatory stage.

A deep anoestrus (animals with more than 150 days open), which is observed in buffaloes that calve between the beginning and the end of the winter. In these cases, the uterus is hypotonic and small and hard ovaries, without clinically evaluating follicles, are present.

Buffaloes that calve after the beginning of the spring are going versus periods characterized by decreasing daylight length, while subjects that calve in the first months of the year are going versus a period of increasing day-length. The latter can be responsible of the deep anoestrus frequently observed in these animals. The changes that occur in the endocrine pattern affect the resumption of ovarian cyclic activity insomuch as that reproduction may be negatively influenced even when the buffaloes become cyclic. This "deep anoestrus" condition can be solved when the animals are exposed to at least two months of the short-day period (Zicarelli, 1994b).

Although several authors ascribed the anoestrus condition to nutrition, contrarily to cattle it plays only a marginal role in buffalo. This can be considered in developing Countries, where the calving season is not modified and the anoestrus is mainly due to alimentary causes.

Another physiological condition of anoestrus is the lactation. In fact, almost all mammalian species tend to suppress the estrus cycle during nursing. This natural condition, that is complete in sow, avoids the establishment of a new pregnancy when the animals are still nursing their offspring. Only few information are available on the effect of suckling on postpartum reproductive efficiency of dairy buffaloes (El-Fouly et al., 1976; Usmani et al., 1985,a; Usmani et al., 1990). Nili-Ravi buffaloes that are allowed to nurse their calves twice daily for two minutes (limited suckling), show a more rapid uterine involution (1 week) compared to non-suckled buffaloes (Usmani et al., 1990). Hence it has been proposed that an oxytocin mediated increase in the frequency and magnitude of uterine contractions may explain this phenomenon. However, some studies performed in cattle do not seem to support this hypothesis (Stewart & Stevenson, 1987). The resumption of follicular development (palpable follicle ≥ 10 mm in diameter), as well as the postpartum intervals to the first progesterone rise and formation of the first palpable CL seems to be longer (1 week) in Nili Ravi (Usmani et al., 1985a) and Murrah (Singh et al., 1979) suckled buffaloes rather than in non-suckled ones (Usmani et al., 1990). Similarly, Swamp buffaloes with unrestricted suckling, once a day suckling or no suckling, show a mean interval from calving to first ovulation of 82, 69 and 50 days respectively (Nordin and Jainudeen, 1991). From studies carried out in beef cattle

(Senger, 2005), it seems that during intense suckling LH concentration is low and LH pulses are observed after a drastic weaning within 2-3 days. Therefore, it is likely that the mammary stimulation does not act on gonadotropin release from pituitary (as accepted in the last years), but inhibits GnRH release from the hypothalamus maintaining basal LH levels during suckling and avoiding the resumption of cyclicity. However, it is worth pointing out that other factors are probably involved in GnRH inhibition, such as visual, olfactory and auditory encounter with the offspring (Senger, 2005). In fact, in dairy cows, differently from beef cattle, which are allowed to suckle their calves, the calf is removed from the mother very soon and the phenomenon of lactational anoestrus is very rare.

Several hormonal treatments have been used in order to remove the anoestrus status, such as progesterone and GnRH. Treatments with progesterone (either by subcutaneous implant, intramuscular injections for 6 days or intravaginal releasing device) have been useful for removing the anoestrus condition in more than 80% of subjects (Borghese et al., 1993; Neglia et al., 2003). When subcutaneous implants or intramuscular injections are used, the treatment-conception interval is variable between 27 and 42 days (Borghese et al., 1993). Treatment with intravaginal releasing device has been used in acyclic buffaloes undergone AI (Neglia et al., 2003), leading to 100% of animals that showed resumption of ovarian activity and 50% pregnancy rate. The utilization of 2 mg subcutaneous osmotic pumps of buserelin (a GnRH agonist) has been able to remove the anoestrus condition in around 70% of animals (Borghese et al., 1993), but the treatment-conception interval has been higher (around 90 days). In any case, whatever treatment is utilized, the conception does not occur with the first ovulation, but since the second (in case of progesterone treatment) or the third (in case of osmotic pumps of buserelin) cycle (Borghese et al., 1993).

Finally, it has to be mentioned that some pathologies can also affect the occurrence of anoestrous. In fact, together with environmental factors, endometritis play a main role by reducing pregnancy condition and causing acyclia. In this case the resumption of ovarian activity occurs only when daylight length decreases and pathology is solved.

5. BUFFALO MALE GENITALIA

5.1. The testis and epididymis: season and reproductive function

The knowledge and understanding of the buffalo bull male anatomy and function is instrumental to the proper and efficient implementation of reproductive technologies in this species. The size of the scrotum in buffalo is smaller than of Bos taurus bull of similar size and weight and has no constriction adjacent to the attachment of abdominal wall. The function of the scrotum is to support the testes and to adjust the testicle temperature a little lower than the body temperature. Sperm production and its morpho-functional quality for fertility assessment, relies on the understanding of physiological parameters mostly linked to the genital apparatus. In fact, the knowledge of the morphology, size and scrotal circumference is of paramount importance to determine to a large extent the reproductive efficiency and to predict the potential for semen production. Testicular weight, a reliable index of semen producing ability, has been shown to increase between 2.5 - 3.0 (68.5 g), 3.5 - 4.0 (96.2 g) and 4.5 - 5.0 (114.2 g) years of age (El-Azab et al, 1978). Variations in the testes weight are markedly greater among younger bulls and decrease with advancement of age. Paired testes weight is highly correlated with body weight. There is evidence that the larger, faster gaining bulls would have larger testes than smaller bulls of comparable age. As with other animal species, gonadal sperm numbers are positively and significantly correlated with the weight of testicular parenchyma (Abdou et al., 1982; Pant et al., 2003). Moreover, the sperm numbers per gram of testicular parenchyma are much higher at 3.5-4 years (85.8 million) and 4.5-5 years (75.7 million) than at 2.5-3 years (50.7 million). A buffalo bull produces, on average, 2.74 billion of spermatozoa daily and the daily sperm production per gram of testicular parenchyma is about 13.74 millions. The parenchymal sperm concentration per weight does not vary appreciably among ages, indicating that 2-3 year-old bulls have already attained the mature rate of spermatogenesis. Nevertheless, variations among bulls appeared to decrease with advancement of age. Daily sperm production increased markedly with age.

In addition, the knowledge of morphological features of the testis is instrumental for the evaluation of the influence that different factors, such as the hormonal fluctuations linked to the photo-neuroendocrine system may have on reproductive efficiency (Seren & Parmeggiani, 1997). The function of the male gonads too, is influenced by the melatonin hormone as the endocrine signal that marks the light and dark hour fluctuation of the day. Such multistep neural pathway is characterized by a domino effect cascade starting with a photoperiod sensitivity to the length and density of the light source, following involvement of the retina, the suprachiasmatic nucleus, the superior cervical ganglia and finally the pineal gland leading to incretion of melatonin. Such neuronal stimulus triggers the rhythmus of melatonin incretion which regulates hypothalamo-hypophysial activity, gonadal function and finally sperm composition and quality (Zicarelli, 1997). Buffalo bulls are characterized by a slower sexual development when compared to cattle bulls. Differentiation of Leydig cells precedes the onset of spermatogonial mitosis, and it seems likely that the production of testosterone by newly differentiated Leydig cells of the prepubertal testis is a prerequisite for initiating spermatogenesis (Rana & Bilaspuri, 2000). Testicular spermatogenic cell divisions begins at approximately 12 months of age and spermatogenesis is reported at around 15 months of age, whereas at the age of 24 months, viable spermatozoa are released and present in the ejaculate (Perera, 2008). Such viability is already very good at this age, allowing for possible processing of spermatozoa for sexing and freezing (Presicce et al., 2005). While Swamp buffalo bulls may reach ~ 26 cm in scrotal circumference at the age of 24 months (Bongso et al., 1984), Indian and Mediterranean breeds exceeds such value by approximately 10 cm, reaching at the same age 34 to 36 cm (Vale et al., 2001). Interestingly, and in consideration of the seasonal fluctuation in reproductive efficiency in buffalo bulls as well, both weight and size of scrotal circumference together with epididymal weight, are slightly but not significantly reduced in the course of the non-mating season when compared to the mating season (Ibrahim, 1985). The duration of one seminiferous epithelium cycle is 16 days, whereas the approximate duration of spermatogenesis is 75 days. It is thought that such long spermatogenic cycle may be in some way behind the low rate of daily sperm production in this species. In adult bulls, the relative distribution of sperm in the three major segments of the epididymis is about 30.4-33.3% in the head, 8.79-20.5% in the body and 49.1-55.5% in the tail. The epididymis of the buffalo is a rather intricately convoluted duct with complicated structure. It is much shorter than that of the bull. Histological assessment of the epididymal duct (Abdou et al., 1985) revealed eight histologically distinct regions, which could be differentiated according to tubular and luminal diameters, cell types and regional differences in their distribution, distinctive features of their principal cells, changes in epithelial height as well as on the height of stereocilia. The caput and proximal corpus are the most active sites at which maturation of spermatozoa is likely to occur. Various maturational biophysical, biochemical and morphological changes have been recorded. Passage of spermatozoa from caput to corpus and then to cauda epididymidis is associated with significant diminution of sperm head length, breadth and area with the head appearing more elongated (Abdel-Rahman, 1980; Abdou et al., 1983). Even more interesting is the finding related to the histological evaluation of seminiferous tubules which showed a maintained spermatogenesis during the non-mating season, and more importantly, a significantly higher epithelial lining, indicative of a possible enhancement of function due to the pressure of sperm accumulation, which is typical of this part of the epididymis (Arrighi et al., 2010). These findings suggest and confirm the potential of buffalo bulls to breed throughout the year, although reproductive function is somewhat compromised during the non-mating season, as confirmed by a large variability in semen quality reported among Nili-Ravi, Murrah and Mediterranean Italian buffaloes (Kumar et al. 1993; Presicce et al. 2003; Saeed et al. 1990). These considerable differences may be explained by the lack of long time selection for semen freezability in this species. In addition to semen quality and its freezability, photoperiod has also been reported to affect sexual activity and bull libido (Sansone et al. 2000). Furthermore, to underline the sensitivity to seasonality in the buffalo species and in particular in the bull, it has been reported a neuro-endocrine interaction between androgen hormones and the autonomic nerve supply in the regulation of male buffalo reproductive functions. In fact, during the mating period, a dense noradrenergic innervations can be observed to supply the vas deferens as well as the accessory sex glands, whereas during the non-mating period the noadrenergic nerves are dramatically and significantly reduced (Mirabella et al., 2007).

5.2. Vas deferens and accessory sex glands

Vas deferentia extend from the tail of the epididymis to the pelvic part of the urethra. They move through the inguinal canal and reach the dorsal surface of the bladder. On the dorsal surface of urinary bladder, the ducts deferens form a fusiform enlargement called “ampulla”, which meets and joins together with the seminal vescicular duct of the same side prior to entering into the urethra. The mucosa of the ampulla contains glandular tissue, and therefore it can be stated that this part of the ducts deferens functions as an accessory sex gland. Other accessory sex glands such as seminal vescicle and prostate glands are less developed when compared to cattle bulls. The bulbourethral glands of male buffaloes are longer but not broader than those of cattle bulls. Such accessory sex glands produce seminal plasma which increases the volume of semen, supplies nutrient to the spermatozoa and acts as a pH stabilizer. The accessory sex glands can be palpated per rectum, but if animals are castrated, their size is reduced. (Kunavongkrit & Koonjaenak, 2006).

5.3. Penis and prepuce

The penis in buffaloes is shorter if compared to cattle bulls, contains very little erectile tissue and is cylindrical in shape with a tapering end. As a consequence, even in the non-erectile state, the penis is firm in consistency, and the organ does not enlarge too much during erection. The hair within the prepuce is absent or scanty and very short. The prepuce itself is tightly and closely adherent to the ventral part of the abdominal wall. It ends few centimeters caudal to the umbilicus where it hangs freely. The preputial cavity in buffalo is only 30 to 35 cm deep compared to the average length of 40 cm in European breeds (Kunavongkrit & Koonjaenak, 2006).

6. REPRODUCTIVE AND PHYSIOLOGICAL CHARACTERISTICS OF BUFFALO BULLS

Buffaloes are generally quiet and easy to handle. They are rarely aggressive towards people but can be very aggressive towards one another. In general, the husbandry of buffalo is not very different from cattle, but buffalo is amazingly versatile. In fact, this species is able to adapt to extreme environmental changes compared to various breeds of cattle and its ability to withstand the environmental conditions prevailing in the tropics is widely recognized. The body temperature of buffaloes is slightly lower than that of cattle, 38.5°C vs 39°C, despite the fact that buffalo skin is usually black, heat-absorbent and only rarely protected by hair. Moreover, they have fewer sweat glands than other bovidae. Hence, they poorly disperse heat by sweating and are fairly sensitive to heat (Nowak, 1999). If buffaloes were worked or driven excessively in the hot sun, their body temperature, pulse rate, respiratory rate and general distress levels would increase more quickly compared to cattle. Therefore, buffaloes usually cool down by wallowing in mud, rather than seeking shade. Wallowing in mud helps them to cool their body temperature because water in mud evaporates more slowly than water on its own, thus extending the effectiveness of cooling when ambient temperature and humidity are high.

6.1. Puberty

Reproductive capability in the male is the result of complex interactions among the hypothalamus, the anterior pituitary gland and gonads. Puberty is related to the age of the bull and environmental factors including availability and food intake. The onset of function of the interstitial (Leydig) cells precedes the formation of spermatozoa, with androgens conditioning the seminiferous tubules to gonadotropic stimulation. The puberty in buffalo bull is as variable as in the female and is determined more by body weight than by age. In healthy bulls, testicular spermatogenic cell divisions starts by approximately 12 months of age and active spermatogenesis can be ascertained from 15 months of age. Although it is reported that the ejaculates contain viable spermatozoa only after 24-30 months of age (Perera, 1999), it is not rare to found sexual mature bulls yet at 15 months. In any case, it seems that buffalo bulls mature more slowly than cattle and have longer time lag between the achievement of puberty and the onset of spermatogenesis.

6.2. Libido and mating behaviour

The term "libido" is commonly used to describe the willingness and eagerness of a male to mount and attempt service of a female, while "mating behaviour" describes the performance of the male in the period immediately before, during and after service (Blockey, 1979; Chenoweth, 1981). Both libido and sexual behaviour are less intense in buffalo than in cattle. In buffalo bull, normal copulation encompasses a sequence of behavioural elements including courtship, erection and protrusion, mounting, intromission, ejaculatory thrust, total reaction time and dismount time (Bhosrekar et al., 1988). Reaction time (the interval from sniffing the vulva by the bull, to the display of flehmen response, to mounting) is ordinarily used to assess both libido and mating behaviour (Chenoweth, 1981). In this species, the reaction time varies greatly between 0.5 and 4.0 minutes (Bhosrekar et al., 1988; Gill et al., 1974; Kushwaha et al., 1955; Rajamahendran & Manickavadivale, 1981) and differs significantly among seasons. In particular, libido seems to be often suppressed during the hottest periods (Hafez, 1992), but conflicting reports indicate the need of further studies on the potential seasonal variation. Breeding soundness depends primarily on the male's health status and welfare, on the function of its endocrine system and its testes, genital tract and accessory sexual glands.

Secondarily it could be influenced by several factors such as age, nutritional level, presence of concurrent affections or diseases, as well as management, and social interactions. All these factors are important to the efficiency in the performance of buffalo bull.

7. SYNCHRONIZATION PROTOCOLS AND AI IN BUFFALO

As reported above, the application of AI in buffalo species is seriously influenced by the difficulties in estrus detection, because of the low signs of estrus behavior. For this reason several pharmacological protocols have been developed in order to manipulate the estrus cycle and, in some cases, to predict the ovulation time. These are based on the utilization of some hormones that can act at different level on the hypothalamus – pituitary – ovary axe. In particular, these protocols can be divided into two categories:

treatments that can control the luteal phase of the cycle, in which are mainly utilized prostaglandins and progesterone analogues;

treatments that can control follicle development and ovulation, by using prostaglandins, progesterone, GnRH, hCG, eCG and estradiol.

7.1. Treatments by prostaglandins

Actually, several prostaglandins analogues (cloprostenol, luprostiol, dinoprost, etc.) are utilized in buffalo species. The effectiveness of these molecules are based on their luteolytic action on the CL, which causes a rapid progesterone decline within 24 hours (Bachalus et al., 1980), leading to ovulation. A rapid decrease in the luteal blood flow has been proposed as one of the main luteolytic actions of prostaglandins and their analogues.

This decrease in luteal blood flow occurs during both normal and PGF2a-induced luteolysis (Knickerbocker et al., 1988; Azmi et al., 1982). Usually, at least in cattle, luteal regression is initiated by an exogenous injection of prostaglandins given after Day 7 of the normal estrous cycle (Schallenberger et al., 1984). This is due to the increase in the intraluteal production of vasoactive substances such as endothelin-1 (ET-1) (Ohtani et al., 1998) and angiotensin II (Ang II) (Hayashi et al., 2001), both of which play important roles in the luteolytic cascade (Miyamoto et al., 1997; Meidan et al., 1999). On the contrary the role of prostaglandins during the first days of the estrus cycle, when an active CL is not present on the ovary, is still unclear, although it has been proposed that they can act on the pituitary, by inducing LH release and on the follicle, by anticipating the ovulation. This unclear aspect needs to be confirmed, although higher pregnancy rate has been recorded in synchronized buffaloes treated by cloprostenol on the day of AI (Neglia et al., 2008).

The protocols, that are based on prostaglandins utilizations, foresee two administrations 11-14 days apart. In fact, as specified above, prostaglandins are able to induce luteolysis only after day 5 of the cycle and within day 17-19 (depending on the length of the cycle). Therefore, one administration would be able to induce the estrus only in animals that show an active CL (diestrus phase). Buffaloes that do not respond to the first prostaglandin administration, because are in estrus, metaestrus (0-3 days of the cycle) or in proestrus (18-22 days of the cycle) are supposed to be in diestrus after 11-14 days and hence would respond to the second administration, since an active CL would be present on the ovary. The interval between the two prostaglandins administrations does not seem to be relevant for increasing pregnancy rate, since with this protocol it is possible to achieve 40-50%, either if it is utilized alone (Khurana et al., 1981; Subramaniam et al., 1989) or in association with GnRH (Neglia et al., 2001).

The main restrictions of this protocol are the period of the year when it is utilized and the difficulty in predicting ovulation. In fact, because of the seasonality (Zicarelli, 1997; Campanile et al., 2009), this protocol is suggested mainly during the breeding season, when a high incidence of buffaloes is cyclic. In this case, double prostaglandins regimen induces estrus and ovulation in around 60-80% of buffaloes, with 50% pregnancy rate (Dhaliwal et al., 1988; Brito et al., 2002), independently by the route of prostaglandin administration (Dhaliwal et al., 1987). On the contrary, it is difficult to record a pregnancy rate higher than 25-30% out of the breeding season (Chohan et al., 1995), despite a high number of buffaloes showing signs of estrus (Sahasrabudhe & Pandit, 1997). This is probably due to the seasonality of the species, since the incidence of CL activity reduction is between 5 and 50% in buffaloes during the transition period (Campanile et al., 2008a). This condition, which is usually accompanied by ovary silence and heats, is characterized by ovulation and subsequent inadequate luteal phase (Zicarelli, 1989). Furthermore, it is worth pointing out that the incidence of heats characterized by a subsequent good luteal phase after treatment with PGF2a is also influenced by bull exposure (Zicarelli et al., 1997). Furthermore, a great variation in the duration of estrus behaviour (36 to 96 hours) and ovulation (60 to 100 hours) after prostaglandin administration has been observed in buffalo (Baruselli, 1994; Porto-Filho et al., 1999). This is particularly evident if buffaloes are treated before or after day 10 of the cycle, confirming that the successful administration of prostaglandin depends on the presence of functional corpora lutea, in a specific phase of the estrus cycle (Porto-Filho et al., 1999). Furthermore, the variation in the follicular population at the moment of CL regression affects the interval between the onset of estrus and the ovulation, constraining the detection of estrus symptoms in order to identify the appropriate moment for artificial insemination (Baruselli, 1994).

Finally, it is worth of note that this protocol has also been used to carry out a preliminary screening (Pre-synch Protocol) of the animals that can be subsequently synchronized, either in cattle (Moreira et al., 2001) and in buffalo (Oropeza et al., 2010). This leads to higher pregnancy rate for both the choose of cyclic subjects and the better response to other subsequent synchronization treatments.

7.2. Treatments by progesterone or progestagens

The utilization of progesterone or progestagens can be carried out by different routes of administration, such as intravaginal, auricular or injectable. These protocols are particularly utilized in buffaloes out of the breeding season, since it has been largely demonstrated that progesterone is able to act on the hypothalamus-pituitary-ovary axe, leading to resumption of ovarian cyclicity in anoestrus animals (Rhodes et al., 2003; Neglia et al., 2003**;** Baruselli, 2001). Several hypotheses have been performed to explain the effect of progesterone or progestagens treatment on hormone concentration and follicular development (Rhodes et al., 2003). In fact, as progesterone concentrations reach sub-luteal levels during treatment, there is an increase in LH pulse-frequency, associated with increasing estradiol synthesis (Rhodes et al., 2002). Therefore, sensitivity of the hypothalamus to the negative feedback effects of estradiol is reduced, the follicular growth is stimulated and the largest ovarian follicle matures and responds to exogenous estradiol or a gonadotrophin (Rhodes et al., 2003). For this reason, treatment with progesterone is often associated with estradiol (benzoate or valerate) and/or Pregnant Mare Serum Gonadotrophin

(PMSG). Another hypothesis is based on some studies performed in vitro on rat pituitary cells, in which it was demonstrated that progesterone augmented GnRH induced LH RNA levels and LH release (Park et al. 1996). Since progesterone in vivo decreases the frequency of GnRH pulses secreted into the hypothalamic–hypophyseal portal circulation (Karsch et al. 1987) and GnRH is also an important regulator of the number of GnRH receptors, it has been hypothesized that progesterone could decrease pituitary responsiveness to GnRH by reducing the frequency of GnRH pulses, which in turn would lead to reduced synthesis of GnRH receptors (Nett et al., 2002). It is likely that exogenous progesterone supplementation may increase LH storage in pituitary gland and augment GnRH induced LH release (Nett et al. 2002). This is supported by the evidence that an increase in pituitary content of gonadotropins following treatment with progesterone is observed in ovariectomized and hypothalamic-pituitary-disconnected ewes (Di Gregorio & Nett, 1995) probably for a double effect, increased protein synthesis and lack of secretion of LH and FSH, due to removal of GnRH.

Usually treatment with progesterone in buffalo is performed by using intravaginal device (containing 1.5 to 1.9 g of natural progesterone) for 10-12 days, and blood concentrations of 4-5 ng/ml are reached (Neglia et al., 2003). Auricular implants, containing norgestomet (17a-acetoxi-11b-metil-

19-norpreg-4-en-3,30-diona), a progesterone analogous, that is more potent than natural progesterone, are also utilized to synchronize estrus cycle and ovulation. Since norgestomet is more potent than natural progesterone, lower doses are required. During the treatment (day 6 or day 7 after insertion), or when the device is withdrawn, it is recommended to perform a prostaglandin administration, in order to remove corpora lutea on the ovaries. The day of prostaglandin and PMSG administration does not seem to affect pregnancy rate after AI in adult buffaloes, that is reported between 30 and 50% (Neglia et al., 2003; Barile et al., 2007). This treatment is also utilized to synchronize estrus cycle in buffalo heifers (Barile et al., 2001). In fact, it is known that the age at puberty in buffalo species ranges from 16–22 to 36–40 months in different Countries (Borghese et al., 1994) and the delay in puberty, and the consequent delay in conception, is one of the problems that causes the low reproductive efficiency of this species. Although many factors may affect age at puberty, such as breed, season climate, nutrition and growth rate, it has been demonstrated that mimicking the hormonal changes occurring around puberty can induce sexual maturity in heifers. Treatments based on progesterone releasing intravaginal device, are able to anticipate the puberty, when applied in animals at peripubertal age (Barile et al., 2001). Therefore, progesterone plus PMSG treatment of heifers may have a strong economic impact on buffalo production because a greater proportion of heifers could be bred earlier than with other synchronization protocols.

7.3. Treatments for the control of follicular development and ovulation

Because of the difficulties in estrus detection in buffalo, the most commonly used protocols of estrus synchronization are based on the control of follicular development and ovulation. These techniques enable fixed time AI, avoiding estrus observation and facilitating the reproductive management of the herd and the use of this biotechnology in field conditions. It is known that in cattle GnRH (or its analogues, such as gonadorelin, buserelin or lecirelin) administration is widely used to manipulate patterns of ovarian follicle development (Twagiramungu et al., 1995). In fact, it allows a LH, FSH and estradiol surges during any stage of the estrous cycle, which will promote the ovulation of a dominant follicle or the luteinization and/or atresia of pre-dominant follicles. Consequently, a new follicular wave emerges two or three days later (Pursley et al., 1995). In particular, plasma LH levels are elevated 6- to 8-fold yet 30 minutes and 3 hours after GnRH agonist injection (Campanile et al., 2008). The response to LH surge is affected by GnRH dose and dominant follicle diameter. In fact, 100 μg of natural GnRH (gonadorelin) are able to induce ovulation in 100% of treated buffaloes compared to 33% obtained with 50 μg

(Rastegarnia et al, 2004). It is still unclear which is the diameter of dominant follicle that is able ovulate in response to exogenous GnRH. In fact, although it has been reported that buffaloes with a dominant follicle diameter of 6.7±2.4 mm do not show ovulation (Baruselli, 2001), a great variability has been observed in several studies (Campanile et al., 2008; Rastegarnia et al, 2004), since follicles with a diameter ranging between 4.0 and 12.0 mm ovulate. Further studies on LH and FSH receptors expression are needed to explain this aspect. On the contrary, the form of GnRH analogue does not seem to affect ovulation and pregnancy rate: either buserelin acetate and lecirelin, a synthetic hypothalamic hormone of prolonged action, obtained through the modification of gonadorelin structure, can be successfully utilized in buffalo species (Baruselli et al., 2001b). In any case, GnRH administration in buffalo induces ovulation in 60–86% of treated animals (Baruselli, 2001; De Araujo Berber et al., 2002; Neglia et al., 2003) and the interval between GnRH administration and ovulation is 33±8.3 hours (De Rensis & Lòpez-Gatius, 2007).

Several synchronization protocols have been developed to control follicular development and ovulation, by extrapolating information in more studied species, such as cattle and sheep. All these methods are based on the ovulation of the dominant follicle and the regression of the CL by using prostaglandin. The most utilized protocol is the Ovsynch-TAI Program (Pursley et al., 1995**;** Baruselli et al, 1999; De Araujo Berber et al., 2002; Neglia et al., 2003), which consists of a GnRH injection on Day 0, a prostaglandin injection on Day 7 and a further GnRH on day 9, with AI performed on day 10 at 60 and 16-24 hours after the prostaglandin and the last GnRH, respectively. In particular, the ovulation or the luteinization of the dominant follicle following the first GnRH administration, causes the presence of the CL and, consequently, high progesterone levels. Around 61% of buffaloes during the postpartum period (meanly 60 days from calving) ovulated in response to the first GnRH analogue (buserelin acetate) administration (Baruselli et al., 1999) and progesterone levels does not interfere in the ovulatory rate of the first GnRH. Furthermore, the administration of a double dosage (20 µg) is responsible of a 10% higher ovulation rate in buffaloes during the postpartum period. Hence, a prostaglandin can be administered 7 days later in order to induce luteolysis, as reported above. Because of the high ovulation rate followed GnRH administration, only few animals show estrus behaviour between day 0 and day 7, whereas 70-80% of buffaloes are in estrus 48-72 hours after prostaglandin (Pursley et al., 1995; Brito et al., 2002). Although buffaloes that ovulate after the first application of GnRH present greater levels of progesterone on day 7 when compared to those that did not ovulate

(2.56±1.02 vs. 1.26±0.82 ng/ml), it has been observed that 100% of the animals shows progesterone levels below 1 ng/ml 48 h after prostaglandin application. The further GnRH administration 36 to 48 hours after prostaglandin (Day 9) aims to increase estrus synchronization and particularly, ovulation synchronization. The response to the first GnRH affects also the ovulation rate. In fact, buffaloes that do not ovulate after the first application of GnRH present shorter intervals from the application of the second GnRH and ovulation, when compared to those that ovulate (around 22 hours vs. 34 hours), probably due to the lower synchronization in follicular wave (Baruselli et al., 1999). The replacement of the last GnRH with LH does not seem to improve synchronization of estrus and pregnancy rate (De Araujo Berber et al., 2002).

It has been demonstrated that the 78 to 90% of buffaloes shows the synchronization of ovulation by the Ovsynch-TAI Program and the pregnancy rate range between 33 and 60% (Baruselli, 2001; Neglia et al., 2003; Paul & Prakash, 2005). These pregnancy rates definitely decrease if the treatment is applied during the transition period (which coincides with the transition from decreasing to increasing daylight length), because of the high incidence of embryonic mortality (20-40%) (Campanile & Neglia, 2007) and during the non breeding season, because of the high incidence of buffaloes in anoestrus condition, that are not able to respond to the treatment

(Baruselli, 2001). Synchronization, and consequently pregnancy rate, by this method can be increased by the presence of a dominant follicle at the first GnRH administration. This can be achieved by an ultrasound examination of the buffaloes when the treatment is initiating (De Rensis et al., 2005) or by treatment with a progesterone releasing intravaginal device between the first GnRH and prostaglandin administration (De Rensis et al., 2005). It may be also possible to apply a pre-synchronization treatment (a double prostaglandin injections 12 days apart) and initiate the Ovsynch-TAI Program 12 days after the second prostaglandin administration (Moreira et al., 2001). It seems that the efficiency of the treatment is also influenced by parity, since higher pregnancy rate is usually achieved in pluriparous subjects rather than in primiparous buffaloes (Baruselli, 2001). Furthermore, the application of this method is advised against heifers, because of the low ovulatory response to GnRH and because the absence of ovulation does not synchronize the development of a new follicular growth wave (Baruselli, 2001).

A similar treatment to the Ovsynch-TAI Program, is the Co-Synch (Stevenson, 2011), during which the second GnRH injection is carried out simultaneously to the AI (72 hours after prostaglandin administration). This method does not allow to achieve higher pregnancy rate than a double prostaglandin treatment when applied in both buffalo heifers and adult animals (Neglia et al., 2001).

7.4. Modified treatments for the control of follicular development and ovulation

Several modifications have been applied to the Ovsynch-TAI Program. The supplementation with progesterone from Day 0 (the Day of the first GnRH) and Day 7 (the Day of prostaglandin administration) allows to obtain 25% higher pregnancy rate only in non-cyclic buffaloes, while no effects are recorded on cyclic subjects (De Rensis et al., 2005). Hence, this selective treatment would be applied after verifying the cyclicity of the subjects (i.e. after an ultrasound examination that can be performed on the day of the first GnRH administration). The administration of cloprostenol (a prostaglandin F2α analogous) on the day of AI after an Ovsynch-TAI Program, has been proved to increase pregnancy rate from 31 to 48%, on day 45 after AI (Neglia et al., 2008). Furthermore, this treatment increases progesterone levels on Day 10 after AI, probably favoring embryo development and attachment. Human Chorionic Gonadotrophin (hCG) is also often utilized in place of GnRH. However, since it can causes the formation of antibodies, its use is recommended only for substituting the second GnRH injection.

7.5. The AI

AI technique performed in buffalo is similar to that described in bovine. An accurate gynecological visit by rectal palpation would be performed before thawing the semen. This is important to assess the estrus status of the animals. In fact, because of the relatively low intensity of estrous behaviour in buffalo (Ohashi, 1994), the ovaries would be accurately palpated to evaluate the presence of a follicle higher than 1 cm. This can be obtained also by the use of ultrasound (Campanile et al., 2009). One of the greatest advantages of this technique is that it is totally non-invasive and so repeated examinations of an animal's reproductive tract can be performed without impairing its breeding potential or having other adverse effects. Buffaloes reproductive organs are most commonly scanned per rectum using a linear-array transducer specifically manufactured for transrectal use. However, specialized applications including ovum pick-up and follicle ablation involve a transvaginal approach using a sector transducer. Linear-array transducers of 5.0 and 7.5 MHz frequency ranges are most commonly used in buffaloes, and most veterinary ultrasound scanners are compatible with probes of different frequencies. Depth of tissue penetration of sound waves and image resolution is dependent upon and inversely related to the frequency of the transducer. Thus, a 5.0 MHz transducer results in greater tissue penetration and lesser image detail, whereas a 7.5 MHz transducer results in lesser tissue penetration and greater image detail. An ultrasound scanner equipped with a 5.0 MHz transducer is most useful for bovine practitioners conducting routine reproductive examinations, however, small ovarian structures such as developing follicles are best imaged with a 7.5 to 12 MHz transducer. During the procedure the operator removed the faecal material from the rectum, introduced the probe and scanned the uterine horns and ovaries. The transducer and the sleeved arm of the examiner are lubricated with an obstetrical lube to facilitate penetration through the anal sphincter and to establish good contact with the floor of the rectal mucosa. Materials used to lubricate the rectum are generally spermicidal, hence it would be avoided to come in contact with the vulva region. The transducer is positioned dorsal to the genital tract and slowly advanced cranially. The cervix, right uterine horn, the left uterine horn and the uterine body have to be examined. The ultrasonographic appearance of the ovaries in the buffaloes show a typical round to oval shape with a fine ecotexture and a discrete ecogenicity. Antral follicles of various sizes appear as nonechogenic structures which could be distinguished from blood vessels in cross-section by the elongated appearance of the latter. Ovulation can be depicted by the absence of a preovulatory follicle that is present at a previous examination and subsequently confirmed by the development of CL at the same spot.

Similarly, uterine body and horns would be accurately palpated to assess the estrous status and, eventually, analysed by ultrasonography, since the ultrasonographic appearance of the uterus is influenced by the stage of the oestrous cycle. In fact, the echotexture of the endometrium is characterized by the presence of non-specular reflections with dark and bright signals seen within the ultrasound image of the endometrium. Uterine echotexture is characteristically dark during the follicular phase (oestrus) reflecting an extensive degree of edema of the endometrium. Furthermore, the uterine horns are maximally curled during follicular dominance and the presence of mucus in the uterine lumen can be easily assessed by ultrasound.

The inseminating device utilized in buffalo species is the same utilized in bovine. One of the main steps for a successfully AI is the semen handling. Frozen semen can be stored indefinitely, if it is maintained constantly at very low temperatures. Liquid nitrogen is at -196°C but the critical temperature is approximately -80°C. Semen which is exposed to temperatures warmer than -80°C (even for a short time) and then returned to the storage tank may be irreversibly damaged. The extent of damage depends upon how long the semen is exposed to the elevated temperatures. The correct thawing recommendation for semen in straws foresee the warming in warm water at 32-35°C for at least 40 seconds. After that the straw would be accurately dried and put in the inseminating device. To avoid disease transmission and contamination protective rods or sheaths have to be used.

Before performing AI, the vulva region would be cleaned with a paper towel to prevent the interior of the reproductive tract from becoming contaminated and possibly infected.

The last fundamental step of the procedure is the deposition of the semen. The semen should be placed in the body of the uterus just in front of the cervix. Uterus body in buffalo species is shorter than in bovine. For this reason, this step has to be carefully carried out. The proper site for depositing semen can be recognized by the change in tissue consistency—from firm and hard in the cervix to soft and spongy in the uterus. However, the utilization of ultrasound can allow to reveal the presence of the follicle on the left or the right ovary. In this way it would be possible to place the semen directly in front of the horn where the dominant follicle is detected. This particular technique is particularly utilized when straws characterized by low sperm concentration are used, such as in case of sexed semen (see below).

8. PREGNANCY LOSS AFTER AI IN BUFFALO COWS

In buffalo species embryonic mortality is considered one of the major causes of poor fertility, especially in the animals inseminated during the period characterized by increasing daylight length. In fact, in Italy, as specified above, the application of the out of breeding season mating technique (Zicarelli, 1997) guarantees milk production in accordance with market requirements, but it forces the breeders to mate buffaloes during the less favourable periods. Embryonic mortality in buffalo species may occur in different stages of pregnancy and it may be due to several causes. On the basis of the last studies, the windows for embryonic mortality in buffaloes can be divided (Campanile et al., 2010) in:

a) early embryonic mortality (EEM), which occurs between Day 15 to Day 24 of pregnancy and incises for around 20%;

b) late embryonic mortality (LEM), between Day 25 and Day 45, which is described in around 40% of pregnant animals at day 25 post-fertilization;

c) foetal mortality (FM), which is observed in 10% of animals and occurs between Day 46 and Day 90 of gestation.

These phenomena largely affect reproductive performances in buffalo species, reducing the fertility, especially in those subjects undergone AI. In fact, it can be hypothesized that oocyte fertilization occurs in around 80% of inseminated buffaloes, but only 35-40% of subject carry out to term the gestation, for the occurrence of pregnancy losses throughout the phases described above.

The seasonal characteristic of this species, causes an increase in the phenomenon of embryonic mortality during the periods characterized by increasing daylight length. It has been observed that embryonic loss in animals mated by AI is 20-40% during seasons characterized by high number of light hours (Campanile et al., 2005; Campanile et al., 2007a; Campanile et al., 2007b), whereas values of around 7-10% or 20% are recorded in during decreasing light days (Baruselli et al., 1997; Neglia et al., 2010) or close to the equator (Vale et al., 1989), respectively.

The high variability in terms of embryonic mortality may be probably ascribed to the different distance from the equator, which influences daylight length throughout the seasons. Furthermore, it is worth pointing out that other causes may be accounted for embryonic mortality. The phenomenon is not always correlated with the breeding season, but with the ovarian resumption after calving and farm reproductive management, embryonic epigenetic expression, environmental conditions (hot, cold, etc.), nutrition, specific and non-specific pathologies, and, obviously, uterine environment (Zicarelli, 2007; Campanile & Neglia, 2007). In fact, embryo survival depends on the physiological regulation of oviductal and uterine function, due to intrinsic errors in maternal physiology or to specific environmental stresses imposed on the mother.

8.1. Early embryonic mortality (EEM)

Embryo development in buffalo species is similar to that described in cattle, although some important differences have been observed. Following ovulation, the oocyte at the stage of Metaphase II, is released into the oviduct, where the fertilization takes place (Campanile et al., 2009).

A progressive increase of oviductal fluid volume has been observed till the ovulation, with a mean production of 0.39, 0.79, 0.96 and 0.61 ml, respectively in diestrus, preovulatory, ovulatory and postovulatory phases (Vecchio et al., 2009). Therefore, also in buffalo species, steroid hormones affect tubal fluid production by modulating ionic transfer cross the oviductal epithelium and may affect oocyte and embryo development.

Following fertilization and fusion of gametes, the zygote undergoes subsequent mitotic divisions, which determines the formation of the blastomeres. Embryonic development in buffaloes is earlier (12 to 24 hours) compared to cattle (Campanile et al., 2009). This probably causes a precocious entry of buffalo embryos into the uterus on around Day 4 to Day 5 after oestrus (Anwar & Ullah, 1998; Karaivanov et al., 1987). In cattle, embryos reach the uterus at around Day 6 after oestrus. Compact morulae in buffaloes are observed on about Day 5 after oestrus and blastocysts typically from around Day 6 (Galli et al., 2001). Hatching of in vivo derived buffalo embryos is reported to occur from Day 5 to Day 7 (Chantaraprateep et al., 1989; Drost & Elsden, 1985; Gasparrini, 2002). The apparent variability in the time of hatching of blastocyst embryos could be related, at least in part, to studies in relatively diverse genotypes amongst Riverine, Swamp and crossbred buffaloes.

The first stage is defined as pre-attachment period, during which the free floating blastocyst undergoes a significant elongation (Campanile et al., 2009). The pre-attachment phase is thought to occur from around Day 13 until 24, period in which the transitory attachment phase starts. With regard to the latter, pregnancy associated glycoprotein β has been reported to increase from Day 25 in buffaloes (Karen et al., 2007). In this phases the first messenger that has to be recorded is the Interferon-Tau (IFN- τ), which is produced by the elongated conceptus. The IFN-τ, was recognized for the first time in sheep with the name of trophoblastin (Martal et al., 1979) and was subsequently isolated in other ruminants (Spencer & Bazer 2002). It plays a fundamental role in this process, by its binding to the endometrium and the inhibition of oxytocin receptors synthesis. In particular, IFN-τ is able to induce the production of several proteins, by binding to the apical portion of the uterine glands. The synthesis of these proteins improves the uterine environment and favours embryo survival (Russo et al., 2009).

The EEM is usually due to the incapability of the embryo to signal its presence to the mother and hence to block the production of PGF2α and oxytocin receptors synthesis (Binelli et al., 2001). For these reasons the luteolysis occurs and usually the animal returns in estrus 21 days after insemination. In this case the interruption of pregnancy is very difficult to evaluate. It has been reported in cattle that the incidence of this phenomenon can reach 30% of the inseminated animals, representing one of the main causes of reproductive failure in this species (Robinson et al., 2008). In buffalo specie a delay in progesterone secretion causes early embryonic loss (Campanile et al., 2010). In support of a role for progesterone in early embryonic mortality, several pharmacological treatments aimed to increase progesterone levels can reduce the phenomenon. Buffaloes treated with PGF2α at the time of AI show greater concentrations of progesterone in circulation on Day 10 and Day 20 compared with untreated buffaloes, and the former animals also had a 16% greater pregnancy rate (Neglia et al., 2008). Also, buffaloes transitioning to seasonal anoestrus that showed an increase in progesterone above normal levels in response to treatment with a GnRH agonist on Day 5 after AI have a higher pregnancy rate on Day 40 than buffaloes in which the treatment did not induce progesterone rise

(Campanile et al., 2008a; Campanile et al., 2007c). In addition, for seasonally breeding herds, concentrations of progesterone are greater in oestrous cycles and early pregnancy during the breeding season compared with non-breeding season (Campanile et al., 1989).

8.2. Late embryonic mortality (LEM)

The transitory attachment is considered of primary importance in ruminants and occurs between 16-18 days of pregnancy until 25-30 in various ruminants. A negative role, throughout this process, is played by a transmembrane glycoprotein called Mucine-one (MUC-1). MUC-1 has been described in several mammalian species, included buffalo (Perucatti et al., 2006). The synthesis of this protein during the non-receptive period for the uterine epithelium is very high, whereas it shows a drastic reduction when the endometrium undergoes the action of progesterone (Perucatti et al., 2006). In fact, it has been demonstrated in cattle that the presence of progesterone for 8-10 days is able to block the receptors on the endometrium and, consequently, the endometrial cells are not yet responsive to progesterone stimulation (Geisert & Malayer, 2000). This process results in the block of MUC-1 synthesis for a negative feed-back mechanism. Hence, the embryo is able to attach the uterine epithelium by the interaction between some adhesive factors (Geisert & Malayer, 2000). In this stage the conceptus projects developed structure like villi into the crypts of uterine glands. The role of these structures favours complete attachment progression and furnishes a temporary anchor and adsorpitive structures for the conceptus. Furthermore, these structures allow the absorpion of the endometrial glandular secrete, a complex of histotrphic substances and proteins (Spencer & Bazer, 2002). These growth factors, enzymes, cytokines, lymphokines, hormones, transport proteins and other substances have a key role in embryo nutrition and development, other than allowing the production of the first signals for the maternal recognition of pregnancy.

A decline in progesterone secretion causes a late embryonic mortality (Day 25 to Day 45). In fact a progesterone concentrations are typically normal on Day 10 after AI but undergo a progressive decline (Figure 4) thereafter (Russo et al., 2009). The decline in progesterone after Day 10 may have implications in viability of embryo and successively in transitory attachment phase In fact, progesterone decreases MUC-1 expression which allows blastocyst attachment and the decline in progesterone from Day 10 associated with late embryonic mortality likely results in continued MUC-1 expression and prevention of normal implantation processes.

Plasma levels in pregnant buffaloes that maintain the gestation until day 45 are higher than those recorded in buffaloes which show late embryonic mortality, since day 10 after AI (Campanile et al., 2005). Pregnant buffaloes have also higher plasma P4 on day 20 than both non-pregnant buffaloes and buffaloes that show embryonic mortality. P4 plasma concentration significantly decrease only in non-pregnant buffaloes between day 10 and 20.

It is still unclear if the diameter of the CL is related to progesterone concentrations, but pregnancy rate after AI is higher in relation to high CL blood flow (Russo et al. 2009). Similarly, preovulatory follicle blood flow, as well as that of the CL on day 10 post-AI, is required for optimal function of the CL and the likelihood of pregnancy in buffaloes (Neglia et al., 2011). In fact, buffaloes with decreased CL vascularisation have greater CL blood resistance index on day 25 after AI and this is associated with reduced foetal growth. The forming embryo, on Day 25 after AI, appears as a c-shaped measuring meanly 0.58±0.1 (Mean ± Standard Error) and growth, between Day 25 until Day 45 after AI of of 0.1±0.0 cm. Lower values of TE (thickness of the embryo) are recorded in subjects that undergo embryonic mortality, compared to those that maintain the pregnancy (Neglia et al., 2011). This peculiar finding may reflect an early sign of impending embryonic mortality.

Gametes quality is one of the main factors involved in the phenomenon of LEM in domestic animals. Oocyte quality is able to affect embryo development and interfere with the following gestation. In buffalo species this phenomenon may be more frequent out of the breeding season and during the seasonal anoestrus, which coincides with day length increase (Campanile et al., 2005) and, consequently, with the resumption of sexual promiscuity in the farms in which the out of breeding season mating technique is applied. More than 50% of buffaloes which undergo LEM show P4 concentrations on days 10 and 20 similar to those of animals which maintain pregnancy (Campanile et al., 2005). Therefore, it is possible that other factors, rather than reduced circulating P4 concentrations, also contribute to embryonic mortality. With this regard, it was reported that oocyte quality, judged as the capacity to result in embryonic development and pregnancy, is worse in buffaloes during the anoestrous period (Abdoon & Kandil, 2001), occurring when daylight length increases (Zicarelli, 1997). It is known that in buffalo species high incidence of atresia is present and the mean recovery of good quality oocytes per ovary is low (Gasparrini, 2002). The maturation and the quality of oocyte depend on the function of the granulosa cells that are sensitive to oxidative stress (Dharmarajan et al, 1999). It is worth mentioning that the antioxidant defence system plays a key role in preventing apoptosis and atresia, thus preserving steroidogenic function of granulosa cells (Cassano et al., 1999), although no significant differences in redox status between pregnant, not pregnant and buffaloes with embryonic mortality have been recorded (Spagnuolo et al, 2007).

8.3. Foetal mortality (FM)

After the transitory attachment of the embryo to the endometrium surface, migration of BNCs and formation of syncytia and trinucleate cells, the formation of placenta takes place (Campanile et al., 2009). This is the third, final stage (Guillomot et al., 1981) that completes embryo attachment. In fact, prior to day 16 in the sheep and day 25 in cattle, the placenta is essentially diffuse (Senger, 2005). At this time the chorion initiates attachment to the caruncules of the uterus. Placenta can be defined as a transient organ that is able to sustain metabolic and endocrine roles.

Buffaloes that undergo foetal mortality (Day 46 to Day 90) have normal progesterone on Day 25 and decreased (Figure 4) progesterone on Days 30 and 45 after AI (Vecchio et al., 2008).

Treatment with progesterone, GnRH agonist and hCG on day 25 after AI maintain progesterone at concentrations similar to pregnant, untreated buffaloes (Campanile et al., 2007b) on days 30 and 45 and reduce the incidence of late embryonic mortality, but have no effect on the rate of foetal mortality on day 70. The onset of progesterone secretion by placental tissue in buffaloes has not been determined whilst in cattle significant placental steroidogenesis occurs from around Day 60 (Hoffmann & Schuler, 2002). It may be hypothesized in buffalo that a lack in progesterone secretion during placentation and progesterone placental secretion could cause the phenomenon.

9 AIM OF TESIS

The aims of the present study were to ascertain, in Mediterranean environment:

a) the influence of season on successful of AI in different years;

b) the relationships between CL vascularization, CL function, and pregnancy outcome to AI in buffaloes, are consistent across the breeding season and the transition period to the non-breeding season.

9.1 EXPERIMENT 1

9.1.1 Materials and Methods

Animals and management

The study was conducted in Southern Italy (between 39.0°N and 41.5°N) between 2009 to 2012 (4 years). A total of 1088 Italian Mediterranean buffaloes underwent AI throughout the experimental period. The buffaloes were maintained in open yards that allowed 15 m^2 for each animal. They were fed once daily as a group pen a total mixed ration consisting of 50–55% forage and 45–50% concentrate, containing 0.90 milk forage units ⁄ kg of dry matter and 15% crude protein ⁄ dry matter once daily.

After calving all buffaloes were clinical examined to esclude any gross abnormalities such as uterine fluid.

Synchronization of ovulation, AI and pregnancy

Starting from 40 Days after calving buffaloes were synchronized by the Ovsynch with timed-AI (OVSINCH-TAI). The protocol used, was similar to that developed for cattle (Pursly et al., 1995) and previously applied in buffaloes (Neglia et al., 2003). Briefly, it consists in the injection of a GnRH agonist (buserelin acetate, 12 μg; Receptal®, Intervet, Milan, Italy) on Day 0. On Day seven all injected buffaloes underewent an ultrasound examination with a portable Sonoace Pico equipped with a 10 MHz linear transducer adapted for trans-rectal examination in large domestic animals. Once the ovary was visualized, the colour-Doppler mode was activated to display signals for blood flow in vessels of the corpus luteum (CL). Only buffaloes with an optimal blood flow and echogenicity received a PGF2α analogue (luprostiol, 15 mg; Prosolvin®, Intervet) and GnRH agonist (buserelin acetate, 12 μg; Receptal®, Intervet, Milan, Italy) again on Day 9. Animals without a functional CL were excluded (NCL). Artificial inseminations were performed by the same operator and each buffalo was inseminated once 20 h after the second injection of GnRH agonist. Because of the relatively low intensity of estrous behaviour in buffaloes (Ohashi, 1994) ovaries were palpated per rectum (immediately before AI) to assess estrous status (follicle >1.0 cm and a tonic uterus with the presence or absence of mucous vaginal discharge). Buffaloes without these characteristic were not mated by AI (NMAI). Both NCL and NAI buffaloes were re-synchronized by the same protocol.

Twenty days after AI inseminated buffaloes received 12 μg of GnRH agonist to induce an ovulation in order to:

a) reduce late embryonic mortality (Campanile et al., 2008; Campanile et al., 2008b) in pregnant buffaloes;

b) re-synchronize not pregnant buffaloes.

Twenty-seven days after AI, buffaloes underwent transrectal ultrasonography to assess embryonic development by visualizing the presence of the embryo and a proper heartbeat rate. Ultrasonography was carried out by the same experienced operator. Pregnancy was further assessed on day 45 after AI by ultrasonography: buffaloes with embryonic development on Day 25, but not pregnant on Day 45, were considered to have undergone late embryonic mortality. Not-pregnant buffaloes on day 27 after AI underwent a further PGF2α analogue and a GnRH agonist injection 2 days later. These animals were further inseminated 20 h after the second injection of GnRH agonist.

Statistical analyses

Data were grouped according to 4 seasons (Period 1 = January-March; Period 2 = April-June; Period 3 = July-September; Period 4 = October-December) for both AI and calving period.

Pregancy rates, incidence of ovulatory response (NCL) and not inseminated buffaloes (NMAI) among diffferent years, periods of AI and periods of last calving were analysed by Chi Square test.

In order to assess if days in milk could affect pregancy rate in the different periods of AI, data of days in milk were analysed by a full factorial Anova model including the following factors and their interctions: year of AI (4 levels= from 2009 to 2012), reproductive status (2 levels= pregnant vs not pregnant), period of AI (4 levels, see above) and period of last calving (4 levels, see above).

9.2 EXPERIMENT 2

9.2.1 Materials and Methods

Animals and management

During the last year, an experiment was performed to further evaluate the effect of the season on CL function and pregnancy rate. A total of 256 animals were selected. In particular, 131 buffaloes (130±5 days in milk) were synchronized during a period (period 1) characterized by decreasing day length (late-autumn to early winter), which is recognized as the breeding season, and 125 buffaloes (152±7 days in milk) were synchronized during a period (period 2) characterized by increasing day length (mid-winter to early-spring) which is recognized as the transition period to the non-breeding season for buffaloes in Southern Italy (Campanile et al., 2005).

Synchronization of ovulation, AI and pregnancy

The synchronization protocol used, Ovsynch with timed-AI (OVSINCH-TAI), was similar to that developed for cattle (Pursley et al., 1995) and previously applied in buffaloes (Neglia et al., 2003). Briefly, it consists in the injection of a GnRH agonist (buserelin acetate, 12 µg; Receptal®, Intervet, Milan, Italy) on Day 0, a PGF2α analogue (luprostiol, 15 mg; Prosolvin®, Intervet) on Day 7 and GnRH agonist (buserelin acetate, 12 µg;

Receptal®, Intervet, Milan, Italy) again on Day 9. Artificial inseminations were performed by the same operator and each buffalo was inseminated once 20 h after the second injection of GnRH agonist. Because of the relatively low intensity of estrous behaviour in buffaloes (Ohashi, 1994) ovaries were palpated per rectum (immediately before AI) to assess estrous status (follicle >1.0 cm) and a tonic uterus with the presence or absence of mucous vaginal discharge. Twenty-seven days after AI, buffaloes underwent transrectal ultrasonography to assess embryonic development by visualizing the presence of the embryo and a proper heartbeat rate. Ultrasonography was conducted with a portable Sonoace Pico equipped with a 10 MHz linear transducer adapted for transrectal examination in large domestic animals. Ultrasonography was carried out by the same experienced operator. Pregnancy was further assessed on day 45 after AI by ultrasonography: buffaloes with embryonic development on Day 25, but not pregnant on Day 45, were considered to have undergone late embryonic mortality.

Morphology of the CL and function

Morphology of the CL and function were assessed by ovarian ultrasonography (see above) on 39 buffaloes in each of Periods 1 and 2. Features of the CL (dimensions and blood flow parameters) were recorded on Days 10 and 20 after AI. Once the ovary was visualized, the image was adjusted in order to have optimal definition of the CL and the image held to measure the long and short axes. The colour-Doppler mode was then activated to display signals for blood flow in the CL and the spectral mode was applied to calculate the resistive index (RI), pulsatily index (PI) and time average medium velocity (TAMV). All Doppler scans were performed at a constant colour-gain setting, velocity setting, and a colour-flow filter setting. The entire CL was scanned in a slow continuous motion. Real-time B-mode⁄colour-Doppler images of the continuous scans were recorded with a digital video-recording system for subsequent analysis.

Progesterone

Function of the CL was determined by measuring circulating concentrations of P4 by RIA (Niswender, 1973; Skaggs et al.,1986) in blood samples collected from the jugular vein into heparinized tubes on Days 10 and 20 after AI. Samples were centrifuged at 800 x g for 15 min and the serum stored at -20°C until required for P4 assay which was carried out at the same time for all samples. Concentrations of P4 > 1.5 ng⁄ml were considered to be indicative of the presence of an active CL (Zicarelli et al., 1997). The minimum detectable amount of progesterone was 2.1 ± 0.1 pg and the intra- and inter-assay coefficients of variation were 6.2% and 11.8%, respectively.

Statistical analyses

Differences in pregnancy rates and embryonic mortality between Period 1 (breeding season) and Period 2 (transition period) were analyzed by Chi-square test. Changes (Δ) in CL dimensions (diameter and area) and P4 concentrations were calculated by subtracting respective values at Day 10 from values at Day 20. Differences between Periods 1 and 2 in CL dimensions, CL growth (Δ), concentrations of P4, and blood flow parameters (TAMV, RI and PI), were analysed by Student's *t*-test. Differences between pregnant and non-pregnant buffaloes in the same ovarian variables as above on Days 10 and 20 after AI were analyzed by Student’s t-test. Two-tailed correlations were performed among CL dimension and growth (Δ), P4 concentrations, blood flow parameters (ΔP4, TAMV, RI and PI) on Days 10 and 20 after AI using PASW Statistic 18.0 (PASW STATISTIC, 2009) statistical software.

9.3 Results experiment 1

9.3.1. Reproductive parameters

Throughout the trial overall fertility rate was meanly 75.5% (821/1088) with an intercalving period of 416±2.4, without differences among the years. In particular, a lower ($P<0.01$) fertility rate was recorded in the first year (161/231; 69.7%) vs. the second (222/272; 81.6%), whereas intermediate values were recorded on the third (210/289; 72.7%) and fourth year (228/296; 77.0%).

The number of service for conception (NSC) throughout the trial was meanly 2.07, varying from 1.87 (2012) to 2.25 (2009 and 2011). The conception rate at the first service (62.4%; 512/1088) was significantly ($P<0.01$) higher than those recorded at other services (Table 1).

Table 1. Number of services/conception (NSC), number of inseminated buffaloes and percentage conceptions on both each service and in total

NSC	1	2	3	4	5	6	7
No inseminated buffaloes	1088	576	371	305	285	285	269
Number of conceptions	512	205	66	20	11	5	2
% conceptions	47.1^{A}	35.6^{B}	17.8^{C}	6.6^{D}	3.9^{DE}	1.8^{E}	0.7^{E}
% total conceptions	62.4	25.0	8.0	2.4	1.3	0.6	0.2

Values in the same row with different superscripts are significantly different (^{A,B,C,D,E}, $P<0.01$).

The conception rate significantly decreased progressively from the second to the seventh service (Table 1). Around 66% (717/1088) of mated buffaloes were pregnant within the second service and these represent the 87.4% (717/821) of the total conceptions (Table 1).

9.3.2. Synchronization efficiency

A total of 3,429 buffaloes were synchronized during the study. Interestingly, 28.3% animals (971/3,429) did not have a functional CL (NCL) after the first GnRH and 21.5% (736/3,429) were excluded on the day of AI (NMAI) because did not show signs of estrus (Table 2). Therefore, 49.8% of buffaloes (n = 1,707) were not inseminated. A higher ($P<0.01$) incidence of NCL and NMAI was recorded during the second and third period, compared to the first and the fourth period (Table 2). In particular, the incidence of not inseminated buffaloes (NCL + NMAI) was lower ($P<0.01$) during the breeding period (Period 4: 259/567; 45.7%) than Period 2 (512/1,014; 50.5%) and Period 3 (603/1157; 52.1%). Data registered in buffaloes synchronized during transitional period (Period 1) showed a intermediate value (333/691; 48.2%).

It is worth pointing out that a higher incidence of subjects that showed a lower ovulatory response after the first GnRH (NCL) was recorded during periods carachterized by a higher daylight length (Periods 2 and 3), compared to Period 1 and 4 (Table 2). Instead, the incidence of not inseminated buffaloes (NMAI) was higher during the transitional period (Period 1) compared to the others (Table 2).

Table 2. Number and percentage of buffaloes that were not inseminated beacuse did not have both a functional CL on day 7 after the first GnRH (NCL) or signs of estrus on the day of AI (NMAI) throughout the experimental period, in different years and different periods.

Period/Year	2009		2010		2011		2012		Total	
	NCL % (n.)	NIS % (n.)	NCL % (n.)	NIS % (n.)	NCL % (n.)	NIS % (n.)	NCL % (n.)	NIS % (n.)	NCL % (n.)	NIS % (n.)
Period 1	19.2	14.3	23.1	30.9	26.7	38.6	21.5	45.1	22.7^{A}	25.4^{Aa}
	(30)	(18)	(37)	(38)	(48)	(51)	(42)	(69)	(157)	(176)
Period 2	24.2	32.7	34.8	24.0	31.3	30.1	28.4	26.5	30.2^{B}	21.8^{B}
	(38)	(60)	(78)	(35)	(100)	(66)	(81)	(54)	(297)	(215)
Period 3	30.3	20.3	33.2	26.7	31.2	34.7	31.7	34.0	31.9^{Bb}	20.2^{B}
	(60)	(28)	(112)	(60)	(103)	(77)	(94)	(69)	(369)	(234)
Period 4	20.4	12.8	25.4	30.9	27.2	10.3	29.2	46.5	26.1^{a}	19.6^{b}
	(20)	(10)	(41)	(37)	(40)	(11)	(47)	(53)	(148)	(111)
Total	24.3^{Xx}	18.1^{X}	30.3^{Y}	19.3^{X}	30.0^{Y}	21.1^{X}	28.1^{y}	26.1^{Y}	28.6	20.8
	(148)	(116)	(268)	(170)	(291)	(205)	(264)	(245)	(971)	(736)

Values in the same row with different superscripts are significantly different (^{X,Y}, $P<0.01$; ^{x,y}, $P<0.05$).
Values in the same column with different superscripts are significantly different (^{A,B}, $P<0.01$; ^{a,b}, $P<0.05$).

9.3.3. Pregnancy rate

Pregnancy rate on 40 Day after AI was lower in Period 1 (transitional period) compared to other periods (Table 3). The incidence of LEM registered in Period 1 (26/176; 15%) was significantly higher than that in Period 2 (21/266; 7.9%; $P<0.05$), Period 3 (23/312; 7.4%, $P<0.01$) and Period 4 (10/161; 6.2%, $P<0.05$). A significantly lower pregnancy rate was also recorded in 2009 and 2011 compared to 2010 and 2012.

Table 3. Number of inseminated buffaloes (AI) and pregnancy rate on day 40 after insemination throughout the experimental period, in different years and different periods.

Year/Period	Period I		Period II		Period III		Period IV	
	AI (n)	G (%)	AI (n)	G (%)	AI (n)	G (%)	AI (n)	G (%)
2009	108	31.5^{AXx}	88	43.2	110	53.3^{abY}	68	51.5^{y}
2010	85	53.0^{B}	111	51.4	165	50.9^{ab}	83	47.0
2011	81	42.0^{AB}	153	45.1	145	45.5^{a}	96	43.7
2012	84	44.0^{ABx}	150	54.0	134	59.7^{by}	61	50.8
Total	358	41.9^{Xx}	502	48.8^{y}	554	52.2^{Y}	308	47.7

^{a,b} means within rows, for respective parameters, with a different superscript differ ($P < 0.05$);
^{A,B} means within rows, for respective parameters, with a different superscript differ ($P < 0.01$);
^{X,Y} means within columns with a different superscript differ ($P < 0.01$).

Differences ($P<0.01$) were found between days in milk in inseminated buffaloes between the four season of the year (Period 1 to Period 4). In fact buffaloes inseminated during the Period 1 and Period 4 showed a higher days in milk. In every case no differences were found between pregnant and not pregnant in each period and no interaction was registered between reproductive status and days in milk.

9.4. RESULTS experiment 2

9.4.1 Late embryonic mortality and pregnancy rate

In Period 1 (breeding season), 62.6% (82/131) of buffaloes had embryonic development on Day 25 after AI and 58.0% (76/131) were pregnant on Day 45, which represented a late embryonic mortality of 7.3% (6/82). In Period 2 (transition period), 59.2% (74/125) of buffaloes had embryonic development on Day 25 after AI and 45.6% (57/125) were pregnant on Day 45, which represented a late embryonic mortality of 23.0% (17/74). There was no difference ($P > 0.05$) between Periods 1 and 2 in the proportion of buffaloes that had embryonic development on Day 25 after AI whilst the proportion of buffaloes pregnant on Day 45 was greater ($P < 0.05$) in Period 1 compared with Period 2. The latter was due to a greater ($P < 0.05$) late embryonic mortality in Period 2 (transition period) compared with Period 1 (breeding season).

For the 78 buffaloes that were intensively observed (39 in each period), 56.4% (22/39) and 53.8% (21/39) showed embryonic development on Day 25 after AI during the breeding season and the transition period, respectively. Pregnancy rates decreased to 53.8% (21/39) during the breeding season and 43.6% (17/39) during the transition period on Day 45 after AI, which reflected late embryonic mortality of 4.5% (1/22) and 19.0% (4/21), respectively. There were no differences (P>0.05) in absolute pregnancy rates on Day 25 and Day 45 after AI.

9.4.2 Morphology of the CL and function

There was no interaction between reproductive status (pregnant or non-pregnant), period (Period 1 and Period 2) and the different parameters measured. Data for pregnant and non-pregnant buffaloes were therefore pooled within each period (Stevens J, 1990).

The dimensions of the CL on Days 10 and 20 after AI did not differ (P > 0.05) between Period 1 (breeding season) and Period 2 (transition period) (Table 4).

Table 4. Corpus luteum (CL) size, area and circulating concentrations of progesterone (P4) during the breeding season (BS) and the transition period to the non-breeding season (TP) in Italian Mediterranean buffaloes. Results are for Day 10 and Day 20 after AI and are presented as means ± sem.

	CL size (mm)		CL area (mm^2)		P4 (ng/ml)	
	Day 10	Day 20	Day 10	Day 20	Day 10	Day 20
BS	19.5 ± 0.6	18.9 ± 0.7	28.5 ± 1.5	27.9 ± 2.1	4.6 ± 0.3^{A}	3.4 ± 0.2^{A}
TP	19.5 ± 0.5	20.0 ± 0.6	29.3 ± 1.3	24.6 ± 2.4	1.6 ± 0.12^{B}	1.8 ± 0.2^{B}

^{A,B} means within columns with a different superscript differ ($P < 0.01$)

In contrast, concentrations of P4 on Days 10 and 20 after AI for all buffaloes were greater ($P < 0.05$) in Period 1 compared with Period 2 (Table 4). Buffaloes pregnant on Day 45 after AI had greater ($P < 0.01$) concentrations of P4 on Days 10 and 20 after AI in Period 1 (4.9 ± 0.4 and 4.1 ± 0.2 ng/ml, respectively) than in Period 2 (1.9 ± 0.2 and 2.4 ± 0.2 ng/ml, respectively). All buffaloes that did not conceive in Period 1 showed a normal estrous cycle whilst for buffaloes that did not conceive in Period 2, 40% (8/20) were acyclic and 15% (3/20) showed a delayed luteal phase.

The change in dimensions of the CL from Day 10 to Day 20 after AI did not differ ($P > 0.05$) between Period 1 and Period 2. Buffaloes in Period 1 showed a greater ($P < 0.01$) decline in concentrations of P4 from Day 10 to Day 20 after AI compared with buffaloes in Period 2 (Table 5).

Table 5. Corpus luteum (CL) growth (Δ size and area) and changes in circulating concentrations of progesterone (ΔP4), from Day 10 to Day 20 after AI, during the breeding season (BS) and the transition period to the non-breeding season (TP) in Italian Mediterranean buffaloes. Results are presented as means ± sem.

	Δ CL size (mm)	Δ CL area (mm^2)	ΔP4 (ng/ml)
BS	-1.2 ± 0.8	0.3 ± 2.2	-1.2 ± 0.3^A
TP	-2.1 ± 1.2	0.9 ± 1.7	0.2 ± 0.1^B

^{A,B} means within columns with a different superscript differ ($P < 0.01$)

The Time Average Medium Velocity (TAMV) on Day 10 after AI was greater ($P < 0.01$) for buffaloes in Period 1 than in Period 2 (Table 6).

Table 6. Corpus luteum time average medium velocity (TAMV), resistive index (RI) and pulsatility index (PI) for Italian Mediterranean buffaloes in the breeding season (BS) and the transition period to the non-breeding season (TP). Results are for Day 10 and Day 20 after AI and are presented as means ± sem.

	TAMV		RI		PI	
	Day 10	Day 20	Day 10	Day 20	Day 10	Day 20
BS	19.3 ± 1.5^{A}	10.4 ± 0.6	0.5 ± 0.0	0.4 ± 0.0	0.7 ± 0.0	0.6 ± 0.0
TP	8.3 ± 0.7^{B}	9.5 ± 0.5	0.5 ± 0.2	0.4 ± 0.0	1.3 ± 0.4	0.7 ± 0.1

^{A,B} means within columns with a different superscript differ ($P < 0.01$)

9.4.3 Conception and CL morphology and function

The following data are combined for Period 1 and Period 2. The dimensions of the CL on Day 10 and Day 20 after AI were greater ($P < 0.01$) for buffaloes that were pregnant on Day 45 after AI compared with buffaloes that were not pregnant (Table 7). Buffaloes pregnant on Day 45 also had a greater ($P < 0.01$) CL area on Day 20 after AI (Table 4). Concentrations of P4 on Day 10 and Day 20 after AI were greater ($P < 0.01$) for buffaloes that were pregnant on Day 45 after AI compared with buffaloes that were not pregnant (Table 7).

Table 7. Corpus luteum (CL) dimensions (size and area) and circulating concentrations of progesterone (P4) on Day 10 and Day 20 after AI. in pregnant (P) and non-pregnant (NP) Italian Mediterranean buffaloes. Results are presented as means ± sem.

	CL size (mm)		CL area (mm^2)		P4 (ng/ml)	
Day post AI	P	NP	P	NP	P	NP
n	38	40	38	40	38	40
10	20.5±0.5^{A}	18.3±0.6^{B}	30.0±1.3	27.6±1.6	3.5±0.4^{A}	2.6±0.3^{B}
20	20.8±0.5^{A}	17.7±0.8^{B}	34.3±1.5^{A}	17.9±2.3^{B}	3.4±0.2^{A}	1.8±0.2^{B}

^{A,B} means within rows, for respective parameters, with a different superscript differ ($P < 0.01$)

Buffaloes that were pregnant on Day 45 after AI showed an increase (0.3 ± 0.5 mm) in size (long axis) of the CL from Day 10 to Day 20 after AI whilst a decrease (- 4.1 ± 1.5 mm) occurred in buffaloes that were not pregnant ($P < 0.01$). Pregnant buffaloes also showed an increase (4.4 ± 1.5 mm^2) in area of the CL from Day 10 to Day 20 after AI whilst the latter buffaloes showed a decrease (- 4.8 ± 2.2 mm^2) ($P < 0.01$). Respective changes in concentrations of P4 from Day 10 to Day 20 after AI did not differ ($P > 0.05$) within buffaloes that were pregnant (- 0.18 ± 0.29 ng/ml) on Day 45 after AI and buffaloes that were not pregnant (- 0.83 ± 0.23 ng/ml).

The CL time average medium velocity (TAMV) on Day 10 after AI was greater ($P < 0.05$) for buffaloes pregnant on Day 45 after AI compared with buffaloes that were not pregnant (Table 8). The CL resistive index (RI) and pulsatily index (PI) on Days 10 and 20 after AI did not differ ($P > 0.05$) between buffaloes that were pregnant on Day 45 after AI and buffaloes that were not pregnant (Table 8).

Table 8. Corpus luteum time average medium velocity (TAMV), resistive index (RI) and pulsatility index (PI) in pregnant (P) and non-pregnant (NP) Italian Mediterranean buffaloes on Day 10 and Day 20 after AI. Results are presented as means ± sem.

	TAMV		RI		PI	
Day post AI	P	NP	P	NP	P	NP
n	38	40	38	40	38	40
10	15.3 ± 1.5^{aX}	10.5 ± 1.3^{b}	0.4 ± 0.0	0.5 ± 0.0	0.9 ± 0.3	1.1 ± 0.3
20	9.9 ± 0.5^{Y}	10.0 ± 0.7	0.4 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	0.6 ± 0.1

^{a,b} means within rows, for respective parameters, with a different superscript differ ($P < 0.05$);
^{X,Y} means within columns with a different superscript differ ($P < 0.01$)

The TAMV values of pregnant buffaloes on Day 10 were higher ($P<0.01$) than those recorded on Day 20.

9.4.4 Correlations

For pregnant buffaloes, there were positive correlations between CL TAMV on Day 10 after AI and P4 concentrations on Day 10 ($r = 0.722$; $P < 0.01$) and Day 20 ($r = 0.446$; $P < 0.01$).

10. DISCUSSION

The aims of the present study were to ascertain, in Mediterranean environment, the influence of the season on the successful of the timed AI and the CL functionality.

The commonest estimate of fertility rate is the percentage of mated or inseminated cows that become pregnant (pregnancy rate) or finally calve (calving rate), on both the total number of adult animals (real fertility) or the number of subjects that delivered on the previous year (apparent fertility).

The results of the study demonstrated that mean fertility rate in buffaloes undergone timed artificial insemination range between 70 and 80% with an intercalving period of meanly 416 days. A higher value of intercalving period (487 days) was reported in 6,052 buffaloes naturally mated with 75% fertility rate (Zicarelli, 2007). It is likely that the application of timed AI improve the reproductive performance in buffalo herds. In fact, data recorded in this study demonstrated optimal values of both fertility and intercalving period. The explanation to this interesting phenomenon may be find in a proper and constant reproductive management that is performed when buffaloes underwent AI. Furthermore, it can not be ruled out that the seasonality of the species may affect also male libido and fertility: it has been reported that sexual activity and libido of the bulls is lower during periods characterized by increasing daylight length (Sansone et al., 2000). Furthermore, to underline the sensitivity to seasonality in the buffalo species and in particular in the bull, it has been reported a neuro-endocrine interaction between androgen hormones and the autonomic nerve supply in the regulation of male buffalo reproductive functions. In fact, during the mating period, a dense noradrenergic innervations can be observed to supply the vas deferens as well as the accessory sex glands, whereas during the non-mating period the noadrenergic nerves are dramatically and significantly reduced (Mirabella et al., 2007). This aspect may be responsible of both lack of conceptions by the bulls and no biostimulation in females, increasing the seasonal anoestrus. Furthermore, it has to be considered that in natural mating conditions the day of insemination is not known, unless the mating is not recorded by the farmer. A high incidence of both late embryonic (LEM) and fetal mortality (FM) are recorded during periods of increasing daylight length. These phenomena are due to a lack of P4 levels increase after Day 10 (LEM) and 25 (FM) post-insemination (Russo et al., 2009). Several studies (Campanile et al., 2007; Vecchio et al., 2008), showed that delayed treatments by trophyc hormones, such as GnRH and hCG, reduced the incidence of LEM and FM, by increasing P4 levels. In our study, the treatment that is performed by GnRH on day 20 post-AI may have resulted in both the re-synchronization of not pregnant animals and the reduction of LEM and FM.

In the last few years fertility rate in dairy cattle dramatically decreased worldwide (Washburn et al., 2002), ranging from 30 to 60% in lactating cows and virgin dairy heifers, respectively (Pursley et al., 1997). Furthermore and increase of days open was observed (Lucy 2001). The stress of high milk production, along with increasing herd size, changes in facilities, management and the use of low forage:concentrate ratio, are probably on the basis of this reduction.

Buffalo breeding in Italy has reached a high grade of innovation, similar to that observed in dairy cattle. Animals are maintained in cement paddocks and a total mixed ration, characterized by at least 0.90 milk forage units and 15% crude protein on dry matter basis is constantly administered (Campanile et al.,2006; Campanile et al, 2010a). It can not be ruled out that in the next years reproductive performance will be influenced, similarly to that observed in dairy cattle breeding. Actually, no data on reproductive parameters are available for buffalo herds in intensive breeding. Therefore, the results of this study represent the first information in this sense.

The number of service per conception (NSC) can be defined as the number of mating that are necessary for the cow to become pregnant. In our study, a mean value of 2.07 service/conception has been recorded throughout the study, varying from 1.87 to 2.25. The NSC depends largely on the breeding system used: it is higher under uncontrolled natural breeding and low where hand-mating or artificial insemination is used. Therefore, the evaluation of NSC in buffaloes naturally mated is difficult to calculate, especially if the seasonality of the species is taken into account: the anoestrus length and the application of the out of breeding season mating technique may affect this parameter (Campanile et al., 2010; Campanile et al., 2009).

The number of services required for conception has increased as well in dairy cows, due to the low fertility. In a recent study (Yamaguchi et al., 2010), the NSC was analysed in relation to conception rate in 8,386 dairy cows bred in 40 commercial herds in Japan. The conception rate from the first to the seventh service was 40.2 to 54.0%. However, although the conception rate at the first service was significantly lower 40.2%, no significant differences were recorded after the second-service. On the contrary, in our study, the conception rate at the first service was higher than those recorded subsequently. This demonstrates that reproductive efficiency in buffalo species is still higher than that actually recorded in dairy cow. The reasons for this interesting result, can be find in the characteristics of the two species. Based on the analyses of large datasets, there is clearly an antagonistic relationship between milk production and reproduction in dairy cattle (Lucy, 2001). High milk production causes a forced negative energy balance: in fact, during early lactation, cows undergo a normal process of nutrient partitioning and adipose tissue mobilization, to face nutrient requirements for maintenance and lactation, which exceed the ability of the cow to consume energy in the feed (Bauman and Currie, 1980). Negative energy balance, therefore, causes weight loss, and decreased BCS, impairing reproductive performance. As other ruminants, buffaloes undergo negative energy balance. However, the lower energy production recorded at the start of lactation in buffalo species, reduces adipose tissue mobilization and hence the negative energy balance. This, together with the low incidcence of post-partum diseases may be accounted for the different results.

In a previous study (Yamaguchi et al., 2010) around 84% of the dairy cows became pregnant between the first and the third service and the average

NSC was 2.2. In our study, 87.4% of the total conceptions was obtained within the second insemination. However, the selection of the buffaloes at the time of prostaglandins administration and AI may explain these results. In fact, around 50% of the synchronized buffaloes were excluded from AI, throughout the study. In particular, 28.3% of the subjects did not show a functional CL after the first GnRH injection. This value is similar to that recorded in previous trials (Zicarelli et al., 1997). In this case, when estrus induction was performed by double PGF2a 12 days apart, only 71.4% of the treated animals received the second dose of PGF2a: this demonstrates that 28.4% of the buffaloes did not show a functional CL. Interestingly, in our study, this phenomenon was particularly evident during the second and third periods, in coincidence with increasing daylight length. In this period, in fact, a high incidence of buffaloes that enter the seasonal anoestrus is usually recorded (Campanile et al., 2010), showing a progressive decline in CL activity (Campanile et al., 2010; Zicarelli et al., 1994b). This would be further confirmed by the evidence that a lower number of buffaloes were excluded in Period 1 and 4 on the day of PGF administration.

No differences were found in all seasons and in all years between reproductive status and days in milk and, thus, the season may affect the pregnancy rate. The comparison among periods showed a lower pregnancy rate in buffaloes inseminated during the transitional period. This data is affected by two factors: 1) a higher percentage of buffaloes that were not inseminated (NCL and NMAI) and 2) a higher incidence of LEM. In fact, the pregnancy rate calculated on synchronized buffaloes was about 24%, without differences among the periods. Therefore, the selection of buffaloes that are involved in AI is particularly important for the final yield in terms of pregnancy rate. Furthemore, the incidence of LEM recorded in period 1. For this reason, experiment 2 was designed to verify embryonic development and CL activity in these two seasons.

In experiment 2 the proportion of buffaloes that had embryonic development on Day 25 after AI did not differ between the breeding season (82/131, 62.6%) and the period of transition to the non-breeding season (74/125, 59.2%). However, a greater proportion of buffaloes were pregnant on Day 45 after AI during the breeding season (76/131, 58.0%) than in the transition period (57/125, 45.6%). This reflected a greater late embryonic mortality during the transition period (23.0%) compared with the breeding season (7.3%). Buffaloes showed a reduced function of the CL during the transition period which would seem to explain, at least in part, an apparent lower capacity to sustain embryonic development. A reduced function of the CL during the transition period could also potentially lower the capacity for embryonic attachment and the establishment of a pregnancy (Campanile et al., 2009; Campanile et al., 2010; Russo et al., 2009). In previous studies in Italian Mediterranean buffaloes maintained under similar conditions as the present study, late embryonic mortality was between 20-40% during the transition period (Campanile et al.,2005; Campanile et al., 2007 a; Campanile et al., 2007 b) and around 7% during the breeding season (Baruselli et al.,1997; Vecchio et al., 2012). A characteristic of CL function during the transition period is the delayed rise in circulating concentrations of P4 (Campanile et al., 2005; Vecchio et al., 2008).

The size of the CL did not differ between the breeding season and the transition period. In contrast, concentrations of P4 on Days 10 and 20 after AI were around 2 to 3 times greater during the breeding season compared with the transition period. The proportion of buffaloes with reduced CL function during the transition period has previously been shown to vary from 5% to 50% (Campanile et al., 2010; Campanile et al., 1992). Rather than absolute size, it would seem that the rate of growth of the CL from Day 5 to Day 10 after AI is related to CL function and concentrations of P4 in circulation (Vecchio et al., 2012). The rate of growth of the CL in buffaloes is related to CL blood flow (Vecchio et al., 2012) and, in a recent study, CL blood flow was related to pregnancy (Russo et al., 2009; Vecchio et al., 2012). In the present study, buffaloes with a greater CL time average medium velocity (TAMV) also had greater concentrations of P4 on Days 10 and 20 after AI.

The reason why blood flow to the CL in buffaloes is greater during the breeding season than in the transition period to the non-breeding season is not known. It was recently shown that the expression of vascular endothelial growth factor (VEGF) by the CL changed during the oestrous cycle and was related to circulating concentrations of P4 in buffaloes, which was also reported in cattle (Papa et al., 2007). It could be speculated that the expression of VEGF by the CL is greater during the breeding season compared with the transition period in buffaloes.

The TAMV values recorded on Day 10 after AI in pregnant buffaloes, independent of period, were higher than those observed on Day 20. This phenomenon has been previously observed in pregnant cattle (Herzog et al., 2011) and buffalo (Vecchio et al., 2012) where TAMV values increased to Day 10 and then decreased to minimal values by Day 18. A major proportion (20-40%) of blood flow to the ovary containing a functional CL in the cow is supplied by the ipsilateral uterine artery through an anastomosis with the ovarian artery. Both the quantity of blood and the direction of flow through this anastomosis changes during the oestrous cycle, perhaps because of changes in uterine blood flow and/or vascular resistance (Ford and Chenault, 1981). These changes are most likely attributable to vasocostriction and indicate reduced uterine perfusion during the phase of pregnancy recognition, which is considered the most critical period during the establishment of pregnancy at Days 15-17 (Herzog et al., 2011; Ford and Chenault, 1981).

11.CONCLUSIONS

The results obtained in this trial supply the first information on reproductive parameters in intensive buffalo breeding with the application of timed artificial insemination. By this study it can be confirmed that the seasonality of the species impairs the reproductive efficiency of the herd, although some important considerations need to be underlined. Firstly, the application of AI in some periods of the year may be more efficient than natural mating, reducing the negative influence of the season on male fertility. This interesting aspect has to be considered and probably applied in the reproductive management of buffaloes in intensive breeding. The synchronization schedule applied in our study may represent a standard protocol to improve the reproductive efficiency and increase the genetic merit of the herd, by utilizing animals at 40 days after calving: in this way, more than 87% of the pregnancy would be obtained within 80 days post partum, further reducing the intercalving period and the incidence of late embryonic and foetal mortality. The remaining not pregnant animals may undergo natural mating or, eventually, culling.

The season also affects CL vascularization and functionality, increasing the incidence of late embryonic mortality and reducing pregnancy outcome to AI, particularly during the transitional period. Further studies are needed to elucidate the reasons for a greater proportion of buffaloes with reduced CL function during this period.

12. REFERENCES

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