Sexed Semen and AI in the Mediterranean Buffaloes  
(*Bubalus bubalis*)

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**ABSTRACT**  
The technology of Artificial Insemination (AI) was rudimentarily attempted for the first time in 1779 by L. Spallanzani in the dog, and it can be considered the very first applied reproductive technology which is nowadays employed worldwide in many animal species, both farm animals and endangered or wild species. Within the buffalo breeding management, young bulls are probably among the easiest mammals to be trained to serve an artificial vagina, although natural mating is still responsible for most of the pregnancies occurring among herds. Recently, an improvement in the efficiency of protocols for synchronization of ovulation has been reported in buffaloes, paving the road for a wider implementation of assisted reproduction in this species and particularly AI. In addition, since the very first report of buffalo calves born following use of sexed semen in conjunction to AI, the technology of sexing sperm cells has become an additional asset in the toolbox of reproductive technologies implemented within the most advanced buffalo farms. The optimization of protocols for synchronization of ovulation and the use of high quality frozen/thawed semen produced by truly superior bulls, in association to acceptable pregnancy rates and birth of live calves, will surely determine the wider diffusion of AI for both non sexed and sexed semen in the buffalo management worldwide for a faster enhancement of the genetic potential and its distinctive production.

**Keywords:** AI, buffaloes, sexed semen

**INTRODUCTION**  
In the field of animal production, the use of AI has always brought within its application a number of advantages related to health, genetics and the overall zoo-economy. From a sanitary point of view, since its implementation this technology has made possible the halt to the transmission of some major venereal infectious diseases, such as *vibrio* and *trichomonas*, that were usually spread through natural mating. On a different hand, by using AI, one bull may produce hundreds of thousands of calves, taking into consideration the number of straws that can be obtained from a single ejaculate following dilution and freezing. In a more global picture, some of the advantages derived from the use of frozen/thawed semen through AI can be identified as: i) the availability of semen even after death of proven sires, and ii) the ease of moving semen to promote genetic improvement in other Countries. 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. In the milk industry, the adoption of AI allows the evaluation of sires through progeny testing of daughters whose productive characteristics can be monitored and confronted. Until few decades ago the use of AI in the buffalo species was considered in its infancy and adopted only by few farmers, and therefore the implementation was only marginal for different reasons for both the river and more significantly for the swamp sub-species. In fact, the diffusion 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 had been kept marginal, due mainly to lack of truly superior progeny tested bulls and to unacceptable low pregnancy rates when using both natural or synchronized estrus (Zicarelli et al., 1997). In some Countries like Italy, buffalo breeding is of particular competitiveness and importance and it has been on the rise in terms of population heads when compared to other more firmly established forms of livestock. Within the buffalo industry there is an ever increasing demand for milk, associated though with the need to cut production costs. In this scenario reproductive biotechnologies may become of stringent importance, by allowing the inclusion of specific selective programs within a more traditional framework of breeding strategies of which AI still retain its primary role. Following the first proven evidence of offspring derived from the use of sexed sperm cells and AI in rabbit (Johnson et al., 1989), the same technology, rooted in the differential amount of DNA content between X- and Y-bearing sperm cells (Garner, 2006), was used with success in cattle and in a number of other production animals (Rath and Johnson, 2008). More recently, artificial breeding in buffaloes has witnessed the introduction of the sexed semen technology, and following the birth of the very first buffalo calves through AI and sexed semen (Presicce et al., 2005a; 2005b), more available and published data has made evident the particular efficiency of such technology in this species (Campanile et al., 2012).

BUFFALO SEMEN QUALITY FOR AI WITH SEXED SEMEN

Buffalo bulls are characterized by a slower sexual development when compared to cattle bulls and, 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). A number of factors such as hormonal fluctuations, are linked to the photo-neuroendocrine system influencing thus the male reproductive efficiency (Seren and 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 hypothalmo-hypophysial activity, gonadal function and finally sperm composition and quality (Zicarelli, 1997). 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). In fact, some studies (Arrighi et al., 2010), 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 (Saeed et al., 1990; Kumar et al., 1993; Presicce et al., 2003). 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, 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 noradrenergic nerves are dramatically and significantly reduced (Mirabella et al., 2007). With regard to parameters usually taken into consideration for semen quality, minimal standards for a classification of “probably fertile” specimen of buffalo semen are: a) over 500,000/mm$^3$ spermatozoa; b) more than 60% of motile sperm with forward progression and c) more than 70% of the spermatozoa conforming to normal morphology. Normal buffalo sperm concentration shows a wide range of variation (600,000 to 1,200,000 cells per mm$^3$ i.e. 800,000 per mm$^3$ on average) and this parameter is highly sensitive to seasonal and nutritional factors. Usually, first ejaculate contains higher number of spermatozoa per ml compared to second ones, and the seasonal and climatic factors have a strong effect on the morphological and chemical seminal characteristics. In temperate regions, it has been reported that better quality semen is produced during the winter and spring while it has been shown deterioration in semen quality during summer and autumn. On the other hand, in tropical regions the quality of semen is found to be good during the rainy season. Moreover, in warm and humid tropical Amazon region, the best time to work with semen freezing is between January and June. Buffaloes are sensitive to heat stress, thus decline in semen quality is a common finding during the hot season of the year. The best manner to overcome the problem of semen quality deterioration due to heat stress during summer is to sprinkle the animals with water during the hotter part of the day or allow the animals to wallow, protect the animals from radiation exchange and hot wind, and keep the animals in ventilated paddock. Free access of the animal to water and shadow is very important, since buffalo have a poorly-developed thermo-regulatory system, causing them to suffer of heat stress during summer. Semen with more than 30% initial dead spermatozoa may not to be good for freezing and semen samples with less than 50% of live spermatozoa may be of questionable fertility even throughout natural breeding. Complex double staining with Trypan Blu and Giemsa has been used in buffalo spermatozoa (Figure 2), to evaluate integrity of acrosome, tail and midpiece (Presicce et al., 2003; Boccia et al., 2007).
SEXED SEMEN AND AI

Following the first study on the detection of buffalo sex chromosomes in spermatozoa by fluorescence in situ hybridization (FISH) by Revay et al. (2003), where the evolutionary conservation of this locus in the water buffalo has been demonstrated, an increasing interest has been shown on the possibility to use sexed semen in the buffalo breeding strategies. A preliminary study was designed to show the possibility to use a significantly reduced number of spermatozoa, and at the same time to obtain acceptable pregnancy rates (Presicce et al., 2004). Subsequently, the same concentration was used to inseminate pluriparous buffaloes with sexed semen aided by a special catheter (Ghent device) designed on purpose to deposit such a reduced sperm number at the utero-tubal junction in order to allow the highest availability of sperm cells at the site of fertilization (Presicce et al., 2005a). This first study showed unequivocally that sexed semen technology in buffaloes allows sperm cell populations to be selectively sorted and enriched with purity similar to what previously reported for cattle bulls. Following these first encouraging results, the impact and feasibility of flow cytometric sorting of X- and Y- sperm cells of Murrah and Nili-Ravi buffalo bulls have been studied (Lu et al., 2006), leading to the born of the first buffalo calves produced by the combined use of in vitro fertilization procedures (IVF) and sexed semen (Lu et al., 2007), showing that this new approach in the toolbox of reproductive technologies, can be used to efficiently exploit valuable sorted semen. The possibility to retrieve immature oocytes from preantral follicles in large ruminants by means of transvaginal guided puncture or ovum pick up (OPU), has open new possibilities for genetic improvement within herds and breeding schemes (Pieterse et al., 1991). Although the efficiency of late stage in vitro embryo production in buffaloes, following OPU and IVF can be considered similar to what reported in cattle (Neglia et al., 2003), the development to term following transfer of fresh or frozen embryos is still unsatisfactory (Neglia et al., 2004; Lu et al., 2007). Nevertheless, the combined use of IVF, OPU and sexed semen has been proven feasible and repeatable, mushrooming new research for a wider field application of these technologies in buffalo (Liang et al., 2008). More recently, very high pregnancy rates following AI with buffalo river type sexed semen into swamp and F1 (River x Swamp) recipients undergoing spontaneous estrus, have been reported (Lu et al., 2010). These last successful results indicate a powerful way to disseminate valuable semen for the acceleration of genetic gain in swamp buffalo too, bearing in mind the different breeding management and local conditions typical of Asian Countries. The first large trial on the use of sexed semen in Mediterranean buffalo heifers has confirmed the similarity of efficiency and pregnancy rates when compared to the use of conventional semen (Campanile et al., 2011). In this study, a modified Presynch-Ovsynch protocol for synchronization of ovulation was implemented. Additional variation within the protocol consisted of an adjunctive administration of GnRH and prostaglandin at the time of AI, in order to verify the beneficial combined effect on possible synchronous ovulation and enhanced internal progesterone production. In the same study, it has been shown that sexed semen deposition into the body of the uterus gives similar pregnancy rates if compared to the deep semen deposition into the uterine horn. This latter finding will facilitate the dissemination and use of sexed semen, since no special skills or
particular technique, as seen in Presicce et al. (2005a), are necessary to obtain acceptable pregnancy rates. More recently, on a similar trial on pluriparous buffaloes subjected to AI with sexed semen during the seasonal and transitional breeding periods, a conventional Ovsynch protocol was employed, reducing thus manpower on handling of animals and reducing the need of exogenous hormonal administration, and at the same time reporting good conception rates non dissimilar from the use of conventional semen (Campanile, 2012).

CONCLUSIONS
Normalcy of buffalo calves at birth, following use of sexed semen in AI schemes has been reported (Presicce et al., 2005b; Campanile et al., 2011). The efficiency of protocols for synchronization of ovulation, tied to good pregnancy rates following use of sexed semen will undoubtedly widen the opportunity of farmers into the selection and adoption of AI and other breeding strategies for improvement of production and reproduction traits.

REFERENCES


