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EFFECT OF SEASON ON REPRODUCTIVE PERFORMANCES IN BUFFALO SPECIES (*BUBALUS BUBALIS*)

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I am strongly inclined to suspect that the most frequent cause of variability may be attributed to the male and female reproductive elements having been affected prior to the act of conception.

Charles Darwin, The Origin of Species.

To My Parents, My Sister, My Friends,

To Bianca, Prof Zicarelli, My Colleagues.

To Everyone who believed in me and still do.

Serena

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APPLICATIONS OF INNOVATIVE REPRODUCTIVE BIOTECHNOLOGIES TO BUFFALO BREEDING

INTRODUCTION

Mediterranean Italian Buffalo

Buffalo is an important economic resource for Italian animal husbandry, especially for the regions traditionally involved in the rearing of this species. The largest concentration of heads can be found in Campania, in the provinces of Caserta and Salerno, where 78% out of 400,000 animals of Italian total amount of buffaloes is bred, but many farms exist also in Latium, in the provinces of Latina and Frosinone, and in Apulia. In these areas, buffalo breeders have constantly grown both in professional and managerial terms, fine-tuning and perfecting breeding techniques and coming to an efficient intensive or semi-intensive approach. In fact, in Italy, the type of farming has changed over time and the scheme with extensive use of meadows and pastures of the past has given way to an intensive farming, with free housing of the animals, modeled on that used for dairy cattle. Dairy buffaloes are kept loose in paddocks close to the milking room, where the cows are submitted to a rigorous control and cleaning of the udder, then mechanically milked twice a day. This advance made the selection of subjects with the purpose of increasing productive and genetic value possible, thus converting a marginal sector into an area with great economic potential. In fact, this breeding has still a positive

trend as it is demonstrated by several investments undertaken by many entrepreneurs. Currently, there is a buffalo population growth of about 70,000 animals per year, so the replacement of other species with buffalo is actually performed in many regions. This process is mainly due to the marked productive qualities, adaptability and rustic nature of the buffalo, on one side, but is also promoted −on the other hand- by a lower environmental impact of this species: in fact, it was shown that buffalo eliminates a smaller amount of nitrogen with manure (54 kg vs. 81 kg/year) compared to dairy cow. This allows to increase the load of animals per hectare. Furthermore, in the total budget of a dairy farm, now more than ever, a careful balance between production costs and management costs is necessarily required in order to keep market competitiveness. This, however, must not definitely affect the parameters that allow to obtain a high-quality raw material (milk), because it represents the *starting point* of a peculiar and strictly linked product, such as mozzarella cheese, which has not only a traditional and cultural value in Italian gastronomy, but above all has been providing employment for many years. In fact, it is worth to highlight that nowadays, all around the world, consumers play a significant role in demanding animal products that are produced to agreed standards for human health, environmental management and animal welfare. Moreover, in the areas mentioned above, the employment impact of this

sector is estimated slightly higher than 5%: this can be considered a respectable value, considering that, in Campania, the employment impact in the sectors of agriculture and animal husbandry represents 3.8%, compared to a National value of 2.8% and 1% in Lombardia region.

The importance and competitiveness of buffalo breeding in Italy, compared with the other more established forms of livestock rearing, is also confirmed by the increase in the national buffalo population in the last ten years (+68.64%; *source: ANASB[1] data, 2008).* The buffalo farming in Italy has been reaching remarkable productive standards thanks to an intense work of selection and research carried out during the past years. The most important business related to buffalo breeding is milk production, all of which is processed into mozzarella cheese. The milk average production recorded in 2009 was over 2150 kg with peaks higher than 5000 kg per standard lactation phase (270 days), with 8.39% fat and 4.61% protein content (*source: ANASB statistical data, 2009; see* Table.1) and with excellent cheese making yields.

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[1] Associazione Nazionale Allevatori Specie Bufalina, i.e. National Buffalo Breeders' Association.

Table. 1 Parameters regarding buffalo population and milk production in the last decade

	Average milk yield (Kg)	Fat (%)	Proteins (%)	Farms checked (n)	Average heads/farm (n)
2000	2.145	8,35	4,74	284	115,5
2001	2.145	8,39	4,66	286	118,6
2002	2.168	8,28	4,73	292	122,4
2003	2.175	8,10	4,65	287	128,8
2004	2.184	8,06	4,68	294	134,1
2005	2.169	8,07	4,69	282	141,5
2006	2.178	8,09	4,67	286	141,3
2007	2.211	8,18	4,66	290	153,2
2008	2.221	8,24	4,66	290	161,3
2009	2.182	8,39	4,61	288	168,5

(*source: ANASB and Anagrafe Nazionale data*)

Since 1977 (year of the establishment of the Herd book of the species, which was managed by ANASB in close cooperation with the Italian Breeders Association - AIA -) up to now, the average production increased by about 600 kg per lactation and the percentage of fat and protein content increased from 6.4% and 4.3% to 8.4% and 4.7%, respectively. These productive levels were achieved by the utilization of breeding systems that better faced the nutritional requirements of the species. Moreover, this enhancement was also due to the best knowledge of the potential production of each buffalo, obtained by milk recording. In fact, the first

step in the breeding and selection activity with regard to any dairy livestock is milk recording of the productivity of each yielding animal. The results of milk recordings, when appropriately merged with the genealogy data, allow a definition of the milk genetic merit of each individual, in particular of the bull, for which we have no other milk record except the production of his related animals (Moioli *et al.,* 2005). Furthermore, those functional controls allowed not only the creation of the genealogical book of the species, an unique example in the World, but also to formulate the EMB (Equivalent mature buffalo) and the genetic indexes that lead a more careful selection of the bulls. In 1988, progeny testing cycles on buffalo bulls were started on the sons of the cows enrolled in the genealogical book. The genetic indexes were estimated by the *Blup*[2] Animal Model method for the following characters: production of mozzarella cheese (PKM), milk production (kg), fat and proteins production (kg and %). All the relationship to each bull (bull's mother, daughters under test) were ensured by a PCR test on biological samples (blood, hair). The productions were calculated out of 270 days of lactation (standard lactation) and heritability values for the estimation of animals' genetic value varied between 0.18 and 0.14, slightly lower compared to those used for dairy cattle. All these efforts spent in selecting a pure breed led in 2000 to the identification and

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[2] In statistics, best linear unbiased prediction (BLUP) is used in linear mixed models for the estimation of random effects.

admission by a Government decree of the "Mediterranean Italian Buffalo" breed, in order to distinguish it from other breeds which were not at the same genetic level. This is a recognition of the peculiarities of the buffalo population bred in Italy: indeed, the Italian female buffalo can be considered exceptional in the international livestock raising scenario. In our country, the profitability of this species is particularly linked to and guaranteed by a peculiar product such as "Mozzarella di Bufala Campana" cheese, that gained a very well defined profile in the market, enhanced and protected by a Denomination of Protected Origin (DPO) trademark and, above all, is recognized in an increasing number of countries. Therefore, currently the selection is aimed to improve the production of mozzarella, which leads indirectly to improve the quantity and the quality of buffalo milk. So, in Italy, the commercialization of milk for mozzarella cheese production represents the main income of buffalo breeding. This is a critical point for farm economy.

However, meat production could also represent a good collateral profit. In fact, buffalo meat shows good quality characteristics according to the usual estimation parameters. In addition, the market interest in buffalo meat has progressively increased due to the negative trend towards bovine meat consumption, which has very recently worsened due to the BSE concern. In spite of the higher costs of buffalo meat production, due to their slower

growth compared to specialized bovine breeds, there are sound reasons to exploit buffalo meat. For several years, rearing buffaloes for meat production has not been an attractive economic channel in our country. The causes of the low interest in this activity are many. In the past, when extensive farming was still the most frequent way of rearing this species, it was very common to find in the market meat of poor quality. This phenomenon occurred because the farmers paid little attention to the breeding of males, whose management costs were higher than production gains. Consequently, they were reared with small amounts of maternal milk, reached the weaning weight of about 60-70 kg and thereafter were left out to pasture. The latter were often marginal pasture and, moreover, affected by the seasonal availability of fodder. This led the males to reach the slaughter weight in about 3 years. Their meat was very tough and characterized by the smell of musk, derived from the habit of the animals to find relief in puddles that they dig themselves into the ground. The gastro-intestinal parasitic infestations and *Pasteurella bubaliseptica* operated a natural selection, on one side and slowed the growth of the surviving males, on the other.

Moreover, in the past years, when the meat came from buffalo calves in a good state of nutrition, was sold as bovine "beef", otherwise it was marketed as buffalo meat. This fraudulent aspect has given rise a false

notion in the consumer, difficult to eradicate. Therefore, the "market" image of buffalo meat, even if requested, was considered poor by the consumer. All this helped to reduce the commercial value of buffalo meat. (Zicarelli, 2001).

Nowadays, the change of EU agricultural policy, the greater awareness of consumers, together with the transformation of management techniques and the continuous efforts by few farsighted farmers and entrepreneurs make the buffalo breeding for meat very competitive. Many studies (Cutrignelli *et al.,* 1996) ; Campanile et al., 2001a; Campanile et al., 2001b; Infascelli, 2004; Infascelli et al. 2005) have reported that buffalo meat, when the animal is properly bred to obtain meat, such as bovine beef, has some particularly interesting nutraceutical features. It is worth to underline that buffalo meat is characterized by nutritional values better by far than other kinds of meat and its calories content is lower than cattle meat. In fact, it is characterized by a low saturated fat content, a high protein level and a large percentage of iron, as it is shown in Table 2.

Table 2. Nutritional features of buffalo meat (100g) compared to other kinds of meat, available in Italian market.

	Protein (%)	Fat (%)	Cholesterol (%)	Iron (%)	Calories (Kcal)
BUFFALO	24.0	1.5	35	2	130
BOVINE	22.0	19.0	80	1	280
HORSE	22.1	7.0	60	4	140
LAMB	26.0	15.0	92	0	241
CHICKEN	29.0	3.0	80	0	152
OSTRICH	24.0	2.0	63	0	105

Source: MIPAF, National Research Institute for Food and Nutrition; http://www.inran.it

As for its digestibility and its good flavor, it is suitable to everyone, especially to children and old aged people. Moreover, table 2 also shows that buffalo meat is low in cholesterol. It is well known that the consumption of animal fats is discouraged by nutritionists, because it leads to the increase of cholesterolemy. The latter, in fact, is mainly due to unsaturated / saturated fatty acid ratio. In buffalo meat, the impact of saturated fat is always lower (45-40%) than unsaturated fatty acids (56-60%), while in bovine beef, this ratio is reversed. In particular, buffalo meat is richer of stearic and oleic acids than beef, two fatty acids neutral to

human serum cholesterol, and, moreover, of linoleic acid, an essential fatty acid (Ferrara and Infascelli, 1994)**.** Currently, considerable attention is given to the role of monounsaturated fatty acids (MUFA), and in particular to the linoleic acid, because able to reduce oxidation of low density lipoproteins (LDL) cholesterol (Parthasarathy et al., 1990). It follows that buffalo meat is above all suitable for those who have necessarily to keep cholesterol blood levels within acceptable limits, such as in particular heart patients and obese persons. Furthermore, mineral and vitamin content is slightly different from bovine beef: in particular buffalo meat is less rich in riboflavin and Ca^{2+}, but contains higher amounts of vitamin B_6, B_{12} and K^+. Finally, buffalo meat has a better water retention and, therefore, is more juicy and more tender than that of other farmed species, as it contains less hydroxyproline, a component of collagen.

Therefore, in order to further promote buffalo meat production and safeguard the consumer, a regulation providing only commercialization of subjects grown in the range of physiological weight gain should be prepared. The animal register of births could represent a good way of verifying if the slaughtering weight agrees with the aforementioned weight gains. The birth concentration in 6–7 months (using or not the out-of-breeding-mating strategy) could cause a discontinuous availability of meat on the market. This problem could be solved by slaughtering the subjects at

different ages and modulating growth through the adoption of proper rationing schemes. In this case, the contemporary presence of animals with different ages should be taken into account planning the stalls in order to guarantee a good available space/head. (Infascelli *et al.,* 2004)

In the last years there has been an increasing interest in the world regarding buffalo breeding, as it is demonstrated by the increase in world buffalo population (+6,44%; Borghese, 2005. *Source: FAO*; http://www.fao.org/docrep/010/ah847e/ah847e00.htm). This occurs especially in tropical countries, in which buffalo is considered an irreplaceable milk producer, because - whereas cattle cannot fully express its own productive potential- is the species that best suits to satisfy animal protein demand, as it is easy-fitting, rustic, long-lived and parasite resistant. Buffaloes have a high capacity for adaptation and are efficient production animals across broad climatic zones. Especially, in tropical countries, the perfect interaction among reproductive seasonality, environmental conditions and forage availability throughout the year allows the buffalo to be able to compensate the loss of bovine milk recorded during the unfavorable season and to produce animal proteins at competitive costs, exploiting pastures that are typically limiting cattle production (Zicarelli, 1994, Campanile *et al.*, 2010). The good feed conversion efficiency of buffaloes and the relatively low maintenance requirements are attributes

which make them ideal in low-input, low-cost production systems (Zicarelli, 1994; Paul et al., 2002). In fact, it is worth to remember that about 98% of the buffaloes in the world is bred in developing countries. They are really interested in Italian dairy genetic "material" to crossbreed the local working buffaloes with the highly productive Mediterranean buffalo, in order to increase animal proteins and meet human needs. According to our experience, this is also demonstrated by the constant increasing request of the Mediterranean buffalo semen and embryos from many countries, in order to improve the morphofunctional features of this interesting species. However, the ever-growing trend of milk request and the necessity to cut down fixed production costs make the adoption of plans to improve milk and meat production essential to farming.

Reproductive biotechnologies

In this scenario the competitiveness of buffalo breeding in Italy is necessarily linked to the use of biotechnologies of reproduction. In fact, these technologies, consenting to plan selective directions in a shorter time, could allow the distribution of elite buffalo genes, the reduction in generation interval and could provide continued genetic gain and increased

production of buffalo meat and milk. Moreover, the use of these technologies is crucially important to meet the genetic requests from developing countries, and then, to crossbreed the local buffaloes with the highly productive Mediterranean buffalo. Reproductive biotechnologies are undoubtedly one of the most emblematic inventions/strategies of applied research in the field of life sciences and animal husbandry. These new technologies have contributed significantly to the evolution of breeding in the last 60 years (Thibier, 2005), preparing the groundwork for a radical transformation of animal husbandry processing and production systems in the coming years. One of the key features of a modern farm in order to be competitive in the market, is certainly to achieve considerable genetic improvement in short time, without neglecting the needs of consumers who are increasingly sensitive to both the quality of product and animal welfare. The availability of genetic material from proven bulls, provides a baseline necessary to continue a more effective selection process, began years ago in an empirical way by farmers. In fact, a critical tool to increase the paternal contribution to the genetic improvement of buffalo herds is represented by the use of the **Artificial Insemination (AI).** This technique offers many advantages over natural breeding: in fact, it allows the choice of the best bulls to improve the genetic make-up of a buffalo herd. AI gives the opportunity to quickly obtain multiple generations of the same bull,

thus allowing a quick evaluation of the genetic value of a given sire, carried out through the productive performances of its daughters. Moreover, it is worth to notice that frozen semen can be transported globally: indeed, the dissemination of AI has been possible thanks to the improved means of cryopreservation, which led to successfully freeze the sperm of bulls. Moreover, it has to be considered that many potentially devastating diseases are spread by sexual contact. Because of the extremely tight controls exerted over both the health of donor bulls and the technical procedures themselves, these risks are vastly reduced with the use of AI. As regarding the cost effectiveness, it is worth to highlight that the cost of an AI straw is on average around 10€, this is nothing compared with the costs of a genetically proven buffalo bull. A bull is expensive to rear, is relatively unproductive, vulnerable to disease or accident and may even be infertile. Last but not least, any bull can be aggressive and is potentially dangerous. This was a major stimulus to the initial setting up of AI services.

Unfortunately, at the moment, in Italy, the use of A.I. is limited to just the 10% of the buffaloes enrolled to the Herd Book, because the efficiency is still low and is significantly affected by the seasonality of the species (Baruselli *et al.*, 1997a; Campanile *et al.*, 2005; Campanile *et al.*, 2007a, 2008). It was observed that embryonic loss in animals mated by AI is 20-

40% during seasons characterized by a higher number of light hours (Campanile *et al.*, 2005; Campanile *et al.*, 2007a; Campanile *et al.,* 2007b.), whereas values of around 7% were recorded in Brazil during decreasing light days (Baruselli *et al.,* 1997b.). Neglia et al. (Neglia *et al.*, 2008) found an increase in pregnancy rate in buffaloes mated by AI during the transitional period and treated by PGF2α analogue (cloprostenol) on the day of AI. In fact, PGFs increase blood flow (Miyamoto *et al.*, 2005) and P4 levels on day 10 after AI (Neglia *et al.*, 2008). Every effort should be made through artificial insemination to increase the number of superior breeding males for accelerating the pace of improvement in water buffalo. To ensure these measures, it is first of all necessary to use sires of known breeding merit as far as possible, but also further investigations are required to assess how to control and enhance the pregnancy rates also in the unfavorable season.

Indeed, different strategies have been developed to reduce embryonic mortality in buffalo cows that at least in part may be attributed to reduced progesterone secretion (Campanile *et al.,* 2007b). Therefore, a study was performed during the seasonal transitional period, which corresponds to mid-winter in the Mediterranean region (Campanile *et al.,* 2007b) to evaluate the effects of P4 secretion promoters [GnRH agonist, hCG and

exogenous P4] administered on day 25 after AI. It was demonstrated that all treatments increased P4 values and reduced embryonic mortality.

Therefore, the use of AI in Mediterranean Italian buffalo should necessarily be "adjusted" to the peculiar physiology of the species to become a routine in the farm, in order to boost the paternal input to genetic improvement.

As regards biotechnologies aimed to improve genetic progress through the maternal contribution in buffalo, we have to mention the **Multiple Ovulation (MO) and Embryo Transfer (ET)** programs. In general, MOET schemes are used to increase the intensity of genetic selection, reducing generation intervals (Smith, 1988), but today they are also commonly used to select bulls to be used for AI (Bondoc et al., 1989; Teepker et al., 1989).

In this case selected donors dams are inseminated with the semen of bulls showing superior traits; as regarding the offspring, females are straightly devoted to the production, while their brothers will wait the production results of the sisters (Smith and Ruane, 1987). Therefore, males are tested and chosen according to the productive performances of their sisters, instead of their offspring, as it was when AI only was used. This makes it possible to genetically test a bull in 3.5 years instead of 5.5 years using traditional progeny test. MOET programs have been increasingly improved over the years and today they represent the technique that produces more

embryos in the world, for a total of 500,000 embryos per year, of which about 440,000 are transferred (Thibier, 1998).

MO consists in inducing multiple ovulations in animals that generally have a single ovulation per cycle by administrating exogenous hormones. Subsequently, the donor dam is covered by the bull or preferably artificially inseminated at the time of ovulation. At 6-7 days post insemination or natural breeding the recovery of embryos is performed in different ways: in the early development stages of the technique, embryos were recovered either surgically, or by laparoscopy, or by transvaginal insertion of a catheter into the uterus. Further development resulted in a technique involving trans-cervical insertion of a three way catheter to flush the uterine horns. Embryos are evaluated under a microscope to assess suitability for direct transfer or for cryopreservation. After estrus synchronization of the recipients, the embryos are transferred in surrogate females for the gestation. However, there are many limiting factors using this technique, among which the most important are the need to use subjects in good genital tract conditions, with patent oviducts, that should neither be pregnant nor lactating, with regular oestrous cycle and after a suitable post-partum period. Using this technique in bovine, the mean embryo recovery per animal is 5, whereas in buffalo there is an average recovery of less than 2 embryos.

Several factors may influence the response to the MO in buffalo species, as a high interindividual and intraindividual variability in the size of the uterine lumen (Zicarelli, 1994), the age of the donors, the number of daily milkings (Zicarelli et al., 1993), the calving interval, the period of year (Zicarelli et al., 1994), and the characteristics of the diet (Di Palo et al., 1994). The low response to MO is likely attributable to the rather low follicular population (1/5 of the cow) and/or to the poor quality of buffalo oocytes.

However, because of the low and inconsistent efficiency of MOET treatments in this species (Misra et al, 1997; Zicarelli et al, 1997), there is a worldwide increasing interest in large-scale *in vitro* production of buffalo embryos for faster propagation of superior germplasm and to enhance genetic progress through the maternal contribution.

Reproductive technologies that offer in buffalo species most of the advantages in this direction are the **Ovum pick-up (OPU)** linked to the ***in vitro* embryo production (IVEP)** which are technologies that allow to obtain from each female a greater number of transferable embryos (Gasparrini, 2002). This procedure permits the repeated production of embryos from live donors of particular value and is a serious alternative to multiple ovulation (Galli *et al.*, 2000).

In addition to the limitations of MOET that have been demonstrated in this species regarding embryo output (Misra 1997; Zicarelli, 1997b), it is worth emphasizing that OPU can be performed on a wider typology of donors, such as non-cyclic animals, pregnant cows, subjects with non-patent oviducts or genital tract infections, and animals that are not responsive to hormonal stimulation, the last representing a high proportion in buffalo (Gasparrini, 2010). Moreover, it can be applied to superior donors of all age starting from 2-month-old calves to very old cows and even pregnant animals without any side-effects on the reproductive career of the donor.

OPU technique consists in an ultrasound guided transvaginal procedure aiming to oocytes recovery from a live donor.

The combined OPU and IVEP technology is currently the most efficient tool for increasing the number of transferable embryos (TE) obtainable per donor over the long term in most species. In buffalo, this technology is even more competitive in terms of embryo yields compared to MOET (Gasparrini, 2002) both because of the lower response to hormonal stimulation together with the poor embryo recovery (Zicarelli, 1997b), and because of the impossibility to repeat continuously the MOET treatments over a long-term since multiple ovulations can be induced only in cyclic animals, and it is likely that buffalo cows enter seasonal anestrus. The competitiveness of OPU-IVEP becomes overwhelming when donors are

selected on the basis of their folliculogenetic potentials. Indeed, the limitation due to the high variability of follicular recruitment, oocyte retrieval, and hence, blastocyst production (the best buffalo in a 6-months OPU trial gave 37 TE with the worst yielding only 1 TE; Gasparrini, 2002), can be overcome, thus resulting in a further increase of the embryo yield. It has been demonstrated that the potential of animals to recruit follicles can successfully be predicted after the first 4 oocyte collection days (2 weeks), and that the number of follicles is correlated to the number of oocytes and blastocysts (Gasparrini, 2002).

However, although the buffalo IVEP system has been greatly improved over the years, leading to high blastocyst yields (Neglia *et al.,* 2003; Gasparrini *et al.,* 2006) and to the production of offspring (Samad *et al.,* 1998; Neglia et al., 2004; Huang *et al.,* 2005; Hufana-Duran *et al.,* 2004; Liang *et al.* 2008) this technology is still far from being routinely practicable.

The low IVEP efficiency recorded in buffalo compared to cattle is in part due to peculiarities of the reproductive physiology of buffalo that are not easily modifiable, such as the low number of oocytes recruitable and their poor quality. In addition, unlike cattle, the reproductive lifespan in buffalo is long (12 years in Italy and even longer in swamp buffalo in China and South East Asia), because, for economic reasons, buffalo cows are usually

slaughtered when they are old or when their fertility and productivity are compromised. This results in a further decrease of the number of competent oocytes recoverable in the case of abattoir-derived ovaries as a source of gametes.

The scarcity of experimental material for buffalo, together with the assumption that the reproductive biology in all ruminants is similar, led in the early attempts, to use the IVEP system in buffalo based solely on information acquired in cattle, with the consequent result of low IVEP efficiency. In fact, it has been demonstrated over the years that improvements in IVEP are possible through the optimization of each procedural step, especially when taking into account species-specific differences, as shown by the higher blastocyst rates reported over recent years (Gasparrini *et al.*, 2006). OPU has been successfully carried out in buffalo by Boni *et al.* (1996), and IVEP efficiency has greatly improved throughout the years (Neglia *et al.*, 2003). It is now possible to adopt a fully *in vitro* system, whereby the immature oocytes are matured through *in vitro* maturation (IVM) technique, followed by an *in vitro* fertilization with capacitated spermatozoa and culture of newly formed zygotes in suitable media for development up to the transferable stage. IVF represents the most economic and efficient method in large quantity embryo production.

Oocyte source and quality

It has previously been described that in buffalo species, one of the major intrinsic limitation of IVEP technology is the low number of immature oocytes that can be recovered per donor. In our experience controlled follicular aspiration of abattoir-collected ovaries allows the retrieval of 2.4 good quality oocytes per ovary on average (Gasparrini, 2000). The slightly lower oocyte recovery (0.4-1.9) reported in other studies (Totey *et al.,* 1992; Madan *et.al.,* 1994; Das *et al.,* 1996; Kumar *et al.,* 1997; Samad *et al.,* 1998; Mishra *et al.,*2008) may be due to differences in breed, older age at slaughter, management and nutritional status (Goswami *et al.,* 1992). The recovery rate is therefore much lower than in cattle in which 10 good quality oocytes are obtained on average per ovary (Gordon, 1994). Similarly, a low number of oocytes is recovered when OPU is performed in buffalo compared to cattle (4.5 vs approximately 10 respectively; Galli *et al.,* 2000). Trials carried out on buffalo cows at different days in milk (Boni *et al.,* 1995, 1996, 1997) suggest that at increasing postpartum period (>500 d) the number of follicles and oocytes decreases. It has also been reported that various biometeorological factors (day length, ambient temperature, relative humidity and rainfall) influence the endocrine system of buffaloes

(Shah, 1988), therefore the oocyte recovery rate can vary according to those parameters.

However, this limitation is currently the major impediment for the diffusion of IVEP in the field, arising from physiological peculiarities of the species, such as the low number of primordial (Danell, 1987) and antral (Kumar *et al.,* 1997) follicles present on the buffalo ovary, as well as the high incidence of follicular atresia (Palta *et al*., 1998), and as such, it is not easily improvable. However, as previously stated, it is possible to increase the number of competent oocytes by selecting donors on the basis of their follicular population (Sá Filho *et al.,* 2005). Furthermore, it has been shown that rBST pretreatment of buffalo donors (Sá Filho *et al.,* 2009) promotes follicular growth (12.2 vs 8.7 total follicles punctured; 9.1 vs 6.5 small follicles), and tends to increase the number of oocyte recovered per session (5.2 vs. 4.1; p=0.07) and the percentage of good quality oocytes (48.8% vs. 40.6%; p=0.07). However, the blastocyst production rate and the number of blastocyst produced per buffalo per session did not vary according to treatment. These results are in agreement with those previously reported in cattle (Bols *et al.,* 1998; Tripp *et al.,* 2000). Therefore, both approaches are successful to increase the number of gametes but only to a limited extend.

Oocyte quality, that is known to affect the IVEP efficiency in most species, plays a determining role in buffalo, further reducing the availability of the

oocytes suitable for IVEP. The oocyte morphology can be used, with a certain reliability, to predict the gamete developmental competence; according to our classification, a progressive decrease of efficiency is recorded from Grade A to Grade D oocytes (Neglia *et al.,* 2003), with Grade A and B considered suitable for IVEP. It follows that the IVEP efficiency would be higher by only processing Grade A oocytes but this is not practical if the final goal is to produce the greatest number of embryos because the limited availability of oocytes often imposes the utilization of all the oocytes recruited, including Grade C. It is worth pointing out that the percentage of good quality oocytes (Grade A and B), is lower in this species compared to others, not exceeding, in our experience 50 % of the total oocytes recovered. It was recently demonstrated that the non invasive brilliant cresyl blue (BCB) staining before IVM can be used to select developmentally competent oocytes for increased in vitro embryo production (Manjunatha *et al.,* 2007a). Indeed, both nuclear maturation rate (86.2 vs 59.2%) and blastocyst rate (33.4 vs 5.2%) were higher in BCB+ oocytes than in BCB – oocytes. However, with 26 mM BCB, approximately 57.2% morphologically selected oocytes had a blue coloration after staining with BCB, indicating that they had finished their growth phase and could be used for IVP. It results that it is possible to increase the efficiency by selecting the oocytes but this in turn further

reduces the number of oocytes that can be processed, that is the real limiting factor in this species. However, on equal blastocyst yields, the percentage of BCB+ oocytes was slightly higher than the percentage of Grade A+B usually recorded in this species.

The oocyte quality may be affected by several factors, such as the aspiration pressure during collection, the source of gametes, the time between collection and processing, the temperature during transportation, season, etc.

In our experience, the oocyte morphology varies with the source of gametes, with an apparent worse quality of OPU-derived oocytes, characterized by fewer layers of granulosa cells, compared to abattoir-derived ones. A different distribution of COCs classes in relation to the oocyte source was also shown in cattle, with a higher incidence of better quality oocytes for abattoir-derived compared to OPU-derived COCs (Merton *et al.,* 2003). As this difference was not due to the OPU equipment or to the collection medium, it was hypothesized that, due to a post-mortem effect, the COCs becomes less tightly connected to the follicle wall and therefore are collected with a more complete morphology. This is even more likely to occur in buffalo because of the poor adhesion of cumulus cells (Gasparrini, 2002).

This feature led us to speculate that also technical factors during OPU, such as the length of the needle, as well as that of the line connected to the suction unit, may result in a greater loss of granulosa cells in buffalo oocytes and, hence in an underestimated evaluation of their quality.

Infact, in contrast to what reported in cattle (Merton *et al.,* 2003), despite their worse morphological appearance, OPU-derived buffalo oocytes have a higher developmental competence compared to abattoir-derived ones (Neglia *et al.,* 2003). These data were also subsequently confirmed by Indian authors who reported both higher blastocyst yields (30.6±4.3 vs 18.5±1.8) and higher blastocyst hatching rates (52.8±4.2 versus 40.2±4.4) following embryo vitrification from OPU-derived compared to abattoir-derived buffalo oocytes (Manjunatha *et al.,* 2008). In many recent OPU trials when we calculate the efficiency of IVEP in relation to the grade A+B COCs, even if all classes are put into IVM, cleavage rate exceeds 100% and blastocyst rate is over 50%. Therefore it may be advisable to use a different morphological classification for OPU-derived buffalo oocytes. An alternative possibility is to evaluate whether the BCB staining method would provide a more appropriate estimation of OPU-derived oocytes.

The improved embryo yield may be accounted for by the OPU-induced modification of the follicular dynamics; resetting the follicular population twice weekly results in increased follicular wave frequency and hence in

the aspiration of follicles before they become atretic, with an improved oocyte "quality". On the contrary, an heterogeneous oocyte population is recovered from pooled ovaries of slaughtered buffaloes. In addition, it is worth reminding that buffaloes are usually slaughtered when they are old and/or hypo fertile. It has been suggested that the better embryo yields recorded in cattle for slaughterhouse compared to OPU-derived oocytes are due to early atresia occurring in the post-mortem which positively affects oocyte developmental competence (Bondin *et al.,* 1997*)*. This does not seem to be the case in buffalo, suggesting that further studies are needed to elucidate whether atresia occurs more quickly or has no positive effect on the oocyte.

It was speculated that the better developmental competence of OPU-derived *vs* abattoir-derived oocytes is related to the shorter exposure to environmental stress. Indeed, abattoir-derived oocytes spend a longer time between excision of ovaries from the peritoneal cavity and laboratory processing and are probably affected by cellular damages due to autolytic processes, especially when they reside in excised ovaries for prolonged periods. It follows that, in the latter case, another important factor to consider is the time interval between ovary collection and processing in the laboratory. In our setting the time lapse between collection of ovaries at slaughter and their arrival at the lab usually varies between 3 and 6 hours.

A retrospective analysis of data collected over 4 years in our lab showed that, although oocyte quality seems improved at the shorter time (3 h compared to 4, 5 and 6 h), as we would expect, as indicated by the higher percentage of grade A and B oocytes together with the lower incidence of degenerated oocytes, cleavage and blastocyst rates were not affected by extending the time interval up to 6 h (Di Francesco *et al.,* 2007).

Furthermore, when OPU is carried out in the field, ie in farms distant from the laboratory, a significant improvement of blastocyst yield can be achieved by reducing the time between oocyte collection and their maturation. In these situations, an increased efficiency is recorded with oocytes searched directly in the farm and immediately transferred in a hepes-buffered *in vitro* maturation (IVM) medium in a portable incubator, compared with those searched in the laboratory, after many hours of their permanence in the follicular fluid (Gasparrini, 2006b).

The oocyte competence also depends on the morphofunctional state of ovaries, with an improved development recorded when oocytes are recovered from ovaries with either a corpus hemorragicum or a CL, in the absence of a dominant follicle (Manjunatha *et al.,* 2007a). In a further experiment the same authors collected ovaries with a CL and a dominant follicle and classified the follicles as dominant, largest subordinate, and subordinate. In this case oocytes from the dominant follicle had a higher

cleavage rate (65.2 %) and transferable embryo yield (30.2%) than those collected from the largest subordinate and subordinate follicles.

Buffalo oocytes are very sensitive to shock temperature so it is important to monitor the temperature carefully during collection as fluctuations can easily occur. When OPU is carried out temperature is controlled by holding the collection tubes in suitable warm boxes. When abattoir-derived oocytes are utilized for *in vitro* embryo production, the ovaries are usually kept in physiological saline, under controlled temperature (30-37°C), during collection and transportation to the laboratory. It has been recently observed, that oocyte developmental competence, evaluated in terms of cleavage and blastocyst rates following IVF, is improved by lowering the temperature range during transportation to 25-29.5°C (Di Francesco *et al.*, 2007).

In vitro maturation (IVM)

A fundamental requirement for a successful fertilization is undoubtedly the appropriate maturational status of the oocytes at the time they encounter the sperm. The oocyte maturation process, that involves both the nuclear and

cytoplasmic compartments, is fundamental for the acquisition of full developmental competence.

Buffalo oocytes can be matured in vitro in complex media, such as Tissue Culture Medium 199 (the most widely employed) and Ham's F-10, supplemented with sera, hormones and other additives. Different sources of serum have been utilized as supplements of IVM medium (Chuangsoongneon and Kamonpatana , 1991; Totey *et al.*, 1993; Chauhan *et al.*, 1998; Samad *et al.,* 1998). Although it has been observed that buffalo oocytes can reach the maturation status even in the absence of hormones (Madan *et al.,* 1994) higher maturation and fertilization rates have been recorded when oocytes are matured in the presence of gonadotropins and 17β-estradiol (Totey *et al.,* 1992; 1993). It is known that hormones interact with receptors located on the follicular cells, and that the signals are transduced into the oocyte through gap junctions or extracellular mechanisms. It results that the presence of cumulus cells is critical for the acquisition of developmental competence during IVM, as confirmed by the significantly reduced cleavage and embryo development of denuded vs cumulus-enclosed oocytes following IVF (Pawshe *et al*., 1993; Gasparrini *et al.,* 2007). This aspect is particularly important in buffalo because of the high proportion of totally or partially denuded oocytes usually recovered in this species. In order to rescue germinal material, it has been proposed to

provide poor quality oocytes with the somatic support by performing IVM on a cumulus cells monolayer, obtaining improved maturation and fertilization rates (Pawshe *et al*., 1993).

In order to reduce IVEP costs, buffalo follicular fluid, a waste product of oocyte collection, has been used in replacement of expensive hormones and serum additives, obtaining comparable maturation, fertilization, and blastocysts rates (Chauhan *et al.,* 1997).

The beneficial effects of several ovarian-derived growth factors, such as IGF-l, IGF-2 and insulin on oocyte maturation, fertilization and development to the blastocyst stage (Pawshe *et al*., 1998) have been also reported in this species. It has also been observed that supplementation of the IVM medium with EGF improves cumulus expansion, nuclear maturation, and cleavage rate of cumulus-enclosed buffalo oocytes without affecting the post-fertilization embryonic development (Chauhan *et al.,* 1999).

Based on the assumptions that buffalo oocytes and embryos, because of their high lipid content (Boni *et al.,* 1992), are particularly sensitive to oxidative damages, the IVM medium has been enriched by thiol compounds, known to act as antioxidants factors, by stimulating glutathione (GSH) synthesis. It is known that GSH plays a critical role in

protecting mammalian cells from oxidative stress, that is a major factor affecting in vitro mammalian embryo development. It has been demonstrated in other species (Telford *et al.,* 1990; Gardiner and Reed, 1994), that the GSH reservoir formed during IVM is the only source of reducing power for the embryos before genomic activation occurs. It was previously demonstrated that cysteamine supplementation during IVM improves blastocyst yield in buffalo (Gasparrini *et al*., 2000), by increasing intracytoplasmic glutathione concentration (Gasparrini *et al.,* 2003), without nevertheless affecting cleavage rate. The addition of cystine, in the presence of cysteamine, to the IVM medium (Gasparrini *et al.,* 2006a) has further increased the GSH reservoir of the oocytes and has significantly improved the proportion of oocytes showing normal synchronous pronuclei post fertilization (81 %), cleavage rate (78 %) and blastocyst yield (30 %). In a recent study the enrichment of IVM medium with other antioxidant factors, such as taurine and melatonin, was evaluated (Manjunatha *et al.,* 2007b). It was found that the addition of 1 mM taurine in the medium resulted in a higher transferable embryo (TE) yield when compared with control (20.6% vs 14.1%). Supplementation of melatonin at 10 and 50 μM concentration in the medium increased maturation rate (90.3% and 88.8% respectively vs 75% in the control) and TE yield (28.4% and 27.2% respectively vs 14%).

Recently it was demonstrated (Pandey *et al.,* 2009) that supplementing IVM medium with mitogenic lectin improves maturation of buffalo oocytes, as indicated by increased cumulus expansion and cleavage rate following IVF, as well as by the up regulation of the transcripts levels of genes involved in different physiological activities like gap junction and cell communication protein (Cx43), cumulus-expansion enabling factor and cell cycle protein (GDF-9), basic growth factor (FGF-4) and cell membrane protein (Fibronectin).

Among factors affecting mammalian embryo development in vitro, the duration of IVM plays a critical role, since an inappropriate timing of maturation results in abnormal chromatin (Dominko and First, 1997), oocyte aging (Hunter and Greve, 1997) and reduced development (Marston and Chang, 1964). Furthermore, although sperm can penetrate oocytes prior to completion of oocyte maturation (Chian *et al.,* 1992), subsequent development is generally reduced. Therefore, it appears that the optimum time for in vitro fertilization (IVF) is at completion of meiosis, that occurs at different times in different species, varying from 18-24 h in cattle (Sirard *et al.,* 1989; Neglia *et al.,* 2001) to 36-48 h in pig (Prather and Day, 1998). Although large variations in the timing of oocyte maturation have been reported in buffalo, with the highest proportion of MII oocytes observed between 16 and 24 h (Yaday *et al*., 1997; Neglia *et al.,* 200; Gasparrini *et*

al., 2008), the majority of the authors inseminate buffalo oocytes in vitro 24 h after the start of IVM. The different oocyte maturation time-scale recorded among buffalo studies may be accounted for by different conditions of IVM and particularly by oocyte quality which, in this species, is also likely affected by seasonal factors. We have recently investigated both the kinetics of oocyte maturation and the influence of the duration of IVM on subsequent embryo development. In this study the attainment of the MII stage has commenced after 18-19 h maturation and the majority of oocytes completed nuclear maturation between 20 and 24 h; furthermore, it has been demonstrated that the duration of IVM affects buffalo oocyte developmental competence, with a progressive decrease of fertilization capability and embryo development as the IVM duration increases from 18 to 30 h (Gasparrini *et al*., 2008). Therefore, the optimal time for IVF in buffalo appears to be at 18 h post-IVM or, in any case, not later than 24 h; in fact, delaying IVF over 24 h has resulted in a significant deterioration of oocyte developmental competence that could be predicted by the poor morphological appearance of oocytes matured for prolonged periods. An earlier aging of buffalo oocytes had been previously hypothesized based on the anticipated accomplishment of maturation, together with the increased incidence of degenerated oocytes at increasing times post-IVM (Neglia *et al.,* 2001). The importance of oocyte aging in this species is also confirmed

by activation studies that showed, in contrast to most other species, a deterioration of post-parthenogenetic embryo development at increasing times post-maturation (Gasparrini *et al.*, 2004a). This aspect is important to be considered when OPU is carried out because usually, to improve cost efficiency, all oocytes batches collected the previous OPU day are fertilized in one session. In the farm setting one OPU session lasts 15-20 minutes and hence, in the case of 15 donors oocytes are put into maturation at different moments in a time lapse of approximately 4-5 h. Based on these observations, we usually fertilize the all oocytes the day after OPU 1 or 2 h before the time of the first lot of maturation so that the earlier collected oocytes will be fertilized at 23-24 h and the latest at 19-20 h.

In vitro fertilization (IVF)

Fertilization has often been considered the most critical step of the IVEP procedures in buffalo, as cleavage rates lower than those obtained in other domestic species have been widely reported (Galli *et al.,* 2000; Neglia *et al.,* 2003; Gasparrini *et al.,* 2004b). In our earlier studies, despite similar maturation rates (87% vs 94% respectively in buffalo vs cattle) significantly lower cleavage rates (65% vs 84%) were observed (Neglia *et*

al., 2003). The overall lower IVEP efficiency recorded in buffalo compared to cattle (26 vs 34 %, respectively) was mainly related to the poor cleavage rate; in fact similar blastocyst yields were obtained in buffalo and cattle (40 %) when the percentages were calculated in relation to the zygotes (Neglia *et al.,* 2003).

Many factors may affect the in vitro fertilization efficiency, such as the adequate in vitro environment for gametes survival, the sperm viability and capability, the appropriate time of insemination, the duration of gametes co-incubation, the presence of cumulus cells and also the acquisition of the oocyte developmental competence during the complex process of cytoplasmic maturation. In fact, it is likely that the fertilization failure is related to inadequacies of the IVF system, but a previous inappropriate maturation of the egg should not be ruled out.

The media commonly utilized for buffalo IVF are Tyrode's modified medium (TALP) and Brackett Oliphant (BO), supplemented by sperm motility inducing factors, such as combined hypotaurine-penicillamine or caffeine. However, significantly higher cleavage and blastocysts rates have been obtained, in a direct comparison trial, by using TALP medium, supplemented with heparin, hypotaurine and penicillamine (Gasparrini *et al.,* 2004b). High sperm motility is required to accomplish fertilization, and this aspect is particularly important when frozen-thawed sperm is

employed. A preliminary selection of motile spermatozoa can be carried out by 1-hr swim-up method or a percoll density gradient. A direct comparison trial (Mehmood *et al.,* 2009) recently reported that swim-up separated sperm show a higher motility and membrane integrity, while the percentage of motile sperm is higher with Percoll separation. Swim-up separated sperm gave a higher cleavage rate (66.8 vs 55.6%) and cleavage index than percoll gradient. However, sperm separated by swim-up showed significant difference among the bulls in cleavage rate and cleavage index ($P < 0.05$), while the Percoll gradient method did not. In our lab both swim-up and percoll gradient have been widely used over the years, with a non evident superiority of swim-up in terms of embryo yield. However, the results above reported are in agreement with our experience, that confirms a marked bull effect with swim-up; in particular, there are bulls that do not respond well to this separation approach, giving low concentrated sperm and hence requiring more straws/IVF. As a consequence, the choice of the method depends on the bull enrolled in the IVF program.

In earlier times the quality of the frozen semen was considered the major factor impairing in vitro fertilization (IVF), based on the demonstration of several damages of the male gamete occurring following cryopreservation (Meur *et al.,* 1988), together with the drastic reduction of cleavage rate reported with frozen compared to fresh semen (Totey *et al.,* 1992).

Currently, the quality of frozen semen has improved, as indicated by similar fertility parameters, recorded for fresh compared to frozen semen (Wilding et al., 2003), suggesting that other factors may negatively affect fertilization.

However, the overall improvement of the quality of cryopreserved sperm has not eliminated another serious impediment, the so-called "bull effect", consisting in the high degree of variation between buffalo bulls in the fertilizing capability in vitro (Totey *et al.,* 1993)**.** It follows that, because only few bulls are characterized by good fertilizing capability in vitro (approximately 10 %), an accurate screening of sperm of several bulls is required in order to identify a suitable semen for IVF programs. Despite the availability of several fertility tests, it is known that currently the most accurate screening still goes through IVF trials, with different bulls tested on the same batch of eggs, that, in this species, because of the poor number of oocytes usable, are very time-consuming. Interestingly, it has been recently found that an easy, quick double staining technique with Trypan-blue/Giemsa (Kovacs and Foote, 1992) can be used to predict the fertilizing capability in vitro of buffalo bulls, as shown by the correlation existent between the percentages of acrosome-intact viable sperm cells at thawing and the blastocyst yields (Boccia *et al.,* 2007a).

Sperm need to undergo capacitation to acquire the fertilizing ability; this process, that in vivo occurs within the female genital tract, must be induced in vitro. Although several agents have been proven to induce sperm capacitation in vitro, heparin is still the most efficient method in the majority of the domestic species. It is not possible to rule out that the process of capacitation, required by spermatozoa to acquire the fertilizing ability is impaired in the currently used buffalo IVF system. In order to investigate whether the capacitation process in vitro can be improved by agents different than heparin, buffalo sperm have been incubated under different conditions and capacitation has been indirectly assessed by evaluating the capability of sperm to acrosome react following incubation with lysophosphatidilcholine (LPC), a fusogenic lipid, known to induce acrosome reaction in capacitated sperm without affecting motility. It has been demonstrated that progesterone induces buffalo sperm capacitation in vitro and may be considered as an alternative capacitating agent for buffalo IVF (Boccia *et al.,* 2006a). Furthermore, it has been demonstrated (Boccia *et al.,* 2007b) that sperm treatment with sodium nitroprusside, a well known generator of nitric oxide in vitro, improves the efficiency of buffalo sperm capacitation in vitro compared to heparin, when the incubation is extended to 2 or 3 h (60.1 vs 44.1 % respectively at 2 h; 68.8 vs 36.6 % respectively at 3h). The most promising results have been obtained by

incubating sperm with some biological fluids, such as buffalo estrus serum (BES) and the follicular fluid (FF) recovered from a pool of dominant follicles (Boccia *et al.,* 2005). In fact, sperm treatment with both BES and FF has resulted in a significantly higher incidence of AR sperm than with heparin treatment (84.3, 94.5 vs 50.1 % respectively). It is likely that factors derived by BES and FF, present in the oviduct environment around fertilization, play a critical role in processing the male gamete *in vivo*.

Subsequently we investigated whether mimicking the oviduct environment could improve the IVF efficiency in this species. In order to do so, we incubated buffalo sperm on a 6-day bovine oviduct epithelial cells (BOEC) monolayer and then we evaluated the capacitation status as well as the fertilizing capability, the latter assessed as penetration rate of bovine oocytes. It was demonstrated that both the capacitation process (Siniscalchi *et al.,* 2010) and the oocyte penetration rate after heterologous IVF (Mariotti *et al.,* 2010a), is enhanced by incubating buffalo sperm in the presence of BOEC. This suggests to carry out IVF of buffalo oocytes on BOEC monolayer to improve the IVF efficiency in buffalo. The higher incidence of advanced embryos obtained when sperm was preincubated for 6 h both in the presence or absence of BOEC may be accounted for by an earlier accomplishment of capacitation, leading to anticipated oocyte penetration. However, in these cases the penetration rate was not improved

compared to the control, suggesting that sperm viability may have decreased and hence that shorter times should be tested in further studies.

Another approach to mimic the oviduct environment for optimizing fertilization is the identification of key molecules and their subsequent incorporation in the in vitro system. Osteopontin (OPN), an acidic single-chain phosphorylated glycoprotein, is one of the protein present in both the oviductal fluid and epithelium in cattle, proven to improve IVEP efficiency and facilitate sperm capacitation [81]. In cattle expression of osteopontin was highly correlated with bull fertility and it was proposed to be a marker to predict male fertility (Monaco *et al.,* 2009). Osteopontin was also detected in buffalo semen, at greater concentration in the seminal plasma than in sperm cells (Pero *et al.,* 2007), suggesting that OPN is produced by the ampullae and seminal vesicles, similar to what was reported for cattle (Cancel *et al.,* 1999). Interestingly, buffalo semen frozen by standard procedures showed a reduction in amount of OPN by up to 50% (Pero *et al.,* 2007). Recently we demonstrated that the supplementation of IVF medium with OPN significantly enhances cleavage rate (71.6 vs 59%) and blastocyst yields (29.9 vs 17.4%) (Di Francesco *et al.,* 2009). It was also recently shown the possibility to significantly improve the efficiency of capacitation in vitro in buffalo species by incubating sperm in the presence of OPN (Mariotti *et al.,* 2010b).

Recently we demonstrated that melatonin (range 10 μM - 1 mM) determines capacitation of buffalo spermatozoa *in vitro,* without affecting their viability (Di Francesco *et al.,* 2010). The capacitating effect was observed at all the tested concentrations, suggesting to extend the range of concentrations to test in future studies, to identify the optimal dose. Furthermore, considering the seasonality of the species and the great differences in fertility attitude of buffalo bulls, it would be interesting to investigate the capacitation effect of melatonin related to both the season and the bull.

It is known that proteolytic enzymes appear to have an essential role in multiple phases of mammalian fertilization. We recently reported that the addition of plasmin, the active enzyme of the plasminogen activation system, to heparin-capacitated buffalo sperm increases the percentage of acrosome reacted spermatozoa and stimulates motility (Venditto *et al.,* 2010). Our results suggest that plasmin may play a role in events surrounding fertilization and suggest to evaluate in further studies whether the addition of plasmin during IVF improves the efficiency in buffalo.

A positive effect of cumulus cells at the time of IVF has been observed in buffalo, as in cattle (Zhang *et al.,* 1995), as demonstrated by the higher cleavage rate and embryo development obtained with cumulus-enclosed oocytes vs oocytes that were freed of their cumulus investment. Recently

we demonstrated that.co-culture of buffalo oocytes deprived of their cumulus at the end of IVM with bovine intact COCs in a 1:1 ratio completely restores their fertilizing capability and post-fertilization development (Attanasio *et al.,* 2010), suggesting that this approach can be used also during IVM for rescuing denuded oocytes, that are recovered in high proportion following OPU in this species.

Another factor that may affect embryo development is the duration of gamete co-incubation during IVF. It has been suggested that prolonged gamete co-incubation under the conditions of IVF, in which high concentrations of spermatozoa are incubated in small volumes of medium, results in the production of high levels of hydrolytic enzymes (Rehman et al., 1994) and free radicals (Aitken, 1994) that damage the oocytes. It has been demonstrated that the optimal sperm-oocyte co-incubation time for maximizing the blastocyst yield in buffalo is 16 h (Gasparrini *et al.*, 2008). Shortening the gamete co-incubation length to 8 h has resulted in a significant reduction of oocyte cleavage, similar to that reported in cattle (Ward *et al.,* 2002; Kochhar *et al.,* 2003). Interestingly, the lower blastocyst development recorded at the shorter durations of sperm-oocyte co-incubation tested were mainly due to the lower cleavage rates, as suggested by the fact that the oocytes that had cleaved developed further and as fast as those in the 16 h group. On the contrary, extending gamete

co-incubation to 20 h has been deleterious because, despite similar cleavage rates, the blastocyst production was reduced both when calculated in relation to COCs and to cleaved embryos. Furthermore, increasing the sperm-oocyte incubation time to 20 h has been found to be correlated to a higher incidence of polyspermy, confirming previous observations (Sumantri et al., 1997).

It is worth pointing out that the above reported results were obtained using semen from one bull previously tested for IVF. Subsequently it was demonstrated that marked differences in the kinetics of sperm penetration exist among buffalo bulls (Di Fenza *et al.,* 2008) and that this parameter is correlated to the blastocyst rate (Rubessa *et al.,* 2009), and hence can be a useful marker to predict the *in vitro* fertilizing ability of buffalo bulls. The great variability in the speed of oocyte penetration suggests to insert this assessment in the preliminary screening of bulls before their utilization in IVF programs. Furthermore, this finding suggests to adapt the gametes co-incubation time during IVF in relation to the bull used.

As previously mentioned, the poor cleavage rate may also be due to the lack of oocyte developmental competence, normally acquired during the maturation process. In order to investigate these aspects oocytes were parthenogenetically activated. In an earlier study we compared in vitro fertilization with oocyte activation using two chemical agents such as

ethanol and ionomycin, both followed by immediate exposure to 6-DMAP for 4 h (Gasparrini *et al.,* 2004a). A significant improvement of cleavage (71 % vs 56 %, respectively) and blastocyst yield (33 vs 23 %, respectively) was obtained with ethanol-induced activation vs IVF, indirectly suggesting that buffalo oocytes had acquired the developmental competence during IVM. However, cleavage and blastocyst rates obtained with ionomycin were intermediate (59 and 26 %, respectively). In addition, the improvement was not as such to make definite conclusions. It has more recently reported that activation with different methods give significantly higher cleavage and blastocyst rates compared to IVF, strongly suggesting that the problem has paternal rather than maternal origin (Mishra *et al.,* 2008). However, we speculate that the evident difference shown by these authors may be due to serious problems with the quality of the sperm utilized in their system since very poor cleavage (< 37 %) and blastocyst rates (< 16 %) were obtained following IVF.

Finally, it is worth pointing out that, after many fruitless attempts to increase cleavage rate in this species, the fertilization efficiency has at last improved, reaching approximately 80 % of cleavage rate, by enriching the IVM medium with cystine and cysteamine. This improvement has been proven to be related to enhanced intracytoplasmic GSH levels (Gasparrini *et al.,* 2006). This interesting finding would indicate that the poor cleavage

rate of this species so far recorded was in part related to an inappropriate maturation of the female gamete. It has been, in fact, suggested that the GSH production is critical for the acquisition of developmental competence of oocytes at a cytoplasmic level (Eppig, 1996) and that the measurement of GSH at the end of IVM can be a reliable indicator of the cytoplasmic maturation (de Matos, 1997).

In vitro culture (IVC)

The in vitro culture system developments for buffalo embryos have imitated those for other ruminant species. Thus, the IVC started from in vivo culture in an intermediate host, such as ligated oviduct of sheep (Galli *et al.,* 1998), which was replaced by the co-cultural systems, which was in turn substituted by defined media in the absence of serum and feeder cells. Buffalo embryos have been co-cultured with cumulus and oviductal cells (Chuangsoongneon *et al.,* 1991; Totey *et al*., 1992; Madan *et al.,* 1994) or with established cell lines such as BRL (Boni *et al.,* 1999). Although many authors still prefer the co-culture system for embryo production in this species, the utilization of defined media for embryo culture has become necessary to comprehend the requirements of buffalo embryos in vitro

which, in turn, would allow the formulation of an optimal species-specific culture system. A well known defined medium, such as the Synthetic Oviduct Fluid (SOF) has been utilized for embryo culture in this species since 1999 (Boni *et al.,* 1999), obtaining higher blastocyst rates compared to the co-culture system with BRL cells (13.5 % vs 7 %). Subsequently, buffalo zygotes/embryos have been successfully cultured either in SOF and in another defined cell-free system, known as Potassium Simplex Optimized Medium (KSOM) with similar embryo development (Caracciolo di Brienza *et al.*, 2001). The great improvement of blastocyst yields (35-40%) achieved in the following years is, according to our experience, due to the optimization of the IVM and in part of the IVF systems rather than to modifications applied to the IVC system. In fact, despite attempts to modify its original composition, at present the original version of SOF remains the most suitable medium for embryo culture in buffalo.

Based on current knowledge on metabolism and particularly glucose utilization of pre-implantation bovine and ovine embryos, we investigated first the effect of glucose removal from the medium and then that of reducing its concentration at different stage of culture in buffalo.

It has been demonstrated (Monaco et al., 2006) that the presence of glucose is absolutely required for in vitro culture of buffalo embryos, particularly during the early embryonic development (up to Day 4). In fact, the lack of

glucose in the IVC medium both for the entire duration of culture or limitedly to early culture (up to Day 4) has seriously compromised blastocyst development (2.4 and 9.6 %, respectively). On the contrary, when glucose was provided during early culture and removed during late culture, blastocyst yields have been high and comparable to the control (SOF), in which glucose was present throughout culture (30.7 and 32.7 % respectively). However, the complete removal of glucose from the medium is unlikely to benefit the embryo, as glucose plays important roles including ribose and NADH production through the pentose-phosphate pathway. Therefore, we subsequently evaluated whether a reduction of glucose concentration in the medium had an influence on buffalo embryos. Reducing glucose up to 0.5 mM (1/3) had no effect whereas when the concentration was reduced to 0.15 mM (1/10) throughout culture and limitedly during early culture a deleterious effect was observed (10% and 23% of TE, respectively vs 38.4%, of the control). On the contrary, decreased glucose concentration limitedly during late culture did not reduce embryo development (32%). This finding indicates that energy requirements of buffalo embryos during IVC are different from those of sheep and cattle, that show a significant rise in glucose uptake just around compaction, i.e. during late culture (Thompson *et al.*, 1991; 1996).

In order to reduce the accumulation of free radicals, ammonium and other catabolites that may affect embryo development, it has been suggested to use the easy expedient to change the medium more times during culture. However, no significant differences in buffalo embryo development have been recorded by changing the IVC medium 3 (Day 1, 3 and 5) or 2 (Day 1 and 5) times during culture, with a tendency of improvement in the latter case (Boccia *et al.,* 2006b). Therefore, in contrast to other species, the addition of fresh medium on Day 3 of culture in buffalo does not exert any positive influence. It is likely that this is related to the higher sensitivity of buffalo embryos to fluctuations of temperature and/or pH that normally occur during a culture change even if to limited extents; it results that it is advisable "not to disturb" buffalo embryos during culture.

Buffalo embryos *in vitro* develop approximately 12-24 h earlier than cattle embryos (Galli *et al.,* 2000) and this pattern of development reflects that observed in vivo, with most of the blastocysts collected by uterine flushing in the hatched stage at 6.5 days after the onset of estrus (Drost and Elsden, 1985). On Day 6 (Day 0 = IVF) it is possible to find embryos in advanced stages of development, including hatched blastocysts but most embryos reach the blastocyst stage on Day 7. A small proportion of embryos are delayed, reaching the blastocyst stage on Day 8 but their quality and viability is poor, as demonstrated by their lower resistance to

cryopreservation (Gasparrini *et al.,* 2001). Totey *et al.* (1996) reported a higher embryo production efficiency along with faster blastocyst production and a higher cell number in case of early cleaved buffalo embryos. The poor viability of slower-developing buffalo embryos has been recently confirmed by their reduced total cell number and by the altered expression profile of developmentally important genes such as HSP-70.1 and GLUT-1 (Rajhans *et al.,* 2010).

This finding implies that although blastocyst rate can be increased, prolonging the incubation time, it is at the cost of embryo viability. Hatching is also a good indicator of embryo quality. Supplementation of the IVC medium with FBS, BSA, and insulin can increase the hatching rate of Day 7 late morulae/early blastocysts (Chauhan *et al.,* 1998). We do not believe it is advisable though to extend the culture time to increase the hatching rate. In our in vitro standard system, hatched blastocysts are observed since Day 6 post-insemination, and the final embryo output is assessed not later than Day 7.

Despite high blastocysts rates pregnancy to term following ET of cryopreserved buffalo embryos is still very poor. This is likely due to poor viability of IVP embryos, resulting from suboptimal culture conditions. Currently, despite many efforts to improve embryo culture systems in different species, the oviduct remains irreplaceable for embryo

development. For this reason we analyzed the composition of oviduct fluid (ODF) in buffalo cows at different estrous cycle phases in order to fulfill the requirements of buffalo embryos in vitro (Vecchio et al., 2009). The analysis of buffalo oviduct fluid composition indicated species-specific differences that may justify modification of the composition of the media used for buffalo IVEP.

Therefore, in buffalo species, the optimization of the IVEP system efficiency is essential for the valorisation of this unique animal resource; nowadays, although the IVEP efficiency has significantly enhanced in the last years in terms of embryo production, (Neglia *et al.*, 2003; Gasparrini *et al.,* 2006), pregnancies to term obtained by vitrified embryos are not high enough to allow the diffusion of this technology in the field (Neglia *et al.,* 2004; Sá Filho *et al.*, 2005; Hufana-Duran *et al.,* 2004) and so unfortunately it is still far from being commercially viable (Gasparrini, 2010). Hence, despite some encouraging results, more studies and investigations are required to improve the efficiency of in vitro embryo and calf production so that the same could be used for breeding programs for the genetic improvement of buffalo, as well as for research in the areas of sexing, cloning, transgenesis, stem cell techniques.

One of the factor that may impair IVEP efficiency in this species is reproductive seasonality.

Seasonality of reproduction

Reproductive seasonality in mammals is the consequence of numerous complex elements based on the underlying physiological mechanisms of the adaptation of animals to local environment. In fact, seasonal breeding is a survival strategy adopted by many wild mammals to ensure that their progeny are born at the most favourable time 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 the offspring's life (Wood *et al.*, 2006).

Although domestication processes generally reduced seasonality of reproduction compared to what is observed in their wild counterparts, a majority of animal-derived products remain accessible only seasonally (Chemineau *et al.*, 2008). Indeed, also domestic animals commonly show seasonal variations in their reproductive activity. In fact, this characteristic is still present in some genetic types of bovine extensively bred, such as

Podolica, Sarda, Maremmana, *Bos Indicus* and the Highland bovine, but also in the horse, sheep, goat and buffalo (Zicarelli, 1997c).

In environments (equatorial zones) where the dark/light ratio is constant throughout the year and so there is no significant annual variation in photoperiod, the major influence on reproduction in mammals is given by fodder accessibility and, subsequently, by the nutritional status. In a recent study, Bronson (2009) reports that when the energetic costs of foraging exceed the calories gained, the result is negative energy balance and this depresses the activity of the GnRH pulse generator. This mode of regulating reproduction is characteristic of all mammals and if severe enough it overrides any factor promoting reproduction. In particular, in sheep and goats, nutritional status affects timing of cessation and initiation of ovulations and follicular development during the periods of transition to anestrus and return to estrus (Estrada-Cortés *et al.,* 2009). In general, reproductive function is influenced by the animal's nutritional condition, particularly the energy status (Imakawa *et al.,* 1986; Schillo, 1992), which includes amount of body energy stores and energy obtained from food consumption on a daily basis (Schneider, 2004).

Beside nutritional status, among the factors affecting the phenomenon of reproductive seasonality, it is worth to emphasize the pivotal influence exerted by the photoperiod. Generally, photoperiod, which entrains the

endogenous circannual rhythms of reproduction, exerts its action through two different but complementary and dependent pathways, by adjusting the phases of gonadal development with external natural conditions and by synchronizing the reproductive period between individuals of the same species (Chemineau *et al.,* 2008)

Buffalo species originated from tropical and subtropical areas corresponding to the large valley of Indo river (currently territory of Pakistan and India) and then it spread in every Continent. The species shows a pattern of reproductive efficiency closely related to the environmental and climatic conditions of breeding. Buffaloes become increasingly influenced by photoperiod with distance from the equator but nutrition remains important (Zicarelli, 1997; Campanile et al., 2010).

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 and Nanda, 1993)

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. It follows that it is a short-day-length species as its reproductive efficiency improves with a negative photoperiod; this demonstrates that the trend towards reproductive seasonality has only been partly influenced by moving into new areas of domestication and breeding.

The influence of photoperiod means that, without intervention, buffaloes have seasonal cycles in conception, calving and milk production (Campanile *et al.*, 2010).
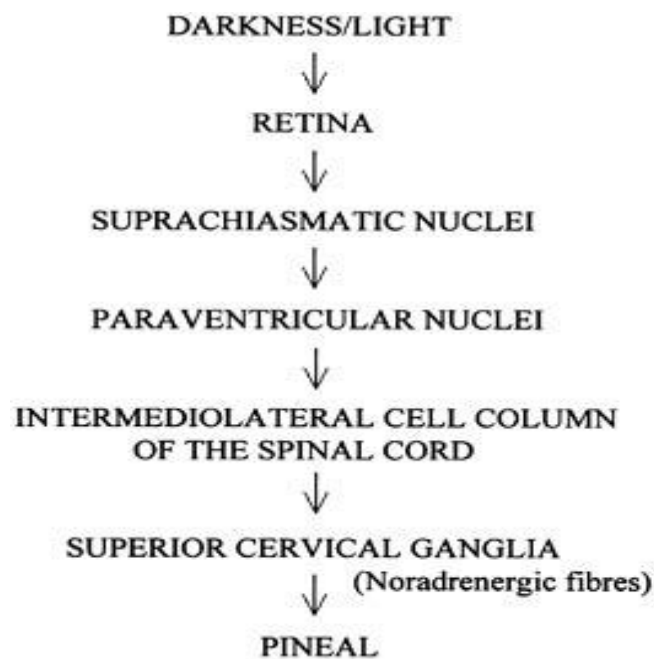
Buffaloes are more affected by reproductive seasonality with distance from the equator (Zicarelli *et al.*, 1997a); in particular, females that calve during the non-breeding season have an extended postpartum anoestrous period with a proportion not resuming ovulation until the following breeding season (Zicarelli, 1997c). 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, 1994), represents one of the causes which gave rise to this adaptation process (Malpaux *et al.*, 1996).

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 1997c).

This reproductive pattern depends on the duration and the intensity of the light source, which is captured by the retina (retinal photoneurons) and subsequently passes through different neural connections: first, it is developed by the suprachiasmatic nucleus (biological clock which regulates the endogenous circadian rhythm). From this level, the information reaches the superior cervical ganglion and then the pineal gland. Therefore, the pineal gland is the main regulatory organ in the seasonality of breeding: it has no efferent projections, and therefore it affects neuroendocrine function by humoral means (Cardinali DP, 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, 1989) *via* exerting a stimulating effect on GnRH secretion by the hypothalamus in short-day breeders. On the contrary, in long-day breeders, such as the horse,

increased melatonin exposure has the opposite effect, inhibiting GnRH release by the hypothalamus. Thus differences in day length are recognized and translated into signals able to turn sexual activity on or off (Figure.1).

Figure 1. Retino-pineal pathway: noradrenergic fibres originating from superior cervical ganglia have their terminals in the pineal.

DARKNESS/LIGHT
↓
RETINA
↓
SUPRACHIASMATIC NUCLEI
↓
PARAVENTRICULAR NUCLEI
↓
INTERMEDIOLATERAL CELL COLUMN
OF THE SPINAL CORD
↓
SUPERIOR CERVICAL GANGLIA
↓ (Noradrenergic fibres)
PINEAL

Over the generations, light stimulus prevailed on reproductive conditioning of photosensitive species, even after being moved from the place of origin. From transfer to zones near to the equator, sensitivity to light stimulus no longer influences reproductive activity due to the constant length of the light/dark ratio during the year. In this case, meeting the nutritional requirements of the buffalo, equine and ovine in the Amazon area, for example, prevails over light stimulus. Transferring Northern-originated

species to the South of the equator results in a seasonal reproductive activity being conditioned more by the light stimulus than by meeting nutritional requirements (Zicarelli, 1997c). In each case, the tendency towards seasonality increases when farther from the equator (Zicarelli 1997a). In Southern Italy, where over 95% of Italian buffalo population is bred, in the month of September there are not lush pastures. In fact, usually, after the middle of June, the pasture is poor and the nutritional status of the subjects in autumn at the onset of lactation and at the resumption of ovarian cycle are poor, unlike the same period of the year in the tropics where there is plenty of fodder, after the wet/rainy season. This can lead to suppose that the buffalo bred in Italy is not indigenous, because it is unlikely that an animal species shows a marked tendency to give birth in a period when forage availability is low and the temperature is not beneficial to the survival of offspring (Zicarelli, 1997c). Furthermore, in buffalo, reproductive seasonality is more evident in older subjects (Zicarelli et al, 1988) that are affected to a greater extent by the "bull effect" and by seasonal anestrus. In some studies carried out on Mediterranean buffaloes bred in Italy, it has been showed that melatonin is the endocrine signal of alternating light and dark. The raising of blood levels of melatonin at 2 hours after sunset is much lower in subjects less sensitive to photoperiod (Di Palo *et al.*, 1993; Parmeggiani and Di Palo, 1994). The high

repeatability of this feature suggests a hereditary basis of the phenomenon, useful for genetic selection purposes. As it has been previously affirmed, in Italy the main economic explanation for buffalo breeding is given by the production of mozzarella cheese, exclusively produced with fresh buffalo milk (Zicarelli, 1992). It has to be observed that in our country the seasonality of the species is a big issue, since it implies that the greater milk production does not coincide with the increased market demand for mozzarella cheese. Years ago, the dairy producers overcame the shortage of milk during the spring and summer using stocks of milk that had been frozen during the winter. Since 1993 with the DCO (Denomination of Controlled Origin) certification, and later on in 1996 with the DPO trademark ("Mozzarella di Bufala Campana"), freezing milk was definitely prohibited, as it adversely affects final product quality. Indeed, mozzarella made with frozen milk shows qualitative and organoleptic characteristics significantly lower than that produced with fresh milk. This resulted in a significant market decline of buffalo mozzarella, which led the technicians working in the field to identify appropriate strategies to reverse the timing of calvings. To solve this obstacle, and then to produce milk in synchrony with the market requirements, a special procedure was defined and developed , the *out of breeding mating season* (OBMS). It consists in the interruption of sexual promiscuity in the herd between September and

December during the first year of application, and from September to March in the following steps of the technique. It allows to modify calving intervals in order to obtain a better distribution of them throughout the year. Currently, the OBMS technique is utilized in more than 60% of the farms in Campania region and offers unquestionable economic advantages. Then again, it is fundamental to point out that this technique, necessary for economic reasons, results in a decline in fertility, as it forces the breeders to mate buffaloes during the less favorable periods.

Aim of the work

In the light of the foregoing introduction, it seemed useful and interesting to investigate the effect of season on reproductive performance in buffalo species at our latitudes. For this reason, aim of this work has been to provide an overview of seasonal influence on buffalo reproductive pattern at different levels: first, we tried to understand how the influence of photoperiod affects gametes because they are the germinal source and the base of any biotechnological process, being it carried out *in vivo* or *in vitro*. Therefore, on the one hand, we have monitored the trend in the recovery of oocytes from slaughtered animals, their quality and developmental competence up to the embryo stage; while on the other hand, we have

analyzed some parameters related to the fertility of buffalo semen during the seasons of the year. Next, we focused our attention on the applications of biotechnologies in the field in different seasons. In particular, we evaluated the efficiency of artificial insemination carried out in two different seasons, ie during autumn and during the transitional period, usually characterized by high embryonic mortality.

Finally , we screened the efficiency of oocytes pick-up from live donors to be devoted to the different procedures of the IVEP, their quality and competence development up to transferable embryos, in three different seasons.

GAMETES

Experiment 1. Assessment of morphological quality and competence to *in vitro* embryonic development of oocytes throughout the seasons

There is now a growing amount of evidence to suggest that while culture conditions during in *vitro* embryo production can impact on the developmental potential of the early embryo, the intrinsic quality of the oocyte is the key factor determining the proportion of oocytes developing to the blastocyst stage (Lonergan et al., 2003). Therefore, an aspect that we believed it was worth investigating to elucidate the effect of the season on reproductive sphere of Mediterranean Italian buffalo was the recovery and morphological quality of the oocytes and, subsequently, their competence *to in vitro* embryo development. In order to do so, an analysis was carried out on data produced from abattoir-derived ovaries in our IVEP lab over 3 years. Four different periods were compared: April-June (spring), July-September (summer), October-December (autumn) and January-March (winter).

Materials and methods

Unless otherwise stated, reagents were purchased from Sigma-Aldrich® (Milano, Italy). IVM medium (B199) consisted of TCM199 buffered with 25mM sodium bicarbonate and supplemented with 10% fetal calf serum (FCS), 0.2mM sodium pyruvate, 0.5 µg/mL FSH (from ovine pituitary), 5 µg/mL LH (from ovine pituitary), 1 µg/mL 17β-estradiol, 50 µg/mL kanamycin and 50 µM cysteamine (Gasparrini *et al.*, 2000). Oocytes were handled on a warm bench in TCM199 with 10% FCS, buffered with 15mM HEPES and 5mM sodium bicarbonate (H199). The fertilization medium was Tyrode albumin lactate pyruvate (Lu *et al.,* 1987) supplemented with 0.2mM penicillamine, 0.1mM hypotaurine and 0.01mM heparin. The IVC medium was synthetic oviduct fluid (SOF) including essential and non-essential aminoacids and 8 mg/mL bovine serum albumin (Tervit et al., 1972, Gardner *et al.,* 1994). Both fertilization and culture media were buffered with 25mM sodium bicarbonate, which was replaced by 20mM HEPES, and 5mM sodium bicarbonate when oocytes and embryos were handled outside the incubator.

Oocyte collection and in vitro maturation

Buffalo ovaries were collected from a local abattoir, transported to the lab within 3–4 h after slaughter at approximately 35°C in physiological saline supplemented with 150 mg/L kanamycin. Cumulus–oocyte complexes (COCs) were recovered by aspiration of 2–8mm follicles using an 18-G needle under vacuum (40–50 mmHg).

The COCs were evaluated on the basis of their morphology and classified in the following categories (see Figure.2):

- Grade A: oocytes with homogeneous cytoplasm and the entire surface surrounded by multiple layers of cumulus cells;

- Grade B: oocytes with homogeneous cytoplasm and with at least 70% of the surface surrounded by multiple layers of cumulus cells;

- Grade C: oocytes with few cumulus cells;

- Grade D: oocytes with obvious signs of cytoplasmic degeneration;

- Naked: oocytes completely free of cumulus cells;

- Expanded: oocytes surrounded by expanded and clustered cumulus cells;

- Small: oocytes with a diameter smaller than 120 μm;

- Misshapen: oocytes with an abnormal shape;

- ZP: zonae pellucidae.
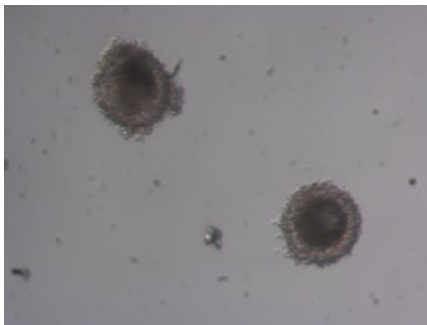
Figure 2. Oocyte grading
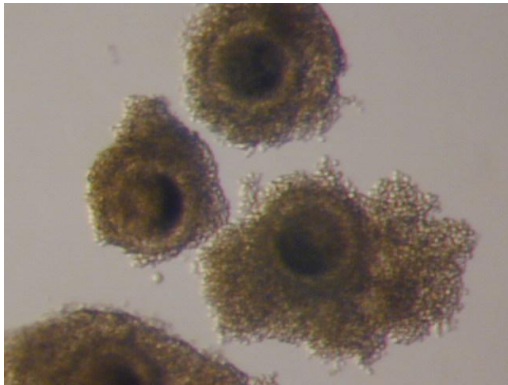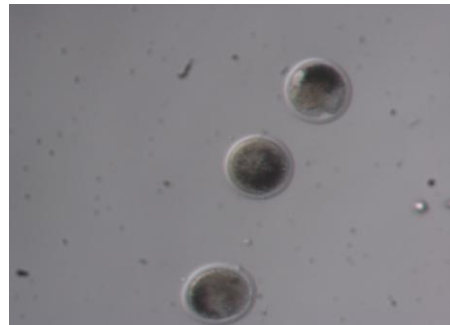

Grade A
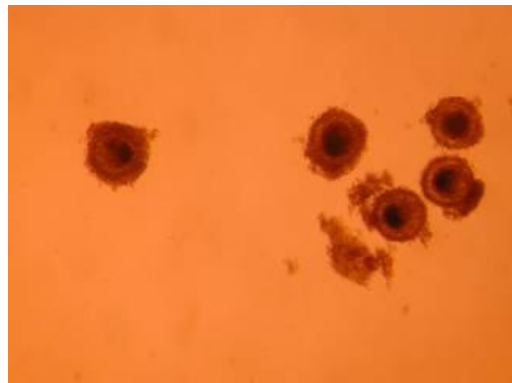
Grade B
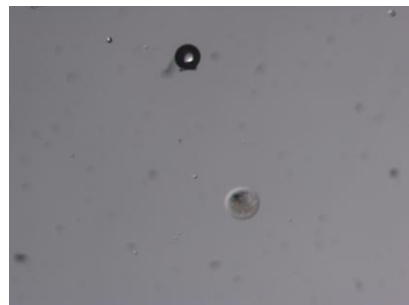

Grade C

Degenerated

Naked

Expanded

Misshapen

ZP

Grades A and B COCS, that are considered suitable for IVEP (Neglia *et al.,* 2003), were quickly selected from the dishes, washed thoroughly in H199, once in the final maturation medium and allocated to 50μL drops (10 per drop) of the same medium under mineral oil. IVM was carried out at 38.5°C for 21 h in a controlled gas atmosphere of 5% CO_2 in humidified air.

In vitro fertilization and culture

Spermatozoa were prepared from frozen-thawed semen, obtained from a bull, which was previously tested for IVF in our laboratory. Sperm were treated by swim-up procedure in Hams F-10 medium for 1 h. After centrifugation of the supernatant, the sperm pellet was re-suspended to a final concentration of 2×10^6 mL^{-1} in the fertilization medium. Insemination was performed in 50 μL drops of fertilization medium under mineral oil (five oocytes per drop) at 38.5°C under humidified 5% CO_2 in air. Twenty hours after IVF putative zygotes were removed from the fertilization medium, stripped of cumulus cells by gentle pipetting, washed twice in HEPES-buffered SOF and allocated to 20 μL drops of sodium bicarbonate-buffered SOF. Culture was carried out under humidified 5% CO_2, 7% O_2 and 88% N_2 at 38.5°C. On day 5 post-insemination (pi) the proportion of

oocytes cleaving was assessed, unfertilized and degenerated oocytes were discarded and the embryos transferred into fresh medium for further 2 days of IVC. Final embryo yield, in terms of tight morulae-blastocysts (TM + BL) and Grade 1 and 2 blastocysts (G 1&2 BL), was evaluated on Day 7 pi. A retrospective analysis was carried out on data recorded over 3 entire years in the IVEP laboratory. In order to study the effects of the season we considered the following 4 periods of the year: April-June (Apr-Jun), July-September (Jul-Sep), October-December (Oct-Dec) and January-March (Jan-Mar). The variability in the number of animals slaughtered during the different months accounts for the non uniform number of cases among the considered periods (Table 3). Per each collection day, we recorded the number of ovaries aspirated and non aspirated (with no follicles or with follicles smaller than 2 mm), the number of total oocytes recovered, as well as the number of the different categories of oocytes previously described. After the follicular aspiration the oocytes were searched under a stereomicroscope, operating a quick selection followed by the IVM of Grades A+B COCs, i.e. those suitable for IVEP, to avoid to compromise their viability by extending the handling time. Afterwards, the dishes containing the discarded oocytes were carefully re-evaluated to establish the incidence of the different categories.

As during the period of the study different experiments were performed in the lab, to evaluate the effect of season on oocyte developmental competence after IVF, we considered only the data of the control group of each ongoing other experiment. This accounts for the discrepancy in the number of replicates/oocytes between the studies on morphological features of the oocytes and those aimed to evaluate the developmental competence into blastocysts. In particular, data on cleavage and embryo yields were obtained from a total number of 785, 987, 486 and 729 Grades A+B COCs, over 32, 35, 18 and 22 replicates, respectively in Jan-March, Apr-June, July-Sept and Oct-Dec.

Statistical analysis

Data were analyzed by the ANOVA procedure of SPSS 17.0 statistical software (2009) according to the following model:

$Y_{IJK} = \mu + A_I + B_J + (AB)_{IJ} + \varepsilon_{IJK}$ where

A = year effect (3 levels)

B = season effect (4 levels)

AB = interaction year x season

As the distribution of variances was not homogeneous and no year effect was found, differences in the percentages of small oocytes among seasons were analyzed by the Kruskal-Wallis test (SPSS 17.0, 2009).

Results

As no differences were observed in terms of meteorological parameters among years, in Table 3 we reported the average values registered in different seasons from the local meteorological centre (National Observatory of Grazzanise, Caserta). Nevertheless, the morphological parameters considered to evaluate oocyte quality showed some fluctuations among years.

Table 3. Meteorological parameters registered in different seasons during the 3 year-trial

	Mean T (°C)	Min. T (°C)	Max. T (°C)	Rain fall (mm)	Humidity (°)	Wind (Km/h)
Jan-Mar	7.6±0.4	3.1±0.2	12.7±0.5	94.4 ±7.1	77.4±1.1	10.8±0.7
Apr-Jun	17.1±0.3	11.3±0.3	23.2±0.7	42.7±12.4	76.6±3.1	9.2±1.0
Jul-Sep	23.3±0.5	17.5±0.2	29.3±0.6	38.6±25.4	74.5±3.0	8.9±0.1
Oct-Dec	13.3±0.7	8.9±1.1	18.7±0.3	107.4±48.7	80.1±1.6	9.1± 1.4

Values are expressed as mean ± SD

Results on oocyte recovery and quality, the latter based on morphological criteria, are shown in Table 4.

Table 4. Oocyte recovery and distribution of different oocyte categories (percentages on the total) among different seasons.

	Jan-Mar	Apr-Jun	Jul-Sep	Oct-Dec
Run	31	32	19	34
N. Ovaries	1257	1388	881	1886
N. total oocytes	5724	7109	4299	9135
N. COC AB	2669	3238	2188	4068
non-aspirated ovaries (%)	3.0±1.2	6.3±1.2	5.8±1.6	7.1±1.2
Oocyte /ovary	4.6±0.27	5.1±0.26	4.9±0.35	4.8±0.26
A+B COCs/ovary	2.1±0.1	2.3±0.1	2.4±0.2	2.1±0.1
Grade A+B (%)	46.4±1.4	46.4±1.3	48.7±1.8	43.3±1.3
Grade C (%)	6.0±0.6	5.5±0.6	6.5±0.8	7.7±0.6
% degenerated	19.9±1.1[ab]	20.2±1.1[ab]	18.1±1.5[a]	23.0±1.1[b]
% expanded	18.3±1.0	17.9±1.0	16.1±1.3	16.8±0.9
% naked	8.1±0.6	7.2±0.6	8.0±0.8	7.2±0.6
% small	0.2±0.1[a]	0.9±0.1[b]	0.9 ±0.2[b]	0.3±0.1[a]
% misshapen	0.8±0.2	1.3±0.2	1.3±0.3	1.3±0.2
% ZP	0.4±0.1	0.5±0.1	0.2±0.2	0.6±0.1

[a,b] Values with different superscripts within rows are different; $P<0.05$. Values are expressed as mean ± SD.

The percentage of degenerated oocytes (Grade D) was affected by the season (P<0.05), the year (P<0.01) and an interaction year x season (P<0.05) was also found. In particular, the percentage of degenerated oocytes increased during Oct-Dec compared to Jul-Sep; however, this percentage was higher in the third year than in the first and the second (17.2 ± 0.9, 19.2 ± 1.1 and 24.5 ± 1.1, respectively).

Finally, the percentage of small oocytes was affected by season, with higher values (P<0.05) during Apr-Jun and Jul-Sep compared to Oct-Dec and Jan-Mar (Table 2).

Although oocyte quality was not much affected, oocyte developmental competence significantly improved during Oct-Dec compared to Apr-Jun, with intermediate values recorded during Jan-Mar and Jul-Sep.

Indeed, the percentage of cleavage, was higher (P<0.01) during Oct-Dec (71.7 ± 3.1) than during Apr-Jun (58.0 ± 2.4), with intermediate values in the other two periods (67.0 ± 3.7 and 64.9 ± 3.4, respectively in Jan-Mar and Jul-Sep), as shown in Figure 3.

Figure 3. Cleavage rate in different seasons in relation to day light hours
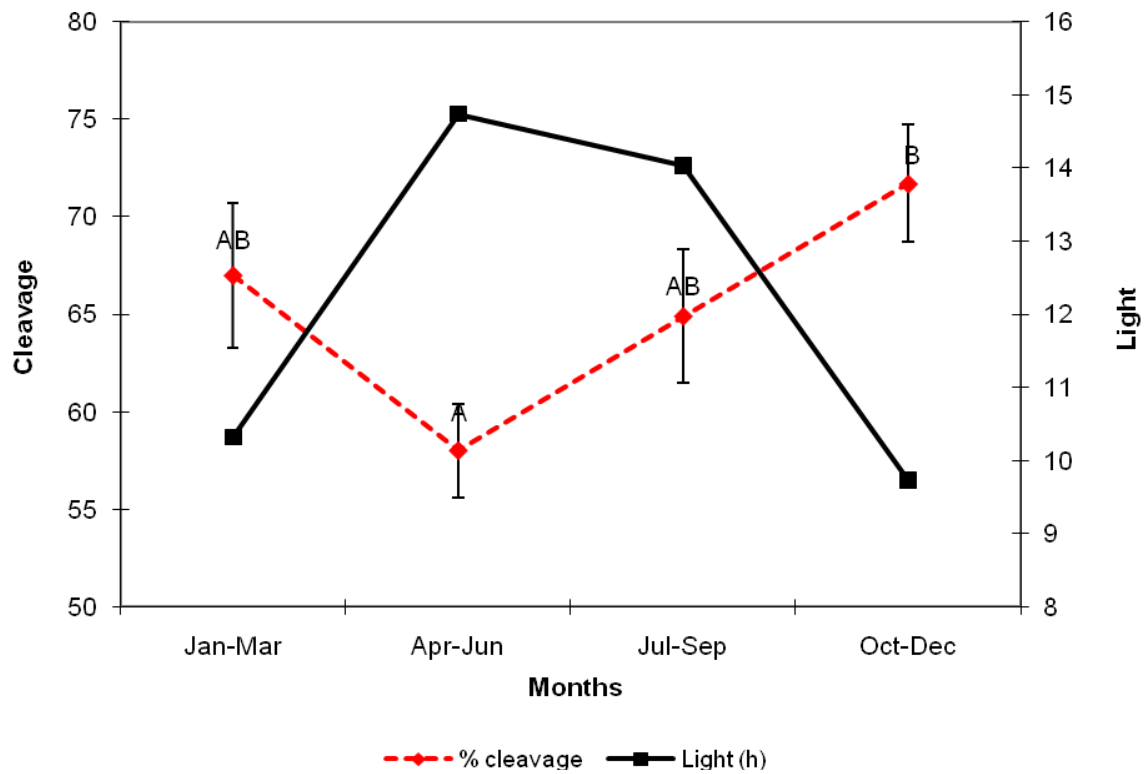
_{A, B,} Values with different letters are significantly different; $P<0.01$

Likewise, the percentages of TM + BL and G 1&2 BL were higher ($P<0.05$) during Oct-Dec (26.5 ± 2.1 and 20.9 ± 2.0, respectively) than during Apr-Jun (18.8 ± 1.6 and 13.7 ± 1.5, respectively), with intermediate values in Jan-Mar (25.4 ± 1.7 and 18.5 ± 1.6, respectively) and Jul-Sep (24.1 ± 2.2 and 14.9 ± 2.1, respectively), as shown in Figure 4.

Figure 4. Percentages of tight morulae-blastocysts (TM + BL) and good quality blastocysts (BL G1&2), calculated out of total COCs, in different seasons in relation to day light hours.
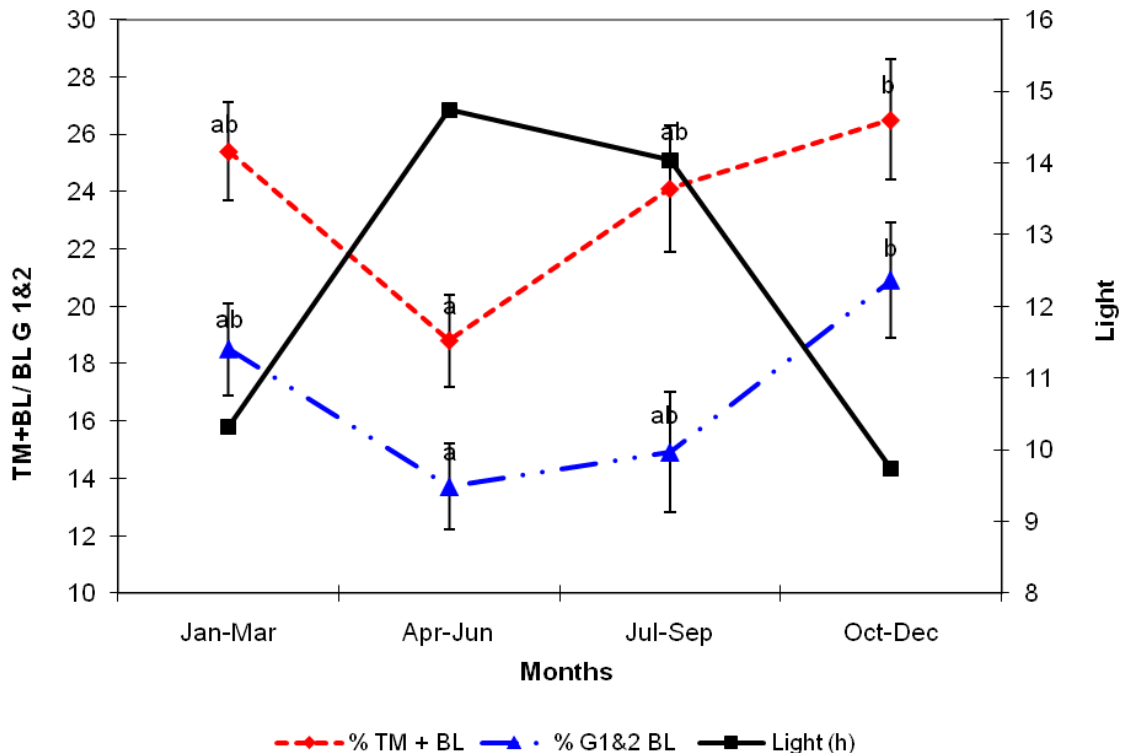
a, b Values with different letters are significantly different; P<0.05

Discussion

The present retrospective study examined whether season could influence oocyte population and quality and in vitro embryo development in buffalo species. The oocyte number recovered per ovary and the number and incidence of good quality oocytes were not affected by season, whereas the developmental competence was improved in autumn compared to spring, as demonstrated by higher cleavage and blastocyst yields.

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The average number of oocytes recovered per ovary was higher than those previously reported by Indian authors following follicular aspiration of slaughterhouse ovaries (Totey *et al.,* 1992; Madan *et al.,* 1994; Das *et al.,* 1996). On the contrary, the average number of Grade A+B oocytes collected per ovary was comparable to that previously observed in Italian Mediterranean buffalo, using slaughterhouse ovaries (Gasparrini *et al.,* 2000). It was previously speculated that the higher recovery rate reported in our setting may reflect the better nutritional status of Italian buffalo cows as the plane of nutrition affects the follicular dynamics (Smith, 1984).

Among all the oocyte categories that we considered, only the small and the degenerated oocytes were affected by season. The lower incidence of small oocytes in both autumn and winter reflects the seasonality of the species: in fact, it has been demonstrated in other species that small oocytes remain at the diplotene stage (Szybeck, 1972; Wassarman and Josefowicz, 1978) and can be considered meiotically incompetent and therefore are not able to acquire the developmental competence. However, it is worth pointing out that the small oocytes had a little weight upon the total population. Unexpectedly, an increased percentage of degenerated oocytes was found in autumn but this was recorded only in one year. As no differences in meteorological parameters were observed among years it is not possible to rule out that this phenomenon is casual. We may speculate that in autumn, as hypophysis is

more responsive, the follicular recruitment and hence the number of small follicles increase. It is known that small follicles, which are poorly responsive to gonadotropins, may undergo atresia through apoptosis more easily (Sugino *et al.,* 1996). This hypothesis is supported by the observations that the number of follicles and of the oocytes is higher in autumn-winter than in spring-summer in buffaloes undergone OPU (Di Palo *et al*., 2001). However, it is also worth noting that incidental factors may play a role, such as the fact that in our country the animals that are slaughtered between September and December, when the price of milk and the market demand for mozzarella cheese decrease, are mainly subjects at the end of the productive career and/or culled for reproductive problems.

However, despite the higher incidence of degenerated oocytes, the various parameters considered for testing IVEP efficiency were improved in autumn; indeed, the percentage of cleavage, as well as the tight morulae-blastocyst (TM + BL) rate and Grade 1 and 2 blastocyst rate (G1&2 Bl), were higher in this period compared to spring, perfectly reflecting the seasonal pattern expressed in vivo by the species at our latitudes (Zicarelli et al., 2007a; 2007b). As clearly shown in Figures 3 and 4, there is an overwhelming evidence that the highest IVEP efficiency corresponds to the shortest day length while the worst efficiency is recorded when the number of daily light hours is the highest.

These results are different from those obtained by Manjunatha et al. (2009) during an OPU trial carried out in India in the low (Apr-Sep) and peak (Oct-Mar) breeding seasons. They also reported an overall improved efficiency in the favourable season that was though essentially due to the increased number of follicles and COCs and, hence in the higher number of blastocysts produced per animal per session, whereas neither the oocyte quality nor the cleavage and blastocyst rate were improved. These authors speculate that the reduction of follicles and COCs during the low season is due to the heat stress; however, temperatures were not so different between the two periods compared, whereas there was a difference in daily light hours, although less evident than at our latitudes. The main difference between these studies is the oocyte source that makes difficult to compare the results. Interestingly, in the present study the blastocyst yield was improved despite the more heterogeneous oocyte population, whereas it was unaffected in the other trial, in which continuous OPU-induced resetting of follicular population should have improved oocyte quality.

Our results agree with those reported in the sheep, another short-day breeder that shares many reproductive traits with buffalo (Zicarelli, 1994; 1997a; 2002), in which a significant decrease in efficiency has been described in the unfavorable seasonal period after IVF (Stenbak et al., 2001).

Summing up the above results, it appears that in autumn, although oocyte quality, assessed on morphological criteria, is not improved, the competence of oocytes to be fertilized in vitro and to develop to the blastocyst stage increases compared to spring, whereas the efficiency is intermediate in summer and winter. This pattern reflects well the reproductive seasonality of buffalo: although the difference in terms of developmental competence was significant only between the two periods with full blown positive and negative photoperiods, there was a tendency to better results in both autumn and winter (negative photoperiod) than in spring-summer (positive photoperiod).

It is interesting to note that the winter months (Jan-Mar) correspond to the so called transitional period, i.e. the period of the year in which the light hours begin to increase, and a higher incidence of embryonic mortality is observed in buffalo after AI (Campanile *et al.*, 2010) The comparable embryo yields recorded in winter and autumn, suggest that other factors may determine the phenomenon rather than oocyte competence. However, since we only evaluated cleavage and blastocyst rates we cannot rule out that the embryo viability and hence the ability to sustain development to term after transfer, in this period is reduced to some extent.

The discrepancy that emerged between the data reporting morphological features of oocytes and those indicating of developmental competence is

difficult to explain. It is known that the morphological classification of gametes is one of many parameters indicative of the relative competence, as a correlation exists between the morphology and the embryo yield. However, this correlation has no absolute reliability so much so that in recent years it has been developed a line of research primarily aimed to identify non-invasive assays for the selection of competent gametes. It is worth remarking that, at present, the best assessment of the oocyte quality is given in retrospect by its ability to be fertilized and to develop into a viable embryo and, even better, by the ability of the embryo to implant and sustain development to term. There are many factors that influence the quality of oocytes, which, under the operating circumstances are not easily controlled and therefore may overlap the seasonal effect here studied retrospectively. On the other hand, this study took place over three years and therefore these data are of some importance from a statistical point of view. It follows that the assessment of oocyte quality, made by observing only the morphology, cannot be viewed as highly reliable for predicting the competence. In addition, it is worth to specify that the microscope screening and the following selection of COCs A + B for IVEP was done quickly to reduce the time of exposure of the gametes to stressful factors: it follows that the two categories considered suitable for embryo production were processed together and therefore it was not examined whether the relationship between A and B

changed among seasons. Although both Grade A and B oocytes are usually considered suitable for embryo production, a significantly higher blastocyst yield is obtained from Grade A vs B oocytes (Caracciolo di Brienza *et al.*, 2001).

We cannot underestimate that the onset of negative photoperiod has actually improved the competence of oocytes recovered. Therefore, the influence of photoperiod, via the hypothalamic pituitary axis, seems to be expressed even at an early stage of oocyte development. In fact, although the oocytes used for IVEP were removed from the follicular environment before the process of gonadotropin-dependent maturation took place and they were released from the endocrine influence, their developmental capacity was influenced by the photoperiod. In other words, the growth of oocytes cannot be attributed solely to autocrine action of the ovary. A proof of this phenomenon can be considered the fact that during the favourable reproductive season the incidence of small oocytes is significantly lower.

Experiment 2. Evaluation of some parameters of sperm fertility throughout the seasons

Fluctuations in bull sperm output can be due to several reasons. Most of the parameters of the ejaculate such as volume, concentration or sperm characteristics (in particular motility, morphology, viability) depend on accessory sex gland secretion and epididymal function (Koonjaenak *et al.,* 2007a). Because spermatogenesis is highly sensitive to even short increases in scrotal temperature, external cues such as seasonality also appear to influence sexual function and so semen quality in bulls. This may occur either through photoperiod (Barth and Waldner*.,* 2002) or through changes in environmental temperature (Fayemi and Adegbite, 1982; Sekoni and Gustafsson, 1987), as it has been recorded in *Bos Taurus* AI sires kept in temperate regions (Januskauskas *et al.,* 1995). For instance, *Bos taurus* bulls have minimum sperm output during midwinter and late summer, concomitantly with the presence of the highest percentages of abnormal spermatozoa (Koonjaenak *et al.,* 2007a). Furthermore, the ability of their spermatozoa to survive freezing is lowest in summer (Söderquist *et al.,* 1997). The age of the bull plays a role in these relationships; young bulls are more affected than older ones. Species and their inherent ability to adapt to tropical or semi-tropical environments is another variable that

influences whether ambient temperature/humidity affects bull reproduction (Koonjaenak *et al.,* 2007a).

Seasonal variations in the semen characteristics of bovine bulls have been described in earlier studies concerning ejaculate volume and sperm concentration (Rekwot et al. 1987; Wildeus and Hammond 1993), as well as morphologically altered spermatozoa (Igboeli and Rahka 1971; Sunby and Torjesen 1978; Ross and Entwistle 1979; Kumi-Diaka et al. 1981; Rekwot et al. 1987; Sekoni et al. 1988; Barth and Oko 1989; Koivisto et al. 1996; Koivisto et al. 2009).

Semen quality of the collected ejaculate, including the ejaculate volume, sperm motility and proportions of morphologically and physiologically normal spermatozoa, determines the quality of the processed (often frozen) semen and, ultimately, the potential fertility level achieved when artificial insemination (AI) is used (Rodriguez-Martinez, 2000).

Furthermore, it should be considered that even if the greater part of spermatozoa survive cryopreservation, many expire in the procedure. The surviving spermatozoa undergo a certain change in viability, presumably related to changes in the plasma membrane and organelles (Watson, 1981; Hammerstedt *et al.,* 1990; Parks, 1997), such as the acrosome, the mitochondria, and the tail (Jones and Stewart, 1979; Thomas *et al.,* 1998). The proportion of spermatozoa with an intact plasma membrane, either as

ejaculated spermatozoa or frozen-thawed, is positively related to fertility (Rodriguez-Martinez, 2003) A spermatozoon must also depict normal progressive motility and this parameter is also affected by cryopreservation, as a consequence of changes inflicted by freezing and thawing on the plasmalemma and the tail. In fact, relationships between post-thaw sperm progressive motility and fertility have also been determined in buffalo species (Koonjaenak *at al.,* 2007b).

Therefore, an accurate evaluation of fertility of bulls used for AI is of utmost importance since a single ejaculate provides several insemination doses and influences the reproductive potential of a herd (Rodriguez-Martinez and Larsson, 1998). It has been now demonstrated that the role of the male gamete is not limited to the fertilization process but, on the contrary, an important paternal contribution is given to the subsequent embryo development, as indicated by the high incidence of early embryo mortality due to genetic and centrosomial defects of sperm in different species (Yanagimachi et al. 2005).

With regard to buffalo, significant seasonal variation was observed in sperm kinematics and hypoosmotic swelling (HOS) reactivity in Murrah buffalo (Mandal *et al.,* 2003). Except for linearity, the mean values of sperm dynamics were higher during summer and rainy season and significantly lower in winter season. Seasonal changes did not appear to

cause deleterious changes in sperm quality in swamp buffalo AI-sires in tropical Thailand (Koonjaenak *at al.,* 2007a). However, another study reported that post-thaw plasma membrane stability and plasma membrane integrity, assessed by flowcytometry, were significantly better in sperm samples processed during winter (November-February) than in samples processed during the other seasons of the year, ie the rainy season (July-October) and summer (March-June). Furthermore, it was demonstrated that frozen–thawed swamp buffalo sperm chromatin integrity is not seriously affected by the seasonal variations in temperature and humidity seen in tropical Thailand (Koonjaenak *at al.,* 2007d).

With regard to buffalo bred at Italian latitudes, it is known that the reproductive seasonality in males is characterized by the reduction of the *libido*. It often happens that, in increasing day length months bulls stop mounting and, hence, do not produce ejaculates. This has not made easy to allow the evaluation of semen in relation to the season. Therefore, it is not possible to rule out that ejaculates collected immediately before and/or after this period of silence of the reproductive activity, are subfertile.

Hence, the objectives of this experiment were to evaluate the effects of season on Mediterranean Italian buffalo (*Bubalus bubalis*) semen comparing some fertility parameters of four bulls (I, II, III and IV) ejaculates, over a period of 24 months, which was divided into summer

(July, August, September), autumn (October, November, December), winter (January, February, March) and spring (April, May, June).

Materials and Methods

Animals

Four Mediterranean Italian mature fertile buffalo bulls (*Bubalus bubalis*) from the studs of Co.F.A. (Cooperativa di Fecondazione Artificiale, via Orezola, Tidolo di Sospiro, Cremona, Italy) were used in this study. The age (mean ± SD) of the bulls was 6.4 ± 1.04 years (range 5.47–7.75 years) at the beginning of the experiment.

In accordance with European Regulation, in the barns, each bull was tied at its individual stand and was fed corn silage ray grass hay, alfa-alfa hay and concentrate. The beds were made of sawdust and the barns were cleaned and disinfected once a month.

Before being put into production, the buffalo sires were kept in quarantine for 3-4 months in another stable and during this period they underwent several tests to meet all the health requirements needed for semen production. They were found to be free from clinical pathologies, including testicular, epididymal and genital tract pathologies.

Semen collection and sperm morphology assessment

Semen was collected by using an artificial vagina twice a week from all sires, with one or two ejaculates per day, according to the market demand and to the *libido* of the bull. For this purpose, the bulls were always handled by the same person. The volume (ml) was assessed manually with a graduated tube, while the concentration (10^9/ml) of each ejaculate was estimated using a spectrophotometer (LKB Biochrom Ltd Novaspec II, Cambridge, England). The percentage of motile sperm was assessed by a visual evaluation of fresh semen, carried out by the same operator, using a phase-contrast microscope (Olympus, Tokyo, Japan) at 100 or 200x magnification, with the sample kept at constant temperature. Furthermore, a morphological analysis was performed in order to detect the appearance and incidence of total sperm abnormalities, hence the sum of primary anomalies (severe defects of head, mid-piece and tail) and secondary anomalies (detached heads, distal and proximal cytoplasmic droplets, bent or coiled tails).

Subsequently, every ejaculate was diluted with the Andromed (Minitüb, Tiefenbach, Germany), an extender without egg yolk, and placed at 5°C for 4 hours. Thereafter, ejaculates were packed into 0.5 ml plastic French Straws, frozen according to standard procedure and then stored in special dewars containing liquid nitrogen at -196°C. Further tests have been carried

out on the frozen/thawed semen and by means of an automatic image analyzer (IVOS Computer-Assisted Sperm Analysis, CASA, Hamilton Thorne, Inc. MA, USA): the percentage of progressive motility was evaluated on spermatozoa with a VAP (average path velocity) that exceeded a threshold value (in buffalo species the VAP is 25.0 µm/sec) and with a straight movement.

For this study, a total number of 295 ejaculates from bulls I, II, III, and IV (60, 75, 80 and 80 respectively) were examined in relation to seasons (n= 53, 107, 98 and 37, respectively for winter, spring, summer and autumn).

Statistical analysis

Data were analyzed by the ANOVA procedure of SPSS 17.0 statistical software (2009). Differences among means were evaluated by Duncan test. In case variances were not homogeneous, within the groups, the Kruskal-Wallis one-way analysis of variance by ranks was used.

Results

Results on the different sperm parameters recorded throughout the seasons regardless of the bull are shown in Table 5.

Table 5. Characteristics of buffalo (Bubalus bubalis) semen in relation to the season.

	Jan-Mar	Apr-Jun	Jul-Sep	Oct- Dec
N. ejaculates	53	107	98	37
Volume (ml)	6.6 ± 0.5^a	9.2 ± 0.4^b	9.7 ± 0.7^b	7.6 ± 0.5^a
Concentration (10^9/ml)	1.02 ± 0.08	0.96 ± 0.07	0.97 ± 0.06	1.10 ± 0.06
Motility (%)	79.7 ± 4.0^a	83.4 ± 6.4^b	86.6 ± 4.6^b	80.7 ± 5.0^a
Total anomalies (%)	36.8 ± 1.3^a	30.4 ± 1.1^b	28.5 ± 1.8^c	34.2 ± 1.4^a
Primary Anomalies (%)	30.2 ± 1.3^a	24.7 ± 1.1^b	21.6 ± 1.8^c	26.8 ± 1.4^b
Progressive motility (%)	47.4 ± 4.5^a	47.5 ± 4.1^a	46.0 ± 4.7^b	51.0 ± 6.3^c

[a, b, c] Values with different superscripts within rows are different; $P<0.05$. Values are expressed as mean ± SD.

The average number of jumps was significantly ($P<0.05$) higher in spring (2.4 ± 0.7) and summer (2.4 ± 0.6) than in autumn (1.8 ± 0.4) and winter (2.0 ± 0.8).

A first difference was observed in terms of volume of the ejaculates among the four bulls, with bulls I and III significantly lower than bulls II and IV (7.4 ± 0.56, 9.7 ± 0.55, 6.9 ± 0.44 and 9.4 ± 0.44, respectively for bulls I, II, III and IV; $P<0.05$). The sperm volume changed with the considered periods of the year, with lower values during autumn and winter in comparison to spring and summer (Table 5).

A significant difference was found in the sperm concentration among bulls: in fact, the bull II showed the lowest values in comparison to the other bulls (1.05 ± 0.03, 0.78 ± 0.03, 1.06 x ± 0.03 and 1.08 ± 0.03, x 10^9/ml, respectively for bulls I, II, III and IV; P<0.05), while no differences were found in sperm concentration among various seasons (Table 5). The motility of the fresh semen was different among the bulls, with the bulls I and III less motile than bulls II and IV (80.2 ± 6.6, 86.4 ± 4.8, 81.6 ± 5.7 and 84.9 ± 4.6, respectively for bulls I, II, III and IV; P<0.05); moreover it was affected by season with semen showing improved (P<0.05) motility during spring and summer compared to the other periods (Table 5). The percentage of total anomalies was different among the four bulls, with bull II giving lower values than all other bulls, and bull III showing higher values than bulls I and IV (29.3 ± 1.6, 27.3 ± 1.6, 38.3 ± 1.3 and 34.1 ± 1.3, respectively for bulls I, II, III and IV; P<0.05). A season effect was also found on the percentage of total anomalies that increased significantly (P<0.05) in winter and autumn compared to spring and summer; furthermore, the lowest incidence of anomalies was found in summer (Table 5). Out of the total sperm anomalies, bull III gave a higher percentage of primary anomalies compared to all other bulls (24.0 ± 1.6, 22.2 ± 1.6, 30.2 ± 1.3 and 26.4 ± 1.3, respectively for bulls I, II, III and IV; P<0.05). The lowest (P<0.05) percentage of primary anomalies was

recorded in summer, the highest (P<0.05) in winter and intermediate values were observed in spring and autumn (Table 5).

A bull effect was also found on post-thaw sperm progressive motility, with bull III having a lower percentage than bulls II and IV, with bull I showing intermediate values (47.6 ± 4.3, 48.8 ± 4.9, 45.5 ± 5.1 and 47.8 ± 4.7; respectively for bulls I, II, III and IV; P<0.05). Finally, the progressive motility was significantly (P<0.05) reduced in summer compared to the other periods; furthermore, an increase (P<0.05) of this parameter was recorded in autumn compared to spring and winter (Table 5).

Discussion

The present experiment estimated different fertility parameters of Italian Mediterranean buffalo sperm collected over 2 years. The ejaculates used in this study were obtained under controlled conditions of management and feeding.

It is known that bovine bulls differ in their ability to produce offspring, both in natural and artificial breeding systems (Saacke *et al.,* 1994), as well as in their capability to fertilize the oocyte *in vitro* (Ward *et al.,* 2001).

A study conducted *in vivo* showed that, using frozen-thawed semen from buffalo bulls with similar post-thaw progressive motility and sperm

morphology, to inseminate superovulated buffalo cows, fertilization and viable embryo recovery rates differed among bulls (Misra *et al.,* 1999). As superovulatory response and total embryo recovery did not differ among donors inseminated with semen from 5 different bulls, these results indicated that individual bulls differ in their contribution to fertilization and also to embryonic development, as determined by viable embryo recovery.

Furthermore, studies previously performed in India in buffalo species had already reported that variability exists in the *in vitro* fertilizing ability of buffalo bull spermatozoa (Totey *et al.,* 1993), confirming the hypothesis of a sire effect at *in vitro* level as well. A strong bull effect was also found in our laboratory, in which only 10% of the tested bulls is on average judged suitable for IVF (Gasparrini, 2002).

The results of the present study, showing a large variability among the four bulls examined, clearly confirmed that there is a strong "bull effect" in terms of sperm quality parameters in Mediterranean Italian buffalo.

In fact, bull III clearly showed the lowest values in comparison to the other three bulls, in terms of volume, motility and post-thaw progressive motility. Moreover, bull III had higher percentages of total and primary abnormalities than the other bulls. On the contrary, bull II, despite a lower concentration, showed good scores in all the other parameters; furthermore, it was the bull with the lowest percentage of total anomalies. It is worth to

point out that bull II is the bull routinely used in our IVF trials. The other two bulls (Bull I and IV) showed intermediate fertility.

However, there are many gaps in our understanding of the male effect in buffalo species: in fact, we speculate that the physical parameters we evaluated in this study are not necessarily effective in predicting the potential of *in vivo* or *in vitro* fertility of a certain buffalo semen sample. Therefore, further studies are suggested to find out the factors responsible for such specific effects in buffalo species. Test models for predicting the outcome of IVF yields in buffaloes might be designed through the assessment of sperm viability and capacitation rates; in addition, *in vitro* embryo production might be conducted trying to enhance reproductive efficiency via modulating gametes co-incubation length or using different capacitating agents according to the potentiality of the individual bull.

In the present study, regardless of the bull, a seasonal factor also appeared. Differences among seasons were reported, but with confounding results. In fact, although sperm concentration did not change among the considered periods, the ejaculate volume and fresh sperm motility increased, while total anomalies and primary anomalies decreased during spring-summer at our latitudes. Sperm concentration did not vary statistically across the year, similarly to what observed under tropical conditions in Thailand (Koonjaenak *at al.,* 2007a). Furthermore, in other trials the ejaculate

volume was not influenced by seasonality in swamp buffaloes (Jainudeen *et al.,* 1982), as well as in Murrah (Bhosrekar *et al.,* 1992) and Nili-Ravi buffalo breeds (Khan *et al.,* 1997).

On the contrary, other authors observed inconsistent variations that were highest in summer (Kapoor, 1973).

Then again, the average percentage of initial motile spermatozoa during the present study surpassed 70%, a figure considered normal for swamp buffalo (Jainudeen *et al.,* 1982; Sukhato *et al.,* 1988; Nordin *et al.,* 1990) and Murrah buffalo (Bhosrekar *et al.,* 1992).

Finally, this study recorded that the mean total proportion of morphologically abnormal spermatozoa was lower in summer (*P < 0.05*) compared to the other seasons: these results are consistent with those found in the literature (Koonjaenak *at al.,* 2007c).

However, the pattern showed by the parameters evaluated on fresh semen does not exactly correspond with the seasonal pattern of buffalo species exhibited in the area of the study. On the contrary, the only parameter we evaluated on frozen-thawed sperm, i.e. post-thaw motility, increased during autumn, season characterized by shorter days at our latitudes.

The variability in the number of ejaculates obtained during the different seasons accounts for the non uniform number of cases among the considered periods and can contribute to explain some of the conflicting

results of the present study. A first observation that is worth to underline is that the germinal material was provided by a sire center, whose policy is strictly linked to the market demand of buffalo semen. Therefore, in general, a bull is put in production only when farmers require semen of a specific bull, thus causing a certain discontinuity in the male jumps. This aspect may account for an improvement of the features when the bull performance is more continuative, i.e. in spring summer during the present experiment.

Furthermore, although the fresh semen motility was assessed by a qualified operator, the evaluation of this parameter is subjective; therefore, these data should be considered with caution. The methods used for the screening of fresh semen motility and post–thaw progressive motility are basically different in their degree of subjectivity. In fact, in our study, the post–thaw progressive motility was determined by CASA, thus providing a more reliable measurement.

It is interesting to remark that this parameter, i.e. the post-thaw sperm motility increased in autumn, thus confirming our expected hypothesis. It is worth pointing out that in cattle this parameter has been considered a more reliable fertility marker than those that we consider operator-depending.

It is recognized that freezability of buffalo semen can also be affected by the season of collection, i.e. by environmental factors like temperature,

rainfall and day length in a particular season. Tuli and Mehar (1983) studied the seasonal variation in freezability of buffalo semen diluted in Tris-based extender. They found that post-thaw sperm motility significantly increased in winter compared to summer season. Sagdeo *et al.* (1991) also studied the seasonal variations in freezability of buffalo bull semen. They analyzed data recorded over a 4-year period and demonstrated that the season significantly affects the post-thaw sperm motility, with highest values in ejaculates frozen in the winter and lowest values in summer. Similarly, Bahga and Khokar (1991) studied the seasonal variations in freezability of buffalo bull semen. They found that post-thaw semen motility was significantly affected by season of collection, being lowest in summer and highest in winter (December–January). It follows that our results on post-thaw motility are in agreement with those reported in all the studies cited above.

 Recently, Argov *et al.* (2007) reported that cattle semen samples, that were considered to be of good quality, collected during summer had alterations in lipid concentration, fatty-acid composition and cholesterol level. In addition, they provided the first evidence for the existence of a very-low-density lipoprotein receptor (VLDLr) in bovine sperm, suggesting a mechanism for sperm utilization of extracellular lipids. Interestingly, the expression of VLDLr was threefold greater in samples collected during the

winter than in those collected in the summer. We speculate that similar modifications may explain, in part, the reduced freezability of buffalo semen collected during summer.

In conclusion, it was demonstrated a strong bull effect indicated by the differences in the majority of the fertility-parameters evaluated both on fresh and frozen-thawed sperm. Furthermore, fluctuations in the fertility parameters were observed in relation to the season. A discrepancy was observed between fertility judged on fresh and frozen-thawed sperm among seasons. Indeed, the majority of the parameters referred to fresh semen improved during spring-summer whereas the post-thaw motility increased during autumn. Among the parameters tested, the latter provides information on the freezability of sperm that hence was improved during autumn, as expected. However, detailed studies should be carried out to establish the biochemical or structural differences in seminal plasma, spermatozoa, or plasmalemma, which might influence the freezability of buffalo spermatozoa during the different seasons of the year.

APPLICATIONS OF INNOVATIVE REPRODUCTIVE BIOTECHNOLOGIES TO BUFFALO BREEDING

Experiment 3. Artificial insemination (AI) performed in different periods of the year

Freezing and thawing of bull spermatozoa has been possible for more than 50 years (Polge *et al.,* 1949) and cryopreserved semen has been used for commercial artificial insemination (AI) for almost as long (Curry, 2000). Since then, AI has been an invaluable tool for spreading desirable genes from selected bulls into a female breeding population (Koonjaenak *et al.,* 2007c).

Despite all the progress made and the efficiency obtained in cattle, AI is not a routine procedure in buffalo farms. In general, reproduction in buffalo is known to suffer from a number of inherent problems that include delayed maturity, a long postpartum period before return to estrus and low conception rates, silent estrous, particularly during summer months (Zicarelli, 1992). The seasonal decline in reproductive activity is manifested by a reduced incidence of estrous behaviour (Esposito *et al.,* 1992), a decrease proportion of females that undergo regular estrous cycles

(Zicarelli 1997d) and generally low conception rates (Zicarelli, 1997c). Actually, according to Baruselli (2007), AI in buffalo has a limited use worldwide especially due the difficulties in the estrous detection and hence, in identifying the most adequate time for insemination. In fact, especially in the period of seasonal anestrous, buffaloes show absence of an evident estrous behavior and a lack of ovulation and progesterone secretion by the ovary. These phenomena led invariably to decreased efficiency when the traditional AI is employed. This way, the use of fixed time artificial insemination (FTAI) is an advantage because it can be scheduled to pre-determined hours of the day, simplifying the management currently required in the traditional AI program.

However, we cannot rule out that the reproductive seasonality observed in buffaloes can interfere in the efficiency of the synchronization of ovulation in protocols for FTAI, as well. It is worth reminding that in our country, in order to meet market demand of buffalo milk to produce mozzarella cheese, it is more convenient to inseminate the animals in a period of the year that is unfavorable to the reproductive activity of the species. It results a lower efficiency, mainly due to a high rate of embryonic mortality (Campanile *et al.,* 2010). Currently, it is not possible to exclude that season plays a role and hence, that the efficiency of the technique may be higher during the favorable period.

Therefore, this experiment was carried out in order to compare the efficiency of AI in two different periods of the year: autumn, that is supposed to be the favorable time for reproductive activity at our latitudes, and mid-winter, when daylight starts to increase towards a clear positive photoperiod. In particular, we evaluated whether the decrease of daily light hours affects the rate of early embryonic mortality (EM), that usually occurs between day 25 and day 45 after AI in buffalo.

Material and methods

Animals and management

The trial was performed between October and December (autumn) and January and March (transitional period) on 131 and 125 pluriparous Mediterranean Italian Buffalo cows, respectively. The buffaloes were on average at 130±63 and 152±82 days *post-partum* on average and were bred in Southern Italy (latitude between 40.5°N and 41.5°N parallel). The animals were selected by clinical examination (rectal palpation of ovaries for follicles and corpora lutea to confirm cyclic status and of the reproductive tract for any gross abnormalities such as uterine fluid) before AI and only those in a healthy reproductive status were included in the study. They were maintained in open yards that allowed 15 m^2 for each

animal. 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 was fed daily in a group pen situation. Only animals with a corpus luteum and ∕or follicle >1.0 cm were selected for synchronizing the estrous cycle and, subsequently, for AI.

Synchronization of ovulations

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 of administration 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 twice, 16 and 40 h after the second injection of GnRH agonist. Because of the relatively low intensity of estrous behaviour in buffaloes, animals 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-five days after AI, buffaloes underwent

transrectal ultrasonography to assess embryonic development. Ultrasonography was conducted with an Aloka SSD-500 unit equipped with a 5.0 MHz linear array probe (Aloka CO., Tokyo, Japan) carried out by the same experienced operator. Pregnancy diagnosis was confirmed on day 45 after AI by ultrasonography. Buffaloes pregnant on day 25, but not on day 45 were considered to have undergone EM.

Statistical analysis

The differences of the AI efficiency in the two periods considered were analyzed by X-square test.

Results

The results of this experiment are shown in Table 6.

Table 6. Efficiency of FTAI in Mediterranean Italian buffalo during autumn and transitional period

	No AI performed	Pregnancies at 25 dd %	Pregnancies at 45 dd %	EM %
Autumn	131	62.6	58.0^a	7.3^a
Transitional period	125	59.2	45.6^b	23.0^b

[a,b] Values with different superscripts within columns are significantly different; P<0.05

In autumn, pregnancy rate on Day 25 after AI was 62.6% (82/131) and declined to 58.0% (76/131) by Day 45, which represented an EM rate of 7.3% (6/82). In the transitional period, pregnancy rate on Day 25 after AI was 59.2% (74/125) and declined to 45.6% (57/125) by Day 45, which represented an EM rate of 23.0% (17/74). No significant differences were found within the two periods in the percentage of pregnancy rate on Day 25, while the pregnancy rate assessed on Day 45 was higher during autumn compared to transitional period (P<0.05). The EM rate also varied significantly (P<0.05) being higher in the transitional period.

Discussion

This study was designed to compare the effectiveness of AI performed in Mediterranean Italian buffalo in two different periods of the year, characterized by different pattern of daylight. In fact, at Italian latitudes, buffalo is a photoperiodic species and females show a decline in reproductive activity from mid-winter to spring in response to increasing day length (Zicarelli, 1997c). Reproductive seasonality may be a major cause of poor fertility in buffalo cows: delayed puberty, silent estrus, and long post-partum ovarian inactivity (Singh, 1988; Singla *et al.,* 1996) are mainly affected by daylight length. In buffalo, a reduced activity of the

corpus luteum (CL) is observed during the increasing daylight length period (Campanile *et al.*, 1992). This condition does not cause always ovarian inactivity: estrous behavior may be present with ovulation, but this may be followed by an inadequate luteal phase (Zicarelli, 1992).

During a period of increasing daylight length (transitional period) a lower function of the CL has been showed (Campanile *et al.,* 2005; Campanile *et al.,* 2007b). In fact, like in cattle and sheep (Garrett *et al.,* 1988. Mann and Lemming, 1999; Mann and Lemming, 2001) it has been demonstrated that in buffalo species, embryonic mortality (EM) is primarily due to a reduced secretion of progesterone (P4) by the CL (Campanile and Neglia, 2007).

Most of these problems result from the use of the "out of breeding season mating" technique (Zicarelli, 1997c; Gasparrini, 2002). In fact, if buffaloes are bred without modification of their natural seasonality and without controlled breeding, an inter-calving period of less than 400 days and a culling rate of less than 12% have been observed in Italy, Brazil, Venezuela, and Argentina (Zicarelli *et al*., 1993). Unfortunately, in our country, the reproductive seasonality of buffalo has strong economic implications as the yearly peak in marked demand of milk occurs in the spring-summer period (not corresponding to buffalo reproductive activity). In order to overcome this issue, the out of breeding season mating (OBSM) technique is widely applied. When the OBSM technique has been applied

for short periods a higher fertility failure rate was observed (30% *vs*15%) compared to the farms in which it has been adopted for longer periods (Campanile, 1997). This is because buffaloes which are less sensitive to photoperiodic effects have been selected over the years; nevertheless, a fertility loss is still recorded. In buffalo species embryonic mortality is considered one of the major causes of fertility loss, especially in the animals that are not mated during their reproductive period.

Due to difficulty to detect estrus in buffaloes Fixed Time Artificial insemination (FTAI) protocols have been widely used. In an earlier trial (Neglia et al., 2003) it was demonstrated that Ovsynch was a more effective synchronization protocol than PRID followed by PG2α and PMSG for FTAI in cyclic buffalo cows, as indicated by the greater pregnancy rates (44.4 vs 30%). However, it was demonstrated that treatment with PRID can induce ovulations in non cyclic animals. In agreement with this finding, Presicce *et al.,* (2005) in another study performed in Italy during the months of increasing day length, reported that on acyclic buffaloes treatment with PRID+PMSG gave higher pregnancy rates than Ovsynch.

In the present study we used Ovsynch-TAI program for FTAI of buffaloes in two different periods of the year, with different pattern of daily light hours.

The results of the present study demonstrated that at Italian latitudes AI efficiency, estimated as pregnancy rates on Day 45 after AI, is significantly improved during the months with decreasing daylight (autumn) compared to the transitional period (58.0% vs 45.6, respectively). As no differences were found in pregnancy at 25 days between the two periods, the increased pregnancy rate recorded in autumn at 45 days was mainly due to the reduced incidence of EM (7.3 % vs 23% in the transitional period). This is a striking and expected example of the seasonality of the species and it is in accordance to other studies performed at different latitudes and under different management and breeding conditions. In fact, the rate of embryonic mortality we found in the transitional period fell in the range previously reported (20-40%) in Italian Mediterranean buffaloes mated by AI during seasons characterized by high number of light hours (Campanile *et al.*, 2005; Campanile *et al.*, 2007a; Campanile *et al.*, 2007b). On the other hand, the rate of embryonic mortality observed in autumn in the present study is similar to that (around 7%) recorded in Brazil during decreasing light days (Baruselli *et al.*, 1997). In the same country, an embryonic mortality rate of 20% was reported for buffaloes close to the equator by Vale *et al.* (1989); it is worth emphasizing that at these latitudes other factors, such as forage availability and rainfall, rather than the light/dark ratio influence the reproductive activity of the species. The

importance of our study consists in having compared the efficiency of AI in periods of increasing and decreasing daylight length during the same year at the same latitudes. In fact, studies from different authors carried out in different countries cannot be easily compared because many other factors, such as management, nutrition and breeding systems play an important role.

Furthermore, the rate of EM we recorded during the transitional period is also comparable to that (20%) recorded in buffaloes naturally mated at Italian latitudes during increasing daylight length (Vecchio *et al.,* 2007).

The conception rate observed in our study was much higher than that (35%) recently reported by Oropeza *et al* (2010) during the breeding season (November-January) following FTAI in Murrah lactating buffaloes managed in a semi–intensive system in the Venezuelan tropics.

Furthermore, the conception rates described in our study in both periods were also higher than those found by Indian authors after AI on spontaneous estrus; they reported a conception rate ranging from 16.67% to 33.33% in relation to the stage of estrus, with the best results when the heifers were inseminated at 18–24 h after estrus. (Kumaresan and Ansari, 2001). However, Honnappagol and Patil (1991), using an analogue of

prostaglandin F2a to synchronize oestrus in cycling Surti buffalo heifers, had a conception rate after AI ranging from 12.5% to 62.5%.

It is worth emphasizing that the pregnancy rate at 45 days we found during autumn is very high (58%) and comparable to that (50%) reported in buffaloes that underwent similar estrus synchronization during a period of decreasing day length in Brazil (de Araujo Berber *et al.*, 2002). The results of both studies indicate that buffaloes have the potential to achieve similar pregnancy rates as cattle (Fricke *et al.*, 1998; Cartmill *et al.*, 2001) after estrus synchronization.

The rate of EM we observed in this study during the transitional period was lower than that previously reported by Campanile *et al.* (2005) during the same period of the year in the same area. In the earlier study pregnancy rate on Day 26 after AI was 63% (131/209) that subsequently declined to 34% (72/209) by Day 40 which represented an embryonic mortality of 45% (59/131). Interestingly, among buffaloes that showed embryonic mortality only 7.6% (10/131) had infectious agents in uterine flushings. Moreover, another interesting result of the same study demonstrated that 51% of buffaloes which showed EM had P4 concentrations on days 10 and 20 post AI similar to those of animals which maintained pregnancy. Therefore, it was concluded that a reduced capacity for P4 secretion can explain around 50% of embryonic mortalities in buffaloes synchronized and mated by AI

during a period of low reproductive activity and that other as yet unidentified factors also have a significant effect on embryonic survival. However, a reduced activity of the reproductive endocrine system, indicated by low circulating concentrations of P4 after estrus synchronization in the remaining animals, certainly plays a critical role. It has been observed that CL activity is mainly influenced by calving season (Zicarelli, 1994b) and depends of daylight length. In fact, this reduces hypothalamus-pituitary activity that consequently influences ovarian activity (Kaker *et al.*, 1981). During the transitional period which corresponds to the midwinter in Italy, the incidence of CL activity reduction is between 5% and 50% in buffalo (Campanile *et al.,* 1992). Quoted publications report that at the basis of EM there is an inadequate functionality of CL and then a reduced concentration of P4 in the transitory period implantation.

Impaired P4 secretion has been linked with a reduced capacity of the developing embryo to secrete Interferon-tau (IFN-τ) at threshold amounts necessary to prevent luteolysis (Wathes *et al.,* 1998).

However, it is worth to underline that, although an early embryonic mortality and a foetal mortality have been described in buffalo, the highest incidence of EM in buffalo species occurs later than in cattle, between 25th and 40th day (Campanile and Neglia, 2007), and this let us exclude that

there is a problem of recognition between mother and foetus. Indeed, this recognition between mother and foetus is due to the production by the embryo of adequate quantities of IFN-τ that blocks luteolysis that would occur, in absence of signals, around 16th-18th day, in order that cycle could re-start. Furthermore, because the phase of implantation in buffalo ends around the 30th day, this late EM occurs when placenta is developing.

However, the observation that reduced P4 concentration was found in approximately 50% of the buffaloes showing EM, suggests that other factors also contribute.

Therefore, EM in buffalo species is a complex phenomenon that it is not yet completely understood and has a multiple etiology. With this regard, it is known that gametes quality, as strongly affecting embryo developmental competence, is involved with the normal prosecution of pregnancy. Therefore, in order to account for about 50% of EM that is not due to reduced P4 secretion, we hypothesized that season may also affects the oocyte developmental competence. The results of Experiment 1, carried out to evaluate the morphology and the developmental capacity of abattoir-derived oocytes collected in various seasons, demonstrated that season affects their developmental competence, as shown by higher cleavage and blastocyst yields recorded in autumn compared to spring, with intermediate results in winter and summer. Interestingly, the embryo yields recorded in

winter and autumn were not statistically different, suggesting that other factors may determine the phenomenon rather than oocyte competence. However, since we only evaluated cleavage and blastocyst rates, we cannot rule out that the embryo viability and, hence the ability to sustain development to term after transfer, in this period is reduced to some extent. In conclusion, the results of the present study clearly demonstrated that season affects the efficiency of AI in Italian Mediterranean buffaloes. Therefore the lower efficiency recorded in this species compared to cattle is mainly due to the need to carry out the inseminations during the unfavorable period of the year. Indeed, the pregnancy rate obtained during the favorable period, i.e. in autumn, was very high, indicating that the efficiency of this technology is greatly competitive when we do not force the natural reproductive pattern of the species.

In our context, in which we are obliged to operate in the unfavorable period to obtain a distribution of calving that meet the market demand, strategies may be, however, adopted to increase the efficiency. It has been observed that the Ovsynch protocol may be improved by administering progesterone for 7 days between the first GnRH treatment and PGF2alpha, as shown by increased fertility during the low season (Ronci and De Rensis, 2005; De Rensis *et al.,* 2005). An interesting strategy was developed to reduce the incidence of EM during the unfavorable season by providing the

inseminated animals for progesterone, to compensate for the reduced CL activity.

In an earlier trial it was demonstrated that a GnRH agonist and hCG, given on day 5 after AI increased P4 concentrations on day 15 but this was not associated with a reduced incidence of embryonic mortality in buffaloes during mid-winter (Campanile *et al.,* 2007a). On the contrary, in a more recent work it was demonstrated that a delayed treatment (on day 25 after AI) with either GnRH agonist, or hCG or exogenous progesterone significantly reduces EM, by increasing P4 concentration (Vecchio *et al.,* 2010)

Experiment 4. Ovum pick-up and *in vitro* embryo production (OPU-IVEP) trials carried out during transitional period, spring-summer and autumn

Repeated transvaginal ultrasound-guided ovum pick up (OPU) represents an opportunity to apply *in vitro* embryo production technologies to animal breeding and can be used to maximize the contribution of genetically superior females to a reproduction program.

This technique is based on a relatively non-invasive system that allows repeated retrieval of oocytes, with minimal trauma to the ovaries and reproductive tract. Moreover, combined OPU - IVEP can be performed regardless of estrous cycle activity of the donor, in animals with occluded tubes or uterine infections, in pregnant subjects or in animals that do not respond to multiple ovulation procedures, the last representing a high proportion in buffalo species (Boni *et al.,* 1994; Gasparrini, 2010).

The application of traditional reproductive technologies like AI, estrus synchronization, MOET has yielded poor results in buffaloes (Madan *et al.,* 1996; Gasparrini, 2002).

For that reason, following successful trials of OPU in cattle, many efforts were made to exploit this technique in buffalo species: it was used for the first time by Boni *et al.* (1994) on deep anoestrous animals (6 buffaloes) under ovarian hypotrophic conditions. Since then, OPU has been successfully carried out several other times in our country (Bony *et al.,* 1995; 1996; 1997; Neglia *et al.,* 2003; 2004) and subsequently in Brazil (Sá Filho *et al.,* 2005; 2009), China (Huang *et al.,* 2005; Liang *et al.,* 2008), Argentina (Pellerano *et al.,* 2007) and India (Manik *et al.,* 2002; Gupta et al., 2006; Manjunatha *et al.,*2008; 2009).

The application of OPU-IVP technology to buffalo can enhance genetic progression through maternal lineage (Gasparrini, 2002), thus overcoming the reduced levels of efficiency linked to the reproductive seasonality pattern of this species.

However, data regarding the effects of seasonal shifts in the activity of the reproductive axis on the quality and the developmental potentials of buffalo oocytes obtained from live donors in temperate climate are lacking.

Hence, the objectives of the present study was to compare the efficiency of OPU – IVEP on Mediterranean Italian buffalo in three different periods of the year: transitional period (January, February and March), spring – summer (May, June and July) and autumn (September, October and November).

Materials and methods

Reagents and media

All chemicals and reagents, if not otherwise stated, were purchased from Sigma (Sigma-Aldrich, Milan, Italy).

Oocyte recovery

The study was conducted in two different farms owned and operated by a single consortium in Campania region in three different periods: transitional period, spring-summer and autumn. It was carried out respectively on 14, 9 and 9 healthy, multiparous and lactating buffalo cows and over 18 puncture session per each period The donors were under controlled nutrition, barnhoused and moved to and restrained in a chute at the moment of the oocyte retrieval session. During the study, the cows did not show any behavioral modification, and ovum pickup treatment did not cause any adverse effects.

Ovum pick up setting consisted of a portable ultrasound unit (SonoAce - PICO, Medison, Cypress, CA, USA) with a convex 4MHz~9MHz probe (mod. CD4-9/10EDN) and a metal guide, to fit on the top 18 gauge

needles, allocated in a properly designed vaginal guide (WTA Ltda., Cravinhos/SP, Brazil). A vacuum pressure of 40 mm Hg was constantly maintained by using a suction unit (K-MAR-5100, Cook IVF Co., Australia) and the aspiration line was continuously rinsed with 25 mM hepes buffered TCM 199 supplemented with 100 USP units ml^{-1} of heparin and 10% Fetal calf serum (FCS) and 1% antibiotic (penicillin and streptomicine complex, pen-strep) during follicular aspiration. The 15 ml Falcon tubes (Becton & Dickinson Co., Lincoln Park, NJ, USA) for oocytes collections were constantly maintained at 37°C. All visible antral follicles were punctured and classified in three categories, according to their size: small (diameter < 0.5 cm), medium (diameter between 0.5 and 1 cm) and large (diameter > 1 cm).

COCs were searched immediately after follicular aspiration by using proper filters (Emcon Technologies, Columbus, IN , USA) the aspirated follicular fluid and aspiration medium, and classified in 7 categories: Grade A (oocytes with more than three layers of cumulus cells and homogenous cytoplasm); Grade B (oocytes with at least 2 layers of cumulus cells and homogenous cytoplasm); Grade C (oocytes partially denuded, but still showing homogenous cytoplasm); degenerated oocytes (oocytes with irregular shrunken cytoplasm); expanded (expanded cumulus oocytes but with homogeneous cytoplasm, typical of the estrous phase); abnormally

expanded (oocytes with particularly clustered cumulus cells) and naked (totally denuded oocytes). Recovery rate (calculated as percentage of the total number of COCs in relation to the total number of follicles), was also recorded.

COCs were washed twice in Hepes-buffered TCM 199 (H 199) with 10% FCS and then allocated in the same medium supplemented with 50 μM cysteamine (Gasparrini *et al.*, 2000) and 0.5 μg ml^{-1} FSH, 5 μg ml^{-1} LH, 1 μg ml^{-1} 17-β-estradiol (Caracciolo di Brienza *et al.,* 2001). Processed oocytes were stored in 15 ml Falcon tubes in a portable incubator at 38.5°C and moved to the lab within 4 to 6 h for *in vitro* embryo production (Gasparrini, 2002).

In Vitro Embryo Production (IVEP)

For *in vitro* maturation (IVM) COCs were individually transferred into 50 μl droplets (10 COCs/droplet) under mineral oil of the final maturation medium, consisting of bicarbonate–buffered TCM 199 (B199) with hormones and cysteamine in the same concentration previously described. The droplets were incubated at 38.5°C for 22 h under controlled gas atmosphere of 5% CO_2 in humidified air.

In vitro fertilization (IVF) was carried out according the method previously described by Parrish *et al.* (Parrish *et al.,* 1986) the day after IVM. Frozen – thawed sperm was prepared by Percoll density gradient The pellet obtained after centrifugation was resuspended to a final concentration of $2x10^6$ ml^{-1} in the fertilization medium, a modified TALP supplemented with 0.2 mM ml^{-1} penicillamine, 0.1 mM ml^{-1} hypotaurine and 0.01 mM ml^{-1} heparin. Fifty µl fertilizing droplets (5 COCs/droplet) covered by mineral oil were incubated under the same gas atmosphere as for *in vitro* maturation.

After 20-22 h of co-incubation with spermatozoa, presumptive zygotes were cultured (IVC) in 20 µl droplets (10 COCs/droplet) of SOFaaBSA (Tervit *et al.,* 1972, Gardner *et al.,* 199) for 7 days in modular chamber with a gas atmosphere of 5% CO_2, 7% O_2 and 88% N_2. At day 5 (day 0= IVF day) cleavage rate was assessed and embryos were transferred into fresh droplets of the same medium for further 2 days of culture. Final embryo output, in terms of tight morulae-blastocysts (TM + BL) and blastocysts (BL) was evaluated at day 7 of culture.

Statistical analysis

Data were analyzed by one way ANOVA procedure of SPSS 17.0 statistical software (2009) and differences among means were compared by Tukey test: when the distribution of variances was not homogeneous, data were analyzed by the Kruskal-Wallis test (SPSS 17.0, 2009).

Results

Our results regarding the follicular population, the recovery rates and the morphological parameters of the oocytes are summarized in Table 7.

The average number of total follicles aspirated per animal per session did not vary among seasons, as shown in Table 7. Furthermore, both the number and the percentage of follicles of different size categories were similar in the three periods. Similarly, the average number of total COCs recovered per animal per session did not show any variation among seasons.

However, the recovery rate (percentage of total oocytes recovered out of the total number of follicles) was affected by season: it increased ($P<0.05$) during autumn compared to spring–summer (Table 7), with intermediate values in the transitional period (mid-winter).

With regard to oocyte quality, no differences were observed in both the number and the incidence (in relation to the total COCs) of the different morphological categories among periods, with the exception of the abnormally expanded oocytes (Table 7). In fact, both the number and the percentage of abnormally expanded oocytes increased significantly (P<0.05) during autumn compared to both the transitional period (mid-winter) and spring–summer (Table 7).

Table 7 Oocyte recovery and distribution of different oocyte categories (percentages on the total) among different seasons.

	Jan-Mar	May-Jul	Sep-Dec
	Mean ± SD	Mean ± SD	Mean ± SD
Large follicles	0.8 ± 0.4	0.6 ± 0.3	0.6 ± 0.4
(%)	(17.6 ± 7.8)	(18.1 ± 11,3)	(18.6 ± 12.9)
Medium follicles	1.3 ± 0.5	1.2 ± 0.5	1.3 ± 0.4
(%)	(27.9 ± 7.6)	(27.5 ± 12.1)	(29.7 ± 12.3)
Small follicles	2.6 ± 0.8	2.8 ± 1.0	2.5 ± 0.9
(%)	(54.5 ± 11.2)	(54.4 ± 16.2)	(51.2 ± 16.0)
Total follicles	4.8 ± 1.1	4.6 ± 1.0	4.4 ± 0.7
Recovery rate %	62.2 ± 15.6^{ab}	49.3 ± 15.0^{b}	63.3 ± 18.3^{a}
Grade A	0.3 ± 0.2	0.2 ± 0.2	0.3 ± 0.4
(%)	(12.6 ± 7.7)	(7.4 ± 7.3)	(7.7 ± 8.6)
Grade B	0.5 ± 0.3	0.4 ± 0.3	0.4 ± 0.5
(%)	(22.2 ± 14.0)	(20.7 ± 16.3)	(16.3 ± 11.8)
Grade C	0.8 ± 0.5	0.7 ± 0.4	0.8 ± 0.4
(%)	(32.9 ± 15.1)	(32.1 ± 15.4)	(30.4 ± 15.7)
Degenerated	0.2 ± 0.1	0.3 ± 0.2	0.3 ± 0.3
(%)	(12.2 ± 7.6)	(15.0 ± 12.5)	(16.4 ± 9.9)
Expanded	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.3
(%)	3.4 ± 5.0	(3.9 ± 5,0)	(3.9 ± 4.9)
Abnormally expanded (%)	0.1 ± 0.1^{a}	0.1 ± 0.1^{a}	0.2 ± 0.2^{b}
	$(2.6 \pm 4.9)^{a}$	$(1.9 \pm 2.6)^{a}$	$(7.5 \pm 7.1)^{b}$
Naked	0.4 ± 0.1	0.5 ± 0.2	0.5 ± 0.4
(%)	(14.9 ± 5.8)	(19.1 ± 10.5)	(17.8 ± 11.1)
Total oocytes	2.3 ± 0.5	2.2 ± 0.5	2.3 ± 0.6
Grade A+B COCs	0.8 ± 0.4	0.6 ± 0.3	0.6 ± 0.5
(%)	(34,8 ± 16,8)	(28,2 ± 17.0)	(24,0 ± 16,7)

[a,b] Values with different superscripts within rows are different; P<0.05. Values are expressed as mean ± SD.

Interestingly, although oocyte categories were not much affected, the oocytes recovered during autumn showed a significant improvement of the developmental competence compared to both spring-summer and the transitional period.

In fact, the percentages of TM + Bl significantly ($P<0.05$) improved during autumn (31.5 ± 17.0) compared to the transitional period (15.4 ± 10.0) and spring – summer (13.0 ± 9.6, Figure 5).

Figure 5. Percentages of tight morulae-blastocysts (TM + Bl) calculated out of total COCs, in different seasons in relation to day light hours
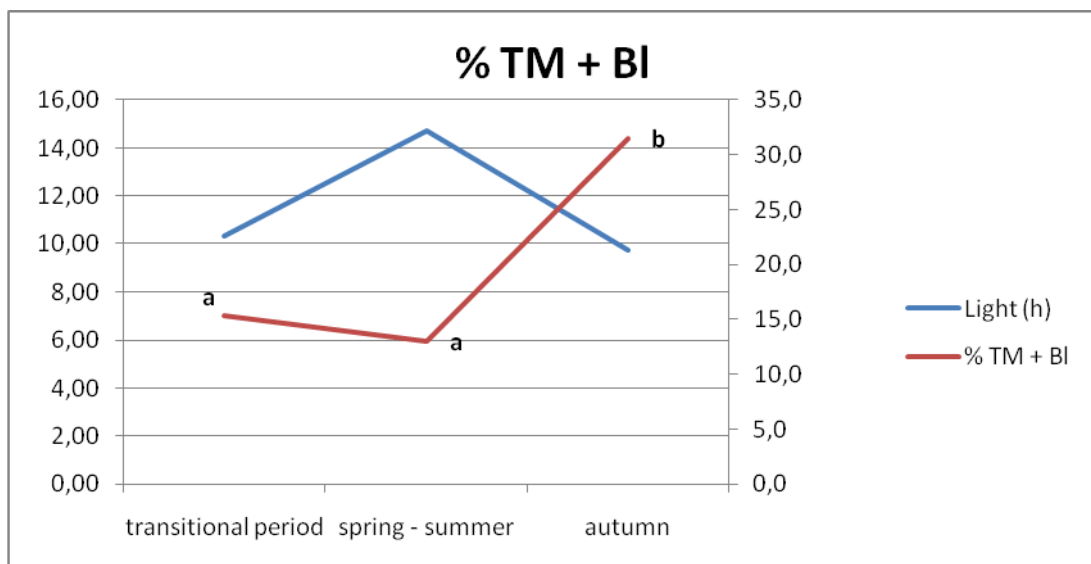
Likewise, the percentages of Bl differ significantly ($P<0.05$) over the three seasons, being higher during autumn than during the transitional period and spring – summer (24.9 ± 17.0, 12.5 ± 7.8, and 10.7 ± 9.7, respectively. Figure 6)

Figure 6. Percentages of blastocysts Bl calculated out of total COCs, in different seasons in relation to day light hours
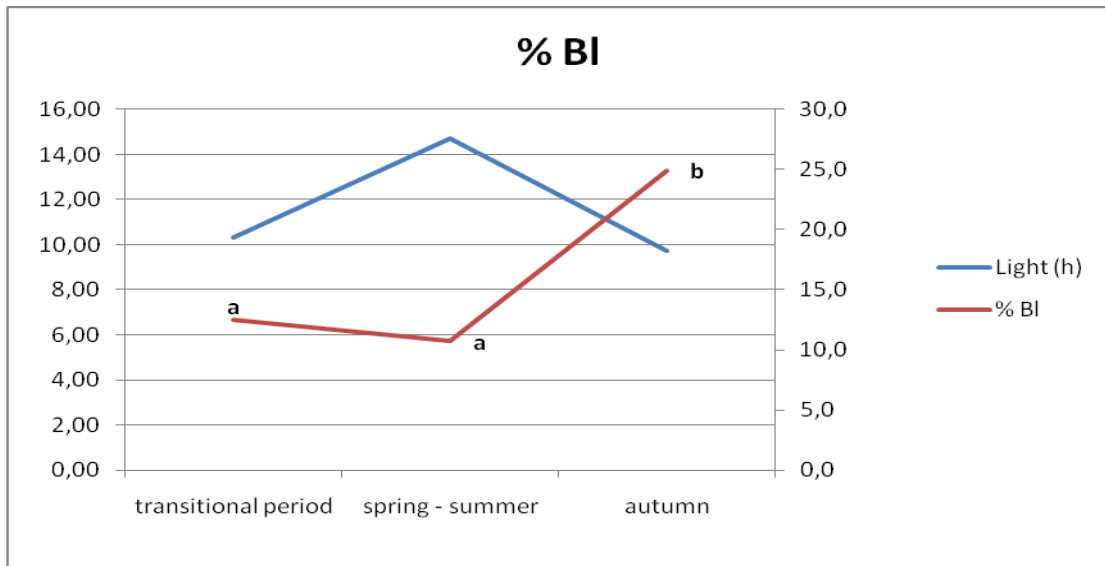
Although the percentage of cleavage did not vary significantly over the three seasons, it is worth to report that it was higher during autumn (62.5 ± 16.1) compared to the percentages recorded during the transitional period (53.0 ± 14.7), and spring-summer (57.6 ± 23.9; Figure7)

Figure 7. Cleavage rate in different seasons in relation to day light hours
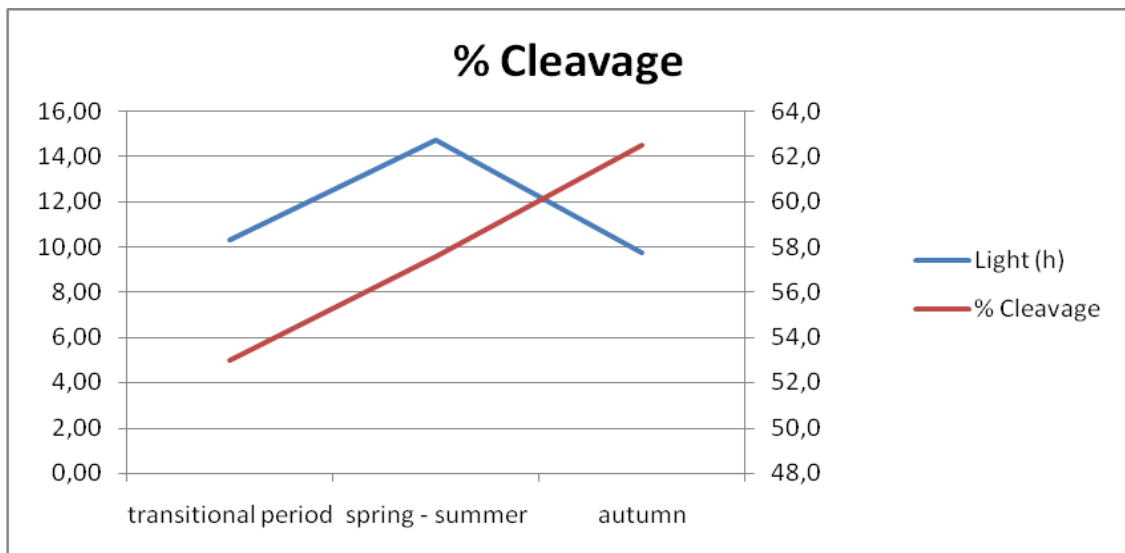
Interestingly, both the percentages of TM + Bl and Bl calculated out of the Grade A+B COCs (P<0.05) were significantly different over the three periods considered, being higher during autumn (138.5 ± 35.4 and 111.8 ± 30.5, respectively) than the transitional period (57.2 ± 14.0 and 45.5 ± 9.5, respectively) and spring–summer (50.8 ± 9.7 and 41.9 ± 9.7, respectively).

Discussion

The present study examined whether season could influence the follicular and oocyte population and, more importantly, the oocyte developmental competence, and hence the overall efficiency of ovum pick–up in Mediterranean Italian buffalo. The results of this study demonstrated an overwhelming seasonal effect, with a significant improvement of the OPU-IVEP efficiency, in terms of the final embryo output, during autumn, i.e. when the daily light hours decrease compared to both mid-winter and spring-summer.

A first result of this study is that season did not affect the follicular population in buffaloes submitted to OPU: in fact, the average number of total follicles, as well as the incidence of follicles of different size (i.e. large, medium and small) did not vary among the three seasonal periods we considered. This result is in contrast with another study performed in Italy

(Di Palo *et al.*, 2001) in which it was observed that both the number of follicles and that of oocytes was higher in autumn-winter than in spring-summer in buffaloes undergone OPU (Di Palo *et al.*, 2001). However, it is worth to point out that the earlier study was performed on the same animals for many sessions and this decline could be explained by a functional exhaustion of the ovary, resulting from repeated punctures.

On the other hand, our results in terms of number of follicles are in agreement with those reported in deep anoestrus buffaloes at the same latitude during 4 months-OPU carried out between autumn and the beginning of the transitional period (Boni *et al.* 1996).

Furthermore, the average number of follicles we observed was similar to that reported by different authors in Murrah buffaloes with reproductive problems (Manik *et al.,* 2002), cyclic Murrah buffaloes (Gupta et al., 2006) and river buffaloes (Manjunatha *et al.,* 2008).

The average number of oocytes recovered per donor per session was also not affected by season. It is worth remarking that the number of total oocytes collected was low (2.2-2.3), confirming that this is the major limitation of the OPU technology in this species. In fact, the majority of the authors reported similar or lower oocyte numbers (Gupta *et al.,* 2006; Manjunatha *et al.,* 2009).

In contrast to these findings, Baruselli *et al.* (2010), recently reported an unexpectedly high number of aspirated follicles per session, and consequently, a greater number of oocytes, in an OPU trial carried out using an intersession interval of one-two weeks. We may speculate that the evident difference in the follicular population may be due to the genetics of the animal, the age (heifers vs adults), the type of breeding and environment, as well as the longer interval between sessions. The latter factor may account for both the greater number of follicles and the lower developmental competence of the oocytes, indicated by the poor blastocyst rate (9 %) recorded in that trial. Indeed, it is known that an extension of the interval increases the number of follicles but also the heterogeneity of the oocyte source, as the phenomena of dominance and atresia in this case occur.

Interestingly, in the present study, despite similar number of both follicles and oocytes, the recovery rate increased significantly in autumn compared to spring–summer, with intermediate values in the transitional period. This was slightly higher than that reported by Boni *et al.,* (1996) in a trial carried out at the same latitude over autumn months. This result is difficult to explain as it is known that many factors may affect the recovery rate, such as the tractability of the animals, the skill of the operator, the efficiency of the aspiration set, etc.

In the present study the incidence of good quality oocytes (Grade A + B COCs) we retrieved was also not affected by season. Interestingly, among all the oocyte morphological categories, only the abnormally expanded oocytes were affected by season, being higher during autumn than during the other two periods. This intriguing datum require further investigations because we do not currently know the biological meaning of these oocytes. It is worth reminding that oocytes of this category are different from those we classified as expanded because they show obvious signs of degeneration. Whether they are expanded oocytes that underwent degeneration or they are oocytes in which the cumulus expansion process was altered we still do not know. Interestingly, an increased incidence of expanded oocytes was recorded during the last period of a 9 months-OPU trial (unpublished data) that coincided with autumn months (September-December). It is worth specifying that in the previous work, however, repeated punctures were carried out for a long time on the same donors. Furthermore, as no differences in the incidence of medium and large follicles were shown in autumn compared to the other periods in the present study, we should rule out that an acceleration of the follicular turn-over in response to decreased light hours occurred, as previously hypothesized. The increased incidence of abnormally expanded oocytes was unexpected because OPU carried out every 3-4 days, by continuously

resetting the follicular population, avoided the dominance occurrence. Therefore, the inappropriate cumulus expansion observed in a certain percentage of oocytes requires further investigations.

The most interesting results of this study were obtained by comparing the oocyte developmental competence in relation to season. The different parameters considered for assessing IVEP efficiency were improved in autumn. Although cleavage rate was not statistically different among periods, the higher values were recorded in autumn. More importantly, both the percentages of the tight morulae-blastocyst (TM + BL) and of the more upgraded embryos (Bl) were significantly higher in autumn compared to the other two periods of the year (spring-summer and mid-winter): this would represent a further confirmation of buffalo sensitivity to photoperiod (Zicarelli, 1997c).

Figures 5 and 6 clearly indicate that the highest IVEP efficiency corresponds to the shortest day length while the worst efficiency is recorded when the number of daily light hours is the highest. These results suggest that the beginning of decreasing daylight has actually improved the developmental competence of the oocytes recovered, despite of their apparent morphology.

Interestingly, embryo production was higher in autumn, either if embryos were calculated on the total recovered oocytes and on grade A+B COCs,

that are the two categories considered suitable for IVEP. It is worth reminding that in this study we processed all the oocytes recovered because a strict selection of the gametes would have resulted in a further reduction of the germinal material. Furthermore, one of the targets was to assess the feasibility of OPU in the field that is strongly linked to the final embryo output. Interestingly, when we calculated the percentage of embryos on Grade A+B COCs the value in autumn exceeded 100% whereas it was around 50% in the other two periods. This is an attractive result on the efficiency, thus meaning that developmental competence improved at any level of oocytes. It also follows that the assessment of oocyte quality, made by observing just the morphology, cannot be viewed as highly reliable for predicting the competence. This suggests to further investigate in research areas aimed to find molecular biomarkers, responsible for the development of oocytes up to the embryo stage. In fact, during the lifespan of the female, biochemical changes occur in the ovarian environment. These changes are brought by numerous endogenous and exogenous factors, including husbandry practices, production demands and disease, and can have a profound effect on ovarian oocyte quality and subsequent embryo development (Fair, 2010)

Furthermore, our results differ with a recent trial carried out in India on six non-descriptive (local breed), Indian river buffaloes (Manjunatha *et al.,* 2009). These authors reported that oocyte developmental competence, indicated by blastocyst production rate, was not affected by season, although the numbers of follicles observed and punctured, oocytes recovered, blastocyst yield per animal per session was significantly affected by breeding season. They speculated that the reduction of follicles and COCs during the low season is due to the heat stress; however, temperatures were not so different between the two periods compared, whereas there was a difference in daily light hours, although less evident than at our latitudes.

The results of this study agree with those reported in the sheep, another short-day breeder that shares many reproductive traits with buffalo (Zicarelli, 1994; 1997a; 2002), in which a significant decrease in efficiency has been described in the unfavorable seasonal period after IVF (Stenbak *et al.,* 2001).

When we compare the results of this experiment to those obtained in experiment 1. on abattoir-derived oocytes, we found many similarities with few differences that it is worth to highlight.

In both studies the number of oocytes recovered (per ovary and per animal) and the number and incidence of good quality oocytes were not affected by season. Among all the oocyte morphological categories, few differences

were found: when the oocyte source was abattoir-derived ovaries a lower incidence of small oocytes was observed in both autumn and winter and unexpectedly, an increased percentage of degenerated oocytes was found in autumn. In this work on OPU-derived oocytes we only observed an increased percentage of abnormally expanded oocytes in autumn. However, the variation in these oocyte categories did not affect at all the IVEP efficiency.

It is more important to look at the developmental competence that in both studies was improved in autumn compared to spring, as demonstrated by higher blastocyst yields. More interestingly, in this study an evident difference was also shown between embryo yields recorded in autumn vs mid-winter.

This is very important because during the transitional period a higher incidence of embryonic mortality is observed in buffalo after AI (Campanile *et al.*, 2010). Furthermore, in experiment 3, we demonstrated that during autumn months pregnancy rate at 45 days is significantly enhanced compared to the transitional period, as a result of the reduced incidence of EM. Therefore, the lower embryo yields recorded in mid-winter compared to autumn, suggest that oocyte competence could play a role in the AI failures observed when daylight hours start to increase. In order to better elucidate this aspect, a further trial is ongoing to investigate

the embryo viability and hence the ability to sustain development to term after transferring the embryos obtained in different periods of the year.

CONCLUSIONS

In conclusion, it was demonstrated that season affects oocyte developmental competence and sperm fertility in buffalo species, hence influencing the efficiency of advanced reproductive technologies, such as AI and OPU-IVEP.

The results of experiment 1 demonstrated that, although oocyte quality assessed by morphological criteria was not much affected, the oocyte developmental competence, i.e. cleavage and blastocyst yields was improved in autumn compared to spring, with intermediate results observed in summer and winter. These results suggest to restrict the period of gametes collection to the autumn months and to avoid spring, when planning OPU trials, in order to save resources and make the benefit/costs ratio more favorable.

In Experiment 2 it was demonstrated that season affects sperm fertility parameters. In particular, our result showed that the freezability of buffalo semen is improved when it is collected during decreasing daylight months, as indicated by the higher post-thaw progressive motility, a parameter that has been previously correlated to embryo yields. Nevertheless, further studies are needed to confirm these results, by evaluating the effective in vitro fertility (embryo yields after IVF) of sperm collected in various

seasons. However, these preliminary results suggest that it is rather preferable to perform AI with semen straws obtained in autumn.

In order to confirm the hypothesis derived from the results of experiment 1, i.e. that it is advisable to limit oocyte collection during autumn, we evaluated the effect of season on the developmental competence of oocytes collected by OPU (Experiment 4). Although we are aware that there is a great difference between aspirating follicles from slaughtered ovaries and from living donors, thus consisting mainly in one aspiration session and repeated picks up, respectively, a similar pattern was observed in experiment 4.

In fact, the results of the OPU experiment carried out in different periods of the year confirmed that the parameters describing oocyte developmental efficiency (cleavage and embryo yields) improved in autumn compared to spring-summer and the transitional period. In this trial, however, the beneficial effect of decreasing daylight length was overwhelming also when compared to the transitional period, i.e. mid-winter, that coincides with the beginning of increasing daily light hours and with the higher incidence of embryonic mortality recorded after AI. In fact, the blastocyst yields in autumn were twice as high as those recorded in winter and spring-summer.

The differences in oocyte developmental competence and hence in embryo yields observed between the experiment 1 and 4 are mainly due to the source of oocytes.

It was previously demonstrated that, despite their worse morphological appearance, OPU-derived buffalo oocytes have a higher developmental competence compared to abattoir-derived ones (Neglia *et al.*, 2003). The improved embryo yield may be accounted for by the OPU-induced modification of the follicular dynamics; resetting the follicular population twice weekly results in increased follicular wave frequency and hence in the aspiration of follicles before they become atretic, with an improved oocyte "quality". On the contrary, an heterogeneous oocyte population is recovered from pooled ovaries of slaughtered buffaloes. In addition, it has been speculated that the better developmental competence of OPU-derived *vs* abattoir-derived oocytes is related to the shorter exposure to environmental stress. Indeed, abattoir-derived oocytes spend a longer time between excision of ovaries from the peritoneal cavity and laboratory processing and are probably affected by cellular damages due to autolytic processes, especially when they reside in excised ovaries for prolonged periods. Furthermore, when OPU is carried out oocytes are searched directly in the farm and immediately transferred in a hepes-buffered in vitro maturation (IVM) medium in a portable incubator, and hence they are less

exposed to thermic shock and to environmental stress compared with those recovered from slaughtered ovaries. For this reason OPU is a more reliable tool to assess the effect of photoperiod because it allows to eliminate the influence of other environmental factors, such as the cold temperatures found during late autumn and winter that may hide the photoperiod effects.

The results of Experiment 4 also provide new insights to comprehend the phenomenon of embryonic mortality in this species, that it is known to increase during the transitional period.

In experiment 3 we demonstrated that the efficiency of AI, evaluated as pregnancy rate on day 45, significantly increased during autumn compared to the transitional period, mainly due to the reduced embryonic mortality. The improved oocyte developmental competence we recorded in autumn may account for the better AI efficiency. On the other hand, it suggests that a seasonal-dependent worsening of the oocyte developmental competence may in part contribute to the increased embryonic mortality recorded during mid-winter.

The lower efficiency of AI recorded in this species compared to cattle is mainly due to the need to carry out the inseminations during the unfavorable period of the year. Indeed, the pregnancy rate obtained during the favorable period, i.e. in autumn, was nearly 60%, indicating that the efficiency of this technology is highly competitive when we do not force

the natural reproductive pattern of the species. In our context, in which we are obliged to inseminate the animals in the unfavorable period to meet the market demand, strategies may be, however, adopted to increase the efficiency.

Our results suggest that in order to improve IVEP efficiency in buffalo, it is advisable to carry out the oocyte collection and the embryo transfer into synchronized recipients during autumn months. However, it will be interesting to verify whether the poorer maternal environment, resulting in greater embryo loss, observed in the transitional period is influenced by the oocyte competence. To answer to this question further studies will be carried out to transfer the embryos produced during autumn into recipients in different seasons.

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