Chapter 6

Reproductive technologies in the buffalo (*Bubalus bubalis*)

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6.1 General introduction to the buffalo

Around 199 million heads around the world and in all continents account for the totality of the two subspecies, breeds, and varieties of domestic buffaloes that can be found. According to some authors, buffaloes perform better than cattle and predominate over the latter species under tropical conditions also because, among other variables, they are considered to be better converter of poor-quality feed resources into milk and meat [1]. These assumptions though are not shared by other authors, as shown in some nutritional studies [2,3]. Actually, it is likely that as milk production increases and high nutritive value diets with low crude fiber content are used, the difference between buffalo and other ruminants regarding gross food digestibility decreases [4]. More realistically, the numeric prevalence of buffaloes in tropical territories is very likely due to the inability of dairy cattle to express its productive potential when reared in humid and hot climate. Conditions pertaining to other aspects such as commercial and political facets make the buffalo farming, like in Italy, more profitable than dairy cattle as buffalo milk is paid to the farmers three to four times higher, and therefore a significant increase in the number of heads has been witnessed around the country over the last decades. It is true that the domestic buffalo can be an ideal transformer of poor-quality forage within marginal areas into animal products to be locally stored and commercialized. Nevertheless, whenever higher quality forage is available, the buffalo can be more selective than cattle under intensive production systems [5]. In the last decades, a varied pattern of development in buffalo both in terms of number of heads and production strategies has been witnessed around the world. Countries characterized mostly by the presence of swamp buffaloes mainly used for draft purposes, like the ones in the Far East such as the Philippines, Vietnam, Bangladesh, Sri Lanka, Thailand, and Indonesia, have seen their heads and production either stabilized or even reduced due to the advancement of mechanization in agriculture [6–9]. On the contrary, in countries where the dominant buffalo is given by the river subspecies such as India, Pakistan, and Italy, an improvement of those features has been reported. The countries where most of the advancement in reproduction and production strategies has been carried out are Brazil in South America, India and Pakistan in the East, and Italy in the Mediterranean area. This is due mainly to the presence of the most productive breeds in terms of milk yield, like Murrah, Nili-Ravi, Surti, and Jaffarabadi in India and the Mediterranean Buffalo in Italy. In addition, these are the countries where most of the efforts in terms of adopted genetic strategies have been and still are implemented, in order to improve both quality and quantity of production traits. In China, where the majority of available buffaloes belong to the less productive swamp type, an attempt at improving milk and meat production was made with the introduction of the river type Murrah and Nili-Ravi in the 1950s–1970s, and more recently by crossbreeding with the local swamp buffaloes [10]. It is noteworthy to mention that in the same 1950s, in Italy the domestic buffalo was on the verge of extinction, due to swamp reclamation and changed economic conditions that would not support buffalo breeding. Despite such economical constraints, from the end of World War II, on the contrary buffalo heads have increased steadily and by the year 2000 the Mediterranean Italian Buffalo breed has been officially recognized, while selection has continued throughout the years thanks especially to the natural isolation and the consequent lack of introduction of other breeds in the country.

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Different production strategies characterize those countries where the least productive swamp subspecies are utilized for both milk and meat production, and in some cases only for meat, as milk is enough only for sustaining calf growth up to weaning. It has to be underlined though that in all Asia, both river and swamp subspecies have always been reared as dual or three purpose animals. On the contrary in other European countries and especially in Italy, there has always been a strong association between the buffalo and its milk production and derived cheese products, of which the mozzarella cheese is certainly the most known worldwide. Recently though, a strong interest in buffalo meat, due to its qualitative characteristics and health benefits, has lifted breeder attention into this parallel production strategy by creating specific protocols for bringing animals to slaughter age and weight [11]. An incredible body of knowledge has been built around buffalo meat and its benefits derived from its consumption in the human species, as opposed to cattle meat, especially when fatty acid composition and cholesterol content are taken into consideration [12]. Although buffalo meat covers only a very small fraction of meat demand in the entire world, in countries where this species is prevalent such as in the Far East, or in countries where for religious beliefs cattle cannot be used for human consumption like India, the availability of buffalo meat has greatly increased for both national and international markets [13].

6.2 The domestic buffalo and its genetic merit

Two are the buffalo species in the world: the African buffalo (*Syncerus caffer*) and the water buffalo (*Bubalus bubalis*), and it is the domestic water buffalo the object of our interest in this chapter. The name buffalo may generate some confusion, as in some parts of the world, like in the United States, often the bison (*Bison bison*, different genus and species) is referred to as buffalo. Of the water buffalo species then, although several haplotypes both at genomic and mitochondrial level have been found to be shared, two distinct subspecies can be described, namely the river and the swamp buffalo, with 50 and 48 diploid number of chromosomes, respectively [14], and their phylogenetic separation predates domestication. As previously synthetically reported, the two subspecies characterize the buffalo species in different countries of the world, and their individual prevalence in each country is mainly dictated by the specific management system that can be found in place, for the milk and meat production system adopted and the recorded yield, and for the interest in enhancing the genetic potential through the implementation of old or newly developed reproductive technologies. Finally, the reason why such varied management system is in place for buffaloes has to be found in the intermingling of a number of different factors such as climate, local geography and economical wealth or constraints, cropping systems, size of farms, and finally the primary purpose for which buffaloes are reared in each different country, among milk, meat, and draught, singularly taken or in combination.

Cytogenetic studies in buffaloes have revealed that the karyotype characterizing the swamp type has derived from the tandem fusion translocation of chromosome 4 (BBU4) and 9 (BBU9) of the river type, giving rise to the large characteristic chromosome number 1 in swamp buffaloes [15]. Their genetic similarity can be inferred by G- and R- banding homologies, in addition to the hypothesis of a common genetic ancestor among all bovids (*urus−Bos primigenius taurus*) as evidenced by centric fusion translocation between two of the ten homologous cattle autosomes, that gives rise to each of the five river buffalo biarmed pairs [16].

The knowledge of a complete genome sequence in farm animals allows a technical and conceptual step to be taken, namely the prediction of phenotypes from available genotypes, reducing thus the time constraints posed by older acquired protocols such as progeny testing. Therefore bulls acquire a genomic breeding value, through the use of genome DNA markers, and semen doses can be sold solely on this basis. In cattle, genome-wide association studies have been widely applied, and therefore either marker-assisted selection or gene-assisted selection constitutes today a strong basis for the application of breeding selection schemes. The buffalo genome has been fully sequenced, although only a very small percentage of genes with known function have been reported so far [17]. In turn, this limited knowledge is reflected into little information for the genetic variability of the species, which prevents a fast and correct genetic improvement. Nevertheless, following comparison with the bovine genome, some knowledge of single-nucleotide polymorphism (SNP) sites in buffaloes has been achieved, bringing to the availability of SNP chips and making it possible to delve into buffalo genetics among all the available breeds and to spot those markers responsible for exerting a greater effect not only on production of the traits of interest, but also on health and efficiency [18]. It has to be underlined though, that still missing the complete notation of the entire buffalo genome, the utilization of the available existing tools for the genetic enhancement of the buffalo species is not yet satisfying. At present, only few genomic studies have been performed in buffaloes. A genome-wide association study (GWAS) was carried out in Italian Mediterranean Buffaloes, in which four candidate SNPs were found in two genomic regions associated with buffalo milk production traits [19]. One region is linked to milk fat and protein percentage and was located on the equivalent of Bos taurus autosome (BTA3), while the other region, linked to total milk yield, fat yield, and protein yield, was located

on the equivalent of BTA14. Interestingly, both of the regions were reported to have quantitative trait loci affecting milk performance also in dairy cattle. Although usually milk production in ruminants is not associated to prolactin, in another study, high resolution melting techniques were developed for genotyping Italian Mediterranean Buffaloes and showed that polymorphisms of the buffalo prolactin gene (PRL) were associated with milk production traits [19]. Hence, PRL could be used as a candidate gene for marker-assisted selection in Italian Mediterranean river buffalo breeding. Regarding reproductive activity, another GWAS study performed in buffaloes showed that a total of 40 suggestive loci (related to 28 genes) were identified to be associated with six reproductive traits (first, second, and third calving age, calving interval, the number of services per conception and open days) [20]. Nevertheless, marker-assisted selection for the traits of interest in the buffalo, with forthcoming increased knowledge and improved efficient technology, can still be seen today for the near future as the best choice for the identification of animals with high breeding value and for planning appropriate breeding schemes [21].

6.3 Reproductive physiology

Buffaloes adapt well to a broad variety of climatic conditions and, like other production species such as horses, small ruminants and some cattle breeds kept under extensive pasture, are tendentially seasonal animals although they can breed all year round. This is an evolutionary strategy adopted in the wild in order to match birth and weaning under the best environmental conditions, to better satisfy nutritional and thermal requirements typical of the species. In fact, being an animal of tropical origin where most of the green pasture is available from the end of the rainy season, coincident with the end of summer, the peak of reproductive activity in natural condition will be therefore witnessed in the semester going from the end of summer into the first months of winter. This will ensure that birth, after around 310−320 days of pregnancy, will coincide with the highest forage availability. The nocturnal release of melatonin from the epiphysis will be triggered by the coincident decrease of daylight hours, and thanks to a domino effect, melatonin in turn will exert an effect on the hypothalamus hypophysis gonadal axis, heading thus toward the beginning of estrous cycles. We can say then that, under such conditions, the buffalo increases its reproductive activity in the period of the year characterized by reduced daylight hours or, even if light hours increase, night hours are still prevalent within the day. Of course, whenever buffaloes are reared in environments where daylight and night light hours are equivalent, reproductive activity is similar throughout the year, and possibly other variables, such as nutritional requirements will represent the key element for reproductive success [22].

Traditionally, the domestic buffalo under farm conditions and in comparison to cattle, has always been considered to be characterized by a reduced reproductive efficiency due to late sexual maturity, seasonality, long time interval calving to first estrus, reduced signs of estrous behavior and conception rate. Up to a very large extent this is true, although recently the efficiency in conception rate following artificial insemination (AI) in conjunction to the newly developed synchronization protocols has approached similar results with cattle, even when sexed semen is utilized. Differently, if we consider the parameters concerning reproductive efficiency in other contexts, where the buffalo is naturally mated without human intervention and/or constraints posed by local governing laws of milk and cheese production and commercialization, then this species can be regarded as highly efficient and productive. As an example of how buffalo reproductive efficiency is put into play, let us consider what happens in Italy, where most of the milk and cheese produced is traditionally required by the market in the time of the year where, by physiologic limits of the species, the least number of calvings are recorded. To offset for such intrinsic species-specific seasonality, a tendency for selecting animals less sensitive to photoperiod is in place in farms, in addition to the practice of skewing the time of the year when most of the natural mating and AI is practiced. Obviously, this contrived protocol may inevitably bring some strong financial loss to the breeders, as a consequence of the attempt at increasing the number of births in the period of the year where naturally a physiological anestrus, typical of this species, will occur. In fact, at these latitudes during increasing day length months, buffaloes sensibly reduce their capacity to reboot an ovarian cycle heading toward a new pregnancy. In this period, if pregnancy is not established, the animal may delay a new fertile follicular development within the ovaries and will possibly restore fertility into the successive autumn to winter months. This will determine a significant and deleterious lengthening of the intercalving period and a strong reduction of milk production. This challenge to buffalo reproductive efficiency is still active in most farms, even though more and more cheese makers are devoting part of their production to being exported in other countries, such as United States and Japan, a context in which the time of the year in which milk and cheese are consumed can also be important for the trade. The strong influence of the environment over reproductive function and success can also be seen, in addition to higher rates of reduced or failed ovarian function as above described, in the case of reported higher embryo mortality. In fact, this is typically

witnessed in the same countries such as Italy where animals are challenged with AI in the period of winter-spring transition, characterized by increasing day length [23].

Despite such species-specific limitation, the buffalo is characterized by a reproductive and productive longevity by far not comparable to cattle. In fact, dairy cattle are ordinarily culled following only few lactations, whereas in contrast usually most buffaloes remain in production for many years and for many lactations mainly due to an intrinsic lower milk production and to a more physiological feed rationing. This is surely due to its more rustic characteristics and shorter time available in the hands of breeders for adopting selection strategies for the characters of interest [24].

Again, an additional element that contributes to lower the overall reproductive efficiency, whenever manipulative technologies are applied to this species, is given by the 10-fold lower content of ovarian primordial follicles when compared to cattle. This accounts for a significant lower number of available antral follicles even following hormonal stimulation within adopted protocols for multiple ovulation and embryo transfer (MOET), and ovum pickup (OPU) in conjunction to in vitro embryo production (IVEP) procedures [25,26]. It is noteworthy to mention though, that prepuberal buffalo calves display the same high number of antral follicles as recorded in cattle calves. In addition, and similarly to cattle, buffaloes are both qualitatively and quantitatively characterized by two to three waves of follicular development within the ovaries in puberal and pluriparous animals. As expected, the pattern of hypothalamic-hypophyseal-gonadal hormonal release is in fact superimposable to other ruminant species too [22]. All the above aspects have to be aligned and put in the correct perspective whenever any of the newly developed reproductive technologies are going to be implemented in this species.

6.4 The role of artificial insemination

The first documented attempt at AI goes back to 1783, when Lazzaro Spallanzani achieved the first pregnancy in a bitch, although it was only at the beginning of the 20th century that the Russian I.I. Ivanov devised the first artificial vaginas for equine and ruminants. Only after the Second World War though, the technology spread over the most important species of zootechnical interest with dairy cattle benefitting the most. Among ruminants, buffaloes have been subjected to AI practice much later than cattle for a number of reasons. Firstly, in most parts of the world the least productive buffaloes have always been considered labor animals, and only residual milk production other than the milk used by the calves is processed for family and local cheese production, and in some countries such as Nepal and India, milk used by families for direct consumption is high, from roughly 50%−80% of the whole production, respectively. Meat from buffalo is made available to families and for local trade only at the end of the animal career within small holders. The small number of animals per owner and the poor management conditions typical of farmers at the village level, especially in Southeast Asia has been a substantial halt to the application of AI. On the contrary, in countries characterized by the availability of the most productive subspecies and breeds, and where intensive management is run, animals have been selected for their production by massal selection, prior to the introduction of reproductive technologies. In these last countries a proper buffalo genetic selection has started few decades ago, and the earlier studies on male and female reproductive characteristics have been performed. Nevertheless, a very slow beginning has characterized the implementation of AI in buffaloes, mostly due to the resistance and doubts posed by the same breeders, skeptical over the years that this technique may prove beneficial for buffalo genetic enhancement.

Therefore, and always in comparison with the cattle counterpart, buffaloes have had less time to be selected for the traits of breeders interest. This can be seen in both males and females. Buffalo bulls are in fact characterized by a poorer sperm quality and larger individual variability in fertility, which is exacerbated in the season of the year of reduced reproductive performance, where also libido is usually affected. The reduced fertility may be attributed to a higher sensitivity to oxidative stress due to an increased lipid peroxidation, in conjunction to a reduced activity of antioxidant enzymes [27]. Attempts have been made in order to overcome the natural sensitivity of buffalo semen to cryopreservation damage, by including in semen extenders proteins derived from the isthmic portion of oviducts [28,29]. Similarly to other ruminant species and mammals in general, nutrition, thermal stress, and other environmental variables may also be responsible for higher or lower semen quality, depending on the intermingling of the variables involved [30].

On the female side, under intensive management and correct and balanced feeding regimen, photoperiod, as previously stated, is known to affect reproductive function. This sensitivity though is different between heifers and pluriparous cows, the first being less sensitive to the light stimulus. Therefore whenever AI protocols are applied in the period of the year characterized by increasing daylight hours, a higher conception rate should be expected from heifers [31]. Along this line, in countries where photoperiod is a critical factor in reproductive efficiency for buffaloes and where higher production is differentially required among seasons, breeders tend to select animals less sensitive to the light

stimulus. On a different note, and likewise cattle, additional nutritional and metabolic factors may negatively affect pregnancy rates following the implementation of AI programs [32].

The very first attempts at synchronization protocols in buffalo heifers and cows have been made by using double prostaglandin administration with variable and inconsistent results. Typically, such well-established protocols would be taken from cattle and strictly applied to buffaloes with the assumption that similar synchronization and conception rate would be achieved. Differently, poor results have been reported initially, discouraging breeders from accepting the introduction of a new concept of genetic improvement in the buffalo species. One of the most important reasons behind such initial failure in AI has to be searched in the physiological difference between cattle and buffaloes control of reproductive function, following exogenous administration of hormones in the course of synchronization protocols. It may be hard to spot the best moment to perform AI in individual buffaloes as, under natural conditions and differently from cattle, they do not frequently express homosexual behavior and are characterized by a reduced frequency of mucus discharge, together with a similar coat color. A vasectomized bull within the herd though [33] usually is effective in significantly increasing sexual behavior among females. The reduction in sexual behavior and signs is even more pronounced when exogenous hormones are applied to synchronize animals, making the heat detection and hence the identification of the best time for AI even more difficult. Following an estrous cycle, which is much more variable in length than cattle, a similar higher variable timing of inner hormonal discharge progression, leading finally to ovulation, is recorded in buffaloes. In early seminal studies by Seren [34], the reproductive hormones (luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol, progesterone, prostaglandin, and prolactin) involved and needed to a fertile ovulation were evaluated during the estrous cycle successive to the one induced by double prostaglandin administration. From that study, it emerged that the interval between the beginning of behavioral estrus and ovulation lies between 50 and 55 hours in the case of single ovulation. As previously reported [35], a high frequency of double ovulations, up to more than 30%, was in fact recorded. In that case the timing to the first and second ovulation would be of 40 versus 110 hours, respectively. In case of a single ovulation the interval between LH peak and ovulation would be of 35 hours, whereas in the event of double ovulations, the interval would be of 30 and 65 hours to the first and second ovulation, respectively. A more recent study following the implementation of two different estrus synchronization protocols has revealed a similar time interval between LH peak to ovulation [36]. Of course, such unexpected variability in hormonal discharge and progression followed by ovulation did explain part of the reasons of unsuccessful attempts of AI at that time.

Far better results have been obtained more recently, through the combined implementation of protocols for synchronization of ovulation and ultrasound monitoring of the reproductive tract and ovarian follicular dynamics. Synchronization protocols have been largely taken from those in use in cattle, especially following the widespread efficiency of the Ovsynch with its various modifications when applied to both heifers and cows [37]. The availability of protocols for the synchronization of ovulation has allowed the possibility to reduce the incidence of double ovulations and to more precisely target the timing of ovulation with a timed fixed AI (TAI). In fact, De Araujo Berber et al. [38] have shown that following the second gonadotropin-releasing hormone (GnRH) administration, most of the ovulations lie between 26 and 38 hours, justifying therefore the insemination of animals up to 20 hours after the second GnRH. The highest efficiency has been reported on cyclic animals with an average, although variable, conception rate of 50%−60% when ultrasound diagnosis is made up to the first 30 days following AI. This protocol, cheap and easy to be performed, is nowadays largely applied to buffaloes. Protocols have been subjected to modifications on acyclic animals by adding progesterone as a removable implant, with a resulting efficiency variable in terms of restoring cyclicity and conception rates [39,40].

The efficiency of AI in conjunction to protocols for synchronization of ovulation has in recent years greatly benefitted by the use of ultrasound technology. A first and important improvement has derived from the significant reduction of mistakes incurred during manual exploration and diagnosis within the reproductive tract. In comparison to cattle, smaller size of the ovaries and corpora lutea (CLs) at times deeply embedded into the ovarian stroma is responsible for misjudgment and clinical false interpretation. In addition, ultrasound technology enables a reliable and correct diagnosis of early pregnancy, as well as detecting embryonic mortality, anticipating though the new course of action to be followed on every single animal. An additional significant aid to reproductive management is given by the possibility to follow the events occurring at the ovarian level with great accuracy. In fact, available ultrasound machines allow easy interpretation of ovarian structures and follicular dynamics, with the result of a better-timed implementation of protocols for synchronization of ovulation and a more suitable selection of animals to be enrolled into AI programs. De Rensis et al. [40] have in fact reported a higher conception rate in the course of Ovsynch programs, whenever a large dominant follicle responsive to the first GnRH administration is found. In the same line, Neglia et al. [41] have found that the ovulation after the first GnRH administration within an Ovsynch protocol results into a larger CL area, a higher

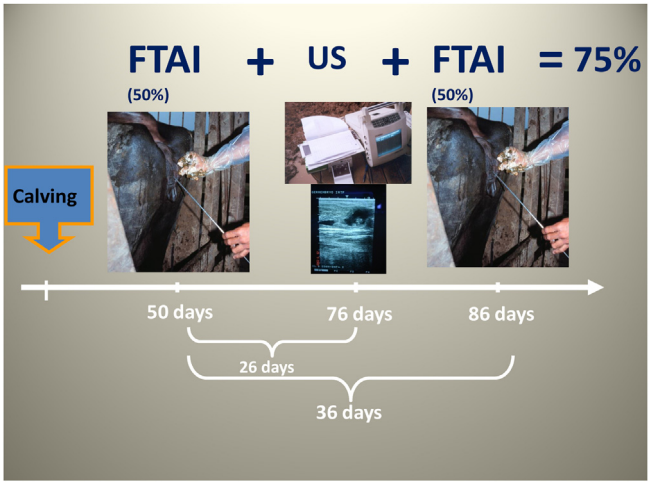

FIGURE 6.1 Pregnancy rates following two consecutive TAIs.

response to the following prostaglandin administration, and finally into a higher response to the second GnRH administration leading to the ovulation of a larger dominant follicle. As expected, the corresponding CLs show a greater progesterone concentration paralleled by significant higher pregnancy rates. Higher efficiency and conception rates are then usually accomplished when such treatments run in parallel to an ideal reproductive management within the herd. In recent years, modifications of TAI programs have mushroomed and tried repeatedly in many farms, usually with such satisfying results, to convince breeders of the usefulness of this technology and make them even more confident as to include synchronization programs into their farm reproductive management. Under this light Rossi et al. [42] have shown that buffaloes, whenever found nonpregnant, can be submitted into a continuously running TAI program without prejudice to their health or future reproductive function, and that the first three consecutive TAI programs hold for the majority of pregnancies established. The same study demonstrated that the intercalving period between animals undergoing TAI programs in comparison to animals following natural mating within the herd is shorter of a little less than 2 months. This translates into an optimization of reproductive efficiency in the herd, higher conception rates, and calving, heading toward a higher economic efficiency of the farm.

The ultrasound technology comes in help when proper assessment of CL functionality is needed. This can be of course accomplished by the ultrasound image referring to size, texture, and shades of gray on the screen. However, an additional important help can be received by the understanding of blood flow through the CL itself, by the adoption of the color Doppler technique. In fact, a reduced CL vascularization on day 5 following ovulation is inductive of significant lower conception rates on day 45, as substantiated by a differential progesterone value made on day 5 [43]. In conclusion, in our experience with the use of ultrasound technology as ancillary aid in well-managed buffalo farms, fixed TAI programs can result in approximately 75% of the animals being pregnant from calving onward up to less than 3 months if a 50% conception rate is assumed (Fig. 6.1).

From all the above, and comparisons over the years run among synchronization protocols, including also the ones incorporating progesterone devices, it can be stated that an ideal synchronization treatment does not exist. In fact, in order to achieve the highest results from AI practice, the following four most important variables have to be taken into account: season of the year, category of animals, metabolic status of the animals and of course, and the protocol for synchronization of ovulation that would fit best the first three variables.

In conclusion, farm and environmental conditions, feeding regimen and availability, season, age and parity of the animals, and implemented protocols are in various degrees responsible for the high variability registered across the years in terms of conception rates following AI.

6.5 Sexed semen

The implementation of sexed semen technology in buffaloes, as for other domestic species, is especially helpful among others for optimizing female replacement and rapidly proliferating the genotypes of interest within the herd. This is especially true also when, due to health problems, there is a need to have an internal turnover of replacement heifers, or when it is mandatory to increase the number of heads in a fast-growing buffalo farm. The technology behind this new tool for genetic improvement relies on the possibility of cell sorting cells bearing differential amount of DNA. In both

cattle and buffaloes this difference in sperm cells bearing either X or Y chromosome is large enough to allow an efficient separation of the two cell populations to obtain a roughly 90% purity within samples. The use of sexed semen has become a potentially powerful tool for the genetic enhancement of *B. bubalis* since the first successful application of the technology in Mediterranean Italian buffaloes [44]. In the intervening years, with a similar number of preselected sperm cells, successful attempts have been carried out in Murrah and Nili-Ravi breeds following either IVEP procedures or AI [45–47]. Finally, the same reduced dose of 2 million live preselected sperm cells has been used in Mediterranean Italian buffalo heifers, showing a similar pregnancy rate when compared to nonsexed conventional frozen-thawed semen. That same study showed that insemination into the body of the uterus ensures higher rates of pregnancies when compared to deposition of preselected semen into the horn ipsilateral to the ovary where ovulation had occurred, possibly as a consequence of more severe trauma received by the horn endometrium by the AI gun [48]. The chronology of studies in buffaloes on preselecting sperm cell populations and first attempts via AI or IVEP procedures can be seen in Fig. 6.2. More recently, in line with a number of ideas tested over the years to select sperm cells to alter sex ratio, a commercial based group in the United States has brought into the market a product that would both increase fertility rate and skew the sex ratio in favor of born female calves in cattle, by adding to conventional frozen/thawed semen a preprepared solution containing molecules enhancing X-bearing sperm cells motility and allowing them to reach the fertilization site earlier than Y-bearing sperm cells, in conjunction to a delayed insemination time as otherwise required by the Ovsynch protocol. Despite the success that has been publicized in cattle, the first attempt in buffaloes has not been equally successful [49], and a more recent trial in buffaloes, in the period of the year characterized by decreasing light hours, has revealed similar results, confirming that even if AI is delayed as requested by the new protocol, both herd fertility and born female calves rates are not altered (Presicce, 2019, unpublished results).

In buffaloes, a similar pregnancy rate with preselected frozen-thawed spermatozoa and conventional nonpreselected cryopreserved semen was recorded, in contrast with reports in cattle, where usually rates are lower down to 60%–80% when compared to controls [50,51]. Although speculative at this point, it could be possible that buffalo semen can better survive to physical and chemical stress during the sorting process. Therefore sexed sperm cells may thus maintain the same fertilizing capacity as controls, as it has also been demonstrated in sheep, by assuming the hypothesis that the sex-sorting process may select a superior population of sperm cells. In fact, it may be possible that cell sorting will select sperm cells against a 15 kDa protein (SLLP1—acrosome indicator protein), which is as a result abundant in control sperm cell samples but absent in sorted population of spermatozoa [52].

FIGURE 6.2 Early chronological events related to sperm cell technology and its implementation in the domestic buffalo (*B. bubalis*).

TABLE 6.1 Projected benefits following use of sexed semen in an intensive buffalo farming system.

Farm turnover 20%; bull effect = 0; mortality = 0; mean farm milk yield = 2550 kg						
	Semen					
	Sexed	Nonsexed				
To get	20♀	20♀				
% conception	40	45				
% top buffaloes to AI	50	90[a]				
Mean milk (kg) of inseminated buffaloes	2800	2550				
Selective differential (kg)	250	50				
Hereditability (30%)	75	15				
Difference from starting mean milk production of 2550 kg						
		Semen				
After years		Sexed	Nonsexed	Milk difference	× head	× 10 heads
		(kg)	(kg)	(kg)	(€)	(€)
4		175	163	12	15	150
8		579	436	143	178	1782

[a]*Half of them will be ♂.*

The use of sexed semen, considering its final cost to the farmer, type of farming, geographical location, and its socioeconomical features, may have different impact worldwide. As an example (Table 6.1), it is interesting to highlight what the benefits may be within an intensive buffalo farming system such as the one in place in Italy where the implementation of sexed semen can be an integral part of the reproductive management of the herd. If we assume some fixed starting conditions such as a farm turnover of 20%, a nonexisting bull effect, and mortality, a heritability for milk production of 30% and a mean farm milk yield of 2550 kg, then a reduced number and more productive buffaloes could be used to obtain the same number of replacement heifers. Over the years, the use of the top production animals and sexed semen would ensure a significant milk difference and a corresponding higher cash gain (Zicarelli, unpublished results).

6.6 In vivo embryo production (multiple ovulation and embryo transfer)

In line with a delayed attention to the implementation of reproductive technologies compared to cattle, the first attempts on in vivo embryo production in the buffalo date back to the beginning of 1980s, with the first report by Drost et al. [53]. As expected, procedures for MOET did rely on already existing protocols of hormone administration in cattle and have been since then improved or modified, in order to enhance the success in terms of ovulating follicles and recovered embryos.

Hormones available such as pituitary extracts usually from porcine origin, equine chorionic gonadotropin, and human menopausal gonadotropin [54–56] are administered in mid-cycle in conjunction to the controlled emergence of a new follicular wave, by hormonal or, more recently, mechanical means [57,58]. Pitfalls in the use of hormonal treatment for control of multiple follicle development are always possible, although a number of strategies, depending on the type of used hormone, have been devised. In buffaloes too, the use of equine chrorionic gonadotropin (eCG) may possibly lead to persistent stimulation due to the prolonged half-life of the molecule, and in order to reduce the incidence of such occurrences, monoclonal or polyclonal antibodies have been administered, although no improvement in terms of produced and recovered embryos have been described when compared to conventional protocols [59,60]. Indeed, nowadays the most used hormones for superovulatory treatment in buffaloes are pituitary extracts of FSH with variable levels of LH content, which may additionally account for the large variability in the final response. Such variability could be overcome by using bovine FSH produced by recombinant DNA technology, although such option has not been employed in buffaloes so far. Overall, the use of both pituitary extracts, eCG or human Menopausal Gonadotropin (hMG), has given over the years similar values in terms of ovulating follicles and recovered embryos in the two buffalo subspecies, river and swamp [61–63].

Nevertheless, despite many efforts carried out over the years, the efficiency of MOET in buffalo is still poor. In addition to the inconsistent response to hormonal stimulation in this species, the major limitation is unquestionably the low number of transferable embryos flushed per donor. Several factors may play a role in the efficiency of MOET programs in buffaloes. Age and parity do not seem to significantly alter the rate of embryo recovery, although in heifers a higher number of ovulations are recorded [56,64]. Likewise cattle, the time of initiation of hormonal administration has to respect the physiological command of follicle development within waves, and therefore it is key to start the protocol at the beginning of the new follicular wave by hormonal or mechanical means, as previously highlighted. With regard to the hormonal status of the animal, higher levels of hematic progesterone are inductive of a more satisfactory follicle development following hormonal administration, and this is confirmed by a higher rate of available CLs and recovered embryos [65,66]. When considering environmental variables, in an earlier study the effects of photoperiod on MOET programs were not in line with the seasonality pattern of the species [67,68]. This unexpected result may be accounted for by the fact that during the unfavorable season only the most fertile cows are cycling and hence can be recruited for MOET, leading to a sort of selection of the most responsive donors. The effect of nutrition on reproductive function has been amply demonstrated in ruminants, and this is substantiated in buffaloes by a higher and more efficient response to reproductive technologies such as AI and MOET programs when body condition score lies between 2.5 and 4 on a scale from 1 to 5 [32,69]. The time to start MOET programs in relation to calving has also been considered and a higher response has been reported when protocols are implemented between 60 and 220 days postpartum [68]. In addition, according to Di Palo et al. [70], better results when implementing MOET programs in buffaloes are achieved when richer diets containing forages are given to the animals, as opposed to concentrates, being the former more physiologically fit to ruminants.

Other attempts at improving the efficiency of MOET programs in buffaloes include the possibility to increase the number of developing ovarian follicles by recombinant-Bovine Somatotropin (rBST) priming on a dosage effect manner [71,72]. Similar improvement can be obtained also by immunoneutralization of buffaloes against inhibin, reflected also in the consequent rate of recovered transferable embryos [73]. An additional improvement has been reported by the combined exogenous administration of a GnRH agonist and LH, restricting thus the timing of ovulation in a shorter period of time [63]. The administration of prostaglandin $F_{2\alpha}$ at the end of the superovulatory treatment has also been implicated in some improvement in the rate of recovered ova and embryos, due to effects on endothelin-1 and angiotensin-II gene expression inhibition within the CL, then by increasing ampulla and fimbriae contractility of smooth muscles and finally by favoring follicle rupture [74]. Nevertheless, there is a clear evidence that the low efficiency of MOET, in terms of embryo yields, in buffalo is not simply due to the reduced follicular reservoir at birth, as hormonal stimulation is effective in inducing the growth of multiple follicles up to ovulation. In contrast, a poor embryo/CL rate is commonly observed (7−9), suggesting an impairment of ovum capture by the oviduct at ovulation. Further studies have confirmed the difficulty in improving the efficiency of MOET programs in buffaloes [75].

It has been previously hypothesized that estradiol plasma level, which under natural estrous cycles is lower in comparison to cattle, is particularly elevated following hormonal treatment resulting in abnormal estradiol to progesterone ratio, affecting the capacity of fimbriae to capture ovulated oocytes, or worse push them back into the peritoneal cavity by reversed peristalsis [76]. Similar assumption has been suggested by Baruselli et al. [77], invoking a lower quality of the ovulated oocytes characterized by a reduced number of surrounding granulosa cells and preventing them to be rightly captured by the fimbriae resulting in a failure entry into the oviduct. We have recently demonstrated that both inappropriate cumulus expansion and disruption of contraction-relaxation of the oviduct contribute to OPU failure in superovulated buffaloes, as a result of impaired steroid synthesis [78]. Indeed, in superovulated buffaloes granulosa cell function was altered, as indicated by the overexpression of *FSHR* and *CYP19A1* and the decreased expression of the STAR protein, leading to lower estradiol synthesis. The altered intrafollicular steroid profile was associated to compromised cumulus expansion in most of the oocytes recovered from periovulatory follicles. Finally, the reduced expression of steroid receptors and vascular endothelial growth factor in the oviduct indicated that the contraction-relaxation of the oviduct in the periovulatory period may also play a role.

6.7 In vitro embryo production

As previously reported, the implementation of MOET programs in buffaloes has brought limited success, despite the attempts over the years at improving the recovery of high-quality embryos. Such lack of efficiency has paved the road to the introduction of another younger strategy, again borrowed from cattle, dealing with the possibility to produce embryos in vitro following procedures devised to mature oocytes recovered from antral follicles and to fertilize them with capacitated frozen-thawed spermatozoa [79]. The mass production of embryos derived from the availability of ovaries from slaughtered buffaloes though would have only a minor impact on the genetic improvement of the species, unless this strategy could target the best available animals. This coupling has been brought on the scene by the possibility to recover

oocytes from the ovaries in live animals, by ultrasound puncture of antral follicles [80]. The combined IVEP/OPU technology has been proved feasible in buffaloes since more than a couple of decades and has resulted, over the world, in live offspring from the transfer of either fresh or cryopreserved embryos [81–85]. In line with the promising results obtained with the use of sexed semen for AI, embryos have also been produced in vitro with sex-sorted sperm [47]. The possibility to link the OPU technology to the IVEP procedures has been proved feasible even in prepuberal buffaloes in which immature and mature oocytes from antral follicles were recovered, with and without hormonal treatment. In that study, the high number of recorded antral follicles within prepuberal ovaries, similar to cattle calves, highlighted the possibility to bypass the intrinsic genetic paucity of antral follicles recorded in adult females, although no attempts were made at fertilizing the recovered mature oocytes [86]. Recently, embryos were in vitro produced from oocytes recovered by laparoscopy from buffalo calves and pregnancies were obtained after embryo transfer [87]. This approach, commonly known as juvenile OPU, would dramatically increase the genetic progress by shortening generation interval. The successes reported in a quarter of century in buffaloes have been and still are hindered though by a series of: (1) in vivo aspects intrinsic to the species, such as lower follicle count and oocyte recovery, partly surpassed by hormonal treatment, high individual variability [88], lower quality of developing CL responsible for inadequate progesterone production to sustain pregnancy, and poor oocyte competence during the long day length season, leading to early and late embryo mortality [89–91]; and (2) in vitro aspects such as reduced embryo quality according to international evaluation standards and corresponding viability, very likely as a consequence of suboptimal culture conditions, together to a reduced capacity and resilience to withstand cryopreservation procedures, possibly due to a higher lipid content in comparison to cattle [92].

Following is a synthetic series of up-to-date findings and recent improvements related to the protocols implemented in buffaloes in order to obtain in vitro embryos: (1) oocytes: in adult animals lower numbers of antral follicles and corresponding retrieved oocytes, the latter usually of lower quality, are recorded. Across the available literature, differences within this variable are reported according to the subspecies or breeds investigated, age, nutrition, and health status. A number of other variables such as time interval between oocyte retrieval and laboratory processing, oocyte holding temperature, time of the year (short or long day length), and environmental temperature, may overall affect the efficiency of the procedure in buffaloes [93]. To enhance quality and number of punctured follicles and recovered oocytes, especially when OPU is integral part of the in vitro procedure, hormonal treatment with FSH and rBST has been evaluated earlier, resulted in limited success [94,95]. More recently, we demonstrated the efficacy of an OPU priming, with FSH treatment in the presence of progesterone, at increasing the number of follicles, oocytes, and, more importantly, transferable embryos. It consists in the ablation of dominant follicle and insertion of a progesterone-releasing device on day 0, followed by 3 days of FSH given twice daily and a coasting period of around 40–44 hours, with OPU carried out on day 6. A remarkable effect was observed in both seasons, with a fourfold increase of blastocysts number. This certainly ensures a better exploitation of the germinal material especially when programming OPU trials; (2) in vitro maturation: nuclear and cytoplasmic maturation in buffalo oocytes is usually completed between 20 and 24 hours after immature oocytes are placed into maturation media. It is worth noting that buffalo oocytes undergo earlier aging, and hence the in vitro fertilization (IVF) should be carried out as soon as possible since 18 hours post-IVM and not later than 24 hours [96]. Indeed, reduced efficiency in terms of both cleavage and blastocyst rates together with higher proportion of degenerated oocytes has been reported at increasing maturation times. Improvements in oocyte maturation have been reported following inclusion into maturation media of growth factors such as insulin-like growth factor (IGF)-1, IGF-2, epidermal growth factor (EGF), and vasoactive intestinal peptide [97–99]. It has furthermore been reported that antioxidants such as thiol compounds promoting glutathione (GSH) synthesis [100–102], melatonin or taurine [103], are fundamental to improve maturational processes and subsequent embryo development. Finally, lectin was reported to ameliorate oocyte maturation and upregulate the expression of genes involved in gap junction, cell communication and cell cycle proteins (Cx43 and growth differentiation factor-9), cell membrane protein (fibronectin), and basic growth factor (fibroblast growth factor-4) [104]; (3) IVF: the fertilization of mature oocytes in vitro is another fundamental and multifactorial milestone whose efficiency depends in turn on a number of finely tuned steps. The most important and limiting factor is the so-called "bull effect," as only roughly 10% of all the bulls screened for IVF will satisfyingly respond with an adequate fertilizing efficiency, despite the fact that the quality of cryopreserved semen has definitely been improved when compared to the past [105]. The most reliable method to screen bulls for IVF is inevitably to test semen against batches of in vitro matured oocytes and assess the rate of late embryo development. Lately, a simple double staining Trypan Blue/Giemsa technique was used, giving evidence of a correlation between acrosome intact sperm cells and blastocyst development rate. Therefore, this staining technique can be considered predictive of the in vitro fertilizing capability of sperm cells [106]. Both Percoll density gradient and swim-up are efficiently used to select motile sperm cells prior to fertilization, although even with this procedure, the one or the other may perform better depending on the individual bull [107]. While in coincubation with matured

oocytes, sperm cells need the support of capacitating agents of which heparin is mostly used also in buffaloes. Other molecules have been tested and proved successful in promoting the capacitation process in buffalo sperm cells, such as progesterone, sodium nitroprusside, and melatonin [108–110]. Finally, factors available in both follicular fluid and serum or present in the oviduct such as osteopontin, also detected in semen in high concentration [111], when included in fertilization media significantly, increase the capacitation process and embryo development [84,112]. The "bull effect" should also be considered when dealing with the time needed for sperm/oocytes coincubation. Although such time has been set at 16 hours according to the results available [96], individual bulls may display a different timing in the kinetics of sperm penetration [113]. Therefore the importance of testing each bull for this parameter prior to entering into fertilizing trials has to be taken into consideration; (4) in vitro embryo culture: differently from cattle, buffalo in vivo and in vitro embryos are known to be characterized by an earlier minor genomic activation at the two- to four-cell stage and a more important one at the four- to eight-cell stage development [114], suggesting from the start of possible differential in vitro culture requirements and explaining a faster development similarly to other domestic ruminants [115]. Initial attempts at culturing buffalo embryos in vitro have relied on the combination of complex media and cell coculture [116]. The need to understand the real requirements of embryos in the course of their early in vitro preimplantation development has brought the attention to the implementation of defined media, which are still today the elective choice [117]. Additionally, since the oviduct represents the only natural milieu where the initial development of the embryos occurs, an understanding of the molecular environment present at the time of embryo presence in the oviduct may well give the hint for the correct adjustments to make in the media used for embryo culture. With this aim, some differences between buffaloes and cattle have been found, following analysis of the oviductal environment in the two species. In fact, the total amount of proteins and their concentration was reported to be lower in buffaloes when compared to cattle, suggesting thus to reduce their level in the formulation for media culture [118]. One of the strategies adopted in cattle in order to reduce ammonium concentration, toxic catabolites, and free radicals in medium for embryo culture, known to be potentially involved in the large offspring syndrome, is to periodically change the same medium during in vitro culture. In contrast, not only this methodology does not improve buffalo embryo development, but on the contrary in our experience it seems that better results are achieved if a minimum amount of manipulation is made on embryos prior to the final evaluation. This is possibly due to the involvement of other variables more likely to affect embryo development during manipulation such as temperature and pH fluctuation. Another significant difference to other domestic ruminants for embryo energy requirement during culture is the need for higher amount of glucose especially in the course of buffalo early development [119,120]. The addition into culture media of cysteamine, taurine, melatonin, or by lowering O_2 concentration, has increased the quality of late-stage embryo development in buffaloes. Such supplementation into culture media has in fact the merit to overcome the oxidative stress in the course of embryo culture as evidenced by a higher expression of antiapoptotic genes in the embryos [121]. Higher quality of embryo development together with increased cryotolerance has been proved successful by inclusion into media of L-carnitine, hyaluronic acid, and leukemia inhibitory factor [122–124].

6.8 Oocyte and embryo cryopreservation

The possibility to store oocytes, similarly to sperm cells, through cryopreservation procedures will ensure the availability of both germ cells whenever wanted and needed for the implementation of in vitro reproductive technologies. The feasibility of cryopreserving buffalo oocytes is of particular interest, as they could be more efficiently harvested and made available in a more suitable manner whenever needed, considering their intrinsic reduced number at birth when compared to cattle [26]. The technology is still far from being commercially viable in all species where it has been tested, and despite the challenges faced due to the injuries received by the oocytes during the cryopreservation procedure, live offspring have been reported in a number of species including humans [107]. Both conventional slow freezing and vitrification procedures on immature oocytes have been characterized by low efficiency in terms of embryo production, due possibly to low permeability of plasma membrane and hence higher sensitivity of chilling injuries. The attention has then shifted to the use of matured oocytes in parallel to an improvement of vitrification technology, characterized by the adoption of very small volume of media and direct contact with liquid nitrogen, such as Cryoloop, Cryotop vitrification, and solid-state vitrification, characterized by a very fast cooling and warming rate, allowing to reduce the concentration of cryoprotectants. Among these vitrification methods, Cryotop proved to be probably the most efficient for in vitro matured bovine and human oocytes [125] and later was successfully used for Swamp and River buffaloes oocytes in IVF [126,127] and nuclear transfer trials [117]. Cleavage rate can be significantly increased by removing granulosa cells from matured oocytes prior to vitrification, followed by coculturing rewarmed oocytes with intact COCs, although late-stage embryo development still remains low [128].

Vitrification has also been the method of choice for cryopreservation of buffalo embryos, and live offspring have been reported from such technology in this species [81,82,85,129]. The importance and the interplay of many variables on the viability of cryopreserved embryos has been amply demonstrated in all animal species. In the buffalo species, cryotolerance is also affected by in vitro maturation and culture conditions, as well as the source of oocytes [130]. It has also been documented in buffaloes that faster developing embryos are more viable and more adequately face cryopreservation procedures as indicated by increased blastocoele re-expansion and hatching [115], and pregnancy rate to term [81]. An additional and possibly a more critical component in buffaloes is represented by the degree of synchrony to be reached between stage of embryo development and recipients, which according to Kasiraj et al. [131] should not exceed 12 hours in the case of in vivo produced embryos cryopreserved by conventional slow freezing, suggesting therefore a need for a more stringent synchrony in case of in vitro produced and vitrified embryos. In addition, it has recently been reported that during the nonbreeding season the cryotolerance of in vitro produced buffalo embryos is negatively affected, possibly due to a reduced oocyte developmental competence [132]. The relatively paucity of information, in comparison to cattle, and the difference among protocols followed by researchers make more difficult to lucidly delineate a clear indication toward the most appropriate and efficient protocol to be used for embryo cryopreservation. In recent years though two different trials have given far better results than ever before by the combination of IVEP, vitrification, and fixed-time embryo transfer into synchronized recipients [85,133], introducing thus a realistic possibility to put this technology into practice for the benefit of buffalo production and genetic improvement.

6.9 More recently developed technologies

The reproductive technologies described so far are instrumental for the development of newly strategic technological steps, for both dissemination of genetic superior animals and/or the introduction of genetic modifications leading to a faster genetic progression or the creation of new genetic lines of animals, for the betterment of animal production and human welfare. Cloning and transgenesis are the relatively new methodologies developed to the scope and applied with varying degrees of efficiency and success across animal species. Somatic cell nuclear transfer (SCNT) is the laboratory procedure meant to clone an individual animal by de-differentiating a somatic cell through nuclear reprogramming. Through the somatic cell methodology though, the first report came from the use of fetal fibroblasts to produce cloned swamp buffalo embryos [134]. Later studies have shown that the expression level of some genes and the epigenetic status can be affected by the sex of the cloned embryos [135]. Within the needed laboratory passages to create a clone, both fertilized zygotes and two-cell embryos can be used as recipient cells [136], and in buffaloes oocytes at metaphase II are ordinarily employed in the process of SCNT as cytoplasmic substrate for cloned embryos production and successful birth of calves [137]. Following preparation of donor and recipient cells, fusion in buffaloes is usually achieved by one or two DC electric pulses [134], and similarly to cattle, a number of successful activation protocols are employed, especially if performed few hours after fusion [138]. Variations of the well-established SCNT cloning protocols, such as the hand-made cloning have also been attempted in buffaloes and pregnancies have been reported [139], together with the birth of calves following vitrification and rewarming of embryos resulting from adult, newborn, and fetal fibroblast donor cells [140]. Lately, nonviable fibroblast from buffalo skin was used for SCNT and embryo production, showing the possibility to use altered nonviable cells for cloning as a possible strategy for rescuing and preserving endangered species [141]. The viability of recently produced cloned buffaloes has also been linked to the DNA methylation pattern and expression of imprinted genes [142]. Adult somatic cells, to be used as donor cells for nuclear transfer, can be induced to be reprogrammed into pluripotent stem cells (iPS) and therefore being characterized by an embryonic stem cell-like state. In buffaloes, recently some attempts have been made in this direction in improving the reprogramming efficiency and the production of iPS [143].

The use of SCNT into the production of transgenic animals has significant higher potential advantages when compared to other approaches. Although transgenic animals have been produced in a number of species, in buffaloes to date, only transgenic embryos have been produced by (1) transfecting buffalo oviductal epithelial cells with enhanced green fluorescent protein followed by transfer into enucleated oocytes [144] and (2) by transfer of embryonic germ-like cells expressing the green fluorescent protein into in vitro–derived buffalo blastocysts [145]. The efficiency of transgenic integration into cloned embryos is being currently pursued by, for example, optimizing electroporation conditions for integration into fetal fibroblast to be used as donor cells [146].

6.10 Conclusion

In the last few decades, the domestic buffalo has greatly benefitted by the implementation of reproductive technologies toward the enhancement of genetic improvement and production. It all started by overcoming the initial difficulties in applying AI to the animals, and the common prejudice of most farmers against the introduction of emerging technologies in a species characterized by evident rustic behavior and a seasonal reproductive performance. Significant progress has been made over the intervening years as clearly highlighted in this review, and we have now a stronger springboard toward higher achievements in buffalo production.

References

[1] Thanh VTK, Orskov ER. Protein digestion and metabolism in buffalo ARSIAL – Regione Lazio, Rome, Italy In: Presicce GA, editor. The buffalo (*Bubalus bubalis*): production and research. Bentham Science Publishers Ltd; 2017. p. 180–95.

[2] Abdullah N, Ho YW, Mahayuddin M, Jalaludin S. Comparative studies of fibre digestion in cattle and buffaloes. In: Proceedings of domestic buffalo production in Asia. Rockhampton, Australia: IAEA; 1989. p. 75–87.

[3] Raghavan GV. La produzione della carne nella specie bufalina. In: Ferrara B, et al., editors. Proceedings II Conv. Internaz. "Allevamento bufalino nel mondo". 1982. p. 620–34.

[4] Bartocci S, Amici A, Verna M, Terramoccia S, Martillotti F. Solid and fluid passage rate in buffalo, cattle and sheep feds diets with different forage to concentrate ratios. Livest Prod Sci 1997;52:201–8.

[5] Zicarelli L. Nutrition in dairy buffaloes. Bubalus bubalis 2001;1–66.

[6] Zicarelli L. Influence of seasonality on buffalo production ARSIAL – Regione Lazio, Rome, Italy In: Presicce GA, editor. The buffalo (*Bubalus bubalis*): production and research. Bentham Science Publishers Ltd; 2017. p. 196–224.

[7] Mingala CN, Villanueva MA, Cruz L. River and swamp buffaloes: history, distribution and their characteristics ARSIAL – Regione Lazio, Rome, Italy In: Presicce GA, editor. The buffalo (*Bubalus bubalis*): production and research. Bentham Science Publishers Ltd; 2017. p. 3–31.

[8] Zicarelli L. Enhancing reproductive performance in domestic dairy water buffalo (*Bubalus bubalis*). Soc Reprod Fertil Suppl 2010;67:443–55.

[9] Zicarelli L. Management of buffaloes. In: Proceedings of world Buiatrics congress, Santiago, Chile; 2010, p. 326–42.

[10] Borghese A, Mazzi M. Buffalo population and strategies in the world. In: Buffalo production and strategies. FAO Regional Office for Europe, REU Technical Series 67; 2005. p. 1–39.

[11] Calabrò S, Cutrignelli MI, Gonzalez OJ, Chiofalo B, Grossi M, Tudisco R, et al. Meat quality of buffalo young bulls fed baba bean as protein source. Meat Sci 2014;96:591–6.

[12] Giordano G, Guarini P, Ferrari P, Biondi-Zoccai G, Schiavone B, Giordano A. Beneficial impact on cardiovascular risk profile of water buffalo meat consumption. Eur J Clin Nutr 2010;64:1000–6.

[13] Murthy TRK, Prince Devadason I. Buffalo meat and meat products – an overview. In: Proceedings of the fourth Asian buffalo congress, New Delhi, India, vol. 28; 2003. p. 194–99.

[14] Di Berardino D, Di Meo GP, Gallagher DS, Hayes H, Iannuzzi L. ISCNDB2000—international system for chromosome nomenclature of domestic bovids. Cytogenet Cell Genet 2001;92:283–99.

[15] Di Berardino D, Iannuzzi L. Chromosome banding homologies in Swamp and Murrah buffalo. J Hered 1981;72(3):183–8 [PMID: 6168678].

[16] Wurster DH, Benirschke K. Chromosome studies in the superfamily Bovoidea. Chromosoma 1968;25(2):152–71 [PMID: 5709393]. <https://doi.org/10.1007/BF00327175>.

[17] Michelizzi VN, Dodson MV, Pan Z, et al. Water buffalo genome science comes of age. Int J Biol Sci 2010;6(4):333–49 [PMID: 20582226]. <https://doi.org/10.7150/ijbs.6.333>.

[18] Iamartino D, Williams JL, Sonstegard T, Reecy J, Van Tassell C, Nicolazzi AL, et al. The buffalo genome and the application of genomics in animal management and improvement. In: The 10th world buffalo congress and the 7th Asian buffalo congress, Phuket, Thailand; 2013. p. 151–58.

[19] Li J, Liang A, Li Z, Du C, Hua G, Salzano A, et al. An association analysis between PRL genotype and milk production traits in Italian Mediterranean river buffalo. J Dairy Res 2017;84(4):430–3.

[20] Li J, Liu J, Liu S, Plastow G, Zhang C, Wang Z, et al. Integrating RNA-seq and GWAS reveals novel genetic mutations for buffalo reproductive traits. Anim Reprod Sci 2018;197:290–5.

[21] Pauciullo A, Iannuzzi L. Molecular genetics and selection in dairy buffaloes: the Italian situation ARSIAL – Regione Lazio, Rome, Italy In: Presicce GA, editor. The buffalo (*Bubalus bubalis*): production and research. Bentham Science Publishers Ltd; 2017. p. 50–68.

[22] Presicce GA. Reproduction in the water buffalo. Reprod Domest Anim 2007;42(Suppl. 2):24–32.

[23] Campanile G, Neglia G, D'Occhio MJ. Embryonic and fetal mortality in river buffalo (*Bubalus bubalis*). Theriogenology 2016;86:207–13.

[24] Peeva R, Ilieva Y. Longevity of buffalo cows and reasons for their culling. Ital J Anim Sci 2007;2007(6):378–80. Available from: https://doi.org/10.4081/ijas.2007.s2.378 (sup2).

[25] Manik RS, Palta P, Singla SK, Sharma V. Folliculogenesis in buffalo (*Bubalus bubalis*): a review. Reprod Fertil Dev 2002;14(5–6):315–25.

[26] Baruselli PS, Presicce GA. Folliculogenesis and ovarian physiology applied to reproductive biotechnologies in buffaloes ARSIAL – Regione Lazio, Rome, Italy In: Presicce GA, editor. The buffalo (*Bubalus bubalis*): production and research. Bentham Science Publishers Ltd; 2017. p. 313–39.

[27] Nair SJ, Brar AS, Ahuja CS, Sangha SP, Chaudhary KC. A comparative study on lipid peroxidation, activities of antioxidant enzymes and viability of cattle and buffalo bull spermatozoa during storage at refrigeration temperature. Anim Reprod Sci 2006;96:21–9.
[28] Kumaresan A, Ansari MR, Garg A. Modulation of post-thaw sperm functions with oviductal proteins in buffaloes. Anim Reprod Sci 2005;90:73–84.
[29] Kumaresan A, Ansari MR, Garg A, Kataria M. Effect of oviductal proteins on sperm functions and lipid peroxidation levels during cryopreservation in buffaloes. Anim Reprod Sci 2006;93:246–57.
[30] Sansone G, Nastri MJF, Fabbrocini A. Storage of buffalo (*Bubalus bubalis*) semen. Anim Reprod Sci 2000;62:55–76.
[31] de Carvalho NA, Soares JG, Baruselli PS. Strategies to overcome seasonal anestrus in water buffalo. Theriogenology 2016;86(1):200–6. Available from: https://doi.org/10.1016/j.theriogenology.2016.04.032.
[32] Campanile G, Neglia G, Di Palo R, Gasparrini B, Pacelli C, D'Occhio MJ, et al. Relationship of body condition score and blood urea and ammonia to pregnancy in Italian Mediterranean buffaloes. Reprod Nutr Dev 2006;46:57–62.
[33] Zicarelli L, Esposito L, Campanile G, Di Palo R, Armstrong AR. Effect of using vasectomized bulls in A.I. practice on the reproductive efficiency of Italian buffalo cows. Anim Reprod Sci. 1997;47:171–80.
[34] Seren E. Periestrous endocrine changes in Italian buffaloes. Agricoltura e Ricerca 1994;17–24.
[35] Zicarelli L, Campanile G, Infascelli F, Esposito L, Ferrari G Incidence and fertility of heats with double ovulations in the buffalo cows. In: Proceedings of the II world buffalo congress, New Delhi, India, vol. 3; 1988. p. 57–62.
[36] Barile VL, Terzano GM, Pacelli C, Todini L, Malfatti A, Barbato O. LH peak and ovulation after two different estrus synchronization treatments in buffalo cows in the daylight-lengthening period. Theriogenology 2015;84:286–93. Available from: https://doi.org/10.1016/j.theriogenology.2015.03.019 Mar. pii: S0093-691X(15)00146-6.
[37] Pursley JR, Mee MO, Wiltbank MC. Synchronization of ovulation in dairy cows using PGF2a and GnRH. Theriogenology 1995;44:915–23.
[38] De Araujo Berber RC, Madureira EH, Baruselli PS. Comparison of two Ovsynch protocols (GnRH versus LH) for fixed timed insemination in buffalo (*Bubalus bubalis*). Theriogenology 2002;57:1421–30.
[39] Neglia G, Gasparrini B, Di Palo R, De Rosa C, Zicarelli L, Campanile G. Comparison of pregnancy rates with two estrus synchronization protocols in Italian Mediterranean buffalo cows. Theriogenology 2003;60:125–33.
[40] De Rensis F, Ronci G, Guarneri P, Nguyen BX, Presicce GA, Huszenicza G, et al. Conception rate after fixed time insemination following Ovsynch protocol with and without progesterone supplementation in cyclic and noncyclic Mediterranean Italian buffaloes (*Bubalus bubalis*). Theriogenology 2005;63:1824–31.
[41] Neglia G, Gasparrini B, Salzano A, Vecchio D, De Carlo E, Cimmino R, et al. Relationship between the ovarian follicular response at the start of an Ovsynch-TAI program and pregnancy outcome in the Mediterranean river buffalo. Theriogenology 2016;86(9):2328–33. Available from: https://doi.org/10.1016/j.theriogenology.2016.07.027.
[42] Rossi P, Vecchio D, Neglia G, Di Palo R, Gasparrini B, D'Occhio MJ, et al. Seasonal fluctuations in the response of Italian Mediterranean buffaloes to synchronization of ovulation and timed artificial insemination. Theriogenology 2014;82(1):132–7. Available from: https://doi.org/10.1016/j.theriogenology.2014.03.005.
[43] Neglia G, Restucci B, Russo M, Vecchio D, Gasparrini B, Prandi A, et al. Early development and function of the corpus luteum and relationship to pregnancy in the buffalo. Theriogenology 2015;83(6):959–67. Available from: https://doi.org/10.1016/j.theriogenology.2014.11.035.
[44] Presicce GA, Verberckmoes S, Senatore EM, Rath D. First established pregnancies in Mediterranean Italian buffaloes (*Bubalus bubalis*) following deposition of sexed spermatozoa near the utero tubal junction. Reprod Domest Anim 2005;73–5.
[45] Lu YQ, Liang XW, Zhang M, Wang WL, Kitiyanant Y, Lu SS, et al. Birth of twins after in vitro fertilization with flow-cytometric sorted buffalo (*Bubalus bubalis*) sperm. Anim Reprod Sci 2007;100(1–2):192–6.
[46] Lu Y, Zhang M, Lu S, Xu D, Huang W, Meng B, et al. Sex-preselected buffalo (*Bubalus bubalis*) calves derived from artificial insemination with sexed sperm. Anim Reprod Sci 2010;119(3–4):169–71. Available from: https://doi.org/10.1016/j.anireprosci.2010.01.001.
[47] Liang XW, Lu YQ, Chen MT, Zhang XF, Lu SS, Zhang M, et al. In vitro embryo production in buffalo (*Bubalus bubalis*) using sexed sperm and oocytes from ovum pick up. Theriogenology 2008;69(7):822–6. Available from: https://doi.org/10.1016/j.theriogenology.2007.11.021.
[48] Campanile G, Gasparrini B, Vecchio D, Neglia G, Senatore EM, Bella A, et al. Pregnancy rates following AI with sexed semen in Mediterranean Italian buffalo heifers (*Bubalus bubalis*). Theriogenology 2011;76(3):500–6. Available from: https://doi.org/10.1016/j.theriogenology.2011.02.029.
[49] Barile VL, Pacelli C, De Santis G, Baldassi D, Mazzi M, Terzano GM. Use of commercial available bovine semen sexing agent in buffalo: preliminary report of the effect on the conception rate. In: The 10th world buffalo congress and the 7th Asian buffalo congress, May 6–8, 2013; p. 52.
[50] Underwood SL, Bathgate R, Maxwell WM, Evans G. Birth of offspring after artificial insemination of heifers with frozen-thawed, sex-sorted, re-frozen-thawed bull sperm. Anim Reprod Sci 2010;118(2–4):171–5. Available from: https://doi.org/10.1016/j.anireprosci.2009.08.007.
[51] Seidel Jr1 GE. Update on sexed semen technology in cattle. Animal 2014;8(Suppl. 1):160–4. Available from: https://doi.org/10.1017/S1751731114000202.
[52] Leahy T, Marti JI, Crossett B, Evan G, Maxwell WM. Two-dimensional polyacrylamide gel electrophoresis of membrane proteins from flow cytometrically sorted ram sperm. Theriogenology 2011;75(5):962–71. Available from: https://doi.org/10.1016/j.theriogenology.2010.11.003.
[53] Drost M, Wright Jr JM, Cripe WS, Richter AR. Embryo transfer in water buffalo (*Bubalus bubalis*). Theriogenology 1983;20:579–84.
[54] Karaivanov C. Comparative studies on the superovulatory effect of PMSG and FSH in water buffalo (*Bubalus bubalis*). Theriogenology 1986;26:51–9.

[55] Singla SK, Madan ML. Response of superovulation in buffaloes (*Bubalus bubalis*) with Super-OV and FSH-P. Theriogenology 1990;33:327.
[56] Misra AK, Tyagi S. *In vivo* embryo production in buffalo: present and perspectives. Ital J Anim Sci 2007;6(Suppl. 2):74–91 <https://doi.org/10.4081/ijas.2007.s2.74>.
[57] Bó GA, Adams GP, Pierson RA, Mapletoft RJ. Exogenous control of follicular wave emergence in cattle. Theriogenology 1995;43:31–40 <https://doi.org/10.1016/0093-691X(94)00010-R>.
[58] Zicarelli L, Boni R, Campanile G, et al. Effect of OPU priming on superovulatory response in Italian buffalo cows. In: 5th world buffalo conference, Royal Palace, Caserta, Italy; October 1997. p. 13–6.
[59] Palta P, Kumar M, Jailkhani S, Manik RS, Madan ML. Changes in peripheral inhibin levels and follicular development following treatment of buffalo with PMSG and neutra-PMSG for superovulation. Theriogenology 1997;48(2):233–40 [PMID: 16728122]. <https://doi.org/10.1016/S0093-691X(97)84070-8>.
[60] Matharoo JS, Cheema S, Ranjna SJ, Mehar HMC, Tiwana MS. Use of hetero and homologous anti-PMSG with PMSG for superovulation and embryo recovery in buffaloes. In: Proceedings of the 5th world buffalo congress, Caserta; 13–16 October, 1997. p. 754–57.
[61] Situmorang P. Superovulation in different buffalo genotypes. Indones J Anim Vet Sci 2003;8:40–5.
[62] Baruselli PS, Mucciolo RG, Arruda R, Madureira RH, Amaral R, Assumpcao ME. Embryo recovery rate in superovulated buffaloes. Theriogenology 1999;51:401 <https://doi.org/10.1016/S0093-691X(99)91960-X>.
[63] Carvalho NA, Baruselli PS, Zicarelli L, Madureira EH, Visintin JA, DOcchio MJ. Control of ovulation with a GnRH agonist after superstimulation of follicular growth in buffalo: fertilization and embryo recovery. Theriogenology 2002;58(9):1641–50 [PMID: 12472135]. <https://doi.org/10.1016/S0093-691X(02)01057-9>.
[64] Boni R, Roviello S, Gasparrini B, Zicarelli L. Pregnancies established after transferring embryos yielded by ovum pick-up and *in vitro* embryo production in Italian buffalo cows. In: Proceedings of V world buffalo congress, Caserta, Italia; 1997. p. 787–92.
[65] Madan ML, Singla SK, Singh C, Prakash BS, Jailkhani S. Embryo transfer technology in buffaloes: endocrine responses and limitations. In: Proceeding of II world buffalo congress, New Delhi, vol. III; 1988. p. 195–211.
[66] Neglia G, Gasparrini B, Vecchio D, et al. Progesterone supplementation during multiple ovulation treatment in buffalo species (*Bubalus bubalis*). Trop Anim Health Prod 2010;42(6):1243–7 [PMID: 20411328]. <https://doi.org/10.1007/s11250-010-9556-8>.
[67] Zicarelli L, Di Palo R, Palladino M, Campanile G, Esposito L. Embryo transfer in Mediterranean *Bubalus bubalis*. In: Proceeding of the international symposium "prospects of buffalo production in the Mediterranean and the Middle East," Cairo, Egypt; 1993. p. 73–5.
[68] Zicarelli L, Boni R, Campanile G, Roviello S, Gasparrini B, Di Palo R. Response to superovulation according to parity, OPU priming, presence/absence of dominant follicle and season. In: V world buffalo congress, Caserta, Italy; 1997. p. 748–53.
[69] Baruselli PS, Madureira EH, Visintin JA, Barnabe VH, Barnabe RC, Amaral R. Inseminação artificial em tempo fixo com sincronização da ovulação em bubalinos. Rev Bras Reprod Anim 1999;23:360–2.
[70] Di Palo R, Campanile G, Esposito L, Barbieri V, Zicarelli L. Characteristics of diet and FSH-p superovulatory response in Italian Mediterranean buffaloes. In: Proceedings of IV world buffalo congress, Sao Paolo; 1994. p. 474–76.
[71] Zicarelli L, Di Palo R, Campanile G, Boni, R, Langella M. rBST + FSH-P in superovulatory treatment of Italian Mediterranean buffaloes. In: Proceedings of IV world buffalo congress, Sao Paolo; 1994. p. 459–461.
[72] Songsasen N, Yiengvisavakul V, Buntaracha B, Pharee S, Apimeteetumrong M, Sukwongs Y. Effect of treatment with recombinant bovine somatotropin on responses to superovulatory treatment in swamp buffalo (*Bubalus bubalis*). Theriogenology 1999;52(3):377–84 [PMID: 10734373]. <https://doi.org/10.1016/S0093-691X(99)00136-3>.
[73] Li DR, Qin GS, Wei YM, et al. Immunisation against inhibin enhances follicular development, oocyte maturation and superovulatory response in water buffaloes. Reprod Fertil Dev 2011;23(6):788–97 [PMID: 21791180]. <https://doi.org/10.1071/RD10279>.
[74] Neglia G, Natale A, Esposito G, et al. Effect of prostaglandin F2α at the time of AI on progesterone levels and pregnancy rate in synchronized Italian Mediterranean buffaloes. Theriogenology 2008;69(8):953–60 [PMID: 18346780]. <https://doi.org/10.1016/j.theriogenology.2008.01.008>.
[75] Zicarelli L. Advanced reproductive technologies for improving buffalo production. In: Proceedings of the first buffalo symposium of Americas, Estacao das Docas, Belèm, Parà, Brazil; 2002. 186–97.
[76] Misra AK, Kasiraj R, Mutha Rao M, Rangareddy NS, Jaiswal RS, Pant HC. Rate of transport and development of preimplantation embryo in the superovulated buffalo (*Bubalus bubalis*. Theriogenology 1998;50:637–49.
[77] Baruselli PS, Madureira EH, Visintin JA, Porto-Filho R, Carvalho NAT, Campanile G, et al. Failure of oocyte entry into oviduct in superovulated buffalo. Theriogenology 2000;53:491.
[78] Salzano A, De Canditiis C, Della Ragione F, Prandi A, Zullo G, Neglia G, et al. Evaluation of factors involved in the failure of ovum capture in superovulated buffaloes. Theriogenology 2018;122:102–8.
[79] Fukuda Y, Ichikawa M, Naito K, Toyoda Y. Birth of normal calves resulting from bovine oocytes, matured, fertilized, and cultured with cumulus cells in vitro up to the blastocyst stage. Biol Reprod 1990;42:114–19.
[80] Boni R, Roviello S, Zicarelli L. Repeated ovum pick-up in Italian Mediterranean buffalo cows. Theriogenology 1996;46(5):899–909 [PMID: 16727954]. <https://doi.org/10.1016/S0093-691X(96)00248-8>.
[81] Neglia G, Gasparrini B, Caracciolo di Brienza V, Di Palo R, Zicarelli L. First pregnancies to term after transfer of buffalo vitrified embryos entirely produced *in vitro*. Vet Res Commun 2004;28:233–326 [PMID: 15372965]. <https://doi.org/10.1023/B:VERC.0000045414.65331.6a>.
[82] Hufana-Duran D, Pedro PB, Venturina HV, Hufana RD, Salazar AR, Duran PG, et al. Post-warming hatching and birth of live calves following transfer of *in vitro*-derived vitrified water buffalo (*Bubalus bubalis*) embryos. Theriogenology 2004;61(7–8):1429–39 [PMID: 15036974]. <https://doi.org/10.1016/j.theriogenology.2003.08.011>.

[83] Huang Y, Zhuang X, Gasparrini B, Presicce GA. Oocyte recovery by ovum pick-up and embryo production in Murrah and Nili-Ravi buffaloes (*Bubalus bubalis*) imported in China. Reprod Fertil Dev 2005;17(2):273.

[84] Boccia L, Di Francesco S, Neglia G, De Blasi M, Longobardi V, Campanile G, et al. Osteopontin improves sperm capacitation and in vitro fertilization efficiency in buffalo (*Bubalus bubalis*). Theriogenology 2013;80(3):212−17.

[85] Saliba W, Gimenes L, Drumond R, Bayao H, Alvim M, Baruselli P, et al. Efficiency of OPU-IVEP-ET of fresh and vitrified embryos in buffaloes. 10th World Buffalo Congress, the 7th Asian Buffalo congress, May 6−8, Phuket, Thailand: 10. Buffalo Bull, 2013;32(Special issue 2):385−88.

[86] Presicce GA, Senatore EM, De Santis G, Stecco R, Terzano GM, Borghese A, et al. Hormonal stimulation and oocyte maturational competence in prepuberal Mediterranean Italian buffaloes (*Bubalus bubalis*). Theriogenology 2002;57:1877−84.

[87] Baldassarre H, Bordignon V. Laparoscopic ovum pick-up for in vitro embryo production from dairy bovine and buffalo calves. In: Proceedings of the 32nd annual meeting of the Brazilian Embryo Technology Society (SBTE), Florianopólis, SC, Brazil; August 16th−18th, 2018. doi: 10.21451/1984−3143-AR2018-0057.

[88] Gasparrini B, Neglia G, Di Palo R, Vecchio D, Albero G, Esposito L, et al. Influence of oocyte donor on in vitro embryo production in buffalo. Anim Reprod Sci 2014;144(3−4):95−101. Available from: https://doi.org/10.1016/j.anireprosci.2013.11.010.

[89] Campanile G, Baruselli PS, Neglia G, et al. Ovarian function in the buffalo and implications for embryo development and assisted reproduction. Anim Reprod Sci 2010;121(1−2):1−11 [PMID: 20430540]. <https://doi.org/10.1016/j.anireprosci.2010.03.012>.

[90] Di Francesco S, Boccia L, Campanile G, Di Palo R, Vecchio D, Neglia G, et al. The effect of season on oocyte quality and developmental competence in Italian Mediterranean buffaloes (*Bubalus bubalis*). Anim Reprod Sci 2011;123(1−2):48−53 PMID: 21168984.

[91] Di Francesco S, Novoa MV, Vecchio D, et al. Ovum pick-up and *in vitro* embryo production (OPUIVEP) in Mediterranean Italian buffalo performed in different seasons. Theriogenology 2012;77(1):148−54 [PMID: 21872310]. <https://doi.org/10.1016/j.theriogenology.2011.07.028>.

[92] Gasparrini B. In vitro embryo production in buffalo species: state of the art. Theriogenology 2002;57:237−56.

[93] Misra V, Misra AK, Sharma R. Effect of ambient temperature on *in vitro* fertilization of bubaline oocyte. Anim Reprod Sci 2007;100 (3−4):379−84 [PMID: 17125942]. <https://doi.org/10.1016/j.anireprosci.2006.10.020>.

[94] Boni R, Di Palo R, Barbieri V, Zicarelli L. Ovum pick-up in deep anestrus buffaloes. In: Proceedings of the 1994 IV world buffalo congress, Sao Paolo, Brazil; 1994. p. 480−82.

[95] Sá Filho MF, Carvalho NA, Gimenes LU, et al. Effect of recombinant bovine somatotropin (bST) on follicular population and on *in vitro* buffalo embryo production. Anim Reprod Sci 2009;113(1−4):51−9 [PMID: 18691835]. <https://doi.org/10.1016/j.anireprosci.2008.06.008>.

[96] Gasparrini B, De Rosa A, Attanasio L, et al. Influence of the duration of *in vitro* maturation and gamete co-incubation on the efficiency of *in vitro* embryo development in Italian Mediterranean buffalo (*Bubalus bubalis*). Anim Reprod Sci 2008;105(3−4):354−64 [PMID: 17481834]. <https://doi.org/10.1016/j.anireprosci.2007.03.022>.

[97] Pawshe CH, Appa Rao KBC, Totey SM. Effect of insulin-like growth factor I and its interaction with gonadotrophins on in vitro maturation and embryonic development, cell proliferation, and biosynthetic activity of cumulus-oocytes complexes and granulosa cells in buffalo. Mol Reprod Dev 1998;49:277−85.

[98] Chauhan MS, Singla SK, Palta P, Manik RS, Madan ML. Effect of epidermal growth factor on the cumulus expansion, meiotic maturation and development of buffalo oocytes in vitro. Vet Ret 1999;144:266−7.

[99] Nandi S, Ravindranatha BM, Gupta PS, Raghu HM, Sarma PV. Developmental competence and post-thaw survivability of buffalo embryos produced in vitro: effect of growth factors in oocyte maturation medium and of embryo culture system. Theriogenology 2003;60(9):1621−31.

[100] Gasparrini B, Neglia G, Di Palo R, Campanile G, Zicarelli L. Effect of cysteamine during in vitro maturation on buffalo embryo development. Theriogenology 2000;54:1537−42.

[101] Gasparrini B, Sayoud H, Neglia G, Matos DG, Donnay I, Zicarelli L. Glutathione synthesis during *in vitro* maturation of buffalo (*Bubalus bubalis*) oocytes: effects of cysteamine on embryo development. Theriogenology 2003;60(5):943−52 [PMID: 12935871]. <https://doi.org/10.1016/S0093-691X(03)00098-0>.

[102] Gasparrini B, Boccia L, Marchandise J, Di Palo R, George F, Donnay I, et al. Enrichment of in vitro maturation medium for buffalo (*Bubalus bubalis*) oocytes with thiol compounds: effects of cystine on glutathione synthesis and embryo development. Theriogenology 2006;65 (2):275−87.

[103] Manjunatha BM, Devaraj M, Gupta PSP, Ravindra JP, Nandi S. Effect of taurine and melatonin in the culture medium on buffalo in vitro embryo development. Reprod Domest Anim 2009;44(1):12−16.

[104] Pandey A, Gupta N, Gupta SC. Improvement of *in vitro* oocyte maturation with lectin supplementation and expression analysis of Cx43, GDF-9, FGF-4 and fibronectin mRNA transcripts in buffalo (*Bubalus bubalis*). J Assist Reprod Genet 2009;26(6):365−71 [PMID: 19629675]. <https://doi.org/10.1007/s10815-009-9314-x>.

[105] Wilding M, Gasparrini B, Neglia G, Dale B, Zicarelli L. Mitochondrial activity and fertilization potential of fresh and cryopreserved buffalo sperm. Theriogenology 2003;59:466.

[106] Boccia L, Di Palo R, De Rosa A, Attanasio L, Mariotti E, Gasparrini B. Evaluation of buffalo semen by Trypan blue/Giemsa staining and related fertility *in vitro*. 8th World Buffalo Congress, Caserta, Italy October 19−22, 2007. Ital J Anim Sci 2007;6(2):739−42 <https://doi.org/10.4081/ijas.2007.s2.739>.

[107] Gasparrini B. Applied reproductive technologies in the buffalo species ARSIAL − Regione Lazio, Rome, Italy In: Presicce GA, editor. The buffalo (*Bubalus bubalis*): production and research. Bentham Science Publishers Ltd; 2017. p. 374−433.

[108] Boccia L, Attanasio L, Monaco E, De Rosa A, Di Palo R, Gasparrini B. Effect of progesterone on capacitation of buffalo (*Bubalus bubalis*) spermatozoa *in vitro*. Reprod Domest Anim 2006;41(4):311.

[109] Boccia L, Attanasio L, De Rosa A, Pellerano G, Di Palo R, Gasparrini B. Effect of sodium nitroprusside on buffalo sperm capacitation *in vitro*. Reprod Fertil Dev 2007;19:276 <https://doi.org/10.1071/RDv19n1Ab322>.

[110] Di Francesco S, Mariotti E, Tsantarliotou M, et al. Melatonin promotes *in vitro* sperm capacitation in buffalo (*Bubalus bubalis*). Proceedings of 36th annual conference of the International Embryo Transfer Society, 9–12 January, Cordoba, Argentina. Reprod Fertil Dev 2010;22 (1):311–12. <https://doi.org/10.1071/RDv22n1Ab311>.

[111] Pero ME, Killian GJ, Lombardi P, Zicarelli L, Avallone L, Gasparrini B. Identification of osteopontin in water buffalo semen. Reprod Fertil Dev 2007;19:279.

[112] Siniscalchi C, Mariotti E, Boccia L, et al. Co-culture with oviduct epithelial cells promotes capacitation of buffalo (*Bubalus bubalis*) sperm. Proceedings of 36th Annual Conference of the International Embryo Transfer Society, 9–12 January, Cordoba, Argentina. J Reprod Fertil Dev 2010;22(1):316. <https://doi.org/10.1071/RDv22n1Ab321>.

[113] Rubessa M, Di Fenza M, Mariotti E, Di Francesco S, De Dilectis C, Di Palo R, et al. Kinetics of sperm penetration is correlated with in vitro fertility of buffalo (*Bubalus bubalis*) bulls. Reprod Fertil Dev 2009;21(I):206–7.

[114] Verma A, Kumar P, Rajput S, Roy B, De S, Datta TK. Embryonic genome activation events in buffalo (*Bubalus bubalis*) preimplantation embryos. Mol Reprod Dev 2012;79(5):321–8 [PMID: 22461405]. <https://doi.org/10.1002/mrd.22027>.

[115] Gasparrini B, Neglia G, Caracciolo, di Brienza V, Campanile G, Di Palo R, et al. Preliminary analysis of vitrified *in vitro* produced embryos. Theriogenology 2001;55:307.

[116] Boni R, Roviello S, Gasparrini B, Langella M, Zicarelli L. *In vitro* production of buffalo embryos in chemically defined medium. Buffalo J 1999;15:115–20.

[117] Caracciolo di Brienza V, Neglia G, Masola N, Gasparrini B, Di Palo R, Campanile G. In vitro embryo production in chemically defined media. In: Proceedings of 1° Congresso Nazionale sull'Allevamento del Bufalo; 2001. p. 341–44.

[118] Vecchio D, Neglia G, Di Palo R, et al. Ion, protein, phospholipid and energy substrate content of oviduct fluid during the oestrous cycle of buffalo (*Bubalus bubalis*). Reprod Domest Anim 2010;45(5):e32–9 [PMID: 19761531].

[119] Monaco E, De Rosa A, Attanasio L, Boccia L, Zicarelli L, Gasparrini B. *In vitro* culture of buffalo (*Bubalus bubalis*) embryos in the presence or absence of glucose. Reprod Domest Anim 2006;41(4):332.

[120] Suárez Novoa MV, Di Francesco S, Rubessa M, Boccia L, Longobardi V, De Blasi M, et al. Effect of reducing glucose concentration during in vitro embryo culture in buffalo (*Bubalus bubalis*). Reprod Fertil Dev 2011;23(1):168.

[121] Elamaran G, Singh KP, Singh MK, et al. Oxygen concentration and cysteamine supplementation during in vitro production of buffalo (*Bubalus bubalis*) embryos affect mRNA expression of BCL-2, BCL-XL, MCL-1, BAX and BID. Reprod Domest Anim 2012;47(6):1027–36 [PMID: 22452597]. <https://doi.org/10.1111/j.1439-0531.2012.02009.x>.

[122] Gasparrini B, Longobardi V, Zullo G, et al. Effect of L-carnitine on buffalo *in vitro* embryo development. In: Proceedings of 20th ASPA congress, Bologna, Italy; 2013.

[123] Boccia L, Rubessa M, De Blasi M, et al. Hyaluronic acid improve cryotolerance of buffalo (*Bubalus bubalis*) in vitro derived embryos. Proceeding of 38th annual conference of the International Embryo Transfer Society, 7–10 January 2012, Phoenix, Arizona. Reprod Fertil Dev 2012;24(I):138. <https://doi.org/10.1071/RDv24n1Ab52>.

[124] Eswari S, Sai Kumar G, Sharma GT. Expression of mRNA encoding leukaemia inhibitory factor (LIF) and its receptor (LIFRβ) in buffalo preimplantation embryos produced *in vitro*: markers of successful embryo implantation. Zygote 2013;21(2):203–13 [PMID: 22892066]. <https://doi.org/10.1017/S0967199412000172>.

[125] Kuwayama M, Vajta G, Kato O, Leibo SP. Highly efficient vitrification method for cryopreservation of human oocytes. Reprod Biomed Online 2005;11(3):300–8 [PMID: 16176668]. <https://doi.org/10.1016/S1472-6483(10)60837-1>.

[126] Attanasio L, De Rosa A, De Blasi M, Neglia G, Zicarelli L, Campanile G, et al. The influence of cumulus cells during *in vitro* fertilization of buffalo (*Bubalus bubalis*) denuded oocytes that have undergone vitrification. Theriogenology 2010;74(8):1504–8 [PMID: 20615538]. <https://doi.org/10.1016/j.theriogenology.2010.05.014>.

[127] Attanasio L, Boccia L, Vajta G, Kuwayama M, Campanile G, Zicarelli L, et al. Cryotop vitrification of buffalo (*Bubalus bubalis*) in vitro matured oocytes: effects of cryoprotectant concentrations and warming procedures. Reprod Domest Anim 2010;45(6):997–1002. Available from: https://doi.org/10.1111/j.1439-0531.2009.01475.x.

[128] Muenthaisong S, Laowtammathron C, Ketudat-Cairns M, Parnpai R, Hochi S. Quality analysis of buffalo blastocysts derived from oocytes vitrified before or after enucleation and reconstructed with somatic cell nuclei. Theriogenology 2007;67(4):893–900 [PMID: 17161454]. <https://doi.org/10.1016/j.theriogenology.2006.11.005>.

[129] Boccia L, De Rosa A, Attanasio L, Neglia G, Vecchio D, Campanile G, et al. Developmental speed affects the cryotolerance of in vitro produced buffalo (*Bubalus bubalis*) embryos. Ital J Anim Sci 2013;12:e80.

[130] Manjunatha BM, Gupta PS, Ravindra JP, Devaraj M, Nandi S. *In vitro* embryo development and blastocyst hatching rates following vitrification of river buffalo embryos produced from oocytes recovered from slaughterhouse ovaries or live animals by ovum pick-up. Anim Reprod Sci 2008;104(2–4):419–26 [PMID: 17689038]. <https://doi.org/10.1016/j.anireprosci.2007.06.030>.

[131] Kasiraj R, Misra AK, Mutha Rao M, Jaiswal RS, Rangareddi NS. Successful culmination of pregnancy and live birth following the transfer of frozen-thawed buffalo embryos. Theriogenology 1993;39(5):1187–92 [PMID: 16727286]. <https://doi.org/10.1016/0093-691X(93)90016-X>.

[132] Kosior MA, Parente E, Salerno F, Annes K, Annunziata R, Albero G, et al. Season affects cryotolerance of *in vitro*-produced buffalo embryos. Reprod Fertil Dev 2019;31(1):139.
[133] Gasparrini B. *In vitro* embryo production in buffalo: Yesterday, today and tomorrow. Buffalo Bull 2013;32(1):188–95.
[134] Parnpai R, Tasripoo K, Kamonpatana M. Development of cloned swamp buffalo embryos derived from fetal fibroblasts: comparison in vitro cultured with or without buffalo and cattle epithelial cells. Buffalo J 1999;15:371–84.
[135] Sandhu A, Mohapatra SK, Agrawal H, Singh MK, Palta P, Singla SK, et al. Effect of sex of embryo on developmental competence, epigenetic status, and gene expression in buffalo (*Bubalus bubalis*) embryos produced by hand-made cloning. Cell Reprogram. 2016;18(5):356–65.
[136] Campbell KH, Fisher P, Chen WC, et al. Somatic cell nuclear transfer: past, present and future perspectives. Theriogenology 2007;68(Suppl. 1):S214–31 [PMID: 17610946]. <https://doi.org/10.1016/j.theriogenology.2007.05.059>.
[137] Shi D, Lu F, Wei Y, et al. Buffalos (*Bubalus bubalis*) cloned by nuclear transfer of somatic cells. Biol Reprod 2007;77(2):285–91 [PMID: 17475931]. <https://doi.org/10.1095/biolreprod.107.060210>.
[138] Simon L, Veerapandian C, Balasubramanian S, Subramanian A. Somatic cell nuclear transfer in buffalos: effect of the fusion and activation protocols and embryo culture system on preimplantation embryo development. Reprod Fertil Dev 2006;18(4):439–45 [PMID: 16737637]. <https://doi.org/10.1071/RD05079>.
[139] Shah RA, George A, Singh MK, et al. Pregnancies established from handmade cloned blastocysts reconstructed using skin fibroblasts in buffalo (*Bubalus bubalis*). Theriogenology 2009;71(8):1215–19 [PMID: 19168209]. <https://doi.org/10.1016/j.theriogenology.2008.10.004>.
[140] Saha A, Panda SK, Chauhan MS, Manik RS, Palta P, Singla SK. Birth of cloned calves from vitrified-warmed zona-free buffalo (*Bubalus bubalis*) embryos produced by hand-made cloning. Reprod Fertil Dev 2013;25(6):860–5. Available from: https://doi.org/10.1071/RD12061.
[141] Duah EK, Mohapatra SK, Sood TJ, Sandhu A, Singla SK, Chauhan MS, et al. Production of hand-made cloned buffalo (*Bubalus bubalis*) embryos from non-viable somatic cells. In Vitro Cell Dev Biol Anim 2016;52(10):983–8.
[142] Ruan Z, Zhao X, Qin X, Luo C, Liu X, Deng Y, et al. DNA methylation and expression of imprinted genes are associated with the viability of different sexual cloned buffaloes. Reprod Domest Anim 2018;53(1):203–12. Available from: https://doi.org/10.1111/rda.13093.
[143] Mahapatra PS, Singh R, Kumar K, Sahoo NR, Agarwal P, Mili B, et al. Valproic acid assisted reprogramming of fibroblasts for generation of pluripotent stem cells inbuffalo (*Bubalus bubalis*). Int J Dev Biol 2017;61(1–2):81–8. Available from: https://doi.org/10.1387/ijdb.160006sb.
[144] Wadhwa N, Kunj N, Tiwari S, Saraiya M, Majumdar SS. Optimization of embryo culture conditions for increasing efficiency of cloning in buffalo (*Bubalus bubalis*) and generation of transgenic embryos via cloning. Cloning Stem Cells 2009;11(3):387–95 [PMID: 19594388]. <https://doi.org/10.1089/clo.2009.0003>.
[145] Huang B, Li T, Wang XL, et al. Generation and characterization of embryonic stem-like cell lines derived from in vitro fertilization buffalo (*Bubalus bubalis*) embryos. Reprod Domest Anim 2010;45(1):122–8 [PMID: 19144015]. <https://doi.org/10.1111/j.1439-0531.2008.01268.x>.
[146] Kumar D, Sharma P, Vijayalakshmy K, Selokar NL, Kumar P, Rajendran R, et al. Generation of venus fluorochrome expressing transgenic handmade cloned buffalo embryos using sleeping beauty transposon. Tissue Cell 2018;51:49–55. Available from: https://doi.org/10.1016/j.tice.2018.02.005.