UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

OVULATION SYNCHRONIZATION PROTOCOLS STUDY WITH ITALIAN MEDITERRANEAN BUFFALO COWS (*Bubalus bubalis*)

Luís Filipe Moreira Martins Esteves

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CHAVE MÓVEL

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ESTUDO DE PROTOCOLOS DE SINCRONIZAÇÃO DA OVULAÇÃO NA BÚFALA ITALIANA MEDITERRÂNEA (*Bubalus bubalis*)

Resumo

A espécie bufalina (*Bubalus bubalis*) tem vindo a crescer em importância nas últimas sete décadas. Em 2019, mais de 82% da população mundial de búfalos encontrava-se em países em desenvolvimento (54% na Índia). Nestes países são mantidos em explorações familiares com 1 a 5 animais para a produção de leite, no Sul e Sudoeste asiático, e para trabalho, no Este e Sudeste asiático, tendo sempre o propósito secundário de produção de carne.

Na Europa, apenas está presente 0,2% da população mundial, encontrando-se 86% desta em Itália, onde em 2017 se atingiu o efetivo de 400.000 animais da raça mediterrânea italiana, com o propósito de produção leiteira para a confeção de mozarela. A *Mozzarella di Bufala Campana* é um produto de denominação de origem protegida (DOP) que liga a sua produção a áreas especificas das regiões de Campania, Lazio, Puglia e Molise, sendo sempre produzida com leite de búfala.

Quando comparada com a espécie bovina, a búfala apresenta uma maturidade sexual mais tardia e tempos de gestação e de anestro pós-parto mais prolongados. A nível anatómico apresenta um cérvix mais curto, canal cervical mais estreito e um útero de menores dimensões que, quando não-gravídico, se encontra na totalidade, enrolado, na cavidade pélvica. A nível ovárico apresenta apenas entre 10.000 a 20.000 folículos primordiais, enquanto a vaca apresenta mais de 100.000 e o corpo lúteo pode não apresentar uma coroa distinta. O seu ciclo éstrico pode variar entre 16 a 32 dias, tendo como média 23,7±3,4 dias, e apresentando 2 a 3 ondas de crescimento folicular. Os comportamentos de cio são menos evidentes nesta espécie, com apenas 37,5% dos animais apresentando sinais externos. Estas caraterísticas da espécie bufalina apresentam-se como obstáculos à eficiência reprodutiva e à utilização da técnica de inseminação artificial, pelo que a monta natural se mantém como a forma mais popular de reprodução nesta espécie.

A búfala é considerada uma espécie poliéstrica sazonal de fotoperíodo decrescente e quando fora da sua época reprodutiva apresenta-se num estado de anestro sazonal e com uma maior taxa de mortalidade embrionária. Em Itália, esta sazonalidade traduz-se pela concentração dos partos no outono-inverno, no entanto, a procura de mozarela atinge o seu pico durante a primavera-verão, quando o preço por litro de leite é bem pago aos produtores, facto que leva à uma baixa rentabilidade das explorações leiteiras. Para se optimizar a produção de leite em contra estação, quando a procura da mozarela é maior desenvolveu-se o método “out-of-breeding-season-mating” (OBSM). Este método consiste na retirada dos

machos durante os meses em que a conceção é indesejada e a sua reintrodução nos grupos de fêmeas, de março a setembro. Este método tem algumas desvantagens como uma baixa na fertilidade e um aumento do intervalo entre partos, levando a uma baixa eficiência reprodutiva, sendo necessário a utilização de ferramentas capazes de induzir o restabelecimento da atividade ovárica durante o anestro sazonal.

A progesterona exerce um feedback negativo no eixo hipotálamo-hipofisário, levando à diminuição da frequência pulsátil da gonadoliberina (GnRH). Isto resulta na promoção do armazenamento de gonadotropinas na hipófise anterior. Quando o estímulo progesterónico cessa, há um pulso de GnRH seguido pela libertação das hormonas luteinizante (LH) e folículo-estimulante (FSH) armazenadas, que vão atuar no ovário, promovendo o crescimento folicular e a ovulação, levando ao restabelecimento do ciclo éstrico.

Protocolos à base de progesterona são o método que tem vindo a dar melhores resultados no restabelecimento da atividade ovárica regular em búfalas acíclicas. Este estudo tem como objetivo avaliar a influência dos dias pós-parto no sucesso de um destes tratamentos, fora da época reprodutiva.

Para este trabalho foram utilizados dados recolhidos pelo Departamento de Medicina Veterinária e Produção Animal da Universidade de Nápoles Federico II, entre abril e julho de 2021. O estudo foi conduzido numa exploração comercial leiteira no sul de Itália, onde o maneio reprodutivo utilizava inseminação artificial e monta natural. As fêmeas adultas eram mantidas em grupos de 60 animais e entre fevereiro e setembro estavam presentes 4 machos por grupo (método OBSM). Por razões de bem-estar animal os animais eram mantidos com um mínimo de 15 m^2/cabeça.

Após respeitado o período voluntário de espera de 40 dias, as fêmeas foram sujeitas a exames ecográficos transretais para avaliação da atividade ovárica e do estado do útero, de 7 em 7 dias. Caso não fosse encontrado um folículo com mais de 7 mm ou um corpo lúteo em 3 exames consecutivos, os animais eram considerados em anestro. Animais em que se diagnosticou uma gestação e quando reavaliados 30 dias depois, foi confirmada a gestação, foram excluídos do estudo. Foram também excluídos todos os animais que apresentavam sinais de inflamação uterina ou outra patologia. O último critério de inclusão foi a condição corporal que deveria variar entre 7 e 8,5, numa escala de 1 a 9. Para o presente estudo, foram selecionadas 246 búfalas adultas primíparas, em anestro, com uma média de 550 Kg de peso vivo, condição corporal média de 7,8 e produção leiteira média de 12,1 Kg de leite por dia.

Todos os animais (n=276) foram sujeitos a um protocolo de sincronização da ovulação com dispositivo intravaginal de libertação de progesterona durante 10 dias e beneficiados por inseminação artificial a tempo fixo (IATF) ao 13º dia. A condição corporal e a área do folículo

pré-ovulatório foram medidas no dia da IATF. Os animais foram divididos em três classes conforme os seus Dias em lactação (DLACT): Classe I (61-90 DLACT; n=86), Classe II (91-150 DLACT; n=102) e Classe III (151-200 DLACT; n=88). Quinze dias após a IATF as fêmeas foram colocadas em grupos com machos, permitindo-se a monta natural. O diagnóstico de gestação foi realizado no 27º dia pós-inseminação. Quando negativo, as fêmeas foram sujeitas a novos diagnósticos de gestação a cada 15 dias. Os animais foram então classificados conforme a gestação resultante da IATF ou se do 1º, 2º ou 3º ciclo éstrico pós-inseminação.

A taxa de conceção total foi de 88%, sem diferenças significativas entre as classes de DLACT, obtendo-se 88, 92 e 80% nas classes I, II e III, respetivamente. Registou-se uma tendência das fêmeas da classe II de revelarem melhor (p<0,07) taxa de conceção por inseminação artificial. Elas apresentaram maiores (p<0,01) folículos pré-ovulatórios no dia da IATF que as outras fêmeas pertencentes às restantes duas classes. A taxa de conceção por IATF foi influenciada pela área folicular (odds ratio=2,24; p<0,05) e pela condição corporal (odds ratio=1,26; p<0,05).

Concluiu-se que os tratamentos à base de progesterona permitem a obtenção de taxas de conceção favoráveis em búfalas acíclicas, fora da época reprodutiva. Sendo os melhores resultados apresentados por fêmeas a meio da lactação (91-150 DLACT) e com uma condição corporal ótima. Os animais nas fases iniciais de lactação (61-90 DLACT) apresentaram um intervalo médio entre o fim do tratamento e a conceção por monta natural superior às restantes, possivelmente devido a um estado de anestro pós-parto prolongado.

Mais informação é necessária para uma melhor compreensão dos fatores que influenciam estes protocolos, para se obter melhores resultados e aumentar a rentabilidade das explorações leiteiras bubalinas. É importante a avaliação de mais fatores e de um aprofundamento do estudo da influência dos dias em lactação, devendo-se avaliar animais provenientes de manadas com genética mais diversificada e não sujeitas ao método OBSM.

Palavras-chave: Búfala Mediterrânea; anestro; progesterona; dias em lactação

OVULATION SYNCHRONIZATION PROTOCOLS STUDY WITH ITALIAN MEDITERRANEAN BUFFALO COWS (Bubalus bubalis)

Abstract

The buffalo species (*Bubalis bubalis*) is known for a seasonal reproductive tendency toward periods of decreasing daylight hours, entering an anestrus state during the out-of-breeding season. In Italy, buffalo milk is mostly used for the production of mozzarella cheese, which has its market demand peak during the summer when the buffaloes express lower reproductive efficiency. For this reason, in order for buffalo farms to present optimum profitability the anestrus state needs to be shortened to decrease inter-calving intervals. Progesterone-based treatments are the method that has presented the best results in achieving the restoration of regular ovarian activity in acyclic buffaloes. This study aimed to evaluate the influence of days after calving on the success of these protocols.

All acyclic animals (n=276) were subjected to an ovulation synchronization protocol with a progesterone-releasing intravaginal device for ten days and fixed-time artificial insemination (FTAI) on day 13 of the protocol. Body condition score (BCS) and preovulatory follicle area were recorded on the day of the FTAI. The animals were divided into three classes regarding their days in milk (DIM): Class I (61-90 DIM; n=86), Class II (91-150DIM; n=102), and Class III (151-200 DIM; n=88). 15 days after FTAI the females were moved into groups with males present and natural mating was allowed. Pregnancy diagnosis was then performed 27 days after FTAI; when the diagnosis was negative, the females underwent more pregnancy diagnoses every 15 days. The animals were then classified as pregnant at FTAI or pregnant at the 1st,2nd or 3rd oestrous cycle post-insemination.

The total pregnancy rate was 88%, with no significant difference among DIM classes, with classes I, II, and III having 88, 92, and 80%, respectively. It was noted that Class II females showed a tendency for higher ($p<0.07$) pregnancy rates at FTAI. The Class II buffaloes presented larger ($p<0.01$) follicles at FTAI than those included in the other two classes. The pregnancy outcome at FTAI was influenced by the preovulatory follicular area (odds ratio=2.24; $p<0.05$) and the BCS (odds ratio=1.26; $p<0.05$) at FTAI.

It was concluded that P_4-based treatments allow the obtention of more favourable pregnancy rates in acyclic buffaloes, with best results being revealed in the females mid-lactation and that presented optimum BCS.

Key-words: Mediterranean buffalo; anestrus; progesterone; days in milk

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List of symbols and abbreviations

® - Registered Trademark

AI - artificial insemination

ART - Assisted Reproductive Technology

BCS - Body Condition Score

CIDR - Controlled Internal Drug Releasing Device

CL - Corpus Luteum

cm - Centimetre

CR – Conception Rate

DF – Dominant Follicle

DIM – Days in Milk

DLACT – Dias em lactação

E2 - Oestradiol-17β

EC - Endothelial Cells

eCG - Equine Chorionic Gonadotrophin

EM - Embryonic Mortality

FGF - Fibroblast Growth Factor

FPr - Prostaglandin F Receptors

FSH - Follicle-stimulating Hormone

FTAI - Fixed-time Artificial Insemination

g - Gram

GH - Growth Hormone

GnRH - Gonadotropin-Releasing Hormone

hCG - Human Chorionic Gonadotropin

IATF - Inseminação Artificial a Tempo Fixo

i.m.- Intramuscular

IBR - Infectious Bovine Rhinotracheitis

IFN-τ - Interferon-Tau

LC - Luteal Cells

LH - Luteinising Hormone

m^2/head – Square Meters per Head

MBC - Mozzarella di Bufala Campana

mm – Millimetre

MY – Milk Yield

ng/ml - Nanograms per Millilitre

NSC - Suprachiasmatic Nucleus

OT - Oxytocin

P/AI – Pregnancies per Artificial Insemination

P_4 - Progesterone

PDO - Protected Designation of Origin

pg/mL - Picograms per Millilitre

PGF_{2a} - Prostaglandin $F_{2\alpha}$

PMSG - Pregnant Mare Serum Gonadotrophin

PR – Pregnancy Rate

PRID - Progesterone Releasing Intravaginal Device

RB - Repeat Breeder

RFM - Retained Fetal Membranes

VEGF - Vascular Endothelial Growth Factor

VWP - Voluntary Waiting Period

1. Introduction

The buffalo species (*Bubalus bubalis*) has seen its importance grow over the years in developed and developing countries alike. In Italy, the primary purpose for breeding these animals is the production of milk for mozzarella cheese. The main hurdle faced by buffalo farms is the seasonal anestrus state manifested by this species when out of their natural breeding season. To overcome this problem, the use of hormonal treatments to reduce the anestrus phase has been gaining importance (Singh and Balhara 2016; Altieri et al. 2020; D'Occhio et al. 2020).

This study aims to help better the understanding of the influence of days after calving, measured as different days-in-milk (DIM), on the success of progesterone-based treatments in the anticipation of the end of this anestrus state.

This chapter will provide an introduction to the study by first discussing the background and context, followed by the research problem, the aim of the study, its significance and limitations, and ending with the structural outline of the dissertation.

The anestrus is a state of sexual inactivity caused by the lack of circulating gonadotropins to promote the final maturation of follicles and consequently ovulation. Buffaloes naturally enter this state during the times of the year with increasing daylight hours. This occurs given that the buffalo shows a reproductive seasonal tendency to deliver calves during "short days" when the demand for mozzarella cheese and buffalo milk is lower. The out-of-breeding-season-mating method allows for greater quantities of milk to be produced during the hotter months by interrupting sexual promiscuity when mating is not desired. This has the disadvantage of animals having longer postpartum anestrus and overall decreased fertility. When the animals give birth during "longer days", to meet the higher market demands for their milk, they tend to undergo a seasonal anestrus state, which is responsible for significant economic losses due to the greater number of open days (calving-to-conception interval) (Zicarelli 1997).

Progesterone (P_4)-based treatments can aid in reversing the anestrus state in buffaloes. However, further information is still needed to fully advance the use of these protocols, to reduce ineffective treatments and unnecessary costs while fully optimising the investments made and raising profitability (Zaabel et al. 2009). The hormonal basis of anestrus has been studied by several authors already, and some studies evaluated the impact of some variables in the outcome of such protocols, with factors like body condition score (BCS), age and season having already been described. Meanwhile, a gap in knowledge regarding other variables, such as days after calving, still exists. This lack of information needs to be corrected in order to achieve the optimisation of the productive lifespan of the animals.

Accordingly, the present study aims to uncover the influence of days after calving on P_4-based treatments in the resumption of ovarian activity in acyclic buffaloes undergoing fixed-time artificial insemination and natural mounting (up to the third oestrous cycle post-insemination) during the out-of-breeding season. The attempt to answer this will be achieved by comparing the success of the therapy utilised in animals in different stages of lactation within the same herd, divided into three different classes according to days-in-milk (DIM). If a significant influence is recorded, it may be beneficial to adapt the selection of animals to be included in these protocols to obtain better results and reduce unjustified costs.

This study has some limitations: as the evaluation of primiparous females coming from a single herd that has been exposed to the out-of-breeding-season-mating (OBSM) method; the sample was not large enough for more than three classes of different DIM to be achieved, nor was the differentiation of the animals by age possible; the author reduced experience; and by only evaluating one complex variable, such as DIM that is influenced by several external factors such as nutrition, season, management and hygienic status, this study will not attempt to answer the problem at hand absolutely but will try to shed some light on it.

The contents of this thesis can be divided into three main parts. In the first part (from chapter 3 to chapter 6), there is a review of published literature regarding the basis of current buffalo production worldwide and its current state in Italy, the female buffalo reproductive anatomy and physiology, its reproductive management with the use of reproductive technologies and the most common pathologies that have a detrimental effect on the reproductive efficiency of the female buffalo. In the second part (chapters 7 and 8), there is a description of the procedures and instruments used in the making of the present study, as well as the presentation of the results of the data analysis. The third and final part (chapters 9 and 10) includes the discussion of the key findings and based on that, recommendations and conclusions are made.

2. Internship Report

The curricular internship to complete the Integrated Master's Degree in Veterinary Medicine, from the University of Lisbon, from the 1st of November 2021 to the 1st of May 2022, was carried out under the European Region Action Scheme for the Mobility of University Students (Erasmus+ Program). Under the guidance of professor Gianluca Neglia (PhD), from the University of Naples, the internship took place in buffalo and cattle farms in the Italian region of Campania (Figure 1).

Figure 1. Map of the provinces of the region of Campania, Italy (from https://www.italyheritage.com/regions/campania/).

During the traineeship, it was possible to acquire knowledge on the buffalo breeding systems and techniques while also gathering information about animal welfare in livestock and morphological and productive characteristics.

It was enabled the development of knowledge on the basis of the evaluation of the reproductive parameters useful to define the correct management of livestock, the application of the main reproductive biotechnologies and the techniques used to reduce the environmental impact of livestock and increase their sustainability.

The internship took over 720 hours, with the observation and assistance of doctor Donato de Nicola (PhD) in performing 1078 artificial inseminations, 759 transrectal palpations (for late pregnancy diagnosis, postpartum evaluations, oestrus detection and reproductive tract evaluation) and 3535 gynaecological ultrasound examinations (for early pregnancy diagnosis, fetal sexing and age determination, evaluation of the reproductive tract with the diagnosis of eventual ovarian and uterine pathologies)

It was possible to perform 1107 transrectal palpations and 15 gynaecological ultrasound examinations.

Additionally, 20 genital and 1 rectal prolapses were corrected, 6 calvings assisted, several samples were collected (blood, milk, feces, fur, abdominal circumference), and many cases of Neonatal Calf Diarrhea, ketosis, hypocalcemia and retained fetal membranes were diagnosed and treated. Some of the other activities performed include animal identification, vaccination, deworming and minor surgery.

3. Buffalo’s importance

3.1 Worldwide

The population of buffaloes worldwide (Graph 1) has been increasing over the past seven decades. Surpassing 100 million animals in 1967 and 200 million in 2016, doubling the number of animals in less than 50 years. In 2019, over 82% of buffaloes were in developing countries, with over half of the world population in India (54%) (FAO 2021).

For centuries, buffaloes have been used in Asia, usually associated with smallholder farms, with only 1 to 5 animals. The main reason for their use is to pull heavy loads in East and Southeast Asia and milk production in South and Southwest Asia while always having the secondary purpose of meat production (Cruz 2007).

Buffalo production in developing countries has increased since these animals can use poor-quality feed and have higher economic returns than other dairy species. Whereas cow slaughter is banned in India, buffalo slaughter is allowed (Singh and Balhara 2016). India is the world leader in milk production and consumption, with almost 50% of the national milk production coming from buffaloes (Subbanna et al. 2021). Therefore, the primary efforts to improve buffalo’s genetics and breeding come from the Government of India by creating plans with this purpose in mind. The first of these plans lasted five years and began in 1951, shortly after the first buffalo calf was born in India by artificial insemination (AI) in 1943 (Singh and Balhara 2016).

According to the last data published by FAO (2021) regarding the year 2019, India had 69% of the world’s production of buffalo milk, with the amount growing and Subbanna et al. (2021) estimated that it would reach 100 million tonnes by the end of 2021. This growth is a response to the population increase, rise in income and purchasing power in Asia, and the corresponding demand for animal-derived products such as meat and milk (Cruz 2007).

3.2 Italy

In 2019, Europe had only 0,2% of all world buffalo population, with 86% being raised in Italy.

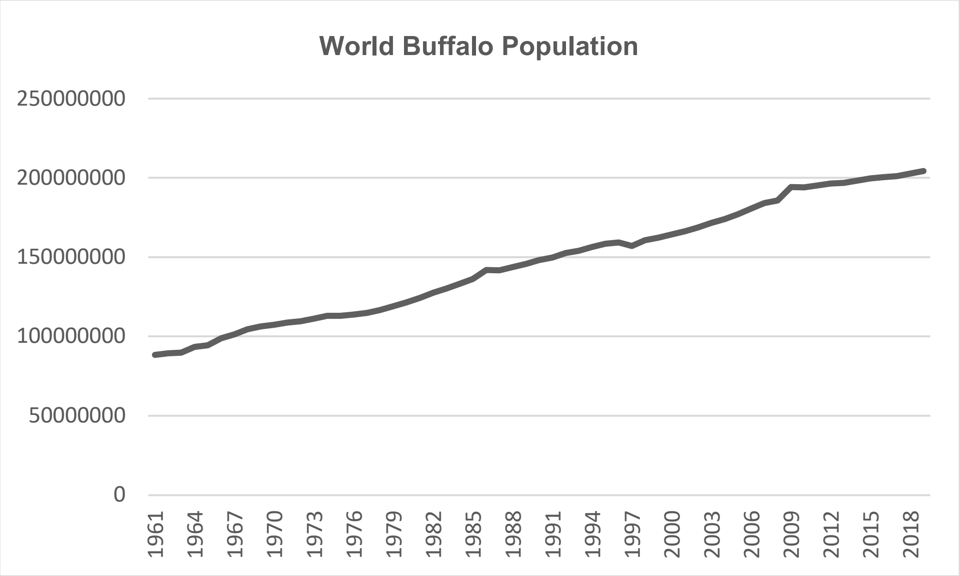
The number of buffaloes in Italy (Graph 2) has been increasing steeply, reaching 200,000 animals in 1999 and doubling by 2017 (FAO 2021). The buffaloes raised in Italy for milk production belong to the river type and are from the Mediterranean breed (Mingala et al. 2017).

While in developing countries, buffaloes are used as an affordable source of nutrients; in Italy, buffalo milk takes importance at the other extreme of the economic scale, being used to produce Mozzarella di Bufala Campana (MBC), an expensive and profitable niche product.

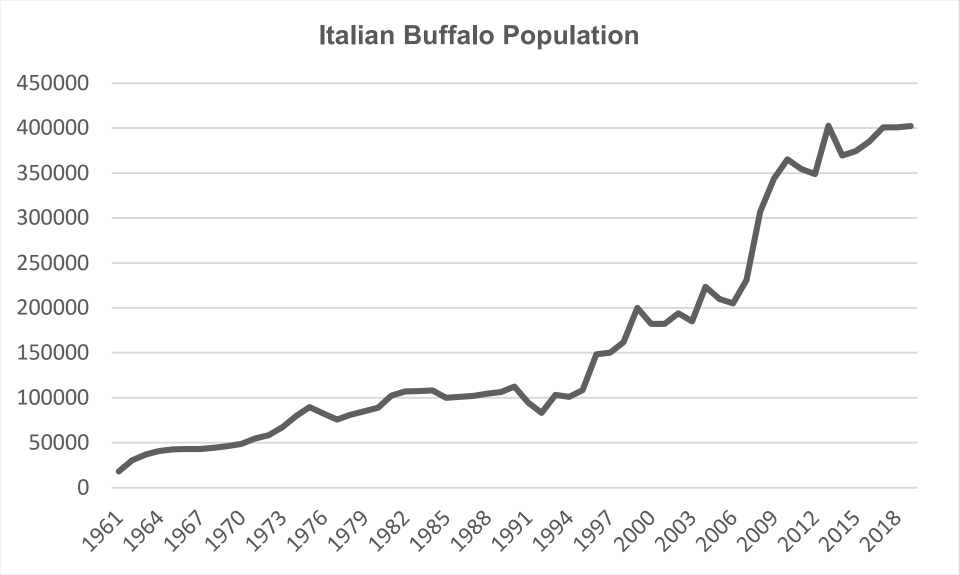
MBC contributes to the economy of the region of Campania (South of Italy) and buffalo production since it has protected designation of origin (PDO) status under European Union regulations (CE 1107/96). The PDO status binds the output of MBC to specific areas of Campania, Lazio, Puglia, and Molise with the exclusive use of buffalo's milk. If produced outside these regions, it may be commercialised without the PDO trademark, as 'Mozzarella (name of the brand) di latte di Bufala', which can reach higher quantity of units produced than the PDO trademarked. This Mozzarella cheese has high demand (peaking in the summer). However, the availability of water buffalo's milk cannot meet the increased demand for this product, making it susceptible to imitation and fraud. These fraudulent behaviours, such as adding cheaper cow's milk, reinforce the value of traceability and detection of adulteration of the MBC (Czerwenka et al. 2010; Altieri et al. 2020).

In the Mediterranean breed lactations of 240-270 days in length and production of 1500 – 6000 litres have been described. An average of 2500 litres/lactation with 4.8% crude protein and 8.5% butirose fat was reported in 2004 on the south of Italy (Zana and Sansinena 2017).

The growth of buffalo production around the globe and its impact on the life of the communities (developed and developing countries alike) makes improving this species' reproductive efficiency essential. The primary restraint to the spread of this species' production lies in its reproductive biology. When compared to cattle, buffaloes have later sexual maturity, longer gestation length, and longer postpartum anestrus. However, the age at first calving has been decreasing throughout the last 30 years with efforts made through genetic selection (D'Occhio et al. 2020). More problems are found when we bring artificial insemination (AI) into the equation. Buffaloes are seasonally reproductive individuals with higher values of embryonic mortality when they are out of their natural breeding season, less visible oestrus behaviour and more variable oestrous cycles. These hurdles make AI less efficient, impairing genetic improvement and subsequent germplasm dispersal worldwide. Even though that natural mating largely remains the most popular form for breeding these animals, AI has seen an increase (D'Occhio et al. 2020; Neglia et al. 2020).



Graph 1. World buffalo population growth 1961-2019 (Original).



Graph 2. Italian buffalo population growth 1961-2019 (Original).

4. Female Buffalo Reproductive Anatomy

The reproductive tract of the female buffalo is very similar to that of cattle (Figure 2). The differences between the two will be discussed in depth in this chapter.

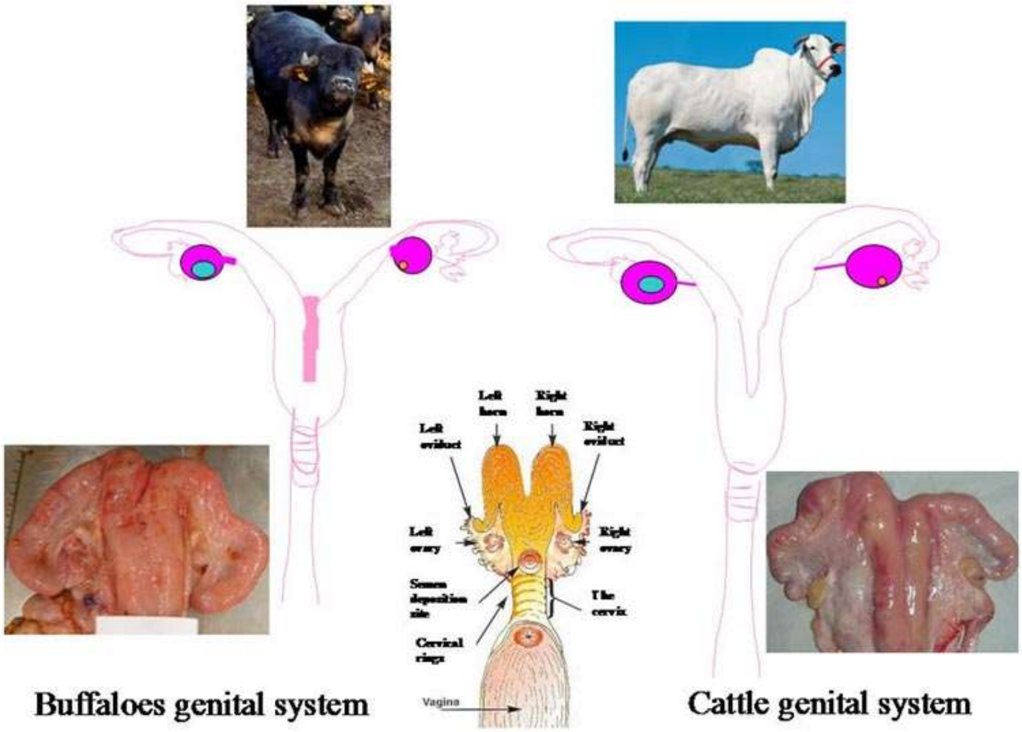


Figure 2. Comparison between buffalo (left) and cattle (right) female genital systems (Carvalho et al. 2014).

4.1 External genitalia

The vulva, vestibule, vestibular glands, and clitoris form the female buffalo's external genitalia. The vulva is similar to cattle in length (approximately 10.5 cm); however, the labia are less firmly opposed, tremendously pigmented, and present a more developed clitoris than cows in its ventral commissure. The vulva has two muscles, the constrictor vulvae and the constrictor vestibuli. Identically to many mammals, the external genitalia of the buffalo contains many sensitive nerve terminals, particularly the subcutaneous connective tissue of the labia majora and minora contains corpuscles like Pacini, Ruffini, and Meissner. These structures are the starting point of nervous reflex arches that regulate some genital functions.

Female buffaloes have a particular morphologic characteristic regarding the position of the urethral opening, which opens on a papillary relief of the mucosa, approximately 1 cm long. Two parallel folds of mucosa originate from the sides of this relief and run caudally, limiting the suburethral diverticulum. This terminal position of the urethra makes its catheterism particularly hard. It makes the orifice visible through the vulva when the animal contracts its pelvis, as in the case of obstetric procedures. Neuroendocrine cells are present in the urethral mucosa, and many show long cytoplasmic extensions oriented towards the mucosa. These cells are dispersed through many body epithelia and produce hormonal peptides and biogenic amines. It is theorised that these urethral neuroendocrine cells affect the regulation of urogenital functions in the buffalo. A group of exocrine glands is organised around the visible excretory duct on the vestibular wall. These structures correspond to the main vestibular glands of cows (Vittoria 1997; Carvalho et al. 2014; Perera and Noakes 2019)

4.2 Vagina

According to Sisson (1953), the length of the cattle vagina averages around 28 cm (25–30 cm), whereas the buffalo vagina is closer to 22.1 cm (Luktuke0 and Rao 1962). The former shows more elasticity and capability for expansion during the oestrus and calving. This ability comes partly from the mucosa being more deeply folded (Vittoria 1997; Dyce et al. 2010; Carvalho et al. 2014).

Many sensitive terminals, such as Pacini and other genital corpuscles, are scattered across the mucosa. In the periorganic connective tissue, there are several bundles of nerve fibres. These bundles cross each other, creating a close network with ganglionic nerve structures with varying numbers of nerve cell bodies (Rosati 1957; Vittoria 1997).

4.3 Uterus

The cervix is smaller and narrower than in cattle, with its length ranging between 5.5 to 5.9 cm and its width from 2.8 to 3.6 cm (Damodoran 1958; Luktuke and Rao 1962), while in cows, the cervix measures 10 cm in length and 6 cm in width (Sisson 1953). This smaller size in female buffaloes presents a more significant difficulty during AI (Perera and Noakes 2019).

The uterus's body length is comprised between 0.8 to 1.5 cm, whereas in cattle, it usually is 3-4 cm long (Sisson 1953; Vittoria 1997).

Starting at the basis of the uterine horns, buffaloes have an intercornual ligament that strongly connects both horns for a prolonged extension. This makes their uterine horns less malleable, making the catheterism for embryo collection harder when compared to cows, which have a similar ligament but are less firmly attached (Sane et al. 1964; Zicarelli 1994). This ligament is responsible for the tightly coiled presentation the nonpregnant bubaline uterus has in the pelvic cavity where entirely it usually lies. In counterpart, cows' reproductive tract usually is located mainly in the abdominal cavity (Sisson 1953; Carvalho et al. 2014).

The nervous network in the uterus is similar to the previously described for the periorganic connective tissue of the vagina (Giordano and Rosati 1956).

4.4 Oviducts

The oviduct extends from the ovary to the ipsilateral uterine horn and is divided into four segments: fimbriae, infundibulum, ampulla, and isthmus. The thickness of the muscular layer increases from the ovarian to the uterine end of the oviduct (Figure 3). Its length is similar to buffaloes and cows and can measure up to 25 cm. Compared to cattle, buffalo oviducts are rougher and less elastic (Taneya et al. 1988; Hafez and Hafez 2000a; Priedkalns and Leiser 2006).

Figure 3. Histologic section of buffalo's oviduct. From left to right: infundibulum, ampulla and istmus (Carvalho 2006).

4.5 Ovaries

Buffalo ovaries differ in shape and size from cattle's. In buffaloes, the ovary is 2.6 cm long, has a round shape and weighs about 3.3 g (Luktuke and Rao 1962; Islam et al. 2018). However, there are reports of its weight being comprehended between 0.5 to 10.9 g (Danell 1987). Whereas in cattle, Sisson (1953) described the ovary as being 3.5 to 4 cm long, having an oval shape and weighing 15 to 20 g.

The number of primordial follicles greatly varies between buffalo and cattle. Danell (1987) revealed the presence of 10.000 to 20.000 follicles in buffalo's ovaries and over 100.000 in cattle.

The dominant follicle (DF) reaches a 12-15 mm diameter, comprises an oocyte and several surrounding theca and granulosa cell layers, and has a fluid-filled cavity (antrum). The buffalo oocyte differs from cattle, the most relevant difference being the more significant amount of lipid droplets (Sharma et al. 2014).

Corpus luteum (CL) in buffalo averages in diameter of 10-17 mm and is deeply inserted into the ovary and may not present a clear crown which makes its correct identification by transrectal palpation harder when compared to the CL of cattle that protrudes from the surface of the ovary (Polding and Lall 1945; Perera et al. 1987).

5. Oestrous cycle and Reproductive Physiology

5.1 Oestrous Cycle

The age at which females reach puberty varies significantly with the conditions in which they are raised and bred and usually happens when they get 55-60% of their adult body weight. After puberty, the oestrous cycle initiates, and under the best conditions, this has been described to happen as soon as 16-18 months of age (Jainudeen and Hafez 2000; Barile 2005).

The oestrous cycle ranges between 16 to 32 days with an average of 23.7±3.4 days (Baruselli et al. 1997; Neglia et al. 2007), and it is divided into 4 phases: proestrus (D18-20 – lasts three days), oestrus (D0/21), metaestrus (D1-4 – lasts 3-4 days), and diestrus (D5-17 – lasts 12-15 days). The proestrus is characterised by the regression of the previous cycle's CL and the follicular growth's final maturation. The oestrus is the period of male acceptance and in buffaloes lasts about 21 hours, but with a large variability from few hours till 3 days. The ovulation occurs 15-18 hours after it has ended, but also in this case, a large variability is recorded. Both these phases form the follicular phase of the oestrous cycle since there is no functional CL. In the metaestrus, from day 1 to 4 after oestrus, the theca interna and granulosa cells of the ovulated follicle suffer luteinisation and give rise to a functional CL. The phase in which the CL is fully functional is called diestrus and goes from day 5 to 17 after oestrus and ends with the luteolysis of the CL. In buffaloes, the CL's lifespan lasts up to 16 days (Jainudeen and Hafez 2000; Robinson and Noakes 2019).

The oestrous cycle regulation in buffaloes is similar to cattle, with control support of the hypothalamic-pituitary-gonadal axis (Figure 4). The hypothalamus produces the gonadotropin-releasing hormone (GnRH) in response to circulating ovarian steroids, such as progesterone (P_4) and oestradiol-17β (E_2) (the most abundant and most potent oestrogen in circulation). GnRH stimulates the production of the gonadotropins follicle-stimulating hormone (FSH) and luteinising hormone (LH), which act on the ovarian follicles, making them grow and ovulate. P_4 and oestrogen are both produced in the ovary and transported by the bloodstream; the functional CL present produces the former in cyclic and pregnant females and the latter produced by growing follicles. Oestrogen exerts positive feedback by stimulating pulsatile release of LH, whereas P_4 acts by diminishing the secretion of LH and FSH by the anterior pituitary, resulting in negative feedback that prevents cyclicity. The cycle resumes by the action of the Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), secreted by the endometrium, which promotes the regression of the CL and the end of the negative feedback mechanism enforced by the luteal P_4 (Singh et al. 2000; Perera 2008; Sharma et al. 2014).

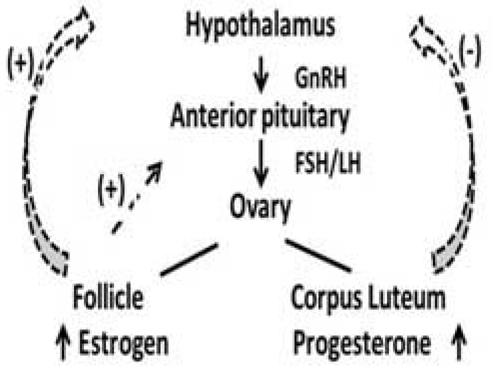


Figure 4. Schematic representation of endocrine control of steroidogenesis in the female buffalo (Sharma et al. 2014).

The preovulatory follicle produces and releases E_2, which reaches its peak in blood concentration around 9-13 pg/mL (in the luteal phase, E_2 values are about 3-7 pg/mL), stimulates a surge of GnRH release by the hypothalamus, which is responsible by the preovulatory surge of FSH and LH. FSH has a peak that reaches 1.6-6 ng/mL and lasts 6-9 hours, and LH values reach 15-25 ng/mL, and this peak lasts about 6-12 hours. During diestrus, the plasma levels of these hormones are 0.2-1.5 ng/mL and 0.5-3 ng/mL, respectively, and a postovulatory FSH surge was rarely recorded. Triggered by this LH surge, ovulation occurs, which is a process where there is an increase in blood flow to the follicles and increased vascular permeability, and the oocyte suffers its final maturation. There is a disruption of the cohesiveness of cumulus cells of the granulosa layer by the action of proteolytic enzymes triggered by the local rise of prostaglandin E_2 and P_4 production by the theca cells. This will result in an increase of follicular fluid with a thinning of the external follicular wall and the formation of a stigma. The stigma is compressed by the contractions of the smooth muscle of the theca externa and is forced to protrude and eventually rupture, releasing the oocyte and follicular fluid. It is also the beginning of the conversion of the follicle into the CL (Seren and Parmeggiani 1997; Hafez and Hafez 2000b; Hafez et al. 2000; Sharma et al. 2014; Robinson and Noakes 2019).

The CL is formed from the follicle, shortly after the ovulation, by the luteinisation of the theca and granulosa cells and angiogenesis. The CL increases to 20 times its initial mass, taking 10-12 days to complete (Robinson and Noakes 2019). Even after fully matured, the CL still has a high cell turnover and is one of the organs with the highest blood supply rate per unit; for this reason, the fibroblast growth factor (FGF) and the vascular endothelial growth factor (VEGF) play a central role on the growth and development of the CL as angiogenic growth factors. This plentiful blood supply is achieved mainly by forming new blood vessels from those pre-existent in the theca layer of the follicle that gave rise to the CL and is essential to support the high metabolic rate of the mature CL (Reynolds and Redmer 1999; Woad and Robinson 2016). The fully formed CL is made of various cells; there are the luteal cells (LC) (responsible for steroid secretion), both small and large, fibroblasts, smooth muscle cells, pericytes and endothelial cells (EC). Large LC represent 30% of the steroidogenic cells of the CL and secrete 70% of the P_4. This cells' secretion is not stimulated by LH, opposite to the small LC that are the remaining 70% of steroidogenic cells and produce only 30% of the P_4 when under the stimulation by LH. EC are the most abundant type of cell in the mature CL representing about half of all the cells present. This means that almost all steroidogenic LC are in immediate contact with one or more capillaries (Weems et al. 2006; Woad and Robinson 2016). LH and the growth hormone (GH) are essential to the formation and function of the CL, with most of the LH receptors being on the small LC and the GH receptors on the large LC. The luteinisation caused by the preovulatory LH surge seems to stimulate the production of ovarian oxytocin (OT) and P_4. The angiogenic growth factors also stimulate P_4 and OT secretion, and high levels of VEGF and FGF in the mature CL are indicators of the functionality of EC and LC. P_4 in the CL stimulates the production of LH receptors and inhibits the start of apoptosis and $PGF_{2α}$ release by the endometrium (Berisha and Schams 2005). During the lifecycle of the functional CL, P_4 plasma levels vary between 5-12 ng/mL and drop to values below 0.5 ng/mL two to three days before the onset of the new oestrus. This drop in P_4 is accompanied by the rise of the $PGF_{2α}$ metabolite 13,14-dihydro,15-keto-$PGF_{2α}$, which increases from the basal value of 50-100 pg/mL to 200-700 pg/mL (Seren and Parmeggiani 1997).

For complete luteolysis, 5 to 8 pulses of $PGF_{2α}$ released by the endometrium are needed. $PGF_{2α}$ is transported directly to the ovary, where it stimulates the secretion of OT by the CL, and the OT stimulates $PGF_{2α}$ secretion, making a positive feedback loop. Luteal regression can be divided into functional and structural regression. Functional regression occurs faster and is established when the secretion of P_4 decreases. Structural regression consists of the physical involution of the CL. The $PGF_{2α}$ receptors of the large LC are the primary target of $PGF_{2α}$, and the large LC decrease in size, followed by the reduction in the size of the small LC. At the same time, there is a disruption of the luteal vasculature and infiltration of immune cells. There is nitric oxide-induced vasodilation of the luteal vasculature. Still, as the luteolysis process advances, there is a loss in blood flow to the CL resultant from the vasoconstriction caused by elevated levels of endothelin and angiotensin II (Robinson and Noakes 2019). Prostaglandin F receptors (FPr) have been found in cattle's mature and immature CL, mainly on LC and the endothelial cells of the large blood vessels. The increase in the CL sensitivity to $PGF_{2α}$ as it matures is likely to occur because of the accumulation of damages to the molecular and cellular infrastructure and not of a lack of FPr in the growing CL (Miyamoto et al. 2009).

Ovarian follicle waves are defined as the recruitment of follicles to grow and the selection of the DF. In buffaloes, there are 2-3 growth waves during each oestrous cycle. The number of follicular waves in each cycle is correlated with its length, which the CL and its lifespan control. Both heifers and adult buffaloes present the same number of follicle growth waves; however, in adults, the development rate is more significant, as well as the size of the DF. The recruited follicles grow to 6-9 mm in diameter under the effect of FSH, with one follicle being selected to develop more than the others from the same wave and suppressing them. This follicle, called the DF, can reach 12-15 mm in diameter. This second growth requires LH to happen. The deviation is defined as when the growth of one follicle (future DF) occurs faster than the others from the same wave. The DF and the second-largest follicle size in buffalo heifers do not vary during the first wave of follicular growth. Dominance only occurs when the largest follicle is at least 1-2 mm larger in diameter than all the other follicles, ceasing the growth of the subordinate follicles, which undergo atresia.

Mid-cycle follicles suffer atresia, and only the DF of the last wave ovulates, and it is the one responsible for the oestrus. Ovulation only occurs on the latest wave of each cycle since a functional CL is present when the previous 1-2 waves arise. The P_4 produced by the CL is responsible for the non-occurrence of ovulation by its inhibitory effect on FSH production by the anterior pituitary; the follicles from these waves undergo atresia. With the absence of the blocking action of P_4, the pulses of GnRH are higher and more frequent resulting in more LH and FSH to support the development of the DF. This also stimulates the production of oestrogen by the DF and the beginning of the positive feedback to the anterior pituitary, which culminates with the ovulation of the DF. The first wave of the next cycle initiates around the day of ovulation. Buffaloes can sometimes present an atypical one-wave cycle; this may be justified by a low number of primordial follicles, a delay in FSH release or an inadequate amount of FSH. The pattern of follicular growth in single wave cycle differs from those of two- and three-wave cycles, since around mid-cycle, the DF regresses in size but maintains its dominance, proceeding to grow slowly from day 15 until ovulation, whereas in the typical cycles, there is no regression between the start of the growth and the ovulation (Jainudeen and Hafez 2000; Presicce 2007; Awasthi 2013; Sharma et al. 2014).

5.2 Oestrus Manifestations and Detection

Oedema of the vulva, hyperaemia of the vestibular mucosa and clear mucoid vaginal discharge (Figure 5) are the main manifestations of oestrus in the genital tract of the buffalo, although they are less evident than in cattle. The mucus is secreted in fewer quantities than in cows and usually does not hang from the vulva, but is accumulated on the floor of the vagina, and is discharged when the animal lies down or when palpated rectally. As in cattle, the uterine horns are coiled and turgid, reaching their maximum tonicity during the oestrus, and the cervix is dilated, allowing insemination. Oestrus behaviour includes mounting, vocalisation, standing reflex, restlessness, raising of the tail, lower feed intake and frequent urination in small quantities. High ambient temperature can cause shorter heat duration, with the signs only being shown during the night or early morning. Oestrus behaviour is significantly less visible in heifers when compared to pluriparous buffaloes; however, no difference in the genital tract manifestations was observed between these two groups (Sharma et al. 2014; Purohit and Rao 2018; Perera and Noakes 2019).

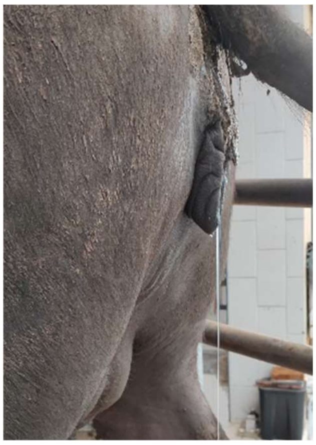


Figure 5. Oestrus clear mucoid vaginal discharge in buffalo (Original).

Oestrus signs in the buffalo are less noticeable and more challenging to detect than in cattle. Seren and Parmeggiani (1997) recorded only 37.5% of buffaloes manifesting external signs of oestrus, with mucus discharged being the only sign recorded, and 16.6% showed oestrus signs during the luteal phase. Oestrus behaviour during diestrus may be due to high values of E_2 produced by the mid-cycle growing follicle and P_4 levels below the oestrus inhibitory threshold (Allrich 1994).

When a bull approaches a female in oestrus, it exhibits a flehmen reaction and licks the vulva and perineum. Vasectomised teaser bulls or androgenised buffalo cows with a chin ball marking device can be used to detect oestrus. Tail paint used on its own is unreliable in the buffalo since homosexual behaviour is rarely recorded in these animals. If the costs are justifiable, more advanced heat detection systems such as pressure sensing radiotelemetric detectors, pedometers, or vaginal electrical resistance measuring probes may be used. Transrectal palpation or ultrasonography can be used to detect oestrus too. A large follicle should be present, as well as a regressing CL, along with palpation of turgid uterine horns; these techniques' efficiency varies according to the technician's experience.

However, the oestrus signs are not consistent in different animals. They are affected by extrinsic factors like photoperiod, humidity, ambient temperature, nutrition, and management, and intrinsic factors like genetics, clinical condition, age, and body condition score. Poor oestrus detection is still an essential factor for low reproductive performance; this can be compensated by removing the need for detection of oestrus by using fixed-time AI (FTAI) with the aid of oestrus synchronization or oestrus induction protocols (Seren and Parmeggiani 1997; Purohit and Rao 2018; Perera and Noakes 2019).

5.3 Pregnancy

Fertilisation occurs at the junction of the isthmus and the ampulla, resulting in a zygote and mitotic cell division initiates after that. The embryo reaches the uterus around 4-5 days after fertilisation, in the early morula stage, and the blastocyst formation is initiated. After the blastocyst hatches (around day 6-8), the embryo becomes dependent on the uterine environment for survival, adequate P_4 production and responsiveness by the uterus are mandatory. To maintain the P_4 production, luteolysis needs to be avoided. This is achieved with the Interferon-Tau (IFN-τ) production by the elongated embryo, which binds to the endometrium and inhibits the synthesis of OT receptors, blocking the production of $PGF_{2\alpha}$. IFN-τ can also attach to the apical portion of the uterine glands, inducing the synthesis of proteins that improve the uterine environment, bettering the chances of embryo survival (Campanile et al. 2009; Sharma et al. 2014; Perera and Noakes 2019).

The implantation process occurs in three different phases. The first one is characterised by a free-floating blastocyst undergoing elongation and reaching the uterine horn. In the second phase, the embryo projects villa-like structures into the crypts of uterine glands. These structures give a temporary adhesion and allow the absorption of the endometrial glandular secrete, which promotes the nutrition and further development of the embryo. The third and final phase of implantation is the formation of the placenta (Campanile et al. 2009; Sharma et al. 2014).

The bubaline placenta is defined as synepitheliochorial and cotyledonary. The placentomes reach 5-10 cm in diameter, and their numbers vary through 70-200 and tend to increase from the early stages of pregnancy to mid-pregnancy and decrease towards the end. The placentome branches less than in cattle, resulting in a different and less complex feto-maternal interdigitation in buffaloes (Figure 6) (Campanile et al. 2009; Sharma et al. 2014; Perera and Noakes 2019).

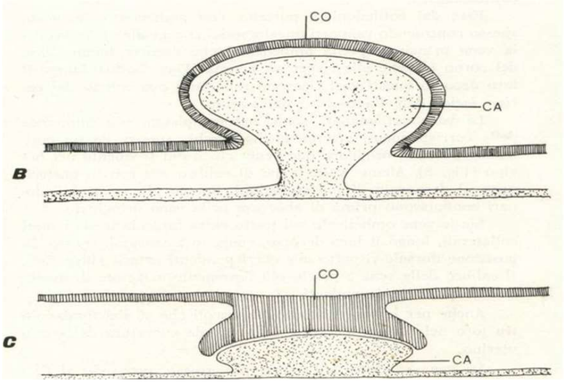


Figure 6. Representation of uterine placentomes, and the interaction between caruncles (CA) and cotyledons (CO) in cattle (B) and in buffalo (C) (Pelagalli et al. 1973).

The gestation length is around ten months, with a mean of 311 days (308-312 days), almost one month longer than that recorded in cattle. This period is also affected by the gender of the calf, parity, and season of calving. Gestation of male calves is 1-2 days longer than that of females. Heifers tend to have longer pregnancies than pluriparous cows. Pregnancies of winter and spring calvings take longer. Nutrition and management are other variables that can affect the duration of gestation. Longer gestations tend to deliver heavier calves, unlike heifers with more prolonged pregnancy and lighter calves (Purohit 2016).

During the first four months of pregnancy, the CL on the ovary ipsilateral to the gravid horn grows and increases in weight and during 30-150 days of gestation appears brownish. Most animals cease cyclical ovarian activity during pregnancy. However, sometimes, in early gestation, follicular growth continues in the ovaries, but the high P_4 bloodstream levels suppress the follicles from reaching an ovulatory size; some animals even exhibit gestational anovulatory oestrus (Purohit 2016; Perera and Noakes 2019).

5.3.1 Pregnancy Diagnosis

Pregnancy diagnosis can be obtained through several clinical methods such as non-return to oestrus, transrectal palpation and transrectal ultrasonography. The non-return to oestrus is the simplest but can over-estimate pregnancy by 10-20%, depending on the accuracy of oestrus detection. If the animal is not pregnant, there is no IFN-τ produced by an embryo to suppress the release of $PGF_{2\alpha}$, and the animal will return to oestrus. The poor oestrus signs expressed by this species heavily impact this method's accuracy, and a gestational oestrus may occur. However, it can give a negative diagnosis before the transrectal palpation or transrectal ultrasonography (Pawshe and Purohit 2013).

Transrectal palpation allows obtaining a positive pregnancy diagnosis through the palpation of uterine enlargement, amniotic vesicle, rebound effect, "membrane slip", placentomes, fetus and fremitus. From the second month of gestation, the pregnant horn feels enlarged and the uterine wall thinner, with the amniotic vesicle being palpable from 30-45 days. In the third month, the uterus is located on the brim of the pelvis, and its ventral side is not palpable; the slipping of the allantochorion ("membrane slip") can be felt, and the rebound effect is present. From the fourth month onwards, the uterus is abdominal, with palpable placentomes and fluctuations, and fremitus can be felt on the hypertrophied middle uterine arteries. During the sixth and seventh months, the fetus usually is out of reach due to the sinking in the abdominal cavity. By the end of the seventh month, the fetus approximates itself to the pelvic cavity, where fetal parts can be easily felt and distinguished as well as solid fetal movements. These movements can be stimulated to confirm fetal vitality by pinching the eyeball or grasping the nose through the rectal wall. The amount of fetal fluid before the 40th day is smaller than in cattle, making the diagnosis of early pregnancies more difficult in the buffalo. This method depends heavily on the technician's experience and is only considered accurate after 46 days of gestation (Pawshe and Purohit 2013; Perera and Noakes 2019).

Ultrasound scanning with a 5.0 or 7.5 MHz linear array transducer can detect a spherical anechoic embryonic vesicle with a height of around 11.7 mm. Sometimes, an echoic embryo can be visible soon as day 19. On day 26, the embryonic vesicle becomes irregular and elongated, and around day 25-29, heartbeat can be visualised to confirm embryonic viability. On day 40, the embryo measures around 33 mm; on day 42, differentiation of head and trunk is present. The genital tubercle becomes visible around day 55, and fetal sexing can be achieved. A complete fetus can be visualised from day 60 onwards, measuring over 64mm in length, and the umbilical cord and placentomes are observable. Although this method has a high sensitivity at day 30, re-evaluation after day 60 should be considered to discard possible embryonic deaths (usually occurring between day 28-60). The accuracy and speed of the diagnosis are affected by equipment used, operator proficiency, animal restraint and parity, with the early diagnosis being easier to make in heifers. The main advantages of performing a transrectal ultrasound scan over the other methods covered are achieving an earlier positive pregnancy diagnosis, evaluation of gestation viability, assessment of the correct development of the fetus and performing fetal sexing (Pawshe and Purohit 2013; Zambrano-Varón 2015).

5.4 Parturition and Puerperium

Parturition represents the end of gestation, and it is impossible to predict the exact time it will happen. However, there are signs of the proximity of parturition. Udder enlargement becomes apparent on the last seven days of pregnancy, being more marked on the final 2-3 days and 1.8 days before calving, the teats become engorged, and the mammary veins tense. Changes in the mammary gland are more noticeable in primiparous than in multiparous. The vulva becomes noticeably oedematous in the last 3-4 days of pregnancy and may present hanging mucus due to the liquefaction of the cervical seal. 24-36 hours before parturition, the vulva becomes highly loose and loses its transverse folds. The appearance of an elevated tail head is an indirect indicator of the relaxation and sinking of pelvic ligaments (sacroiliac and sacrosciatic), which occurs 2-3 days before calving to the sacroiliac ligament and 12-24 hours prepartum to the sacrosciatic ligament. Watery diarrhoea may be evident in the last two days of gestation. The correct evaluation of these signs and good data management can help to estimate the expected time for calving.

As labour approaches, the animals appear restless, isolate themselves from the herd, reduce their food and water intake, and begin to have uterine contractions. Similar to cattle, labour in buffalo is divided into three stages. The first stage is defined by the dilation of the cervix and can take up to 2 hours to fully dilate. The delivery of the calf marks the second stage. First, the allantochorion reaches the vulva, usually already ruptured, followed by the anterior legs of the fetus and the amnion; at this time, abdominal contractions are strong and easily noticeable. The most usual presentation of the fetus is the anterior longitudinal in the dorsosacral position. The calf usually is delivered in 30-60 minutes but may take up to 6 hours, especially in primiparous animals; the umbilical cord is ruptured before the calf reaches the ground. The newborn calf is sniffed and licked by the mother. The third and last stage of labour is the expulsion of the fetal membranes, which takes 4.3-9.1 hours (Das et al. 2013; Perera and Noakes 2019).

The return of the reproductive tract to its non-pregnant state is known as puerperium and begins immediately after parturition. The main events are uterine involution and restoration of regular cyclic ovarian activity. Involution of the uterus is complete when both uterine horns are equal in size, content-free, located in the pelvis, and usually takes 20-42 days. Lochia discharge usually lasts up to 3 weeks without problems during calving. The lochia changes as the puerperium advances, changing from pure blood (first 24 hours), chocolate colour (day 2-3), dirty amber (day 4-5), light grey to whitish-yellow, gradually decreasing in volume. The functional luteolysis of the CL of pregnancy is rapid, marked by the fast drop in P_4 after calving. However, structural luteolysis is slower and usually completed by day 30 postpartum. The first postpartum ovulation occurs around day 60. Shorter lengths of puerperium enable shorter inter-calving periods and are a crucial component in bettering the herd productivity (Das et al. 2013; Perera and Noakes 2019).

5.5 Melatonin and Reproductive Seasonality

The buffalo is considered a photoperiod-negative seasonally polyoestrous species. This came to be through natural selection of the calves born in the most favourable conditions, meeting nutritional and heat requirements for survivability (Zicarelli 1997).

The length and density of the light stimulus stimulate a multi-step neural pathway involving the retina, the suprachiasmatic nucleus (NSC), the superior cervical ganglia (SCSG) and the pineal gland. The NSC regulates the endogenous circadian rhythm, and this processed stimulus is forwarded to the pineal gland by the SCSG. The pineal gland is the primary regulatory organ in seasonality and acts by transducing the neuronal information into the rhythm of melatonin secretion that, through complex mechanisms, influences the hypothalamic-pituitary-gonadal axis. Darkness promotes melatonin release, and light suppresses it. This mechanism has been studied in-depth by Lincoln (1979) in the ovine species and is believed to be similar in the buffalo. What has been shown in the buffalo is that the administration of melatonin correlates to an increase in LH secretion. Melatonin also has a substantial antioxidant property that protects sperm, oocytes and embryos from free radical damage and apoptotic mechanisms and stimulates steroidogenesis. Females treated with melatonin respond better to oestrus synchronization protocols during the summer (Zicarelli 1997; D'Occhio et al. 2020).

The high melatonin levels produced during the night and their persistence are correlated to the photoperiod. However, animals that show different seasonal behaviour tend to show different circadian modifications of melatonin levels. Buffaloes raised on farms that show a seasonal trend (more than 15% of females unable to conceive in autumn) show a strong correlation between the plasmatic levels of melatonin and the photoperiod. Distinctly, animals raised on farms with calvings more evenly distributed throughout the year do not show an apparent melatonin increase during the night. This pattern is maintained even when individuals with a clear tendency toward seasonality are transferred to another farm where the animals exhibit less sensitivity to the photoperiod. Accordingly, seasonality tends to lie in the individuality of each animal. Heifers show a lesser difference in day/night melatonin values compared to adult females (Seren and Parmeggiani 1997; Zicarelli 1997; Vale et al. 2019).

In Italy, this photoperiod-negative seasonality translates into the concentration of calvings in the autumn and winter (Zicarelli 1997).

During the spring and summertime, the market demand for mozzarella cheese reaches its peak, coinciding with the lowest calving incidence (and milk production). This presents a tremendous economic hurdle for buffalo production since the price during spring-summer is substantially higher than during autumn-winter, forcing some breeders to modify the calving calendar to meet market demand for milk and enable higher profitability. To achieve this modification, the out-of-breeding-season-mating (OBSM) technique must be successful (Zicarelli 1997).

The OBSM method or calving “deseasonalisation” consists in interrupting sexual promiscuity during the months when conception is undesired (October-February) and leaving the bull in the herd from March to late September, concentrating the calving calendar between late January and early August. This method can be applied in two diverse ways: drastically or gradually (diluted in 3 years). How the OBSM strategy is enforced depends significantly on the degree of herd seasonality, availability of pubescent heifers, and the commercial conditions of the farm's area. The OBSM method reduces fertility and increases the inter-calving interval, reducing the herd’s efficiency, mainly in farms where this technique has been used for less time. The better results this strategy gets over time are obtained from the culling of the animals that do not respond well to it, enforcing selection over the individuals with less tendency to reproductive seasonality. The animals that respond better to the OBSM method are the ones that show a lower increase in melatonin values after sunset. For this reason, it may be possible to use melatonin plasma levels to mark the animals’ sensitivity to the photoperiod and their responsiveness to the OBSM technique. Other than the OBSM method, assisted reproductive technology (ART), such as oestrus synchronization protocols coupled with FTAI, has successfully combat the fertility decrease caused by longer day lengths (Zicarelli 1997; D’Occhio et al. 2020).

5.6 Anoestrus

Anoestrus is a state of sexual inactivity with the absence of oestrus expression. The ovaries are presented as moderately inactive; as discussed before, ovarian cyclicity is diminished by an ongoing gestation and longer photoperiods, leading to the anoestrous state. Other physiological reasons for this phenomenon are the presence of offspring/lactation and nutrition. Lactation anoestrus results from the inhibition of gonadotropin secretion, which determines the release of basal levels of LH. This occurs to prevent another gestation during the calf’s nursing and is influenced by the calf's visual, auditory, or olfactory recognition. Negative energy balance can also cause anoestrus, as the animal prioritises its energy to maintain basal metabolism, circulation, neuronal activity, and thermoregulation, reducing milk production, locomotion, growth and ceasing reproductive activity (Zicarelli 1997; Prasad and Purohit 2015).

6. Oestrus Synchronization Protocols/Reproductive Technologies

The buffalo is considered to have low reproductive efficiency due to late puberty, poor oestrus expression, seasonal anoestrus, high incidence of embryonic losses, and long inter-calving periods. The control of ovarian follicular development and ovulation has seen some success and continues to be one of the key strategies to improve reproductive efficiency in buffaloes. Controlling ovulation allows the performance of FTAI, overcoming the difficulty of oestrus detection in this specie. Most oestrus synchronization protocols are based on those developed for cattle and can take two approaches: manipulating the peripheral P_4 concentration or manipulating follicular growth and timing of ovulation.

Protocols that aim to manipulate the peripheral P_4 concentration are based on the control or the mimicry of the CL lifespan by shortening or prolonging the luteal phase using prostaglandins ($PGF_{2α}$) or progestogens, respectively. In comparison, protocols that aim to manipulate follicular growth and timing of ovulation are based on recruiting ovulatory follicles and preventing the further development of persistent dominant follicles regardless the oestrous cycle stage present at the time of treatment (Barile 2016; Baruselli et al. 2018; Ahmad and Arshad 2020).

6.1 Manipulating Peripheral Progesterone Concentration

6.1.1 Prostaglandin $F_{2α}$

$PGF_{2α}$ and its analogues are an effective and economical way to promote the luteolysis of the CL and have seen some use to help better oestrus detection and enable ART. $PGF_{2α}$-induced luteolysis can only occur in the presence of a functional CL (Day 5 to 17 of the oestrous cycle) and takes 24h to cause a drop in circulating P_4. Ovulation occurs up to 6 days after $PGF_{2α}$ depending on the size and responsiveness of the CL, circulation P_4 levels and the phase of follicular growth at the time of $PGF_{2α}$ administration. The animals with lower P_4 levels and smaller CL showed lesser responsiveness to $PGF_{2α}$ treatment. At the time of the $PGF_{2α}$, animals with a dominant follicle will return to oestrus in 2-3 days, and those that do not, will take around five days. In the one-shot protocol, there is only one injection of $PGF_{2α}$ after ultrasound confirmation of the presence of a CL, and the animal begins the oestrus around 60h later. No difference in oestrus behaviour and endocrine changes was recorded between luteolysis after prostaglandin treatment and natural oestrus. The two-shot protocol (Figure 7) comprises 2 $PGF_{2α}$ injections 11-14 days apart, does not need prior ultrasound CL identification and maximises the number of females in oestrus after the second $PGF_{2α}$. Females can undergo FAI 72 and 96h after the second injection of $PGF_{2α}$ or be inseminated at observed oestrus. However, buffaloes' poor oestrus expression and high variability of prostaglandin-only treatments in the interval between treatment and ovulation, translate in a poor efficiency of FTAI protocols. The time from the treatment to oestrus varies from 48 to 144h and to ovulation from 60 to 156h. With values of 85-90% of oestrus induction post-treatment and 50% pregnancy rate (PR) during the breeding season being recorded. Values over 25-30% of PR when out of the breeding season are rare. Synthetic and natural prostaglandins show the same results. Prostaglandin-only protocols need the presence of a functional CL; because of this, they do not result in prepubertal heifers or anoestrus buffalo cows and should be used during the breeding season to avoid the acyclic animals during days with longer photoperiods. The two-shot protocol can sometimes be used as a preliminary screening (Pre-synch) of animals to select those females responsive to the treatment to be synchronized (Barile 2016; Baruselli et al. 2018; Neglia et al. 2020).

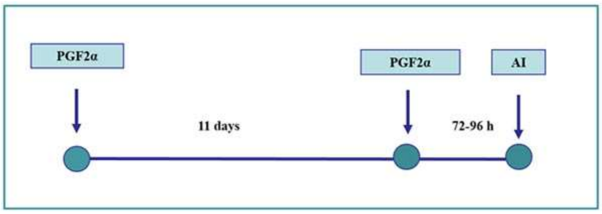


Figure 7. Two-shot protocol with two $PGF_{2\alpha}$ injections 11 days apart and AI 72-96 hours after the second injection of $PGF_{2\alpha}$ (Barile 2016).

6.1.2 Progesterone and progestogens

Progestogens can be administered orally (melengestrol acetate), subcutaneously (ear implants of norgestomet) or with an intravaginal progesterone delivery device (PRID/CIDR), which is the most practical method and the only one that can be used in Italy. The most commonly used intravaginal progestogen devices are the PRID® (Progesterone Releasing Intravaginal Device) and the CIDR® (Controlled Internal Drug Releasing device), with 1.55 g and 1.38 g of progesterone, respectively (Neglia et al., 2003; Barile 2016).

In the buffalo, Progestogens and their synthetic derivatives (progestins) have been shown to induce oestrus in pre-pubertal heifers and buffalo cows during postpartum anestrus, bettering reproductive efficiency in the herd by reducing the age at which puberty is reached and inter-calving time. Additionally, allow FTAI and fertility improvement when out of breeding season, which is the primary reason for its use in Italy. High values of P_4 exert negative feedback by reducing the frequency of GnRH pulses, leading to a reduction in the synthesis of GnRH receptors and, therefore, a lower sensitivity of the pituitary to GnRH. This results in a lower secretion and augmented storage of gonadotropins and higher GnRH-induced LH release. The lack of gonadotropin secretion prevents follicular maturation and ovulation, preventing oestrus (Zaabel et al. 2009; Barile 2016; Neglia et al. 2020).

When the treatment ends, the circulatory concentration of P_4 drops, stopping the negative feedback and promoting GnRH release, followed by LH and FSH release, with the ovulation of the DF present. To synchronize a group of animals in disregard to the stage of the oestrous cycle that they are in, it is needed to apply a treatment with progestogens or progestins for a period as long as the natural luteal phase to prevent the possibility of the CL outliving the therapy. However, long-term treatments show low conception rates (CR). For this reason, short-term therapies (7-10 days) (Figure 8) are preferred. However, they do not interfere with spontaneous luteolysis, and so a luteolytic agent should be included in the protocol, which can be E_2 or $PGF_{2\alpha}$. Equine Chorionic Gonadotrophin (eCG) [or Pregnant Mare Serum Gonadotrophin (PMSG)] is a follicle-stimulating hormone and when administered at the moment of intravaginal device (IVD) removal induces oestrus in acyclic buffaloes, promoting the efficiency of the ovulation synchronization protocols during the out-breeding season (Barile 2016; Neglia et al. 2020).

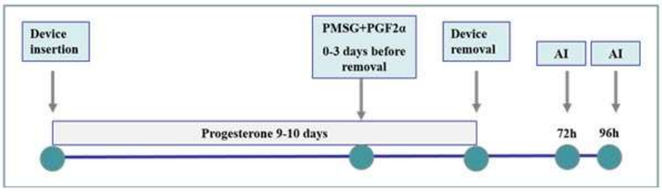


Figure 8. Short-term P_4 therapy with the association of $PGF_{2\alpha}$ and PMSG with double AI at 72 and 96 hours after intravaginal device removal because of the variability of the time of ovulation (Barile 2016).

When treated with 1.4-1.6 g of natural P_4 with a PRID/CIDR for 9-10 days, values of circulating P_4 reach 4-5 ng/mL. However, circulating P_4 levels vary with the quantities of endogenous and exogenous P_4 as well as with the amount metabolized by the liver, which is correlated to the dry matter intake, being a reason for the variability in results in different studies (Neglia et al. 2020).

Barile (2016) suggests that FTAI after PRID treatment should consider this species' seasonality for better results. During the spring (out of breeding season), AI should take place 72 and 96 h after PRID removal, and during autumn (during breeding season), 48 and 72 h after removal.

In cattle, the reuse of CIDR is possible. However, Zaabel et al. (2009) evaluated the efficacy CIDR P_4 and its reuse in buffalo cows in the treatment of postpartum anestrus. They recorded induction of oestrus in 42.9% of the animals treated with CIDR for 7 days + intramuscular (i.m.) injection of $PGF_{2\alpha}$ on the 6th day; 85.7% in animals treated with CIDR for 14 days + i.m. injection of $PGF_{2\alpha}$ on the 13th day; and 100% in animals treated with CIDR for 7 days + i.m. injection of $PGF_{2\alpha}$ on the 6th day + i.m. injection of GnRH 24h after CIDR removal. These results suggest that seven days may not be enough to promote an efficient synthesis and storage of GnRH to induce follicular development and ovulation; this may be covered by the additional i.m. injection of GnRH, which showed very promising results in this study, unlike the reuse of the CIDR that showed poor results.

However, more recently, Gutiérrez-Añez et al. (2018) when comparing two different P_4-releasing intravaginal devices in synchronization protocols for FTAI, did not find any significant difference between new and reused (one or two times). The only significant difference recorded was between the use of 1.0 g of P4 (DIB®) and 1.38 g of P4 (CIDR®), with PR obtained being higher in the animals under treatment with DIB®. This possible reuse of the IVD without compromising the FTAI results, could help lower costs and improve the profitability of farms.

6.2 Manipulating Follicular growth and Timing of ovulation

FTAI programs need an ovulation inducer to synchronize the ovulation post-luteolysis; these typically are GnRH, LH, human chorionic gonadotropin (hCG) and E_2 esters. By using an association of prostaglandin and GnRH, it is possible to synchronize the follicular wave and consecutive oestrus in cattle. The GnRH-induced LH release results in the ovulation or luteinisation of the DF, leading to the emergence of a new follicular wave, with the following $PGF_{2\alpha}$ administration promoting CL regression. The low values of circulation P_4 promote the development of a new DF and the onset of oestrus and ovulation (2-3 days after) (Barile 2016; Baruselli et al. 2018).

These insights about the follicular growth and manipulation of follicular waves led to the development of the oestrus synchronization program called Ovsynch by Pursley et al. (1995). The Ovsynch program (Figure 9) is comprised of an injection of GnRH (Day 0), an injection of $PGF_{2\alpha}$ (Day 7), and a second injection of GnRH to promote the synchronization of ovulation (Day 9), allowing FTAI 16-20h after the last GnRH administration. This program has been instrumental in enabling FTAI in cattle without the need for oestrus detection (Barile 2016).

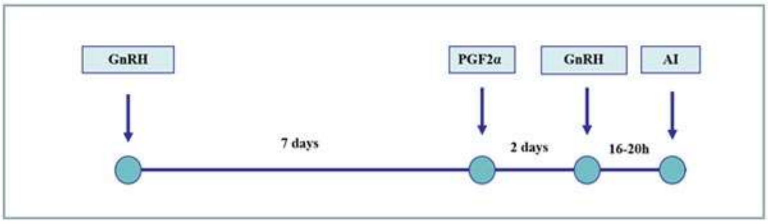


Figure 9. Ovsynch program with a first GnRH administration followed by $PGF_{2\alpha}$, 7 days later, and a second GnRH, 2 days after the $PGF_{2\alpha}$, and a single AI 16-20 hours after the last administration (Barile 2016).

To work as intended, a DF needs to be present at the time of the first GnRH administration; if a DF is not present or is physiologically immature, as it happens during the recruitment phase or early selection, it will not work, and as such follicular status should be evaluated by ultrasound or the animals should be pre-synchronized. This limitation is the reason why it is not advised to use the Ovsynch program on pre-pubertal heifers and acyclic animals (Barile 2016; Neglia et al. 2020). Baruselli (2001) documented a CR of 48.8 % during the breeding season (autumn-winter) and 6.9 % during the non-breeding season (spring-summer) in Murrah buffaloes and this can be justified by the higher percentage of acyclic buffaloes when out of the breeding season. De Rensis et al. (2005) recorded 35.7 % of CR in cyclic buffaloes and only 4.7% in acyclic buffaloes. Derar et al. (2012) evaluated the effect of parity and recorded ovulation after the second administration of GnRH in 100% of buffalo heifers and 88.8% of buffalo cows. Heifers showed higher values of P_4 on day 7. Buffalo cows ovulated earlier (10.4±7.6h) than heifers (22.6±5.4h), and the CR was 62.5% in heifers and 22.7% in adults. The low CR of buffalo cows could be explained due to a less functional CL and premature ovulation and they concluded that the Ovsynch program could be better in buffalo heifer than in buffalo cows.

Neglia et al. (2016) recorded that 60.4% of buffaloes ovulated after the first administration of GnRH. These animals showed a smaller area of the ovulatory follicle, a larger CL area, and a greater blood concentration of P_4 on day 0. Luteolysis after $PGF_{2\alpha}$ administration was evidenced in these females more than in the ones that did not ovulate after the first injection with GnRH (98.6% and 84.4%, respectively); the same occurred with the response to the second GnRH (92.8 % and 80.4%). On day 27 after FTAI, 75.7% of animals that responded to the first GnRH were pregnant, and only 30.4% of those who did not, late embryonic mortality was also higher in the latter (13.2% and 28.6%). The animals that ovulated with the first GnRH had the largest follicle, smaller than those that did not ovulate, indicating that they were in the early stages of the oestrous cycle (days 5-9), because less follicular growth had occurred to that point. It was concluded that ovulation after the second GnRH and being at oestrus at the time of FTAI were not as predictive of pregnancy per AI (P/AI) as whether the female ovulated after the first GnRH or not, being this the better predictor of P/AI. This is explained by the optimum state of the oocyte to undergo FTAI and develop embryonically resultant of the second GnRH administration, present in animals that responded positively to the first GnRH.

Campanile et al. (2008) evaluated the use of a GnRH agonist at day five after AI during the non-breeding season, with 60% of buffaloes ovulating and forming an accessory CL, increasing P_4 secretion and PR. It was recorded a significant variability in the size of the follicles that ovulated (4.2-13.0 mm with an average of 8.9 mm) and in those that did not (4.0-12.0 mm with an average of 8.7 mm), suggesting heterogeneous maturational stages and gonadotropin receptors status in ovulatory-size follicles.

The hCG has similar biological activity as LH; however, it persists longer in the circulatory system, and the CL formed after its use tends to have greater P_4 production. This could translate into a higher CR; however, Carvalho et al. (2007) did not evidence any positive effect with the substitution of the last GnRH on the Ovsynch program with hCG, but higher plasmatic P_4 concentrations were recorded as expected.

The eCG promotes the final follicular development and the production of P_4 and improves ovulation response to synchronization protocols. This results in larger DF and corresponding CL diameters, higher P4 concentration in the following diestrus and, consequently, better pregnancy outcome. It can be administrated seven days after the beginning of the treatment or at the time of CIDR/PRID removal with AI 72 and 96h after. By associating eCG with a P_4-based protocol, it is possible to induce the cyclicity of anoestrous buffaloes. E_2 has a very limited ability to induce luteolysis (3-5 days after ovulation) and has been the target of restrictions in EU regulations; for these reasons, $PGF_{2\alpha}$ is the most used luteolytic agent. When used correctly, all these ovulation inducers can produce positive results, with different intervals from administration to ovulation: LH (24h), hCG (24h), GnRH (26-28), and E_2 benzoate (44h). When compared to GnRH, it has been shown that hCG will promote the higher secretion of P_4 and that using E_2 will result in a higher peak of LH (Barile 2016; Baruselli et al. 2018; Neglia et al. 2020).

Some variations of the Ovsynch program are possible such as the association of P_4 from day 0 to day 7 of the treatment, resulting in an increase of 25% in the PR in non-cyclic buffaloes. The "G6G protocol" is made by adding a $PGF_{2\alpha}$ and a GnRH 8 and 6 days before the Ovsynch program, increasing PR and ovulation to the first GnRH of the Ovsynch. The Ovsynch-plus program results from the association of an injection of eCG three days before the Ovsynch and has shown better results in anovulatory buffaloes. The addition of an administration of $PGF_{2\alpha}$ on the day of the AI has also been shown to improve PR. The substitution of the last GnRH injection of the Ovsynch program by LH on the same day or by E_2 24h after $PGF_{2\alpha}$ (Heatsynch protocol) did not show better results. The two-shot protocol of $PGF_{2\alpha}$ (Pre-synch) can also be used to ensure the presence of a DF at the time of the first GnRH administration (De Rensis and López-Gatius 2007; Neglia et al. 2020).

7. Male Buffalo and Semen Production

Buffalo bulls are chosen to become semen donors based on progeny records and pedigree. Milk yield and weight at birth are highly heritable traits, and the same is not seen with reproductive traits. For bull selection, a complete breeding soundness evaluation needs to be made, which includes a physical evaluation, the animal needs to have a good body conformation, scrotal circumference should be measured, good elasticity of the scrotal skin and the testes size uniformity confirmed, and semen samples evaluated. Before being selected for an AI Centre, bulls need to be tested for brucellosis, campylobacteriosis, Bovine Virus Diarrhea, Leptospirosis, Tuberculosis and any other disease present in the area. This occurs around 18 months of age (Vale et al. 2014; Henry et al. 2017; Perera and Noakes 2019).

Semen collection is usually made with the aid of an artificial vagina. Electroejaculation is also possible. However, transrectal massage showed poor results in volume and concentration with higher pH in the semen collected. The artificial vagina should be kept at 44-46ºC, and it should be made a collection of 2 ejaculates with an interval of 30 minutes. 1-4 collections/week/bull can be made without a noticeable drop in fertility. After collection, the semen needs to go immediately to a 37ºC water bath, and its quality evaluated. Buffalo semen is supposed to be milky white to creamy with a light blue tinge in colour, 1.3-4.5 mL of volume (young bulls: 1-3mL; adult bulls: up to 8 mL), and a slightly acidic pH (6.2-7.0). The ejaculate should be evaluated under the microscope: gross motility is lower when compared to cattle, with an evaluation of 2.4-4 (on a scale of 0 to 5) being normal; individual sperm motility is also evaluated, which is a visual estimate of the percentage of motile sperm; viability is evaluated as eosin nigrosin stained smears; and abnormalities of the head, middle piece and tail counted. 25-66% of static ejaculates are average, meaning that freshly collected semen does not show any mass activity but regains motility when an extender is added. As with the female buffalo, buffalo bulls are affected by the season and the climate, having lower libido and producing less semen and poorer quality ejaculate during the summer (out of breeding season) (Vale et al. 2014; Henry et al. 2017; Perera & Noakes 2019).

Buffalo semen, compared to cattle's, has a higher concentration of calcium, chloride, alkaline phosphatase and acid-soluble phosphorus and lower a concentration of total electrolytes, protein, cholesterol, ascorbic acid, citric acid and fructose. For this reason, extenders for semen preservation need to be adapted to buffalo semen. At 5ºC, semen is conserved for 72h, and citrate-based extenders are best for liquid storage of buffalo semen. For cryopreservation, extenders are made of a permeable cryoprotectant (glycerol, propylene glycol, ethylene glycol or dimethyl sulfoxide), non-permeable cryoprotectants (egg-yolk), antibiotics (benzylpenicillin 1000 IU/mL and streptomycin sulphate 1000 µg /mL), and some additives. Some common combinations are lactose-egg yolk-glycerol, lactose-fructose-egg yolk-glycerol and tris (hydroxy-methyl-amino-methane)-egg yolk-glycerol. Egg yolk concentration has little to no benefit on post-thaw motility in being over 5% and augments viscosity, diminishing forward motility; glycerol is kept between 5 and 7% (Vale et al. 2014; Perera & Noakes 2019).

Extended semen is then placed in 0.25 or 0.5 mL straws, with Patil et al. (2020) demonstrating good results with a dilution of up to 12 million sperm in a 0.25 mL straw, which allows the production of more semen doses per ejaculate than the 20 million sperm in a 0.25 mL straw that is generally accepted as enough to produce acceptable fertilization. They did not evidence any significant differences in conception rate between the 12 and 20 million sperm per 0.25 mL straw.

Sexed-semen is obtained by separating X and Y-bearing sperm using high-speed flow cytometric cell sorting and stored with 4 million sperm in 0.25 mL straws. 38.8-49.8% CR is expected with insemination and an accuracy of 90% in the sex of the calf (Vale et al. 2014; Perera & Noakes 2019).

The semen is exposed to freezing temperatures of the nitrogen vapours (-120ºC to -140ºC), 4 centimetres above liquid nitrogen, for 10-20 minutes. Then it can be conserved in the liquid nitrogen (-196ºC) for an undetermined time. When manipulating frozen semen, it is essential not to expose it to temperatures higher than -80ºC (critical temperature). Semen must be thawed prior to its use by being immersed in water at 37–40 ºC for 10-20 seconds, and excess nitrogen should be flicked out of the straw before immersion. It is expected for post-thawed semen to maintain 35-60% of motility and to have a shorter lifespan in the female reproductive tract than fresh semen. After being thawed, semen should not be exposed to temperatures below the temperature of the water-bath (Vale et al., 2014; Perera & Noakes 2019).

7.1 Artificial Insemination Technique

AI (Figure 10) is considered a reliable technology for bettering genetic progress and helping venereal diseases control (Baruseli et al. 2018).

If using frozen semen, after it is thawed, the straw must be dried prior to being loaded into the insemination gun. Protection sheet must be used at all times, and sanitary smock should be used when possible. The AI technique in buffaloes is the same as the one used in cattle, with the rectovaginal method being the most commonly used, in which one hand is inserted into the rectum and grasps the cervix through the rectal wall. The catheter is then passed into the vagina and manipulated through the cervix, and the insemination is made in the uterine body. When using sexed-semen, AI in the uterine body has seen better results than AI in the horn ipsilateral to the ovary that had ovulated, possibly to the more significant trauma inflicted during the latter AI technique. This technique requires much practice to be commercially viable. Previous cleaning of the vulva should be made with single-use paper tissue to prevent potential infections (Vale et al. 2014; Parkinson and Morrell 2019; Perera & Noakes 2019; Presicce et al. 2020).

Figure 10. Artificial insemination technique in the buffalo (Original).

8. Female Infertility

Buffalo female infertility is a product of delayed sexual maturity, prolonged postpartum anestrus, high rates of repeat breeders and abortions.

Repeat breeders (RB) result from fertilisation failure or early embryonic death due to anatomic defects that do not allow the passage of gametes, ovulation dysfunction or genital infections. A female can be considered RB after three consecutive AI without conceiving and without any change of the interoestrual interval. This is more common in larger herds and less in heifers than in buffalo cows, up to the third calving, since RB animals are generally culled from the herd (Purohit et al. 2016; Perera and Noakes 2019).

Non-specific uterine infections, clinical or subclinical endometritis, are the leading cause of RB in buffaloes and are due to a lack of hygiene in the management of calving, persistent infections from the postpartum, or the introduction of pathogens in the vagina. The most commonly isolated bacterias are *Trueperella pyogenes, E.coli, Fusobacterium necrophorum and Bacteroides spp.* (Perera and Noakes 2019). Salzano et al. (2020) found that 90% of RB buffaloes, animals that did not conceive within 300 DIM, had the presence of moderate or severe endometritis. Other common causes for RB are nutritional deficiencies, luteal dysfunction with retarded ovulation or anovulation, and problems with the AI technique like the lack of experience of the inseminator, insemination occurring mid-cycle, and impaired quality of semen (Perera and Noakes 2019).

Embryonic mortality (EM) rates are one of the most important causes of infertility in the buffalo, especially when out of breeding season, with 20-40% of animals experiencing embryonic loss, after AI, with higher rates between day 28 and 60 of gestation. Insufficient P_4 production is the reason behind most early embryonic deaths, but it can also be due to oviductal and uterine dysfunction or when the mother is exposed to stress. Early diagnosis is vital by allowing rebreeding and can be made by measuring a drop in circulating P_4 or transrectal ultrasound evaluation. Treatments in early pregnancy stages that are used with good results in cows do not show the same results in the buffalo. This is probably due to EM occurring later in the buffalo. However, treatments with P_4 starting on day 25 of gestation have shown some promising results in lessening EM rates (Campanile and Neglia 2007; Purohit et al. 2016).

Standard abortion rates are usually around 1-6% and have infectious and non-infectious causes (Perera and Noakes 2019).

Specific infections that lead to abortion in buffaloes (Figure 11) are the same as in cows, however, representing a lesser problem in the former. Even though the occurrence rates of these infections are low, they still represent significant economic losses by the costs of the calf that is lost, reduction in productivity, testing and treatment, and the substitution of the mother if it has to be culled. Bovine viral diarrhoea, infectious bovine rhinotracheitis (IBR), brucellosis, bovine venereal campylobacteriosis, listeriosis, leptospirosis, neosporosis and trichomonosis are the most critical infections in the buffalo. IBR can cause abortion storms that can be responsible for abortion rates going as high as 60%. Trichomonosis is more easily transmitted in herds that use natural mount for breeding since the bulls show no symptoms and the protozoan parasite persistently infects the prepuce (Mahechanadani et al. 2018; Perera and Noakes 2019).

Abortion can be a result of several non-infectious causes. Trauma during the early stages of pregnancy, such as AI during gestational oestrus, heat stress that leads to fetal hyperthermia causing hypotension, hypoxia and acidosis, genetic or chromosomal abnormalities, ingestion of poisonous plants, inadequate nutrition, hormonal imbalance or administration of abortifacient drugs are some of the most common non-infectious causes of abortion in buffalo (Kumar et al. 2015).

Figure 11. An aborted buffalo fetus (Original).

Retained fetal membranes (RFM) are the most common disorder in postpartum. After parturition, the reduction in blood flow to the uterus and contractions of the uterus results in a loosening of the fetal cotyledon. However, if fetal membranes are still present 8-12 h after birth, they are considered retained. This is considered to be a bigger problem in buffaloes than in cows. RFM present a significant risk for uterine bacterial infections. If these infections go untreated, they may give origin to an acute systemic reaction, with loss of appetite, hyperthermia, drop in milk production, and delayed resumption of regular ovarian activity postpartum. Metritis and endometritis represent the most important postpartum disorder in the buffalo since they are responsible for significant economic losses due to the longer open days and inter-calving intervals (Azawi 2013).

Genital prolapse (Figure 12) is the third or fourth most common problem in the buffalo; protrusion of the vagina through the vulva is common during late gestation and postpartum and may even occur in non-pregnant buffaloes. Uterine prolapse is only evidenced in postpartum. Genital prolapse occurs due to a relaxation of the constrictor vestibuli muscle and atony of the vaginal musculature. Rough manipulation during calving and postpartum stimulates contractions and predisposes the occurrence of prolapse. Hypocalcaemia, prepartum vaginal prolapse and dystocia are the buffalo's major risk factors for uterine prolapse (Purohit et al. 2019).

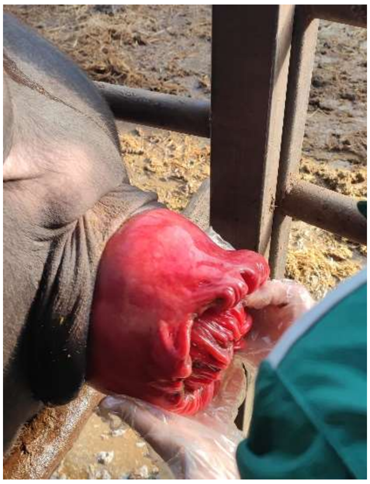


Figure 12. Vaginal prolapse in a postpartum buffalo (Original).

Ovarian problems are less common in buffaloes than in cattle, with cystic ovaries representing only 6% of reproductive failure in the buffalo (Perera and Noakes 2019).

9. Experimental Work

9.1 Materials and Methods

The focus of this study was to evaluate the influence of days after calving on the efficiency of P_4-based treatments in correcting the anestrus state in buffaloes during the out-of-breeding season. This research is a retrospective work based on data collected between April and July 2021 by the Department of Veterinary Medicine and Animal Production of the University of Naples Federico II. It qualifies as inductive research as the theory is built on the data collected. By basing the theory on the data evidence, some subjectivity is ruled out.

9.1.1 Farm

The present study was conducted on a commercial farm with a population of about 900 buffaloes, located in the South of Italy (between 39.0ºN and 41.5ºN). Included in this study were 276 primiparous adult buffaloes with a weight of around 550 Kg, and body condition score (BCS) averaging 7.8 on a 1 to 9 scale, and 12.1 Kg of average MY.

9.1.2 Reproductive management

The reproductive management utilised both AI and natural mating. Adult female buffaloes were kept in groups of 60 animals in cement open yards with at least 15 m^2/head. At this farm, mating was only allowed from February to September to concentrate deliveries and maximum milk production from January to August (OBSM technique). A 40 days voluntary waiting period (VWP) is respected, after which natural mating is allowed by the introduction of 4 buffalo bulls per group, allowing a proportion of male and females of 1:15.

9.1.3 Experimental design and animal inclusion criteria

Only acyclic primiparous lactating buffaloes were selected for this study during the increasing photoperiod season (April to July).

After respecting the VWP of 40 days, all animals underwent gynaecological examinations by transrectal ultrasound to assess ovarian activity and uterine status. Evaluations were spaced by seven days. Only the females that did not show the presence of CL or a follicle larger than 0.7 cm in any of the three consecutive examinations were classified as being in anestrus and included in the study. The animals that showed inflammation of the uterus or indication of being affected by any other condition or did not meet the previously described criteria were excluded from the present study. Animals in which pregnancy was recorded underwent diagnosis confirmation two times (30 days apart) and then were excluded. The last inclusion criterion was the BCS between 7 and 8.5. The females included were subjected to ovulation synchronization protocol based on a P_4-releasing intravaginal device and FTAI.

The protocol carried out on the acyclic buffaloes was the following: On day 0, these animals received a GnRH agonist (buserelin acetate, 12 µg; Receptal®; Intervet, Milan, Italy) injection with the insertion of a 1.38g P_4 intravaginal device (CIDR®; Zoetis SRL, Rome, Italy). The device was removed ten days after and administrated cloprostenol (500 mg of Estrumate®; MSD Animal Health, Milan, Italy) to promote luteolysis and 750IU of eCG (Folligon; Intervet MSD, Milan, Italy). A second GnRH agonist was administered 48 hours later to synchronize the ovulation. FTAI was performed 16 h after the last GnRH, and on the same day, an ultrasound examination (MyLab 30Gold®; Esaote, Genova, Italy) was completed, and the size of the preovulatory follicle was recorded. The area of the preovulatory follicle was calculated based on the measurements of its major and minor axis with the following formula:

$$\boldsymbol{Formula\ 1: Follicle\ area = \frac{major\ axis}{2} \times \frac{minor\ axis}{2} \times \pi}$$

The AI technique was performed by the same veterinarian using frozen and thawed straws from the same buffalo bull.

Sperm progressive motility and viability were confirmed by analysis under a light microscope of a sperm straw from the same lot as the semen used for AI. Fifteen days after

AI, the inseminated animals were transferred to a larger group with bulls present to allow natural mating. On days 27, 45 and 90 after AI, underwent pregnancy diagnosis and two confirmations, respectively. If the buffaloes were not recorded as being pregnant, an ultrasound pregnancy diagnosis was carried out every 15 days. After insemination, the buffalo cows were categorised as pregnant at FTAI or pregnant at the 1st, 2nd, and 3rd oestrous cycles. This categorisation was only possible by the pregnancy diagnosis being performed with ultrasound since all the other alternatives did not provide reasonably confident differentiation between pregnant at FTAI or pregnant at the subsequent oestrous cycles. A schematic representation of the protocol and experimental design can be visualised on Figure 13.

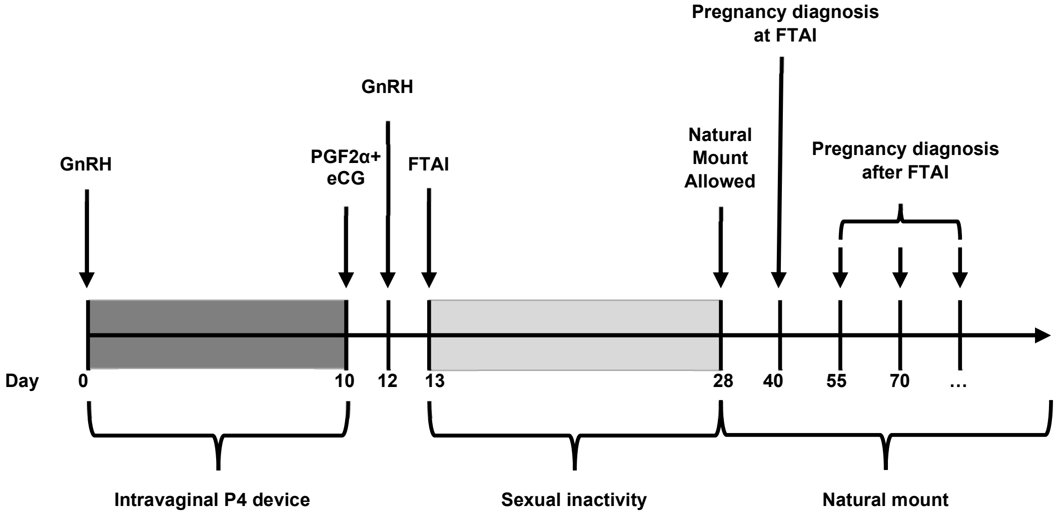


Figure 13. Representation of the ovulation synchronization protocol used and the experimental design of the study

9.1.4 Statistical analysis

Statistical analysis was performed with SPSS (28.0) (SPSS Inc., Chicago, IL, USA) on Windows 10. The buffalo cows were divided into three classes according to the postpartum time after their first calving and the phase of lactation they were at, represented by their days in milk (DIM): from 61 to 90 DIM (n=86), from 91 to 150 DIM (n=102), and from 151 to 200 DIM (n=88), the catabolic phase of lactation, and the first and second anabolic phases of lactation, respectively.

Pregnancy rates were recorded according to the oestrous cycle post-synchronization the animals were in, DIM classes (I, II, and III) and the different months and then compared using nonparametric tests (Pearson and X-square). Analysis of Variance (ANOVA) was utilised to evaluate the difference between groups.

Multiple linear regression (stepwise procedure) was performed to evaluate the relation between DIM, BCS, follicular area, and the interval from treatment to conception. Multiple logistic regression (stepwise procedure) was used to assess the odds ratio for the pregnancy outcome by FTAI, with DIM, MY, BCS, follicular area, and month of calving included in the model as continuous variables.

Statistical significance was accepted if the difference of *p*<0.05.

9.2 Results

In order to evaluate the impact of days after calving in the reversion of the anestrus state in buffaloes by the use of P_4- based ovulation synchronization protocols, the data obtained from 276 acyclic primiparous animals is described in this chapter.

Firstly, the pregnancy rates obtained at FTAI, the three consecutive oestrous cycles post-insemination and the total at the end of the study are presented. Secondly, the pregnancy rates differentiated by the DIM classes are compared, followed by the comparison of the intervals between the mean interval from the end of treatment to conception (disregarding pregnancies at FTAI) within the same classes. Then are presented and compared the results of the follicle area between animals pregnant at FTAI and their non-pregnant counterpart, also divided into the three DIM classes. Pregnancy rates obtained in the different months of the study are presented after. The results of the linear regression analysis of the BCS and follicular area are presented, and finally, the results of the multiple logistic regression results are presented.

At the end of the study, a total PR of 88% was obtained. 61% (148/242) of pregnancies were obtained at FTAI, 22% (54/242), 10% (24/242) and 7% (16/242) were obtained at the first, second and third post-insemination oestrous cycles, respectively (Table 1). The only statically significant (*p*<0.05) different value was the PR after FTAI when compared to the pregnancy rates obtained from the three following oestrous cycles. The 34 non-pregnant females after the third post-insemination oestrous cycle were not subjected to any further clinical examinations.

Table 1. Pregnancy rates after FTAI, and natural mating during the first, 2nd, and 3rd oestrous cycles postinsemination. Different superscripts above values within the same row mean that the difference is statistically significant (a, b, p<0.05).

Animals	FTAI	1st	2nd	3rd	Total
Buffaloes inseminated (n)	**276**	**128**	**74**	**50**	**276**
Number pregnant (n)	**148**	**54**	**24**	**16**	**242**
Percentage Pregnant (%)	**54^a**	**42^b**	**32^b**	**32^b**	**88**

Cumulative Pregnancy Rate (%)	**54**	**73**	**82**	**88**	**88**

When the total PR was compared among classes of animals with different DIM at the start of the treatment, no significant differences were recorded. Total pregnancy rate recorded by classes I (61-90 DIM), II (91-150 DIM), and III (151-200 DIM) were 88, 92, and 80%, respectively. On the other hand, the PR at FTAI was numerically higher in Class II when compared to Class I (PR at FTAI: I=48%; and II=60%), however not statistically significant ($p=0.07$). In the first cycle post-insemination, Class II revealed a significantly ($p<0.01$) higher PR when compared to the other two classes. Buffaloes in class I showed a significant ($p<0.01$) lower BCS than those in classes II and III (BCS: I=7.6±0.1; II=7.8±0.1; and III=8.1±0.1).

If the buffaloes pregnant at FTAI are ignored, the females in Class I showed a significantly ($p<0.05$) higher mean interval from the last GnRH administration to the confirmation of pregnancy than those in classes II and III (I=48.2±4.1; II=27.5±2.4; and III=34.6±4.7 days).

At the day of FTAI, the preovulatory follicle area was measured and was significantly ($p<0.01$) greater in animals in Class II than those in other classes (Table 2). There was no significant difference recorded in the size of the pre-ovulatory follicle between pregnant and non-pregnant buffaloes within the different classes. However, buffaloes that became pregnant after FTAI had significantly ($p<0.01$) larger follicles when compared to those who did not.

Table 2. Recorded areas of the dominant follicles on the day of the FTAI in pregnant (P) and non-pregnant (NP) buffaloes according to the different days in milk at the beginning of the treatment (Class I:<90 DIM; Class II: 91-150 DIM; Class III: >151 DIM). Different superscripts above values within the same column (x,y) and the same row (a,b) mean that the difference is statistically significant (p<0.01). The standard error was <0.05 in all data values.

Class	**P FTAI (cm)**	**NP FTAI (cm)**	**Total (cm)**
I	**1.2^{x}**	**1.1^{x}**	**1.2^{x}**
II	**1.6^{y}**	**1.5^{y}**	**1.6^{y}**
III	**1.2^{x}**	**1.2^{x}**	**1.2^{x}**
Total	**1.4^{a}**	**1.3^{b}**	**1.3**

When comparing PR in buffalo cows treated in the different months of the year, few differences were recorded. However, females treated in June had a significantly ($p>0.05$) higher PR than those treated in July. Furthermore, the PR at FTAI and by natural mating during the first cycle post-insemination was significantly ($p<0.05$) higher in June compared to May.

The multiple linear regression analysis revealed a significant ($p<0.01$) correlation between BCS at FTAI and the follicular area ($r^2=0.24$), which followed the resulting equation:

$$\boldsymbol{Equation}\ 2\text{:}\ \boldsymbol{Follicular\ Area} = -\mathbf{0.30} + (\mathbf{0.26} * \boldsymbol{BCS})$$

Lastly, the multiple logistic regression analysis (Table 3) revealed the PR outcome at FTAI was influenced by the follicular area (odds ratio=2.24; $p<0.05$) and BCS (odds ratio=1.26; $p<0.05$) at FTAI, with no other variable having significant influence.

Table 3. Results of the logistic regression for the risk of pregnancy at FTAI. Considered factors: days in milk (DIM), body condition score (BCS), dominant follicle area (FL area), month of calving (Month calv.), and milk yield (MY).

Variable	**Coefficient**	**Odds Ratio**	**95% Conf.Int.**	**p Value**
Constant	**-2.43**	**0.09**	**64.35**	**0.47**
DIM	**0.15**	**1.16**	**1.81**	**0.51**
BCS	**0.1**	**1.22**	**2.31**	**0.03**
FL area	**0.7**	**2.01**	**3.79**	**0.01**
Month calv.	**0.19**	**1.21**	**1.8**	**0.35**
MY	**0.04**	**1.04**	**1.14**	**0.29**

9.3 Discussion

Anoestrus is one of the main obstacles to the reproductive efficiency and profitability of buffalo farms, occurring naturally during the out-of-breeding season. Solutions to improve fertility during the months of longer days are a main goal. Hormonal treatments with P_4 were used with success over the past few years to obtain shorter inter-calving intervals by overcoming the anoestrus state and restoring ovarian activity. To optimise the use of these ovulation synchronization protocols, it needs to have a great understanding of the animal response. Some variables such as days after calving have not been studied in detail, which is the main focus of the present study.

The buffalo species is known to show a reproductive seasonal activity towards the months with decreasing daylight hours, naturally concentrating the calvings in the autumn and winter in Italy. This occurs in order to enhance the chances of survivability of the offspring by having births during the most favourable conditions of less heat stress and higher feed availability, with free-range conditions.

Long periods of darkness promote melatonin release, and light suppresses it. This hormone’s administration promotes LH release in the buffalo. For this reason, melatonin has been the hormone most associated with the reproductive seasonality in the buffalo and other species alike. During the hotter months, the longer photoperiod inhibits melatonin release and these animals enter a state of sexual inactivity with a pause in ovarian activity.

Buffalo milk is mainly used for the production of mozzarella cheese, which the market demand peaks during spring and summer. The natural mating period of the buffaloes is in disagreement with the demand for buffalo milk, becoming the biggest hurdle to buffalo production in Italy. To combat this, the OBSM method is used, which consists of the interruption of mating during the months of October through February and allowing it from March to September, concentrating calvings in the January-August period. However, the use of this option results in decreased fertility and longer inter-calving intervals since the animals are bred out of their natural mating season. This further emphasises the importance of treatments which can restore ovarian cyclicity.

The anestrus state has been correlated to insufficient secretion of LH by the anterior pituitary. The lack of circulating gonadotropins (FSH and LH) is responsible for the absence of follicular maturation and subsequent ovulation. The most used method to re-establish ovarian activity during the out-of-breeding season are P_4-based treatments (Carvalho et al. 2016). These treatments take advantage of the negative feedback exerted by the high values of circulating P_4 on the hypothalamus-anterior pituitary axis by reducing the frequency of GnRH and LH pulses (Karsch et al. 1987). This promotes a greater storage of gonadotropins in the anterior pituitary. This greater storage results in a higher GnRH-induced LH release. When the exogenous P_4 stimulus is removed, there is a great drop in circulating P_4, and the negative feedback ceases, resulting in a GnRH pulse. Followed by the release of the stored LH and FSH, and the promotion of follicle growth and ovulation with the restoration of oestrus and the return to regular ovarian activity.

Follicular growth naturally occurs in response to circulating gonadotropins, however, in acyclic buffaloes, FSH and LH activity was secured by the administration of eCG included in the protocol. The association of eCG at the time of exogenous P_4 removal increases follicular growth, ovulation rate, P_4 concentration 4 days after FTAI, and pregnancy per AI rates (Carvalho et al. 2013).

In this study, the influence of days after calving on the efficiency of P_4-based treatments in the correction of the anoestrus state in buffaloes during the out-of-breeding season was evaluated. At the end of the third oestrous cycle post-insemination a PR of 88% and a significant influence of the follicular area and BCS at FTAI, on the pregnancy outcome were the key findings.

In the present work, with the use of a P_4-based treatment in acyclic buffaloes it was possible to obtain a PR of 54% at FTAI, and 88% by the end of the study, meaning that the therapy was effective in obtaining a favourable conception rate and restoring cyclicity. These results are in agreement with others previously published papers in the buffalo species, regarding treatments with P_4 during the out-of-breeding season (Neglia et al. 2003; Zaabel et al. 2009; Neglia et al. 2018).

It must be noted that a PR of 73% (202/276) was obtained by the end of the first post-insemination oestrous cycle, which is on accordance with the results obtained in a previous study in Nili-Ravi buffaloes that underwent synchronization and subsequent resynchronization protocols with intravaginal P_4 (Arshad et al. 2017). And the 88% (242/276) of cumulative PR obtained by the end of the third oestrous cycle is also similar to the data published by Neglia et al. (2018) regarding to four consecutive resynchronization protocols in buffalo heifers during the out-of-breeding season. The data from both these mentioned studies result from resynchronization protocols with FTAI. In contrast, the values obtained from the present study only made use of hormonal treatments to obtain the first ovulation. Although, similar results were obtained, making it possible to conclude that after the reestablishment of ovarian activity by the use of P_4-based treatments in acyclic buffaloes, at least the first three naturally-obtained cycles were fertile and allowed conception by natural mating.

Buffaloes included in class II (91-150 DIM) tended (p=0,07) to have a better pregnancy outcome at FTAI and in the first oestrous cycle post-insemination than the animals in Class I (61-90 DIM). These animals also presented a longer mean interval from the end of the treatment to conception, when only accounting for the pregnancies obtained with natural mating. Class I buffaloes were under a natural postpartum anoestrus state, which is not influenced by the photoperiod, as it happens in the seasonal anoestrus, but is aggravated by the negative energy balance that animals suffer during the early lactation. This energy deficit condition has been associated with later postpartum resumption of ovarian activity and impaired follicular growth; this happens because the animal shifts its energy use to maintain basal metabolism, circulation, neural activity, and thermoregulation. This hypothesis is further confirmed by the low BCS presented by the animals in Class I when compared to those in Class II. Low BCS has been shown to be responsible for longer postpartum anoestrus intervals in Murrah buffaloes (Kumar et al. 2020) and lower conception rates in Nelore cows that underwent P_4-based treatments followed by FTAI (Pereira et al. 2018). However, females in

Class II were under a seasonal anoestrus state at the beginning of the treatment, which is a natural condition that inhibits mating and calving under unfavourable conditions of survivability for the offspring. This state usually ceases when the animals are exposed to decreasing photoperiods.

Class II females had higher BCS than those from Class I counterparts and showed a larger preovulatory follicular area on the day of FTAI. Larger preovulatory follicles at the time of FTAI have been positively correlated with better ovulatory response to P_4-based protocols and pregnancy outcomes in buffaloes during the breeding season (Monteiro et al. 2016). The results obtained in the present study further corroborate this, where animals that got pregnant by FTAI had larger preovulatory follicles than those that did not. This explains the tendency recorded of animals in Class II to have a greater PR at FTAI.

Because of the present study, some recommendations can be made toward the practical applicability of P_4-based protocols in buffaloes during the out-of-breeding season. These treatments represent a useful tool to allow a sooner return to oestrus that can be used to improve animal fertility and profitability by allowing the obtainment of favourable pregnancy outcome by FTAI and/or natural mating during the most desired months, which would be out of reach if only the natural ovarian cycles were used. It must be noted that the females in the mid-stage of lactation (91-159 DIM) may present better results than those in the earlier phases. However, animals in the earlier stages of lactation were also able to produce satisfactory PR when out of their natural breeding season. More importantly, BCS is an easily evaluated condition which has a great influence on the results and could be a relevant factor in the process of female selection.

The present retrospective study only evaluated animals from a single herd of buffaloes, divided into three DIM classes. Further investigation with different herds resulting from different genetic backgrounds and larger samples is still needed for a more complete understanding of the impact that different DIM may or may not have in re-establishing ovarian activity with hormonal treatments, and the overall efficiency of these therapies in this species. Herds that have not been subject to the OBSM method may also provide a better understanding of the true response to these protocols. Differentiation of the females by age and parity and their division into more than three classes regarding DIM may help in attaining deeper knowledge. This may evidence the optimum time to administer these therapies. Likewise, other studies regarding different variables are essential to enhance the proficiency of the use of ovulation synchronization protocols as a mean to obtain an earlier return to normal sexual activity in the buffalo, and this way improve fertility and profitability of buffalo farms.

The main takeaways from the present study rely on the fact that P_4-based ovulation synchronization protocols to FTAI keep on proving to be a useful tool in reverting the anoestrus state in buffaloes during the out-of-breeding season. The follicular area and BCS have a strong influence on the pregnancy outcome after these treatments. Regarding the different DIM, animals during the mid-lactation phase (91-150 DIM) showed better results at FTAI and at the first oestrous cycle post-insemination, and animals in the early lactation stage (61-90 DIM) may reveal longer intervals between treatment and the conception.

9.4 Conclusion

This chapter will begin by providing a summary of the findings regarding the main aim of the study, followed by comparing the results obtained with existing theories and will end with some recommendations for further research.

This study aimed to evaluate the influence of days after calving on P_4-based ovulation synchronization protocols on acyclic female buffaloes during the out-of-breeding season. The results indicate that females in mid-lactation (91-150 DIM) revealed a better pregnancy outcome at FTAI and on the first oestrous cycle post-insemination and that those in early-lactation (61-90 DIM) experienced longer intervals between the end of treatment and pregnancy by natural mating.

The relevance of this study relies on contributing to the body of knowledge regarding anoestrus treatments in the buffalo species. The findings in the present work agreed with the previously published theories, further confirming the strong influence of BCS and follicular area on the success rate of FTAI protocols and the possibility of obtaining positive results of conception with the use of hormonal treatments when correctly applied during the out-of-breeding season

More research with a focus on other variables is still needed to develop the knowledge on acyclic buffaloes and the treatments available to allow higher profitability of buffalo farms. Additionally, the influence of days after calving must be studied further in different buffalo populations with diverse genetic backgrounds and across several ages and parities. P_4 measurements should be also used to prove its usefulness, in comparing the functionality of CL obtained through these protocols and the ones naturally occurring during the breeding season.

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