15 Application of Fixed-Time Artificial Insemination in Water Buffaloes

Juan Carlos Gutiérrez-Añez, Adriana Camacho de Gutiérrez, and Héctor Nava-Trujillo

Abstract

To date, no reproductive biotechnology has had such a massive and significant impact on herds' genetic progress and technological transformation as Artificial Insemination (AI). Nonetheless, the water buffalo is characterized by a low intensity of estrus signs, affecting the detection of animals in estrus, compromising the success of conventional artificial insemination on a large scale, thus, limiting genetic progress through this technique. Furthermore, low reproductive performance in water buffalo has also been described, mainly attributed to a late onset of puberty, reproductive seasonality, long calving intervals, and estrus' low expression. Therefore, several protocols have been developed for fixed-Time Artificial Insemination (TAI) to improve water buffalo's reproductive performance and omit the need for heat detection. These hormonal treatments allow controlling follicular dynamics and luteal function, synchronizing estrus and ovulation, and, most importantly, avoiding the complicated detection of estrus in this species. Herein, we provide the basics for fixed-Time Artificial Insemination (TAI) application in water buffaloes, including the fundamental knowledge and aspects of applying the technique successfully. Basic concepts, advantages, disadvantages, physiological mechanisms of the hormonal protocols to

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synchronize and induce the ovulation, as well as a brief description of factors affecting the efficiency of the fixed-TAI programs, are included in an easy way fashion for clinicians, technicians, and veterinary or animal sciences students.

Keywords

Buffalo · Time Artificial Insemination · Estrus · Ovulation

15.1 Introduction

So far, no reproductive biotechnology has had such a massive and significant impact on herds' genetic progress and technological transformation as Artificial Insemination (AI). The genetic progress and production levels achieved in the dairy industry through AI seem to have no limits, yet AI has still not been widely used globally by the water buffalo's industry worldwide. Even though buffalo's reproductive biology is more likely similar to that of cattle, there are unique characteristics and meaningful differences in applying this biotechnology to improve buffalo's productivity effectively.

In the livestock industry for both cattle and water buffalo, to obtain optimal productivity and profitability, it is essential to achieve optimal reproductive performance. To accomplish this goal, factors like the environment, nutrition, health, and general herd management should be controlled. Some authors have described lower fertility in water buffalo (*Bubalus bubalis*) than cattle (*Bos indicus* and *Bos taurus*). Low reproductive performance in water buffalo is mostly attributed to a late onset of puberty, reproductive seasonality, long calving intervals, and estrus' low expression (Drost 2007). Reproductive seasonality observed in water buffalo prolongs the postpartum anestrus periods, compromising their reproductive performance (Nava-Trujillo et al. 2019a). This seasonality has been attributed to environmental factors, primarily associated with the photoperiod, generally negatively affected by the increase in day length (Presicce et al. 2005). Besides, the high temperature-humidity index during summer favors the appearance of thermal stress due to the restricted cooling mechanism in this species. Limited thermoregulation through skin evaporation in the water buffalo is linked to sweat glands' low density (Das and Khan 2010).

Furthermore, the water buffalo is characterized by a low intensity of estrus signs, affecting the detection of animals in estrus, compromising the success of conventional artificial insemination on a large scale, thus, limiting genetic progress through this technique. Some factors associated with the low expression of estrus signs in water buffalo are nocturnal behavior (Das and Khan 2010), infrequent homosexual behavior (Perera 2011), variable duration of estrus (5–27 h), and variable occurrence of ovulation amongst animals (24–48 h: mean 34 h) after the onset of heat (Perera 2011). Several protocols have been developed for fixed-Time Artificial Insemination (TAI) to improve water buffalo's reproductive performance and omit the need for heat detection. These hormonal treatments allow to control follicular dynamics and luteal function, synchronize estrus and ovulation, and, most importantly, avoid the complicated detection of estrus in this species (Carvalho et al. 2016; Monteiro et al. 2016; Gutiérrez-Añez et al. 2018).

Protocols used in water buffalo are adapted from TAI protocols in cattle. The application of cattle ovulation synchronization protocols to water buffaloes have resulted in lower pregnancy rates (De Rensis and López-Gatius 2007; De Rensis et al. 2005; Karen and Darwish 2010; Rossi et al. 2014), when are compared to those obtained in cattle (Pursley et al. 1997a; Sá Filho et al. 2011; Bó and Baruselli 2004; Wiltbank and Pursley 2014). Substantial evidence of lower pregnancy rates after fixed TAI in water buffalo than cattle is observed when those protocols are applied in buffaloes during seasonal anestrus (De Rensis and López-Gatius 2007; Karen and Darwish 2010; Rossi et al. 2014). However, new protocols considering buffalo's physiology for ovulation synchronization and fixed TAI in this species have resulted in a high pregnancy rate index. In that sense, in this chapter, we summarized some of the significant advances on fixed-TAI protocols in water buffalos for both breeding and non-breeding season.

15.2 Overview of the Water Buffalo's Estrous Cycle and Reproductive Performance

As in the cow, the estrous cycle in the buffalo is divided into two phases, 1. Estrogenic phase, also known as the follicular phase, and 2. The progestational phase is also referred to as the luteal phase. The follicular phase is divided into proestrus and estrus, while the luteal phase is divided into metestrus and diestrus. The estrous cycle duration ranges from 16 to 33 days, with the highest concentration between 21 and 24 days. Unlike cows, the duration of heat in water buffalo could vary from 8 to 32 h, and heat symptoms are less pronounced than in cattle (Drost 2007). They are also characterized by nocturnal sexual behavior, with little or rare homosexual activity and slight mucus discharge, which makes it difficult to correctly identify females in heat, bringing as a consequence a high silent ovulation rate, also referred to as silent heat when compared to cattle (Perera 2008). A particular estrus sign known as tail curling when exerting pressure on the pelvic area is a secondary sign present in this species that is not present in cattle. This maneuver could also be considered a practice in animals under suspicion of heat in heat detection programs.

Water buffaloes can be defined as short-daylight seasonal polyestrous animals, but under some special conditions, they can reproduce throughout the year (Perera 2011). Although in tropical climates, the length of photoperiod during a day is relatively constant (~12 h of light and 12 h of dark), other factors, such as the rainfall changes, seem to influence the cyclical reproductive pattern, according to the availability and quality of forage (Perera 2011). Nevertheless, one has to consider that seasonal reproductive activity in water buffalo (*Bubalus bubalis*) is defined by exogenous (photoperiod, climate, nutrition, management) and endogenous (hormones, genotype) factors (D'Occhio et al. 2020).

In Venezuela, a tropical-located country, the water buffalo's reproductive pattern is characterized by seasonal breeding, with a reproductive activity that could cover mostly from September to February, followed by a calving season markedly from September to December, with strong peaks during October and November (Nava-Trujillo et al. 2019a, b). This seasonal behavior could promote prolonged anestrus periods during the unfavorable season (March, April, May, June, July) until the resumption of ovarian activity in the next favorable season, reducing their reproductive performance (Gutiérrez-Añez, Data not published). Similar effects of climate and nutrition have been revised by Perera (2011) on the reproduction patterns in India, In the Amazon region of Brazil, and even also in Italy, a country with a temperate climate, where buffalos are farming under intensive or semi-intensive systems under a constant balanced diet throughout the year, a seasonal reproductive pattern, is also observed. Causes of anestrus in the water buffalo during summer in temperate climates or under heat stress in tropical and sub-tropical could be associated with elevated blood prolactin concentrations (hyperprolactinemia), which in turn reduced the gonadotropin secretion (Roy and Prakash 2007; revised in D'Occhio et al. 2020), and the progesterone profile (Roy and Prakash 2007). In temperate climates, reproductive activity is influenced by photoperiod and mediated by melatonin secretion (Borghese et al. 1995) and melatonin receptor 1A (MTNR1A) gene expression (Carcangiu et al. 2011; D'Occhio et al. 2020). Reduced oocyte quality and developmental competence in Italian Mediterranean buffaloes submitted to in vitro embryo production (IVEP) during spring and summer are observed compared to autumn and winter (Di Francesco et al. 2011). Additionally, the resumption of postpartum ovarian activity, and subsequent conception, can be affected by various factors such as breed, nutrition plan, milk production, lactation, uterine involution, delivery season, or seasonality (Barile 2005).

15.3 Conventional Artificial Insemination Vs. Fixed-Time Artificial Insemination

The deficiency of finding an acceptable method for estrus detection in the water buffalo, accompanied by the high variability in the duration of the heat that influences the timing of ovulation amongst animals, makes the conventional AI in this species suboptimal when using frozen semen. Buffalo sperm, subjected to freezing and thawing, appear to have a shorter fertile lifespan within the female tract than fresh semen (Moioli et al. 1998). These aspects could be a reason for the low conception rates found in this species in AI programs under natural or spontaneous heat.

The commercial application of fixed TAI in buffalo herds has been possible thanks to understanding the ovarian follicular dynamics, studied through ultrasound (Baruselli et al. 1997), and knowledge of endocrine control and hormonal profiles during the estrous cycle in this species (Terzano et al. 2012). These protocols for the treatment of anestrus and oestrus synchronization have had various success rates, providing effective pregnancy rates, ranging from 35% to 60%, comparable and in some cases higher than the parameters achieved in buffaloes reproduced under natural estrus.

15.4 Fixed-Time Artificial Insemination (TAI) Programs

15.4.1 Definition

In simple and practical terms, the fixed TAI, also known as timed AI after synchronization of ovulation (Wiltbank and Pursley 2014), is a technology that allows pre-established Artificial Insemination (AI) without the need for heat detection. Generally, the fixed TAI is implemented in batches of animals simultaneously under a pre-planned scheme or schedule. It translates into a convenient way to decide the number of animals to inseminate and choose the most appropriate time to do it (season, month, day, and hour). Fixed TAI is possible thanks to implementing a hormonal protocol for ovulation synchronization.

From the technical point of view, the fixed TAI could be defined as reproductive biotechnology that, based on the physiological knowledge of the female's estrous cycle, allows combining several hormones, which will be administered at a fixed or pre-established regime, allowing synchronizing the follicular development and the ovulation in more than 85% of treated females. The result is the programmed or systematic insemination carried out in a group of animals in a previously defined time, without the need for heat detection.

15.4.2 Advantages

- Eliminates the need for imprecise heat detection in buffalo species.
- Optimizes the use of semen and makes the AI technique more efficient.
- It allows to massively increase the use of AI, thereby accelerating the genetic progress of the herds.
- Fixed TAI favors individualizing the crossing of each female with unrelated bulls, thus minimizing the risk of consanguinity.
- It allows controlling reproductive seasonality, thus promoting the possibility of stabilizing milk/cheese production and economic income of the farm by achieving a convenient distribution of calvings throughout the year.
- It is helpful for the treatment of postpartum anestrus.
- It helps reduce days open and increases the reproductive performance of the herds.
- In the case of heifers, it allows advancing their reproductive seasonality (30–60 days), minimizing the risk that this category of animals after the first calving will remain open in the following season, considering that in primiparous buffaloes, the resumption of ovarian activity is dramatically affected.
- It improves reproductive controls since the TAI enters as a technological package that requires management improvement of the farm (records, management, sanitary program, food, among others).
- It enhances the productivity and profitability of the livestock business.

15.4.3 Disadvantages

- Drug cost.
- Technical staff requires basic training in the application and management of hormones.
- Big batches of animals (over 50) require well-organized and planned logistics, with high time-consuming and challenging work. Enough supporting staff is required.
- Three to four activities are implemented chronologically, including reproductive status checking and animal selection, hormone administration, and artificial insemination, necessary to perform the whole program. Climatic problems and logistics issues could affect the rigid schemes of activities.

15.5 Fixed-TAI Protocols During the Reproductive Season

15.5.1 GnRH and $PGF_2\alpha$ Based Protocols (Ovsynch)

One of the most critical constraints of using Prostaglandin F2-alpha (PGF2α) in reproductive control is high estrus and ovulation interval variability. There is not a proper synchronization because prostaglandin only regulates the lifespan of the corpus luteum and not the follicular development (Pursley et al. 1997b). The Ovsynch protocol, originally designed for cows (Pursley et al. 1995), consists of combining Gonadotropin-releasing hormone (GnRH) and PGF2α to control the lifespan of the corpus luteum, the follicular development, and the ovulation. This protocol has been adopted to buffaloes and currently is the most used protocol for the synchronization of ovulation and fixed TAI in water buffaloes, and numerous studies have been published (De Araujo Berber et al. 2002; Baruselli et al. 2003; Neglia et al. 2003; Campanile et al. 2005; De Rensis et al. 2005; Ali and Fahmy 2007; Karen and Darwish 2010; Oropeza et al. 2010; Derar et al. 2012; Di Francesco et al. 2012; Ghuman et al. 2012; Rossi et al. 2014; Ghuman et al. 2014; Hoque et al. 2014; Gupta et al. 2015; Waqas et al. 2016; Hussein et al. 2016; Neglia et al. 2016; Arshad et al. 2017; Ramoun et al. 2017; Rathore et al. 2017; Sharma et al. 2017).

15.5.2 Mechanism of Action of Ovsynch-Based Protocols

The physiological mechanism of Ovsynch-based protocols is schematically shown in Fig. 15.1. The Ovsynch protocol could be considered the precursor of the fixed TAI programs in cattle. It consists of two GnRH applications and one PGF2α. The first application of GnRH (day 0) induces ovulation of the dominant follicle present at this moment, followed by the further formation of a new corpus luteum and or the atresia of small follicles, causing the emergence of a new follicular wave. The application of PGF2α (day 7) causes luteolysis and a fall in progesterone levels. Forty-eight hours later, the second application of GnRH on day 9 causes ovulation of

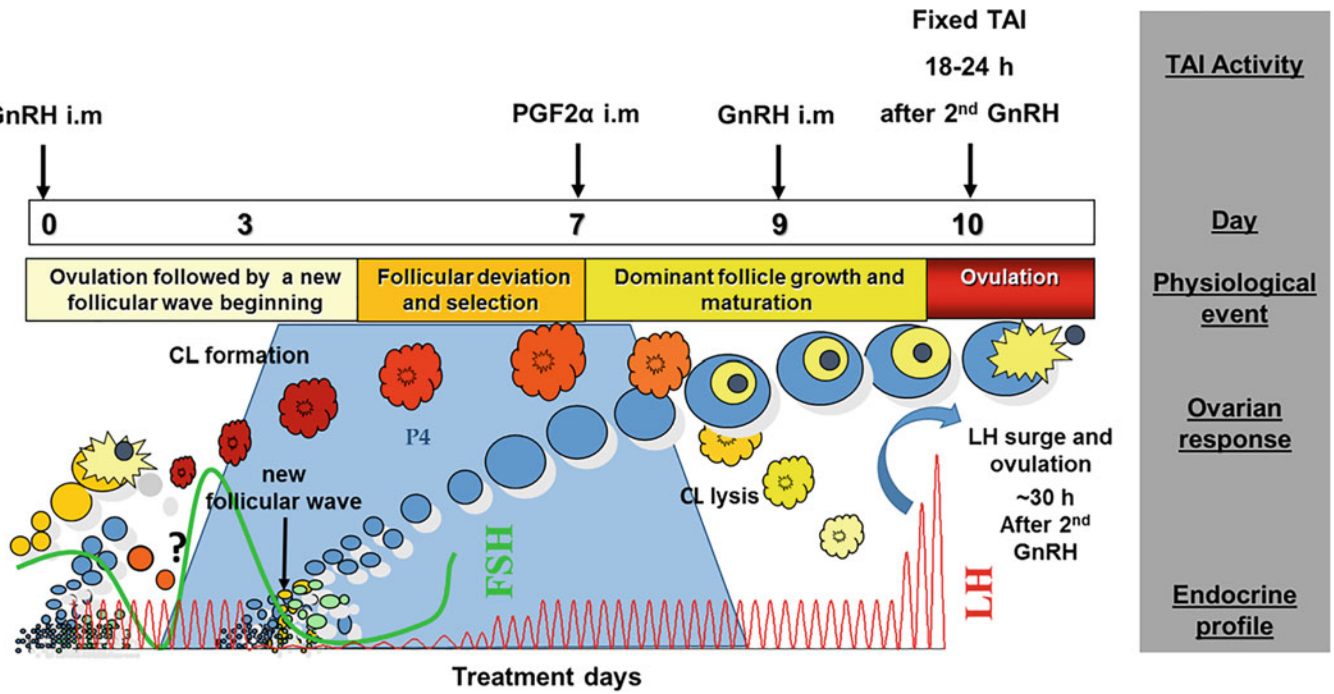

Fig. 15.1 Schematic representation of the physiological mechanism of action of Ovsynch protocol for fixed-time artificial insemination in water buffaloes. Application of gonadotropin-release hormone (GnRH) on day 0 induces the ovulation of a dominant follicle or luteinization of subordinate follicles (question mark). After ovulation, a CL (corpus luteum) starts to form, followed by a new follicular wave starting ~ on day 3. On day seven (day 7), Prostaglandin F2-alpha (PGF2α) administration induces the lysis of the CL formed, thereby decreasing P4 concentrations. On day nine (day 9), the second GnRH doses induce a uteinizing hormone (LH) surge, followed by ovulation ~30 h after. Fixed-Time Artificial Insemination (TAI) is performed 8–12 h prior to ovulation, allowing sperm transport and capacitation

the dominant follicle originated after the first GnRH injection, with insemination taking place at day ten (10), 18–24 h after the second GnRH, and without the need for estrus detection (Pursley et al. 1995).

The efficiency of the original Ovsynch protocol in buffalo in anestrus has been low; however, modern Ovsynch-based protocols have been developed; improving its efficiency in both cyclic and anestric buffalo, representing an additional alternative to progestin treatments. Some modifications of the original Ovsynch protocol will be discussed in this chapter.

15.5.3 Hybrids-Ovsynch Protocols in Water Buffalo

Some modifications of the Ovsynch have allowed obtaining acceptable results when used in acyclic buffaloes or during the low reproductive activity season. However, there are still scarce published studies on these, and therefore more research is necessary.

15.5.4 Cosynch

The Cosynch modification consists of artificial insemination on day nine (9) simultaneously with the second GnRH injection, reducing 1 day of labor (Geary and Whittier 1998). Conception rate of 62.5% and up to 75% when buffaloes received a dose of 400 IU of equine chorionic gonadotropin (eCG) 3 days before the first injection of GnRH (Cosynch-Plus) when applied to postpartum multiparous buffaloes during the reproductive season has been reported (Kumar et al. 2016). Recently, a modification of the Cosynch-Plus protocol applied to multiparous buffaloes in anestrus and during the low reproductive activity season presents encouraging results. This modification included the administration of 400 IU of eCG 3 days before the first application of GnRH, with or without intravaginal progesterone supplementation for 7 days. On day seven, the correspondent dose of $PGF2\alpha$ was injected. The protocol was completed with a dose of 2000 IU of human chorionic gonadotropin (hCG) on day 9 (replacing GnRH) at the time of the first insemination and the second insemination was performed 24 h later (day 10). When intravaginal progesterone was not inserted, 53.8% of the conception rate was observed, whereas it fell to 33.8% when administered. After subsequent insemination, the pregnancy rates were 69.2% and 86.6%, respectively (Dhaka et al. 2019).

15.5.5 Double Ovsynch

The Double Ovsynch is a pre-synchronization and consists of repeating a second Ovsynch 7 days after the first one is finished, inseminating 16 h after the fourth GnRH injection. Hoque et al. 2014, evaluated this protocol and reported higher ovulation and conception rates than traditional Ovsynch (83.3% and 44.4% vs. 72% and 28%, respectively; $P < 0.05$).

15.5.6 Ovsynch Plus

Ovsynch Plus includes a dose of eCG (500 IU) applied 3 days before the Ovsynch protocol. When it was applied to acyclic buffaloes during the low reproductive activity season, only a numerical improvement in the conception rate was observed compared to the traditional Ovsynch (34.5% vs. 23.1%, $P > 0.05$) (Sharma et al. 2017). Rathore et al. 2017 reported similar results during low reproductive activity season (Ovsynch Plus: 28% vs. traditional Ovsynch: 24%, $P > 0.05$).

15.5.7 Double Synch

Mirmahmoudi and Prakash 2012, applied the Double synch protocol (a dose of PGF2α 48 h before the first GnRH injection of Ovsynch) during the low reproductive activity season (April–May, in India). The authors reported an ovulation rate of 100% after the second GnRH injection, an interval to ovulation of 23.2 ± 1.0 h (range 20–28 h), and a conception rate of 58.1% ($P < 0.05$); this latter was higher than in buffaloes inseminated under spontaneous estrus (27.3%).

15.5.8 G6G

This protocol consists of the application of a dose of PGF2α and GnRH 8 and 6 days before starting the Ovsynch protocol respectively and allowed to increase the progesterone levels on Ovsynch day seven; subsequently, the diameter of the ovulatory follicle at the moment of insemination (12.5 ± 0.3 mm, $P = 0.04$) and ovulation (14.8 ± 0.3 mm, $P = 0.02$) compared to the traditional Ovsynch (11.6 ± 0.3 and 13.7 ± 0.3 respectively), additionally a tendency to improve the conception rate was observed (56% vs. 32%, $P = 0.08$) (Waqas et al. 2016).

15.5.9 Melatonin and Progesterone Supplementation

Treatment with melatonin in the form of subcutaneous implants (18 mg / 30 Kg) before Ovsynch in anestrus buffalo heifers and during the low reproductive activity season increased the conception rate at first insemination (30% vs. 0%) and accumulated pregnancy (50% vs. 20%) (Kavita et al. 2018).

Progesterone supplementation through an intravaginal device (PRID) between days 0 (first application of GnRH) and 7 (application of PGF2α) of Ovsynch in postpartum buffaloes and during the long photoperiod season improved the conception rate regarding control (46.51% vs. 27.71%) (De Rensis et al. 2005).

Furthermore, progesterone supplementation would be ideal for the treatment of acyclic buffaloes during the long photoperiod season since a higher conception rate was reported in this group compared to traditional Ovsynch (30% vs. 4.7%, $P = 0.04$), and this effect was not observed in cyclic buffaloes (51.5% vs. 35.7%, $P = 0.077$) (De Rensis et al. 2005). These results coincide with the 50% pregnancy recently reported in Egypt in cyclic multiparous buffaloes during the season of low reproductive activity (April–October) (Ramoun et al. 2017).

15.6 Fixed-TAI Protocols During Both the Reproductive and Non-reproductive Season

15.6.1 Progesterone (P_4)-Based Fixed-TAI Protocols

Progestogens constitute a group of hormonal products primarily used mainly to synchronize the heat in cattle. Initially, for more than four decades, the route of administration of progestogens was restricted to repeated intramuscular injections for several days. Later, other routes of administration such as oral (Melengestrol Acetate), subcutaneous implants (Norgestomet), and intravaginal Progesterone (P4) devices were used. So far, the intravaginal route has been the most widely disseminated and preferred in both cattle and buffalo. Various intravaginal devices impregnated with P4 or progestogens used in different countries across the world; thus, some includes the CIDR (Controlled Internal Drug Release; Zoetis, Zoetis Animal Health, Parsippany, NJ, USA), PRIDDelta (Progesterone Releasing Intravaginal device; CEVA-Santé Animale, France), DIB [Dispositivo Intravaginal Bovino (Bovine Intravaginal Device, Sintex, Argentina), and PregnaHeat-E (Viateca, Venezuela).

The P4-based protocols in the buffalo species have the advantage of being efficient during both reproductive season (favorable) and non-reproductive season (not favorable). Progesterone devices for ovulation synchronization are combined with Estradiol (E2) (Carvalho et al. 2014; Monteiro et al. 2016), gonadotropin-releasing hormone (GnRH) (Gutiérrez-Añez et al. 2018), Prostaglandin F2-alpha (PGF2α), and equine chorionic gonadotropin hormone (eCG) (Carvalho et al. 2014; Monteiro et al. 2016, Gutiérrez-Añez et al. 2018). Most of these programs derived from the adaptations made to the hormonal protocol in cattle, based on the similarities in the waves of follicular dynamics (Perera 2008; Baruselli et al. 1997) and hormonal profiles in the course of the estrous cycle as described in cattle (Presicce 2007).

Various variants and modifications of the protocols have been evaluated based on the days of the permanence of the device (seven, 8 and 9 days), the concentration of P4, hormones to control follicular development (E2-P4, GnRH, eCG, FSH), luteal phase (P4, PGF2α) and ovulation [E2, GnRH, porcine luteinizing hormone (pLH)] (De Rensis et al. 2005; Carvalho et al. 2014; de Araujo Berber et al. 2002; Monteiro et al. 2016; Carvalho et al. 2017; Gutiérrez-Añez et al. 2018). Other variables, such as hormonal dose and time of insemination after removing the device, have also been studied.

15.6.2 Mechanism of Action of P_4-Based Protocols

The physiological mechanism of P4-based protocols for ovulation synchronization and fixed TAI is schematically shown in Fig. 15.2. These products act like an artificial corpus luteum, inhibiting the pulsatile secretion of luteinizing hormone (LH) and, therefore, ovulation. However, the hypothalamus continues the synthesis of GnRH, consequently accumulating it. When the source of P4 is removed, the progesterone blockade on the hypothalamus ceases, triggering the massive release of GnRH and gonadotropins, initiating a normal, ovulatory, and a potentially fertile new cycle.

When estrogens and progesterone or progestogens are combined, the following occurs: estrogens in the hypothalamic-pituitary axis inhibit the release of a follicle-stimulating hormone-releasing hypothalamic hormone (FSH-RH, or FSH-GnRH), suppressing it at the level of the pituitary the synthesis and secretion of follicle-stimulating hormone (FSH). On the other hand, controlled progesterone secretion by the device in the hypothalamic-pituitary axis inhibits the release of luteinizing hormone-releasing hypothalamic hormone (LH-RH, or LH-GnRH); suppressing at the level of the pituitary gland the synthesis and secretion of luteinizing hormone (LH). Consequently, the suppression of the secretion of gonadotropins (FSH and LH) forces that the follicle (s) that is (are) present at the beginning of the protocol (day 0), either in the recruitment or selection stage (dependent on FSH), deviation or dominance (mainly dependent on LH), initiate the atresia due to the absence of the necessary hormones (gonadotropins) that stimulate and maintain their growth.

Once the estrogen administered exogenously is metabolized by the animal (generally 48–72 h in the case of EB), the inhibition for FSH release at the pituitary level ceases. Likewise, follicle atresia will result in a progressive decrease in the levels of endogenous inhibin and estradiol produced by the dominant follicle. The decrease in inhibin and endogenous estradiol (both inhibitory factors of FSH secretion) results in their inhibitory action cessation. It systematically allows the generation of FSH pulses and the restart of a synchronized new follicular wave 3–4 days later in treated animals, ensuring the emergence and ovulation of a new and more viable follicle containing a competent ovum at the device removal time. Generally, the device is kept for 8–9 days, allowing the development and maturation of a dominant preovulatory or Graff follicle until the time of device removal.

The incorporation of equine chorionic gonadotropin (eCG) at the end of the TAI protocol produces an increase in the follicular growing rate, stimulating final follicular growth and oocyte maturation, increasing the ovulation rate, progesterone concentrations, and subsequent pregnancy rate. Application of PGF2α on P4-based protocols allows the lysis of the luteal tissue (corpus luteum or luteinized follicles), avoiding the negative feedback that luteal tissue and basal levels of P4 may

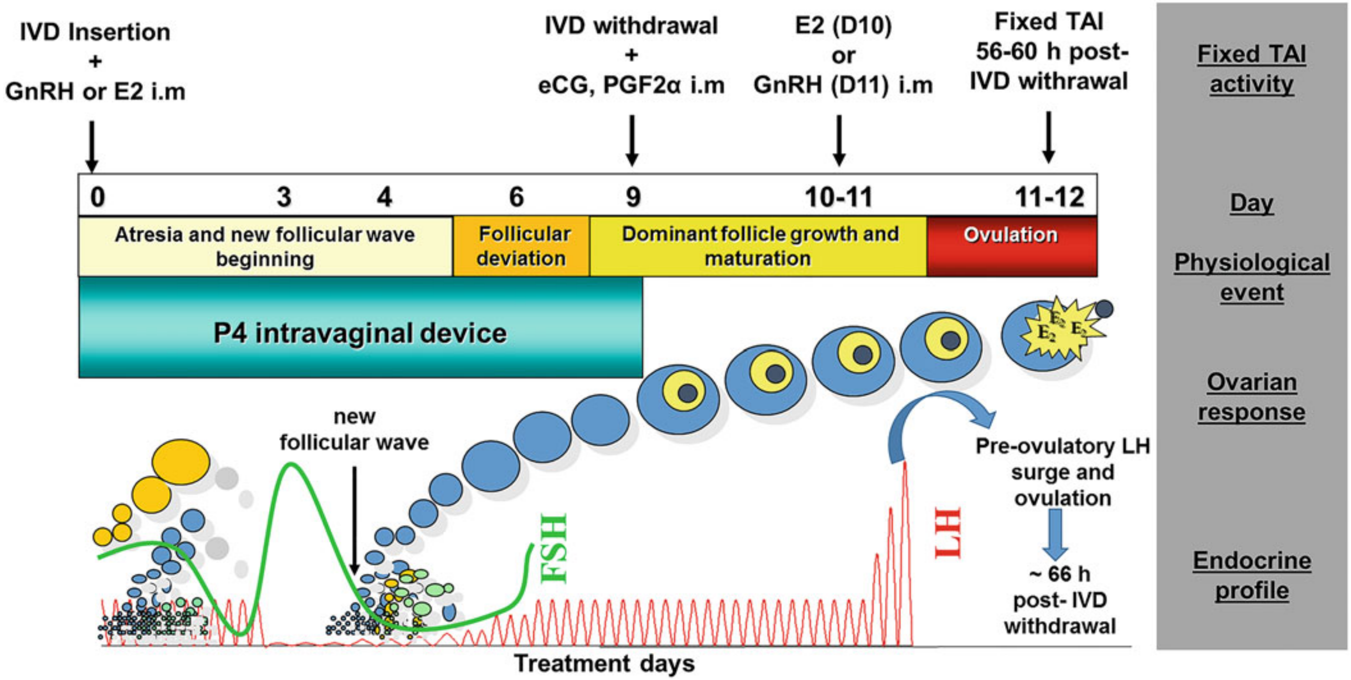

Fig. 15.2 Schematic representation of the physiological mechanism of action of progesterone-based protocol for fixed-time artificial insemination in water buffaloes. On day 0, the insertion of progesterone (P4) intravaginal device (IVD) plus the application of gonadotropin-release hormone (GnRH) or estradiol (E2), usually 2 mg of estradiol benzoate (EB), induces the atresia of the follicles present. A new follicular wave begins 3–4 days after. On day nine (day 9), the administration of equine chorionic gonadotropin (eCG) favors the maturation of the dominant follicle. At the same time, Prostaglandin F2-alpha (PGF2α) induces the lysis of any luteal tissue (luteinized follicles or corpus luteum), thereby a decrease in P4 concentrations, which could interfere with the preovulatory surge of luteinizing hormone (LH). On day ten (D10), the application of E2, usually 1 mg of EB, or on day eleven (D11) administration of GnRH, induces a

surge of LH, followed by ovulation ~66 h after the IVD withdrawal. Fixed-Time Artificial Insemination (TAI) is performed 8–12 h prior to expected ovulation, allowing sperm transport and capacitation. All treatments begin with the insertion of the P4 intravaginal device (day 0), accompanied by the simultaneous injection either of an estrogen ester, usually estradiol benzoate (EB), or gonadotropin-releasing factors (GnRH). These hormones cause atresia or ovulation of the dominant follicle [atresia in the case of estradiol, or ovulation (preovulatory follicle) or atresia (small follicle) in the case of GnRH], thus preventing the possible persistent or aging follicles from ovulating, which can negatively interfere with fertility exert in the pulses of LH surge during the final maturation of the follicle or during ovulation.

Finally, an ovulation inducer is administered after removal of the device (Estradiol or GnRH), which stimulates the preovulatory surge of LH and the synchronization of ovulation; allowing in this way the establishment of a single moment to carry out the AI without the detection of heat in all treated animals. A study carried out by Carvalho et al. (2017), indicated that both estradiol benzoate administered either 24 or 36 h and GnRH at 48 h induced comparable follicular responses, ovulation, and pregnancy rates in the buffalo cows and heifers.

15.6.3 P_4/Estradiol (E_2)-Based Fixed-TAI Vs. P_4/GnRH/$PGF_2\alpha$-Based Fixed-TAI

The protocols which combine estradiol (E2) and progesterone (P4) have been used effectively with adequate pregnancy rates during both breeding and the non-breeding season in water buffalos under tropical conditions (Carvalho et al. 2013, 2016; Monteiro et al. 2016). Nevertheless, it is essential to consider that the uses of oestradiol-17β, as well as its related esters, aimed to estrus synchronization of food-producing animals, have been prohibited due to public health regulations in the European Union (EU) (Lane et al. 2008), and are omitted in the list of medications approved by the Food and Drug Administration of the United States (FDA, USA 2017).

Under the mentioned restriction above, an alternative to substitute the estradiol-related compounds in the synchronization protocols must be considered. The estradiol substitution by GnRH in the P4-based protocols is an alternative that allows synchronizing the follicular waves in both cattle and buffaloes. The use of fixed-time artificial insemination (TAI) protocols could adapt to the particular conditions of each country or region and respond to farmers' needs, but at the same time, it must consider and ensure the health of consumers of animal products as a prevailing condition regarding its uses.

In dairy buffalos subjected to E2-P4-based fixed-time artificial insemination has been a reported high pregnancy rate (64.0%) during the breeding season (Monteiro et al. 2016), and over fifty percent (55.9% and 52.7%) during seasonal anestrous (Carvalho et al. 2013, 2014), comparable with the results observed in a trial conducted with GnRH (Gutiérrez-Añez et al. 2018). In those experiments mentioned above, the devices were maintained for 9 days, followed by GnRH administration to induce ovulation 48 h after device withdrawal (Day 11). However, in those studies, Estradiol Benzoate (EB) was injected on day 0 instead of GnRH, and the fixed-time artificial insemination was performed 16 h post-GnRH injection (day 12), instead of 8–12 h post-GnRH administration as was done in the experiment by Gutiérrez-Añez et al. (2018).

In the last years, our research group has been focused on assessing the efficacy of a novel ovulation synchronization and TAI protocol (Fig. 15.3) based on the combination of P4, GnRH, and PGF2α on pregnancy rate in water buffalo cows

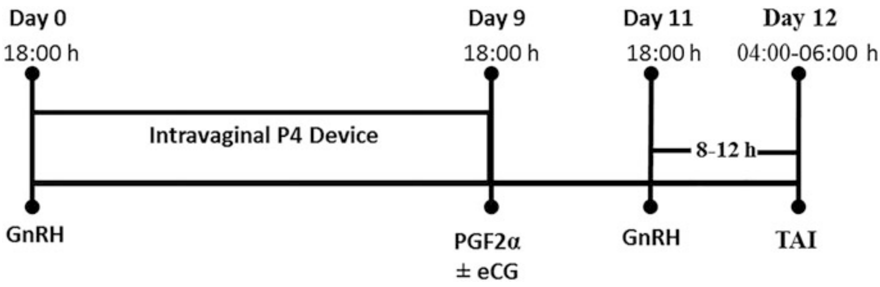

Fig. 15.3 Schematic diagram showing the P4 + GnRH/PGF2α/GnRH-based protocol for ovulation synchronization and fixed-time artificial insemination (TAI) during the non-breeding and the breeding season in dairy buffalo cows (Adapted from Gutiérrez-Añez et al. 2018). GnRH: gonadotropin-release hormone; eCG: equine chorionic gonadotropin; PGF2α: prostaglandin F2-alpha. Application of eCG at the time of intravaginal device withdrawal could be optional, but it is recommended its application under low body condition score, profound anestrous, and in primiparous cows

treated during both breeding (October to December) and non-breeding season (June to August), under tropical conditions.

Differences in pregnancy outcomes among similar protocols based on P4 intravaginal devices, combined with E2 or GnRH and PGF2α, could be attributed to many factors, including P4 concentration, protocol length, and device permanence, time for TAI after device removal or after induction of ovulation by GnRH, semen quality, management, environmental conditions, breeds, and cyclicity, among others, are discussed in this chapter.

15.6.4 Addition of Equine Chorionic Gonadotropin (eCG)

Incorporating equine chorionic gonadotropin (eCG) into ovulation synchronization and TAI protocols increases the maximum diameter of dominant follicles, ovulation rate, corpus luteum diameter, P4 concentrations, and pregnancy rate of buffaloes during the non-breeding season (Carvalho et al. 2013). Usually, the eCG is administered at the moment of the device withdrawal in a dose of 400–500 UI (Fig. 15.3).

In one study performed in non-cyclic and cyclic multiparous Murrah buffalo cows, the treatment with eCG within a CIDR-based protocol was able to increase the ovulation rate (eCG: 84.4% vs. control: 57.6%) but did not improve pregnancy rate in non-cyclic (eCG: 38.1% vs. control: 21.1%) and cyclic cows (eCG: 35.7% vs. control: 45.5%) (Murugavel et al. 2009). Perhaps the absence of statistical differences in pregnancy rate in that study might be due to the small number of animals per group. Likewise, in another study currently performed by our research group (data unpublished), the addition of the eCG could not increase the pregnancy rate significantly, including in the non-breeding season. This result suggests that the protocol based on P4, GnRH, and PGF2α was efficient in controlling both ovarian

Table 15.1 Pregnancy rates in dairy water buffaloes synchronized with a protocol based on one P4 intravaginal device combined with GnRH, PGF2α, and either with or without eCG (P4 + GnRH/PGF2α ± eCG/GnRH) during both breeding and non-breeding season (Gutiérrez-Añez et al. data unpublished)

Effect	N	Pregnancy rate (%)	*P* value
Non-breeding season			
+eCG	32/51	62.74	0.65
-eCG	31/55	56.31	0.68
Subtotal	**63/106**	**59.4**	**0.72**
Breeding season +eCG	33/53	62.26	0.70
-eCG	29/48	60.41	0.75
Subtotal	**62/101**	**61.3**	**0.60**
Total	**125/207**	**60.39**	

+eCG: With equine chorionic gonadotropin (eCG). *–eCG*: without equine chorionic gonadotropin (eCG). *NS*: No significant differences within season and groups ($P > 0.05$)

follicular dynamics and ovulation, obtaining a satisfactory pregnancy rate in lactating buffalo cows during the breeding and the non-breeding seasons (Table 15.1).

We assumed that possibly the absence of effect of eCG on the pregnancy rate in such experiment was due to the homogeneous and good body condition score (BCS) in all groups of buffalos treated (3.2 ± 0.2), as well as to the advanced postpartum period (147.9 ± 23.4) and parity (3.5 ± 0.6). In cattle, it has been documented that the effects are not always observed in cyclic animals but are evident in animals by which the LH secretion and ovarian activity is compromised; for instance, during the early postpartum period, under seasonal heat stress, or in animals with a low body condition score or in anestrous (De Rensis and López-Gatius 2014). On the other side, Sales et al. (2016) found that the eCG treatment increased final follicular growth, ovulation rate, and fertility in *Bos indicus* cows submitted to TAI protocols, especially in primiparous cows.

15.6.5 Progesterone Concentration and Reuse of Intravaginal Devices

High circulating P4 concentrations, either from endogenous or exogenous sources, decrease LH pulse frequency (Bergfeld et al. 1995; Carvalho et al. 2014). These reports suggest that high levels of P4 in protocols could negatively affect the ovulation and pregnancy rates in both cattle and buffalos. In a recent study performed by Gutiérrez-Añez et al. (2018) using the mentioned protocol above (Fig. 15.3) with intravaginal devices containing different P4 concentrations combined with GnRH and PGF2α was found a higher pregnancy rate in buffalo cows treated with 1.0 g of P4 (DIB, 62.7%), compared to those cows treated with 1.34 g of P4 (CIDR, 40.0%). These differences could be attributed to the difference in P4 concentrations, chemical composition, or the rate of P4 absorption, suggesting that

1.0 g of P4 was sufficient to control follicular development and ovulation, which resulted in an adequate pregnancy rate (Carvalho et al. 2016; Gutiérrez-Añez et al. 2018). Furthermore, in the experiment by Gutiérrez-Añez et al. (2018), reusing the intravaginal device (two and three times) did not negatively affect the pregnancy rate. Likewise, Carvalho et al. (2014) stated that low circulating P4 concentrations observed in buffaloes treated with used P4 devices (once or twice) are sufficient to promote adequate ovarian follicular growth and pregnancy outcomes as observed in buffaloes treated with new 1.0 g P4 devices.

15.6.6 Protocol Length and Device Permanence

Lower pregnancy rate (27–45%) in both Mediterranean (Neglia et al. 2003; De Rensis et al. 2005) and Murrah buffaloes (Murugavel et al. 2009), using a combined P4 for 7 days, GnRH and PGF2α protocol (PRID: 1.55 g of P4 and CIDR: 1.38 g of P4, respectively) have been observed. Apparently, in water buffalo, the protocols which maintaining the intravaginal progesterone devices for 9 days, accompanied by a GnRH injection 48 h later, and performing the fixed TAI 8–16 h post-GnRH (Gutiérrez-Añez et al. 2018; Monteiro et al. 2016; Carvalho et al. 2014), have shown to be more efficient to control the follicular dynamics, to synchronize the ovulation and produce higher pregnancy rates than the 7-day P4 protocols (Neglia et al. 2003; De Rensis and López-Gatius 2007; De Rensis et al. 2005; Murugavel et al. 2009; Bhat et al. 2015).

This theory could be physiologically reinforced, considering that in P4-based protocols, since the follicular wave emergence (3–5 days) after starting the hormonal treatment, until the ovulation should be necessary from 8 to 10 days approximately. Baruselli et al. (1997) have informed diameter of ovulatory follicles (1.55 ± 0.16 and 1.34 ± 0.13 cm), the duration of the growing phase (7.39 ± 1.55 and 5.50 ± 1.22 days), the static phase (6.88 ± 2.37 and 5.30 ± 1.34 days), and follicular linear growth of the ovulatory follicle (0.131 ± 0.01 and 0.172 ± 0.02 cm/d) for buffalo cows of 2- and 3-wave cycles, respectively. Application of GnRH 48 h after the device withdrawal (day 11) has been documented to induce ovulation between 25 and 28 h later (73–76 h after device removal (Baruselli et al. 2003; Carvalho et al. 2016). These observations suggest that artificial insemination should be performed 60–64 hours after the intravaginal device's withdrawal (8–12 h before the ovulation), which provides the necessary time for sperm transport and capacitation process (Fig. 15.2).

15.7 Evaluation and Factors Affecting the Efficiency of the Fixed-TAI Programs

When implementing a fixed-TAI program, several factors such as season (Favorable-Unfavorable), parity, postpartum period, body condition score, semen quality, general health status, and the reproductive tract condition should be considered.

A higher conception rate was reached during the season of high reproductive activity (48.80% vs. 6.90%, $P < 0.05$); in buffaloes with a body condition score ≥ 3.5 (on a scale of 1 to 5) compared to those with a body condition score < 3.0 (54.03% vs. 31.1%; $P < 0.05$); and in multiparous (51% vs. 35.5%, $P < 0.05$) (Baruselli et al. 2003). These results agree with those reported by de Araujo Berber et al. 2002, with a conception rate of 61.7% for multiparous and 30.8% for primiparous ($P < 0.05$). Hoque et al. 2014 observed that both ovulation and conception rates were higher in multiparous in comparison with primiparous (83.3% and 33.3% vs. 42.8% and 14.3%, respectively) and buffaloes with a body condition score $\geq$ of 3.5 (on a scale from 1 to 5) in comparison with those with a body condition score < 3.0 (79% and 31.6% vs. 50 and 16.7%, respectively). Besides, in Italy, a higher pregnancy rate was observed during the high reproductive activity season (58%) in comparison with the low reproductive activity season (45.6%) (Di Francesco et al. 2012). On the contrary, Rossi et al. (2014), when analyzing the fixed-TAI programs carried out for three consecutive years in Italy, did not find significant differences in the pregnancy rate according to the body condition score, days in milk or days postpartum, milk production, age (parity or number of calvings) and year of implementation of the TAI program.

Ovarian structures on day 0 could affect the results. Baruselli et al. 2003 observed that a high ovulation rate after the second dose of GnRH was related to a follicle with a larger diameter at the first GnRH (day 0) and high levels of progesterone at the moment of PGF2α dose (day 7). De Rensis et al. 2005 observed that buffaloes with follicles $\geq$10 mm at day 0 had a higher conception rate than those with $\leq$10 mm. Ghuman et al. 2014 observed that buffaloes becoming pregnant had higher progesterone levels at day 0 (2.23 $\pm$ 0.29 ng/mL) than non-pregnant (0.55 $\pm$ 0.24 ng/mL, $P < 0.05$). Souza et al. 2015 observed that buffaloes with corpus luteum at day 0 had a larger follicle on day 10 (13.9 $\pm$ 0.3 mm vs. 12.3 $\pm$ 0.3 mm, $P < 0.01$), higher ovulation rate (87.8% vs. 52%, $P < 0.01$) and higher conception rate (65.3% vs. 20%, $P < 0.01$) than buffaloes without corpus luteum. While Neglia et al. 2016, observed that buffaloes ovulating after the first GnRH had a higher conception rate (75.7%) compared with those buffaloes that did not ovulate (30.4%, $P < 0.05$) and in the same way, a difference was observed in progesterone levels at day 0 between pregnant and non-pregnant buffaloes (1.90 $\pm$ 0.2 vs. 1.40 $\pm$ 0.2, ng/mL; $P < 0.05$), which coincides with that reported by Souza et al. 2015. Besides, the selection of buffaloes at the moment of insemination according to the ovulatory follicle diameter could also improve the conception likelihood. Campanile et al. 2005 synchronized 243 postpartum multiparous buffaloes during the transition to the season of low reproductive activity. At the moment of insemination, 34 buffaloes were excluded by not having a follicle >10 mm of diameter, and only 209 buffaloes were inseminated; a pregnancy of 63% at day 26 post-insemination was reached, however, given that the trial was carried out during the transition to long days, pregnancy decreased to 34% at day 40, which represents late embryo mortality of 45%. Late embryo mortality is one of the major limitations of reproductive efficiency in buffaloes inseminated during the transition to long days (Nava-Trujillo et al. 2019c). Therefore, the traditional Ovsynch protocol could be an excellent choice to apply during the short photoperiod season, when anestrus is low, in multiparous buffaloes, which has lower anestrus incidence, and those buffaloes with good body condition score.

On the other hand, the establishment of pregnancy results from the sum of the effects of various factors; some are not even evident and are not considered, as is the case of mastitis. Clinical mastitis before the first service increased the interval to conception (148.79 ± 12.66 vs. 76.1 ± 2.89 days), with longer intervals when mastitis occurred between the first service and the pregnancy diagnosis (232.47 ± 17.96 days, $P < 0.05$) (Manimaran et al. 2014). A positive genetic and phenotypic correlation between somatic cells score and age at first calving, calving interval, number of services per conception, and days open has been reported (de Camargo et al. 2015). In addition, clinical and subclinical mastitis affect conception rate after estrus synchronization. Conception rates at day 25 and 45 post-insemination were lower in buffaloes with clinical (28% and 16%, respectively) or subclinical mastitis (55.56% and 44.45%, respectively) compared to healthy buffaloes (69.57% and 60.87%, respectively, $P < 0.05$) (Mansour et al. 2017). Similarly, a reduction of 16.11 and 36.2 percentual points in conception rate at 40–45 days was observed by consequence of subclinical and clinical mastitis in comparison with healthy buffaloes (55%, $P < 0.05$) (Mansour et al. 2017).

These consequences could be due to the reduction in the diameter of preovulatory follicle observed in buffaloes with clinical and subclinical mastitis (12.40 and 10.25 mm, respectively) in comparison with healthy buffaloes (14.35 mm, $P < 0.05$), and consequently a reduction in estradiol level at the day of estrus and a reduction of corpus luteum diameter and progesterone level was observed in buffaloes with clinical and subclinical mastitis (Mansour et al. 2017). Also, the worst impact of mastitis occurred when it is present between 15 days before and 30 days after insemination, and this could be a consequence of the reduction in the diameter of the corpus luteum and the lower production of Progesterone (Mansour et al. 2017). The negative impact of mastitis on fertility could be related to the higher cytokines (TNF-α, IL-6, IL-1β, and IFN-γ) observed in buffaloes with clinical and subclinical mastitis (Mansour and Zeitoun 2019). Therefore, when planning a fixed-time insemination program, it is necessary to consider the health of the udder at the beginning of the hormonal treatment and on the day of insemination to avoid mastitis's adverse effects reducing the cost for each pregnancy. Furthermore, given the few published works in this area, further research in this field is required.

15.8 Conclusions

Water buffalo production systems have significant challenges, one is a genetic improvement, and the second is reducing non-productive days. Advanced age at first calving and prolonged calving to conception interval are attributed to this species. Implementing a reproductive control program, including fixed-TAI programs, is a fundamental key to reducing non-productive days, increasing reproductive efficiency in the short term, accelerating genetic progress, and ultimately increasing the buffalo systems' profitability. This chapter has detailed the recent advances in protocols for synchronization of ovulation and fixed TAI. We also have shown different possibilities to start and maintain such programs in different scenarios according to the buffaloes' characteristics, the season of the year, and the system's productive purpose.

We also have described that the establishing protocols that combine P4, GnRH, PGF2α, and eCG out or during the buffalo breeding season generate satisfactory results. However, there is no "magic" and "perfect" protocol. Each situation and condition deserve certain adjustments that should always consider the possible factors that affect the fixed-TAI programs' efficiency, including season, animal health status, nutritional situation, and cost, considering that assisted reproductive technologies supplement good management and not replace it. Finally, basic knowledge on buffalo's reproductive physiology combined with recognizing the different factors that affect the fixed-TAI programs' efficiency reinforces either the likelihood to obtain good fixed-TAI outcomes or gives a better insight when the results were not as we were expecting.

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