# Technologies Related with the Artificial Insemination in Buffalo

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**Abstract:** In buffalo oestrus behaviour has a lower intensity than in cows and is much more difficult to detect, limiting the application of artificial insemination (AI) program. Several methods of heat detection have been developed for use in cattle; these include visual observation, heat mount detectors, tail paint, chin-ball markers, teaser animals and electronic devices. In buffalo, unlike cattle, the female are receptive to mounting activity mainly by the bull and occasionally by other cows. Consequently unless a buffalo bull is to be left running with the herd it can be difficult to know when oestrus is occurring. The presence of a teaser bull is helpful to identify buffaloes in heat; in this case the standing oestrus is the most reliable sign referable to a next ovulation. Other heat detection aids utilized in buffalo include: pedometers; vaginal probes; pressure sensitive telemetry device (Heat Watch®). In order to increase the use of AI easy management schemes, that not require the identification of oestrus, have been studied. These schemes are based on the manipulation of the hormonal events occurring during the oestrous cycle as: manipulate peripheral progesterone concentration (by PGF2α or progesterone releasing device); manipulate follicular growth and timing of ovulation (by GnRH and PGF2α). A brief description of these technologies, with special reference to synchronization protocols to apply fixed time AI in buffalo, are presented in this review. The potential application of predetermining the sex of offspring will be also discussed, with reference to the techniques available for commercial practice in buffalo.

**Keywords:** Oestrus detection, teaser bull, automatic system, hormonal treatment, sexing technology.

## INTRODUCTION

An accurate knowledge of the regulatory mechanisms involved in the oestrus cycle is necessary to increase the reproductive efficiency of the buffalo. Current knowledge of the basic pattern of changes in hormone profile during the oestrus cycle and the basic pattern of follicle development, are important to develop models for improving reproductive efficiency, particularly when controlled breeding techniques using synchronization and superovulation protocols are utilised.

The average length of oestrus cycle has been reported to be 21 days in the riverine type. Several factors such as climate, temperature, photoperiod, nutrition, have been shown to affect the length of oestrus cycle and the degree of heat expression. Oestrus behaviour in buffalo has a lower intensity than in cows and is therefore much more difficult to detect. Acceptance of the male is considered as the most reliable oestrus indicator. Frequent urination, bellowing, vulvar swelling and mucus discharge are referred to be salient signs of oestrus but their expression, in buffalo, is extremely weak. Ovulation can not be predicted from oestrus behaviour signs because even when they are shown, they are not reliable. For these reasons the application of artificial insemination is limited in buffalo, considering that a high conception rate depends mainly on insemination at a correct time relative to ovulation. Moreover, although buffaloes are polyoestrus, their reproductive efficiency shows wide variation throughout the year. Buffalo cows exhibit a distinct seasonal change in displaying oestrus, conception rate and calving rate, therefore buffaloes calving during unfavourable season may not resume the ovarian activity until the following favourable season [1].

## AIDS TO OESTRUS DETECTION

The oestrus period in the animals is the time during which the female will stand to be mounted by a male. Changes in the levels of circulating hormones, particularly oestradiol from the developing follicle, induces the behavioural changes associated with oestrus. In the cattle, the other cows of the herds will begin to take interest in the female in oestrus (sniffing her and resting their chin on her back) and will became more likely to mount her [2].

There are several methods of heat mount detector that have been developed for use in cattle, where mounting behaviour is prevalent when a cow is in heat [3-5]. However, none are substitutes for visual observation. The main problem of automatic oestrus detection is to reduce the presence of false positive alerts, thus the combination of more methods is desirable [4, 6, 7].

### Heat Mount Detectors

One of the most common detectors, the Kamar®, consists in an adhesive pad which is glued to the cow's

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ISSN: 1927-5196 / E-ISSN: 1927-520X/12 

rump, just forward of the tail head. The device is made up of a cylindrical plastic container of red dye inside a clear plastic capsule. The cylinder is hidden by an opaque cover under the capsule. Prolonged pressure (at least 3 seconds) from the brisket or chest of mounting animals will squeeze the dye out of the cylinder and the originally white detector will turn to red. This timing mechanism helps distinguish between true standing heat versus false mounting activity.

Another commercially available aid to heat detection is the Estrotect™. This device is self-adhesive and is placed between the hip and tail head. This heat detection aid was designed to indicate when cows are in standing heat in contrast to only showing when a cow has been mounted once for two or three seconds. The silver device surface is removed *via* friction each time a cow is mounted. When more of the fluorescent colour indicator is visible than the silver scratch-off surface, the closer the cow is to standing heat.

An economical heat detection aid used by much U.S. dairy industry is called "*tail-chalking*" and involves only the small expense of an oil-based paint stick in a wide variety of colours. Cows are mark by making a line from the tail head. When a cow is just coming into heat and is being ridden but will not stand, the chalk will be slightly smeared. When she is in standing heat and being ridden repeatedly, the paint will be mostly rubbed off.

In the buffalo, the females in oestrus are receptive to mounting activity mainly by the bull and occasionally by other cows. Unless a buffalo bull is to be left running with the herd it can be difficult to know when oestrus is occurring so that artificial insemination can be carried out. Moreover, in the buffalo the oestrus detection aids, such as pressure sensitive indicators placed on the sacrum like Kamar® or Estrotect™ mount detector or tail-chalking detection aid, are unsatisfactory because wallowing or rubbing interfere with their efficiency.

To solve the problem of heat detection in buffaloes we can make use of other techniques to control oestrus such as: teaser bull, automated systems or hormonal treatments.

### Teaser Bull

Teaser is a bull that is used to make sexual advances to cows and detect those that are on heat without being able to fertilize them. Various surgical treatments have been attempted to prevent intromission so physical mating could not occur [8, 9]. These include penectomy, penis deviation and a device in the prepuce to prevent erection. The disadvantages of these procedures are that they require a long period of recuperation and the bull will soon lose libido due to discomfort at the surgical site upon erection of the stump, and in any case most of these methods are unacceptable because of animal welfare considerations. More commonly bulls are altered through vasectomy (that consist in doing a caudal epididymectomy) so they are able to copulate but not to impregnate cows [10]. They are more effective if they are equipped with some device for marking the mounted cows, like a chain ball marker. This device is strapped on the underneath side of the chin of the bull. The marker consists of a paint reservoir with a steel ball valve, similar to a ball-point pen. When bull rides another animal, the chin ball marker is activated, and paint marks are left on the in-heat animal's back.

The presence of a teaser bull is helpful to identify buffaloes in heat; in this case the standing oestrus is the most reliable sign referable to a next ovulation. Utilizing a teaser bull and inseminating the animals after the end of heat, Baruselli [11] obtained a conception rate ranging from 40.44% and 60.68% depending on farms. Zicarelli *et al*. [12] reported that exposure to a vasectomised bull increase the pregnancy rate in buffaloes inseminated at spontaneous (42.5 vs 18.9%) or induced oestrus (51.1 vs 33.3%). Similar results were found from our group in buffaloes inseminated at spontaneous oestrus in presence of a vasectomised bull: pregnant cow rate was 56% in total and 40% at first oestrus [13].

### Automated Systems

New approaches are being developed in cattle to provide automated systems of detection of oestrus using electronic technology, such as pedometry and pressure sensing radiotelemetric HeatWatch® system [14-17].

### Pedometer

This device is used along with a computer to determine how far an animal has walked. Animals in heat are usually restless and may walk long distances since cows in heat are more mobile and walk 2 to 4 times as far as non-oestrous cows. The pedometer is attached to the metatarsus of a leg proximal to the fetlock. It measures the average number of steps taken per hour since the previous milking. The receiver is attached to a pole in the milking parlour and the data are recorded daily during milking. The recorded data from each cow are processed by a software program to produce a graph that flags any deviation in the data of a given day from that cow's own daily averages over the last 10 days.

Studies on the efficiency of pedometers in buffalo oestrus detection have been carried out in Italy by Di Palo *et al*. [18]. These Authors found pedometer very useful for AI, providing a greater number of alerts for spontaneous oestrus to be inseminated (conception rate at AI was 40%). The same researchers evaluating the sensibility of the system in the different months of the year, found that the number of detected oestrus decreased in the months with long day length, indicating that oestrous behavioural was less intense thus lowering the system signal efficiency [19]. They suggest that in order to increase the sensitivity of the pedometer oestrus detection system, the setting parameters should be modified in relation to the season.

### HeatWatch® System

Heat Watch® is a pressure sensitive telemetry device that is glued to the cow's tail head. The device sends a radio telemetry message to a receiver to be recorded by computer every time a cow is mounted. The message contains the date, time and animal ID. A cow is confirmed in heat if she is mounted 3 times within 4 hours.

In buffalo this heat detection system needs to be associated with the use of a teaser bull because the buffaloes are receptive to mounting activity mainly by the bull and occasionally by other cows. A study on oestrus detection using HeatWatch® system has been carried out in Brazil by Baruselli [20]. The Author reports that the distribution of mountings during the day did not present significant differences showing that buffalo present a homogeneous distribution of oestrus during the 24 hours of the day.

### Vaginal Electrical Resistance Probe

During oestrus there is a raise in the volume and ionic content of vaginal mucus, consequently the electrical resistance of vaginal mucous increases. Several probes are available for measuring vaginal electrical resistance (VER). One or two daily readings for several successive days are recommended for assured oestrus detection that depends on identifying the period when resistance is minimum and the following sharp rise. The rise in resistance indicates ovulation within a few hours and is the best period for insemination or mating. However, the animal handling requirements for twice daily measurement and the lack of automation make this method of limited applicability.

The use of VER probe to predict oestrus and ovarian activity has been studied by Gupta and Purohit [21] on Indian buffaloes. They proved that VER can be used successfully to predict the stage of oestrous cycle, ovarian status and ovulation; insemination at a low VER distinctly improves the conception rate in buffaloes (81.48 vs 16.66% with 26 and 40 ohms respectively).

## HORMONAL TREATMENTS

In order to increase the use of AI, easy management schemes, that not require the identification of oestrus, have been studied. These schemes are based on the manipulation of the hormonal events occurring during the oestrous cycle [22, 23, 24] as:

- manipulate peripheral progesterone concentration
- manipulate follicular growth and timing of ovulation

### Manipulate Peripheral Progesterone Concentration

Progesterone produced by corpus luteum (CL), exerts negative feedback on the release of gonadotrophin so that the endocrine events leading to the maturation of the preovulatory follicle and succeeding ovulation is inhibited until the progesterone drop and the regression of CL. Controlling the lifespan of CL it is possible control ovulation and synchronize oestrus. There are two ways to control or mimic the lifespan of CL:

a) by inducing premature luteolysis using luteolytic agents as the prostaglandins;

b) by simulating CL function by long-term administration of progesterone or progestogens, followed by sudden withdrawal.

#### *a) Induction of Luteolysis*

The luteolytic agents available on the market are derivates of prostaglandin $F2\alpha$ ($PGF2\alpha$). The $PGF2\alpha$ is naturally secreted by the uterus in the event that there has been no implantation during the luteal phase, causing regression of CL (luteolysis) and decreases of progesterone. This results in a rise in gonadotrophins and estradiol that leads to a return into oestrus. Injection of exogenous PGF2α or one of its analogues causes regression of CL within 24-72 h and oestrus and ovulation follow within 2-3 days. CL is responsive to the administration of luteolytic agents only during the midluteal phase, i.e. between days 5 and 17 of the cycle. This has to be taken into account when luteolytic agents are utilized to induce and synchronize oestrus. Utilized protocols include:

- PGF2α one-shot method
- PGF2α two-shot method

**PGF2α One-Shot Method**

With this method only those cows having a functional CL will be injected. These cows should show sign of oestrus within 2-3 days. This method has the disadvantage that animals have to be palpate or scan prior to be injected.

**PGF2α Two-Shot Method**

This method was planned to synchronize groups of cows at random without known their exact ovarian status. All females scheduled to be synchronized are injected on day 0 and on day 11 of the treatment. The basics of this schedule is that at the time of the 1st injection of PG will be responsive only those animals that have a functional CL (i.e. day 5 -17 of the cycle). These animals will ovulate and at the time of the 2nd injection will be about day 8 of the next cycle. The animals that did not respond at the 1st injection (i.e. those between day 18 and 4 of the cycle) would have a responsive CL at the time of the 2nd injection. Hence, all animals will be in the midluteal phase at the time of the 2nd injection of PG.

Animals may be inseminated either at fixed time (72h after 2nd PGF2α) or at observed oestrus.

Various Authors have recorded the use of PGF2α or one of its analogues in oestrus control in buffalo, often using an 11 day interval between two consecutive doses. Chohan *et al.* [25], in buffaloes synchronized with PGF2α, reported a fertility rate at AI of 22.86% in the low breeding season and 53.33% in the peak breeding one, concluding that the use of PGF2α to synchronize oestrus should be done in animals having a functional corpus luteum and preferably during the peak breeding season. Nevertheless, Sahasrabudhe and Pandit [26] reported that a high percentage of suboestrus buffaloes expressed oestrus after PGF2α treatment during the hot season. The detection of oestrus after prostaglandin treatment, however, had posed problems because external sign of oestrus were found by some workers to be less apparent than at spontaneous oestrus. Baruselli [20] detected a greater variation in the duration of oestrus manifestation after the administration of prostaglandin; moreover, he found that the phase in which prostaglandin was administered interfered in the interval from administration and the beginning of oestrus signs and ovulation; in fact this interval was shorter when PGF2α was given during early luteal phase of the oestrous cycle in the presence of a dominant follicle. Similarly, Brito *et al.* [27] found that the efficacy of PGF2α for causing luteolysis and ovulation was dependent upon progesterone plasma concentration and CL size before treatment. In addition, the interval from treatment to ovulation and the characteristics of the ovulatory follicle were dependent upon follicular status before treatment.

Therefore protocols using fixed time insemination and only prostaglandin treatment have not presented good results in buffaloes.

***b) Use of Progestins***

Progestins are exogenous or synthetic hormones that act as endogenous progesterone that is the hormon produce by CL during the diestrus phase of oestrous cycle. The administration of progesterone or its derivates extends the period of time in which progesterone is present into the circulation and prevents animal from coming into heat. Gonadotrophins release and ovulation is suppressed until progesterone withdrawal. In order to synchronize randomly a group of females without regard for the stage of oestrus cycle, it is necessary to treat them with progesterone for a period corresponding to the length of natural luteal phase, otherwise CL might live longer than the progesterone treatment. However, the long-term progesterone treatment (i.e. 16 days like a natural luteal phase) leads to a low conception rate, probably due to the adverse effects in the intrauterine environment. Therefore a short-term treatment (7-12 days) is preferable but in this case it is necessary to include a PGF2α in order to eliminate any natural CL.

Some of progestins utilized for synchronize oestrus include: melengoestrol acetate (MGA), Norgestomet and intravaginal progesterone device.

The **MGA** is a synthetic analogue of progesterone in a form that is orally active, so it can be administered in the feed. However, this way of administration presents problem of drug dosage monitoring because it must be ensure uniform daily consumption of feed during oral administration of MGA, otherwise oestrus synchronization may be variable. Moreover there could be the possibility of milk residue and for this reason it is utilized more in prepubertal heifers. With this method, animals are fed at rate of 0.5 mg/head/day for 14 days. Oestrus occurs between 2 and 5 days after removal of MGA but females should not be inseminated at this time due to low conception rate. Nineteen days after MGA removal animal are injected with PGF2α that will cause the luteolysis of functional CL and return to oestrus. Females will be inseminated at the detected oestrus or at fixed time (72h after PGF2α).

The **Norgestomet** is a synthetic analogue of progesterone and consists of an impregnated silastic implant containing 3 mg of Norgestomet. The implant is inserted subcutaneously beneath the skin of ear. At the time of implantation an intramuscular injection of 5 mg oestradiol valerate in combination with 3 mg of Norgestomet is given. The implant will be withdrawal after 9 or 10 days. Two days before implant removal animals are injected with PGF2α in order to eliminate any natural CL. Insemination can be done 48 (heifers) or 56 (cows) h after implant removal.

The **intravaginal progesterone devices** are present on the market by the name of **PRID®** (Progesterone Releasing Intravaginal Device) and **CIDR®** (Controlled Internal Drug Releasing device). The PRID is a stainless steel spiral device covered with an inert silicone rubber matrix containing 1.55 g of progesterone. CIDR is a T-shaped device with flexible wings impregnated with1.38 g of progesterone in elastic silicone moulded over a nylon spine. The device (PRID/CIDR) is inserted into vagina using a special applicator. A string is attached for easy removal. When the device is inserted into the vagina, progesterone is slowly released over the treatment period. Under the influence of progesterone, normal pituitary gonadotrophin output is inhibited and the ovarian cycle is interrupted. The removal of the device results in the rapid decline of plasma progesterone and the onset of oestrus in animals responding to treatment.

Natural or synthetic progesterone containing devices (injections, intravaginal pessary, ear implants along with estradiol, PMSG and prostaglandin) have been used successfully to improve synchrony of oestrus and conception in buffaloes [20, 28-30]. The synchronization protocols, however, are efficient if buffaloes are cyclic and therefore if they are used during the breeding season (autumn). In the spring season there is a higher variability between the oestrus beginning and the ovulation time and it is more difficult to establish the correct time for AI.

Our previous work showed that the use of a progesterone pessary (PRID), associated with PMSG and prostaglandin, is able to control ovulation and induce a good rate of synchronization in buffaloes. Using this synchronization treatment schedule in the peak breeding season (autumn) and the low breeding one (spring), we found no differences in the fertility rate between the two seasons considered: in fact the CR was 46.2% and 44.3% in autumn and in spring respectively [31]. We found that the use of PMSG increases the fertility that is related to the doses utilised; in fact CR was 26% in buffaloes in which PRID + 500 IU PMSG were used and 17.5% in buffaloes in which PRID was used without gonadotrophin [32]. To better define the proper time for AI following the PRID synchronization treatment we have evaluated the time of LH peak, after pessary removal, in two different seasons [33, 34]. On the basis of these results we can suggest that 72 and 96 h after PRID removal are more appropriate time for AI in synchronized buffalo cows in the low breeding season, while 48 and 72 h could be better in the autumn. In fact, utilising 2 AI schedules at 72 and 96 h during the spring season we have obtained a CR of 51 % and 56.7% [32, 35]. Therefore the use of PRID associated with PMSG and prostaglandin can be successfully employed to increase the effectiveness of AI programmes improving the fertility rate in the low breeding season too.

## Manipulate Follicular Growth and Timing of Ovulation

### *GnRH Protocols*

The GnRH (Gonadotrophin Releasing Hormone) is a naturally occurring hormone that stimulate the release of LH and FSH by the anterior pituitary gland. These two hormones act on the ovary, stimulating follicular development (FSH) and ovulation (LH). The use of GnRH has been associated to that of prostaglandin, in order to decrease the variation in the ovulation time after prostaglandin treatment. One program that has been extremely successful for insemination of cows at a fixed time without the need for detection of oestrus is the **Ovynch** program. Ovulation synchronization can be obtained using GnRH

+ prostaglandin after 7 days + GnRH after 48 h. This system synchronizes follicle maturation with regression of the corpus luteum before the GnRH-induced ovulation and timed insemination. The animals are inseminated 16–20 h after the second injection of GnRH. The limitation of this protocol is that it works better when the animals are cyclic, so it is not recommended in heifers unless they have reached puberty, and it is not recommended in buffaloes during the non-breeding season.

Baruselli [20] using Ovsynch protocol had a CR of 48.8% in buffaloes inseminated during the breeding season (autumn-winter) and 6.9% in that inseminated during the non-breeding season. Similarly, De Rensis *et al.* [38] using Ovsynch have a higher conception rate in cyclic compared to non-cyclic buffaloes (35.7 vs 4.7%). Other Authors using the Ovsynch protocol reported a CR at AI ranged from 56.5% [39], if used during the breeding season, to 36.0% [40] and 42.55% [41] if used in the period of transition to seasonal anoestrus.

### Hormonal Treatment Schedule and the TAI (Timed Artificial Insemination)

The oestrus synchronization and timed artificial insemination protocols more utilized in buffalo are Ovsynch and PRID or CIDR, this last associated to PGF2α and PMSG. In succession are reported the treatment schedule and the time of AI in the two protocols (Figures **1** and **2**).

In the Ovsynch protocol buffaloes are treated with 150 µg gonadorelin (GnRH) + 150 µg cloprostenol (PGF2α) on day 7 +150 µg gonadorelin (GnRH) on day 9. Buffaloes are artificially inseminated after 16 hours from the 2nd injection of GnRH. In the Progesterone based protocol buffaloes are treated with an intravaginal device containing natural progesterone (1.55 g in PRID or 1.38 g in CIDR) for 10 days + 1000 IU PMSG (Pregnant Mare Serum Gonadotrophin) and 150 µg cloprostenol (PGF2α) on day 7 after device insertion. Buffaloes are artificially inseminated at 72 and 96 h from device removal. The Progesterone based protocol is to prefer during the low breeding season (spring-summer) when the buffaloes are non-cyclic. The Ovsynch protocol is efficient if the buffaloes are cyclic and therefore during the breeding season (autumn) [41].

## SPERM SEXING TECHNOLOGIES

Sexing technology takes advantage of the physiological differences between X and Y spermatozoa, favouring the presence of the desired

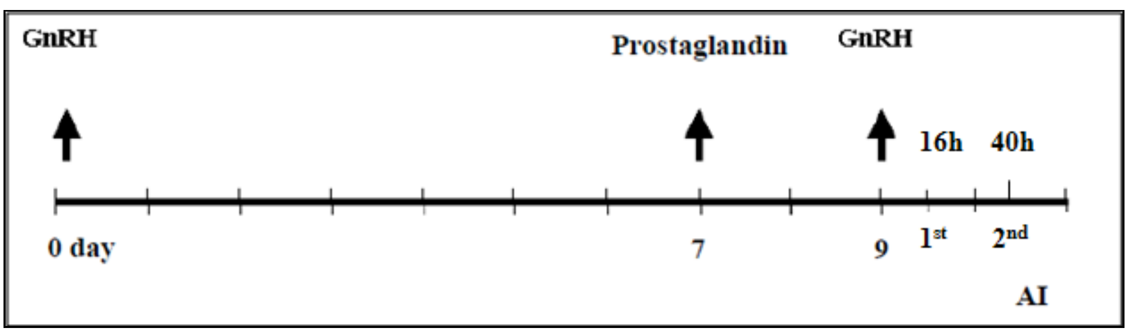


**Figure 1: Treatment schedule and timed AI in the Ovsynch protocol in buffaloes:** one insemination at 16h after the 2nd injection of GnRH is sufficient, but sometime a second insemination 24h later is utilized.

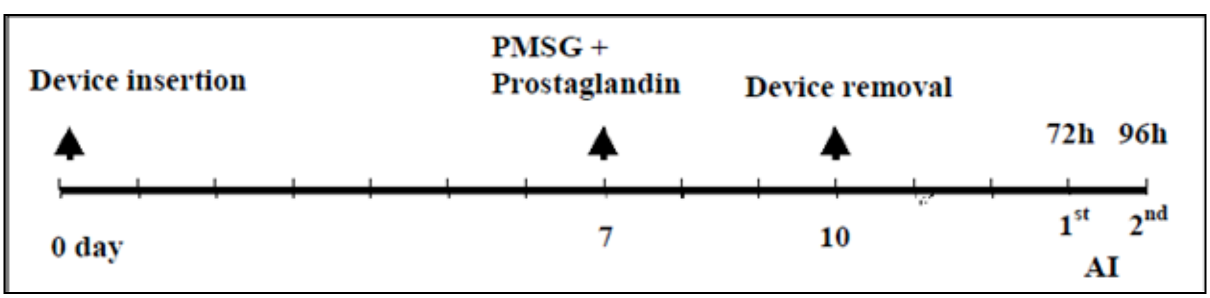


**Figure 2: Treatment schedule and timed AI in PRID/CIDR protocol in buffaloes:** two inseminations are necessary because of a large variability in the ovulation time with this protocol; 72 and 96 hrs after device removal are appropriate time for the insemination.

kind of spermatozoa in the moment of fertilization and deviating the physiological sex ratio [42]. Although the basic principles controlling the sex of mammalian offspring have been known for a relatively long time, recent application of flow cytometry sorting system led to differentiation and separation of living X and Y chromosome-bearing spermatozoa [43]. This procedure, based on difference in spermatozoa DNA content, brought to a commercialization of this sexing technology. A number of companies now offer sex-sorted bovine sperm, subsequently, the use of sexed semen for AI is being widely commercialized in cattle. However, a great disadvantage of this approach is the high cost of the semen doses and the reduced pregnancy rates when compared with no-sexed semen. Even though the efficiency of this technology continues to get better, at present, sexed semen is only available from relatively few selected bulls. Recently, this new strategy is being introduced in buffalo too [44].

Because dairy heifer calves are significantly more valuable than bull calves, a reliable method of swaying the bovine sex ratio in favour of females is desirable in the dairy industry. At present, there is no inexpensive method for commercially separating X- and Y-bearing bovine sperm cells. An American company produced a post-thaw semen treatment product that attempted to alter the bovine sex ratio in favour of females. Although the ingredients are undisclosed, the manufacturer published internet resources supporting its efficacy [45]. The product works by enhancing the fertility of the X-chromosome bearing (female) sperm and slowing the motility of the Y-chromosome bearing (male) sperm. When inseminated, the sperm sort in the reproductive tract of the dam. The result is more ova fertilized by the X-chromosome bearing (female) sperm. The sex ratio would increased by 20-25%. This product requires a delay in timing of insemination, therefore using Ovsynch protocol cows have to be bred 24 hours after GnRH. If the results indicate from the producer company will be corroborate, the product could be an alternative approach to the sex pre-selection of offspring, with the advantage to be used with the semen of whatever bull.

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Received on 29-03-2012 Accepted on 18-05-2012 Published on 01-09-2012

DOI: http://dx.doi.org/10.6000/1927-520X.2012.01.02.02