COMPARISON OF 5-DAY VERSUS 7-DAY PRID®DELTA CO-SYNCH PROTOCOLS IN BEEF CATTLE

By

JIGDREL DORJI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Veterinary Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Veterinary Science

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Chairman : Mark Hiew Wen Han, PhD
Faculty : Veterinary Medicine

The main advantage of timed artificial insemination (TAI) protocols is that they allow artificial insemination without the need for heat detection. CO-Synch programs are the type of TAI programs where second dose of GnRH is injected concurrently at the time of TAI. However, literature of CO-Synch programs from tropical regions of the world is unavailable. The objectives of this study were to compare 5-day CO-Synch (5DCOS) and 7-day CO-Synch (7DCOS) plus progesterone-releasing intravaginal device (PRID®Delta) in terms of estrus response rate (ERR), electrical resistance of vaginal mucus (ERVM), and progesterone (P4) profile in beef cattle. A total of 26 Brangus-cross cattle (18 postpartum cows and 8 heifers) were randomly assigned to two groups to receive either the 5DCOS or 7DCOS protocol. Animals in both treatment groups received P4 device and GnRH on days D-7 and D-5, respectively for 7DCOS and 5DCOS. On day 0 (device removal), cattle in 7DCOS received a single dose of prostaglandin (PGF2α), while cattle in 5DCOS received two doses 8 hours apart (initial dose upon device removal). The second dose of GnRH was injected at the scheduled TAI; 58 and 72 hours after PGF2α. The result showed ERR was proportionally higher in cows in 7DCOS than 5DCOS, but the difference was not significant (80 vs. 37.5%, $p > 0.05$). Likewise, the ERR in heifers was not significantly different (75 vs. 50%, $p > 0.05$). No significant differences were observed in EVRM across days in both cows and heifers, and also ERVM failed to distinguish cattle in estrus and non-estrus. P4 concentration was relatively constant throughout the treatment period in both cows and heifers ($p > 0.05$). 7DCOS in cows and 5DCOS in heifers observed comparatively high P4 values, but the differences between days were not significant ($p > 0.05$). In conclusion, both protocols effectively induced estrus response in both cows and heifers and maintained a consistently high circulating P4. However, ERVM was not effective in identifying cattle in estrus in a TAI program.

Keywords: Timed artificial insemination, beef cattle, electrical resistance of vaginal mucus, estrus response rate, PRID®Delta.

SDG: GOAL 1: No Poverty, GOAL 2: Zero Hunger

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains Veterinar

PERBANDINGAN ANTARA PROTOKOL PRID®DELTA CO-SYNCH 5 HARI DAN 7 HARI PADA LEMBU PEDAGING

Oleh

JIGDREL DORJI

Januari 2025

Pengerusi : Mark Hiew Wen Han, PhD
Fakulti : Perubatan Veterinar

Kelebihan utama protokol permanian beradas bermasa (TAI) ialah keberkesanannya tanpa keperluan mengesan biang. Objektif kajian ini adalah untuk membandingkan CO-Synch 5 hari (5DCOS) dan CO-Synch 7 hari (7DCOS) serta alat intravaginal yang mengandungi progesteron (PRID®Delta) dari segi kadar tindak balas estrus (ERR), rintangan elektrik mukus vagina (ERVM), dan profil progesteron (P4) pada lembu pedaging. Sebanyak 26 lembu kacukan Brangus (18 ekor lembu yang pernah melahirkan anak/lembu matang dan 8 ekor betina dara) secara rawak dibahagikan kepada dua kumpulan untuk menerima sama ada protokol 5DCOS atau 7DCOS. Haiwan dalam kedua-dua kumpulan rawatan menerima alat P4 dan GnRH pada hari ke-7 dan ke-5, masing-masing untuk 7DCOS dan 5DCOS. Pada hari 0 (pengeluaran alat P4), lembu dalam kumpulan 7DCOS menerima satu dos prostaglandin (PGF2α), manakala lembu dalam 5DCOS menerima dua dos pada selang waktu 8 jam (dos pertama diberi apabila alat dikeluarkan). Dos kedua GnRH diberikan semasa permanian beradas (AI) yang dijadualkan; 58

dan 72 jam selepas. PGF2α. ERVM dan pemerhatian visual estrus dilakukan dari hari pengeluaran alat sehingga hari AI yang dijadualkan. Didapati ERR menunjukkan kadar yang lebih tinggi pada lembu dalam kumpulan 7DCOS berbanding 5DCOS, tetapi perbezaannya tidak ketara (80 berbanding 37.5%, $p > 0.05$). ERR pada lembu betina dara dalam dua kumpulan tersebut juga tidak jauh berbeza (75 berbanding 50%, $p > 0.05$). Tiada perbezaan ketara diperhatikan pada EVRM sepanjang pemerhatian dilakukan pada kedua-dua lembu matang dan lembu dara, dan juga ERVM gagal membezakan lembu dalam estrus dan tidak estrus. Kepekatan P4 adalah konsisten sepanjang tempoh rawatan pada kedua-dua lembu matang dan lembu betina dara ($p > 0.05$). Lembu matang 7DCOS dan lembu betina dara 5DCOS menunjukkan nilai P4 yang agak tinggi, tetapi perbezaan antara hari adalah tidak ketara ($p > 0.05$). Kesimpulannya, kedua-dua protokol berkesan mencetuskan tindak balas estrus dalam kedua-dua lembu matang dan lembu betina dara serta mengekalkan P4 di dalam badan yang tinggi secara konsisten. Walau bagaimanapun, ERVM tidak berkesan dalam mengenal pasti lembu dalam estrus di dalam program TAI ini.

Kata Kunci: Permanian beradas bermasa, lembu pedaging,rintangan elektrik mukus vagina, kadar tindak balas estrus, PRID®Delta

SDG: MATLAMAT 1: Tiada Kemiskinan, MATLAMAT 2: Tiada Kelaparan

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The members of the Supervisory Committee were as follows:

Mark Hiew Wen Han, PhD
Senior Lecturer
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Nurhusien Yimer Degu, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

ZALILAH MOHD SHARIFF, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 8 May 2025

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LIST OF ABBREVIATIONS

5DCOS	Five-day CO-Synch
7DCOS	Seven-day CO-Synch
ACTH	Adrenocorticotropic hormone
AI	Artificial Insemination
BCS	Body condition score
CIDR®	Controlled internal drug release
CL	Corpus luteum
CR	Conception rate
E2	Estradiol
EB	Estradiol benzoate
eCG	Equine chorionic gonadotropin
ERCM	Electrical resistance of cervical mucus
ERR	Estrus response rate
ERVM	Electrical resistance of vaginal mucus
ETT	Embryo transfer technology
FSH	Follicle stimulating hormone
FTAI	Fixed time artificial insemination
GnRH	Gonadotropin releasing hormone
hCG	Human chorionic gonadotropin
LH	Luteinizing hormone
MGA	Melengestrol acetate
P4	Progesterone
PBS	Phosphate Buffer Saline
PGF2α	Prostaglandin F2 alpha

PMSG	Pregnant mare serum gonadotropin
PPP	Pusat Pertanian Putra
PR	Pregnancy rate
PRID®Delta	Progesterone releasing intravaginal device Delta
TAI	Timed artificial insemination
UPM	Universiti Putra Malaysia

CHAPTER 1

INTRODUCTION

1.1 Background

Estrus synchronization is a valuable reproductive management tool that plays a key role in optimizing breeding practices in cattle industries. By inducing synchronized ovulation in heifers and cows at a predetermined time, timed artificial insemination (TAI) protocol makes artificial insemination (AI) and embryo transfer easier to perform (Lamb & Mercadante, 2016; Nowicki et al., 2017; Thatcher et al., 2001). Using gonadotropin releasing hormone (GnRH) and prostaglandin F2α (PGF2α), the first successful TAI program was developed and given the name "OV-Synch" (Pursley et al., 1995). Since then, additional TAI protocols have been developed, such as the 7-day CO-Synch protocol and the 5-day CO-Synch protocol, which decreases the number of animal handlings and give beef producers the chance to incorporate them into their reproductive management programs (Colazo & Mapletoft, 2014).

The use of intra-vaginal progesterone devices during ovulation synchronization is a common practice as low levels of progesterone are linked to decreased fertility in high-yielding dairy cows (Colazo & Mapletoft, 2014). Progesterone (P4) supplementation during a TAI program offers several benefits such as enhancing ovulation synchronization by reducing the likelihood of the occurrence of ovulation before the TAI (Bisinotto et al., 2015).

This increases the proportion of pre-pubertal heifers and cows that have their cycles initiated (Lamb et al., 2007; Smith et al., 2011; Stevenson & Lamb, 2016) and further prevents premature estrus expression (Kim et al., 2003).

Response to estrus synchronization is essential for maximizing reproductive efficiency because cows and heifers that exhibit estrus behaviors before TAI were found to have higher pregnancy rates. Richardson et al. (2016), conducted a meta-analysis and found that the likelihood of pregnancy was 27% greater in cows and heifers that expressed estrus before TAI. Likewise, Pohler et al. (2016) found that cows seen in estrus prior to AI had pregnancy losses between days 30 to 100 that was lower than those that did not express estrus.

On the other hand, there are several benefits of identifying cows in estrus. Firstly, one could decide to use expensive sexed semen for cows expressing estrus and less expensive conventional semen for cows not expressing estrus to reduce the cost. Secondly, this helps the selection of embryo recipient cows during embryo transfer technology, as cows that expressed estrus had a greater chance of establishing pregnancy than those that did not (Pereira et al., 2016). Moreover, higher estrus response indicates a successful estrus synchronization program which increases the probability of successful conception (Reith & Hoy, 2018) while fewer estrus response delays genetic progress and overall productivity (Boer et al., 2010).

Several estrus detection tools are available for use such as pedometer, radio-telemetric system, pressure sensing devices as well as monitoring vaginal temperature and milk progesterone levels. However, not all these tools are adequately sensitive and reliable enough to replace the frequent visual observation (Nebel et al., 2011). Previous research has indicated that the electrical resistance of vaginal mucus (ERVM) decreases during estrus (Senger, 1994) and found to coincide with luteinizing hormone peak (Aboul-Ela et al., 1983). Similarly, the electrical resistance of vaginal mucus has been used to detect estrus and determine the optimal time for artificial insemination (Fazleabas et al., 1990; Meena et al., 2003). Prior studies on ERVM have mostly focused on normal estrous cycle and very limited studies are available that looked at TAI programs. An investigation by Zuluaga et al. (2008) found that the ERVM was unable to distinguish between cows with and without large follicles, making it impossible to predict when AI should be conducted in the 7-day CO-Synch plus CIDR® program.

1.2 Problem Statement

Comparative studies on fertility parameters, particularly in the context of 5 and 7-day CO-Synch protocols have been conducted previously. However, the primary focus of these studies was the use of the CIDR® (Controlled Internal Drug Release, 1.38 g P4) device as the primary source of progesterone and conducted in the temperate and subtropical regions of USA and Canada. Additionally, studies on ERVM have been predominantly conducted during natural estrus, resulting in a significant knowledge deficit regarding ERVM in context of TAI programs. Furthermore, with the introduction of new progesterone device: Progesterone Releasing Intra-Vaginal Device-Delta (PRID® Delta; 1.55g P4), a novel intra-vaginal progesterone delivery device in the Malaysia there is a need of study to assess the fertility parameters using PRID® Delta in Malaysian dairy and beef industries. Therefore, the current study aimed to fill this significant gap in the scientific literature by conducting a comparative analysis of the 5 and 7-day CO-Synch + PRID®Delta protocols in terms of estrus response rate (ERR), ERVM trend, and progesterone profiles in beef cattle.

1.3 Research Objectives

1. To compare estrus response rates (ERR) between 5 and 7-day CO-Synch + PRID®Delta protocols for cows and heifers.
2. To compare the trend of electrical resistance of vaginal mucus (ERVM) between 5 and 7-day CO-Synch + PRID®Delta protocols for cows and heifers.
3. To compare ERVM of scheduled AI between cattle expressing estrus and those not expressing estrus behaviors.
4. To compare progesterone profile between 5DCOS and 7DCOS protocols in cows and heifers.

1.4 Research Hypotheses

Objective 1:

H0: There is no difference in the ERR between the 5DCOS and 7DCOS protocol in cows and heifers.

Ha: There is a difference in ERR between the 5DCOS and 7DCOS protocol in cows and heifers.

Objective 2:

H0*:* There is no difference in the ERVM trend between the 5DCOS and 7DCOS protocol in cows and heifers.

Ha*:* There is a difference in the ERVM trend between the 5DCOS and 7DCOS protocol in cows and heifers.

Objective 3

H0**:** There is no difference in the ERVM values between cows and heifers that expressed estrus and those that did not.

Ha: There is a difference in the ERVM values between cows and heifers that expressed estrus and those that did not.

Objective 4

H0**:** There is no difference in the progesterone profile between the 5DCOS and 7DCOS protocols in cows and heifers.

Ha**:** There is difference in the progesterone profile between the 5DCOS and 7DCOS protocols in cows and heifers.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview

The global human population is expected to witness a growth of approximately 2 billion people in the next three decades from the current 8 billion to an estimated 9.7 billion by 2050 (United Nations, 2021). Global meat consumption is rising annually and it is estimated that by the year 2050, it could range from 460 million to 570 million tons with an estimated increase by 19% from 2019 to 2050 (Falcon et al., 2022). Therefore, a proportionate increase in the global meat production is required to address the rising consumption. Assisted reproductive technologies such as estrus synchronization and artificial insemination would provide rapid increases in cattle numbers to meet global beef demand and enhance food security.

Although CO-Synch programs have shown promising breeding outcomes in temperate regions of the world such as the United States of America and Canada, such protocols have not been studied in Malaysia. This lack of research could be due to various factors, where the major dairy and beef companies focus more on already established reproductive management practices without relying on research and development leading to less emphasis on research in this area. As significant amount of high quality beef is still imported, Malaysia faces a unique challenge which may result in producers emphasizing on quantity over quality especially for beef intended for daily consumption which does not require higher quality demanded by specialty beef cuts. This sentiments among producers can lead to deteriorating interest among local beef producers in investing for advanced reproductive technologies and research.

2.2 Beef Consumption in Malaysia

Data from Statista Research Department (2023) showed that Malaysia's cattle stock as of the year 2021 was 0.7 million which is 0.04 million lesser than in 2012. Although, the current cattle number indicates an increase compared to the year 2019 (0.66 million), there is a general trend of decline over the 10 year timeframe. Beef consumed in Malaysia mainly consists of meat derived from cattle and buffalo. However, production from domestic sources is inadequate to reduce imports due to the growing demand for beef and beef products (Ariff et al., 2015; Hamir et al., 2022) as it is the most demanded meat in Malaysia. Although there is a general preference towards purchase of imported beef, unknown slaughtering methods (halal preferred) and issues related to cattle smuggling has cast a negative perception on imported beef amongst consumers (Hasan, 2022). Local beef is now preferred because it is halal and fresher than imported beef (Hamir et al., 2022).

2.3 Reproductive Anatomy of Cow

For an efficient reproductive management of cattle, good knowledge on reproductive anatomy of both male and female cattle is important (Whittier, 1988). The understanding of reproductive tract structures such as vagina, cervix, uterus, and ovaries helps with the interpretation of estrous cycle physiology and reproductive pathology (Lefebvre & Gnemmi, 2009).

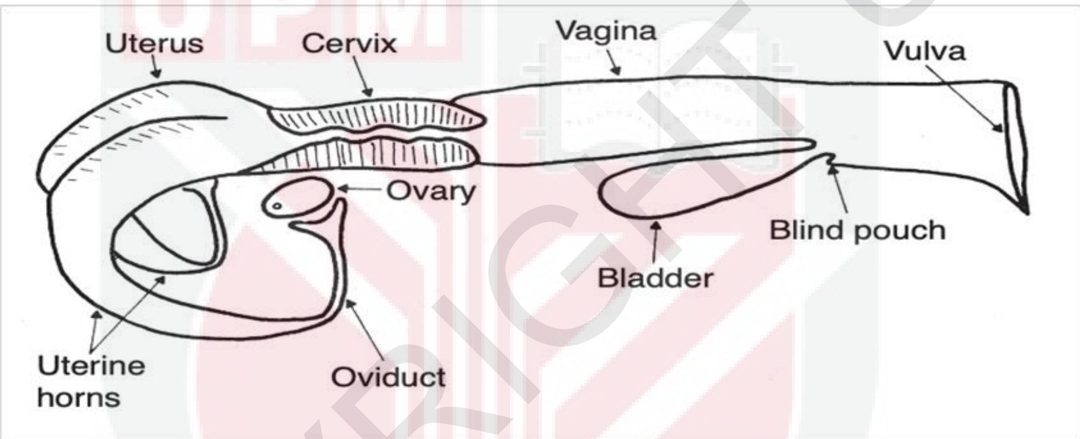

Figure 1: The Anatomy of the Reproductive Tract of Cow. Adapted from Rich & Turman (1981)

2.3.1 Vulva and Vagina

The vulva is the outermost opening of the cow's reproductive tract, which serves as the entry point for the bull's penis and AI gun, aids in the expulsion of the fetus, and provides an exit point for urine (Ball & Peters, 2008). The vagina is located between the caudal aspect of the cervix and vestibular border at the external urethral opening (Nabors & Linford, 2014).

2.3.2 Cervix

The cervix is situated at the pelvic floor, usually a firm, cylindrical and mobile structure measuring 7 to 10 cm in length and 3 to 4 cm diameter. Parity and cyclicity affects its size and location in the body and it appears lobulated due to the presence of 3 to 4 cervical mucosal rings (Lefebvre & Gnemmi, 2009). The hard muscular sphincter serves as a barrier between internal and external genitalia (Nabors & Linford, 2014). During estrus, the cervix relaxes due to hormonal changes and facilitates the introduction of an AI gun. It remains closed due to a cervical mucus seal during pregnancy to prevent exposure of the developing fetus to infectious organisms.

2.3.3 Uterus

The uterus of a cow has a short body (3 cm) with two uterine horns, 30 to 40 cm in length, which remain attached at the cornual bifurcation supported by the inter-cornual ligament (Lefebvre & Gnemmi, 2009). The uterus plays very important roles such as transport of spermatozoa from the site of ejaculation to the site of fertilization in the oviduct. It also regulates implantation, pregnancy, and parturition (Hafez & Hafez, 2000).

2.3.4 Ovary

A female’s primary reproductive organ is the ovary. They are suspended at the end of the oviduct with the help of the broad ligament. The main function of ovary is to produce ovum and secrete estrogen and progesterone which are major hormones of reproduction. It is oval in shape measuring 2.5-3.8 cm in length containing thousands of primary follicles consisting of germ cells which have the potential to become a mature egg (Rich & Turman, 1981).

2.4 Stages of Estrus and Hormonal Regulation of Estrous Cycle

The bovine estrous cycle (Figure 2) is an important aspect of female cattle reproduction characterized by hormonal and physiological changes that effects reproduction. The cycle lasts an average of 21 days ranging from 18-24 days (Crowe, 2022) which includes different phases: the diastral and follicular phase. Diastral phase starts after ovulation and lasts about 18 to 14 to 18 days characterized by formation of corpus luteum (CL) whereas, follicular phase lasts about 4 to 6 days at which the CL is broken down leading to maturation of follicles to pre-ovulatory follicle. Estrus is a period of sexual receptivity that lasts about 10 to 18 hours with ovulation ensuing in about 10 to 12 hours after the estrus (Lamb et al., 2010; Rathbone et al., 2001)

The estrous cycle is primarily controlled by several hormones (Figure 3). Gonadotropin-releasing hormone (GnRH) from the hypothalamus causes the synthesis and release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from anterior pituitary gland. FSH is responsible for the growth of ovarian follicles, while luteinization of ovulatory follicle and ovulation is triggered by LH (Visser & Themmen, 2014). Once ovulation has occurred, the Graafian follicle then becomes the corpus luteum, which is the source of progesterone (P4), required for maintaining pregnancy. The amount of circulatory P4 level during pregnancy play crucial role as it supports secretion from endometrial gland and fetal development. If the fertilization did not occur, the endometrium secretes prostaglandin F2α to breakdown the CL to begin the next follicular wave (Lonergan & Forde, 2015).

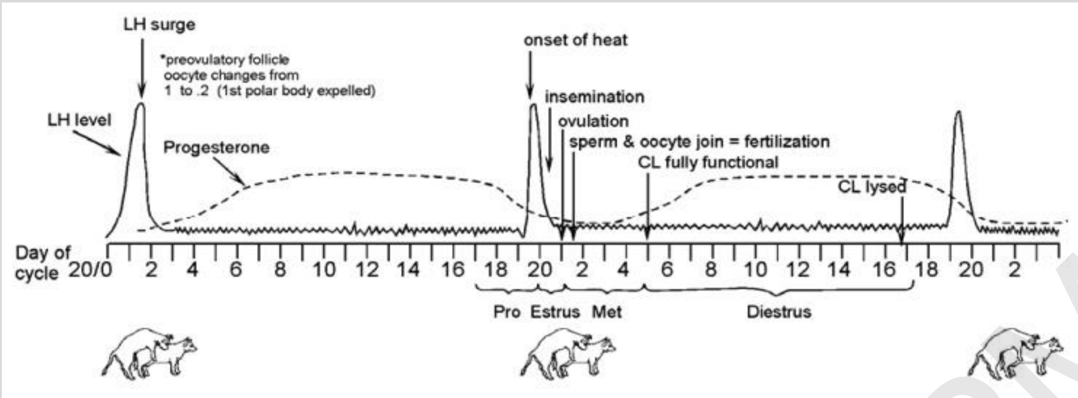

Figure 2: Phases of the Estrous Cycle with Luteinizing and serum Progesterone Hormone. Adapted from (Larson & Randle, 2008)

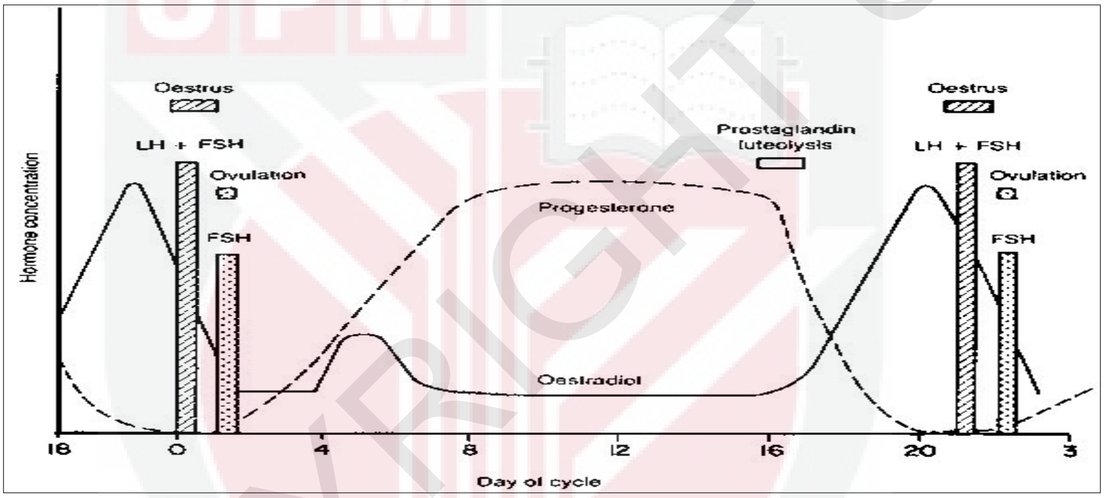

Figure 3: Pattern of the Major Reproductive Hormones during Estrous Cycle. Adapted from (Kubovicová et al., 2008)

2.5 Estrus Synchronization

Estrous synchronization involves manipulating the estrous cycle to bring large numbers of females into heat in a short, predefined time (Yizengaw, 2017) at the same time (Salverson & Perry, 2005). Understanding the underlying mechanisms and physiology of regulation of the estrous cycle is key to develop and apply estrus synchronization protocols. The natural estrous cycle can be artificially manipulated using exogenous hormones so that estrus and ovulation can be controlled to facilitate AI at optimal time. Initially, estrus synchronization was achieved by the use of PGF2α by eliminating the CL which required estrus detection to identify cows for artificial insemination. Synchronization of estrus reduces the calving season, improves calf uniformity (Lamb et al., 2007). According to Salverson and Perry (2005), estrus synchronization protocols are grouped under 4 classes: 1) Prostaglandin-based, 2) GnRH-based, 3) Progestin-based, and 4) combinations. However, use of PGF2α, GnRH and progestins alone will synchronize either luteal or follicular phase or not both the phases. Therefore, many synchronization protocols combine the previous three hormones to control and regulate both follicular growth and CL.

2.6 Hormones Utilized for Estrus Synchronization

Estrous synchronization is crucial for enhancing reproductive performance in cattle, allowing more concentrated breeding season and herd fertility management. The primary hormones involved in this process include prostaglandins (PGF2α), gonadotropin-releasing hormone (GnRH), and progestins.

Prostaglandins (PGF2α) are utilized to induce luteolysis, causing regression of the corpus luteum (CL) and to initiate a new follicular wave (Yizengaw, 2017). This action is important for synchronizing estrus, as it facilitates timing of AI to coincide with ovulation. However, the utilization PGF2α alone can only synchronize the luteal phase, necessitating the use of other hormones to control both the luteal and follicular phases of the estrous cycle effectively.

GnRH plays a primary role in causing the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary gland, which are essential for follicular growth and ovulation (McArdle, 2007). By administering GnRH at strategic points in the estrous synchronization protocol, breeders can effectively synchronize ovulation, thereby improving the time for AI.

Progestins are often delivered via intravaginal devices, which are used to maintain a controlled hormonal environment that supports follicular development and also prepares the uterine environment for pregnancy (Hameed et al., 2021). By combining these hormones in various protocols, they allow for a more comprehensive approach to estrus synchronization, regulating both the follicular and luteal phases of the estrous cycle. The various hormones and their roles in cattle reproduction is shown in Table 1

Table 1: **Hormones and their Role in Reproduction**

Hormone	**Endocrine Gland**	**Function**
P4	Corpus luteum	Prevent estrus and ovulation Prepares uterus and maintain pregnancy
PGF2α	Uterus	Luteolysis
GnRH	Hypothalamus	Control LH Secretion and LH surge Induce ovulation
FSH	Anterior pituitary	Initiate follicular wave
LH	Anterior pituitary	Induce ovulation Oocyte maturation Luteal tissue formation
E2	Ovarian follicle	Estrus expression Induction LH surge Transport of spermatozoa

2.7 Artificial Insemination (AI)

Artificial insemination refers to the procedure of introducing semen into the female reproductive tract by means excluding natural service (Schultz et al., 2020). It is an old assisted reproductive technology (ART) widely practiced globally for upgradation of breeds and rapid transfer of valuable genetic materials. The first successful AI technology was demonstrated in dogs by Lazzaro Spallanzani in 1784 (Foote, 2010). It offers several advantages over natural breeding. The historical reason for AI was to control transmission of venereal diseases while currently it is viewed as a method of improving genetic material (Parkinson, 2019).

In Malaysia, the first artificial insemination in cattle using frozen semen from ampoules of Holstein bulls were imported from Australia in 1963 (Raymond & Saifullizam, 2010). In the tropical countries, such as in Malaysia, beef cattle breeding is predominantly natural using Bulls (Azizah et al., 2014). Furthermore, the method of breeding and rearing are primarily focused on small holders (less than 50 heads) rather than medium and commercial (more than 50 heads) according to Saufi et al. (2023) inferring tremendous potential to increase beef cattle population. Beef cattle serve an important protein source however, there is greater demand for beef than supply in Malaysia. Furthermore, the imported beef per kilogram is cheaper than locally available beef making it an important issue in food security (Ariffin et al., 2023). The Malaysia's struggle to attain beef sufficiency could be due to lack of grazing areas and scare feed sources (Saufi et al., 2023). In order for Malaysia's beef industry to realize beef sufficiency, reduce import, feed and fodder availability issue needs to be addressed first. Although the use of assisted reproductive technologies in beef cattle has been implemented however, literature on success rates is currently inconclusive. For example, a pregnancy rates ranging from 24.1% to 36.6% have been achieved in a timed artificial insemination program (Malik et al., 2012).

Over the past decades, Artificial Insemination (AI) technology in cattle has observed significant advancements enhancing genetic progress, breeding efficiency, and overall productivity in cattle reproduction. These advancement have been associated with innovative technologies in the handling of semen, detection of estrus, and techniques of AI and as well as its integration with genomic selection and machine learning. Technological advancement have helped in genetic distribution and increasing pregnancy outcomes as a result of improved processes in terms of semen collection, processing, and preservation of semen indefinitely through cryopreservation. Further, breeders were able to control the over the sex of offspring preventing undesirable calf production through the use of sex-sorted semen (Mikkola et al., 2024; Praveen et al., 2024). Furthermore, analysis of luteinizing hormone in urine has also improved AI timing. Most importantly, with an advent of timed AI (TAI) programs has enabled breeding without concerning the need of estrus detection process that enabled rapid genetic gain (Sales et al., 2024).

Another important advancement in cattle breeding is integration of machine learning and genomic selection. Using such technology has helped breeders in choosing the best embryos. This advancement has further allowed breeders in selecting excellent breeding animal at a young age (Mikkola et al., 2024). Although technological advancement have been observed in the field of animal breeding, challenges still remain in terms of success rates of AI specifically in developing countries. Factors such low-quality semen, lack of insemination skills, and environmental conditions have negatively impacted pregnancy outcomes with AI success rates performing below average implying requirement of further research and innovation (Mekonnen & Mariam, 2024).

In the review paper by Monteiro et al. (2023), globally, pregnancy rates have improved over the last 27 years. For instance, use of progesterone implants increased pregnancy rates from 44.3 to 54.3%, while use of second PGF2α in 5DCOS increased from 53.2 to 60.9%. Similarly, GnRH injection at the time of TAI increased pregnancy rates from 54.7 to 59.2% particularly in cows. Similarly, Dias et al. (2020) observed a pregnancy rate of 55.5% in 2018 and 52.8% in 2019, suggesting improvement in AI success rates. Their findings underscore the importance of technological advancements in the field of artificial insemination.

2.8 Timed Artificial Insemination (TAI) Protocols

Artificial insemination (AI) involves semen deposition into the female reproductive tract which needs to be coincided with natural estrous cycle of the animal. Whereas, timed artificial insemination (TAI) is a program that utilizes exogenous hormones to control natural estrous cycle without the need of estrus detection (Baruselli et al., 2018). TAI protocols control follicular growth, regulate lifespan of CL, and stimulate ovulation of preovulatory follicle enabling AI to be performed at predetermined time (Colazo & Mapletoft, 2014). The first successful TAI protocol was developed using GnRH and PGF2α in dairy cows commonly known as OV-Synch (Pursley et al., 1995).

Currently, two types of TAI protocols are commonly utilized which are GnRH and E2 based (Bó & Baruselli, 2014). GnRH-based timed-AI programs involves sequential administration of initial GnRH, PGF2α, and subsequently a second dose of GnRH (Bridges et al., 2012) while EB is utilized instead of the initial GnRH in an estradiol-based TAI protocol. The initial GnRH injection, before the PGF2α therapy, is designed to cause ovulation of the dominant follicle and restart follicular growth, resulting in synchronized development of dominant follicle that is ovulated later by a second GnRH dose administered 2-2.5 days after the PGF2α injection (Bridges et al., 2008).

2.8.1 OV-Synch

The OV-Synch protocol was the first TAI reproductive management tool developed for lactating dairy cows (Kim et al., 2003). It constitutes two doses of GnRH 9 days apart and PGF2α on day 7, followed by fixed-time artificial insemination (FTAI) 24 hours after the second GnRH (Pursley et al., 1995). Such protocol allows AI without the need for time and labor-intensive estrus detection especially in farms with large cattle herds (Nowicki et al., 2017). OV-Synch is an alternative approach to detect heat and also used for treating reproductive diseases of the ovary (Nowicki et al., 2017). There are several modifications in the OV-Synch protocol such as to increase the duration between PGF2α and the second GnRH to 56 hours or to perform AI along with GnRH 56-66 hours after PGF2α (known as CO-Synch) (Ambrose et al., 2010). Currently, supplementing P4 in the form of intra-vaginal inserts in a TAI program has become a common practice.

2.8.2 CO-Synch Protocols

The CO-Synch program involves sequential use of GnRH and PGF2α to regulate the growth of follicles, control the life span of CL and proestrus duration (interval from luteolysis to LH surge), and ovulation. Intravaginal inserts are often used between the first GnRH injection and PGF2α (Bridges et al., 2008). The term CO-Synch has been used due to fact that the second GnRH is injected concomitantly at the time of TAI.

2.8.3 Seven-Day CO-Synch

After the successful development of the OV-Synch protocol, other TAI protocols were developed to suit the requirement of farms. One modified version of the OV-Synch protocol is the 7-day CO-Synch in which TAI is performed simultaneously with the second GnRH injection (Geary et al., 2001). As the second GnRH is concomitantly given at TAI, this protocol reduces the number of animal handlings to three from four in the original 7-day OV-Synch protocol. Thus, it is more commonly used in beef cattle due to the large herd size. There are several variations in the period between PGF2α and TAI. The

7-day CO-Synch begins with a GnRH injection, PGF2α 7 days later, and another GnRH dose concomitantly on TAI Day after 60–66 hours (Lansford, 2018), 54-66 hours (Bridges et al., 2012), 60 hours (Bridges et al., 2008) or 72 hours (Randi et al., 2021) of progesterone device removal.

2.8.4 Five-Day CO-Synch

The length of proestrus has been found to positively correlate with increased pregnancy rates (Bó et al., 2016; Day, 2018; de la Mata et al., 2018). Therefore, to provide a longer proestrus, the time interval between first GnRH to PGF2α in 7-day CO-Synch was reduced to five days resulting in a 5-day CO-Synch (Bridges et al., 2008). An increased pregnancy rate in a lengthened proestrus duration was a result of a lower incidence of ovulating immature follicles and increased gonadotropin exposure in the preovulatory follicle. This leads to an increase in estradiol concentration before ovulation (Day, 2018). However, due to the reduced duration from initial GnRH to PGF2α, researchers have recommended delivering two doses of PGF2α to completely lyse the young 5-day-old CL. Therefore, studies have been conducted to determine the need of two doses of PGF2α in a 5-day CO-Synch program. Bridges et al. (2012) conducted a study to determine if two doses of PGF2α concurrently at the time of progesterone device removal was efficient to result in a successful pregnancy and luteolysis of CL. The highest TAI pregnancy rate of 55% was recorded when two PGF2α injections were given 8 hours apart compared to a single large dose (51%) and single PGF2α (48%). Although the TAI pregnancy rates were no different between single large dose and 2 separate doses 8 hours apart, significantly lower pregnancy rate was observed in groups with single PGF2α. This indicates the need of either a single large dose or 2 doses at 8 hours interval. Furthermore, a luteolysis rate of 97%, 94%, and 89% for 2 doses (8 hours apart), single large dose, and single dose respectively were reported. In the current study, two doses 8 hours apart were used.

To establish a reasonable timing for AI in 5-day CO-Synch + CIDR® protocol, TAI at 56 hours was compared with that of 72 hours after P4 device removal in beef heifers (n = 1098) at nine locations (Kasimanickam et al., 2012). An average of 10.3% higher pregnancy rate was observed in beef heifers inseminated 56 hours compared to 72 hours. However, Bó et al. (2016) suggested the optimum time for AI in 5-day CO-Synch to be between 56-66 hours (beef heifers) and 72 hours (beef cows) after the P4 device removal. In a similar study by Busch et al. (2008), postpartum beef cows had TAI-PR of 67% when inseminated at 66 hours compared to 54 hours (61%) after insert removal. However, the reproductive outcome was affected by several factors other than timing of artificial insemination alone. Lamb et al. (2010) stated that the performance of TAI procedures was influenced by geographical characteristics such as pasture, nutrition, breed makeup, health status, postpartum period, climate, and geographical regions.

2.9 Behavioral Signs of Estrus

For successful accomplishment of AI and conception, accurate identification of estrus signs is crucial. Numerous studies on estrus behavioral signs have been done (Layek et al., 2011; Reith & Hoy, 2018; Roelofs et al., 2005). However, identifying animals in estrus precisely is still a major problem. Various behavioral estrus signs were documented in literature that differs among species, environment, and management level of farms. In the study by Roelofs et al. (2010), sniffing, chin resting, mounting, and standing to be mounted were observed in Holstein-Friesian cows. Apart from behavioral signs, discharge of mucus, vulvar tumefaction, and hyperemia of vulvar mucous membrane were observed by (Layek et al., 2011). Although the primary sign of estrus in cattle is standing to be mounted, the number of animals showing such estrus behavior was unfortunately low (Dobson et al., 2008; Gaude et al., 2021; Roelofs et al., 2010). Reith and Hoy (2018) listed additional behaviors such as aggression, increased activity, and agnostic behaviors by animals in estrus and they claimed that these parameters had a stronger indication of estrus than standing behavior. Wang et al. (2020) recorded estrus signs such as flehmen reaction, restlessness, vulva sniffing of another cow, chin resting, an attempt to mount other cows, and standing to be mounted in Holstein dairy cows through a video camera.

2.10 Factors Affecting Estrus Behavior

There are several factors contributing to the expression of sexual behaviors in dairy cattle. Individuals differ greatly in the intensity and length of estrus behavior during the estrous cycle (Orihuela, 2000). Landaeta-Hernández et al. (2002) assessed the effect of genetic, environmental and social factors affecting estrus expression of beef cattle in subtropical climate. It was reported that temperature humidity index (THI), social dominance and breed had significant effect on estrus expression. The estrus behaviors and intensity were also suppressed in herds with strong social dominance (Galina et al., 1996). According to Dobson et al. (2008), cows suffering from severe lameness exhibits 50% lower estrus intensity with few ovulations. Similarly, the number of cows in estrus at any given time also affects estrus expression. For example, the study by Hurnik et al. (1975) observed increased mounting behavior from an average of 11.2 to 52.6 times when the number of cows in estrus increased from one to three respectively. Estrus behaviors are also influenced by floor surfaces. Britt et al. (1986) observed increased estrus duration, mounting activity, and standing estrus on dirt compared to concrete floor. A similar report was later published by Vailes and Britt (1990) whereby mounting activity in Holstein cows were four times more on dirt compared to concrete. Although many factors contribute to estrus behaviour, BCS and cyclicity of estrus before breeding were important. Beef cows with BCS >4 (9-point scale) had greater estrus expression than BCS ≤4. Similarly, expression of estrus was greater for anestrus cows before breeding season compared to cycling cows (78±5% vs. 70±5%) (Richardson et al., 2016).

2.11 Importance of Estrus Expression before AI

Although current TAI programs obviate the need of detecting estrus expression, identifying females expressing estrus behavior in a TAI program can offer certain advantages. Importance of estrus expression before TAI program is well-established in literature. Richardson et al. (2016), revealed 27% greater pregnancy rates in cows that were observed in estrus before TAI compared to those not observed in estrus. Furthermore, pregnancy loss at day 30 to 100 was minimized in cows expressing estrus behaviors before TAI (Pohler et al., 2016). Estrus expression before TAI modifies uterine environment, increases accessory sperm counts and fertilization rates, and also increases overall embryo survivability (Richardson et al., 2016). Perry et al. (2007) demonstrated an increased PR, follicle size, and estrogen concentration in beef heifers that have expressed estrus behaviour prior to TAI than those who did not.

Detecting estrus in a TAI program help breeders in allocating valuable semen to cows that express estrus and less expensive semen to cows that did not express estrus because the chances of conceiving in former was higher. Similarly, it will further help breeders in deciding to use sexed semen and conventional semen. In general, sexed semen has lower conception rate than conventional semen. To optimize cost per pregnancy of a cow, prioritizing sexed semen to cows that expressed estrus can enhance the chances of conception. Furthermore, the chances of establishing pregnancy are higher in embryo recipient cows that express estrus before embryo transfer compared to recipient cows that did not express estrus (Pereira et al., 2016).

2.12 Methods and Tools of Estrus Detection

The persistent obstacle to efficient reproductive performance in dairy cows is inaccurate heat detection (Reith & Hoy, 2018). This remains a major problem despite extensive knowledge of reproductive physiology and heat detection tools (Roelofs et al., 2010). Dairy herd reproduction is determined by heat detection efficiency, which is a particular concern in high-production dairy herds. As a result, there are fewer heifers and eligible cows that are artificially inseminated thus prolonging age at first calving and resulting in low pregnancy rates (Tibary et al., 2019).

Visual observation of estrus behavior is a highly reliable method. However, due to increased cattle numbers in modern cattle industries, this method incurs huge expenses due to the large amount of time and labor required for visual observation. Moreover, accurate identification of cows in estrus requires skills and knowledge to differentiate true estrus from other social behaviors. One hundred percent accuracy in estrus detection was achieved by HeatWatch®/Radiotelemetry in comparison to 97.6% for visual observation (Xu et al., 1998). Although highest accuracy is obtained by Radiotelemetry system of estrus detection, such technology may not have practical application to farmers in developing countries due to the cost and expertise required for operation. Other estrus detection tools available such as pedometer, KAMAR®

patches, neck collars, tail paint, chin ball markers, Draminski estrus detector, and PRID®Delta patches are available for use. Despite abundant estrus detection tools, the challenge of identifying cows in estrus still remains a big issue (Roelofs et al., 2010).

2.13 Electrical Resistance of Vaginal Mucus (ERVM)

Studies on ERVM have been done previously in ewes (Talukder et al., 2018; Theodosiadou et al., 2014), sows (Hidalgo et al., 2015), cows (Ahmed et al., 2017), buffaloes (Gupta & Purohit, 2001), camels (Vyas et al., 2009), and bitches (Günzel et al., 1986). Measuring the electrical resistance of vaginal and cervical mucus is possible due to circulating estradiol and progesterone that cause a shift in the ionic balance of vaginal and cervical mucus (Bowers et al., 2005). The onset of estrus is characterized by an increase in estrogen levels, followed by the synthesis of adrenocorticotropic hormone and aldosterone. This leads to an increase in sodium chloride (Ahmed et al., 2017), magnesium, calcium, and other cations that affect its electrochemical reactivity (Jotanović et al., 2011). According to Fehring (1996), the dominant follicular estrogen effect causes the synthesis of adrenocorticotropic hormone and aldosterone, which causes the release of NaCl in the saliva and cervix. In a review by Leidl and Stolla (1976), lower ERVM values were observed during estrus which corresponds to the time of high E2. Estrogen was responsible for the reduction in vaginal mucus resistance, as no significant changes in levels were observed in ovariectomized animals while lower levels were recorded in intact animals treated with estrogen. This low ERVM during estrus is associated with the highest concentration of LH and total estrogen in blood plasma (Leidl & Stolla, 1976).

The majority of studies conducted were based on observations in cows in spontaneous heat. There are only limited studies on the ERVM of cows in an ovulation synchronization program evaluating effectiveness in determining the best time for AI. Zuluaga et al. (2008) found that ERVM measurements were not meaningful in determining the optimal timing for insemination because they failed to identify the presence or absence of large follicles in a TAI program. Furthermore, ERVM is not sufficiently reliable in large farms. It was observed that the lowest ERVM values varied between animals at different times of estrus and this makes it an inappropriate method for determining the timing for artificial insemination (Cavestany & Foote, 1985).

2.14 Factors Affecting ERVM Readings

Several studies have been conducted that correlate ERVM levels with those of estrus and resulting pregnancy outcome (Aboul-Ela et al., 1983; Ahmed et al., 2017; Fazleabas et al., 1990; Meena et al., 2003). Most studies reported consistent observations of ERVM levels decreasing as the animal approached estrus. However, the actual reported values varied depending on the type of device used. Ovatec (Electronic Animal Management Inc., Co &, NY) recorded resistance values as low as 30 Ω during estrus (Rorie et al., 2002). In another study, ERVM during natural estrus was recorded at 82.2 ± 2.2 (mean ± SD) Ω (Tadesse et al., 2011), while measurement values from Draminski estrus detector (Olsztyn, Poland) varied from 150 to as high as 280 Ω (Ahmed et al., 2017) in natural estrus. Similar range in the readings (150 to 290 Ω) were also recorded by DeKa et al. (2022) prior to AI in crossbreed cattle. In another study (Jotanović et al., 2011), the same device recorded 162.9 ± 5.04 Ω at AI and resulted in 87.5% pregnancy rate in Simmental cows. Much higher readings (353.33 ± 135.46 Ω) were obtained in does after a second dose of prostaglandin (11 days apart) (Triagil et al., 2020). Apart from the type of devices, factors such as depth of probe insertion, orientation, area of probe in contact with vagina mucosa, and vaginitis all affected ERVM values (Kitwood et al., 1993). The EVRM values obtained from posterior vagina are more variable than the values obtained from the anterior vagina during estrus (Kitwood et al., 1993). In addition to the above mentioned factors, the pressure of the device against the mucous membrane also affects the values (Leidl & Stolla, 1976).

2.15 Relationship between ERVM and Pregnancy Rate

Ahmed et al. (2017) studied the relationship between EVRM at estrus and post-insemination pregnancy rates. Significantly higher pregnancy rates were observed when ERVM ranged from 150 to 220 Ω (80 to 83.3%) compared to 221 to 280 Ω (36.4%). However, DeKa et al. (2022) reported a higher pregnancy rate when cows were inseminated at 201 to 250 Ω . Jotanović et al. (2011) divided the cows into three groups based on ERVM readings; G1: less than 179 units, G2: 180 to 320 units and G3: > 320 units. Pregnancy rates of 87.5%, 73.6%, and 75.0% were reported respectively with no statistical significance between groups. However, pregnancy rate was proportionately higher in the group with the lowest ERVM values. A significant association between vaginal mucus resistance and conception rate ($p < 0.01$) was also seen in which the lowest range (26 to 30 Ω) had the highest conception rate of 84% (Meena et al., 2003).

2.16 Factors Affecting Reproductive Performance

Numerous intrinsic and extrinsic variables such as BCS, heat detection efficiency, semen quality, correct AI time, and efficiency of the AI technician contribute to the success of synchronization program (Bihon & Assefa, 2021). One of the major challenges to increase reproductive efficiency in cows is the anestrus phase that follows calving (Montiel & Ahuja, 2005) whereby failure to resume the cyclicity in cattle within 60-80 days postpartum is a frequent problem (Opsomer et al., 1996). According to Carvalho et al. (2022), the body condition score at calving determines the success of artificial insemination program.

2.16.1 Body Condition Score (BCS)

The impact of body condition scores on reproductive performance in cattle has been extensively studied (Dechow et al., 2002; D'Occhio et al., 2019; Nazhat et al., 2021; Pryce et al., 2001; Wang et al., 2019). The BCS of a cow is a measurement of her body fat content and is widely regarded by producers and animal scientists as a crucial component of dairy cow management (Roche et al., 2009). A visual assessment of BCS is a useful tool for determining subjective levels of body energy reserves in cattle (Pfeifer et al., 2021). It is a useful visual assessment method on the farm to describe an animal's energy reserves. The reproductive success of cows is expected to be influenced by their BCS at critical times of the year (Weik et al., 2020). A more accurate indicator of a cow's nutritional status is the body fat level in beef cows at various stages of their reproductive cycle. This plays a significant role in the cow's ability to reproduce and overall performance (Herd & Sprott, 1986). Rae et al. (1993) reported a significant association between BCS and PR in cows with BCS 4 and 5 (scale 1-9) with a pregnancy rate of 59% and 90%, respectively. This indicates a positive association between these variables. A similar finding was reported by Vedovatto et al. (2022) where cows with BCS >5 at the start of the FTAI program had higher PR, behavioral expression of estrus, follicular development, and embryo size. A study by Anzar et al. (2003) also reported a significant association between BCS and conception rate in cattle and buffalo, indicating that BCS is an important management tool to improve reproductive performance. Additionally, Carvalho et al. (2022) found a higher PR to FTAI in Bos indicus cows with BCS of ≥5 regardless of parity. The onset of estrus and calving interval is also influenced by body condition score. For a beef cow to maintain a 12-month calving interval, she must conceive within 82 days postpartum. However, due to inadequate body condition, cyclicity is not resumed thus prolonging the calving interval (Eversole et al., 2009a). Overall, animals should have a BCS greater than 5 (on a scale 1-10) for optimal pregnancy outcomes (Lamb et al., 2007).

2.16.2 Estrus Detection Efficiency

Poor detection of estrus animals is one of the most critical issues affecting reproductive efficiency in the cattle industry (Reith & Hoy, 2018; Senger, 1994). Efficient and accurate identification of heat is essential for artificial insemination and embryo transfer operations (Rorie et al., 2002). The traditional method of heat detection by visual observation for mounting behavior is a very reliable approach. However, the time that cows are ready to mount accounts for only 1% of the total duration of heat and the detection efficiency is often around 50-70% (Rorie et al., 2002). The method of visual observation is time consuming and requires a lot of experience and expertise (Adenuga et al., 2020). In today's modern dairy and beef industry, TAI protocols that synchronize ovulation has overcome the need for heat detection.

2.16.3 Semen Quality

Semen volume, motility before filling, motility after thawing, and spermatozoa concentration are some of the important parameters of semen quality (Filipčík et al., 2023). Increasing sperm concentration through an increase in the quantity of healthy sperm increases the rate of conception (Morrell et al., 2018). A bull's fertility is more significant than a cow's because it can produce up to 40 female offspring naturally or hundreds of thousands through artificial insemination (Kastelic, 2013). Rabidas et al. (2012) conducted a study to correlate the semen quality of four bulls with the non-return rate (NRR) to estrus in 2016 cows. A significantly higher NRR was observed for bulls with higher sperm motility, viability, and normal morphology.

2.16.4 Timing of Artificial Insemination

The purpose of fertilization is to make sure that the caudal region of the oviductal isthmus, which serves as the cow's primary sperm reservoir, has a sufficient supply of competent, capacitive, motile sperm to ensure fertilization during ovulation (Diskin, 2018). Pursley et al. (1998) studied 732 lactating dairy cows to determine an optimal time point for AI by inseminating 0, 8, 16, 24, or 32 hours after the second GnRH following the OV-Synch protocol. The cows inseminated at 0, 8, 16 and 24 hours had a similar pregnancy per insemination (P/AI) and calving rate but those in the 32 hour group saw a decrease. In addition, the authors reported decreased P/AI, CR, and higher pregnancy loss when AI was performed after ovulation. In a study by Dransfield et al. (1998), the conception rate was greater when AI occurred 4-12 hours after the onset of estrus.

2.16.5 AI Technician Efficiency

An experienced and qualified AI technician is required to execute the AI procedures efficiently. There is a positive association between technician efficiency and PR in AI programs. Hossain et al. (2016) reported a significant difference in the PR achieved by two technicians with 5 and 8 years of experience (51.24% and 60.20% respectively). A similar study by Paul et al.

(2013) also reported a significantly higher PR among experienced AI technicians compared to less skilled AI technicians.

2.17 Progesterone Devices in Estrus Synchronization

One of the causes of decreased fertility, particularly in high-yielding dairy cows, is insufficient circulating P4 concentration (Colazo & Mapletoft, 2014). Progesterone supplementation during timed AI procedures increases ovulation synchronization by reducing the likelihood of ovulation before the AI day (Bisinotto et al., 2015). According to Kasimanickam et al. (2014), the main objective of P4 supplementation via an intravaginal device between the administration of GnRH and PGF2α is to prevent premature estrus, LH surge, and ovulation in heifers lacking endogenous progesterone. Inclusion of P4 in protocols increases the proportion of response in prepubertal heifers and cows and initiates cyclicity (Smith et al., 2011, 2005; Stevenson & Lamb, 2016). This also avoids low-intensity heat and incomplete luteal regression (Kim et al., 2003). Furthermore, these P4-based programs, whether cyclic or acyclic, allow for successful regulation of reproduction in high-production dairy cows (López-Helguera et al., 2017) since reduced P4 concentration before FTAI is associated with reduced fertility and vice versa (Wiltbank et al., 2014). A brief exposure to progesterone is required to reprogram the reproductive axis and return to the normal estrous cycle. Progestin pretreatment before the onset of ovulation can therefore establish the state of the estrous cycle and reduce the frequency of short estrous cycles in herds with high numbers of prepubertal heifers or anestrus cows (Smith et al., 2013). It was reported previously that at least three days of progesterone exposure is necessary to restore estradiol responsiveness of hypothalamus in cows with a follicular cyst (Gümen & Wiltbank, 2005). High incidence of double ovulation in high milk-producing cows is due to reduced P4 circulation in the blood brought on by high feed intake and increased P4 metabolism by the liver. Circulating P4 concentration depends on a balance between hepatic metabolism and corpus luteum secretion (Wiltbank et al., 2014). In contrast, low progesterone is associated with multiple ovulations in cows can result in twin pregnancies, which is detrimental to cow health, productivity, and reproductive efficiency (Macmillan et al., 2018). Gunn et al. (2016) compared the effect of the 5-day CO-Synch protocol in weaned beef cows with or without the use of a progesterone device (CIDR). The authors reported a higher PR in cows with CIDR than without ($p=0.05$, 62.3% vs. 50.7%). A similar study by Ghuman et al. (2014) compared OV-Synch with or without the P4 device in terms of ovulation synchronization and CR in silent heat buffalos. A higher CR was observed in animals with OV-Synch P4 based protocols compared to those without the progesterone device (66.7% vs. 30%). Similarly, Kawate et al. (2004) compared conception rate using standard OV-Synch protocols with or without using the CIDR device in Japanese black beef cows and reported a higher CR (72.5% vs. 47, 7%; $p = 0.03$). Wiltbank and Pursley (2014) discovered that before AI, cows with low progesterone levels had much lower fertility than cows with high progesterone. Only 37.1% of the low progesterone cows were pregnant at day 29 while 51.0% of the high progesterone cows were pregnant, suggesting the need for a higher progesterone concentration before TAI to improve cow fertility.

CHAPTER 3

MATERIALS AND METHODS

3.1 Study Location

The use of beef cattle for this experiment was approved by the Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia (IACUC No. UPM/IACUC/AUP-R036/2022). The study was conducted at Pusat Pertanian Putra, Universiti Putra Malaysia, Serdang, Lat: 2° 59’ 29.8’’ N and Long: 101° 43’ 58.83’’ E, about 54 m above sea level in the state of Selangor. The area has an average ambient temperature and a relative humidity of 30°C and 87.5% respectively (Malik et al., 2012). This study was conducted from August to November 2022.

3.2 Animals and Management

A total of 26 healthy Brangus crossbreed beef cattle constituting 18 postpartum cows and 8 heifers were included in the study. Both cows and heifers were included in this study assuming that they may respond to treatments differently as a result in differences of age, history of reproduction, and physical maturity. The daily diet comprised of freshly cut Napier grasses and palm kernel pellets (2 kg/animal/day) with occasional water-soaked rice straw hay mixed with molasses. Throughout the experimental period, the animals were housed in concrete paddocks and had ad libitum access to water and mineral licks. Cattle were vaccinated against Foot and Mouth Disease (every 6 months) and Hemorrhagic Septicemia (annually) while deworming was performed every 3 months with albendazole oral suspension. The experimental animals remained in the paddock during the entire experimental period. Although the farm has maintained 5 breeding bulls, they remained in pasture throughout the day except during night they were kept tethered at least 10 meters away from the experimental animals.

3.3 Selection of Animals

One day before the study began, all recruited cattle underwent a trans-rectal palpation and ultrasonography of the reproductive tract (5.0 MHz linear endo-rectal probe, Draminski® 4VET mini, Poland) to ensure a normal reproductive tract and to rule out pregnancy. A thorough physical examination including the temperature, heart rate, respiration rate, rumen motility and quality, mucous membrane colour, hydration status, and body condition score (Eversole et al., 2009) was performed to ascertain the general health condition of the animals (Appendix 2).

3.4 Experimental Design and Assignment of Treatments

Eighteen postpartum cows and eight heifers were randomly divided into two groups to receive either of the treatment protocols: 5-day CO-Synch + PRID®Delta (5DCOS) or 7-day CO-Synch + PRID®Delta (7DCOS). The schematic diagram for the study is presented in (Figure 4). Two cows from

5DCOS group were eliminated from the study (1 dead due to pneumonia and other lost during experiment). The cattle in both treatment groups received an intra-vaginal progesterone releasing device, PRID®Delta (1.55g P4, Ceva Santé Animale, Libourne, France) and 1ml intramuscular injection of GnRH (Cystorelin®, 100 µg, Ceva Santé Animale, Libourne, France) on D -5 and D -7 respectively for 5DCOS and 7DCOS protocols (Figure 5). PRID®Delta was removed on D0: 5 and 7 days after initiation of the treatments for 5DCOS and 7DCOS respectively. On D0 (device removal), animals in the 5DCOS group received two doses (5ml each) of PGF2α (Enzaprost® T, 25mg Dinoprost, Ceva Santé Animale, Libourne, France) 8 hours apart (initial dose at PRID removal) while 7DCOS received a single dose. TAI was scheduled 58 hours (D2) and 72 hours (D3) after PRID®Delta removal for 5DCOS and 7DCOS respectively. In both groups, a second dose of GnRH was given at the predetermined time of AI (Figure 5).

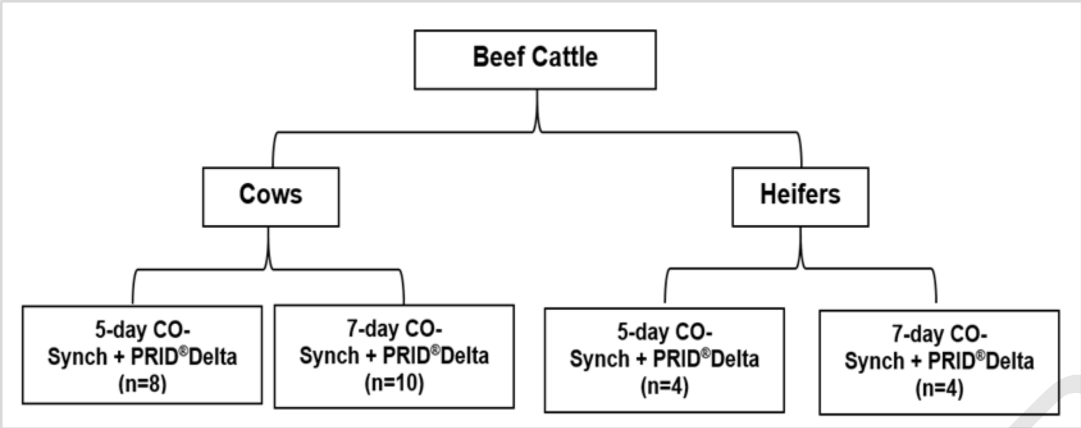

Figure 4: Study Design and Treatment Allocation for Cows and Heifers

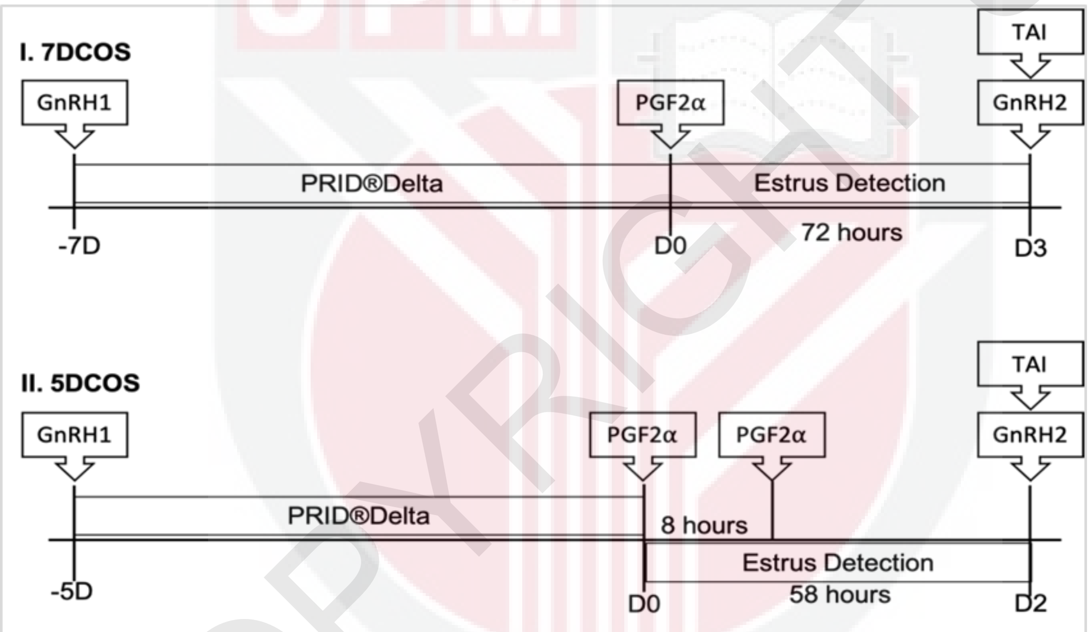

Figure 5: Five-Day and 7-Day CO-Synch Estrus Synchronization Protocols. GnRH: Gonadotropin Releasing Hormone, TAI: Timed Artificial Insemination, PRID®Delta: Progesterone Releasing Intra-Vaginal Device-Delta.

3.5 Blood Sampling

All blood samples were taken in the morning (0800 - 0930 hours) prior to feeding. Blood samples at D0 represented parameters prior to device insertion, whereas all other samples represent samples during P4 treatment. A total of 5 samples (D0, D1, D3, D5, D7) from animals in the 7DCOS and 4 samples (D0, D1, D3, D5) from 5DCOS were collected via coccygeal blood vessels using 21G needle in a 5 ml syringe and then transferred into plain tubes. Blood samples were left undisturbed for an hour and transported to the laboratory in a cooler box within two hours of collection. The plain tubes were then centrifuged at 3000 rpm for 15 min (Hettich® Zentrifugan EBA21 Centrifuge, Germany). The serum was transferred into cryovials (SPL Lifesciences, Korea) and kept at -20°C until assayed.

3.6 Enzyme Linked Immunosorbent Assay (ELISA) for Progesterone and Estradiol Quantification

The quantification of serum P4 was achieved using Pan-species Progesterone ELISA kit (Cloud-Cone Corp., USA) (Appendix). Absorbance to concentration was converted using webpage www.myassay.com.

Figure 6: Quantification of Progesterone Concentration in Serum Samples by Enzyme Linked Immuno-Sorbent Assay method.

3.7 Estrus Detection and Methods

Twice daily (0500 - 0700 and 1700 - 1900 hours) visual observation of estrus behavior was carried out by a single researcher beginning from the time of PRID®Delta removal until the day of scheduled AI. To complement visual observation, PRID®Delta breeding indicator patches (Rockway Inc, USA) were placed midway between the hip and the head of the tail, perpendicular to the spine on the day of PRID®Delta removal. The patches were evaluated on the day of AI based on a scale from 0 to 3 depending on the extent of color exposed after initial application: 0= undisturbed, 1= <50% exposed, 2= >50% exposed and 3 = fully exposed (Figure 7 & 8). Animals with scores of 2 and 3 were assumed to have expressed estrus behavior (Zwiefelhofer et al., 2021). Behavioral signs such as nudging, chin resting, aggressive behavior (butting), sniffing genital area, and chasing herd mates were considered as estrus signs. Additionally, physiological changes such as swollen vulva/vulvar edema, vulvar mucus discharge were also considered. The percentage of estrus response rate was calculated:

$$\mathrm{ERR}(\%) = \frac{\text{No. of animals detected in estrus}}{\text{Total number of animals synchronized}} \times 100$$

Figure 7: PRID®Delta Patches used in the Study

Figure 8: An activated PRID®Delta Patch and the Site of Placement

3.8 Measurement of the Electrical Resistance of Vaginal Mucus (ERVM)

There is currently no documentation of standard data on ERVM from cows around the world although ample of studies were conducted. In this study, Draminski estrus detector (Draminski Electronics in Agriculture, 17 Owocowa, 10-860 Olsztyn, Poland) was used to measure the ERVM daily beginning from PRID® removal until the scheduled day of AI. The probe was washed in clean water and disinfected using a chlorhexidine solution prepared in a separate bucket. The probe was then dried with paper towels. With cows secured in the crush, the vulvar lips were spread apart and the probe was inserted at 45o angle. Once the tip of the probe was inside the vaginal canal, it was aligned horizontally and gently advanced until cervical resistance was felt. Three concurrent readings were taken after rotating the probe at 360o. The final daily recording was an average of the three measurements. The probe was washed clean, disinfected and dried with papers towels before a measurement was taken from a different cow.

Figure 9: Draminski Estrus detector used for recording the Electrical Resistance of vaginal Mucus (ERVM)

Figure 10: Recording Electrical Resistance of Vaginal Mucus using the Draminski Estrus Detector in a cow

3.9 Statistical Analyses

All data analysis was carried out using IBM-SPSS version 29. Microsoft Excel and Word version 2016 were used to generate figures and tables respectively. Estrus response rates between protocols were compared using Chi-Square test because comparison involved two categorical data (Yes/No versus Protocol type). However, Fisher's exact test was interpreted as 75% of cells (2 by 2 contingency table) had expected count less than 5. Differences in the electrical resistance of vaginal mucus and progesterone concentrations on different days within the group were analyzed using Friedman's repeated measures within subject ANOVA which is non-parametric test for one-way repeated measures ANOVA which is appropriate for related samples that account for repeated measures on the same subjects where data deviated from normality. Pairwise comparison was performed via Friedman's 2-way ANOVA by ranks (Friedman's post-hoc test) whenever Friedman test showed significant difference. The electrical resistance of vaginal mucus at the scheduled AI day between estrus positive and negative, as well as progesterone concentration of days between protocols were compared using Mann-Whitney U test which is a non-parametric version of t-test because the data came from two independent groups and did not follow normal distribution. A p value of ≤ 0.05 was considered statistically significant.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Animal Characteristics

The characteristics of cows and heifers included in the study are presented in Table 2 and Table 3 respectively.

Table 2: Descriptive statistics for Age, Parity, Body Condition Score (BCS), and Days Postpartum (DPP) of Cows at the start of the Synchronization Program

Parameters	N	Mean	SD	Min	Max
Age (year)	18	6.61	1.65	3.0	9.0
Parity	18	3.67	1.28	1	5
BCS	18	3.56	0.62	3	5
DPP (day)	18	378.11	211.51	86.0	830.0

Body condition score (1-9 scale: 1 = Emaciated; 9 = Obese).

Table 3: Descriptive Statistics for Age and Body Condition Score (BCS) of Heifers at the Start of the Synchronization Program

Parameters	N	Mean	S. D	Min	Max
Age (year)	8	3.13	0.84	2	4
BCS	8	3.38	0.52	3	4

Body condition score (1-9 scale: 1 = Emaciated; 9 = Obese)

4.2 Estrus Signs and Behaviors

Figure 11 shows various estrus behaviors displayed during the visual observation period expressed as percentage of cases. From the 26 cattle (18 postpartum cows and 8 heifers) enrolled in the experiment, 15 (10 cows and 5 heifers) exhibited various estrus behaviors, representing an overall 58% estrus response rate. The incidence of standing estrus was not observed in any of the animals which was further confirmed with the assessment of PRID®Delta patches during the day of TAI.

The figure below shows various estrus signs and behaviors observed in experimental animals during the visual observation period.

A. Sniffing genital region B. Nudging mates

C. Chin Resting D. Butting behavior

G. Vaginal Discharge

E. Chasing Mates   F. Vulvar swelling

Figure 12: Estrus Signs and Behaviors recorded during the study

4.3 PRID®Delta Patch Scores

Table 4 shows the results PRID®Delta patches scores assessed at the time of TAI for cows and heifers respectively. Cattle with the scores of 2 and 3 were considered to have expressed behavior. Of the total 18 cows and 8 heifers included in the study, only 1 cow had the core of 2 with majority below score of 2. Whereas in heifers, all of the scores were 0 and 1.

Table 4: PRID®Delta scores of the Cattle expressed as frequency and percent

Animal Category	Score	Frequency	Percent (%)
Cows	0	8	44.4
	1	9	50.0
	2	1	5.60
	3	0	0.00
Heifers	0	4	50.0
	1	4	50.0
	2	0	0.00
	3	0	0.00

0: colour undisturbed, 1: < 50% colour activated, 2: > 50% colour activated, 3: colour fully activated. Only scores of 2 and 3 were considered to have expressed estrus

4.4 Comparison of ERR in Cows and Heifers

The percentage of cows and heifers that showed estrus signs are presented in (Figure 13). Out of 18 cows enrolled in the study, 11 cows (61.11%) exhibited various estrus signs while 7 cows (38.89%) did not exhibit any estrus signs. Similarly, from the 8 heifers enrolled in the experiment, 5 (62.5%) showed estrus signs. A Fisher's exact test revealed no statistically significant difference between the treatment protocols in both cows and heifers.

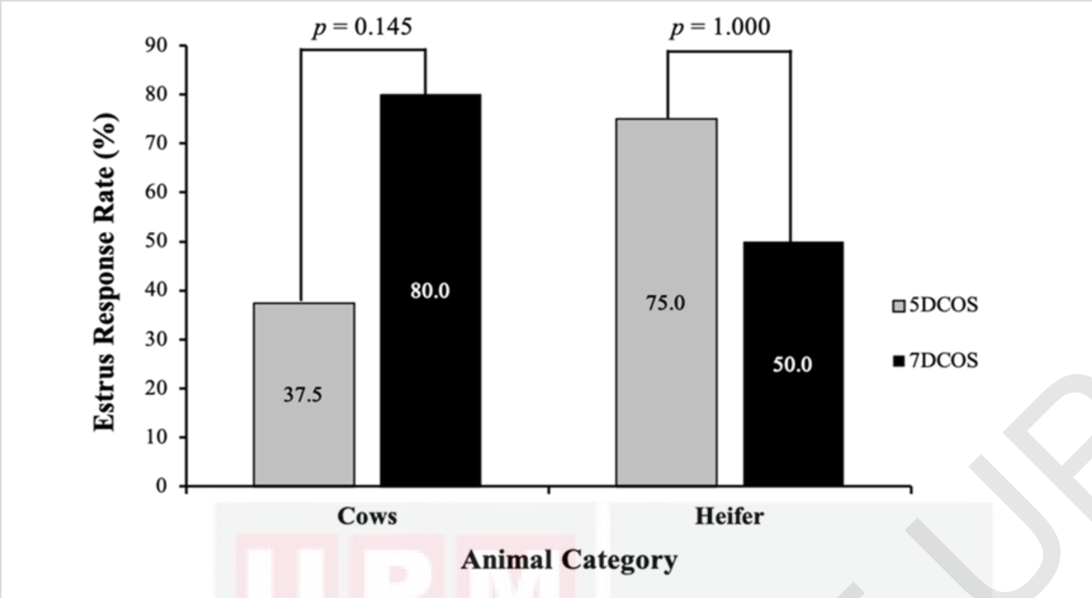

Figure 13: Estrus Response Rate in Cows and Heifers by Treatment groups (p > 0.05, exact 2-sided significance)

There are a number of advantages to carrying out heat detection even though timed artificial insemination protocols do not require it. Effective synchronization is indicated by a high estrus response rate, which also raises the likelihood of successful breeding and conception (McNally et al., 2014; Reith & Hoy, 2018). Furthermore, lack of response to treatment delays genetic progress and reduces overall farm productivity (Boer et al., 2010). In a meta-analysis conducted by Richardson et al. (2016), cows that exhibited estrus before timed artificial insemination had a 27% higher pregnancy rate than cows that did not exhibit estrus. Additionally, estrus detection helps allocate valuable semen to cows showing estrus as they have a higher chance of conceiving. Pohler et al. (2016) reported that cows that showed estrus before TAI had fewer pregnancy losses between days 30 and 100. Similarly, Pereira et al. (2016) found that pregnancy loss due to timed AI was greater in cows that were not in estrus than in cows that were in estrus. Furthermore, pregnancy rates per embryo transfer in recipient cows was greater in those observed in estrus compared to those not observed in estrus (Madureira et al., 2022).

The result of this study showed that estrus response rates to 5DCOS and 7DCOS + PRID®Delta had no significant difference in both postpartum cows and heifers (Figure 13). Therefore, we failed to reject the null hypothesis that there is no significant difference in ERR between the two protocols. It is important to highlight that there is a lack of previous research directly comparing 7DCOS and 5DCOS protocols using PRID®Delta as an intravaginal progesterone device. The authors were in the opinion that the fertility parameters from the use of PRID®Delta and CIDR® would be different because previous studies have shown superior performance of PRID®Delta compared to CIDR®, complicating comparison of our results with CIDR based studies. A higher estrus response (Zwiefelhofer et al., 2021) and higher circulatory P4 concentration (Van Werven et al., 2013) was observed in PRID®Delta than CIDR®. In a CIDR-based comparison between 5DCOS and 7DCOS protocol, Whittier et al. (2013) reported significantly higher estrus response in 7DCOS than 5DCOS protocol (61.1 vs. 52.9%) which is contrary to our findings. The lack of significance in our study could be due to reduced statistical power to detect the small differences as a result of low sample size. Nevertheless, our results from postpartum cows revealed proportionately higher estrus response to 7DCOS (80.0%) than 5DCOS (37.5%) protocols. A higher proportion of estrus response in 7DCOS compared to 5DCOS could be a result of longer proestrus duration (72 vs. 58 hours). This longer proestrus duration may have allowed cows to develop larger follicles, which may lead to increased estrogen production, resulting in a higher number of cows in estrus in the 7DCOS group (Whittier et al., 2013).

Result from heifers in the current study is consistent with the results of Ahmadzadeh et al. (2015) in their comparison between modified 5DCOS protocols (without initial GnRH with single PGF2α) and 7DCOS protocols, which achieved an ERR of 52% and 56% for 5DCOS and 7DCOS respectively ($p=0.49$). However, it is important to emphasize that the sample size of this study is small ($n = 4$ each) and the results may not be generalizable to a larger population. Therefore, further studies with a larger sample are required to validate our current findings.

The impact of the body condition score on reproductive performance in beef and dairy cattle has been extensively studied. For a beef cow to maintain 12 months calving interval, she must conceive within 82 days postpartum. In our study, the mean ± SD for DPP was 378 ± 212 days indicating prolonged anestrus. Despite having 5 bulls maintained in the farm for natural breeding, the cows failed to become pregnant indicating the presence of factors affecting pregnancy such as inadequate body condition, whereby cyclicity is not resumed which prolongs the calving interval (Eversole et al., 2009). Increased pregnancy rates, greater estrus expression, follicular development, and increased embryo size were observed in cows of BCS more than 5 (Vedovatto et al., 2022). Similarly, the study by Andersen et al. (2020) observed greater estrus expression in beef cows with greater BCS. For optimal pregnancy rates, a BCS greater than 5 (1-10 scale) have been recommended (Lamb et al., 2007). In this study, the body condition of postpartum cows varied from 3 to 5 with only one cow with BCS of 5 (1/18). In the heifer category the BCS ranged from 3 to 4 (Table 3) which is lower than the recommended score of 5 or more which may have affected the estrus expression in our results.

The method of heat detection, in terms of accuracy, can significantly affect ERR. Several methods for heat detection have been developed and are being widely used. However, visual observation is one of the methods that is often used in conjunction with other methods. The accuracy of visual observation approximately ranges from 50 to 70% constituting only 1% of the total estrus duration (Rorie et al., 2002). Furthermore, it has been reported that 37% of standing behavior was undetected by visual observation but were detected by radio-telemetry (Stevenson et al., 1996).

In this study, twice daily (0500 to 0700 and 1700 to 1900 hours) visual observation combined with PRID®Delta patches were used to detect estrus beginning from P4 device removal until scheduled AI. Sniffing genital region, nudging of mates, chin resting, butting behavior, mate chasing, edematous vulva, and vaginal discharge were some of the observed secondary estrus signs during the experiment period (Figure 12). Mucus discharge, restlessness, and nudging of mates were observed more frequently than vulvar edema, mounting activity and chin resting (Figure 11). Standing to be mounted is considered the most reliable sign of estrus. However, there is a clear trend that such behavior is not shown by many cows (Dobson et al., 2008;

Gaude et al., 2021; Moore et al., 2021). Additionally, researchers have focused more on secondary signs such as agnostic behaviors, aggression, and mounting activity (Reith & Hoy, 2018). Assessment of PRID®Delta patch score at the scheduled AI day revealed that standing estrus did not occur in any of the cows (score 0 and 1). While only 7.1% of the cases (1/26) exhibited mounting activity. Low incidence of mounting activity and the absence of standing estrus in our current study may be due to concrete paddock floor and limited space where animals were housed during the experimental period. A lower incidence of mounting and standing estrus on the concrete floor was also previously reported by Britt et al. (1986).

4.5 Comparison of Electrical Resistance of Vaginal Mucus (ERVM) Trend in Cows and Heifers

In postpartum cows, Friedman's test revealed no significant difference in the medians between days in both protocols ($p > 0.05$) for ERVM, indicating consistent values across days (Figure 14). However, the values in the 7DCOS group were numerically higher than the 5DCOS group. In both protocols, the highest ERVM value was recorded on day 0 (after device removal) and the lowest on day 1 after device removal. In the heifer category, the Friedman test showed a significant difference in ERVM across days. However, post hoc multiple comparisons using the Bonferroni correction revealed no significant difference between days ($p > 0.05$) indicating consistent values across days in both protocols. In contrast to cows, the median ERVM of heifers was numerically higher in the 5DCOS group than in the 7DCOS group. The highest ERVM was recorded at D0 and D3 in the 5DCOS and 7DCOS groups, respectively. However, the lowest value was observed on D3 in both treatment groups (Figure 15).

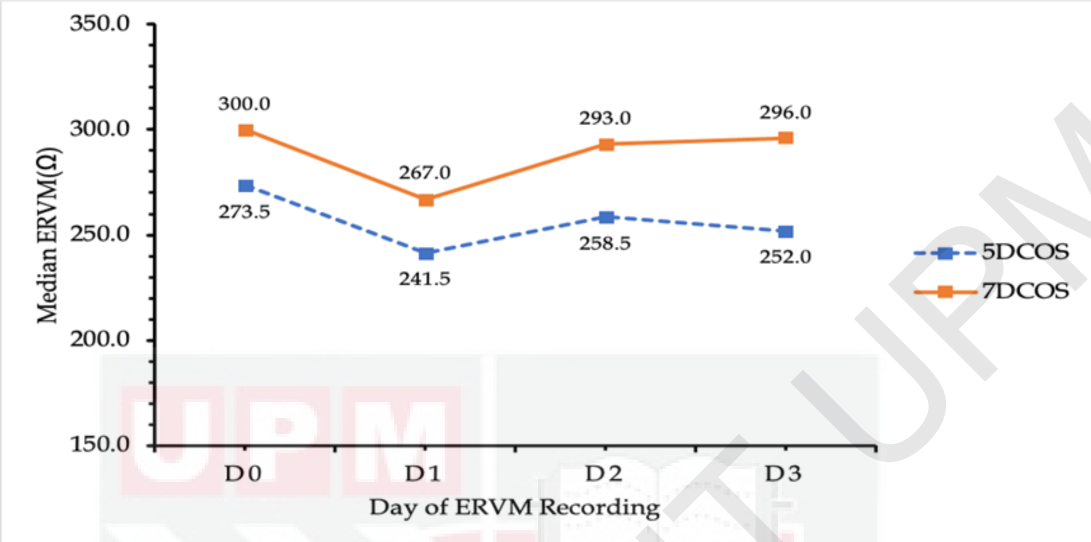

Figure 14: Median ERVM of Cows across days for 5DCOS and 7DCOS protocols ($p > 0.05$). D0 represents the day of PRID®Delta removal and D2 and D3 represents the predetermined time of AI for 5DCOS and 7DCOS respectively

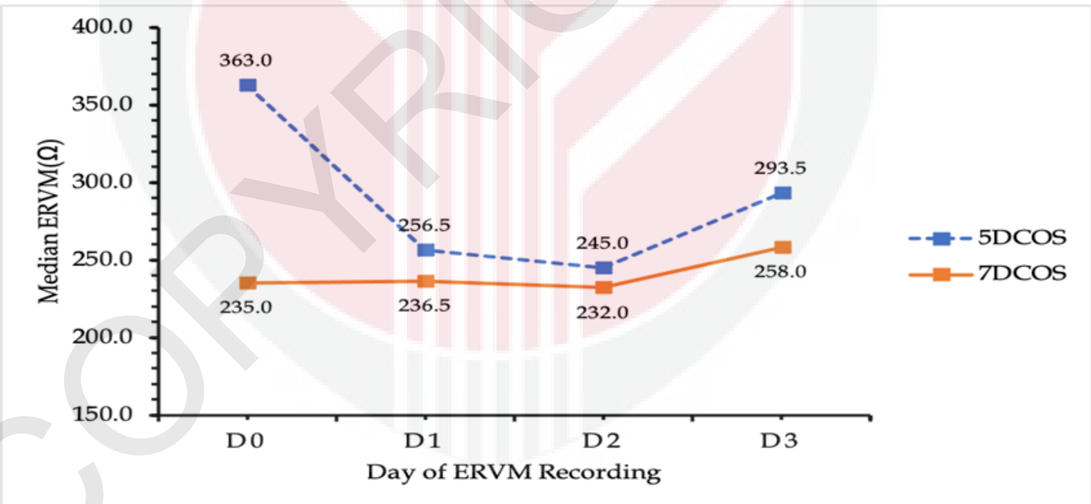

Figure 15: Median ERVM of Heifers across days for 5DCOS and 7DCOS protocols ($p > 0.05$). D0 represents the day of PRID®Delta removal and D2 and D3 represents the predetermined time of AI for 5DCOS and 7DCOS respectively

4.6 Comparison of ERVM of Cows and Heifers based on Estrus Status

The ERVM values on the day of TAI for cows that did and did not express estrus was compared using Mann-Whitney U test. The test results revealed no significant difference in the ERVM values (Table 5) between the cows that showed estrus signs (median = 281.0, n = 11) and those that did not show estrus signs (median = 285, n = 7), U = 36.50, Z = -0.181, p = 0.878. Therefore, we failed to reject the null hypothesis. Similarly, in heifers, the test results showed no significant difference between the group that exhibited (median = 243.40, n = 5) and that did not exhibit estrus signs (median = 250.00, n = 3), U = 7.00, Z = -0.150, p = 0.881.

Table 5: **Median ERVM (Ω) of the day of TAI for Cows and Heifers that showed and did not show Estrus signs.**

Category	Estrus response	Median	*p*-value
Cows	Yes	281.0	0.878
	No	285.0	
Heifers	Yes	243.40	0.881
	No	250.00	

The results from ERVM across days showed non-significant changes in both cows and heifers between protocol types indicating consistent values across days. The role of estrogen in reducing the resistance of vaginal mucus has been established previously (Leidl & Stolla, 1976). As the animal progresses to the day of AI from the day of PGF2α injection/device removal (D0), increasing circulatory estradiol concentration as a result of growing dominant follicle reduces the electrical resistance of vaginal mucus. However, the results showed no significant difference among values across days which is consistent with the findings of Zuluaga et al. (2008) in cows synchronized with 7-day CO-Synch protocol. However, other studies (Fazleabas et al., 1990; Meena et al., 2003) reported that ERVM values decreased during estrus and pregnancy rates were higher in cows that had low ERVM values. However, it is important to note that, these studies were conducted in natural estrus rather than in a TAI program.

In this study, the highest median ERVM values were recorded on the day of P4 device removal in both the protocols for cow (Figure 14). However, in heifers, highest values were recorded on the day of P4 device removal and on the day of AI in 5DCOS and 7DCOS respectively (Figure 15). Increased ERVM values during P4 device removal may be due to the presence of exudates and a relatively low circulatory estradiol and high progesterone concentration. This finding is in agreement with the report of Triagil et al. (2020) who also reported higher ERVM values after P4 device removal. Although the overall median ERVM measurements across days were numerically higher in cows in 7DCOS group, the differences were non-significant. Relatively lower ERVM in 5DCOS groups compared to 7DCOS groups may be due to the presence of younger follicles with high estrogenic capacity resulting in higher estradiol concentration during the pre-ovulatory phase (Bridges et al., 2008). In contrast, the median ERVM in heifers was numerically lower in 7DCOS compared to the 5DCOS group. Due to lower sample size in heifers ($n = 4$/group), fewer data points may have led to less accurate estimation of the true variability and therefore requires further exploration with a larger sample size.

In both cows and heifers, ERVM values failed to differentiate animals that expressed and did not express estrus ($p > 0.05$) indicating its unreliability as a method of estrus detection (Table 5). A consistent decrease in ERVM values during estrus has been reported (Ahmed et al., 2017; Fazleabas et al., 1990; Gupta & Purohit, 2001; Leidl & Stolla, 1976; Meena et al., 2003). However, huge variations in the values reported were observed. The ERVM in this study ranged 147 to 487 Ω. According to Leidl and Stolla (1976), an absolute ERVM values from different studies is not comparable despite the use of standardized equipment and technique because of the pressure of instrument applied on vaginal mucous membrane affects the results obtained. Furthermore, pathological conditions of the reproductive organs such as follicular cysts and endometrial inflammation resulted in values similar to estrus.

In this study, the mean ERVM in cows ranged from 147 to 363.0 Ω and 203 to 487 Ω for 5DCOS and 7DCOS respectively. While in heifers, ERVM ranged from 237 to 463 Ω and 197 to 433 Ω for 5DCOS and 7DCOS respectively. The values recorded in our study are much higher than the values reported using the same device (Ahmed et al., 2017; DeKa et al., 2022) but similar to values reported by Triagil et al. (2020) in does synchronized with a prostaglandin-based synchronization protocol. The type of device utilized also affects the values recorded. The Ovatec (Electronic Animal Management Inc., Co &, NY) recorded 30 Ω (Rorie et al., 2002) and 82.2 ± 2.2 Ω (mean ± SD) (Tadesse et al., 2011) resistance during estrus. Furthermore, the values were found to vary depending on the location of the probe inserted (Řezáč, 2008). The values recorded from posterior vagina are variable compared to those obtained from the anterior vagina (Kitwood et al., 1993). As such, high variability in the values obtained in this study could be due to measurements taken from the posterior vagina.

Results in this study suggest that ERVM values are unreliable and not sensitive for estrus detection due to inconsistent trends and failure to differentiate estrus and non-estrus animals. However, the low sample size may have affected the results and therefore, may not be suitable for extrapolation to other populations. Further studies with a larger sample is required to validate the present findings.

4.7 Comparison of Progesterone Profile Trend in Cows and Heifers

Friedman’s test was performed to determine significant differences among repeated measurements across days. The result from 7DCOS group revealed non-significant difference, $\chi 2$ (4) = 2.240, = 0.692. The highest and lowest median P4 concentration was 13.24 (D5) and 10.33 ng/ml (D3) respectively. Similarly, in 5DCOS group, the result revealed non-significant difference, $\chi 2$ (3) = 0.750, p = 0.861. The highest and lowest median P4 concentration was 17.00 (D3) and 15.36 ng/ml (D1) respectively. Details of the P4 concentration are presented in Figure 16

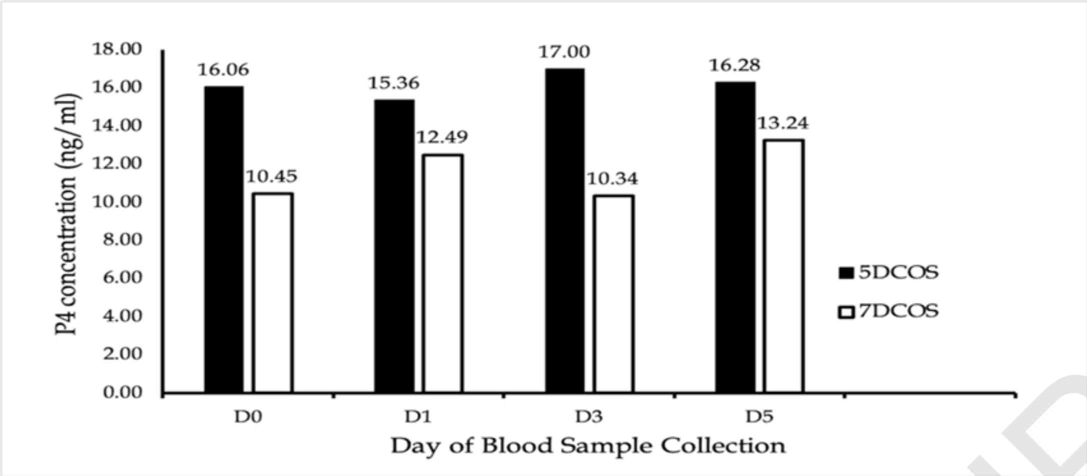

Figure 16: Comparison between two Estrus Synchronization Protocols for median Progesterone Concentration in Cows during PRID®Delta treatment (*p* > 0.05)

Similarly in heifers, Friedman's k- related sample analysis was conducted to determine significant differences among P4 concentration across days. The result from 7DCOS group revealed no significant difference, $\chi 2$ (4) = 6.400, p = 0.171. The highest and lowest median P4 concentration was 22.14 (D0) and 16.69 (D3) ng/ml respectively. The P4 concentration across days in 5DCOS also revealed non-significant difference 2 (3) = 2.700, p = 0.440. The highest median P4 concentration recorded was 16.64 ng/ml (D5) and the lowest median concentration was 12 ng/ml (D3). The overall P4 concentration in heifers between protocols is presented in Figure 17.

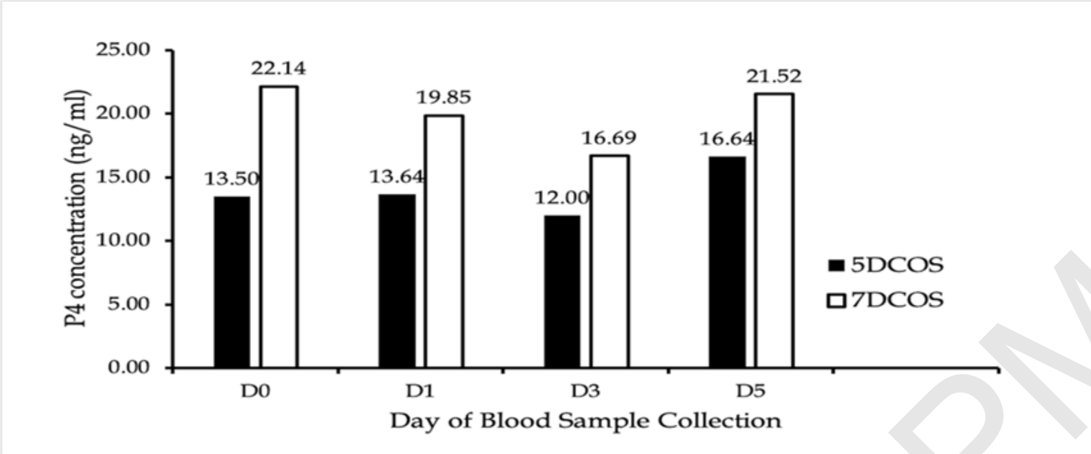

Figure 17: Comparison Between two Estrus Synchronization Protocols for median Progesterone Concentration in Heifers during PRID®Delta treatment (*p* > 0.05)

Improved embryo quality and better conception outcomes have been associated with higher levels of circulating P4 during follicular growth (Bisinotto et al., 2010). In lactating dairy cows, low blood P4 levels were associated with higher risk of pregnancy losses between day 29 and 60 after AI (Cunha et al., 2008). Increased dry matter intake in high producing cows caused increased metabolism of P4 in liver. Thus, intra-vaginal supplementation to elevate circulatory P4 is crucial in lactating cows during synchronization (Wiltbank et al., 2008). It has been proposed that during routine synchronization programs, plasma concentration above 2 ng/ml must be maintained via intravaginal device until removal (Rathbone & Burke, 2013).

In this study, the recorded progesterone concentrations appear notably higher than the findings reported by Van Werven et al. (2013), that observed a maximum mean progesterone concentration slightly above 5 ng/ml within 24 hours after device insertion in non-lactating, ovariectomized Holstein cows. The substantial disparity in progesterone levels observed can be attributed to a combination of factors including both endogenous progesterone production and external supplementation via the progesterone-releasing device. It is important to acknowledge that the methodology employed for quantifying serum progesterone also contributes to these variations. While the study by Van Werven et al. (2013) utilized a gold standard radioimmunoassay (RIA) to measure the hormone, our study employed general or pan-species P4 ELISA Kits from Cloud Clone Corp., USA which may have contributed to the observed differences.

Throughout the duration of our study, the median progesterone concentrations remained relatively stable from the time of P4 device insertion until removal in both protocols in cows. Notably, the 5DCOS group exhibited a slightly higher median progesterone concentration in comparison to the 7DCOS group (Figure 16), while heifers in the 7DCOS group displayed higher median progesterone levels than those in the 5DCOS group (Figure 17). Despite these variations, no significant differences were observed between the two synchronization protocols across different days for both cows and heifers ($p > 0.05$). This suggests that both protocols effectively maintained similar progesterone concentrations throughout the study.

This pattern in our results contrasts with findings reported by Van Werven et al. (2013) and Crabtree et al. (2018) whereby progesterone concentrations reached their peak within a short period, typically within four hours or one day after device insertion, and then gradually decreased until the device was removed. In this study, blood sampling was restricted to one sample daily and which makes difficult to compare with their results. However, it is important to emphasize that due to the low sample size of this study, statistical power to detect small differences may have been compromised. G* Power analysis (version 3.1) revealed a total required sample size of 134 (n =67 each) for a moderate effect size of 0.5 (d) at a power of 0.8 and alpha error probability of 0.05.

CHAPTER 5

SUMMARY, CONCLUSION, LIMITATION, AND RECOMMENDATIONS FOR FUTURE RESEARCH

5.1 Summary of the Findings

The objectives of this study were to compare ERR, ERVM, and P4 concentration between 5DCOS and 7DCOS protocols supplemented with PRID®Delta in beef cows and heifers. The two protocols were hypothesized to differ in ERR and P4 concentration. EVRM values were also hypothesized to differ significantly between cows exhibiting and not exhibiting estrus. In both cows and heifers, no significant difference was observed in ERR, ERVM across days, ERVM at AI for estrual and non-estrual cows, and P4 concentrations across and between days. Thus, the null hypotheses were accepted.

5.2 Conclusion

1. The percentage of ERR for 5DCOS and 7DCOS was 37.5% and 80.0% respectively. Whereas in heifers, it was 75.0% and 50.0% respectively for 5DCOS and 7DCOS. The difference was not statistically significant in both cows and heifers. Therefore, it was concluded that both the protocols induced similar ERR in both cows and heifers.

2. The median ERVM was relatively constant in both cows and heifers beginning from P4 device removal until the day of AI and did not differ significantly in both cows and heifers. Cows and heifers that exhibited and did not exhibit estrus did not differ significantly. Thus, ERVM was not useful to detect estrus in a TAI program.

3. The median P4 concentration beginning from device insertion till removal remained elevated throughout the treatment period. The concentration in 5DCOS group in cows and 7DCOS group in heifers was relatively higher. However, the differences were not statistically significant. Comparison of P4 concentrations between days revealed no significant difference between protocols in both cows and heifers. It is concluded that both protocols maintained similar P4 circulatory concentrations in cows and heifers.

4. Due to double PGF2α dose required in 5DCOS protocol, this results in extra handling animals and additional cost due to purchase of hormone which may restrict breeders to implement the protocol. Thus, other reproductive parameter such as pregnancy outcome resulting from 5DCOS and 7DCOS must be considered in view of extra cost in 5DCOS.

5. Small farms wishing to implement new reproductive technologies could use the findings as a guide to start their pilot programs by conducting trials in small scale to gauge its effectiveness prior to large scale implementation. Furthermore, study on CO-Synch programs in tropical countries such as Malaysia is currently unavailable and the findings from this study, although of

low sample size could serve as a baseline information to the research community and cattle producers.

5.3 Recommendations for Future Research

While this study provides valuable insights on estrus synchronization using PRID®Delta based on ERR, ERVM, and P4 profiles in beef cattle, there remains several avenues for further study to gain better understanding and further enhance the reproductive management of cattle. Some recommendations to be considered for future research:

1. Due to the low sample size, statistical power to detect effect or differences may have been missed due to reduced power. Therefore, future studies may consider incorporating larger sample size.

2. The success of TAI program very much depends on the BCS of animals at the start of TAI program. In the current study, only 1 out of 26 cattle had BCS of 5 (1-9 scale) which is lower than the recommended BCS. Thus, future research may consider animals with a BCS 5 and above (1-9 scale) for overall success of the TAI program because research indicates improved reproductive performance with BCS greater than 5. Additionally, future research could focus on how varying BCS scores affect efficacy of CO-Synch protocols and overall fertility which could help strategize cattle management in terms of nutrition and health.

3. This study focused on Brangus cross beef cattle. Future studies could consider investigation in other breeds of cattle including dairy cows.

4. Due to logistical issues, estrus was visually observed in a compact paddock on the concrete floor which may have affected the overall estrus response and behavioral expression of cattle. Therefore, future research could be conducted in open field or pasture setting as cattle in open pastures/space have more opportunities for social interactions that leads to heightened estrus behavior. Moreover, open pasture provides them more space to move freely which may be suppressed in closed enclosure.

5. To understand relationship between progesterone profile and estrus signs, as well as electrical resistance of vaginal mucus (ERVM), correlational studies could be conducted to establish temporal correlation and variations in hormone profiles relative to estrus expression and ERVM values.

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APPENDICES

Appendix 1: Pictorial guide for body condition scoring in beef cattle.
Adapted from Eversole et al. (2009).

BCS 1: Emaciated with muscle atrophy and no detectable fat. Tail head and ribs project predominantly. Animal physically weak.

BCS 2: Poor condition with muscle atrophy and no detectable fat. Tail head and ribs prominent

BCS 3: Thin condition. Slight muscle atrophy. All ribs visible. Very little detectable fat

BCS 4: Borderline condition. Outline of spine slightly visible. Outline of 3 to 5 ribs visible. Some fat over ribs and hips

BCS 5: Moderate, good overall appearance. Outline of spine no longer visible. Outline of 1-2 ribs visible. Fat over hips but still visible

BCS 6: High moderate condition. Ribs and spine no longer visible. Pressure applied to feel bone structure. Some fat in brisket and flanks

	
BCS 7: Good, fleshy appearance. Hips slightly visible but ribs and spine not visible. Fat in brisket and flanks with slight udder and tail head fat.	**BCS 8:** Fat, fleshy and over-conditioned. Bone structure not visible. Large patchy fat deposits over ribs, around tail head and brisket.

	
BCS 7: Good, fleshy appearance. Hips slightly visible but ribs and spine not visible. Fat in brisket and flanks with slight udder and tail head fat.	**BCS 8:** Fat, fleshy and over-conditioned. Bone structure not visible. Large patchy fat deposits over ribs, around tail head and brisket.

Appendix 2: Guidelines for assigning body condition scores in beef cattle

Reference point	**Body Condition Scores**								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Physically weak	Yes	No	No	No	No	No	No	No	No
Muscle atrophy	Yes	Yes	Slight	No	No	No	No	No	No
Outline of spine visible	Yes	Yes	Yes	Slight	No	No	No	No	No
Outline of ribs visible	All	All	All	3-5	1-2	0	0	0	0
Outline of hip and pin bone visible	Yes	Yes	Yes	Yes	Yes	Yes	Slight	No	No
Fat in brisket and flanks	No	No	No	No	No	Some	Full	Full	Extreme
Fat udder and patchy fat around tail head	No	No	No	No	No	No	Slight	Yes	Extreme

Adapted from Eversole et al. (2009)

Appendix 3: Sample data recording sheet used during the study.

Animal ID: B904	IACUC NO: UPM/IACUC/AUP-R036/2022	
Pre-Experimental Data		
Day 0	DOB/Age	6/2/2019 (3.6 yrs)
	Date of commencement	27/09/22
	Physical and Clinical Examination	
	Respiration rate (bpm)	22
	Body condition score (1-9)	4
	Heart rate (bpm)	52
	Rumen motility -/2 min	3
	Rumen motility quality	Poor Ok Good
	Dehydration (%)	0
	Lymph node	left prefemoral lymphnode assymetric
	Rectal temperature	37.5°C
	Vulvar capillary refill time	Instant
	Vulvar mucous membrane color	Pale pink
	Category (Cow/Heifer)	Cow Heifer
	Parity	
	Pregnancy status	Yes No
Ultrasound	Left Ovary	Growing follicles observed
	Right Ovary	"
Time of PRID Insertion		10.07 am

Daily Monitoring (7-day Protocol)

Day	0	1	2	3	4	5	6	7	8	9	10
Date	27/9										
Vulvar discharge	-	-	-	-	-	-	-	-	-	-	-
PRID-Retention	✓	✓	✓	✓	✓	✓	✓	✓	✗	✗	✗
Rectal Temp	37.5	37.1		37.9		37	✗	38	37.9	37.5	37.2
Blood sample	✓	✓		✓		✓		✓	✓	✓	✓

5-Day Protocol

Day	0	1	2	3	4	5	6	7	8	9	10	11
Date												
Vulvar discharge												
PRID-Retention												
Rectal Temp												
Blood sample												

Date of PRID Removal: 04/10/22

Time of PRID removal and PGF	9.55 AM
Time of Second PGF (5DCOS)	-

Estrus Detection and ERVM

Day		D5/D7 PRID Removal	D6/8	D7/9	D8/10
Date					
ERVM Readings		1 240	1 210	1 230	1 290
		2 240	2 210	2 240	2 290
		3 220	3 220	3 310	3 280
ERVM Average					
Estrotect Score		0			
Estrus Signs Observed	AM	No behavioural signs observed			
	PM				

Appendix 3. Enzyme linked Immune-Sorbent Assay (ELISA) procedures.

Progesterone (P4)

Reagents supplied by manufacturer of the Kit

Reagents	Quantity	Reagents	Quantity
Pre-coated ready to use 96 well strip plate	1	Plate sealer for 96 wells	4
Standard	2	Standard Diluent	20 ml
Detection Reagent A	1	Assay Diluent A	12 ml
Detection Reagent B	120 µl	Assay Diluent B	12 ml
Reagent Diluent	300 µl	Stop Solution	6 ml
TMB Substrate	9 ml		
Wash Buffer (30x concentrate	20 ml		

Other materials required but not supplied

No	Materials
1	Mmicroplate reader with 450 ± 10nm filter.
2	Single or multi-channel pipettes with high precision and disposable tips
3	Microcentrifuge Tubes.
4	Deionized or distilled water
5	Absorbent paper for blotting the microplate
6	Container for Wash Solution.
7	0.01mol/L (or 1×) Phosphate Buffered Saline (PBS), pH7.0-7.2.

1. Standard Reagent Preparation and Assay Procedure

A. Standard Reagent Preparation

1. Standard was reconstituted with 2ml of Standard Diluent and kept for 10 mins at room temperature.
2. A total of 6 microcentrifuge tubes were arranged. Five for Standards and one for Blank.
3. All tubes except standard were filled with 0.6 ml of Standard Diluent.
4. Triple dilution series by transferring 300 µl of reconstituted standard to four other tubes except Blank tube.

B. Detection Reagent A (12 ml)

1. The Detection Reagent A was reconstituted with 150 µl of Reagent Diluent and kept for 10 mins at room temperature.
2. The Detection Reagent A was further diluted by transfer ring 50µl of it into 4950 µl of Assay Diluent A to prepare 1:100 working solution (Total required = 5000 µl).

C. Detection Reagent B (12ml)

1. Detection Reagent A was briefly centrifuged and diluted to working concentration of 1:100 by transferring 100 µl of it in 9900 µl of Assay Diluent B (Total required =10000 µl).

D. Preparation of Wash Solution

20 ml of Wash Buffer was mixed with 580 ml of deionized water to make a total volume of 600 ml.

2. Assay Procedure

1. 50 µL each of dilutions of standard (read Reagent Preparation), blank and samples was added into the designated wells, respectively.

2. Immediately added 50 µl of Detection Reagent A to each well.

3. Shaken the plate gently (using a microplate shaker). Covered with a Plate sealer and incubated for 1 hour at 37^{o} C.

4. Aspirated the solution and washed 3 times with 350 µl of 1X Wash Solution.

5. Added 100 µl of Detection Reagent B working solution to each well and incubated for 30 minutes at 37oC after covering it with the Plate sealer. Washed 3 times with Wash Solution.

6. Added 90 µl of TMB Substrate Solution to each well. Covered with a new Plate sealer and incubated for 25 minutes at 37^{o}C.
7. Added 50 µl of Stop Solution to each well. Mixed the liquid solution by tapping the side of the plate.

E. Absorbance Reading and Hormone concentration

The absorbance was measured using Tecan Infinite M200 plate reader at 450 nm (Institute for Bioengineering of Catalonia [IBEC] Spain). Preparation of

Standard Curve and Concentration of hormones were performed using online website (www.myassays.com).

BIODATA OF STUDENT

Dr. Jigdrel Dorji was born in the Pemagatshel district, in the Nagtsheri village of Bhutan in 1987. He received his primary education at Tsebar Pilot School in 1996. He completed his middle and higher secondary education at Pemagatshel Middle Secondary School and Nangkor Higher Secondary School in 2006 and 2008 respectively. In 2016, he obtained his undergraduate degree (BVSc & AH) from Guru Angad Dev Veterinary and Animal Sciences University in Punjab, India.

Following the completion of his undergraduate studies, Dr. Jigdrel Dorji served as an assistant lecturer from 2016 to 2021 at the College of Natural Resources, Department of Animal Science, Royal University of Bhutan. Subsequently, he joined Universiti Putra Malaysia, to pursue a master's degree in veterinary science (MVSc) with a specialization in Theriogenology and Cytogenetics. His research was conducted under the supervision of Dr. Mark Hiew Wen Han at the Department of Veterinary Clinical Studies, Faculty of Veterinary Medicine, Universiti Putra Malaysia.

LIST OF PUBLICATION

Dorji, J., Hiew, M. W. H., & Yimer, N. A review of pregnancy rates in beef cattle via timed artificial insemination utilizing CIDR-based 5- and 7-day CO-synch protocols. *Journal of Tropical Agricultural Sciences (*Accepted*)*