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‘The Occurrence of Twin Pregnancies After Using an Intravaginal Progesterone Device in a Hungarian Dairy Herd.’

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Contents

Abbreviations

DOS – Double OvSynch

PRID – Progesterone Releasing Intravaginal Device

CIDR – Controlled Internal Drug Release

CL – Corpus Luteum

FTAI – Fixed Timed Artificial Insemination

P/AI – Pregnancies per Artificial Insemination

DIM – Days in Milk

PGF2a – Prostaglandin F (2 alpha)

P4 – Progesterone

TMR – Total Mixed Ration

TAD – Twice A Day

1. Introduction

Twinning has long since been an undesirable trait in dairy herds due to the negative repercussions on cow and calf health [1]. Twin calving has been proven to increase the risk of peripartum disorders such as ketosis, dystocia, retained placenta, and embryonic loss [2]. As veterinarians our research into minimising the outcome of twinning in timed reproductive protocols is crucial to improving the soundness of our national dairy herds. The effects of twin calving have been shown to extend into the following lactations and reduce the productive lifespan of the cow [3]. This coupled with increased veterinary intervention significantly reduces herd profitability and places an elevated economic stress on the herd [3]. Previous studies have shown that up to 10% of cows produce twins at least once in their lifetime and the risk of calving twins is higher in those who have previously twinned before [4]. This statistic suggests that more emphasis should be placed on the prevention of conceiving twins via hormonal treatment rather than invasive manual intervention required for the reduction of twins at pregnancy diagnosis [5].

In this paper, I examine the issue of twin pregnancies on a Holstein dairy farm, focussing on the role of progesterone supplementation using intravaginal progesterone implants (PRID) that could potentially minimise twinning rates in a reproductive protocol. Nowadays, the use of these PRID devices is very frequent on modern Holstein dairy farms for synchronisation protocols. Some studies mention that using progesterone devices increases twin pregnancy, often due to reduced incidences of embryonic loss [6]. The aim of this study is to compare the occurrence of twin pregnancy using progesterone device with other hormonal synchronisation protocols in large dairy farms.

This paper reviews key factors resulting in twinning and explores the consequences it has on both cow and calf health. It further discusses the economic consequences of twins on profit and alternative methods suggested to reduce twinning in dairy herds.

2. Literature review

2.1 The Physiology of Twinning

The literature identifies the Holstein dairy cow as a typically mono-ovular species. This means that it normally ovulates one egg per estrus cycle, producing one offspring per calving. Twinning occurs when double ovulation takes place during the reproductive cycle, where more than one dominant follicle arises from a follicular wave. Twins can be either monozygous or dizygous in nature. This means that they arise from the fertilisation of one oocyte that cleaves into two embryos or the fertilisation of two separate oocytes respectively[1]. Twins can be unilateral (in both the left or right uterine horn) or bilateral. Those occurring unilaterally showing significantly higher levels of embryonic loss. Similarly, unilateral twin pregnancies have been recorded 3.45 (1/0.29) times more likely to undergo the loss of one embryo than bilateral twin pregnancies [7].

Monozygotic twins are genetically and phenotypically identical as they arise from the fusion of the same oocyte and sperm. Dizygotic twins are as genetically alike as siblings of the same parents as they arise from the fertilisation of two separate oocytes and sperm. Dizygous twins account for most twins in the dairy industry [8] and so we must begin to fully understand the follicular dynamics of the dairy cow's reproductive cycle to address the issue of twinning[1, 9].

The bovine estrus cycle is made up of a series of follicular waves. During these waves, 'deviation' of a dominant follicle takes place. The exact mechanism behind the occurrence of two dominant follicles or 'codominance' is not fully understood and further research into its origin would aid our studies. However, it has been shown that waves that developed codominant follicles differed in hormones as well as follicular dynamics [10]. This allows us to use hormones to manipulate the estrus cycle and therefore develop reproductive protocols that potentially minimise twinning.

2.2 Factors Influencing the Risk of Twinning

Twinning is a multifaceted phenomenon, a double-edged sword with many possible causative agents. Here we will discuss the impact of increased milk production, parity, season and progesterone levels on twinning rates. Research suggests that the Holstein dairy cow exceeds many beef breeds with twinning rates higher (3-5%) than those (1%) in beef herds [11].

2.2.1 Increased Milk Production and Parity

The increasing rate of twinning over recent decades has been mainly attributed to growing levels of milk production. Cows with high milk production levels are shown to have three times higher levels of double ovulation. As we all know milk production increases with parity in dairy cows with many cows reaching peak milk production around lactation number 5. Experts believe that high milk producing cows have higher feed intake and therefore increased blood flow to the liver leading to increased metabolism and elimination of steroid hormones. This has led to the conclusion that there is correlation between increased double ovulation and low levels of P4 in the bloodstream [12]. As a result, Fricke et al developed the more suitable Double-OvSynch protocol which aims to achieve hormone levels that minimise the occurrence of double ovulation. These protocols differ between farms and management systems and should be uniquely developed by the supervising veterinarian and farmer. Management systems can vary from timed AI (TAI) protocols to those who still rely on heat detection systems. PRID sync and Double OvSynch are examples of these synchronisation protocols developed. These synchronisation programmes are proven to increase conception rates, increase embryo survival rates and decrease incidences of double ovulation [12].

2.2.2 Season

The perception that seasons and environmental factors have an impact on increased twinning rates has been around for the last century. Previous studies have concluded that external stimuli such as temperature, photoperiod and quality of nutrition play a significant role in the occurrence of twinning. A study conducted by D.P. Ryan *et al.* found a marked increase in twin calves during summer calving months, concluding that there was greater embryo mortality during the hot versus cool months [9]. An increased rate of twinning has been observed during warmer calving months, with elevated incidences of twins being reported during the months of spring or autumn [13]. This can perhaps be attributed to increased feed

consumption during the colder months when summer calves were conceived. This underscores previous assumptions that increased feed intake leads to increased metabolism of steroid hormones and supports the fact that reduced P4 at conception may lead to increased incidences of double ovulations and therefore twins [12]. Other studies have shown that summer heat stress plays a main role in the lower conception rates and decreased embryo survival rates observed in high producing dairy herds [14], contributing to the lowered incidences of twins calving during winter months.

2.2.3 Corpus Luteum and Progesterone

The corpus luteum is an essential structure to the bovine reproductive cycle and pregnancy relies on its regression. A persistent corpus luteum can lead to unsuccessful fertilisation. Therefore, PGF2a hormone is an essential part of reproduction protocols ensuring luteolysis takes place. The CL secretes P4 (also by the placenta), an essential pregnancy hormone and its serum concentration levels have been proven to be dependent on the size of the CL. There are two morphological types of CL, CL homogenous and CL cavitary. The first being a complete body of luteal tissue and the latter an internal cavity surrounded by luteal tissue[15]. There are conflicting studies that the cavitary CL produces higher levels of P4 concentrations [16] and some reports that there were no differences [17].

Research suggests that heifers had higher P4 levels with a cavitary CL and showed higher pregnancy rates that those with CL homogenous. The authors therefore concluded that higher P4 levels increased pregnancy rates because of increased chances of survival during the crucial pregnancy recognition period [15]. P4 hormone is an important pregnancy hormone that supports embryo survival during critical pregnancy implantation and establishment. This can be seen here as its increased concentration elevated pregnancy survival rates amongst heifers. This increased embryo survival rate may also be applicable to increasing survival rates amongst twin pregnancies with P4 supplementation at pregnancy diagnosis [18]. This suggests further investigation into the CL cavitary's role in the occurrence of twinning amongst dairy cows would be informative.

Furthermore, there is correlation between number of days in milk (DIM) and number of CL on the rate of pregnancy loss in twin pregnancies. An increase in fertility or a decrease in embryo loss is associated with the presence of 2 CL, perhaps due to increased P4 levels [19]. The increased number of CL results in increased P4 concentrations and supports the fact that increased P4 at critical pregnancy recognition plays a role in embryo survival rates. As stated

before, serum P4 levels can be seen to decrease as milk production increases due to increased metabolism of steroid hormones. The volume of milk reaches its peak mid lactation cycle and therefore we can conclude that the number of DIM clearly plays a role in P4 levels.

Progesterone plays a critical role in the events leading up to and maintaining pregnancy. It plays a role in establishing oocyte quality and preparation of the endometrium for implantation. Elevated levels of P4 during follicular growth are associated with increased fertility and after ovulation with advancement in conceptus elongation and higher pregnancy rates in some cattle. The timing and levels of increased P4 have been deemed critical in its impact on the follicular cycle and reproductive outcomes[18].

Elevated P4 before the timed AI has been seen to decrease double ovulation and increase fertility. Slight increases in P4 at TAI has led to decreased fertility and circulating levels of P4 after conception is essential for embryo growth and pregnancy maintenance [18] as mentioned above. This allows us to assume that the hormonal manipulation of the reproductive cycle by increasing P4 levels before TAI will allow us to minimise twinning rates. It also indicates to us the optimum time of removing the P4 device to reduce P4 levels to an optimum level at TAI.

2.2.4 Estrus Expression

Detection of estrus is one of the major challenges in the reproductive management of dairy cows. Therefore, efficient, and accurate detection of estrus is vital to good reproductive performance in a dairy herd where artificial insemination is used. FTAI protocols have been developed to eliminate the need for estrus synchronisation protocols which are dependent on inaccurate and unreliable estrus detection [20, 21]. The physical expression of estrus is heavily influenced by the temperature, environment (housing systems and flooring surfaces) [22]. It usually exhibits itself in the form of increased activity with 21.4% decrease in locomotion being noted in multiparous cows [23]. This has been concluded to be as the result of increased clearance of estradiol (primarily believed to be responsible for estrus expression) in high producing cows [24]. This correlates with the increased occurrence of double ovulation and twinning in multiparous cows. Similarly, evidence has been found that increased milk production results in reduced estrus expression, presumably accounting for this increased estradiol clearance. This suggests that there is a connection between decreased estrus expression and the increased chances of twins [25]. The phenomenon of visual estrus detection is no longer implemented due to impracticality on large scale farms. Fully

automated sensor-based technologies that continuously record very detailed information on the cows have been developed to make estrus detection more reliable [22]. Therefore, we can conclude that the elimination of the need for estrus detection using FTAI protocols that supplement these steroid hormones, may aid in reducing twinning rates in dairy cows.

2.3 Negative Impact of Twinning on Calf Health

2.3.1 Increased Incidence of Embryonic Death

Despite efforts to reduce the above risks, twinning still occurs and as a result embryonic loss often takes place. Embryonic death refers to the losses that occur between fertilisation and the completion of the stage of differentiation at approximately day 45. It can be categorised based on the timing of occurrence; Very Early Embryonic death (day 0- day 7), Early Embryonic Death (day 7 – 24), Late Embryonic Mortality and Early Foetal mortality (days 24 to 28). Placental establishment is usually complete after day 60 and chances of embryonic loss are significantly reduced from then onwards [26]. Early embryonic loss often evades clinical diagnosis. Many factors have proven to cause embryonic mortality such as vitamin A deficiency, expression of lethal genes up to day 5, P4 deficiency, iodine deficiency and infectious diseases such as Leptospirosis [27]. However, our focus is the effect of twinning on embryonic death and survival rates, there is an increased risk of embryonic loss in cows carrying twins, it has been found to be three to nine times greater than for cows carrying singletons [28].

'Spontaneous reduction' of twin embryos refers to our inability to clinically prevent the loss of twin embryos with rates of 11.2% to 28.4% previously recorded in dairy herds [5, 7, 19, 29, 30]. Previous studies have suggested that it is because of insufficient P4 support for placenta establishment due to unbalanced vascular supply in shared placentas for twins [7].

Higher incidences of dystocia in twin calves have been recorded [31]. Those who survive this dystocia experience lower passive immunity transfer, higher postnatal mortality, and higher indicators of physiological stress [32]. This combined with decreased birth weight due to shorter gestation lengths in twin pregnancies can lead to poor future performance amongst the herd. As a result, twin calves are left more susceptible to future infections such as infection with Coxiella Burnetii, an infection becoming more and more prevalent amongst dairy herds.

This poses a potential zoonotic risk causing ‘Q Fever’ amongst humans especially as it is spread via placental membranes and amniotic fluids. The increased intervention required with twin calving’s leaves farm workers exposed to this flu-like disease [33]. This provides further evidence that the prevention of twin calving not only plays a role in animal health, but also in protecting human health.

2.3.2 Bovine Freemartin Syndrome

The appearance of freemartins in bovids otherwise known as ‘Bovine Freemartin Syndrome’ was first detailed in 1917. Freemartins occur in dizygotic twins in which one female and one male calf is born, this female calf is deemed infertile and cannot be used for further breeding [34]. The intimate nature of the vasculature of the female and male calf and eventual vascular anastomoses leads to masculinisation of the female tubular reproductive tract (see figure 1 below). The possibility of the development of an XX/XY chimera has been suggested with reports of both female and male internal reproductive organs. Chimeras are individual animals that contain two cell types originating from separate zygotes. Due to the earlier development of the male testicular organs, secretion of anti-müllerian hormone from the male inhibits the development of the female paramesonephric ducts [35]. Genital tract defects such as hypoplastic uterine horns or discontinuity between the uterus and the vagina can be seen. The external genitalia often appear normal or abnormal, enlarged clitoris and distorted vulva with coarse hairs present at the ventral commissure can often be observed [36].

Bovine Freemartin Syndrome occurs in different forms; heterosexual twins, single-born sterile heifers, and heifers born with ‘Acardius amorphus’[37]. Single born freemartins (sterile heifers) have been reported and concluded to be as the result of the early embryonic death of the male co-twin in utero. The concept of single born freemartins is difficult as the death of a co-twin causes release of endotoxins resulting in the death of the female calf due to the intertwined vasculature, however survival has been recorded [38].

The occurrence of twin calves periodically results in a stronger twin and freemartins is no exception. It has been compared to ‘twin transfusion syndrome’ in humans which occurs in monozygotic twins more often and results in unequal sharing of blood supply, asymmetrical foetal growth, and foetal mortality, although none have been reported in bovids to our knowledge.

Only 80% to 95% of females born co-twin to a male are freemartins, the other 5-20% of cases in which a fertile female is born as a twin to a male is thought to be because of the failure of fusion of foetal membranes or timing of membrane fusion after a pivotal stage in reproductive organ differentiation [39]. More research could be applied here into the reason behind this and the potential interventions that could be used to prevent the effects of male hormones on female reproductive development.

The continuous replacement of unproductive dairy cows with new breeding heifers is an essential part of any bovine reproductive programme, therefore the occurrence of sterile female calves has a clear negative economic impact on the herd. For this reason, methods of early diagnosis of freemartin have been suggested using PCR analysis detecting the XY cells in cases suspected to have the Bovine Freemartin Syndrome. Other diagnostic methods include; palpation, karyotyping, blood typing, or fluorescence identification of Y-chromosome directed probes (FISH). Although these methods may help us predict freemartin lines, they are not used as a method of preventing twinning on dairy farms due to increased costs, timing, and reliability[40].

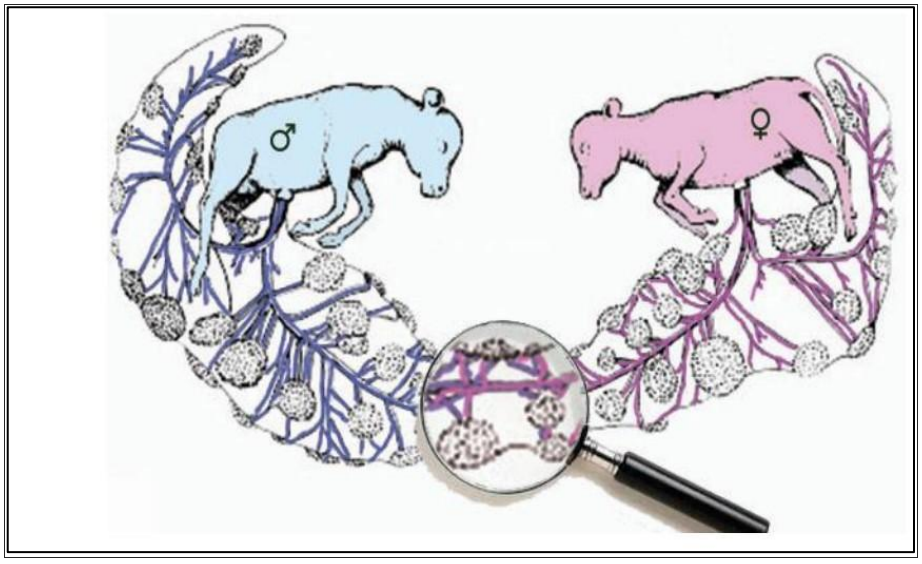

Figure 1: A schematic representation of chorion-allantois anastomosis between two cattle foetuses of different gender, according to literature [41].

2.4 Negative Impact on Cow Health

2.4.1 Decline in Body Condition and Production

The physical burden of carrying twins often takes a toll on the body condition of the dam. Minimising the number of cows carrying twins with hormonal interventions such as PRID sync protocol will reduce the amount of cows calving with insufficient BCS. Furthermore, the BCS and drying off period should be carefully managed in twin bearing cows to avoid future production and health issues. It is not surprising that there is a negative correlation between the number of calves born and the body condition score of the dam. Cows carrying twins have shorter gestation lengths and therefore shorter drying off periods, resulting in poor body condition score upon calving [42]. Scanning to allow early diagnosis of twin pregnancies is suggested to allow early drying off and sufficient time for dam to meet the recommended BCS of three at calving time. Although considered a period of rest, the drying off period is a time of expedited foetal growth. A change in nutritional requirements coupled with milk production post calving causes the cow to enter a state of negative energy balance (NEB). This can only be exacerbated during twin calving. This deficiency in energy can lead to a compromised immune response resulting in metabolic and microbial diseases such as; milk fever, endometritis, ketosis, displaced abomasum and retained placenta [43]. This research suggests that investment into a FTAI protocol that uses hormonal supplementation to minimise twinning will result in future savings due to reduced incidences of metabolic diseases.

Proper nutritional management of cows diagnosed with twins can help reduce these negative clinical consequences of twin calves [42]. Ketosis in dairy cows can appear as a consequence of the above-mentioned NEB accompanying twin calving. It can manifest itself clinically or sub-clinically, signs include diminished appetite, decreased milk production, loss of weight, hypoglycaemia, and hyperketonemia [44]. It can lead to decreased milk production and compromise the future reproductive performance of the dam. Similarly, it increases the number of services per conception in following lactations and increases the number of days open [2, 45]. For these reasons, this study suggests that intervention is needed to prevent twinning in dairy cows.

2.4.2 Retained Placenta and Dystocia

Retained placenta also known as 'retained foetal membranes' occurs when a cow fails to fully expel its foetal membranes beyond 24 hours post calving. Cows with retained foetal membranes are at higher risk of metritis, displaced abomasum and ketosis. Increased incidences of uterine infections, foot problems and mastitis were found in cows with retained placentas[46], with higher rates of retained placentas recorded during the summer months compared to winter [47]. These require veterinary intervention and therefore increased economic losses on dairy herds affected.

78.9% of incidences of the dystocia resulting from twins result from abnormal presentation of the head and(or) legs for one or both twin foetuses at parturition. Increased incidences are marked in unilateral twin cases [32]. Diagnosis of twin pregnancy before calving allows timely administration of obstetrical assistance to enable safe delivery of twin calves and to increase their neonatal survival [32]. Furthermore, dystocia was positively correlated with the number of dead calves at birth and with the total weight of calves born. These difficult calvings have been linked to increased incidences of metritis and endometritis recorded in cows carrying twins [42]. This provides further evidence of the negative impact that twinning has on cow health and supports the conclusion that twinning must be minimised amongst dairy cows.

Health problem	Low-concentrate diet				High-concentrate diet			
	Twin	Single	Odds ratio	95% Confidence interval	Twin	Single	Odds ratio	95% Confidence interval
Metritis	22.6	3.2	2.27	0.03–0.35	17.5	7.5	1.35	−0.04–0.24
Endometritis	16.1	0	2.33	0.03–0.29	10	2.5	1.39	−0.03–0.18
Retained placenta	25.8	0	3.03	0.10–0.41	27.5	0	3.57	0.14–0.41

Figure 2: The above table showcases the increased incidences of reproductive tract illnesses associated with twin versus single pregnancies on both low and high concentrate diets [42].

2.5 Economic Impact of Twins

Reduction in profit can be seen on dairy farms with poor reproductive protocols that do not implement strategies that reduce twinning rates amongst the herd. Increased culling rates coupled with reduced milk production in subsequent lactation cycles are the main culprits [48]. Early diagnosis of twin pregnancies by transrectal ultrasonography can allow management to decide whether to do nothing, terminate pregnancy or perhaps attempt embryo reduction. The increased costs of scanning cows to determine twin pregnancy is outweighed by the increased benefit of a confirmed pregnancy diagnosis. This allows cows found empty at pregnancy diagnosis to return to resynchronisation protocols reducing days open and days to return to estrus [49].

Contrary to the logical thought that more calves results in more profit, the negative impacts of twinning on cow and calf health previously discussed contradicts this statement. According to studies, the approximated the rate of loss to be anywhere from $97 to $225 depending on the type of twin pregnancy (unilateral vs. bilateral), parity, and DIM when the twin pregnancy occurred [3]. The negative economic impact is because of an elevated occurrence of retained placenta and vulval discharges with an extended calving to conception interval by 33 days [45].

As mentioned before, increased culling rates due to twinning is a major contribution to economic loss on dairy farms. A study completed on a Holstein Friesian dairy farm confirmed our knowledge that udder diseases, future reproductive failure and decreased fertility caused higher culling rates in cows who previously calved twins [50]. This provides further evidence that twinning is not a desirable trait in dairy cows due its adverse effects on cow and calf health, further reducing farm profit. Furthermore, this advocates an additional need to minimise twinning rates in dairy herds.

2.6 Methods to Reduce Twinning

Due to this negative impact on cow fertility, studies have been conducted to prevent twinning. For example, a study has proven that cows receiving FTET (Fixed time embryo transfer) showed no incidence of twinning compared to those who received FTAI. Single frozen thawed in vitro produced (IVP) embryos were used as opposed to fresh to allow easier on farm handling. This showed that IVP embryo transfer proved valuable in avoiding twin pregnancy [51]. Although there is evidence to suggest that there are lower fertility rates in cows inseminated with FTET using IVP embryos as opposed inseminated with in vivo embryos [52]. The number of farms using FTET via IVP embryos is increasing globally probably due to it becoming a more affordable procedure. It also allows for the selection of genetically superior dams. Proper handling and adequate training of personnel is paramount in this procedure and potentially influences the outcome of insemination.

Another technique proven to reduce the incidence of twinning is the follicular drainage of the subordinate follicle at FTAI, supported by a GnRH treatment 7 days post AI. Recent studies have introduced this idea of embryo reduction to decrease the occurrence of twinning on dairy farms. This involves transrectal ultrasound-guided aspiration of the allantois-amniotic fluid or manual rupture of the amnios at pregnancy diagnosis [5]. More research needs to go into the development of this strategy [53]. These procedures contain risks and a decision needs to be made as to whether the benefits exceed the risks.

Both these techniques are said to reduce pregnancy loss as well as minimise risk of twinning, although questions still arise around whether these benefits outweigh the extra labour and costs. Due to the ethical concerns, increased labour intensity and costs of the above methods other approaches may be more appealing to dairy farmers to adopt in an aim to reduce twinning rates. In addition, the invasive intervention of these procedures adds further stress to the dam. This may cause unpredictable alterations in ovulation or inhibit the estrus cycle. The introduction of increased P4 levels to manipulate ovarian function during the development of the preovulatory follicle in order to reduce double ovulation, is definitely a more economical and achievable method on everyday farms globally.

3. Materials and Methods

3.1 Aim of this study:

The aim of this study was to determine the frequency of twin calving using PRID delta intravaginal implant and to compare the incidence of twin pregnancies in a Hungarian dairy herd between dairy cows using the PRID sync protocol and those using Double OvSynch protocol. This study aims to enable farm owners and managers to make informed decisions on which protocol to implement based on the frequency of twin calving and the increased management and hormonal product costs.

3.2 Animals and procedures:

All data used was collected from *BoviSync* System used on the farms.

This study was conducted on three large commercial dairy farms in Western Hungary from January until December 2022. A total of 2623 freshly calved cows were recorded during this time. All farms (1,2,3) came under the same management and the total number of cows were as follows; farm 1 n=904, farm 2 n=1157, farm 3 n=612. Farm 1 and farm 2 contain both cows and heifers while no heifers are recorded on farm 3. All treatments and data were regularly recorded in the farm management software *BoviSync* system and regularly reviewed by reproductive advisors. Data is recorded both manually and electronically. Data of cows producing stillborn calves were not recorded in the online system, these include calves who are born dead (after day 260 of gestation) or die within 24 hours after birth. The reproductive protocol and pregnancy detection were identical on each farm. The animals are kept in large modern free stall barns and were fed a TMR diet that that met or exceeded the nutritional requirements for Holstein Friesian cows and heifers, TAD.

Figure 3: The table below displays the herd size and milk production data.

Farm	Total number of cows (including heifers)	Milk production (Av L/ day)	Milking system used
1	904	37.51	Manual
2	1157	37.5	Robotic
3	612	36.33	Manual

Reproduction protocol

Cows were treated with DOS starting at 47-53 days in milk (DIM) to receive fixed-time AI (FTAI) at 75-80 DIM. Pregnancy diagnosis was done with transrectal ultrasonography at 30 days post insemination (PI), and regardless of corpus luteum (CL) presence, PRID sync was used if the cow was not in calf. All gynaecological examinations and pregnancy diagnoses were performed by the same trained personnel. Parturition and AI dates, parturition number, breeding technique (AI or ET), milk production at oestrus, AI number, pregnancy, presence of twins and pregnancy loss were recorded for each animal. The protocol followed a calendar system beginning on the Friday of each week allowing each member of the farm team to easily follow each step and record it in the BoviSync system. See below for a summary of DOS and PRID sync used, (Figure 4) and (Figure 5).

Double OvSynch protocol:

The protocol begins on a Friday with the first GnRH (1) injection given IM, PGF2a (1) is given a week later followed by a second GnRH (2) injection 3 days later. The second OvSynch protocol begins one week later with GnRH (3) with 2 PGF2a (2 + 3) injections given the following week. As you can see from the figure below the second PGF2a injection is given on day 26 of the protocol, 24 hours after the previous. This ensures complete regression of the CL, decreasing the risk of codominant follicles which often lead to twins in multiparous cows due to overexposure to luteal pulses. Complete regression of the CL is also needed to ensure the odds of a normal and healthy pregnancy. Another GnRH injection is given the following evening with FTAI performed 16 hours later.

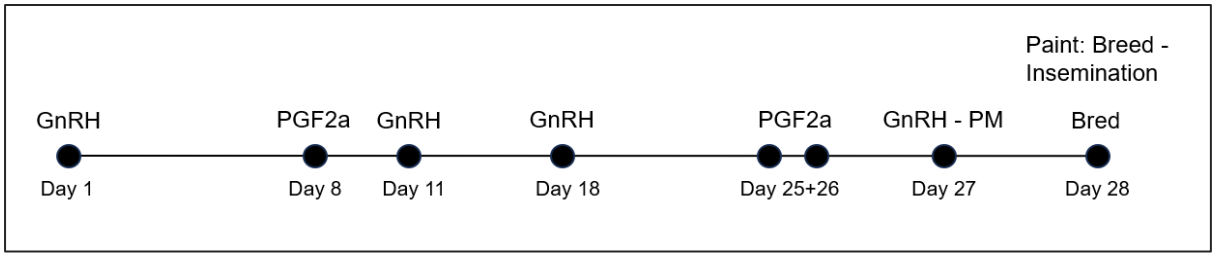

Figure 4: Represents the DOS protocol used on all farms as described above.

PRID sync protocol:

PRID delta is a commercially available intravaginal progesterone device produced by a company named CEVA. It has a unique triangular shape aimed to increase surface area and a P4 content of 1.55g. Administration of this single-use device was done using a clean applicator and a sterile glove by trained personnel. The use of intravaginal P4 devices like PRID delta have long since be incorporated into synchronised breeding programmes amongst dairy cows for decades due to their increase in reproductive efficiency and improved embryo quality.

Figure 5: Describes the PRID Sync protocol used in cycling and non-cycling cows on all farms. Cows found empty at pregnancy diagnosis (Day 30) received GnRH injection and PRID delta was inserted. The device was removed one week later and PGF2a injection given, followed by GnRH 56 hours later, TAI was performed 16 hours later.

Body Condition Score:

BCS was regularly monitored and managed by workers on each farm. The cows were scored on a body condition score of 1-5, 1=thin to 5 =fat. Cows achieving scores of 2.5–3.5 were in good condition [54] with sufficient energy resources to maximise ovulation and conception rates.

Artificial Insemination:

AI was performed using the AM/PM rule by the veterinarian on site.

Pregnancy diagnosis:

Transrectal ultrasonography was used to detect pregnancy by the on-farm veterinarian and recorded to allow cows left open to return resynchronisation protocols.

4. Results

Twinning data from BoviSync system:

The total number of twins over the three farms was n=51, excluding stillborn calves. The data collected includes details such as the sex of the calf and the culling date of cows. The distribution of twins amongst the three farms is represented on the below figure and is divided into those using DOS only and those who received PRID sync protocol.

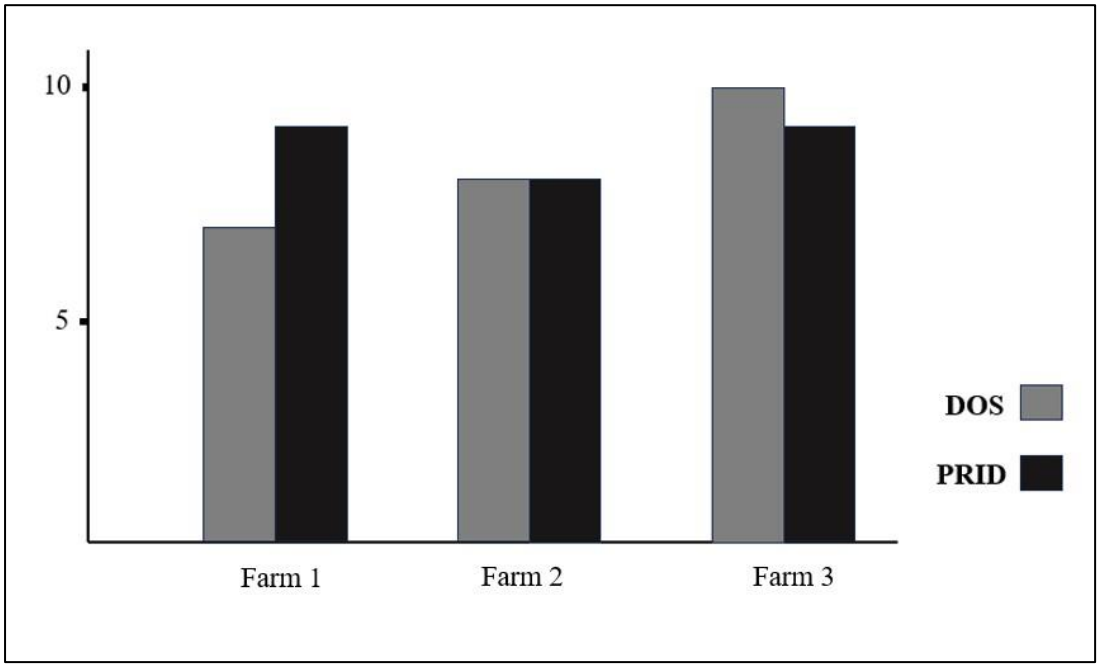

Figure 6: The above bar chart represents the distribution of twins on each farm depending on which protocol they received; DOS or PRID sync.

	Farm 1	Farm 2	Farm 3
Total number of twins	16	16	19
DOS	7	8	10
PRID sync	9	8	9

Figure 7: The table above shows the distribution of twins amongst the three farms, including the distribution of twins depending on which reproductive protocol was used: DOS or PRID sync.

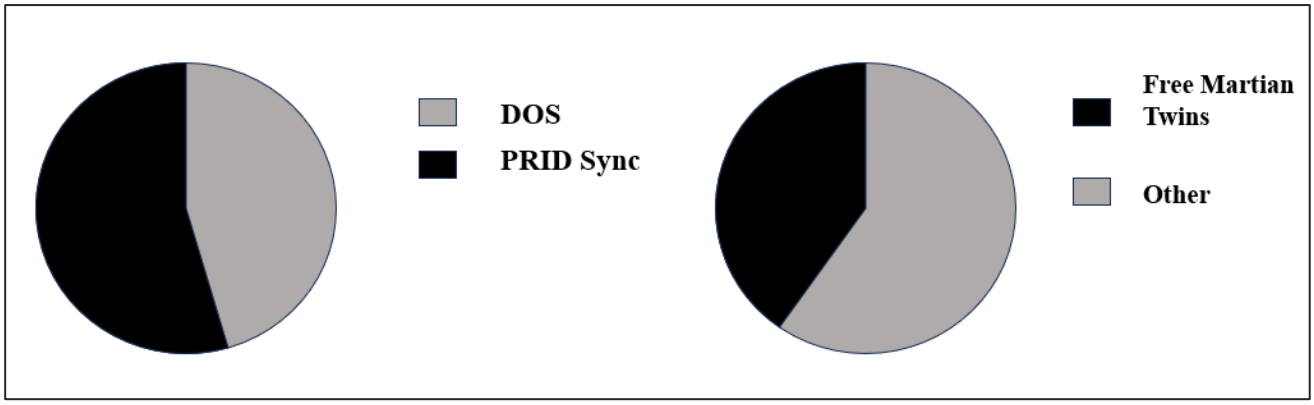

Figure 8: The first pie chart describes the distribution of twins over all 3 farms with DOS and PRID showing similar twinning rates, DOS = 25 and PRID = 26 respectively. The second pie chart represents the number of freemartin calves.

Increased likelihood of freemartin will have a negative economic impact and reproductive consequences on the herd. The total number of freemartin calves recorded was n=23.

Protocol	Number of Twins	% of total twins
DOS	25	49
PRID 1	9	17.64
PRID 2	13	25.49
PRID 3	3	5.88
PRID 4	1	1.96

Figure 9: The table above shows correlation of number of twins produced in relation to the reproductive protocol used.

Gender of Twins	Number of twins
FF	20
FM	23
MM	8

Figure 10: The table above shows the ratio of genders distributed amongst twins.

Twinning data recorded manually on farm 1:

In the next part of this study, we looked at the manual data recorded on farm 1. This was data that was recorded in a folder by the farm workers before it was reviewed by the supervising vet and management team. It included the date of calving, the tag number of the dam, the sex of the calf and if it was stillborn or alive. This was important as stillborn calves were not recorded in the BoviSync system and therefore an accurate picture of twin births could not be reached from that data. According to the manual data, of the 57 total twin calves born on farm 1, 19 of them were stillborn. There was an even distribution of heifer and bull calves with 17 sets of heifers, 14 sets of bull and 22 sets of mixed amongst the 57. Unfortunately, 4 sets were unaccounted for and were marked 'dead' by the workers.

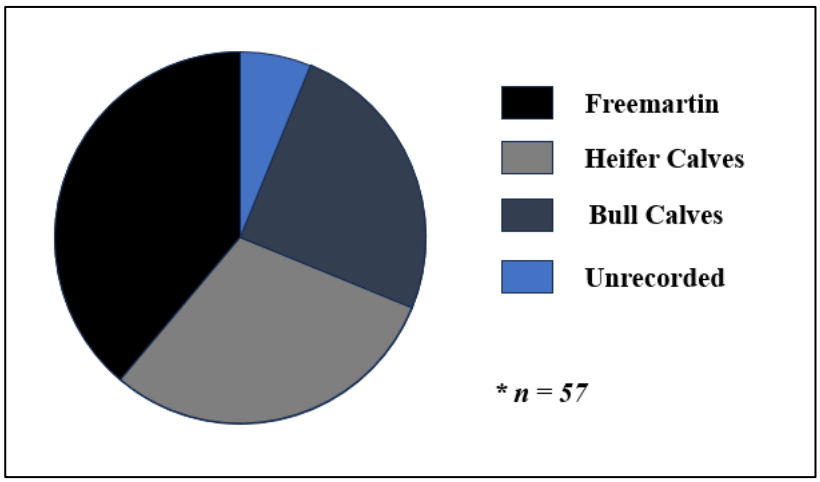

Figure 11: The pie chart represents the distribution of calves according to their genders on farm 1. It also shows the calves unaccounted for according to the manual data provided.

5. Discussion

In this study the researcher aimed to compare the twinning rates of dairy cattle using DOS and the PRID sync protocol. During this study all cows received a DOS protocol and those found open at pregnancy diagnosis (day 30) were entered into the PRID sync protocol. Based on the above results we can see that the rate of twin pregnancies did vary between the DOS and PRID protocols. The overall twinning rate for all three farms was 1.9% according to BoviSync data which is lower than twinning rates of 3 to 5% previously recorded in literature [11]. This suggests that the implementation of estrus synchronisation protocols such as DOS and PRID sync aids in minimising twinning rates amongst dairy cows.

Cows receiving their second round of PRID sync protocol calved the highest percentage of twins using PRID at 25.49% of the 51 twins produced (Figure 9). This is lower than the percentage of twins produced from the DOS protocol of 49% of the 51 twins produced. This supports the fact that increased circulating P4 levels around critical times in follicular development decreases the occurrence of double ovulations and therefore the number of twins. Other studies have been performed which support this. A study on the effects of PRID on the pregnancy rates in dairy cows in Mexico found that the addition of an exogenous P4 source reduced the incidence of twinning amongst multiparous cows [55].

Furthermore, a study completed in North-Eastern Spain explored the influence of different estrus synchronisation protocols on the outcome of twinning in dairy cattle. They concluded that the introduction of PRID device to an estrus synchronisation protocol did not increase the incidence of twinning[56]. The overall twinning rates were 17.9%, differing from the previous range of 15 to 37% of double ovulations amongst literature [57, 58]. They explored the theory that double ovulations very often does not manifest itself in the form of twin calves. This may be attributed to the increased likelihood of embryonic loss or the fact that double ovulations may also result in the conception of a single embryo. This study also considered other variables that may contribute to twinning including environmental factors.

As discussed earlier, insufficient P4 at critical points in the estrus cycle play a crucial role in increased twinning rates in high producing dairy cows. An obvious solution is the introduction of a simple intravaginal insert supplying a continuous exogenous source of P4. The result of this study's research supports this as decreased twinning was observed in PRID protocols.

Approximately half of cows conceived on the first DOS protocol with half being subjected to PRID sync protocol when found open at day 30 regardless of CL presence. 557 of the 1283 cows subjected to the PRID sync protocol conceived on the first round of PRID supporting evidence that P4 supports ovarian follicular development previously mentioned. Of these 9 twins were produced, a relatively low number and perhaps can be accounted to the parity of the dam. Some studies mentioned that using progesterone devices increase twin pregnancy rates amongst cows, the addition of an exogenous source of P4 has been around since the 1970s and functions to suppress LH pulses and delay onset of ovulation. The removal of a P4 device decreases circulating, stimulating follicular maturation and ovulation. As previously discusses elevated P4 levels around time of AI have been found to reduce fertility rates however the elevation of P4 before AI around the time of follicular development has been shown to reduce double ovulation and the occurrence of co-dominant follicles [18].

Sub-optimal levels of circulating P4 have been extensively related to such losses during the late embryonic/early fetal period and that P4 in the preimplantation period improves the viability of both co-twins. This same study indicated that P4 supplementation at the time of pregnancy confirmation perhaps increases the risk of twin pregnancy, perhaps due to increased survival rates, although further studies are needed to confirm this [6].

Farm 3 has the lowest average milk production per day of 36.33L, approximately 1.2L less than the other farms. Despite this farm 3 interestingly produced the highest number of twins, 19 out 51. This may be attributed to the fact that no heifers are housed on farm 3. Therefore, this supports conclusions that increased parity often leads to increased risks of twinning. A study conducted by Lopez et al in 2005 found that 50% of cows producing above 50 kg/day of milk production had double ovulation compared to less than 40% of cows producing less than 40kg/day [59]. This was solidified by previous studies concluding that milk production is the primary factor affecting the incidence of double ovulation in lactating dairy cows and may explain the effect of parity on twinning rate [18, 59, 60].

Many studies have been conducted to compare the fertility rates using different estrus synchronisation protocols. Supplementation of P4 during OvSynch protocol improved both the pregnancy rates and embryo survival rates of cows after TAI [18]. Similarly, progesterone supplementation given to cows with reduced fertility improves their pregnancy rates [61]. However, a study conducted in 2009 found that progesterone supplementation at

pregnancy diagnosis with the presence of two CL had a negative effect on pregnancy survival perhaps due to the mechanisms associated with P4 feedback [62].

Free Martin

According to the above results, the total number of freemartins was 23 out of the 51 twins produced, approximately 45.19% of twins produced. As mentioned in the literature review most twins produced are dizygous in nature meaning they arise from the fertilization of two separate oocytes. Literature shows the rate of FM to be up to 92% amongst these dizygous twins [41]. If the researchers results of this study are compared with that study, the rate of FM is considerably low amongst the dairy cows in the above study.

Furthermore, 14 of the FM were produced from the PRID sync protocol, higher than that of the DOS protocol, 60.9% and 39.1% respectively. Interestingly, P4 supplementation is previously recorded to have no effect on the gender outcome of the fetus[6]. This study suggests that there may be some correlation between hormonal supplementation and gender outcome. There is no previous evidence published to support this to my knowledge.

As previously mentioned, the phenomenon of FM has been well explored over the last century. More recent studies on FM in cattle focuses on methods of detection such as PCR quantification of the SRY gene[63]. Other studies focus on ways to reduce the number of FM in dairy herds by utilisation of embryo reduction methods [64]. Therefore, the result of the above study suggests future research should consider the impact of hormones on the gender outcome of reproductive protocols. This would further extend into the impact of hormones on the rate of FMs amongst cows.

In the above study, 20 out of the 51 twin calves born were twin heifer calves. Based on literature one can assume these calves will have lower growth rates, impaired immune function and consequent reduced fertility [32]. Therefore, despite the fact these heifer calves are technically born ‘fertile’, it is unlikely they will be reintroduced to the herd for breeding purposes. As a result, these calves will be sold to the beef industry and replacement heifers bought in. The occurrence of FMs in a dairy herd reduces the number of replacements heifers and compromises the future fertility of the herd. This also limits the genetic integrity of the herd and causes economic losses. This supports previously stated conclusions that the incidence of twinning places an economic burden on dairy farms.

Economics

The economic welfare of dairy herds relies heavily on effective reproductive protocols. Increased hormonal costs and cows unresponsive to estrus protocols provides unnecessary added expenses. Furthermore, increased culling rates due to increased incidences of metabolic disorders and subsequent poor future reproductive performance place negative impacts on farm profits. The increased need of obstetrical assistance and veterinary intervention in twin calving only further contributes to economic losses.

In the above study, on farm 1 the total cost of DOS and PRID fertilisation protocols was 2.660.620 HUF and 4.802.364 HUF respectively. The total cost of hormonal treatments on all three farms was 16.970.156 HUF in total, supporting the fact that reproduction is a serious investment on all dairy farms.

In the above study, of the total number of cows who calved twins (n=51), 24 cows were culled. This was a 47% culling rate which supports previous evidence that increased twinning rates leads to increased culling rates. These are significantly higher compared to the culling rate of those who calved singles 27.8% and the overall culling rate of the herd 28.7%. These culling rates are due to decreased future reproductive performance and increased incidence of reproductive disorders as described earlier.

Similarly, increased embryonic loss due to incidences of twinning is economically undesirable. Return rates to estrus are lowered and increased hormonal supplementation is required. According to the manual data recorded on farm 1, 19 out of the 57 twin calves born were stillborn. This supports previous evidence that there is an increased risk of embryonic loss with twin calving [4]. Increased risks of perinatal mortality also decrease the number of replacement heifers eligible in the herd.

6. Conclusion

Further investigations into the physiological and genetic background of twins have allowed us to make informed decisions into the use of reproductive protocols on large dairy farms. From the research in this study, the researcher can conclude that the addition of an exogenous source of P4 in the form of PRID delta implant minimises the number of twins produced compared to those receiving DOS protocol. This has been attributed to sufficient levels of circulating P4 at stages of critical follicular development. This study also supports the claim that incidences of twinning are increased in multiparous cows. Furthermore, this study suggests that hormonal intervention may influence the gender outcomes of reproductive protocols. However, more research should be done to support this. Therefore, it can be concluded that the prevention of twinning using more affordable and accessible methods will improve the economic well-being of our dairy herds.

7. Summary

In this study, the rate of twin pregnancies amongst 2623 freshly calved cows on a Holstein Friesian dairy farm in Western Hungary was surveyed. Cows on all 3 farms were subjected to a DOS protocol, those found open at pregnancy diagnosis received PRID sync protocol. The overall twinning rate on these farms was 1.9% according to BoviSync data which is lower than twinning rates of 3 to 5% previously recorded in literature [11]. This suggests that the implementation of estrus synchronisation protocols such as DOS and PRID sync aids in minimising twinning rates amongst dairy cows. This study also compared the rate of twinning using DOS versus PRID sync protocol. The results showed higher rates of twinning amongst cows receiving DOS when compared to PRID sync protocol round 2, 49% and 25.49% respectively. This supports previous evidence that addition of P4 to a FTAI protocol at critical stages in ovarian follicular minimises the rate of twinning [55]. Furthermore, 45.19% of the twin calves produced in the above study were FM, this is significantly lower than the 92% previously recorded in literature[41]. This study suggests further investigation into the possibility of hormonal influence on the gender outcome of calves. The occurrence of FM as well as the increased culling rates of 47% amongst those cows who calved twins in the above study, places an increased loss on farm profits. In conclusion, this study suggests the addition of P4 supplement to a reproductive protocol like PRID sync is an affordable and appealing solution to minimising twinning amongst dairy cows. This would be a step in the right direction to ending the negative impact that twinning has on the fertility of our dairy industry.

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Thesis progress report for veterinary students

Name of student: Rachel Kathleen Carter

Neptun code of the student: CEX6E3

Name and title of the supervisor: Dr. Attila Dobos

Department: Department of Veterinary Forensics and Economics

Thesis title: The Occurrence of Twin Pregnancies After Using an Intravaginal Progesterone Device in a Hungarian Dairy Herd.

Consultation – 1st semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2023	02	21	Processing data collected	
2.	2023	03	15	Processing data collected	
3.	2023	04	26	Processing data collected	
4.	2023	05	22	Writing Methods and Materials of thesis	
5.	2023	06	26	Writing Results of thesis	

Grade achieved at the end of the first semester: jó (4)

Consultation – 2nd semester

Timing				Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2023	09	01	Writing the Introduction of the thesis	
2.	2023	09	15	Writing the Literature Review of the thesis	
3.	2023	10	02	Writing the Discussion of the thesis, conclusion of results	
4.	2023	10	13	Writing and finalising the Summary of the thesis	
5.	2023	11	03	Finalisation of the thesis	

Grade achieved at the end of the second semester: elégséges (2)

The thesis meets the requirements of the Study and Examination Rules of the University and the Guide to Thesis Writing.

I accept the thesis and found suitable to defence,

.....
signature of the supervisor

Rachel Carter

Signature of the student:

Signature of the secretary of the department: Krisztina Bodor

Date of handing the thesis in 17. November 2023.