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The use of artificial insemination, embryo transfer, and intracytoplasmic sperm injection in sport horse reproduction

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Abstract

The sport horse industry relies on advanced reproductive techniques to enhance breeding efficiency and genetic progress. This retrospective study delves into the use of Assisted Reproductive Techniques (ART) in sport horse breeding, focusing on Artificial Insemination (AI), Embryo Transfer (ET), and Intracytoplasmic Sperm Injection (ICSI). By extensively reviewing relevant literature and incorporating data obtained from Boleybawn Horse Farm (BHF), Co. Wicklow, Ireland, this paper aims to assess the methods and influencing factors associated with these ART methods, evaluate their efficacy, and compare our findings with existing research.

The comprehensive literature review revealed the diverse approaches and methodologies used in AI, ET, and ICSI within the sport horse industry. This paper discusses the influence of various factors, such as reproductive management practices, and timing of procedures, on the success of these techniques.

Incorporating data from BHF, this paper assesses the real-world effectiveness of ART in sport horse reproduction employed in a small sport horse breeding operation in Ireland. This involved a critical examination of success rates, pregnancy outcomes, and the overall utility of these techniques from both quantitative and qualitative data obtained by the author. By comparing our empirical findings with existing literature, the author aims to provide a comprehensive overview of the efficient use of ART in the sport horse industry in Ireland as it relates to the operations at Boleybawn Horse Farm.

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Abbreviations

AI	Artificial insemination
ART	Assisted reproductive technique
AV	Artificial vagina
BHF	Boleybawn Horse Farm
DSF	Dominant Stimulated Follicle
ET	Embryo transfer
ICSI	Intracytoplasmic sperm injection
IVF	In vitro fertilisation
IVP	In vitro produced
OPU	Ovum pick-up

1. Introduction

Assisted Reproductive Techniques (ART) have become more popular in equine sport horse reproduction in recent years, including Artificial Insemination (AI), Embryo Transfer (ET) and Intracytoplasmic Sperm Injection (ICSI). The sport horse breeding industry in Ireland is not as developed as other countries due to the high production of thoroughbred foals for the racing industry. According to *Weatherbys Group*, Irish thoroughbred foals that have been conceived only by natural cover can be registered to the "General Stud Book" for eligibility to race[1]. As a result, ART are not applicable to the racing industry, thereby leading to the underdevelopment of ART in Ireland. However, in recent years sports horse breeding, where the restriction of natural cover is not a factor, has become increasing popular and accordingly the use and application of ART in Ireland has begun to develop and evolve.

1.1. Artificial Insemination

AI can be used for both the sole production of foals in breeding mares or as part of the embryo transfer procedure minimizing the danger of natural mating and decreasing the chances of spread of sexually transmitted diseases.

AI is a valuable tool used in the equine reproduction industry as it allows breeding facilities to put AI programmes in place to optimise the conception rates while controlling and improving the genetic characteristics of the offspring. It was remarked that AI could have conception rates equivalent if not superior to those of natural service [1]. The superiority of AI over natural mating was further supported when a study confirmed that the number of registered foals for AI was 4.3% higher than that of natural mating in Celle State stud, Germany [2].

Furthermore, AI can overcome any geographical limitations accompanying natural cover. Methods used to preserve the semen collected during AI programmes allow for the transport of semen to different breeding facilities worldwide [3].

[1] Sourced from https://www.weatherbys.co.uk/Weatherbys/media/PDFs/gsb-conditions.pdf, accessed 4th of November 2023.

AI can also allow for a more structured breeding regime to be employed as it can allow operators to use semen more appropriately by inseminating the mare as close to ovulation as possible resulting in higher pregnancy rates [3] and requires lower insemination volumes therefore reducing the required load of the stallion [1, 4]. The mares in these breeding programmes can be synchronised using pharmaceuticals such as Prostaglandins and hCG ensuring they ovulate for insemination [1].

1.2. Embryo Transfer

ET has transformed the sport horse industry by offering a multitude of invaluable applications. Beyond its role in enhancing genetic diversity by enabling the propagation of superior bloodlines, ET allows competition mares to continue their careers while producing offspring therefore avoiding the age-associated decline in fertility [5]. ET also addresses the challenges faced by older mares with excellent bloodlines who may struggle to carry a pregnancy to term [6]. These benefits have reshaped the landscape of equine breeding, offering breeders and enthusiasts exciting opportunities to optimize their breeding programs.

Equine ET was first reported in 1970 but has increased steadily in recent years [7]. The reason for this increase is due to changes in the American Quarter Horse Association (AQHA) in 2002 where they removed the ban on the number of foals that can be produced by a mare in one breeding season [6]. Further developments on the success of the non-surgical transfer of embryos have also aided in the increased popularity of ET [8].

1.3. Intracytoplasmic Sperm Injection

A newer development in the assisted reproductive techniques known as Intracytoplasmic Sperm Injection (ICSI) has become increasingly common in sport horse reproduction.

ICSI is used in combination with Ovum Pick-Up (OPU) to produce quality embryos in vitro. The use of OPU-ICSI programmes has become increasingly popular in recent years [9]. The increased interest in ICSI stems from the fact that ICSI overcomes the need for superovulation which, as will be discussed later is not applicable in the equine industry [10]. Furthermore, traditional In Vitro Fertilisation (IVF) is not successful in the equine industry due to the morphology of the equine embryo as well as the failure of capacitation of the equine spermatozoa and therefore no penetration to the oocyte in vitro [11, 12]. ICSI involves the injection of only one spermatozoa into the oocyte to promote the production of blastocysts [13].

In the past, ICSI was originally adopted to facilitate reproduction in sub-fertile mares or older mares with the inability to conceive or to maintain pregnancy to term [9]. Also, ICSI can be used to produce offspring from mares that have reproductive tract issues such as oviduct fibrosis or uterine infections [9]. As well as this, ICSI can utilize semen of poor quality therefore allowing the use of superior stallions with desirable genetics that are said to be sub fertile [13]. ICSI may also utilise semen of low post-thawing motility following cryopreservation as the sperm is injected [9]. This is particularly important for maximising the breeding potential of stallions with less-than-ideal sperm quality.

Recently however, ICSI has been used in fertile mares as a management strategy as it offers greater flexibility and convenience compared to traditional assisted reproductive techniques. Oocytes can be collected and transported to a laboratory, which can be beneficial in various scenarios, such as when the mare and stallion are in different locations, avoiding the need for them to be physically present for mating, as well as avoiding the need to adequately line up recipient mare who may not be available [14, 15].

Furthermore, oocytes can be recovered from mares in which an unexpected death occurs as oocytes can be excised from ovaries post-mortem. This can be especially important in the case of high-value mares with exceptional genetics. It allows for the use of these genetics in offspring despite the death of the mare [16].

2. Objectives

There is little doubt that these assisted reproductive techniques have transformed equine reproductive technology, making it possible to overcome various fertility issues and streamline the breeding process, even in cases where logistical challenges or unexpected events arise. It has become an invaluable tool for preserving and enhancing equine genetics and breeding programs.

The beneficial effects of the assisted reproductive techniques are clearly outlined above however there are several influencing factors that operators should consider when optimising the use of ART's in the equine breeding industry.

This paper will review the use of assisted reproductive techniques (AI, ET, ICSI), including the factors influencing each of the techniques, an analysis of the methods used to perform the technique and the outcome of the ART used including embryo recovery rates, pregnancy rates, early embryonic loss, and foaling rates as they relate to the small equine breeding operation in Ireland.

Given the analysis of the BHF data, and the suggested importance of ET at BHF, (see Fig 1 below), the author felt it more appropriate to analyse the use of AI on the farm as it relates to ET, as AI is the first step in the ET procedure. Additionally, it is clear from the data that BHF put more emphasis on the production of foals from ET, than AI alone. As a result, and while the author will review the literature on AI and ICSI as it relates to the BHF data, much of the authors subsequent discussion will focus on the ET protocol and its efficacy.

Considering this and using the knowledge the author gained from the literature review, the author will perform a retrospective analysis of both quantitative and qualitative data obtained from BHF in support of, or the contrary to, information obtained during the literature review.

3. Literature Review

3.1. Artificial Insemination

3.1.1. Artificial Insemination Procedure

Before insemination, thorough preparation of the mare's reproductive tract is essential. The mare's tail is wrapped, and a thorough cleaning of the vulva and perineal area is completed to minimise the risk of contamination during the procedure. A plastic sleeve is placed over the operator's hand and the hand is lubricated with a sterile non spermicidal lubricant. The sterile catheter or insemination pipette is gently inserted through the mare's cervix guided by the operator's index finger and into the uterine lumen. The extended semen is slowly deposited into the uterus and a small amount of air can be pumped through to ensure all the semen has been expressed from the insemination pipette [3, 17, 18].

While the technique outlined above is a common method used to inseminate mares, according to [18] there has been another method adopted for the use of deep intrauterine insemination in cases when sperm viability is low or of poor quality. With this technique, the deposition of the semen is guided by an ultrasound transrectally to the ipsilateral uterine horn to the dominant follicle. According to [19] pleasing results were obtained from this insemination technique when low-quality semen with lower spermatozoa quantity was used.

3.1.2. Influencing Factors

The AI outcome can be influenced by several factors. To achieve a successful result in the AI programmes one must consider a complex interplay of factors that must be precisely managed. A comprehensive understanding of these interrelated factors is crucial for achieving reliable AI results in the equine breeding industry.

3.1.2.1. Management of Mare

The timing of AI in mares is a critical aspect of equine reproduction, and it requires a deep understanding of the mare's reproductive cycle. Mares have a unique and relatively short window of fertility, typically occurring during the estrus phase, commonly known as "heat." Accurately pinpointing this window is essential for the success of AI [20, 21, 22].

3.1.2.1.1. Behavioural Changes

Observation of behavioural changes is one of the primary methods used to detect estrus, commonly exhibited upon teasing the mare in the presence of a stallion. During oestrus, mares typically exhibit increased receptivity to the stallion, and a tendency to stand still when mounted by other horses. Additionally, they may frequently urinate and raise their tail, exposing the perineal area. Physical signs such as the presence of a clear, watery vaginal discharge and softening and relaxation of the cervix can also indicate oestrus [17, 18].

3.1.2.1.2. Ultrasonographic Monitoring

Another aspect used for pinpointing this window for successful outcome of AI is transrectal ultrasonographic monitoring which tracks the development of ovarian follicles represented as fluid-filled sacs containing the mare's eggs (oocytes). During oestrus, one or more dominant follicles will grow on the ovaries. These follicles can be observed measured and monitored using ultrasound [18]. Ultrasonography also allows for the assessment of uterine changes during estrus. The uterine lining may become develop a characteristic appearance known as "uterine oedema." As the dominant follicle nears its maximum size, close monitoring is essential. Ovulation typically occurs within 24-48 hours after the follicle reaches its peak size. The precise timing can vary among mares, so continuous monitoring is crucial [18, 23].

3.1.2.1.3. Hormonal Manipulation

Manipulating the ovulation of mares in AI programme is a common practice in equine reproduction to synchronise the mare's reproductive cycle with the desired breeding schedule. Some of the more common agents used in practice are prostaglandins, GnRH analogues, and hCG [24]. Prostaglandins are commonly used to induce luteolysis and initiate a new follicular wave, which can help synchronise ovulation in mares. This allows breeders to plan insemination at a specific time. GnRH analogues like deslorelin are administered to trigger ovulation. hCG similar in function to LH and can be used to induce ovulation in mares. It is often used when a mare has a mature follicle (>30mm) but has not ovulated naturally. [25].

3.1.2.1.4. Timing of AI in relation to predicted ovulation.

A number of studies [20, 21, 22] have been performed on the optimal timing of AI in relation to predicted ovulation. The studies conducted compared whether pre-ovulatory insemination, insemination at the exact time of ovulation or post-ovulatory insemination is the best for producing viable pregnancies.

In the first study [20], the author examined the timing of AI in mares and its impact on pregnancy and embryo loss rates. Pregnancy rates were significantly lower when mares were inseminated 36-24 hours before ovulation compared to those inseminated closer to ovulation. Mares inseminated within 24 hours before ovulation had high pregnancy rates (60%), followed by those inseminated 0-8 hours (66.7%) and 8-16 hours (70.1%) post-ovulation. However, it was discovered that the issue with post-ovulatory insemination was the increased incidence of early embryonic loss. Early embryonic loss was highest when mares were inseminated 24-32 hours after ovulation. Although acceptable pregnancy rates for mares inseminated 16-24 hours post-ovulation were obtained, early embryonic loss increased markedly compared to inseminations before 16 hours post-ovulation. In conclusion, the study highlights that the timing of AI in mares in the 16-hour window around ovulation (0-16 hours before or after) resulted in the highest pregnancy rates with acceptable embryo loss rates [20].

Secondly, [21] showed that insemination closer to ovulation, within a time frame of up to 3 days before ovulation yielded higher pregnancy rates and lower rates of embryonic loss. The study also supported the theory that the incidence of embryonic loss is greater in post-ovulatory insemination. Additionally, this research included insemination on the day of ovulation which surprisingly yielded lower pregnancy rates than 3-day pre-ovulatory inseminations.

The findings of the third study [22] are consistent with those of the previous two, both suggesting that post-ovulatory inseminations come with a higher risk of early embryonic loss and unsuccessful fertilisation therefore lower pregnancy rates. According to this study, the optimal window for insemination was 18 hours before or after ovulation, while keeping in mind higher embryonic loss rates with inseminations performed post ovulation [22].

Finally, another study [26] was performed on the influence of the insemination regime on pregnancy rates in AI programmes. This study found that the number of inseminations per oestrus, the timing of insemination in relation to ovulation, and the type of semen used all played significant roles in determining the pregnancy rates in mares, providing valuable insights for optimising equine breeding practices. It has been previously described that normal practice in breeding studs includes the insemination of mares showing estrus signs every 48 hours until estrus signs are no longer detected [3, 26]. However depending on the facilities available, when regular veterinary monitoring is provided, a solitary insemination per estrus yields pregnancy rates equivalent to multiple inseminations, provided it falls within the window of 24 hours before and 12 hours after AI for cooled semen, or 12 hours before and 12 hours after for frozen-thawed semen [26].

Therefore, by carefully timing AI to coincide with the mare's peak fertility, breeders can increase the likelihood of conception, resulting a more efficient breeding programs. Proper timing maximises the chances of success and helps reduce the cost and resources required for each breeding attempt, making it a pivotal factor in equine reproductive management.

3.1.2.2. Management of Semen

Two factors in relation to the semen must be considered for the AI to result in an embryo: the quality of the semen and the handling of this semen by the operator. This includes the practice of semen preservation and getting the right balance of insemination dose and volume.

3.1.2.2.1. Semen handling & Quality analysis

Spermatozoa are extremely sensitive to the environment predominantly light and temperature. Stallion semen when exposed to inadequate temperatures can undergo cold shock which results in damage and consequently death of the spermatozoa [27]. To prevent this, all equipment that is in contact with the semen must be heated to 37-39 degrees Celsius [6]. All materials used during the collection, preservation and quality analysis of the semen should be washed cleaned and dried with non-residual non-toxic soaps [28].

After collection, the semen is examined to assess its quality. There are many conflicting results on the link between seminal characteristics such as motility and morphology in the stallion fertility [29]. In a study [30] it was proven that semen with a large amount of major morphological defects was correlated with a decline in stallion fertility. An important aspect leading to these conflicting results is the inability to reproduce the same seminal characteristic from the same stallion in a single ejaculate [29, 31]. However it is widely accepted that the most important characteristics in terms of fertility are both motility and morphology of the spermatozoa as it was concluded these two parameters are greatly compatible with fertility [32].

3.1.2.2.2. Semen Preservation

Handling semen after collection is a critical part of the equine breeding process to ensure its viability and quality for AI. Semen is extremely sensitive to environmental conditions and therefore careful handling must be ensured [27].

Semen processing after collection entails numerous steps including diluting and adding extenders, centrifugation, cooling, and storage of the semen. The spermatozoa in particular the sperm plasma membrane may be damaged by these processes highlighting the need for extenders to protect the spermatozoa and ensure the longevity of the semen [33].

As indicated by the literature cryopreservation of semen in stallions is very challenging. Comparing cryopreservation in the bovine industry where the adaptability of the semen to the freezing technique is a priority, the equine industry focuses on the desirable characteristics of the stallion (performance, conformation, and breeding) and therefore even stallions with poor quality semen are sought after for cryopreservation [34]. This can be a possible explanation for the discovery that between 20-50% of stallions used in the AI programmes for cryopreservation have semen that is unacceptable for the AI [35]. Furthermore, stallion semen is less robust than bull semen and results in wide discrepancies in the ability to withstand the stresses of cryopreservation [34].

Consequently, the reduced fertility of semen samples post-thawing is a major factor that discourages people from opting for frozen semen [36]. As a consequence of the reduced fertility of frozen semen, the pregnancy rates obtained when using cryopreserved semen for AI are undesirably low [3].

3.1.2.2.3. Insemination Dose & Volume

Overall, the key to achieving successful pregnancy outcomes in equine AI is to strike the right balance between insemination dose and volume, tailoring them to the individual mare and the quality of the stallion's semen.

Insemination dose is an influencing factor because it directly affects the number of sperm available for fertilization. In the past, the acceptable insemination dose used in AI was 500 $x10^6$ progressively motile sperm (PMS) however in recent years due to improvements in semen extenders and better management data presented in the study indicates that the dose may be effectively used at 100 x 10^6 PMS in fertile stallions. However 100 x 10^6 PMS is the threshold level and below this is not recommended [37]. In line with the previous study, the authors of the next study also concluded that the insemination dose can be reduced from 500 x 10 PMS to 300 x 10 PMS without interfering with resulting pregnancies. The results from this study proved that pregnancy rates were not lower when a lower insemination dose was used [38].

Insemination volume refers to the amount of fluid (usually semen extender) used to deliver the sperm into the mare's reproductive tract. The volume helps ensure the even distribution of sperm, aiding in their journey toward the site of fertilisation. Typically between 10 and 25ml of semen is used in the AI programmes [3]. Evidently, the sperm number is of greater importance in fertilisation and the resulting pregnancy than the volume itself. This was proven in a study performed where large insemination volumes with low concentrations of spermatozoa yielded lower rates of embryos in comparison to a smaller insemination dose with higher sperm concentrations [4].

3.2. Embryo Transfer

3.2.1. Embryo Recovery

Non-surgical embryo recovery is a less invasive method used to retrieve equine embryos compared to traditional surgical procedures. It involves flushing the embryo from the donor mare's uterus using specialised equipment and a flushing medium [39].

The donor mare's reproductive cycle is closely monitored to pinpoint the optimal time for embryo recovery, typically around 7-8 days after ovulation when the embryo has reached the uterus [39]. A veterinarian inserts a specialised catheter through the mare's cervix and into the uterus. The catheter is connected to a collection system usually Y-shaped with an inflow and outflow line. A flushing medium is prepared, which is a sterile solution containing essential nutrients and electrolytes to support the embryo's viability during recovery [39]. According to [40] Dulbecco's phosphate-buffered saline, accompanied by fetal bovine serum as a protein source, is typically used as a flushing medium. Around 1-2L of the flushing medium is gently introduced into the uterus through the catheter. This medium flows through the uterine horns, surrounding and suspending the embryo within the fluid. A gentle pressure is applied by massaging per rectum to encourage the flushing medium to flow through the uterine horns and tubes, carrying the embryo along with it. The flushing procedure is repeated 3 or 4 times to ensure the optimal chance of retrieving the embryo [40]. Further developments in the technique resulted in the medium being left to sit for 3 minutes when flushed into the uterus, the idea being that even if the medium does not contact the embryo, the mobility of the embryo will result in it moving into the medium [41]. The embryo is collected into a filter or mesh at the end of the catheter and is then further assessed [39, 40].

3.2.2. Embryo identification and assessment

Once the flushing medium suspected to contain the embryo is drained from the uterus, the operator must ensure around 50ml is kept in the embryo filter and is transferred to a dish to be analysed under the microscope [40]. Magnification under the stereomicroscope will allow for the quality of the embryo to be determined. The quality of each embryo can be ranked on a scale of 1 to 4 deeming the embryo excellent to degenerate respectively [42]. Additionally, with the microscopic examination, the developmental stage of the embryo can be decided ranging from morula to expanded blastocyst depending on the day of recovery [40]. The importance of examining the morphology of the embryo is highlighted in [43] where the morphology was indicative of the prognosis of ET resulting in a viable pregnancy. Embryos with abnormalities resulted in a higher incidence of embryonic death [43].

3.2.3. Transfer procedure

Upon detection of the embryo in the medium, the embryo is transferred to a holding medium and subject to a series of washes to rinse the flushing medium. The embryo is loaded into the straw ensuring that on either side of the embryo in the fluid there are air spaces, the fluid pockets act as a lubricant and ensure the embryo is washed out of the transfer pipette [40].

Both surgical (involving incisions either in the ventral midline or flank approach) and non-surgical methods of ET are available [44]. In recent years the popularity of the non-surgical method has increased as it is cheaper, less invasive and excludes the need for general anaesthesia [40]. According to [45], transcervical ET is the preferred method by most operators performing this procedure.

Transcervical ET is performed with a "cassou" type pipette which is fixed with an outer chemise to protect the pipette and consequently the embryo from bacterial contamination of the vulva and vagina. The pipette is advanced gently through the vulva vagina and the cervix into the uterus lumen. Transrectal palpation to guide the pipette ensuring it is deposited close to the uterine horn is often used to assist [40, 45].

3.2.4. Influencing Factors

3.2.4.1. Management of the Donor

The age of the donor mare plays a significant role in determining both the quality and recovery rate of embryos. Generally, younger mares tend to have a higher likelihood of producing better-quality embryos with a greater potential for successful implantation and development. This is attributed to the fact that reproductive efficiency decreases with the increasing age of the mare [46]. In conjunction with the donor age, [5] proved that mares aged between 3 and 10 years had higher embryo recovery rates than mares aged 13 to 25 years old. In addition to this, it was shown that older mares had a longer interovulatory interval than younger mares meaning fewer embryos would be produced per cycle in older mares [5].

Furthermore, when embryo recovery rates were compared in mares grouped according to age, a decreasing trend in embryo recovery rate was observed with increasing age [47]. Aside from the embryo recovery rate, the embryo quality concerning donor age also showed that older mares tended to produce embryos of decreased quality in terms of size and development [48]. While older mares can still produce viable embryos, their recovery rates may be lower, and the embryos themselves may have a higher likelihood of abnormalities.

Although one of the major advantages of ET is the ability to breed from mares that are still competing, it has been recently hypothesised that donor mares that are in consistent work and competing during the breeding season produce embryos in lower amounts [49]. Heat accumulation during exercise can damage the ovarian follicle and lead to the production of degenerate oocytes and therefore failure to conceive or produce an embryo [49]. Recently it has been recommended that proper management of the mare intended to donate embryos is crucial. A break from competing and strenuous exercise around the periovulatory period can be necessary to optimise the embryo recovery [8].

As outlined in Section 3.1.2 above the influencing factors of AI play an important role in the recovery of embryos from the donor mare. One of the biggest influencing factors is the type of semen used. It was detected that frozen semen produced extremely low recovery rates [48].

Finally, an aspect of importance when managing the donor mare in an ET programme is the timing of the uterine flush to obtain the embryo in relation to the predicted ovulation. The timing is important as the embryo has been proven to enter the uterus from day 6 after ovulation, knowing the time of ovulation will allow us to flush the uterus after day 6 as it will not be present before [50]. According to [40] it is common practice to flush the uterus on day 7 or 8 post ovulation. However, it has also been shown that embryos flushed on days 8, 9 or 10 after ovulation result in lower pregnancy rates because of their larger size and therefore they are susceptible to technical damage that results in a lower pregnancy rate [43, 51]. Additionally, knowing the age of the embryo post-ovulation can be used effectively to synchronise with the recipient [52].

3.2.4.2. Embryo Transfer Technique

Upon review of the literature, there has been several studies providing evidence that the ET technique used, has a great impact on the pregnancy rate and therefore the success of the implantation [53–56]. As mentioned above, both surgical and non-surgical methods can be used for ET however a common trend noted is that non-surgical ET methods have lower pregnancy rates [53, 55]. Reported reasons for the lower pregnancy rates with non-surgical ET include the influence of the operator's experience, where it was found that operators with less experience result in lower pregnancy rates [53, 56]. In support of this, it was concluded that non-surgical ET in Argentina had high pregnancy rates due to the high level of experience the operators had. Argentina is one of the leading countries in performing ET in polo ponies and therefore results in a high level of experience [57].

Furthermore, the manipulation of the cervix in the transcervical ET, where the pipette is passed into the uterine lumen results in the production of prostaglandins, oxytocin and other inflammatory mediators compromising the uterine environment and therefore leads to an unsuccessful implantation [40]. With the compromised uterine environment, it was also proven that non-surgical ET was at a higher risk of bacterial contamination [40]. For these reasons, a new technique was invented to effectively increase the pregnancy rate of transcervical ET, it included the use of “Polanskey Speculum” and “Wilsher Equine Transfer Forceps”. The idea is that the sterile speculum can be used for more accurate visualisation and deceased contamination. The forceps are used to grasp the cervix gently and pull to straighten the cervical canal for easier insertion of the pipette decreasing the manipulation [58].

In more recent years, two studies [54, 56] were performed to assess if this new technique truly yields higher pregnancy rates. In both studies, the authors compared the conventional transcervical technique with the use of the Wilsher forceps. In the first study, it was concluded that the use of the Wilsher forceps resulted in over 90% pregnancy rates and also proved that the operator experience did not influence the outcome with the use of the forceps adding another advantage [56]. In support of this, the second study [54] confirmed that higher pregnancy rates were obtained using the forceps.

3.2.4.3. Management of Recipient mare

Managing the recipient mare in equine ET is important for the success of the procedure. These mares play a critical role in nurturing and supporting the developing embryo until birth. Proper recipient mare management ensures a conducive uterine environment and optimal conditions for embryo implantation and subsequent pregnancy. Key aspects include selecting recipients of appropriate age with suitable uterine characteristics, timing the ET accurately, and monitoring the mare's health throughout gestation [40].

Recipient mare's general health is as important as the reproductive health. Data has been analysed and proven show that the maternal environment can impact the foal's body shape, immune system, energy balance, bone and joint health, and various functions like the thyroid and adrenal glands [59].

Screening of the reproductive health of the recipient mare is important as pregnancy rates can be affected in case of inadequate uterine health or abnormal pathologies that can be detected on the transrectal ultrasound [60]. The detection of intrauterine fluid after ovulation has been proven to affect the successful implantation and results in lower pregnancy rates [61]. Recipient mares between the ages of 3-12 years of age are more desired due to the negative correlation between age and reproductive efficiency [40, 43]. Older recipient mares were proven to have a higher incidence of endometrial inflammation compromising the uterine environment, leading to decreased pregnancy rates and an increase in early embryonic loss [62].

The third component when screening recipient mares is the size in comparison to the donor or predicted "genetic size" of the embryo. Although sometimes underrated as an important factor it has been convincingly shown that disparities in the "genetic size" of the embryo and the recipient mare have significant implications for various aspects of both intrauterine and post-natal development. When the maternal size is not suitable, it can result in either fetal undergrowth or overgrowth, depending on the direction of the size difference [63].

Ultimately a very important aspect of the management of the recipient mare is the synchronisation of the oestrus cycle, more precisely the ovulation of the recipient, ensuring the uterine environment is ready for the implant of an embryo. The variation of oestrus length between mares can make it difficult to synchronize donor and recipient accordingly [40]

In the past, the window for embryo implantation in a recipient was confined to 1 day before ovulation up to 3 days after ovulation about the donor mare's ovulation [64]. According to a recent study, this window can be extended up to 5 days after ovulation of the donor mare and consequently have acceptable pregnancy rates [65]. The demonstrated issue with extending the window further from the ovulation of the donor is the decline in embryo survival further into the pregnancy and the increase in embryonic loss [66].

To synchronise the recipient mares in this time window there are two options. The large-scale breeding farms use transrectal ultrasound every day to track the stages of the estrus through examination of the ovaries and uterus, while others use pharmacological substances to manipulate the cycle [67]. Prostaglandins, progestogens, estradiol, combined with hCG or GnRH analogues are often used in the protocol for synchronization [68].

3.2.5. Outcomes

3.2.5.1. Embryo Recovery Rates

After a review of the literature, it was evident that the embryo recovery rate for donor mares is characterised by considerable variability. Several studies have shown different ranges for embryo recovery some as low as 30% per cycle and ranging up to 80% embryo per cycle [6, 40, 41, 69]. In relation to the uterine flushes and resulting embryos achieved it was proved an embryo recovery percentage between 55 and 80% can be achieved [41, 69]. This fluctuation in embryo recovery rate varies significantly due to a wide range of factors related to the donor mare as discussed in section 3.2.4.1.

3.2.5.2. Pregnancy Rates, Early embryonic Loss & Foaling Rates

As well as embryo recovery rates, pregnancy rates following the transfer of the embryo to the recipient also vary over a wide range. A pregnancy rate ranging between 27%-71% can be seen in a study performed to assess the impact of embryo age and the synchrony of the recipient use in the ET procedure [65].

After the ET in the recipient mare, pregnancy checks are typically performed at intervals to monitor the progression of the pregnancy and confirm its viability. According to the literature, different intervals for transrectal ultrasound pregnancy checks can be used ranging between 7– 14 days after the ET in the mare [43, 48, 52, 70]. The second pregnancy check occurs around 35-60 days after the ET. This check serves several purposes, including confirming the continued viability of the pregnancy and assessing fetal development. It is also an opportunity to detect any early embryonic loss or other pregnancy complications [43, 52, 70].

Early embryonic loss occurs when a developing embryo fails to establish or maintain pregnancy in the mare's uterus during the early stages of gestation. A trend in the studies performed assessing the early embryonic loss showed that between the first and second pregnancy check after ET a pregnancy loss between 9% to 15.5% [43, 48, 52].

Although foaling rates are typically used to assess the fertility and reproductive efficacy of the mare, the literature is scarce on the comparison between pregnancy rates and foaling rates after ET except for one useful study [52]. In this study, it was proven that a pregnancy loss of 7% occurred between the second pregnancy check and the predicted foaling [52].

3.2.6. Limitations

3.2.6.1. Superovulation

Most mares participating in ET programmes are single-ovulating mares. Multiple ovulation has been shown to increase the embryo recovery rate resulting in more embryos implanted and therefore more successful pregnancy rates [69]. Numerous studies have explored various pharmacological agents with the aim of inducing superovulation, but these endeavours have encountered recurring challenges and shortcomings [71]. More research is needed to produce an agent capable of stimulating superovulation in mares while upholding the standards for embryo quality and viability [10].

3.2.6.2. Cryopreservation

Despite its potential to enhance breeding and genetic preservation efforts in the equine industry, cryopreservation of equine embryos is less advanced than that of other domestic animals. This can be attributed to a combination of factors, including the unique biological characteristics of equine embryos, the challenges posed by their large size and structure, and a relative shortage of research and development efforts dedicated to refining equine-specific cryopreservation techniques [40].

It is widely reported that successful pregnancy rates can be obtained from cryopreserved embryos of smaller size (<300 micrometres) usually collected from the donor mare 6 days after ovulation.[72–75]. The issue with using embryos that must be collected on day 6 after ovulation is, as stated above the embryo enters the uterus at about 6 days to 6.5 days after ovulation [50]. Meticulous observations and prediction of ovulation must be performed to ensure the embryo is collected at day 6, embryo in utero grow extremely quickly and if a day 7 or 8 embryo is obtained the successful results of cryopreservation decrease dramatically [76]. A study [77], proved that expanded blastocysts resulted in much lower pregnancy rates after cryopreservation than early blastocysts therefore highlighting the importance of obtaining embryos early after ovulation.

Larger embryos (day 7 or day 8 after ovulation) of >300 micrometres are less viable post-thawing and result in lower pregnancy rates according to the literature [73, 74, 75].

Many breeders still prefer to use fresh embryos, when possible, to maximize their chances of success in equine ET procedures despite the ongoing efforts to improve the success rates in the context of cryopreserved equine ET [40].

3.3. Intracytoplasmic Sperm Injection

3.3.1. Procedure

In vitro production of embryos in mares is a complex and specialised process involving several steps, including obtaining the oocyte through the procedure known as ovum pick up (OPU), maturation of the oocyte in culture to ensure it is equipped for spermatozoa, the injection of single spermatozoa through intracytoplasmic sperm injection and finally the transfer of this in vitro produced embryo into the recipient mare [78].

3.3.1.1. Ovum Pick-Up

Ovum pick-up is the first step in vitro embryo production and involves the collection of oocytes (eggs) from the mare's ovaries. Before OPU, the mare is typically subjected to hormonal treatments such as eFSH to stimulate multiple follicles to develop. This enhances the chances of collecting a greater number of oocytes in a single OPU session [79].

There are two methods for collecting ova in mares. The traditional approach involves a flank incision, which, while highly invasive, yields a high oocyte recovery rate. However, in recent times, there have been advancements in transvaginal ovum retrieval, a procedure guided by ultrasound to aspirate follicles through scraping and flushing techniques. Follicular fluid containing oocytes is aspirated, and the oocytes are collected. The transvaginal method has demonstrated its effectiveness, with a recovery rate ranging from 5 to 12 oocytes per OPU session, which naturally varies based on the number of follicles present [13].

There are two categories of oocytes obtainable through OPU. The initial category is the pre-ovulatory follicle or dominant stimulated follicle, which is stimulated by hormonal treatment, as previously discussed. The second category comprises immature follicles that can be collected at any point in the reproductive cycle. Advancements in the aspiration of these immature follicles have opened up significant opportunities for better mare scheduling and enhanced potential for generating embryos [15].

3.3.1.2. Oocyte In Vitro Maturation

In vitro maturation (IVM) of the aspirated oocytes is crucial in the ICSI procedure. It involves culturing the oocytes in a medium to encourage nuclear maturation to metaphase 2, which is essential for fertilisation. In the case of a well-performed IVM nuclear maturation rates of 60-80% are to be expected [13]. Information on the types of media used in IVM will be discussed in more detail later in Section 3.3.2.2.

3.3.1.3. Intracytoplasmic sperm injection

Following in vitro maturation, only oocytes that have successfully reached the MII stage are selected for the ICSI procedure. These oocytes are characterised by the presence of a visible polar body, indicating their readiness for fertilisation [13]. ICSI outcomes remained variable and inconsistent until the development of the piezo drill technique [80]. This technique involves the injection of the spermatozoa into the oocyte disrupting both, the oocyte membrane and the spermatozoa plasma membrane therefore avoiding the need for capacitation [78]. Following the injection of spermatozoa, the embryo is subject to culture prior to embryo transfer. The blastocyst can develop any time between 7 and 10 days of culture. As soon as signs of the blastocyst development are evident the embryo should be implanted by nonsurgical embryo transfer into the recipient mare [78].

3.3.2. Influencing Factors

As with the previous assisted reproductive techniques used in the equine industry, OPU and ICSI programmes are susceptible to a range of influencing factors associated with the procedures. The final outcomes such as oocyte maturation rate, blastocyst development, pregnancy success and the birth of a viable foal are greatly impacted by these factors as discussed below.

3.3.2.1. Management of Donor Mare

While a rise in maternal age is commonly linked to a decline in reproductive capability in various assisted reproductive techniques as discussed above in Section 3.2.4.1, that does not hold true in the OPU- ICSI programmes [9, 81].

Previously, it was concluded that advancement of maternal age has been associated with decline in oocyte morphology particularly in relation to the zona pellucida and perivitelline space. The decline in oocyte quality can lead to a decreased developmental ability [79]. However, in contrast to this, a more recent study [9] performed proved that although increase in maternal age resulted in fewer oocyte collected during the OPU session, it did not result in decreased blastocyst rate from the aspirated oocytes. Furthermore, in support of the latter it was shown that blastocyst maturation rate as well as developmental capability of the in vitro produced embryo was not affected by the age of the donor mare. Therefore it can be concluded that maternal age does not significantly decrease the likelihood of blastocyst development and therefore pregnancy success [81].

Although superovulation is not successful in mares, it was hypothesised that the use of hormonal treatments can increase the number of follicles produced and obtained and therefore increase the chances of blastocyst development and finally embryos implanted [79]. In this study it was shown that the use of eFSH treatment resulted in the recovery of more follicles and oocytes from the donor mare.

3.3.2.2. Oocyte Management

As discussed above two types of oocytes can be obtained from the mare through OPU for maturation. The blastocyst maturation rate and storage of the two types of oocytes greatly influences the pregnancy outcome for ICSI [15, 82].

Dominant Stimulated Follicle (DSF) that are acquired after GnRH analogue administration often result in high recovery rates of up to 85%. Moreover, DSF are believed to undergo the natural selection process, and therefore lead to a notably higher blastocyst rate. [15]. Although unfavourably, when DSF is selected for OPU, it often results in only or 2 oocytes being recovered as there is no superovulation in mare but each of these oocytes have a higher developmental capacity [82].

Immature follicles undergo OPU and due to increased number of oocytes allow for greater potential for embryo production. However immature follicles result in lower blastocyst rate of around 30% as not all the follicles mature in vitro [15]. Nevertheless, it was concluded that there is a higher incidence of producing blastocyst from multiple small follicles in comparison to pre ovulatory follicle per OPU session [82].

The storage conditions between both DSF and immature follicles is where the major influential characteristics are seen. After OPU is performed of the dominant stimulated follicle it is of extreme importance that this oocyte is stored in a maturation medium of 37 degrees. If this is not performed blastocyst rates are extremely low [15]. The development of OPU from immature follicles revolutionised the ICSI programme as it was discovered that these obtained oocytes can be stored at room temperature overnight without any detrimental effects to the blastocyst maturation rate [15].

As maturation of the oocyte to a suitable level for fertilisation is vital in ICSI, the maturation medium is important. Many of the maturation medium are based upon the M199 medium [13]. Within the medium the most important component is glucose as it has been stated that mature oocytes have a high metabolic rate especially glycolytic activity which can be related to the embryonic development [83]. Other constituents including protein sources such as fetal bovine serum and hormones such as LH and FSH can be used to optimise reproductive conditions in the culture medium [13]. In a study [84], comparing two media used for oocyte maturation it was confirmed that TCM-199 had higher maturation rate of oocytes at 36hrs compared to that of Ham's F-10, however upon further investigation it was proven that at 48hrs of culture the maturation rates were even. The composition of the maturation medium is still undergoing investigations to optimize the ICSI outcome.

While emphasis is placed upon the maturation of the oocyte nucleus to metaphase 2, the development of the oocyte cytoplasm synchronously is also vital for blastocyst formation and can obscure oocyte quality and embryo development [13, 83].

3.3.2.3. Embryo Management

The timing of embryo transfer of the blastocyst following ICSI can be influential of the pregnancy rates and success of the in vitro produced embryo. Interestingly transfer of the ICSI embryo on day 4 after ovulation of the recipient mare resulted in the highest pregnancy outcomes in comparison to day 5 or day 6 after ovulation. Furthermore the embryonic vesicle when ET performed day 4 after ovulation was bigger and more developed [81]. In support of this, it was confirmed that day 4 after ovulation in the recipient mare resulted in higher pregnancy rates than day 5 or 6 [85].

3.3.3. Pregnancy rates & early embryonic loss

Pregnancy success regarding OPU- ICSI programmes can range between 60-80%, though in recent years the pregnancy success has been steady at 70% [13, 81]. Early embryonic loss of around 20% has been associated with ICSI embryos before 60 days gestation [13]. In support of this, it was stated that a 15% loss of in vitro produced embryos will be lost between the ET and foaling [81].

3.3.4. Foaling rates & viability

The final successful outcome of the ICSI programme is the birth of a live foal. Foaling rates from OPU – ICSI – ET programmes are high at approximately 60% [81, 86].

Upon review of the most recent literature a significant majority of ICSI foals have displayed good health and normal clinical conditions. Moreover, when examining the characteristics of the placenta and foal size, there are minimal differences between ICSI foals and those conceived naturally. Consequently, as of now, there is no evidence suggesting that in vitro produced embryos pose a risk to the well-being or survival of the foals after birth [81, 86]. Nevertheless, it is crucial to underscore that conducting more extended studies to confirm that there are no long-term effects that could potentially affect their health or performance in the future [81, 86].

While viability and appearances of the foals are in line with the natural mating, the literature has indicated that in vitro produced embryos result in an unbalanced sex ratio of the live foals [81]. One of the contributing factors to this skewed sex ratio was the rate of in vitro embryo development. Specifically, it was observed that transferring day 7 IVP blastocysts had a higher likelihood of resulting in colts while in contrast, transferring day 8 IVP blastocyst yielded a more balanced outcome. This suggests that, according to our system, male equine embryos tend to develop more rapidly in vitro compared to their female counterpart [81].

4. Materials and Methods

4.1. Materials

In this retrospective study, quantitative and qualitative data was obtained from Boleybawn Horse Farm (BHF) over the course of the previous three breeding seasons, spanning February to late August in the years 2021, 2022, and 2023.

The data used in this research provides an overview of reproductive activities within the farm during these specified seasons, including the most recent records of assisted reproductive techniques employed.

4.2. Methods

The methods employed in this retrospective study was a simple analysis and deduction of relevant calculations from the quantitative data provided in spreadsheet form, supported by the authors assessment and interpretation on the qualitative data obtained from two in-person interviews with the proprietor of Boleybawn Horse Farm. This qualitative data was collated, based on both the proprietor's subjective opinion on how things work on BHF and from diaries and journals that he has kept regarding his ART operations and productivity.

Using the materials provided and by applying the methods described the author will assess the efficacy and impact of both ET and ICSI on pregnancy rates and foaling rates among the equine population at BHF. By comparing the recorded data with existing literature and industry standards, this study aims to shed light on the success and challenges associated with the application of assisted reproductive techniques in equine breeding practices for this small family run business.

5. Data & Results

From the outset the author would like to acknowledge that with respect to the quantitative data obtained the sample size is not as large as she would have liked. This is because BHF is a small family run business and has only recently started to record and document data as related to its use and application of AI, ET and ICSI techniques.

In this regard the author is working with three years' worth of data relating to 2023, 2022 and 2021, providing an overall sample size of 61 pregnancies, yielding 47 foals, a foaling success rate of 77%. The dataset serves as the basis for the comparative analysis of pregnancy rates and foaling rates with the existing literature, thereby contributing valuable insights into the application and outcomes of assisted reproductive techniques at BHF.

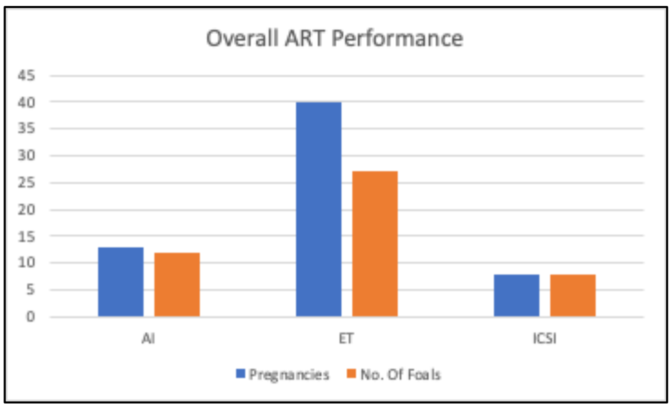
5.1. Three Year ART Performance

It can be seen from the table and graphs below that in the context of the use an application of ART on BHF, ET seems to be a very important protocol representing 66% of all pregnancies and 57% of all foals born.

Overall 3 Years								
Type	No.		ART	Pregnancies	No. Of Foals	Success Rate	% of Pregnancies	% of Foalings
Pregnant Mares	61		AI	13	12	92%	21%	26%
Foalings	47		ET	40	27	68%	66%	57%
Success Rate	77%		ICSI	8	8	100%	13%	17%

Table 1 Summarised data over the three years

Overall, the number of foals born to ET is 27, compared to 12 from AI and 8 from ICSI.



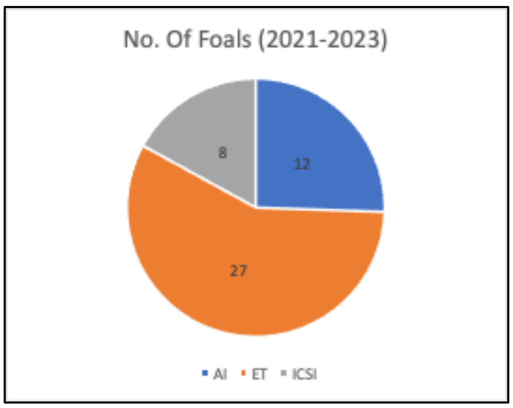


Figure 1 Performance by ART on BHF over three years

However, notwithstanding the numbers of pregnancies yielded by each ART, it is worth noting that from the data provided, ET has a lower overall success rate of 68% when compared to ICSI at 100% and AI at 92%. For comparative reasons, the author has defined success rate as being the number of foals born from the number of pregnancies conceived.

Given the suggested importance of ET at BHF much of the authors subsequent discussion will focus on the ET protocol and its efficacy.

5.1.1. Additional ET Data

The table below summarises some of the additional ET data that the author gleaned from both the quantitative and qualitative data, relevant to her further discussion at Section 6 below.

Additional ET Data	
Total Flushes	85
Total Embryos	63
Total Pregnancies	40
Total Foals	27
Total Embryonic Loss	11
ERR	74%
Pregnancy Rate	63%
Foaling Rate	43%

Table 2 Additional ET related data extracted for the three years

This allowed the author to calculate Embryo Recovery Rates (ERR), Pregnancy Rates and Foaling Rates for the overall three-year period, comparing these rates to those suggested by the literature.

5.2. Annual Breakdown of Data

Similarly, the following three sections breakdown and present the data for each of the three years, 2023, 2022 and 2021.

5.2.1. 2023 Data

2023 Data								
Type	**No.**		**ART**	**Pregnancies**	**No. Of Foals**	**Success Rate**	**% of Pregnancies**	**% of Foalings**
Pregnant Mares	22		AI	5	5	100%	23%	26%
Foalings	19		ET	10	7	70%	45%	37%
Success Rate	86%		ICSI	7	7	100%	32%	37%

Table 3 Summarised data for 2023

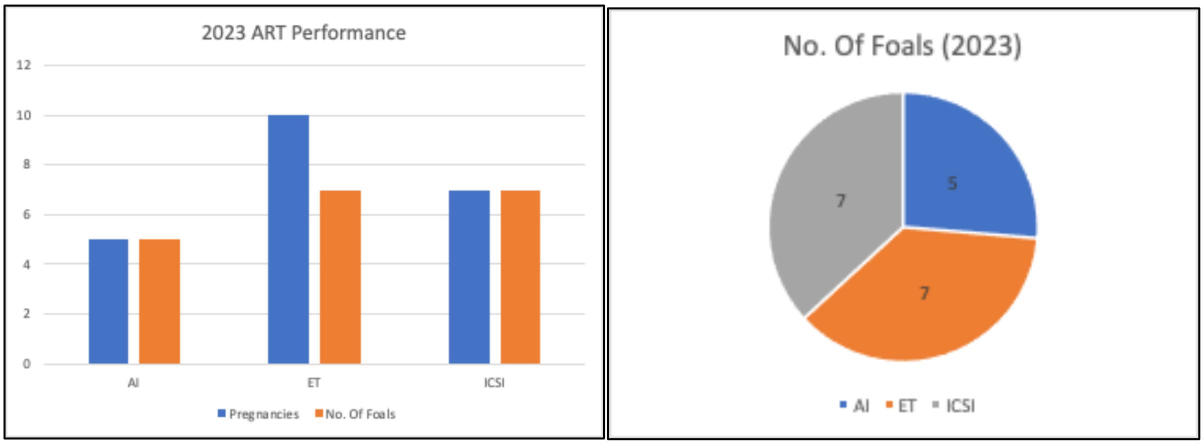


Figure 2 ART performance on BHF for 2023

5.2.1.1. *Additional ET Data & Embryonic Ratio's (2023)*

Additional ET Data	
Flushes	21
Embryo's	16
Pregnancies	10
Foalings	7
Embryonic Loss	3
1st Stage Loss (9.5 days)	2
2nd Stage Loss (28 Days)	1

Embryonic Ratios	
ERR	76%
Pregnancy Rate	63%
Foaling Rate	44%
ETR	63%
1st Stage Loss (9.5 days)	50%
2nd Stage Loss (28 Days)	44%
Embryos Lost	38%

Table 4 Additional ET data and associated ratios for 2023

Please note that 1st Stage Loss is a loss that occurred between the 1st and 2nd scans, taken at 9.5 days. 2nd Stage Loss is a loss that occurred between the 2nd scan taken at 28 days after the ET and foaling.

5.2.1.2. *Additional ICSI Data (2023)*

Additional ICSI Data	
Embryos	13
Pregnant Mares	7
Foals	7
Sex (M)	4
Sex (F)	3
Pregnancy Rate	54%

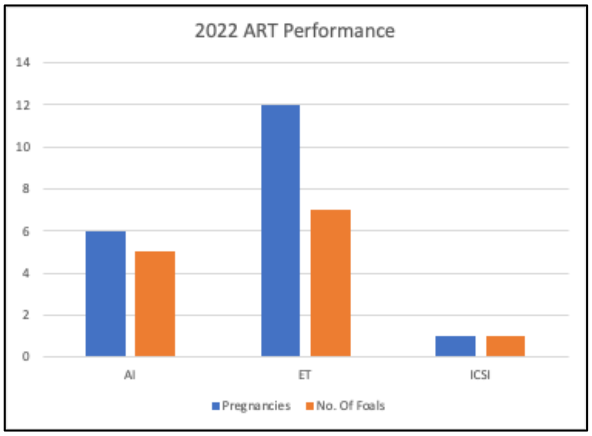
Table 5 Additional ICSI data for 2023

5.2.2. 2022 Data

Notably, 2022 was the first year that BHF introduced ICSI as part of the reproductive techniques.

2022 Data								
Type	**No.**		**ART**	**Pregnancies**	**No. Of Foals**	**Success Rate**	**% of Pregnancies**	**% of Foalings**
Pregnant Mares	19		AI	6	5	83%	32%	38%
Foalings	13		ET	12	7	58%	63%	54%
Success Rate	68%		ICSI	1	1	100%	5%	8%

Table 6 Summarised data for 2022



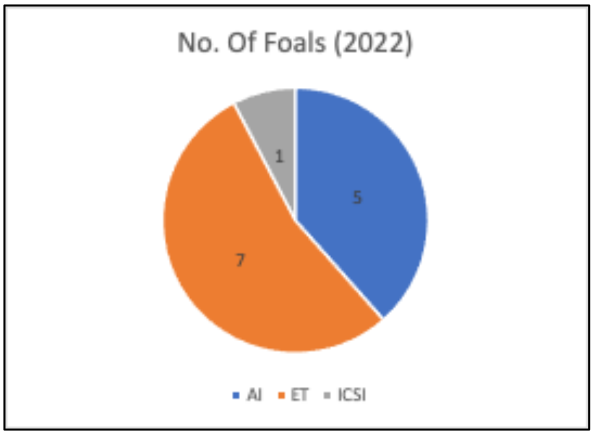


Figure 3 ART Performance for 2022

5.2.2.1. Additional ET Data & Embryonic Ratio's (2022)

Additional ET Data	
Flushes	27
Embryo's	20
Pregnancies	12
Foalings	7
Embryonic Loss	5
1st Stage Loss (9.5 days)	n/a
2nd Stage Loss (28 Days)	n/a

Embryonic Ratios	
ERR	74%
Pregnancy Rate	60%
Foaling Rate	35%
ETR	60%
1st Stage Loss (9.5 days)	n/a
2nd Stage Loss (28 Days)	n/a
Embryos Lost	40%

Table 7 Additional ET data and associated ratios for 2022

It is also worth noting that embryonic loss that year was severely impacted by an aspergillus infection within the bedding material at 30 times the tolerable level causing several abortions. As a result, the author has not used embryonic loss in this year as a measure of reproductive efficacy.

5.2.2.2. Additional ICSI Data (2022)

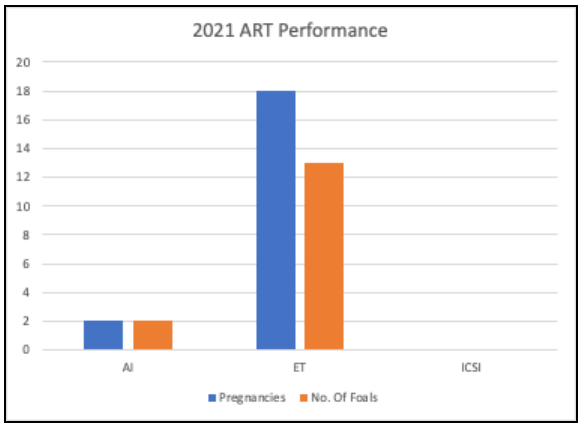
Additional ICSI Data	
Embryos	2
Pregnant Mares	1
Foals	1
Sex (M)	0
Sex (F)	1
Pregnancy Rate	50%

Table 8 Additional ICSI data for 2022

5.2.3. 2021 Data

2021 Data								
Type	**No.**		**ART**	**Pregnancies**	**No. Of Foals**	**Success Rate**	**% of Pregnancies**	**% of Foalings**
Pregnant Mares	20		AI	2	2	100%	10%	13%
Foalings	15		ET	18	13	72%	90%	87%
Success Rate	75%		ICSI	0	0			

Table 9 Summarised data for 2021



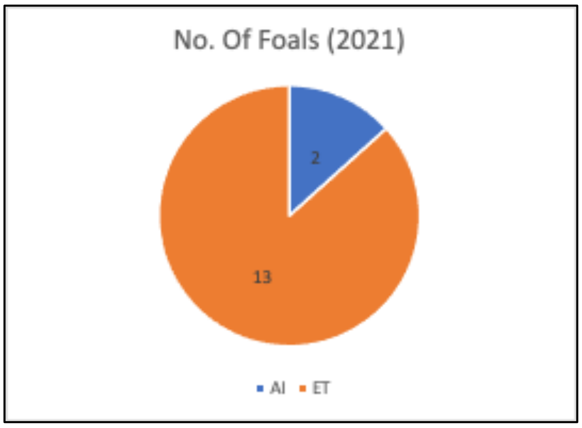


Figure 4 ART Performance data for 2021

5.2.3.1. Additional ET Data & Embryonic Ratio's (2021)

Additional ET Data	
Flushes	37
Embryo's	27
Pregnancies	18
Foalings	13
Embryonic Loss	3
1st Stage Loss (9.5 days)	2
2nd Stage Loss (28 Days)	1

Embryonic Ratios	
ERR	73%
Pregnancy Rate	67%
Foaling Rate	48%
ETR	67%
1st Stage Loss (9.5 days)	59%
2nd Stage Loss (28 Days)	56%
Embryos Lost	33%

Table 10 Additional ET data and associated ratios for 2021

Please note that the raw data recorded 5 losses in this year. However, two of those five losses accounted for stillborn foals. In the authors opinion stillborn foals do not account as an influential factor directly on the ART protocols on the farm. As a result, the author has excluded these two stillborn foals from the embryonic loss count thereby using a number of 3 embryonic losses as a measure of the ET protocol efficacy.

5.3. Quality of Foals – BHF Grading System

Foals born are classified according to a grading system on the farm that is based on the subjective assessment of the weight of the new-born foal. Foals are subsequently classified as Grade 1, being small, Grade 2, being medium and Grade 3, being large.

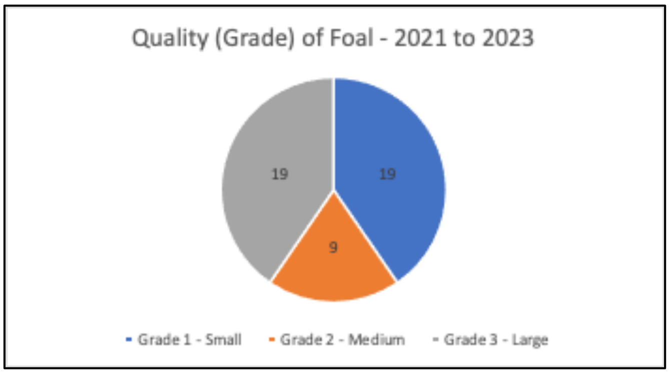


Figure 5 Summary of the grades of foal achieved at BHF

This subjective assessment is made against a normal baseline-weight of around 50Kg to 70Kg, which the farms grading system classifies as Grade 2. Therefore, it is most desirable for new-born foals to be Grade 2 or Grade 3.

	Quality of Foals - Grading Classification			
	Total Foalings	47		
	Grade 1 - Small	19	40%	
Desirable Quality	Grade 2 - Medium	9	20%	60%
	Grade 3 - Large	19	40%	

Table 11 Number of foals by grade

From the overall 47 foaling’s, 40% were classifies as Grade 1, 20% as Grade 2 and 40% were classified as Grade 3. This in turn means that 60% of foals born through ART were considered of desirable quality.

5.3.1. Breakdown of Foal Quality by ART

The table and graph below show the number and quality of foal produced by each of AI, ET and ICSI respectively.

	AI	ET	ICSI
Grade 1	0	14	5
Grade 2	5	4	0
Grade 3	7	9	3
	12	27	8

Table 12 Number of foals born by quality and by ART

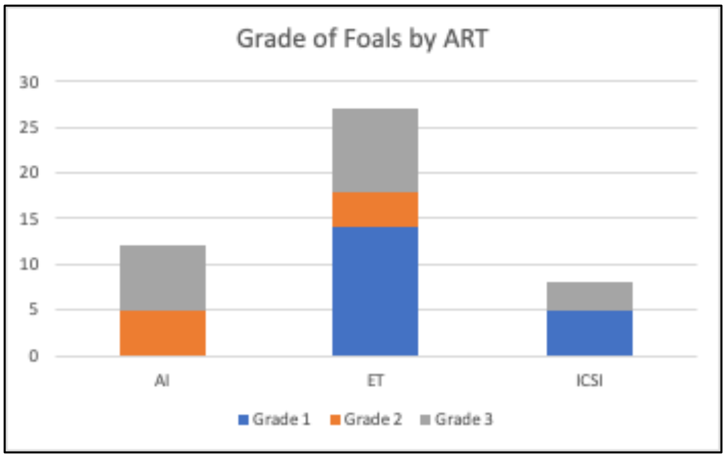


Figure 6 Grade of foal broken down by ART

5.3.2. Breakdown of Foal Quality by ET

In the case of the 27 ET foals born to BHF, 52% were classified as small (Grade 1), 15% as medium (Grade 2) and 33% as large (Grade 3). Overall, 48% of ET foals were considered of desirable quality.

ET (27 Foals)			
Grade 1	52%	Small	
Grade 2	15%	Medium	48%
Grade 3	33%	Large	

Table 13 Breakdown of foal quality by ET

5.3.3. Breakdown of Foal Quality by AI

In the case of the 12 AI foals born to BHF, 0% were classified as small (Grade 1), 42% as medium (Grade 2) and 58% as large (Grade 3). Overall, 100% of ET foals were considered of desirable quality.

AI (12 Foals)			
Grade 1	0%	Small	
Grade 2	42%	Medium	100%
Grade 3	58%	Large	

Table 14 Breakdown of foal quality by AI

Breakdown of Foal Quality by ICSI

In the case of the 8 ICSI foals born to BHF, 62% were classified as small (Grade 1), 0% as medium (Grade 2) and 38% as large (Grade 3). Overall, 38% of ICSI foals were considered of desirable quality.

ICSI (8 Foals)			
Grade 1	62%	Small	
Grade 2	0%	Medium	38%
Grade 3	38%	Large	

Table 15 Breakdown of foal quality by ICSI

6. Discussion

Given the limited data available, the author will discuss only those aspects of AI, ET and ICSI, as related to the literature review and for which evidence is found in either of the quantitative and qualitative data.

Furthermore, given the analysis of the BHF data, and the suggested importance of ET at BHF, (see Fig 1), the author felt it more appropriate to discuss the use of AI on the farm as it relates to ET, as AI is the first step in the ET procedure. Additionally, it is clear from the data that BHF put more emphasis on the production of foals from ET, than AI alone. As a result, and while the author will discuss the use of AI and ICSI as it relates to the BHF data, much of the subsequent discussion will centre around on the ET protocol and its efficacy.

Therefore, the author will discuss the following aspects of AI, ET and ICSI, by way of insights and observations as to the ART operations of Boleybawn Horse Farm:

- Assisted Reproductive Techniques and Protocols
- Embryo Recovery Rates
- Pregnancy Rates and Embryo Transfer Rates
- Embryonic Loss and Foaling Rates
- ICSI

6.1. ART Protocols

6.1.1. AI Protocol

The author has observed little variation between the AI procedure used in Boleybawn in comparison to the literature outlining the conventional AI method using the insemination pipette [17, 18].

Within the farm, AI is performed using semen imported primarily from Holland, Belgium, and Germany. This semen is preserved by chilled methods. The farm abstains from using frozen semen as the reliability of post thaw fertility is so low [34–36]. It is also stated by the proprietor that frozen semen requires more scanning for ovulation, every 4hrs as opposed to chilled semen when ovulation checks are performed every morning.

The insemination dose and volume are factors controlled by the importing studs; however, the farm uses a standard policy with insemination dose of 500 x 10^6 PMS (Progressively motile sperm) per dose and most often the semen is in 10-20ml aligned with the literature [3, 37]. While the semen is subject to quality analysis prior to dispatch, upon arrival at Boleybawn it is assessed under microscope to assess the progressively motile sperm as motility is an important parameter of sperm in relation to fertility [32].

The mare is closely monitored every 24hrs from the onset of oestrus. In line with the literature the donor mares are subject to teasing and transrectal ultrasound to accurately monitor the estrus cycle and allow to accurately pinpoint the ovulation window [20–22]. On the farm the protocol for AI is as follows; the donor mare is given Prostaglandin analogue; the mare is then monitored using transrectally ultrasound every morning before 8am to check for the oedema and follicle of 35mm or greater. Upon detection of these the mare is subject to hCG analogue which is expected to ovulate within 48hrs. The use of these pharmaceuticals for manipulating the ovulation in the mares is in support of the literature [24]. The mare is then inseminated with chilled semen pre- ovulation within this 48 window evidently yielding good pregnancy rates and embryo recovery as stated in the literature [20, 21].

6.1.2. ET Protocol

Donor mares are used from around 4 yrs to 15yrs with exceptions based on the ability in producing offspring. Donor mares over 15years are usually less productive and often require more flushing attempts to achieve an embryo [5, 47]. Donor mare on the farm are kept in continuous work and no changes are made to the training programmes periovulatory despite contradictory results in the literature [49].

The author observed that the embryo recovery process on the farm is aligned with that from the literature review and performed by a specialist veterinarian (one of very few in Ireland) [39–41]. AI of the donor mare follows the same protocol as above. Flushing donor mares occurs at 7½ days after ovulation, followed by implantation into a synchronised recipient mare which is common practice as outlined by the literature [40]. The flushing of the donor mare for frozen embryos is performed between 6½ and 7 days after ovulation however is not common practice on the farm due to cryopreservation limitations [76].

It is noteworthy that a large herd of about 100 recipient mares on the farm span a wide age range, from 2-year-olds to 14-year-olds. It was reported by the proprietor that recipients mares greater than 14 years tend to have higher incidences of reproductive issues such as uterine system and inflammation which according to the literature is believed to be true [62]. The operational procedure at Boleybawn Horse Farm involves aligning donor mares with recipient mares, and the specific timing for implantation is contingent upon the type of embryo used. Recipient mares are scanned everyday by transrectal ultrasound. Due to the large number of recipient in the herd no hormonal manipulation is used in the recipients [67]. The protocol involves aligning 1 donor mare to 3 recipients for optimal outcomes.

The non-surgical transcervical ET procedure on the farm is in line with the literature performed by a veterinarian with the insemination pipette [40, 45]. The timing of the transfer can be dependent on the type of embryo used on the farm. Mostly the transfer of fresh embryos is performed and involves transferring the embryo from 0 to +3 days following ovulation of the donor. 0 is the day of ovulation of donor anytime up until 3 days after it. According to the literature this synchronisation resulted in acceptable pregnancy rates and can be deemed good practice [64]. The proprietor had stated that when necessary recipients may be used that ovulate up to 5 days after the donor which according to the literature still results in the acceptable pregnancy rates [65].

The ET pregnancy in the recipient mare is then monitored with 4 ultrasound scans. First as highlighted above the pregnancy is detected 9 ½ day (Scan 1) after ET. Following this a heartbeat scan is performed 28 days (Scan 2) after the transfer to ensure viability of the embryo. The timing of the scans performed in BHF are relatively in line with the literature however scan 2 seems to be done earlier than indicated [43, 48, 52]. It is with these scans we can assess the embryonic loss in line with the literature [43, 52]. After this, mares are scanned a further 2 time during gestation in order the monitor the pregnancy. At the end of the breeding season around August a follow up scan is performed and finally in December a final monitory scan is performed to assess the progress of the pregnancy.

6.1.3. ICSI Protocol

ICSI is a new ART adopted by BHF in the last two years as evident from the data, foals have been born by ICSI in 2023 and 2022. ICSI protocol is at early stages at BHF and as such is still evolving in terms of best practice.
However, in the case of ICSI at BHF, transvaginal aspiration (TVA) is performed by a specialist veterinarian in ovum pick up. Due to the speciality of this assisted reproductive technique, the OVP is performed by a veterinarian on the farm and the oocytes are shipped to a breeding facility in Italy.

The farm uses immature pre-ovulatory follicles of about 1-2cm. Interestingly much of the literature debates between the use of dominant stimulated follicle and immature pre-ovulatory follicles [15, 82]. The author suggests that the reason for the selection of immature oocytes may be related to the transport of immature oocyte at room temperature overnight having no adverse reactions on the blastocyst rate as stated in the literature [15]. From this observation the author suggests that the OPU of immature follicles could be superior to the OPU of DSF follicles for farms located at a distance from the ICSI centres, thereby adding additional insight to the ongoing debate between DSF and immature follicles for OPU.

After the OPU session the oocytes are placed in medium and transferred to a specialised centre in Italy. After 48hrs and upon proper maturation of the oocyte to the appropriate stage, the oocytes are injected with spermatozoa by the Piezo drill technique and left to culture for 8-10days. The embryos that have reached the desired day 8-10 IVP blastocyst stage are subject to cryopreservation.

The cryopreserved IVP embryo is then transferred to the recipient mare between 4 ½ and 5 ½ days after ovulation of the recipient. Interestingly however better pregnancy rates have been obtained in the literature for implantation 4 days after ovulation [85].

6.2. Embryo Transfer

6.2.1. Embryo Recovery Rate (ERR)

The embryo recovery rate for BHF was qualitatively suggested by the proprietor as being around 80%. The author observed that this rate was at the very highest end of the scale when compared to that found in the literature, which stated an effective ERR range of between 55% and 80% [41, 69].

Furthermore, according to much of the literature [6, 40], ERR's are mainly discussed in the context of the number of donor mares' ovulations and cycles. Unfortunately, this information was not provided in the quantitative data obtained. However, in examining the data in more detail the author observed that the number of flushes performed in a season was available.

Interestingly, according to two studies from the author's literature review [43, 48] the ERR was discussed in the context of uterine flushes performed and this allowed the author to calculate a 74% ERR from the BHF data for the three-year period 2021 to 2023, thereby somewhat validating the proprietor's high qualitative suggestion of 80%

In trying to better understand this high ERR, the author observes that this could be because of how semen is handled, semen preservation being one of the influencing factors in ERR. In Section 6.1.1 above the author draws attention to the fact that BHF prefers the use of chilled semen as opposed to frozen semen. This is supported by [48] that suggested that it can be predicted that with the use of frozen semen the ERR's would be reduced. As BHF does not use frozen semen then the author suggests that the high ERR's achieved by the farm support the literatures assertion that chilled semen is better than frozen semen.

Another influencing factor is the exercising regime, where the literature [49] suggests that exercising donor mares around the periovulatory period led to decline in ERR. In section 6.1.2 above the author draws attention to the exercising regime employed by BHF, where the exercise regime of the donor mare remains unchanged around the time of ovulation. Given the high ERR achieved by BHF, as discussed above, this would seem to contrast with the literature [49].

6.2.2. Pregnancy Rates

Given the high ERR found as evidenced by the data the author next considered ETR and Pregnancy Rates. Examining the data, pregnancy rates over the 3 years from 2021 to 2023 indicated an effective implantation of around 63%. According to the literature [65] this overall rate is within the effective range for pregnancy.

In section 6.1.2 above the author draws attention to the fact that all donor mares are synchronised within 4 days of ovulation of the donor mare (0 to +3 days). In each of the three years, this approach to synchronisation resulted in pregnancy rates of 63%, 65%, 67%, for 2023, 2022, 2021 respectively. Given these pregnancy rates the author asserts that the synchronisation process of the donor and recipient mare is adequate and in line with the literature [65], resulting in what the author considers is an effective ET procedure on the farm.

Furthermore as discussed in Section 6.1.2 above, the author draws attention to the fact that BHF uses conventional cassou pipette ET techniques in line with those found in the literature [40, 45], which is an influencing factor in the pregnancy rates of ET.

As suggested by the literature these pregnancy rates may be improved by changes in the technique. Outlined in the literature [54, 56] it was proved pregnancy rates were higher for mares undergoing ET via the Wilsher Forceps and Polansky Speculum. The operator's experience was also confirmed as an influencing factor, therefore the technique may be adjusted to increase pregnancy rates accordingly [53, 56]. Given the average pregnancy rate (63%) obtained in BHF with the cassou pipette method, alongside the consequential loss of embryos, the author suggests that a potential change in the transfer technique, as discussed here, might result in a more favourable pregnancy rate.

6.2.3. Embryonic Loss & Foaling Rates

Following successful implantation studies have been performed on the embryonic loss of these embryos over 3 stages of the gestation. According to the literature [43, 52] it a noted trend is the declining pregnancy rates from the 1st scan to the 2nd scan (1st stage loss) and declining pregnancy rates from the 2nd scan to foaling (2nd stage loss). The data provided for BHF indicated similar results to the literature where embryonic loss for the seasons were calculated by the author according to the number of embryo transfers performed.

For example, in 2023, there were 16 embryos transferred following the flushing process. 10 of these embryos (63%) resulted in pregnancy at the 1st scan (9 ½ days after ET). Between the 1st scan and the 2nd scan (28 days after ET) 2 embryos were lost. This left 8 viable embryos (50% of the original embryos transferred) after the 2nd scan. Between the 2nd scan and foaling a further 1 embryo was lost, leaving a remaining 7 viable embryos (43% of the original embryo's transferred) resulting in 7 foals. According to the literature [43, 52] it is expected that between 9-15% of embryos are lost between the first and 2nd scan and 7% lost between the 2nd scan and foaling. Therefore, with embryonic loss rates of 13% (63%-50%) between the 1st and second scan and 7% (50%-43%) between the 2nd scan and foaling, the BHF data would support the reported literature.

Notably, the data from the 2022 season was excluded from the embryonic loss analysis due to the occurrence of an exceptionally high loss of foals during gestation. This elevated loss in 2022 was attributed to circumstances unrelated to the typical influencing factors. From the authors discussions with the proprietor, it was revealed that an aspergillus infection within the bedding material was the underlying cause, leading to abortions. Consequently, the author has deemed this season's data as unsuitable for assessing the embryonic loss as related to ET protocol for 2022.

2021 results showed similar embryonic loss results to 2023, however the embryonic loss between scans and foaling's proved to be less than that suggested by the literature [43, 52]. Therefore, from the authors perspective indicates a more effective ET protocol on the farm during this *year*. It is worth noting that 2 ET foals, although born, were stillborn in this year. However, in the authors opinion these stillborn foals should not be counted as an embryonic loss associated with any influential factor of the ET protocol on the farm. As a result, the author has excluded these foals from the assessment of embryonic loss this year.

6.3. ICSI

The increased popularity in ICSI in recent years is evident from the BHF data where 7 ICSI foals were born in the year 2023 in comparison to one ICSI foal in 2022 and 0 ICSI foals in 2021.

However, from the limited availability of ICSI related data the authors assessment of the ICSI protocol and related outcomes proved more difficult. More data needed to be provided on the number of OPU – ICSI sessions in order to determine the successful embryo production from the number of oocytes acquired. From the authors perspective the data was therefore deemed to be inadequate for comparing the number of embryos achieved through IVP to the original number of oocytes cultured.

6.3.1. Pregnancy Rate and Embryonic Loss

Nevertheless, from the 13 IVP embryos known from the data, the pregnancy rate for the year 2023 was 53% which is slightly lower than the 70% average stated by the literature [13, 81] .The author proposes that this lower pregnancy rate could be because the ICSI protocol is at early stages at BHF and as such is still evolving in terms of best practice. For example, the author has observed that ICSI embryos on BHF are transferred between 4½ and 5½ days whereas the literature [81, 85] states that day 4 resulted in higher pregnancy rates than day 5.

Surprisingly, given the literature [13], according to the 2023 ICSI data for BHF, embryonic loss did not occur. In the authors opinion this is likely on account of low number of mares that became pregnant from the ICSI embryos.

6.3.2. Sex Ratio

According to the ICSI literature in general [81], an unbalanced sex ratio was discovered with more colts being born than fillies. However, in this same study, a more balanced sex ratio was discovered using day 8 IVP embryos, with the study indicating 54% male and 46% females. While acknowledging the small sample size, similar sex ratios are found in the BHF data with 4 colts and 3 fillies (57% and 43% respectively) born from the 7 ICSI foals using the BHF ICSI protocol (after day 8 IVP embryos) as described in section 6.1.3 above. While this would seem to support the literature [81], the author is cautious about drawing any definite conclusions at this stage of ICSI evolution at BHF.

6.3.3. Foal Size & Viability

The limited literature available on the viability of ICSI foals stated that the majority of ICSI foals are born of normal size and condition [81, 86]. Upon examination of the BHF data regarding ICSI foals born in 2023 and 2022, and considering the subjective application of the grading classification, the author observed that only 38% of the ICSI foals were born of desirable size and quality (see Table 15).

Conversely, the BHF data indicates 68% of foals born to ICSI are subjectively graded as being of an undesirable quality (i.e., Grade 1) and therefore is contrary to the literature, suggesting that ICSI foals are born less viable and smaller than foals conceived naturally. Similarly, given the size of sample, the author is cautious about drawing any definite conclusions at this stage of ICSI evolution at BHF.

6.4. Other Observations and Discussion Points

6.4.1. BHF Grading Classification

Having reviewed the available data, there is a suggestion that foals born exclusively through the AI alone tend to exhibit Grade 2 and 3 characteristics (superior quality as defined by BHF), denoting these as medium and large healthy foals at birth respectively (see Section 5.3). However, the author suggests that it is worth noting that the review of the scientific literature did not provide concrete evidence to substantiate the claim that foals born through AI alone are consistently of superior quality in terms of weight and size compared to ET-born foals. The factors influencing foal development are multifaceted, and while the data may suggest trends, any conclusive determinations should be made with caution, considering the numerous variables at play in equine reproduction.

Conversely, according to the same data it appears that Grade 1 foals, indicative of smaller size, are more prevalent following the transfer of embryos from a donor mare into a recipient mare in ET. The BHF data hints at the possibility of the ET procedure being associated with a higher incidence of developmental issues in foals, resulting in smaller and less mature offspring irrespective of the donor. However, the author has not found any definite link or study to suggest that all ET's result in smaller foals.

However, from reviewing the scientific literature [63] there is evidence that the size compatibility between the donor mare and the recipient mare is an influencing factor that impacts the development of the foal. For example, in the 2023 BHF qualitative data, it is noteworthy that a large (Grade 3) ET foal was born from a comparatively small recipient mare measuring 158 centimetres. This specific case revealed that the foal exhibited a minor parrot mouth deformity, indicative of underdevelopment potentially resulting from the size mismatch.

Furthermore, according to the literature [59] the general health of the recipient mare is also of importance to the outcome of ET. According to the BHF data, where mares with poor health conditions (i.e., ringbone, placentitis, fractured pedal bone) were used as recipients, this resulted in the birth of foals that were classified as small (Grade 1). This highlights the impact that the general health of the recipient mare has on the quality of the foal produced as stated in the literature.

6.4.2. Embryo Cryopreservation

Cryopreservation represents a major disadvantage in the ET procedure. As stated in the literature [73, 74, 75] operators, such as BHF, tend to avoid the use of frozen embryos due to the decreased pregnancy rates. This unpopularity of the frozen embryos can be seen in Figure 7 below where 12½ % of BHF implanted embryos that established a pregnancy over the three-year period were frozen embryos. This would seem to support the literature.

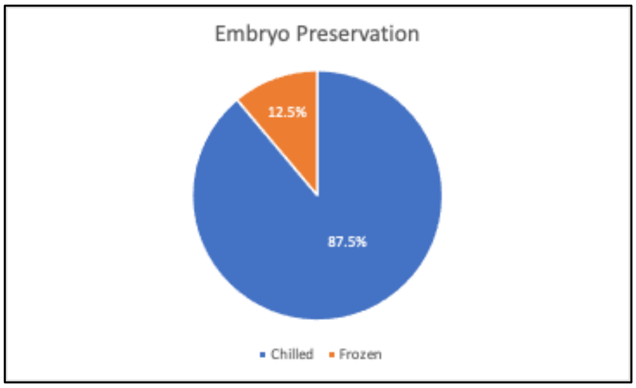


Figure 7 Embryo Preservation

Interestingly, the literature indicates that the scanning of mares for frozen embryos takes more effort in terms of additional scanning time, to ensure that the embryo is collected (flushed) at day six after ovulation [76]. As a result, the author suggests that for a small family run business like BHF, this additional effort may prove to be too much of a workload.

6.4.3. Donor Mare Age and ERR

In the context of an influencing factor, that of the age of the donor mare, the only information contained with the BHF data was an age range stated to be between 4 and 15 years.

According to the literature [48] the age of the mare, particularly donor mares over 15 years, has a negative impact on the ERR. In the absence of specific age-related data, the author was unable to substantiate the literature on this regard. However, given the high ERR that was evident in the data, it could be inferred that the absence of donor mare over 15 years is beneficial to this outcome.

6.4.4. Imported Semen

In section 6.1.1. above the author draws attention to the fact that BHF use imported semen. It is worth noting that BHF also harvest semen from their own stallions. However, this semen is not used in any of the ART protocols used by BHF, but rather is sold on to other stud farms. In the authors opinion this is a business decision by BHF where the imported semen is deemed more valuable due to the genetic and performance characteristics associated with the stallion it was taken from. This improves the potential value of the embryo and is therefore more likely to yield greater profits than using locally harvested semen.

7. Conclusion

Overall, the author concludes that much of the ART methods and associated protocols employed at Boleybawn Horse Farm are in line with what the academic literature suggests. The data assessed in this retrospective study showed that ART methods are valuable and efficiently used in the sport horse breeding industry in Ireland as in the case of this small family run farm it has led to the production of 47 foals from 61 mares, over the last 3 years. Therefore, it can be concluded that the farm's operations are likely optimised as best they can to ensure the best outcomes, demonstrated by an overall 77% success rate associated with the use and application of ART on the farm.

A more efficient and effective analysis of the assisted reproductive techniques on the farm would certainly have benefited from having a broader sample size; but more so, a more detailed and deeper level of data related to each of the three techniques. For example, as related to AI, it would have been beneficial to have data relating the number of inseminations performed. In terms of ET, more precise information relating to the donor mare (i.e., exact age of each donor mare, number of ovulations) would have been very helpful. As related to ICSI, and notwithstanding the fact that this is a very new and evolving technique on the farm, data related to the number of OPU sessions performed and number of oocytes flushed could lead to a more accurate assessment of the performance of this technique.

As a result, the author would advise that Boleybawn Horse Farm consider keeping more precise data related to the three techniques. Furthermore, this deeper level of data should ideally be systemised or computerised (as opposed to journals and diaries) allowing the quick retrieval and analysis of information, thereby allowing real-time or near real-time assessment of performance. This in turn would facilitate more informed decision making relating to what may need to be improved on the farm using the assisted reproductive techniques.

It is apparent from the data (Fig 1) that that there is a very uneven distribution of the use and application of the three techniques used with greater emphasis on embryo transfer. In the context of the farms emphasis on ET (accounting for 66% of all pregnancies), and noting that ET can be considered less successful (68% success rate) than AI (92%) and

ICSI (100%) on the farm, it can be concluded that this technique (ET) is critical to the farm's business, as indicated by the number of foals produced (27), accounting for a higher percentage (57%) of the overall foals born on the farm, when compared to AI only (26%) and ICSI (17%).

Furthermore, the use and application of ET allows the donor mare to continue to work and compete while producing offspring and minimising the dangers of natural mating. This another reason why ET is the preferred techniques on the farm.

As a result, it can also be concluded that, given the preference and criticality of ET, the farm should pay more attention to the influencing factors that affect ET to maximise its investment in ET as the primary technique on the farm. That said, while influencing factors have been proven to play a pivotal role, it is also important to keep within the limits of these influencing factors outlined to achieve the most successful outcomes.

Notwithstanding the importance of ET to Boleybawn Horse Farm, the author concludes from the evidence provided within the data that ICSI (100% success rate) is potentially a very promising ART and one to watch in the future. However, it should be noted that the pregnancy rate associated with ICSI on Boleybawn Horse Farm is only 54% and with improvements in this new evolving protocol, there is opportunity for the farm to bring pregnancy rates and success rates into closer alignment. With more research, and more foals born through ICSI in the sports horse industry in Ireland, it could be concluded that ICSI become more prevalent in small family run businesses like Boleybawn Horse Farm.

As a final concluding remark, the author suggests based on the evidence presented herein, and with extensive knowledge of the influencing factors that ensure these methodologies are used appropriately, that AI, ET and ICSI are effectively used in sports horse reproduction.

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Thesis progress report for veterinary students

Name of student: Kyra Synnott

Neptun code of the student: MJDD90

Name and title of the supervisor: Prof. Dr. Ottó Szenci

Department: Department of Obstettics and Food Animal Medicine Clinic

Thesis title: *The use of artificial insemination, embryo transfer, and ICSI in sport horse reproduction*

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Consultation – 1st semester

Timing				Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2022	05	23	Selection of thesis topic	[signature]
2.	2022	06	27-28	Preparation of thesis	[signature]
3.	2022	09	01	Preparation of thesis	[signature]
4.	2023	06	15	Progress of thesis	[signature]
5.					

Grade achieved at the end of the first semester: 5 (five)

Consultation – 2nd semester

Timing				Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2023	08.	23.	Discussing the progress of the thesis	[signature]
2.	2023	10.	28-31.	Discussing the progress of the thesis	[signature]
3.	2023	11.	01.	Discussing the final version of the thesis	[signature]

4.					

Grade achieved at the end of the second semester: 5 (five)

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,

Dr. Ottó Szenci

....

signature of the supervisor

Signature of the student: Kyra Symott..............

Signature of the secretary of the department: Tódor Emese...................................

Date of handing the thesis in 15 Nov. 2023..........................