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Dairy-Beef Integration: Use of assisted reproduction techniques to accelerate genetic gain and increase value of beef production in dairy herds

This thesis is submitted to University College Dublin in fulfilment of the requirements for the degree of Doctor of Philosophy in the **School of Agriculture and Food Science.**

by

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Statement of Original Authorship

I hereby certify that the submitted work is my own work, was completed while registered as a candidate for the degree stated on the title page, and I have not obtained a degree elsewhere on the basis of the research presented in this submitted work.

Alan Crowe ____________________________

Acknowledgements

As I sit here writing this piece for my thesis, I am struck by the fact that this four year journey has suddenly come to an end. It has been a rollercoaster ride for sure, with ups and downs and some bumps in the road. But the good times always outweighed the bad, be it a full Irish after a hard morning scanning or working with cows, or a lunch in Annie May's, I count myself lucky to have had such great friends and colleagues to enjoy it all with. I would not have gotten to this point without you all, and for that, I will forever be in your debt.

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Thesis abstract

The contribution of the calf enterprise to the profit of the dairy farm is generally considered small, with beef bull selection on dairy farms often not considered a high priority. This is rapidly changing, however, as the rapid rate of expansion of the dairy herd in some countries is now plateauing and improvements in dairy herd fertility combine to reduce the proportion of dairy breed calves required on dairy farms. This presents the opportunity to increase the proportion of beef breed calves born, increasing both the value of calf sales and the marketability of the calves. Assisted reproductive technologies such as in vitro production (IVP) of embryos can contribute to accelerated genetic gain by allowing an increased number of offspring to be produced from genetically elite dams (both dairy and beef breeds to produce bulls suitable to use on dairy cows). The expected benefits of these collective developments include accelerated genetic gain for milk and beef production, and transformation of the dairy herd calf crop to a combination of good genetic merit dairy female calves and premium quality dairy-beef calves. The aim of this review is to describe how these technologies can be harnessed to intensively select for genetic improvement in both dairy breed and beef breed bulls suitable for use in the dairy herd.

In Chapter 2, pregnancy outcomes in lactating dairy cows following timed AI or timed ET using either fresh or frozen IVP embryos from dairy or beef breeds were compared. Oocytes collected by transvaginal ovum pick-up on elite dairy and beef donors, and oocytes collected from the ovaries of slaughtered commercial beef heifers were fertilized in vitro with sperm from elite dairy and beef breed bulls. The resulting grade 1 blastocysts were transferred either fresh or frozen into synchronized lactating Holstein Friesian cows. A control group of the synchronized cows received AI. Pregnancy outcomes were compared on d 32 and d 62 by transrectal ultrasound examination. Overall, pregnancy per service event was similar between AI and ET. Cows that received AI or fresh ET had greater pregnancy success and less pregnancy loss than cows that received frozen ET. In Chapter 3, using blood samples from a subset of the recipient/AI cows from Chapter 2 and the ultrasound scanning results from Chapter 2, pregnancy outcomes were determined on d 7 (estimated based on serum Progesterone (P4)), d 18 (Interferon Stimulated Gene 15 (ISG15)), d 25 (Pregnancy Specific Protein B (PSPB)), d 32, d 62, d 120 (all ultrasound) and at parturition. This allowed a comparison of pregnancy loss between the AI and ET treatments from the day of initial AI/ET, right up to full-term parturition. Cows assigned to both AI and fresh ET had greater probability of becoming pregnant and reaching full-term parturition compared with cows assigned to frozen ET. Cows that had greater serum concentration of P4 on d 7 had greater peripheral blood mRNA abundance of ISG15 on d 18, indicating a stronger maternal response to embryo derived IFNT. These cows also tended to have a greater serum concentration

of PSPB on d 25, which in turn was associated with greater likelihood of reaching full-term parturition. Chapter 4 used daily PSPB measurement, to compare the incidence of pregnancy loss to the timing presumptive conceptus attachment (pCA), in order to compare the timing of pCA for cows that received AI with conventional or X-sorted semen or frozen IVP-ET. Cows that received frozen-thawed IVP-ET had later pCA than cows that received conventional AI. This potentially helping to explain the greater pregnancy losses observed in cows that received TET. Finally, Chapter 5 compared the gestation length, calf birth weight, calving difficulty and overall health status of calves derived from either the AI or ET events in Chapter 2. Calves that originated from IVP-ET had moderately increased BW and GL than calves born from AI. In some instances, larger BW led to calving difficulty/dystocia. Beef calves had greater BW than dairy. Calves derived from IVP-ET were as healthy and had similar survival post-partum as calves derived from AI. Given the importance of fertility in seasonal, pasture-based dairy systems, use of IVP-ET could potentially be used on a small proportion (the genetically elite cows) of the national herd. Limitations in fertility and increased pregnancy loss following frozen ET and the potential for greater birthweight using beef breed embryos mean it is a high risk strategy for farmers to aim to produce 100% beef breed calves in the near future. However, with the use of fresh ET, and careful selection of sires and dams, IVP-ET has great potential to transform the way high genetic merit AI sires and dams are produced in Ireland in the future.

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ELITE-BEEF) using transvaginal ovum pick-up, OPU). Following OPU, COCs from each donor were transported to the IVF laboratory where they completed maturation, approximately 24 h after the time of OPU. On one day per week for 6 weeks, COCs were collected from the ovaries of beef heifers of known pedigree following slaughter at a commercial abattoir (ET-COMM-BEEF; n = 119 heifers). Ovaries were removed from the reproductive tract in the abattoir and stored in flasks of PBS at 35 °C until arrival at the laboratory where all surface visible follicles were aspirated to recover the COCs. In vitro maturation (IVM) was conducted as described for those collected from the live donors. Following IVF, presumptive zygotes were cultured in vitro (IVC). The resulting grade 1 blastocysts were either transferred fresh (all d 7) or frozen (d 6, d 7 or d 8) for on-farm thawing and direct transfer (ET). Timed AI was carried out for 243 control cows and ET occurred on d 7 after synchronized estrus for 863 cows.

Figure 2.2: Protocol for synchronization of recipients for timed artificial insemination (AI) or timed embryo transfer (ET). On d −10 relative to the planned breeding date, a 2-mL i.m. injection of GnRH analog was administered, and a progesterone-releasing intravaginal device (PRID) was inserted. On d −3, a 5-mL i.m. injection of PGF2α was administered. On d −2, a second 5-mL i.m. injection of PGF2α was administered and the PRID was removed. On d −1 (32 h after PRID removal) a second i.m. injection of GnRH was administered. Timed AI and ET were carried out 16 h and 7 d later, respectively. Pregnancy diagnosis (PD) was carried out by ultrasound scanning on d 32-35 and d 62-65.

Figure 2.3: (A) Mean blastocyst yield per session as a percentage of presumptive zygotes placed in culture (●) and total number of blastocysts per OPU/IVF session (●) by ET-DAIRY donor (D1 to D12 and ET-ELITE-BEEF donor (B1 to B16) that were used for ≥ 6 OPU sessions. Donor affected blastocyst yield (Dairy, P = 0.001; Beef, P = 0.001) and the number of blastocysts per OPU/IVF session (Dairy, P = 0.014; Beef, P = 0.002). (B) Mean blastocyst yield per session as a percentage of presumptive zygotes placed in culture (●) and total number of blastocysts per OPU/IVF session (●) by ET-DAIRY sire (D1 to D5 and ET-ELITE-BEEF sire (B1 to B5) that were used for ≥ 10 IVF sessions. Sire affected blastocyst yield (Dairy, P = 0.025; Beef, P < 0.001) and the number of blastocysts per OPU/IVF (Dairy, P = 0.011; Beef, P < 0.001).

Figure 2.4: Mean blastocyst yield per OPU/IVF session for ET-ELITE-BEEF donors (n = 9 OPU sessions) and ET-DAIRY donors (n = 7 OPU sessions). Blastocyst yield was not affected by session number for ET-DAIRY donors (P = 0.235), but tended to be affected by session number for ET-ELITE-BEEF donors (P = 0.069).

Figure 2.5: Representative images of blastocyst survival and hatching post-freezing/thawing. Frozen blastocysts (n = 107, 3 replicates) were thawed and cultured in vitro for 72 h. Survival (re-expansion) and hatching were recorded at 24, 48 and 72 h post thawing. Representative images of blastocysts were captured at 10 min, 24 h, 48 h and 72 h post thawing. For more details, see text and Table 2.3. Scale bar represents 100 µm.

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Figure 2.7: Serum progesterone (P4) concentrations on d 7 after synchronized estrus for all cows that were assigned to be receive AI or ET. (A) Box and whisker plot indicating variation in serum progesterone concentration on d 7 for cows that received AI or ET, and for cows that were rejected for ET following transrectal ultrasound examination on d 7. Progesterone concentration differed between cows that were rejected and cows that received AI or ET ($P < 0.0001$). The ends of the whiskers represent the maximum (Quartile 3 + 1.5*Inter-quartile range) and minimum (Quartile 1 - 1.5*Inter-quartile range) P4 concentration for each group. The upper and lower quartiles make up the boundaries of the box. The height of the box represents the interquartile range, and the median is indicated by the horizontal line within the box. The arithmetic mean serum P4 concentration for each group is indicated by the circle within the box and outliers are identified by the circles outside of the ends of the whiskers. (B) Interquartile ranges in d 7 serum P4 concentrations in cows that received AI or ET that were non-pregnant (blue) or pregnant (red) 32 d after estrus. Mean serum P4 concentration did not differ between cows that were diagnosed pregnant or non-pregnant on d 32 after the service with AI or ET ($P = 0.113$). (C) Interquartile ranges in d 7 serum P4 concentration in cows serviced using AI or ET that were non-pregnant (blue) or pregnant (red) 62 days after estrus. Mean serum P4 concentration did not differ between pregnant and non-pregnant (d 62) cows serviced using AI or ET ($P = 0.262$). (D) Distribution of d 7 serum P4 concentrations in lactating dairy cows that were pregnant (red) or non-pregnant (blue) on d 32 after AI or ET service events.

Figure 3.1: Schematic outline of the synchronization, service event (AI or ET), blood sample collection, ultrasound diagnosis of pregnancy, and recording of calf delivery. Cows received an

intramuscular (IM) injection of GnRH (Ovarelin, CEVA Santé, France) and had a progesterone-releasing intravaginal device (PRID Delta) inserted into the vagina on d -10. Cows received IM injections of prostaglandin-F2α (Enzaprost, CEVA Santé, France) on d -3 and -2, with PRIDs removed on d -2. Cows received GnRH again on d -1. Cows were assigned to receive either TAI on d 0 or TET on d 7. Blood samples were collected on d 7 for determination of serum progesterone (P4), on d 18 for determination of peripheral blood mRNA abundance of interferon-stimulated gene-15 (ISG15) and on d 25 for determination of pregnancy specific protein B (PSPB). Pregnancy diagnosis was conducted using transrectal ultrasound on d 32, d 62 and d 125. Parturition date was recorded for all cows (mean = d 280).

Figure 3.2: Incidence and timing of embryonic loss following timed artificial insemination (AI) or timed embryo transfer (ET) with fresh or frozen in vitro produced embryos. The data-points indicate the predicted probability of pregnancy/service event at d 7, 18, 25, 32, 62, 125 and 280 (parturition). Values not sharing a common letter (a–b) differ ($P < 0.05$). Open shapes and dashed lines indicate ‘assumed’ pregnancy results (see text for further details). The probability of pregnancy and 95% CI values are included in Table 3.4.

Figure 3.3: Incidence and timing of pregnancy loss following timed artificial insemination (AI) or timed embryo transfer (ET) with fresh or frozen in vitro-produced embryos. The pie charts illustrate raw data from the subset of cows (n = 536) for which d 18 and d 25 pregnancy data were available. Percentage pregnancy loss within the windows of d 0 - d 1 (AI only), d 1 - d 7 (AI only), d 7 - d 18, d 18 - d 25, d 25 - d 32, d 32 - d 62, d 62 - d 280 and the percentage of cows that reached parturition at full-term are indicated. Note that these raw values differ slightly from the predicted probability of pregnancy loss included in Table 3.4. Pregnancy was diagnosed by measurement of mRNA abundance of interferon-stimulated gene 15 in peripheral blood on d 18, serum pregnancy specific protein B concentration on d 25 and ultrasound scanning on d 32, d 62 and d 125 (see text for details). For cows that received AI, the hatched quadrants are based on ‘assumed’ pregnancy on d 0, d 1 and d 7.

Figure 3.4: Relationships between d 7 serum progesterone (P4) concentration (by quartile) with mean d 18 interferon-stimulated gene-15 (ISG15) mRNA abundance in peripheral blood and mean d 25 serum pregnancy-specific protein B (PSPB) concentration . The whiskers around each mean indicate the 95% CI. Progesterone quartile was associated with mean d 18 mRNA abundance of ISG15 in peripheral blood ($P = 0.01$) and the mean d 25 serum PSPB concentration ($P < 0.0001$).

Figure 3.5: Scatterplots of the relationship between (A) d 7 serum progesterone (P4) concentration and d 18 interferon-stimulated gene-15 (ISG15) expression in peripheral blood, (B)

d 7 serum P4 concentration and d 25 serum pregnancy-specific protein B (PSPB) concentration, and (C) d 18 ISG15 expression in peripheral blood and d 25 serum PSPB concentration. Varying pregnancy outcomes are displayed in separate sets of data points: Non-pregnant cows, cows deemed pregnant on d 18 based on ISG-15 but had undergone pregnancy loss by d 25, cows deemed pregnant on d 25 based on PSPB concentration but had undergone pregnancy loss by d 32, cows pregnant on d 32 but had undergone pregnancy loss before parturition and cows that reached full-term parturition. The solid red vertical and horizontal lines indicates the cut-off values for P4, ISG15 and PSPB, respectively, that were used for pregnancy determined by ROC curve analysis (see text for details). The relationships between serum P4, peripheral blood mRNA abundance of ISG15 and serum PSPB were explored, but the R2 was <0.1 in all cases with the exception of the relationship between P4 and PSPB (R2 = 0.13) and the relationship between ISG15 and PSPB (R2 = 0.12).

Figure 3.6: Day 18 interferon-stimulated gene-15 (ISG15) expression in cows predicted pregnant on d 18 (left) and d 25 pregnancy-specific protein B (PSPB) concentration in cows predicted pregnant on d 25 (right) for each treatment. Cows that received fresh ET tended to have a greater mRNA abundance of ISG15 than cows that received frozen ET (P = 0.097). Cows that received AI and fresh ET had greater serum concentrations of PSPB than cows that received frozen ET (P = 0.006 and P < 0.0001 respectively). The solid red horizontal lines indicates the cut-off values for ISG15 (0.00131) and PSPB (4.4307 ng/mL), respectively, that were used for pregnancy determined by ROC curve analysis (see text for details). When a cow was predicted to be non-pregnant on d 18 and subsequently predicted to be pregnant by PSPB on d 25 (n = 58) or predicted non-pregnant on d 25, and subsequently diagnosed pregnant by ultrasound on d 32 (n = 11), the earlier diagnosis of pregnancy status was corrected for the purpose of the data analyses.

Figure 4.1: (A) Experimental design. Lactating dairy cows were bred by timed artificial insemination with conventional (TAI-C) or X-sorted (TAI-S) semen or by timed embryo transfer (TET) of a frozen-thawed in vitro-produced embryo. Oocytes were collected weekly from the ovaries of elite dairy donors using transvaginal ovum pick-up (OPU). Following OPU, cumulus-oocyte-complexes were transported to the laboratory where they completed in vitro maturation (IVM), approximately 24 h after the time of OPU. Following in vitro fertilization (IVF), presumptive zygotes were cultured in vitro (IVC) and resulting grade 1 frozen-thawed blastocysts were transferred by direct transfer on d 7 (d 0 – d of TAI). (B) Experimental timeline of the synchronization, service event (AI or ET), blood sample collection, ultrasound diagnosis of pregnancy, and recording of calf delivery. Cows received an intramuscular (IM) injection of GnRH (Ovarelin, CEVA Santé Animal, France) and had a progesterone-releasing intravaginal device (PRID

Delta, CEVA Santé Animal) inserted into the vagina on d -10. Cows received IM injections of prostaglandin-F2α (Enzaprost, CEVA Santé Animal) on d -3 and -2, with PRIDs removed on d -2. Cows received a second GnRH injection on d -1. Cows were assigned to receive either TAI on d 0 or TET on d 7. Blood samples were collected on d 7 for determination of serum progesterone (P4), on d 17 for determination of a baseline concentration of pregnancy-specific protein-B (PSPB) and on d 19 - d 28 for determination of PSPB concentration. Pregnancy diagnosis was conducted using transrectal ultrasound on d 32, d 62 and d 120. Full-term parturition date was recorded for all cows (mean ± std. dev. = d 278.1 ± 5.5).

Figure 4.2: Mean d of presumptive conceptus attachment (pCA) following timed artificial insemination with conventional (TAI-C) or X-sorted (TAI-S) semen or timed embryo transfer (TET) with a frozen-thawed in vitro-produced embryo. Treatment affected timing of pCA ($P = 0.031$). Cows that received TAI-C had earlier pCA than cows that received TET ($P = 0.027$). Bars with different letters indicate a difference between treatments ($P < 0.05$).

Figure 4.3: Calving/service event (left) and pregnancy loss from time of presumptive conceptus attachment (pCA) to calving (right) for cows with early pCA (≤ d 20, n = 137) compared with cows that had late pCA (≥ d 21, n = 79). Both calving/service event and pregnancy loss differed between cows with early and late pCA ($P < 0.0001$).

Figure 4.4: Mean daily serum pregnancy-specific protein B (PSPB) concentrations categorized by cows that had presumptive conceptus attachment (pCA) (■) and cows that had no evidence of pCA (-●-). The whiskers around each mean indicate the 95% CI. The first day with an increase in serum PSPB of ≥12.5% relative to the baseline value (d 17) followed by two more consecutive day of ≥12.5% increase from the previous day was defined as the day of pCA.

Figure 4.5: Mean daily serum pregnancy-specific protein B (PSPB) concentrations categorized by pregnancy status in lactating dairy cows following timed artificial insemination with conventional (TAI-C) or X-sorted (TAI-S) semen or timed embryo transfer (TET) with a frozen-thawed in vitro-produced embryo. The whiskers around each mean indicate the 95% CI. (A) Cows that had presumptive conceptus attachment (pCA) and reached full-term parturition (-●- - TAI-C, -▲- TAI-S, -■- TET). The values presented are back-transformed model-adjusted least square means. Mean daily serum PSPB concentration did not differ between treatments (treatment effect $P = 0.50$; treatment × time effect $P = 0.099$). (B) Cows that had pCA but underwent pregnancy loss between pCA and reaching full-term parturition (-●- - TAI-C, -▲- TAI-S, -■- TET). The values presented are back-transformed model-adjusted least square means. Mean daily serum PSPB concentration was affected by treatment (treatment effect $P = 0.053$; treatment × time effect $P = 0.38$). * indicates

greater serum PSPB concentration for cows that received TAI-C than cows that received TET (all P < 0.05) and † indicates a tendency for greater serum PSPB concentration for cows that received TAI-S than cows that received TET (0.05<P<0.1). (C) Raw data for serum PSPB concentrations for all cows across each of the three treatments TAI-C (—), TAI-S, (—) or TET (—) that had evidence of presumptive conceptus attachment (pCA) and reached full-term parturition (-■, -■, -■), evidence of pCA and underwent pregnancy loss between pCA and full-term (-Δ-, -Δ-, -Δ-) or had no evidence of attachment (-*-, -*-, -*-).

Figure 4.6: Mean daily serum serum pregnancy-specific protein B (PSPB) concentration for cows gestating male (-▲) and female (-●-) fetuses. The values presented are back-transformed model-adjusted least square means. The whiskers around each mean indicate the 95% CI. There was no difference in serum PSPB concentration observed on any day of PSPB sampling between cows gestating male and female fetuses (P = 0.63), but an interaction between fetus sex and day was detected (P = 0.026).

Figure 5.1: Experimental design. Oocytes collected using oocyte pick-up (OPU) from the ovaries of elite dairy donors (n = 40 Holstein Friesian (HF) and Jersey (JE); ET-DAIRY), elite beef donors (n = 21 Angus (AA); ET-ELITE-BEEF) and by aspiration of follicles from ovaries that were harvested from beef heifers (with at least 50% beef breed genetics) of known pedigree following slaughter at a commercial abattoir (COMM-BEEF, n = 119 donors, comprised of AA (n = 92), Limousin, (n = 18) and other (n = 9)). Following OPU/oocyte aspiration, COCs from each donor were transported to the IVF laboratory where they completed maturation, approximately 24 h after the time of OPU. Following IVF, presumptive zygotes were cultured in vitro (IVC). The resulting grade 1 blastocysts were either transferred fresh (all d 7) or frozen (d 6, d 7 or d 8) for on-farm thawing and direct transfer (ET). Lactating HF cows were synchronized using an 8-day P4 Ovsynch protocol, and timed AI was carried out for 243 control cows and ET occurred on d 7 after synchronized estrus for 863 cows. In six herds, recipient cows were randomly assigned to receive AI, fresh ET-DAIRY, frozen ET-DAIRY, fresh ET-ELITE-BEEF or frozen ET-ELITE-BEEF. In one herd, recipient cows were randomly assigned to receive AI, fresh ET-ELITE-BEEF or frozen ET-ELITE-BEEF. In the remaining three herds, recipient cows were randomly assigned to receive AI, fresh COMM-BEEF or frozen COMM-BEEF. Gestation length, calf mortality and calving difficulty/dystocia data were recorded for all calves born (n = 442) on all of the herds enrolled in the original study (n = 9).

Figure 5.2: Variation between birth weights (BW) of Angus (AA), Holstein Friesian (HF) or Jersey (JE) calves born following the timed artificial insemination (AI) or timed embryo transfer (ET) with

fresh or frozen in vitro-produced blastocysts. For each treatment and breed, × indicates the mean BW, the line intersecting the box indicates the median BW, the box indicates the interquartile range of BW and the whiskers indicate the 5th and 95th percentiles of BW.

The mean birth weights included are raw BW for all calves in each breed and treatment for which BW was recorded.

a-b Different superscripts indicate different mean BW between treatments and breeds ($P < 0.05$).

* Indicates a tendency for mean BW to differ between treatments and breeds ($0.05 < P < 0.1$).

Figure 5.3: Variation between gestation length (GL) of Angus (AA), Holstein Friesian (FR), Jersey (JE) or Limousin (LM) calves born following the timed artificial insemination (AI) or timed embryo transfer (ET) with fresh or frozen in vitro-produced blastocysts. For each treatment and breed, × indicates the mean GL, the line intersecting the box indicates the median GL, the box indicates the interquartile range of GL's and the whiskers indicate the 5th and 95th percentiles of GL's.

The GL's included are raw GL's for all calves in each breed and treatment. The n for each of the groups AI-AA, AI-LM and ET-Frozen-LM was too small to analyze as independent variables within treatments. However, across breeds, LM calves had longer GL than all other breeds ($P < 0.05$).

a-b Different superscripts indicate different mean GL between treatments and breeds ($P < 0.05$).

* Indicates a tendency for mean GL to differ between treatments and breeds ($0.05 < P < 0.1$).

Figure 5.4: Percentage of calves within each breed and treatment that fell into each of the four points on the calving difficulty score scale (1 = unassisted calving, 2 = minor assistance, 3 = considerable difficulty or 4 = veterinary assistance/caesarean section). Calving difficulty score was affected by treatment ($P = 0.05$).

The data included are raw numbers and percentages included to illustrate the breakdown of dystocia within each breed and treatment. The n in each group is included to aid the interpretation of the figure.

Figure 5.5: Blood biochemistry results for calves of beef (Angus) and dairy (Holstein Friesian and Jersey) breeds that were derived from timed artificial insemination (dairy only) and timed embryo transfer with fresh and frozen in vitro-produced embryos.

a-d Different superscripts indicate differences in amounts of metabolites between treatments ($P < 0.05$).

* Indicates a tendency for amounts of metabolites to differ between treatments ($0.05 < P < 0.1$).

Table 5.1: Mean (± 95% CI) birth weight (BW), gestation length (GL), calving difficulty score (CD) and perinatal mortality (within 14 d of birth) following timed artificial insemination (AI) or embryo transfer (ET) with fresh or frozen in vitro-produced embryos. HF: Holstein Friesian, JE: Jersey, AA Aberdeen Angus.

Table 5.2: Mean (± 95% CI) birth weight (BW) for calves within each quartile of gestation length (GL) following timed artificial insemination or embryo transfer with fresh or frozen in vitro-produced embryos.

Table 5.3: Blood biochemistry results for calves of beef (Angus) and dairy (Holstein Friesian and Jersey) breeds that were derived from timed artificial insemination (dairy only) and timed embryo transfer with fresh and frozen in vitro-produced embryos.

Table 5.4: Blood hematology results for calves of beef (Angus) and dairy (Holstein Friesian and Jersey) breeds that were derived from timed artificial insemination (dairy only) and timed embryo transfer with fresh and frozen in vitro-produced embryos.

Chapter 1: Literature Review

Invited Review: Use of assisted reproduction techniques to accelerate genetic gain and increase value of beef production in dairy herds

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1.1 Abstract

The contribution of the calf enterprise to the profit of the dairy farm is generally considered small, with beef bull selection on dairy farms often not considered a high priority. This is likely to change in the future, however, as the rapid rate of expansion of the dairy herd in some countries is set to plateau and improvements in dairy herd fertility combine to reduce the proportion of dairy breed calves required on dairy farms. This presents the opportunity to increase the proportion of beef breed calves born, increasing both the value of calf sales and the marketability of the calves. Beef embryos could become a new breeding tool for dairies as producers need to reassess their breeding policy as a consequence of welfare concerns and poor calf prices. Assisted reproductive technologies can contribute to accelerated genetic gain by allowing an increased number of offspring to be produced from genetically elite dams. There are three general classes of donor females of interest to an integrated dairy beef system: (i) elite dairy dams, from which oocytes are recovered from live females using ovum pick-up, and fertilized in vitro with semen from elite dairy bulls; (ii) elite beef dams, where the oocytes are recovered from live females using ovum pick-up, and fertilized with semen from elite beef bulls; and (iii) commercial beef dams (≥ 50% beef genetics), where ovaries are collected from the abattoir post-slaughter, and oocytes are fertilized with semen from elite beef bulls that are suitable for use on dairy cows (resulting embryo ≥75% beef genetics). The expected benefits of these collective developments include accelerated genetic gain for milk and beef production, and transformation of the dairy herd calf crop to a combination of good genetic merit dairy female calves and premium quality beef calves, with a greater portion of beef genetics than typical F1 beef × dairy calves. The aim of this review is to describe how these technologies can be harnessed to intensively select for genetic improvement in both dairy breed and beef breed bulls suitable for use in the dairy herd.

1.2 Introduction

‘Beef on dairy’ is currently a hot topic. Traditionally, in a dairy herd, all cows and heifers were bred to a dairy breed bull; the required number of heifer calves were kept as replacements with the remaining surplus (mostly male) dairy calves being sold for a low value. This traditional situation has evolved such that it is now possible to target only the top elite females in the herd to generate replacements, allowing new approaches to increase the value of the non-replacement calves for beef production.

Dairy and beef production are inextricably linked. Although the main source of revenue in dairy herds is from milk sales, beef output from the sale of cull cows and surplus calves represents 10-20% of the gross income in most production systems (van der Werf et al., 1998). Gestation and parturition are prerequisites for the initiation of lactation, but in all dairy herds the total number of calves born is greater than the required number of replacement females. Hence, in most herds, ≥60% of the calves born are destined for beef production, despite the fact that their genetics have been selected for dairy production. This results in animals of low economic value, in turn leading to welfare and environmental concerns. As a strategy to increase calf value, many dairy producers are increasingly mating dairy dams not required to generate replacement females (either surplus to requirements or genetically inferior) to beef sires (Berry et al., 2019).

For the small proportion of dairy herds with elite genetic merit dams, male dairy calves are also of potential value as future bulls to be used for artificial insemination (**AI**). The next generation of AI bulls can be identified shortly after birth using genomic testing, allowing elite bulls to be purchased by animal breeding companies, producing semen for sale from as young as 12 months of age (Murphy et al., 2018). Use of conventional (i.e., not sex-sorted) semen results in approximately 52:48 male to female sex ratio at birth (Xu et al., 2000, Roche et al., 2006, Berry and Cromie, 2007), resulting in a large surplus of male dairy calves. Thus, animal breeding companies can screen large numbers of potentially elite male dairy calves of interest using genomic testing. While this has provided long term genetic gain, there are unintended consequences of this approach that present welfare, social and environmental concerns (Ritter et al., 2019, Shivley et al., 2019, Haskell, 2020).

Biological and physiological constraints limit the speed at which animals can reproduce. Young bulls must reach 9–12 months of age before they achieve puberty and produce fertile sperm. Similarly, while female calves are born with all of their oocytes in their ovaries, they too must wait until puberty to ovulate a fertile oocyte. While attainment of puberty can be accelerated somewhat in both males (Dance et al., 2015, Bollwein et al., 2016, Byrne et al., 2018, Kenny and Byrne, 2018) and females (Perry, 2016, Cardoso et al., 2020, Heslin et al., 2020) by judicious early-life nutritional management, the time taken to reach this developmental milestone nonetheless restricts the speed at which generations can turnover. For instance, the current generation intervals for Holstein cattle in the US are ~2.5 years for sires and dams of bulls and ~4.5 and ~5 years for sires of cows and dams of cows, respectively (Garcia-Ruiz et al., 2016). However, the biological boundaries to generation interval are currently being pushed; it is now possible for a heifer calf to be the mother of a bull destined to become an AI sire before she herself has reached puberty or ever lactated. Holstein calves can now be born from oocytes aspirated from prepubertal females as young as 2 month and fertilized *in vitro* by the sperm of 10-month-old bulls. This process, known as velogenetics, discussed later, was first described in 1991 (Georges and Massey, 1991), but it was not until the development of low-cost, high-density genotyping chips (SNP chips) and genomic selection that it became practicable. Now, instead of waiting until a bull is approximately 7 years old for progeny test results, semen from bulls is routinely made available at 1.5-2 years old.

Several generations of assisted reproductive technologies (**ARTs**) have been applied to dairy cattle breeding. The more 'traditional' ARTs such as AI, multiple ovulation embryo transfer (**MOET**), *in vitro* embryo production (**IVP**), precise pharmaceutical regulation of oestrus and ovulation facilitating timed AI (**TAI**) and timed embryo transfer (**TET**) and sex-sorting of sperm to produce offspring of the desired sex are already well established in the toolbox accessible to farmers. Others, including *in vitro* gametogenesis (Hayashi, 2019, 2021) have undergone rapid development in association with stem cell biology, opening many possibilities in this field. However, future global regulation and public acceptance of some of the newer technologies, in particular modern molecular techniques such as genome editing, remains uncertain (Van Eenennaam et al., 2020).

In this review, we briefly outline dairy breeding goals, the integration of the beef and dairy industries, and the historical development of ART in cattle and describe how these techniques can be harnessed to intensively select for genetic improvement in both dairy breed and beef breed bulls suitable for use in the dairy herd. In an era with ever increasing focus on maximising efficiency and reducing waste (Place and Mitloehner, 2014, Burggraaf et al., 2020), an exciting new development is the potential to produce and transfer 100% beef breed embryos into surrogate dairy dams that are not suitable for generating replacements. The expected benefits of these collective developments include accelerated genetic gain for milk and beef production, and transformation of the dairy herd calf crop to a combination of high genetic merit dairy female calves and premium quality beef calves. This structural change takes advantage of the new tools mentioned above that are now easily available to producers for animal breeding (in particular, sex-sorted sperm and IVP embryos), and will help to increase the efficiency of dairy and beef production.

1.3 Dairy cattle breeding goals

For centuries, animal breeders have intentionally selected the parents of the next generation based on their concept of the ‘ideal’ or ‘best’ animals in the current generation. The rate of genetic improvement is controlled by four main factors: (i) the selection intensity, a measure of how choosy breeders are in selecting the best animals; (ii) the selection accuracy, a measure of the confidence that the selected animals are indeed the best; (iii) the genetic variation in the trait under consideration - the greater the variation, the greater the scope to select animals that are well above the population average, and (iv) the generation interval, a measure of how quickly the superior genes of the selected parents in the current generation can be propagated into the next generation.

Genetic selection has been a very successful tool for the long-term improvement of livestock, and the rapid adoption of genomic selection, first postulated in 2001 (Meuwissen et al., 2001) and first introduced in 2008 with the development of the first high-density genotyping chip for agricultural species (Matukumalli et al., 2009), has doubled the rate at which some dairy cattle populations are improving (Garcia-Ruiz et al., 2016). By avoiding the delays associated with progeny testing and phenotypic measures, genomic selection leads to an increase in genetic gain due to shorter generational intervals, as well as savings in cost. The rate of genetic gain through genomic selection is doubled by using bulls at two years of age instead of five, with a decrease in cost of up to 92% by avoiding progeny testing (Schaeffer 2006). As such, genomic selection is the most important technology adopted by the dairy industry since the introduction of AI about 75 years ago, playing a critical tool in addressing declining Holstein cow fertility associated with intensive selection for milk in the previous decades (Garcia-Ruiz et al., 2016, Taylor et al., 2016).

Selection indices for different dairy production systems and breeding strategies vary from country to country. Historically, selection within the global dairy industry focused exclusively on increasing milk production, but negative associations with fertility prompted a move away from single-trait selection to more balanced breeding objectives (Berry et al., 2016). Nowadays, selection emphasis has shifted away from traits focused on animal productivity toward those related to efficient resource utilization and improved health, welfare and resilience. Cole and VanRaden (2018) summarized traits included in 21 total merit indices from the US and 16 other countries. While current selection indices differ within and across countries due to variations in economic conditions including payment schemes, traits recorded and breeds used, common trait groups include yield (e.g., milk volume, fat and protein yield), longevity (e.g., productive life), fertility (e.g., non-return rate, days open, calving interval), udder health (e.g., somatic cell count, clinical mastitis), calving traits (e.g., calving difficulty, stillbirth), milking traits (e.g., milking speed) and conformation (e.g., udder conformation, feet and leg score) (Cole and VanRaden, 2018).

In terms of genetic improvement, genes can flow through one of four different pathways in a population: (i) sires of bulls, (ii) sires of cows, (iii) dams of bulls, and (iv) dams of cows (Rendel and Robertson, 1950). The aim of breeding companies is to maximise selection intensity of paths (i) and (iii), while the farmer controls (ii) and (iv) when making breeding decisions. More than 70% of all US dairy cows are bred by AI, and because nearly all of the female calves produced have historically been retained as herd replacements, selection differentials and generation intervals for the sires of bulls and sires of cows pathways have contributed the most to selection response (Garcia-Ruiz et al., 2016). These authors measured the impact of genomic selection on selection differential and generation interval in US Holstein cattle using this four-path model, and compared the observed results with those predicted by theory (Schaeffer, 2006). This analysis demonstrated that rates of annual genetic improvement in US Holstein dairy cows had increased from 50% to 100% for moderately heritable yield traits and from 300% to 400% for lowly heritable fitness traits.

Good reproductive performance in the dairy herd is essential to improve the integration of the dairy and beef sectors, as it ensures a greater proportion of dairy cows are not required to produce dairy replacements and are available to increase beef production from the dairy herd. This can be further accelerated when sexed dairy semen is used to generate replacements (Murphy et al., 2016, Ettema et al., 2017). There is a growing body of evidence that using bulls with greater genetic merit for fertility traits can improve herd fertility phenotypes and reproductive performance (Cummins et al., 2012, O'Sullivan et al., 2020, Rojas Canadas et al., 2020a,b). In addition, the availability of low cost high density genetic marker panels (SNP chips) as well as the emergence of low-cost whole genome sequencing has provided marked improvement in our understanding of the biology of fertility in the dairy cow by allowing identification of genes associated with large effects on fertility and the dissection of the biology of gametogenesis, fertilisation and embryo development and maternal embryo interaction (Cole and VanRaden, 2018).

Since about 2000, balanced breeding objectives have been implemented that incorporate fertility and longevity traits, and phenotypic fertility performance in the Holstein breed has recently begun to improve (Butler, 2013, Garcia-Ruiz et al., 2016). The trend worldwide is to move towards more rounded selection indices and in many cases, breeding cows to have reduced costs of production is as important as improving total income (VanRaden, 2004, Miglior et al., 2005, Coleman et al., 2010, Ramsbottom et al., 2012, Byrne et al., 2016). In Ireland, since 2001, the Economic Breeding Index (**EBI**) has evolved to combine milk production, fertility and health and management traits (Veerkamp et al., 2002, Berry et al., 2007). Animals with greater EBI produce less total milk kg per year, but greater milk solids (i.e., fat and protein) reflecting the importance of milk solids in the Irish payment scheme (Ramsbottom et al., 2012, O'Sullivan et al., 2020). Cows with greater EBI, and specifically greater EBI fertility sub-index, maintain greater body condition throughout lactation (Coleman et al., 2010, Cummins et al., 2012, O'Sullivan et al., 2020), which is a key phenotype that facilitates superior fertility performance and greater longevity in the herd. The current EBI places a strong emphasis (35%) on fertility traits, reflective of the economic importance of phenotypic fertility performance, particularly in a seasonal pasture-based production system (Roche et al., 2018).

In the US, fertility traits and calving ease have been included in the USDA national genetic index (Net Merit Index, **NM$**) since 2003 with the aim of increasing the total profitability of US dairy cows (VanRaden, 2004). The latest revision includes milk traits (with a negative value for milk volume but a positive value for milk solids), health traits (somatic cell score, udder health, lameness etc.), fertility (daughter pregnancy rates, cow and heifer conception rates), calving traits (calving ease, etc.) and cow longevity (livability and survival in a productive herd) (VanRaden et al., 2018). It is likely that the NM$ of the future will continue to select cows that are efficient producers of milk, are healthy and have long productive lives and have good fertility (Cole and VanRaden, 2018).

1.4 Welfare concerns arising from current breeding strategies – a driver for change

The health and welfare of unwanted male calves is a significant issue in the dairy industry worldwide, and represents a major reputational risk to the industry (Ritter et al., 2019, Haskell, 2020). The value and fate of male dairy calves varies significantly between countries (e.g., bobby calves in New Zealand/Australia, veal, finished beef). Furthermore, as mentioned above, the carcass characteristics and meat quality in finished dairy breed steers is inferior compared with beef breed steers (Berry et al., 2018).

Due to their low economic value, early rearing and health management practices are less stringently implemented for male dairy calves compared with more valuable female dairy calves. This presents animal welfare risks and damages the public perception of dairy production (Renaud et al., 2017). In New Zealand and Australia, seasonal calving and the absence of a well-established industry for raising male dairy calves, means the majority are transported long distances to be slaughtered within days of birth (Cave et al., 2005, Thomas and Jordaan, 2013, Boulton et al., 2020). In Ireland, a substantial proportion of male dairy calves are transported to mainland Europe for either veal or beef production. For example, in 2019, the total number of calf births registered to a dairy sire was 798,926. Of these, 115,885 dairy breed calves under 6 weeks of age were exported live to continental Europe, of which 114,063 (98%) were bull calves. This amounted to 28% of the dairy bull calves born in 2019 being live exported under 6 weeks of age (Department of Agriculture, Food and The Marine, 2019b). Even though these animals could all potentially enter the human food chain and provide a source of high-quality animal protein, long distance travel is a welfare concern, and slaughter of young calves is unacceptable to a majority of consumers.

Solutions to this issue are likely to vary between regions, and may include use of sexed semen to generate replacements and beef semen for all other inseminations (i.e., to markedly reduce the number of male dairy calves), use of dual purpose breeds (i.e., where male dairy calves have a recognised beef merit), targeting premium meat products and ensuring high welfare standards. In addition, it is likely that new systems of beef production will be required, especially in countries with seasonally concentrated spikes in dairy calf births in late winter and early spring. For example, in New Zealand, the potential to finish male calves of dairy origin at 8 to 12 months is being explored (Pike et al., 2019). Of note, economic modelling has indicated that the resulting beef would need to command a price premium to break even with existing conventional steer or bull beef enterprises (Hunt et al., 2019). Pike et al. (2019) reported that meat quality attributes related to tenderness scores were good, suggesting that there may be options to explore specialised premium markets.

1.5 Beef from the dairy herd – integrating beef and dairy

The global dairy market was valued at 718.9 billion U.S. dollars in 2019 and is projected to grow to 1032.7 billion U.S. dollars by 2024 (Global Dairy Market Report, 2020). One of the primary factors supporting the market growth is the rising demand for milk and milk-based ingredients, which can be attributed to population growth, rising incomes, and health consciousness. Expanding dairy herds, particularly in some European countries following the removal of the EU milk quota regime in 2015, coupled with improved fertility performance brought about through changes in selection indexes (Ma et al., 2019) and improvements in management practices (Carvalho et al., 2018), means that a greater proportion of cattle slaughtered for beef production originate from dairy herds. Thus, revenue attainable from the sale of surplus calves can significantly impact dairy farm profitability.

Use of sexed dairy semen to generate replacement females and beef semen on the remaining animals not required for replacements is growing in popularity (Ettema et al., 2017, Berodier et al., 2019), facilitating genetic gain in the dairy industry while enhancing the beef value of surplus calves (Bittante et al., 2020). For example, in the US, the number of matings between beef bulls and dairy cows more than doubled in the period from 2015-2019 (McWhorter et al., 2020). Similarly, in 2018, 45% of Irish calves from Holstein Friesian dams were sired by beef bulls (Department of Agriculture, Food and The Marine, 2019a), an increase from 32%, five years previously (Department of Agriculture, Food and The Marine, 2014; Figure 1.1). While beef-cross calves have greater economic value than dairy bred calves, further gains can be made by using 100% beef breed genetics through embryo transfer (see below).

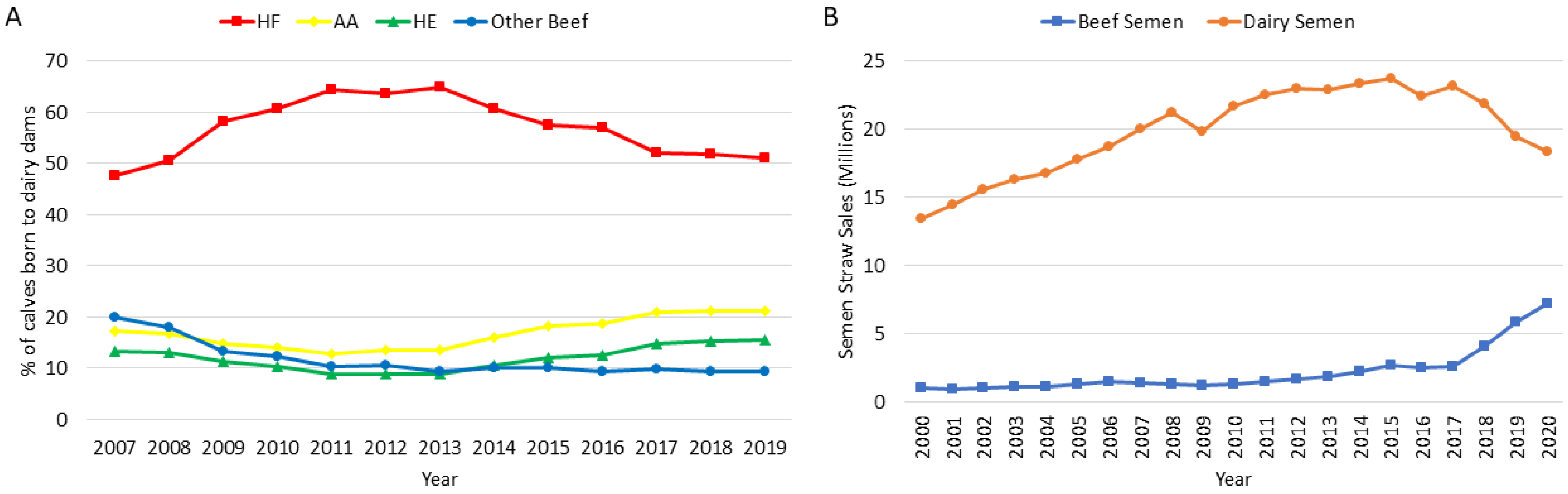

Figure 1.1 (A) Percentage of calves born from Holstein Friesian dairy dams in Ireland between 2007 - 2019 displayed by sire breed (HF = Holstein Friesian; AA = Aberdeen Angus; HE = Hereford). Data from Animal Identification and Movement (AIM) System - Bovine Statistics Annual Reports (https://www.gov.ie/en/publication/467e3-cattle-aim/#aim-bovine-statistics-annual-reports). (B) Total domestic semen sales in the USA between 2000 and 2020. Data from National Association of Animal Breeders (NAAB, www.naab-css.org/semen-sales).

The two key traits of interest to dairy farmers when selecting beef bulls for use on their herds are calving ease and gestation length. Conversely, beef farmers that purchase these calves are interested in terminal traits such as age at finishing, carcass weight and carcass conformation. Berry et al. (2019) described the introduction of a dairy-beef index (**DBI**) to rank beef bulls for use on dairy females, based on genetic potential to efficiently produce a high-value carcass while having minimal repercussions on milk, health and reproductive performance of the dairy female. This index helps dairy farmers to select the most appropriate beef bulls, but is also used by beef bull breeders that breed the next generation of beef bulls to meet the demands of dairy producers. Some 65% of the emphasis in the index relates to calving performance (calving difficulty, gestation length and calf mortality) reflecting the needs of the dairy farmer; the remaining weighting is on carcass merit (26%), feed intake (8%) and docility (1%), reflecting characteristics desired by the beef farmer and processor.

Superior growth performance and carcass traits are achievable with appropriate selection of beef bulls for use on dairy females with only a very modest increase in collateral effects on cow performance (2-3% greater dystocia and 6-d longer gestation length) (Berry et al., 2019). While the DBI evaluates traits related to calf growth and dam performance, it does not include traits related to bull fertility. McWhorter et al. (2020) evaluated sire conception rate for beef breed sires, predominantly Angus, used to inseminate dairy cows and heifers. Mean conception rates were similar in cows (33.8% vs. 34.3%) and heifers (53.0% vs. 55.3%) for insemination events with beef vs. Holstein sires, respectively. Beef sires were used more frequently in problem cows, which may explain some of the minor difference in conception rate. Hence, greater usage of beef bulls is possible without detriment to timing of pregnancy establishment.

Controlled studies have consistently demonstrated superior carcass characteristics from beef x dairy crossbred animals compared with dairy breed contemporaries (Keane and Drennan, 2008, Campion et al., 2009). In an analysis of 53,838 calves (<12 wk of age) born to dairy cows, Mc Hugh et al. (2010) reported that male calves with a beef breed sire had greater value relative to male calves with a dairy breed sire (Mc Hugh et al., 2010). For example, each 1% increase in the proportion of Belgian Blue or Charolais breed composition was worth an extra €1.86 and €1.99, respectively. Similarly, based on an analysis of 117,593 carcass records from the progeny of dairy cows, Berry et al. (2018) reported a greater carcass value of Angus x dairy crosses compared with purebred dairy animals or dairy x dairy crosses. Using field data, Berry and Ring (2020a) quantified the physical and financial performance of male progeny from different sires: (1) a dairy sire; (2) a sire that excelled in a total merit index encompassing calving performance and beef performance traits (DBI); or (3) a sire that exceled in a subindex based solely on calving performance (**CLV**). The authors concluded that sires that were highly ranked on DBI produced progeny that had heavier and better confirmation carcasses compared with the progeny from both high CLV beef sires and dairy sires, and that using highly ranked DBI sires could increase dairy herd profit by 3-5% compared with CLV.

Beef-sired calves are more prone to dystocia, which can have consequences for subsequent dairy cow performance. Berry and Ring (2020b) used field data to examine the potential repercussions of the beef-cross pregnancies on subsequent performance of the dairy female in the absence of calving difficulty. A total of 1,764,075 singleton calving events from 896,629 Holstein-Friesian dairy cows in 7,353 herds were used in the analysis to quantify the associations between sire beef merit (sire breed and genetic merit for carcass weight and conformation) and subsequent cow milk production and phenotypic reproductive performance (Berry and Ring, 2020b). Although some statistically significant associations were detected, the actual size of the associations was biologically small (Berry and Ring, 2020b). For example, service sire accounted for only 1% of the phenotypic variation in milk kg, fat kg and protein kg, and service sire had negligible effects on phenotypic reproductive performance.

1.6 Assisted reproductive technologies - impact on genetic gain

The idea of using ARTs to accelerate genetic gain in dairy breeds and improve the beef quality of non-replacement offspring in dairy herds is not new (Bekman et al., 1994, Hanekamp, 1999). The technologies and strategies available to accelerate genetic gain in dairy breeds and in beef breeds suitable for crossing with dairy breeds are summarized in Figure 1.2A and 1.2B. While insemination with beef semen will result in the generation of a calf with an expected 50% beef breed composition, the opportunity also exists to generate embryos with 100% beef breed composition (using slaughterhouse ovaries from beef heifers to provide oocytes for IVP-ET) to maximize the value of the calves surplus to replacement requirements (Figure 1.2C).

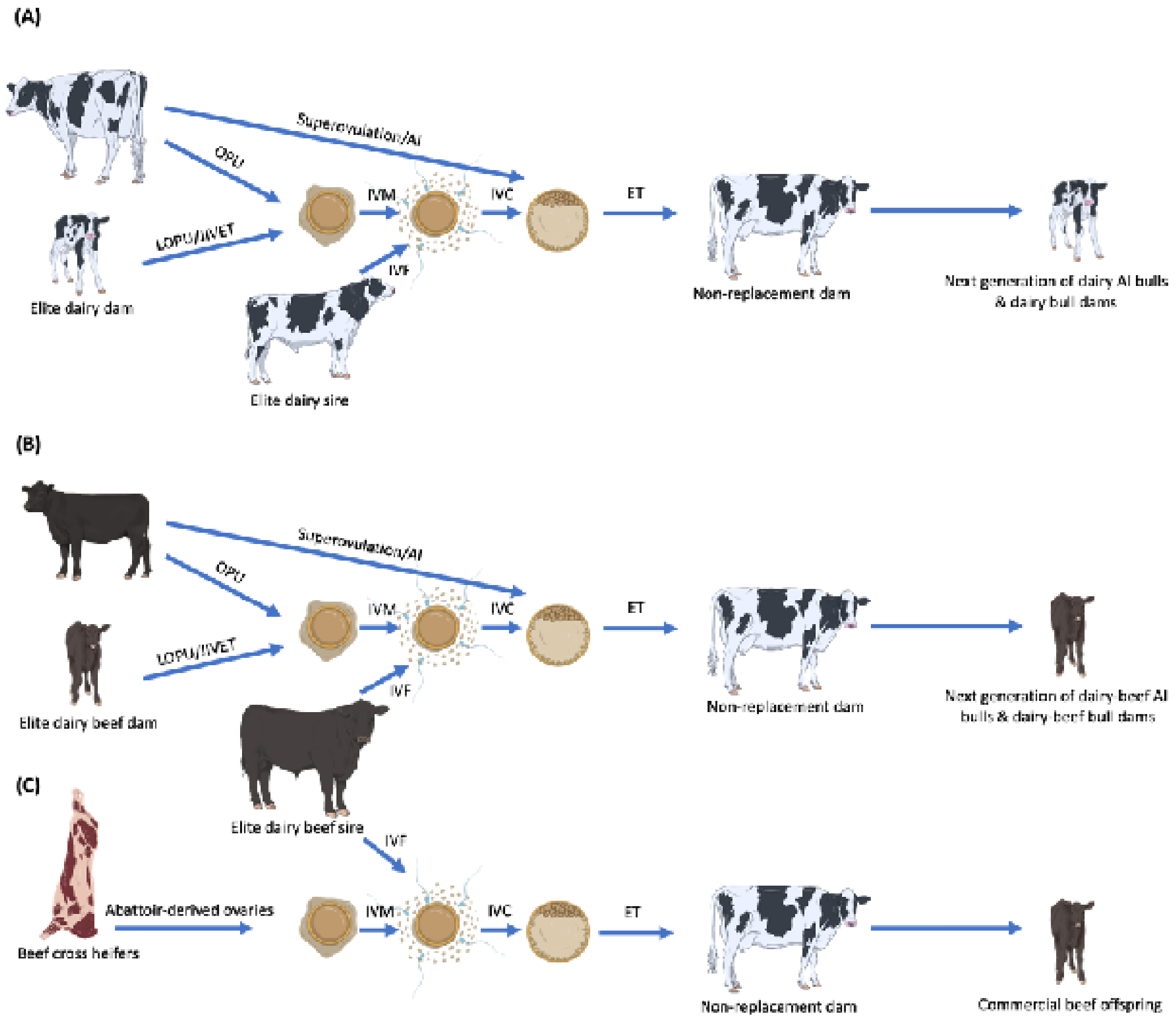

Figure 1.2 Use of assisted reproductive technologies to generate the next generation of elite dairy, elite dairy-beef and commercial beef calves. (A) To create the next generation of dairy AI bulls and dairy bull dams, embryos are generated from elite dairy dams by superovulation/AI or by using ovum pick-up (OPU) or laparoscopic OPU (LOPU)/juvenile in vitro fertilization and embryo transfer (JIVET) to collect oocytes followed by in vitro maturation (IVM), fertilisation (IVF) with semen from elite dairy bulls and culture (IVC) to produce embryos for transfer to recipient dairy dams not required to generate replacement females (either surplus to requirements or genetically inferior). (B) To create the next generation of dairy-beef AI bulls and dairy-beef bull dams, superovulation/AI or OPU/LOPU followed by IVF with semen from elite dairy-beef bulls (e.g., Angus) is conducted on elite dairy-beef dams to generate embryos for transfer to recipient dairy dams. (C) To produce commercial beef animals, ovaries collected post-slaughter from beef-cross heifers are used as a source of low-cost oocytes. These oocytes are fertilized with semen from elite dairy-beef bulls (different breed to maximise heterosis), resulting in embryos suitable for transfer to recipient dairy dams. The male and female offspring are all commercial beef animals, with female offspring also being a potential source of oocytes after slaughter.

Several generations of ARTs have been applied to domestic animal breeding and can impact one or more of the factors affecting the rate of genetic improvement (Lonergan, 2007). As mentioned earlier, many of these technologies are already well established in the toolbox accessible to farmers and breeding companies and have potential value for dairy genetic gain (more calves per elite donor, more dam-sire combinations). Although the rate of improvement could be accelerated using newer technologies (cloning, genome editing), both approval to use these technologies by regulatory authorities and public acceptance currently remains uncertain (Van Eenennaam, 2019, Bishop and Van Eenennaam, 2020).

The application of reproductive technologies in dairy cattle breeding has been recently reviewed (Moore and Hasler, 2017, Ferre et al., 2020). Artificial insemination has revolutionised dairy cattle breeding since its widespread adoption. Artificial insemination, however, has only resulted in increased selection intensity in the sire to progeny selection pathway. Considerable gains in selection intensity, and thus accelerated genetic gain, could be achieved by applying similar principles to the dam to progeny selection pathways. In contrast to high genetic merit sires, which produce billions of fertile gametes at each ejaculation and can sire thousands of offspring during (and even after) their lifetime, the contribution of genetically superior cows to a breeding program is limited by the fact that they are (usually) monovulatory, have a 9-month gestation followed by a necessary period of uterine involution, and that they have a relatively short (~5 years) productive herd life span.

1.7 Superovulation

Manipulating reproduction by repeated superovulation of the donor animal, recovery of the resulting embryos, and their transfer to surrogate recipients provides an opportunity to substantially increase the impact of superior females on a breeding program. Embryo transfer is growing in importance in US dairies. It can be used to mitigate the adverse effects of summer heat stress on cow fertility, and can increase the number of genetically elite female calves born. However, the costs of embryo production must decrease and pregnancy rates must increase to drive greater adoption (Hansen 2020).

Techniques for superovulation and ET for cattle were developed in the 1940s and 1950s (Casida et al., 1943, Rowson, 1951, Willett et al., 1951, Dziuk et al., 1958); however, large-scale ET operations were not established in North America until the 1970s, in Europe until the 1980s, and in South America until the 1990s (Hasler, 2014). Despite sustained research focused on methods to increase the number of ovulations and viable embryos recovered from the donor female, the average yield of transferable embryos produced per superovulatory cycle (6 to 8) has not changed markedly during the last 50 years. The incidence of embryo death by day 7 after estrus and insemination can be as high as 50% in high producing dairy cows (Sartori et al., 2010). Given that all of the myriad biological and technical reasons for embryo death by d 7 are avoided when a blastocyst-stage embryo is transferred into the recipient female, one would expect that pregnancy success would be greater for ET than for AI. Pregnancy success is generally similar for both technologies, however, implying that either technical aspects of ET have yet to be optimized or that underlying female fertility associated with embryo death before d 7 also cause it to die later in pregnancy (Hansen, 2020). Success of ET is under some degree of genetic control (Jaton et al., 2016; Parker Gaddis et al 2017), implying that it is possible to select for better outcomes.

The association between the size of the ovarian reserve and fertility in female cattle has recently attracted attention due to the validation of two reliable markers of the reserve: (i) the number of follicles recruited during waves of follicular development (antral follicle count, **AFC**); and (ii) circulating concentrations of anti-Müllerian hormone (**AMH**). Using ultrasonography, the peak number of follicles recruited per wave has been shown to be highly variable between animals but highly repeatable within individual animals (Burns et al., 2005, Gobikrushanth et al., 2017). Similarly, growing evidence indicates that AMH concentrations vary minimally during estrous cycles in cattle, implying that AMH concentrations can be reliably determined with a single blood sample on a random day of the cycle (Gobikrushanth et al., 2017). This allows accurate phenotypic evaluation of the ovarian reserve in cattle based on a single assessment of either AFC or blood concentrations of AMH. Furthermore, a strong positive correlation has been demonstrated between the variation in AFC, AMH, and total number of morphologically healthy follicles and oocytes in ovaries of young adult cattle (Ireland et al., 2011). In addition, peripheral concentrations of AMH are positively correlated with response to superstimulation and number of collected embryos (Rico et al., 2009). Hence, prior identification of dams that are most likely to have a strong response to ovarian superstimulation by measuring either AFC or AMH can aid identification of the best candidate donors for MOET. Other markers may arise in time. For instance, recently, heifers with divergent ovarian responses exhibited differential expression of plasma extracellular vesicle-miRNAs which may prove useful as a potential biomarker to predict superstimulation response (Gad et al., 2020).

1.8 In vitro embryo production

The techniques and challenges associated with IVP have been the subject of numerous comprehensive reviews (Bavister, 2002, Hansen, 2006, Lonergan and Fair, 2008, Sirard, 2018). Developments in *in vitro* oocyte maturation and sperm capacitation, fertilization, and embryo culture during the 1970s and 1980s led to the birth of calves following the transfer of blastocysts produced completely *in vitro* in 1987 (Lu et al., 1987, Gordon, 2003). In the early days of IVP, the sole source of oocytes was from ovaries collected from heifers or cows post-slaughter. While an excellent resource for research, these female cattle represent the commercial tier of the population, and are typically of average genetic merit; hence, the application of IVP in breeding programs was limited. The development of transvaginal ovum pick-up (**OPU**) during the late 1980s by Pieterse et al. (1988) allowed repeated oocyte recovery from live (elite) donor females and opened up the possibility of carrying out *in vitro* fertilization (IVF) with semen from elite males to produce genetically valuable embryos in large numbers, thus providing an alternative to MOET by combining OPU, IVP, and ET (Kruip et al., 1991). The practice of IVP has grown rapidly since the 2000's, with large-scale commercial operations established primarily in South and North America. Data collated annually by the International Embryo Technology Society indicate that around 1.1 million bovine embryos were transferred worldwide in 2019, the latest year for which figures are available (Figure 1.3). Approximately one-third of these were *in vivo*-derived embryos from MOET, while the remaining two-thirds were produced *in vitro* (Viana, 2020). Of all the IVP embryos transferred in 2019, the majority were in South America (50%) and in North America (40%) (Viana, 2020).

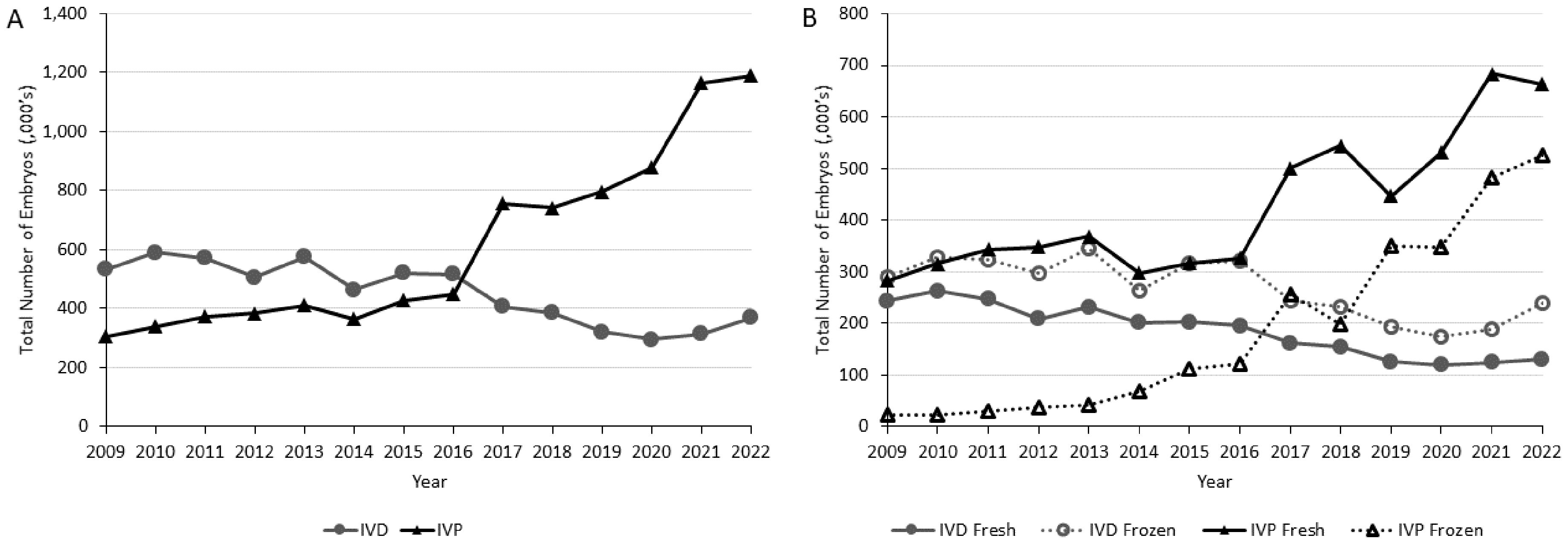

Figure 1.3 Number of in vivo and in vitro produced bovine embryos transferred in the years 2009-2022. (A) Total number of embryos transferred, (B) Relative numbers of fresh and frozen embryos. Data collated by the International Embryo Technology Society (www.iets.org).

The use of embryo technologies in Brazil increased dramatically in the past 20 years and the country was the largest producer of bovine IVP embryos in 2016, where it has almost fully replaced superovulation (MOET) as the technique of choice for embryo production (Viana et al., 2017). There are several reasons for this marked increase: (i) the willingness to rapidly adopt new technologies and develop the logistics required for use on a commercial scale; (ii) the large number of antral follicles available for aspiration in many Zebu breeds (Nelore) and Zebu-hybrids (Brahman), effectively balancing the relatively poor overall efficiency of OPU-IVP; (iii) poor and inconsistent ovarian response to exogenous FSH stimulation commonly observed in Zebu breeds; and (iv) the scale effect; while IVP has high fixed costs, it facilitates optimization of the use of high-cost semen straws, and also an improvement in the logistics of recipient synchronization and management, due to a better predictability of oocyte yield per donor. Consequently, when used on a large scale, the cost per pregnancy from IVP can be lower than from conventional ET (Viana et al., 2012). Furthermore, the increase in dairy IVP in Brazil was mainly driven by the commercial availability of sex-sorted semen (Pontes *et al*., 2010).

According to data collected by the American Embryo Transfer Association (AETA) Statistical Committee (Demetrio and Wehrman, 2019), dairy cows yield an average of 15.7 oocytes and 3.3 viable embryos per OPU session. Approximately 80-90% of immature bovine oocytes undergo nuclear maturation *in vitro*, about 80% undergo fertilization, 30-40% develop to blastocyst stage, and around 50% of the transferred embryos establish a pregnancy. While issues with cryotolerance (i.e., freezability) of IVP embryos, embryo/fetal loss and calf birth weight remain to be fully resolved (Ealy et al., 2019), IVP embryos are here to stay as a tool for genetic improvement in dairy herds (Sanches et al., 2019). Over the past decade, the success of commercial IVP has significantly improved, as greater blastocyst rates, better cryotolerance, greater pregnancy rates, reduced pregnancy loss and decreased incidence of offspring with large birthweights have been reported (Demetrio et al., 2020). Nevertheless, embryos generated *in vitro* still differ from their *in vivo* produced counterparts (Hansen, 2020). The quality of the oocyte at the start of the process is the key factor determining the proportion of oocytes developing to the blastocyst stage (Lonergan and Fair, 2016). Continual refinement of post-IVF culture media, known to have a major impact on blastocyst quality (Rizos et al., 2002, Lonergan et al., 2003), will undoubtedly improve success rates in the future.

1.9 Use of juvenile in vitro embryo production and transfer (JIVET) to shorten generation interval

The use of genomic selection allows the identification of genetically elite dams at birth, resulting in growing interest in producing embryos from calves that are too immature to respond to traditional superovulation and flushing protocols. One of the main differences between MOET and OPU-IVP-ET is that the latter can be performed earlier in the life of the heifer than MOET, leading to shorter generation intervals. In addition, OPU-IVP allows the potential for each oocyte to be fertilized by a different sire, whereas MOET typically involves insemination with semen from a single sire per flushing.

The ovaries of young animals are characterized by much greater numbers of antral follicles compared with older donors (Desjardins and Hafs, 1969), and hence more oocytes are typically recovered from young animals per OPU session (Landry et al., 2016). Although viable embryos can be produced, the success rate of OPU-IVP with prepubertal heifer donors is generally poorer compared with that achieved with postpubertal and mature female donors (Baruselli et al., 2016).

Laparoscopic OPU (LOPU) in calves followed by *in vitro* embryo production and transfer into adult recipients – to produce 'calves from calves' – has great potential for accelerated genetic gain through significant shortening of the generation interval. This allows the production of progeny from prepubertal females as young as 2 to 6 mo of age, yielding an average of ~22 viable oocytes, ~20% transferable blastocyst rate, and >50% pregnancy rate (reviewed by Baldassarre, 2021). This technique, sometimes referred to as juvenile *in vitro* embryo production (JIVET), exploits the fact that, although prepubertal females are incapable of ovulation, waves of follicular growth occur and the recruited follicles can be stimulated with exogenous gonadotropins to produce competent oocytes for aspiration, followed by *in vitro* embryo production (Currin et al., 2017, Baldassarre and Bordignon, 2018, Baldassarre et al., 2018).

Hormonal stimulation of prepubertal donors is critical given the fact that their hypothalamus–pituitary–ovary axis is not yet fully functional. Oocytes collected from 2 to 6 mo-old Holstein calves exhibited greater rates of development to the blastocyst stage following longer gonadotropin stimulation (3 days) compared with either shorter duration (2 days) or no stimulation, which was associated with a greater proportion of larger follicles (Currin et al., 2017) yielding more competent oocytes (Baldassarre et al., 2018).

1.10 Sex-sorted semen

Sexed semen involves the sorting of X and Y sperm cells by flow cytometry and reliably produces a 9:1 female to male sex ratio, reducing the number of male dairy calves (Holden and Butler, 2018). As farmers move towards greater usage of sex-biased semen on genetically superior females to generate replacements, there is a corresponding increase in beef semen usage (to produce crossbred beef calf offspring) or perhaps an opportunity to further increase calf value by beef ET (to produce offspring with ≥75% beef breed genetics). Increasing the dam-side selection pressure by breeding replacement females from only genetically superior heifers and cows in the herd could accelerate herd genetic gain by up to 15% (De Vries et al., 2008). This is only feasible, however, with widespread uptake of sexed semen from the best bulls.

Once the finite requirement for female offspring pregnancies has been achieved using sex-sorted sperm for insemination, there is increased scope for beef AI or beef ET. This resulting increase in the number of beef bred calves from the dairy herd (Murphy et al., 2016, Ettema et al., 2017) increases the marketability of the non-replacement calf crop, and these calves have greater likelihood of achieving desired market specifications at slaughter (Wolfova et al., 2007, Berry and Ring, 2020a, Twomey et al., 2020). The usage of sexed semen has steadily increased during the last decade, especially in systems with year-round calving. Hutchinson and Bickhart (2016) reported that usage of sexed semen in heifers in the US increased from 9% in 2007 to 31% in 2015. More recently, similar trends have been reported in a survey of 3,200 US dairy farms (700,000 cows), highlighting reduced usage of conventional Holstein semen, replaced by parallel increases in both sexed Holstein semen and beef semen (Agsource Dairy, 2019; Figure 1.4A). Even more striking, a recent survey of UK breeding companies indicated that for the first time farmers are buying more sexed dairy semen than conventional semen (Agriculture and Horticulture Development Board, 2020; Figure 1.4B). In the 12 months to March 2020, sales of sexed semen made up 51.3% of all dairy semen sales, up from 31.9% the previous year, and only 17.9% in 2017. This rapid change is likely due to a combination of improving P/AI with sexed semen, more competitive pricing of sexed semen relative to conventional semen and greater scrutiny and monitoring of the welfare and fate of dairy breed bull calves.

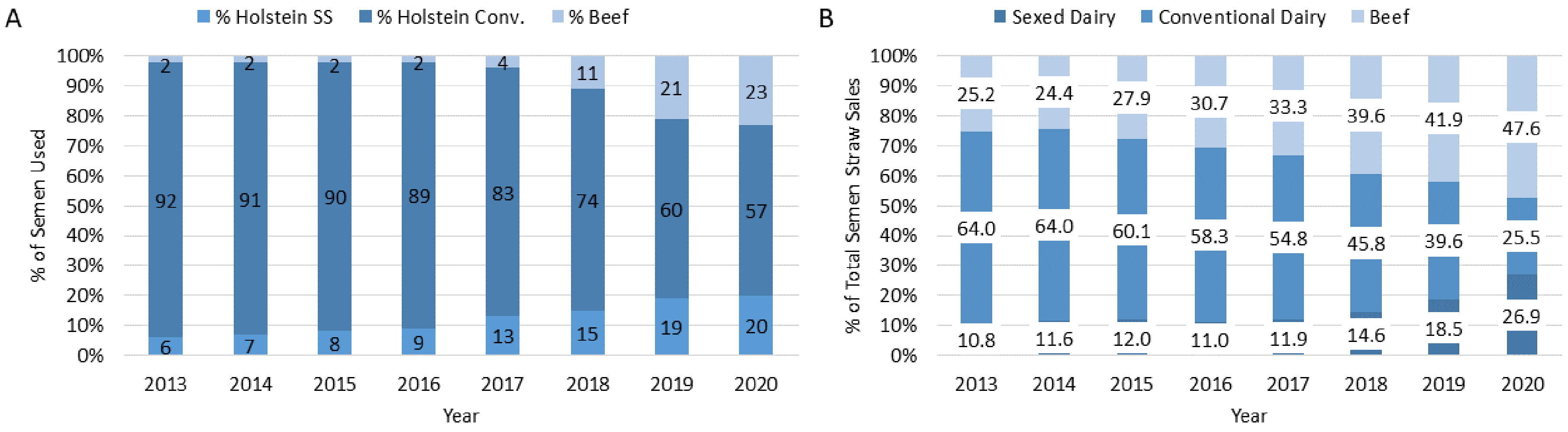

Figure 1.4 (A) Relative proportions of Holstein sexed semen, Holstein conventional semen and beef semen used to inseminate Holstein females in Wisconsin from 2013 - 2020. Source: AgSource, 2020 (M. Lauber, P. Fricke). (B) Breakdown of semen sales in the UK 2013-2020 (% of total semen sales). Sexed dairy semen, conventional dairy semen and beef semen are included. Data from the Agriculture and Horticulture Development Board (AHDB)(https://www.thecattlesite.com/news/55869/jump-in-use-of-sexed-dairy-beef-semen/).

There are several drivers of these changes. Sexed semen availability is now much greater than it was in the early 2000’s and, importantly, the best bulls are now generally available sexed (and sometimes exclusively available as sexed). This was not always the case. Genomics has facilitated earlier identification of elite sires since 2009, and is now increasingly applied to dam selection also. About 40% of the semen used on dairy cattle nowadays is either beef semen or sexed semen. The sudden surge in popularity potentially comes from low value for dairy bull calves, large dairy heifer inventories and the high cost of raising replacement animals.

The drivers of the use of sex-sorted semen different among countries. Adoption of sexed semen and beef semen for use in lactating cows in the US are strategies to right-size replacement inventories, set lower culling rates, and add value to calves not needed as replacements in a scenario where increases in reproductive efficiency through improved management, adoption of technology, and the development and implementation of hormonal fertility programs lead to an overproduction of replacement heifers. In Europe, while the attractiveness of sexed semen is still associated with being able to breed replacement females from the elite dams, its use, in combination with beef semen, to overcome the over production of unwanted low value male dairy calves is a major driver. Usage of hormonal fertility programmes for whole herd reproductive management is much less common in Europe compared with North America, but yet the uptake of sexed semen is steadily increasing. In the UK, for example, the Agriculture and Horticulture Development Board (AHDB) and National Farmers Union (NFU) published a national dairy calf strategy in 2020, which aims to eliminate calf euthanasia by 2023 and increase the number of male calves entering the beef supply chain (https://ahdb.org.uk/GB-calf-strategy, accessed July 7 2021). A recent survey conducted in Ireland examined the attitude of Irish dairy farmers to male dairy calves (Maher et al. 2021). In that study, the three highest ranked options for managing the number of male dairy calves were to increase exports, encourage greater use of sexed semen, and improve the beef merit of these calves. Of note, the majority of respondents (85%) indicated that dairy farmers had a responsibility to reduce the number of male dairy calves.

At present, the P/AI achieved with frozen-thawed conventional semen continues to be better than sex-sorted sperm (Drake et al., 2020, Maicas et al., 2020), although the gap has been reported to be considerably smaller if the sex-sorted sperm is distributed as fresh liquid semen (i.e., not cryopreserved; Xu et al 2014). It is likely that the gap in P/AI between conventional and sex-sorted sperm will continue to close as the technologies for creating sex-biased semen improve in the years to come, fostering greater uptake and usage of sex-sorted sperm.

Sexed beef semen is now becoming increasingly available, and is being actively marketed in some countries for use on dairy cows (e.g., UK; https://www.cogentuk.com/news/sexed-male-beef-semen-an-industry-game-changer, accessed July 07 2021). We are not aware of any detailed economic appraisal of the merits of this approach and whether this will gain traction remains to be seen. The big driver of future uptake will depend on the price differential between male and female beef cross calves, and hence greater ability to market the non-replacement calves.

1.11 Use of ART to reduce (avoid) generation intervals – in vitro breeding

A bottleneck of genomic selection is that the generation intervals are still reliant on the mating of individual animals, and thus on the amount of time required for the individuals to reach puberty. Large increases in genetic gain can be achieved when reproductive technologies (MOET, JIVET) are combined with genomic selection (Granleese et al., 2015). These technologies allow increased selection intensity on females while reducing the age at which animals are selected and thus decrease generation intervals.

Thirty years ago, Georges and Massey (1991) introduced the concept of *velogenetics*. This comprised use of IVF of prepubertal or even fetal (Betteridge et al., 1989) oocytes using sperm from (progeny-tested) bulls to rapidly introgress markers for important traits into new genetic backgrounds by using repeated backcrossing. The proposed strategy presented two main advantages over the status quo at that time: (i) markedly reduced dam generation interval; and (ii) ability to monitor the segregation of markers in each backcross without a requirement for phenotypic expression of the trait of interest. Velogenetics could be used for several generations without adult animals and without recording the phenotype of interest until an animal with the desired marker configuration was developed. Eight years later, this concept was further developed by Haley and Visscher (1998), who proposed two modifications. The first was termed *nuclear velogenetics*, which relied on *in vitro* culture of embryos, selection of embryos based on markers and using nuclear transfer from cultures of interest to generate embryos for transfer to recipients; fetal oocytes could be harvested in utero and matured, fertilized, and cultured *in vitro* to repeat the cycle. The second was termed *whizzogenetics*, which also relied on *in vitro* culture of embryos, selection of embryos based on markers but then selected cultures of interest were induced to undergo meiosis, and the resulting cells were fertilized *in vitro* and the cycle of embryo culture repeated until the desired marker configuration was achieved. Then, nuclear transfer from selected cultures could be used to generate new embryos for transfer to recipients (Haley and Visscher, 1998). If strategies like velogenetics and whizzogenetics can reduce the generation interval by a factor X, then they can also increase the genetic gain by the same factor X, but only if accuracy of selection is not affected (Meuwissen, 2003). At that time, however, selection strategies relied on measures of phenotypic performance and genetic markers only explained a part of the genetic variance.

The velogenetics and whizzogenetics concepts were developed before high density QTL maps were available, and before widespread use of genomic information in genetic selection programs. In the years since, SNP arrays and next generation sequencing have allowed identification of thousands of QTL's linked to traits of economic importance, and facilitated the development and successful implementation of genomic selection in dairy cattle (Meuwissen et al., 2001, Hayes et al., 2009). Nevertheless, successfully increasing the frequency of favourable alleles for large numbers of QTL's, even with the advent of genomic selection, will remain slow.

The emerging technology of *in vitro* gametogenesis, where the entire germline can be recapitulated *in vitro* (Hikabe et al., 2016), is expected to eliminate the bottleneck in genomic selection (Hou et al., 2018, Goszczynski et al., 2019). The successful *in vitro* generation of germ cells from embryonic stem cells (ESCs) in mice (Hikabe et al., 2016) (reviewed by Hayashi, 2019, 2021) and the recent efficient derivation of ESCs from bovine blastocysts (Bogliotti et al., 2018) will potentially enable a paradigm shift in livestock breeding in the near future. Building on the concepts of velogenetics and whizzogenetics, Goszczynski et al. (2019) outlined a potential methodology, which they termed *in vitro breeding*, that may soon be feasible to leverage the new ART and genomic tools to accelerate genetic gain. This strategy uses genomic selection to identify elite sires and dam combinations, from which large numbers of embryos are generated. Embryonic stem cell (ESC) cultures are derived from the blastocyst inner cell mass (Bogliotti et al., 2018), and the embryonic stem cells are genotyped to allow estimation of genomic merit of each cell line for the traits of importance for a particular breeding objective. After identifying the best cell lines, functional oocytes are derived from the embryonic stem cells through germ cell differentiation, and these are used in repeated rounds of IVF, generation of embryonic stem cells, selection of the best cell lines and germ cell differentiation. Assuming the time required for germ cell differentiation in cattle (procedure not yet developed) is similar to mice (2 to 3 months), the authors estimated that a complete breeding cycle could be completed in 3 to 4 months. It is noteworthy that the *in vitro* breeding strategy is amenable to combination with genome editing to promote favourable alleles and with gene drive to generate homozygosity for the edited allele. Simulation studies have indicated that both genome editing (Jenko et al., 2015) and gene drive (Gonen et al., 2017) can markedly accelerate genetic gain. The use of established, new and emerging ARTs combined with genetic and genomic tools will soon revolutionize dairy and beef cattle breeding.

1.12 Implications of ART for Inbreeding

The use of any reproductive technology to selectively focus on a limited pool of (elite) genetics could result in an increase in inbreeding rate, and this includes existing commercially available technologies including AI (+/- sexed semen), MOET and IVP-ET that have already been in use for some time. A simulation study conducted by Thomasen et al. (2016) examined how genomic selection of dams (0 or 2,000 genotyped heifers per year) interacted with reproductive technologies (0 or 50 selected donors) with different reliability values for genomic prediction (0.36 or 0.50). Stochastic simulation was used to vary key inputs: (1) the number of donors (25, 50, 100, 200); (2) the number of calves born per donor (10 or 20); (3) age of donor (2 or 14 mo); and (4) number of sires (25, 50, 100, 200). Greater reliability for the genomic prediction estimates and use of greater numbers of donors and sires limited the inbreeding rates. It is important that use of ART's for generating breeding stock is appropriately implemented to provide sustainable breeding schemes for the future.

1.13 Economics of in vitro embryo production

Sanches et al. (2019) concluded that IVF is becoming an economically viable practice in large scale dairy programs. Nonetheless, only a few studies are available that have examined the economics of the use of IVP-ET in dairy herds. IVP embryos are costly compared to a straw of semen, and transfer of IVP embryos, particularly frozen-thawed embryos, can lead to lower reproductive performance compared with AI through increased pregnancy losses. The economic incentive to implement IVP-ET in a dairy herd is based on the ability to generate offspring with superior genetic merit compared with use of AI, but the cost to produce a pregnancy with an IVP embryo is significantly greater than the cost of AI. De Vries and Kaniyamattam (2020) reviewed estimates of the net benefit of using IVP-ET versus AI in dairy herds, and reported that the most profitable use of AI and IVP-ET is often a combination of both technologies; more IVP should be used when the value of the surplus calves is greater and the cost of IVP-ET is lower. Benefits are maximized when superior donors and recipients are accurately identified, reducing the generation interval and achieving greater embryo production efficiency.

Regarding the potential gains that might be achieved, use of IVP-ET can greatly decrease the genetic lag (difference in genetic merit between the average cow in the herd and the best available sires). IVP-ET results in a high selection intensity (a small number of genetically elite animals provide many calves for the next generation), a short generation interval (donors are typically heifers or young cows) and may have increased accuracy through the use of genomic testing for donor and recipient selection.

One major disadvantage of IVP-ET is the greater cost per breeding and pregnancy compared with AI. Ribeiro et al. (2012) calculated a difference in the cost of a female pregnancy to be $329 higher for IVP-ET than for AI using sexed semen. That study did not include additional genetic gain benefits from IVP-ET, however. Thomasen et al. (2016) reported that the greatest increase in economic value of genetic gain in a closed population was achieved when JIVET was used along with genomic selection in the bull-dam part of the population. Combining IVP-ET with genomic testing was profitable in almost all scenarios examined when the cost of producing a calf (potential future sire) by IVP-ET ranged from $500 to $1500.

Kaniyamattam et al. (2017) compared an exclusive (100%) IVP-ET system and artificial insemination for genetic, technical, and financial herd performance. For the IVP-ET system to be at least as profitable as the comparable AI system during a 15-yr investment period, the surplus calves from the IVP-ET system needed to be sold at premium prices. The break-even price of fresh embryos was estimated to be $84 for the exclusive IVP-ET system, which is significantly lower than the current market price for IVP-ET. This resulted in the same profit as the AI system, which maximized NM$ for a 15-yr investment period and in which heifer calves were sold at a premium price. In a subsequent study (Kaniyamattam et al., 2018), the percentage of pregnancies derived from IVP-ET was varied from 0 to 100% to find the optimal proportion of pregnancies from IVP-ET and AI to maximize profitability across a range of prices for embryos and surplus dairy heifer calves. Importantly, some use of IVP-ET was profitable in many realistic combinations of embryo prices and surplus dairy heifer calf values. The profit at yr 15 after the start of the IVP-ET program was maximized when 40% of the total pregnancies in the herd came from IVF-ET. Lower prices for IVP-ET or greater value of surplus dairy heifer calves increased the optimal proportion of IVP-ET that should be used.

While not assessed directly in those studies, the use of IVP-ET to generate a calf with increased beef merit would also contribute to farm profitability and add to the justification for using the technology. Ettema et al. (2017) reported that the potential returns from increasing beef semen usage is herd-specific. In herds with above-average management levels for calf survival, longevity, and reproductive performance, economic performance can be improved by combining the use of sexed dairy semen and beef semen, but only when the effect of the changes in the genetic merit of the female dairy calves was included in the calculations. In reality, a combination of technologies will likely be most profitable in a given scenario. For example, Clasen et al. (2021) reported that a combination of genomic testing, sexed semen, beef semen and terminal cross breeding improved the total economic return in simulated Swedish Red and Swedish Holstein herds. In that study, the greatest total economic returns were achieved in scenarios where the breeding tools were used most, whereas the greatest genetic returns depended on phenotypic reproductive performance.

1.14 For thesis purposes only, not included in the publication

1.14.1 Pregnancy Establishment

Several major developmental events occur in the first week after insemination and ovulation, including fertilization, the first mitotic divisions of the embryo, the timing of which is associated with developmental competence (Lonergan et al., 1999), activation of the embryonic genome at the 8- to 16-cell stage (Rabaglino et al., 2023), morula compaction and blastocyst formation. Fertilization and the first 3 to 5 days of embryonic development occur in the oviduct, which plays a central role in early embryo development. Following hatching from the zona pellucida on around d 8 to 9, the blastocyst undergoes a change in morphology from a spherical to ovoid shape during a transitory phase preceding the elongation or outgrowth of the trophectoderm to a tubular, then filamentous form that usually begins between d 12 and 14. Around this time, the trophectoderm cells of the conceptus begin to secrete significant amounts of IFNT, the maternal pregnancy recognition factor in cattle, which ultimately blocks the uterine luteolytic mechanism to ensure maintenance of a functional CL and production of P4 (Bazer and Thatcher, 2017). Conceptus elongation during the second week after fertilization is driven by increasing circulating P4 concentrations that induce changes in the endometrial transcriptome (Forde et al., 2009, Spencer et al., 2016), which are reflected in changes in the uterine lumen fluid composition (Simintiras et al., 2019a, Simintiras et al., 2019b). Conceptus attachment to the endometrium typically first occurs around d 20-21 post-fertilization (Wathes and Wooding, 1980). During this period, trophoblast giant binucleate cells develop within the chorion to migrate and fuse with the uterine surface epithelium to form syncytial plaques. These binucleate cells produce PSPB, which migrates from the conceptus, across the newly forming placenta, into maternal circulation. Recent studies have highlighted that the timing of conceptus attachment, as assessed by increasing concentrations of PSPB in maternal circulation, is strongly associated with subsequent pregnancy loss in lactating dairy cows (Middleton and Pursley, 2019, Middleton et al., 2022, Santos et al., 2023). From approximately d 26, the conceptus becomes identifiable by transrectal ultrasound (Fricke, 2002, Romano et al., 2006). Fetal development continues through the second trimester, and formation of internal organs is largely complete by 180 days (Valadão et al., 2018). The majority of fetal growth occurs in the last trimester, particularly during the final month when it will reach full size (Prior and Laster, 1979).

1.14.2 Pregnancy diagnosis as it currently is done

Historically, non-pregnant dams were (and still are) identified when they return to estrus and more recently, using transrectal ultrasonography to confirm absence of a viable conceptus (i.e., embryo and associated extraembryonic membranes). More recently, the use of chemical tests on blood or milk samples (i.e., pregnancy-associated glycoproteins, PAGs) from approximately d 28 of pregnancy onwards has been commercialized (Branen et al., 2011). Until relatively recently, the period from artificial insemination (AI) or embryo transfer (ET) to this first pregnancy diagnosis represented a ‘black box’ due to the inability to either visualize or detect the conceptus. For successful pregnancy establishment, the developing conceptus must signal its presence by secreting sufficient interferon-tau (IFNT) for maternal recognition of pregnancy to occur. The maternal response to this conceptus-derived IFNT can be measured by quantifying the mRNA abundance of interferon-stimulated genes (ISGs) in circulating blood as early as d 18 after estrus (Stevenson et al., 2007, Gifford et al., 2008, Green et al., 2010, Dalmaso de Melo et al., 2020). Similarly, concentrations of PAGs secreted from the binucleate trophoblast giant cells of the bovine conceptus can be determined in blood or milk samples collected from the gestating dam and used to assess pregnancy status from d 25 after AI (Green et al., 2000). These developments allow earlier determination of pregnancy status and characterization of the timing of early pregnancy losses.

1.14.3 Timing and causes of pregnancy losses

The incidence of embryo loss during the first week post-breeding can be captured by recording the proportion of non-viable embryos recovered following non-surgical uterine flushing on d 6 to d 7 post-breeding; this has been estimated to be as high as 50% in some reports (Sartori et al., 2010, Wiltbank et al., 2016). The causes of early embryo loss before d 7 likely reflect compromised oocyte quality due to a variety of factors including postpartum negative energy balance and body condition loss (Maillo et al., 2012), postpartum disease (Ribeiro et al., 2016), heat stress (Hansen et al., 2001), and inadequate circulating P4 concentrations (Forde et al., 2012). According to Wiltbank et al. (2016), pregnancy loss from d 8 to d 27 averaged approximately 30%, and failed or delayed trophoblast elongation accounted for 7% of pregnancy loss in the second week after estrus. On d 15 after detected estrus and insemination, Berg et al. (2022) recovered conceptuses and uterine luminal fluid from 406 lactating dairy cows, and estimated pregnancy success to be 59.1%. Of 259 conceptuses recovered, 19 (7.3%) had failed to elongate beyond the spherical or ovoid stage and were deemed non-viable, contributing to pregnancy failure during the second week after estrus. Delayed or failed conceptus elongation during this window is associated with reduced IFNT secretion and a failure to elicit an adequate response from the endometrium for maternal recognition of pregnancy (Sánchez et al., 2019). Conceptus attachment to the endometrium typically first occurs around d 20-21 post-fertilization (Wathes and Wooding, 1980). During this period, trophoblast giant binucleate cells develop within the chorion to migrate and fuse with the uterine surface epithelium to form syncytial plaques. These binucleate cells produce PSPB, which migrates from the conceptus, across the newly forming placenta, into maternal circulation. Recent studies have highlighted that the timing of conceptus attachment, as assessed by increasing concentrations of PSPB in maternal circulation, is strongly associated with subsequent pregnancy loss in lactating dairy cows (Middleton and Pursley, 2019, Middleton et al., 2022, Santos et al., 2023). In cows that had conceptus attachment later than d 21 post-ovulation, the likelihood of pregnancy loss was four times greater compared with cows that had conceptus attachment on d 20 or 21 (Santos et al., 2023). From approximately d 26, the conceptus becomes identifiable by transrectal ultrasound (Fricke, 2002, Romano et al., 2006). Inadequate placentation is a potential explanation for embryonic mortality after d 28 of gestation, since placentome formation occurs between d 25 and 50 of gestation in cattle (Seo et al., 2023). Similar to the d 25 to d 32 window of time between pregnancy diagnoses, poor placentome development increases the risk of pregnancy loss up to d 62. Wiltbank et al. (2016) reported that the main causes of pregnancy loss between d 28 and d 60 were defects in placentome and/or vascular development (losses of ~12%). Domingues et al. (2023) reported that approximately 50% of pregnancy loss is initiated by embryonic death and 50% by luteal regression. Their review estimated that the typical incidence of pregnancy loss between d 28 and d 60 is between 5 and 20%. Pregnancy losses after d 62 are generally small in lactating dairy cows. Previous studies have estimated the incidence of pregnancy loss during the interval from d 60 to d 90 to be approximately 2% (Wiltbank et al., 2016, Albaaj et al., 2023). Pregnancy losses can be greater in specific categories of cows, particularly those carrying twins in the same uterine horn (Garcia-Ispierto and López-Gatius, 2019).

1.15 Conclusions

Because of their poor future beef value, the majority of male dairy calves have low economic value. This is now leading to major concerns regarding the welfare and survival of these calves. In addition, ruminant production is being placed under increasing scrutiny for the environmental impact of milk and meat production. The suite of ART tools that are now available can be harnessed to provide a step change in the efficiency, environmental footprint and public image of milk and beef production.

Assisted reproductive technologies can contribute to accelerated genetic gain by allowing an increased number of offspring to be produced from genetically elite dams. There are three general classes of donor females of interest to an integrated dairy-beef system: (i) elite dairy dams, from which oocytes are recovered from live females (potentially multiple times) using OPU, and fertilized *in vitro* with semen from elite dairy bulls; (ii) elite beef dams, where the oocytes are recovered from live females using OPU, and fertilized with semen from elite beef bulls; and (iii) commercial beef dams (≥ 50% beef genetics), where ovaries are collected from the abattoir post-slaughter, and oocytes are fertilized with semen from elite beef bulls that are suitable for use on dairy cows from (ii) above (resulting embryo ≥75% beef genetics). For (i) and (ii), the embryos can be genotyped to calculate the genomic merit, the best embryos transferred to surrogate dams, and the best male and female offspring retained to sustain the cycle of continued genetic gain. For (iii), the major challenge will be to be economically competitive versus beef AI (pregnancy establishment, embryo loss, calf value). For a dairy farmer to switch from using beef AI to beef ET, the resulting calf would need to attract a greater economic value at two weeks of age. For the beef farmer to spend more money on an ET calf vs. an AI calf, either the slaughter value needs to be greater (larger carcase, better conformation, premium price) or the cost of getting to slaughter needs to be less (finished at an earlier age, better growth rates, better feed efficiency etc.). What phenotype differences can be expected between calves that have 50% beef breed + 50% dairy breed genetics vs 100% beef breed genetics? How much selection intensity can be placed on the oocyte donor? Can beef bulls be selected that are specifically suited for IVP and ET into surrogate dairy dams? There are many gaps in our current knowledge. Research on the cost-benefit of using beef ET vs beef AI is required, incorporating comparative data on pregnancy establishment, calving performance, post-natal growth rates, post-slaughter carcass characteristics and meat quality attributes.

In an ideal scenario, future AI bulls are generated by design, relying on OPU from elite dairy dams, and IVF to generate blastocysts suitable for transfer to recipients. Within commercial dairy herds, only elite dairy sexed semen would be used to generate female replacements. Collectively, this would account for approximately one third of the pregnancies in the dairy herd. The remaining two thirds of the dairy herd would either be (i) inseminated following observed oestrus or timed-AI using suitable beef bulls (50% beef genetics in resulting offspring); or (ii) have an embryo transferred (≥75% beef genetics) on day 7 after oestrus. This scenario would change the face of dairy and beef production, removing the male dairy calf as a major welfare concern, and increasing the value of beef output from the dairy herd.

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Thesis Objectives

The objectives of the work described in this thesis were:

1.To compare pregnancy outcomes in lactating dairy cows following timed AI or timed ET using either fresh or frozen IVP embryos from dairy or beef breeds.

2.To characterize the incidence and timing of pregnancy loss from initial service event to parturition following TAI or TET with fresh or frozen IVP embryos by using a combination of detection of cows returning to estrus, chemical assays for Interferon Stimulated Genes (ISGs) and Pregnancy Associated Glycoproteins (PAGs) and transrectal ultrasonography.

3.To determine the timing of presumptive conceptus attachment (pCA) and subsequent incidence of pregnancy loss in seasonal-calving pasture-based lactating dairy cows following TAI with conventional (TAI-C) or X-sorted (TAI-S) semen or TET with a frozen-thawed IVP embryo.

4.To compare the gestation length, calf birth weight, calving difficulty and overall health status of calves derived from either AI or ET, with ET treatments comprising fresh and frozen IVP embryos of both beef and dairy breeds.

Chapter 2: Fertility in seasonal-calving pasture-based lactating dairy cows following timed artificial insemination or timed embryo transfer with fresh or frozen in vitro produced embryos

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2.1 Abstract

The objective was to compare pregnancy per service event (P/S) in lactating dairy cows following timed artificial insemination (AI) or timed embryo transfer (ET) using either fresh or frozen in vitro produced (IVP) embryos. Oocytes were collected once per week for up to 9 weeks using transvaginal ovum pick-up from elite dairy donors (ET-DAIRY; n = 40; Holstein Friesian and Jersey) and elite beef donors (ET-ELITE-BEEF; n = 21; Angus). Both ET-DAIRY and ET-ELITE-BEEF donors were comprised of heifers and cows. In addition, oocytes were collected from the ovaries of beef heifers of known pedigree following slaughter at a commercial abattoir (ET-COMM-BEEF; n = 119). Following in vitro maturation and fertilization, presumptive zygotes were cultured in vitro to the blastocyst stage. Grade 1 blastocysts were either transferred fresh or frozen for on-farm thawing and direct transfer. 1106 recipient cows (all lactating, predominantly Holstein Friesian) located on 16 herdlets were blocked based on parity, calving date and economic breeding index, and randomly assigned to receive AI (n = 243) or ET (n = 863) after estrous synchronization with a 10-d Progesterone-synch protocol. Cows assigned to ET were further randomized to receive fresh (n = 187) or frozen (n = 178) ET-ELITE-BEEF embryos, fresh (n = 169) or frozen (n = 162) ET-DAIRY embryos, or fresh (n = 80) or frozen (n = 87) ET-COMM-BEEF embryos. Pregnancy was diagnosed using trans-rectal ultrasound on d 32 to 35 after synchronized ovulation and confirmed on d 62 to 65, at which time fetal sex was determined. Pregnancy per service event at d 32 was not different between AI (48.8%) and ET (48.9%) and did not differ between dairy and beef embryos (50.3% vs 48.1%, respectively). However, P/S was less on d 32 following transfer of frozen embryos (41.6%) compared with fresh embryos (56.1%). Pregnancy loss between d 32 and 62 was greater for ET (15.1%) compared with AI (4.7%), with greater losses observed for frozen beef (18.5%), fresh beef (17.3%) and frozen dairy (19.2%) compared with fresh dairy (6.0%) embryos. Serum P4 concentration on d 7 was associated with P/S at d 32 and d 62. Cows in the quartile with the least serum P4 concentrations (Q1) had less probability of being pregnant on d 32 (33.4%) compared with cows in the three upper quartiles for serum P4 (45.7%, 55.6% and 61.2 for Q2, Q3 and Q4, respectively). Sex ratio (M:F) at d 62 was skewed towards more male fetuses following ET (61.1:38.9) compared with AI (43.2:56.8) and was consistent with the sex ratio among in vitro blastocysts (61.2:38.8). In conclusion, P/S was similar for AI and ET, although pregnancy loss between d 32 and d 62 was greater for ET than for AI.

2.2 Introduction

Until relatively recently, artificial insemination (AI) with conventional (non-sorted) semen from high genetic merit dairy bulls was the only option available to commercial dairy farmers to impregnate cows and to generate replacement heifers. While the use of conventional semen has provided long-term genetic gain, its use results in approximately 52:48 male-to-female sex ratio at birth (Xu et al., 2000, Roche et al., 2006, Berry and Cromie, 2007). While a small proportion of these male calves (around 0.1%) are genetically elite and of value as potential future AI bulls (De Vries et al., 2008), the majority of male dairy calves have low economic value because of their poor future beef value. These surplus male calves present welfare, social and environmental concerns (Ritter et al., 2019, Shivley et al., 2019). As a consequence, the use of both sex-sorted dairy semen and conventional beef semen in the dairy herd is increasing (reviewed by Crowe et al., 2021). Use of sex-sorted dairy semen on the dams with the best genetic merit to generate replacement females and beef semen to generate all remaining pregnancies (non-replacement calf crop) is growing in popularity (Bittante et al., 2020, Pahmeyer and Britz, 2020, Cabrera, 2022), facilitating genetic gain in replacement stock while enhancing the beef value of surplus calves. For example, in the United States, the number of beef breed AI straws used on dairy herds more than doubled in the period from 2015 to 2019 (McWhorter et al., 2020). Similarly, in 2018, 45% of calves derived from Irish Holstein Friesian dams were sired by beef bulls (Department of Agriculture, 2019) an increase from 32%, 5 years previously (Department of Agriculture, 2014). Although beef-cross calves (derived from AI or natural service) have greater economic value than male dairy calves, further gains are potentially feasible if the percentage of beef-breed genetics in non-replacement stock could be increased to ≥75%.

Widespread uptake and usage of female sex-sorted semen on the best genetic merit dams may inadvertently decelerate genetic gain as a result of fewer elite male dairy calves from which to choose potential AI sires. One solution to this undesirable side-effect of sex-sorted semen usage would be to generate future AI bulls by design, using ovum pick-up (OPU) to harvest oocytes from elite dairy dams, and in vitro embryo production (IVP) to generate blastocysts suitable for transfer to recipients. In seasonal, pasture-based, systems of production, however, excellent fertility in the short (12-week) breeding season is essential. The economic consequences of poor fertility, manifested as poor pregnancy per service event (P/S) and/or excessive embryo loss after conception, are amplified in such systems compared with year-round calving systems (Shalloo et al., 2014).

The overall objective was to compare pregnancy outcomes in lactating dairy cows following timed AI or timed ET using either fresh or frozen IVP embryos from dairy or beef breeds. Specifically, we tested the hypotheses that cows that received a fresh embryo would have similar P/S as cows artificially inseminated with conventional semen, and that both AI and ET with fresh embryos would achieve better P/S than that achieved with frozen embryos.

2.3 Materials and methods

All experimental procedures involving animals were approved by the Teagasc Animal Ethics Committee and authorized by the Health Products Regulatory Authority in Ireland, in accordance with Statutory Instrument No. 543 of 2012 under European Union legislation (Directive 2010/63/EU) for the Protection of Animals used for Scientific Purposes. The experimental design is illustrated in Figure 2.1. Experimental procedures with animals were conducted between March and August 2021.

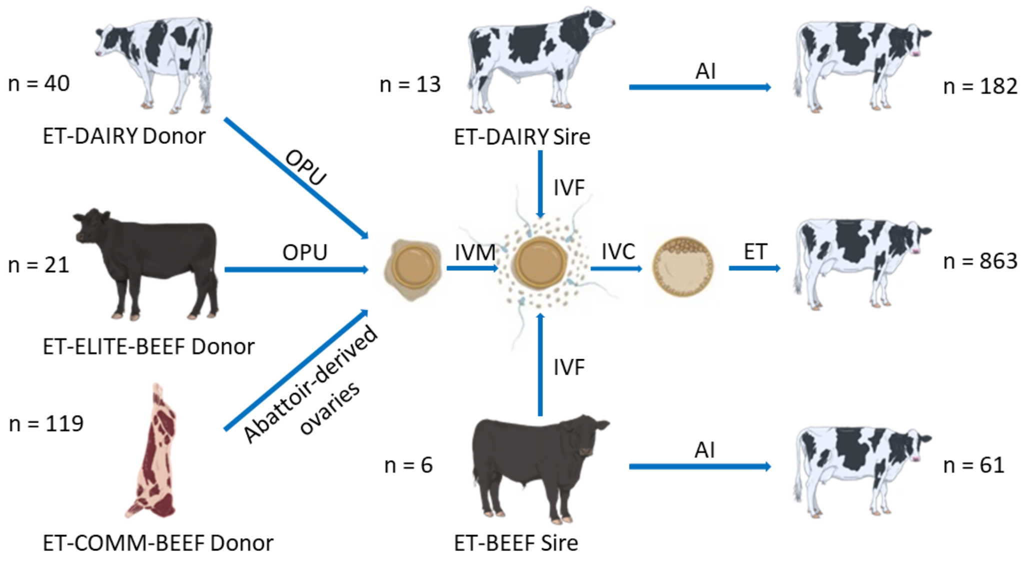


Figure 2.1: Experimental design. Oocytes were collected weekly from the ovaries of elite dairy donors (n = 40 Holstein Friesian and Jersey; ET-DAIRY) and elite beef donors (n = 21 Angus; ET-ELITE-BEEF) using transvaginal ovum pick-up, (OPU). Following OPU, COCs from each donor were transported to the IVF laboratory where they completed maturation, approximately 24 hours after the time of OPU. On one day per week for 6 weeks, COCs were collected from the ovaries of beef heifers of known pedigree following slaughter at a commercial abattoir (ET-COMM-BEEF; n = 119 heifers). Ovaries were removed from the reproductive tract in the abattoir and stored in flasks of PBS at 35 °C until arrival at the laboratory where all surface visible follicles were aspirated to recover the COCs. In vitro maturation (IVM) was conducted as described for those collected from the live donors. Following IVF, presumptive zygotes were cultured in vitro (IVC). The resulting grade 1 blastocysts were either transferred fresh (all d 7) or frozen (d 6, d 7 or d 8) for on-farm thawing and direct transfer (ET). Timed AI was carried out for 243 control cows and ET occurred on d 7 after synchronized ovulation for 863 cows.

2.3.1 Live donors used for oocyte collection

Oocytes were collected weekly from the ovaries of elite dairy donors (n = 40 Holstein Friesian (HF), and Jersey (JE); ET-DAIRY) and elite beef donors (n = 21 Angus (AA); ET-ELITE-BEEF) using transvaginal OPU. The ET-DAIRY donors were a mixture of maiden heifers (n = 7 HF, n = 1 JE; mean age (± SD) 13.3 ± 0.4 months), and cows (n = 22 HF, n = 10 JE; mean parity (± SD) 3.7 ± 1.9; mean DIM (± SD) on the day of the first OPU session 42.1 ± 13.6). The ET-ELITE-BEEF donors were a mixture of heifers (n = 15; mean age (± SD) 14.1 ± 1.2 months) and non-lactating cows (n = 6; mean parity (± SD) 2.3 ± 1.5; mean number of days since last parturition (± SD) on the day of first OPU session 627 ± 148.5). The ET-DAIRY donors were sourced from the Next Generation Herd, which was established at Teagasc Moorepark as a sentinel research herd (top 5% of females nationally for Economic Breeding Index, EBI) to investigate the anticipated phenotypic performance of future animals selected using the EBI (O'Sullivan et al., 2020). The ET-ELITE-BEEF donors were sourced from two herds at the upper end of the Dairy Beef Index (DBI) for the AA breed (Berry et al., 2019).

Ovum pick-up was conducted once per week on each donor for up to 9 weeks, with a total of 21 OPU days during the study. Immediately before OPU, donors received caudal epidural anesthesia (5 ml Procaine Hydrochloride; Adrenacaine, Norbrook Laboratories, Monaghan, Ireland). The rectum was emptied, and the vulva and perineal area were cleaned. Subsequently, the ultrasound transducer (ExaPad, C614P Micro-convex probe 128 element 5.0 – 7.5 MHz; IMV Imaging, Bellshill, United Kingdom) was placed in the vagina, while ovaries were manipulated and positioned per rectum for follicle puncture. Follicles were punctured using an 18-guage 3-inch needle, the follicular fluid was aspirated (Bovine Follicular Aspiration Pump; WTA, College Station, Texas, USA) at a vacuum pressure of approximately 100 mm Hg and cumulus oocyte complexes (COCs) were collected into a 50 ml tube maintained at 35 °C in a tube heater (MicroQ Technologies, Scottsdale, AZ, USA). OPU sessions lasted < 15 min per animal.

All media used for oocyte and embryo handling were proprietary media from Vytelle LLC (Hermiston, OR, USA). Throughout the entire process, oocytes/embryos from individual donors were processed separately. Following OPU, COCs from each donor were identified using a stereomicroscope in a mobile laboratory at the farm. They were then transferred to 2-mL plastic tubes (one tube per donor; Becton Dickenson (BD), Franklin Lakes, New Jersey, USA) containing 1 ml maturation medium (Vytelle LLC), covered by 500 µL of mineral oil in a portable incubator (iQ2, MicroQ Technologies, Scottsdale, AZ, USA) and transported to the IVF laboratory. In the laboratory, the tubes were transferred to an incubator maintained at 38.5 °C with 5% CO2 in air to allow completion of maturation, approximately 24 h from the time of the OPU.

2.3.2 Heifers used for oocyte collection post-slaughter

On one day per week for 6 weeks, COCs were collected from the ovaries of beef heifers of known pedigree following slaughter at a commercial abattoir (ET-COMM-BEEF; n = 119 heifers). These heifers were comprised of AA x HF crossbreds (n = 90), Limousin (LM) x HF crossbreds (n = 18) and other beef or beef x dairy crossbreds (n = 11). The mean age (±SD) at slaughter was 24.6 ± 1.6 months, and the mean carcass weight (± SD) was 291.9 ± 28.7 kg. Ovaries were removed from the reproductive tract in the abattoir and stored in flasks of PBS at 35 °C until arrival at the laboratory (approximately 3 hours post slaughter). Once in the laboratory, all surface visible follicles were aspirated using a 10-ml syringe and 18-guage needle to recover the COCs. In vitro maturation of oocytes was conducted as described for those collected from the live donors.

2.3.3 In vitro fertilization and embryo culture

Frozen thawed semen straws from 13 dairy-breed bulls (n = 7 HF and n = 6 JE), 6 beef-breed bulls (n = 5 AA and n = 1 LM) were used for the fertilization of oocytes harvested from ET-DAIRY donors, ET-ELITE-BEEF donors and ET-COMM-BEEF donors, respectively. A motile sample of sperm was obtained by density gradient separation (Vytelle LLC). Matured COCs were washed in one drop of washing medium and one drop of fertilization medium (Vytelle LLC), transferred in groups of up to 30 oocytes to droplets of fertilization medium (Vytelle LLC) under mineral oil and inseminated with a concentration of approximately 1 million sperm/ml. Gametes were co-incubated for approximately 18 to 24 hours in an atmosphere of 5% CO2 in air at 38.5 °C. Presumptive zygotes were cultured in vitro in first-step culture medium (Vytelle LLC) in an atmosphere of 5% CO2, 6% O2, 89% N2 at 38.5 °C. On d 4, cleaved embryos were transferred to second-step culture medium (Vytelle LLC). Resulting grade 1 blastocysts, classified according to guidelines of the International Embryo Technology Society (Barfield and Demetrio, 2022), were either transferred fresh (all d 7) or frozen (on d 6, d 7 or d 8) for on-farm thawing and direct transfer.

2.3.4 Embryo cryopreservation

Grade 1 blastocysts and expanded blastocysts were removed from culture on d 6 pm (n = 87), d 7 am (n = 220), d 7 pm (n = 42) or d 8 am (n = 49) and exposed to a freezing first-step and freezing second-step medium (Vytelle LLC). Embryos were loaded into 0.25-mL yellow direct transfer straws, placed in a central column surrounded by four columns of the freezing second-step medium separated by air bubbles. After loading, the straws were placed in a freezing machine (EFT-3002, Beltron Instruments, CO, USA) that had been previously stabilized at -6 °C. Two minutes after being placed in the machine, crystallization ("seeding") of the columns immediately below and above the embryo column was conducted. The freezing curve was then initiated, lowering the temperature 0.5 °C per min to -35 °C, after which straws were immersed directly into liquid nitrogen, where they were stored until on-farm thawing for direct transfer into the recipients.

2.3.5 In vitro assessment of embryo survival post freeze-thawing

To obtain an estimate of embryo survival post-thawing, representative samples of frozen blastocysts (n = 107, 3 replicates) were thawed (10 sec in air followed by 30 sec in water at 35 °C) and cultured in vitro, as described above, for 72 hours. Survival (re-expansion) and hatching were recorded at 24, 48 and 72 hours post thawing.

2.3.6 Recipient synchronization for timed AI and timed ET

Recipient cows (all lactating, predominantly HF but including a small number of JE or HF x JE crossbreds; mean parity = 2.9 +/- 1.5) were located in 10 herds, broken into 16 herdlets, with some herds (n = 6) having two separate cohorts of cows enrolled on the synchronization protocol followed by AI and ET two weeks apart, and others (n = 4) having a single cohort of cows enrolled on the synchronization protocol followed by AI and ET. Recipients, within each herdlet, were blocked based on parity, calving date and EBI and randomly assigned to receive AI or ET with an ET-DAIRY, ET-ELITE-BEEF or ET-COMM-BEEF embryo. Recipients were synchronized using a modified 10-d Progesterone-synch protocol (Figure 2.2.) as described by Drake et al. (2020). On d −10 relative to the farm mating start date, a 2 mL i.m. injection of GnRH analog (Ovarelin®, 100 µg of gonadorelin diacetate tetrahydrate; Ceva Santé Animale, Libourne, France) was administered, and a progesterone-releasing intravaginal device containing 1.55 g progesterone (PRID® Delta; Ceva Santé Animale) was inserted. On d −3, a 5 mL i.m. injection of PGF2α (Enzaprost®, 25 mg of dinoprost trometamol; Ceva Santé Animale) was administered. On d −2, a second 5 mL i.m. injection of PGF2α was administered and the PRID was removed. On d −1 (32 h after PRID removal) a second i.m. injection of GnRH was administered. AI was carried out 16 h later and ET occurred on d 7.

The industry target for pregnancy to first service in seasonal-calving systems in 60%. Based on previous publications, the P/S for cows following transfer of a frozen IVP embryo has been reported to be 15 to 20 percentage points less than P/S for cows following either AI or transfer of a fresh IVP embryo (Carrenho-Sala et al., 2016, Pereira et al., 2016, Hansen, 2020). For the current study, a power test indicated that 150 cows per treatment were required to have an 80% chance of detecting, as significant at the 5% level, a decrease in the pregnancy per service event from 60% (target for AI and fresh ET treatments) to 45% in the frozen ET treatments. To mitigate against cows being dropped from the study for various reasons, a greater number of cows was initially enrolled.

A total of 1197 recipients were synchronized as described above, of which 243 (20.3%) were assigned to receive AI (16 h after second GnRH) and 954 (79.7%) were assigned to receive ET on d 7 after presumptive estrus. Treatments were split equally across all herdlets. In 12 of the herdlets, cows assigned to AI (n = 183, 20%) were inseminated using frozen-thawed semen from dairy sires and cows assigned to ET (n = 648) were further randomized to receive ET-ELITE-BEEF (20% fresh, 20% frozen) or ET-DAIRY (20% fresh, 20% frozen) blastocysts. On the remaining four herdlets, cows assigned to AI (n = 60) were inseminated with semen from a beef sire. On one of these four herdlets, cows assigned to ET (n = 48) all received ET-ELITE-BEEF (40% fresh, 40% frozen) blastocysts and on three herdlets, cows assigned to ET (n = 167) all received ET-COMM-BEEF (40% fresh, 40% frozen) blastocysts. On the day of scheduled ET, recipient reproductive tracts were examined by trans-rectal ultrasound to assess corpus luteum (CL) status, presence or absence of cystic structures and uterine health status. Following this examination, 91 cows (9.6%) were deemed unsuitable for ET on the basis of having no dominant follicle or CL present (n = 41), abnormal ovarian structures (presumed follicular cyst or luteal cyst; n = 26) or small CL (< 15 mm diameter; n = 22), and two cows were removed for non-compliance with the synchronization protocol. Thus, the final number of recipients that were suitable for embryo transfer was 863. The uterine horn ipsilateral to the ovary bearing the CL was identified at the time of the ultrasound scan. For cows with corpora lutea on both ovaries (n = 2), the uterine horn ipsilateral to the ovary bearing largest CL was identified.

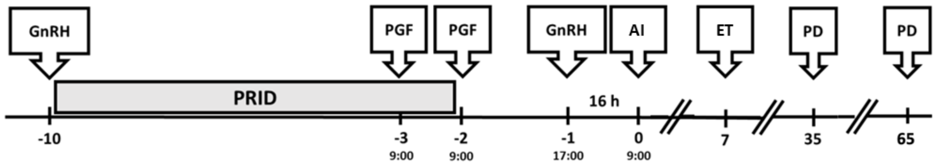


Figure 2.2: Protocol for synchronization of recipients for timed artificial insemination (AI) or timed embryo transfer (ET). On d −10 relative to the planned breeding date, a 2-mL i.m. injection of GnRH analog was administered, and a progesterone-releasing intravaginal device (PRID) was inserted. On d −3, a 5-mL i.m. injection of PGF2α was administered. On d −2, a second 5-mL i.m. injection of PGF2α was administered and the PRID was removed. On d −1 (32 h after PRID removal) a second i.m. injection of GnRH was administered. Timed AI and ET were carried out 16 h and 7 d later, respectively. Pregnancy diagnosis (PD) was carried out by ultrasound scanning on d 32-35 and d 62-65.

2.3.7 Embryo Transfer

Embryos for fresh transfer were removed from culture on the morning of d 7 (d 0 = day of IVF) and loaded into transportation medium (Vytelle LLC) in a clear straw. Fresh embryos were maintained at 38.5 °C in a portable incubator (MicroQ Technologies) during transport from the laboratory to the farm where the recipients were located. At the farm, fresh straws were loaded into ET guns, which were then placed into a temperature-controlled gun warmer (IFT Instruments, Montevideo, Uruguay) at 35 °C until immediately before transfer. Straws containing frozen embryos were removed from liquid nitrogen, held in air for 10 sec and immersed in water at 35 °C for 30 sec. The straw was dried and loaded into an ET gun, which was then placed into a gun warmer until transfer. All embryos were transferred to the uterine horn of the recipient ipsilateral to the ovary bearing the CL, or to uterine horn ipsilateral to the ovary bearing the largest CL if both ovaries had a CL present. All transfers were conducted by one of two experienced technicians.

2.3.8 Pregnancy diagnosis and fetal sexing

Returns to estrus were recorded by visual observation of estrous activity and/or tail paint removal. Pregnancy status was diagnosed in cows that had not returned to estrus by trans-rectal ultrasound scanning on d 32-35. For cows that were diagnosed pregnant, a second ultrasound examination was conducted at d 62-65 after synchronized ovulation to determine whether the pregnancy had been maintained or lost. Fetal sex was determined at the ultrasound exam on d 62-65 in 436 pregnant cows based on the detection and location of the genital tubercle. Fetal loss was calculated as the percentage of those cows pregnant on d 32 that were no longer pregnant on d 62.

2.3.9 Determination of progesterone concentration on d 7

On the day of ET, blood samples were collected into serum tubes (BD Vacutainer, BD, Plymouth, UK) by coccygeal venipuncture from all cows enrolled in the study (n = 1197), including those that received AI, ET and those rejected for ET, to measure serum progesterone (P4) concentration. Blood samples were stored at 4 °C for 24 h before centrifugation at 1922 x g at 4 °C for 15 min. Using a Pasteur pipette, serum was separated and stored at -20 °C until analysis of P4 concentrations by solid-phase radioimmunoassay using a PROG-RIA-CT Kit (DIAsource ImmunoAssays S.A., Louvain-la-Neuve, Belgium) according to the manufacturer´s instructions. The sensitivity of the assay was 0.05 ng/ml. The inter-assay coefficients of variation for quality control samples were 12.1% (low), 7.4% (medium), and 5.5% (high), respectively. The intra-assay coefficients of variation were 16.3% (low), 13.0% (medium), and 8.7% (high).

2.3.10 Sex determination of IVP embryos

Day 7 (n = 63) and d 8 (n = 40) IVP blastocysts produced in 3 replicates (i.e., 3 independent days of ovary collection) using abattoir-derived ovaries as the source of oocytes were snap-frozen, and crude DNA lysates were prepared by adding 20.0 µl of PCR Buffer supplemented with Proteinase K (1 mg/ml) to each sample. The samples were incubated at 56 °C for 30 min with gentle shaking and then heat inactivated at 94 °C for 10 min. A 20.0 µl PCR assay targeting the Amelogenin gene was prepared using 5.0 µl of the crude lysate, 0.5 µM of each primer, AML-X and AML-Y, 1 X DreamTaq PCR buffer and 0.5 Units of DreamTaq DNA polymerase (ThermoFisher, Waltham, Massachusetts, USA). The PCR amplification consisted of an initial 5-min activation at 95 °C, followed by 25 cycles of amplification; denaturation (60 sec at 95 °C), annealing (60 sec at 60 °C) and extension (90 sec at 72 °C), followed by a final incubation at 72 °C for 10 min and a hold at 4 °C. The resulting PCR products were analyzed using gel electrophoresis on a 2.5% agarose gel and the sex of each blastocyst was determined. Male blastocysts produced two PCR products at 241 bp and 178 bp and female blastocysts produced a single PCR product at 241 bp (Gokulakrishnan et al., 2012).

2.4 Statistical Analysis

All statistical analyses were conducted using SAS v. 9.4 (SAS Institute, Cary, NC). Before analysis, data were assessed for normality and transformed using optimum Box-Cox transformations where necessary (TRANSREG). Data relating to oocyte collection data, blastocyst percentage and blastocyst number were analyzed using generalized linear mixed models (GLIMMIX) with repeated measures. Several models were constructed to compare the oocyte collection and embryo production between donors and embryo production between sires used. Due to parity structure differences between elite dairy donors and elite beef donors, data were analyzed separately for each type of donor. In each model assessing oocyte collection, the model fixed effects included donor, parity of donor (nulliparous vs parous) and the collection number. In each model assessing blastocyst rate (%) and total blastocyst yield (n) per OPU session, the model fixed effects included donor, parity of donor and sire.

A total of 1197 cows were enrolled in the study and synchronized for either AI or ET. Before the d 32 pregnancy diagnosis, 97 cows were removed from the study: two cows had no recorded service event, 91 cows were deemed unsuitable on the day of ET, three cows were culled for reasons unrelated to the study and one cow died due to reasons unrelated to the study; therefore, 1100 cows were included in the d 32 analysis. Before the d 62 pregnancy diagnosis, a further 4 cows were removed: three cows were culled for reasons unrelated to the study and one cow was sold; therefore, 1096 cows were included in the d 62 analysis. Pregnancy data were analyzed using generalized linear mixed models, with a binary distribution specified. P/S did not differ between AI services that used dairy or beef semen ($P = 0.969$), and these services were combined and reported as AI. P/S did not differ between the ET-ELITE-BEEF and ET-COMM-BEEF ($P = 0.395$), and these services were combined and are reported as ET-BEEF. Multiple variables and interactions were included as fixed effects and retained in the final GLIMMIX models when $P \leq 0.25$. Several models were constructed to compare the type of service event: (1) AI vs. ET (all cows); (2) AI vs. ET-BEEF vs. ET-DAIRY; (3) AI vs. Fresh ET vs. Frozen ET; and (4) AI vs. ET-BEEF-Fresh vs. ET-BEEF-Frozen vs. ET-DAIRY-Fresh vs. ET-DAIRY-FROZEN. In each model, treatment, parity and serum P4 concentration (categorized into quartiles; Q1: < 5.79 ng/ml, n = 274; Q2: 5.79 - 7.36, n = 274; Q3: 7.37 - 9.42, n = 274 and Q4: > 9.43, n = 275) were included as fixed effects and herd was included as a random effect. Box-and-whisker plots were used initially to visualize the variation in serum P4 concentrations on d 7 after synchronized estrus in cows assigned to AI vs. ET, including a separate plot for cows that were rejected for ET. Cows that were deemed unsuitable for ET on the scheduled day of transfer were then added back into the same models to determine the effect of treatment on pregnancy/cow synchronized (P/Sync). For assessing the effect of each type of service event on the incidence of embryonic loss, 538 cows that were diagnosed pregnant on d 32 were available for inclusion in the analysis. The GLIMMIX model included treatment and parity, with herd included as a random effect. For all binary outcome variables analyzed, the GLIMMIX model output values for treatment means are reported, which equate to the predicted probability of that event. Finally, an additional analysis was undertaken on the data related to frozen ET only. For the 397 cows that received frozen ET, a Chi-Square test of independence was completed to determine the association between the day of culture (age) of the embryo at the time it reached blastocyst stage and was cryopreserved and the associations with P/S on d 32 and 62, and with embryo loss between d 32 and d 62.

2.5 Results

2.5.1 Factors associated with oocyte recovery and in-vitro embryo production

The oocyte recovery rates and in vitro embryo development results are summarized in Table 2.1 and Figure 2.3. The donor used for OPU was associated with the mean number of oocytes collected per OPU session (mean: 15.6, range: 4.3 to 36.3), the mean number of viable embryos per OPU/IVF session (mean: 3.8, range: 0 to 15) and with the percentage of oocytes that developed to transferable embryos (mean: 25.0%, range: 0% to 75%). The yield of transferrable embryos per donor per OPU/IVF session remained relatively constant over time (Figure 2.4). Blastocyst developmental rates per sire are presented in Table 2.2 and Figure 2.3. The sire used in IVF was associated with blastocyst yield (mean: 23.1%, range: 5% to 46%) and the number of transferable embryos per IVF session (mean: 3.6, range: 0.5 to 7.1).

Table 2.1: Oocyte recovery and embryo production from ET-DAIRY, ET-ELITE-BEEF and ET-COMM-BEEF donors.

	n OPU/aspiration sessions	Total oocytes in IVM	Mean oocytes/donor/session (range)	Total embryos in IVC	Total transferable embryos	% transferable embryos	Mean transferable embryos/donor/session (range)
ET-DAIRY							
COWS	148	2621	17.7 (4.3-36.3)	2360	657	27.8	4.4 (0.0-9.3)
HEIFERS	26	311	12.0 (5.0-19.0)	270	37	13.7	1.4 (0.0-4.0)
TOTAL	174	2932	16.9 (4.3-36.3)	2630	694	26.4	4.0 (0.0-9.3)
ET-ELITE-BEEF							
COWS	40	773	19.3 (11.8-29.8)	712	366	51.4	9.2 (2.3-15.0)
HEIFERS	104	1508	14.5 (7.4-25.2)	1310	288	22.0	2.8 (1.5-6.0)
TOTAL	144	2281	15.8 (7.4-29.8)	2022	654	32.3	4.5 (1.5-15.0)
ET-COMM-BEEF	119	2187	18.4 (1.0-86.0)	2042	333	16.3	2.8 (0.0-16.0)

Table 2.2: Effect of sire used for IVF on outcomes related to embryo development. Table includes sires used for ≥ 10 IVF sessions.

Sire[1]	n IVF sessions	Total embryos in IVC	Total transferable embryos	Blastocyst Rate %*	Mean transferable embryos/IVF session*
D1	21	259	37	14.3	1.8
D2	11	167	25	15.0	2.3
D3	29	487	146	30.0	5.0
D4	39	556	214	38.5	5.5
D5	12	223	85	38.1	7.1
B1[2]	85	1354	236	17.4	2.8
B2[3]	56	945	170	18.0	3.0
B3[4]	35	462	121	26.2	3.5
B4[4]	48	689	222	32.2	4.6
B5[4]	33	503	229	45.5	6.9

[1] Sires are grouped by type: D = ET-DAIRY; B = ET-BEEF.

[2] B1 was used for IVF on oocytes from both ET-COMM-BEEF (n = 63 IVF sessions) and ET-ELITE-BEEF (n = 22 IVF sessions) donors; data are included here together (abattoir and ovum pick-up from live donors).

[3] B2 was used for ET-COMM-BEEF only.

[4] B3 - B5 were used for ET-ELITE-BEEF oocytes only.

*Blastocyst rate (P = 0.025) and the mean number of transferable embryos per IVF session differed by sire (P = 0.011).

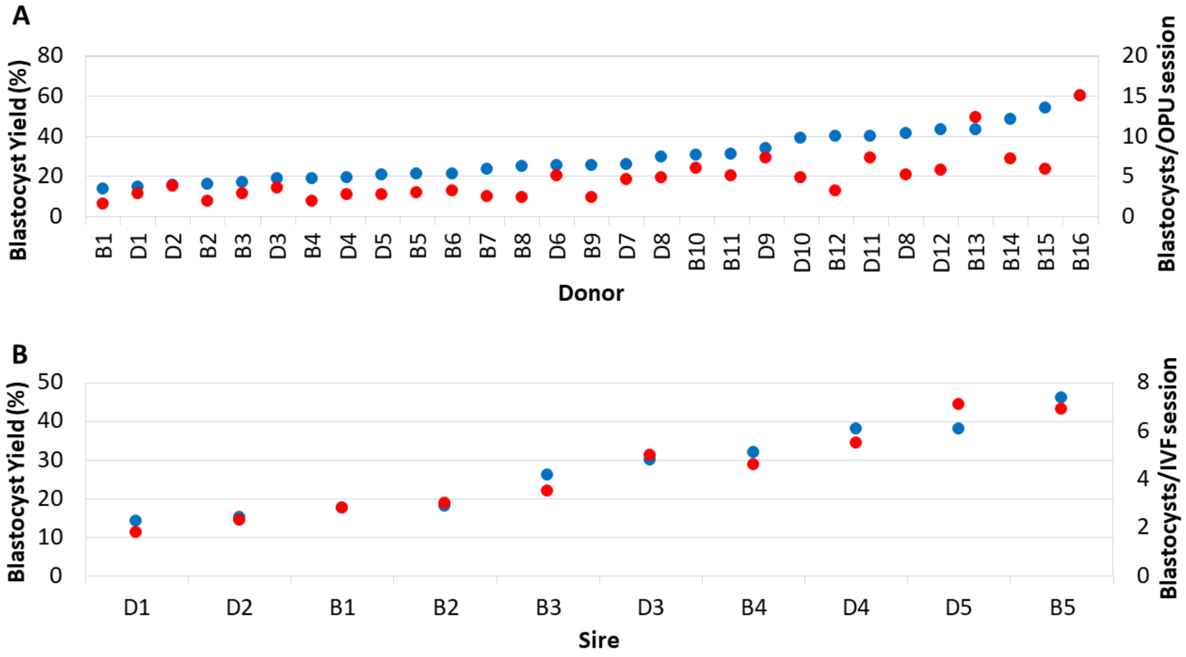


Figure 2.3: (A) Mean blastocyst yield per session as a percentage of presumptive zygotes placed in culture (●) and total number of blastocysts per OPU/IVF session (●) by ET-DAIRY donor (D1 to D12 and ET-ELITE-BEEF donor (B1 to B16) that were used for ≥ 6 OPU sessions. Donor affected blastocyst yield (Dairy, P = 0.001; Beef, P = 0.001) and the number of blastocysts per OPU/IVF session (Dairy, P = 0.014; Beef, P = 0.002). (B) Mean blastocyst yield per session as a percentage of presumptive zygotes placed in culture (●) and total number of blastocysts per OPU/IVF session (●) by ET-DAIRY sire (D1 to D5 and ET-ELITE-BEEF sire (B1 to B5) that were used for ≥ 10 IVF sessions. Sire affected blastocyst yield (Dairy, P = 0.025; Beef, P < 0.001) and the number of blastocysts per OPU/IVF (Dairy, P = 0.011; Beef, P < 0.001).

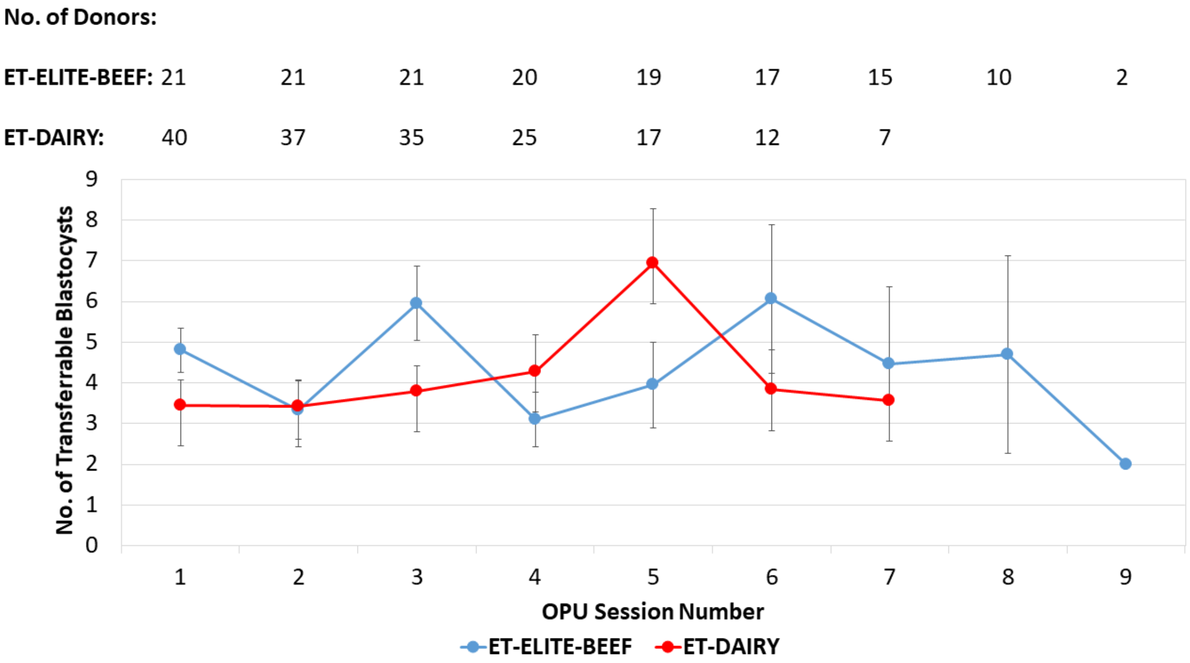


Figure 2.4: Mean blastocyst yield per OPU/IVF session for ET-ELITE-BEEF donors (n = 9 OPU sessions) and ET-DAIRY donors (n = 7 OPU sessions). Blastocyst yield was not affected by session number for ET-DAIRY donors (P = 0.235), but tended to be affected by session number for ET-ELITE-BEEF donors (P = 0.069).

2.5.2 Blastocyst survival post-thawing

Blastocyst survival and hatching results following freezing/thawing are summarized in Table 2.3 and illustrated in Figure 2.5. By 24 h after thawing, 94.4% of blastocysts had re-expanded and 19.6% had hatched. By 48 h, 96.3% had re-expanded and 51.4% had hatched. At the final assessment, 72 h after thawing, 96.3% had re-expanded and 72.0% had hatched.

Table 2.3: Survival and hatching at 24, 48 and 72 h post freezing/thawing of in vitro produced d 7 or d 8 bovine blastocysts.

	n	24 h		48 h		72 h	
		Survival (%)	Hatching (%)[1] [%][2]	Survival (%)	Hatching (%)[1] [%][2]	Survival (%)	Hatching (%)[1] [%][2]
d 7	65	61 (93.8)	10 (15.4) [16.4]	63 (96.9)	31 (47.7) [49.2]	63 (96.9)	45 (69.2) [71.4]
d 8	42	40 (95.2)	11 (26.2) [27.5]	40 (95.2)	24 (57.1) [60.0]	40 (95.2)	32 (76.2) [80.0]
Total	107	101 (94.4)	21 (19.6) [20.8]	103 (96.3)	55 (51.4) [53.4]	103 (96.3)	77 (72.0) [74.8]

[1] as a percentage of total blastocysts

[2] as a percentage of surviving blastocysts

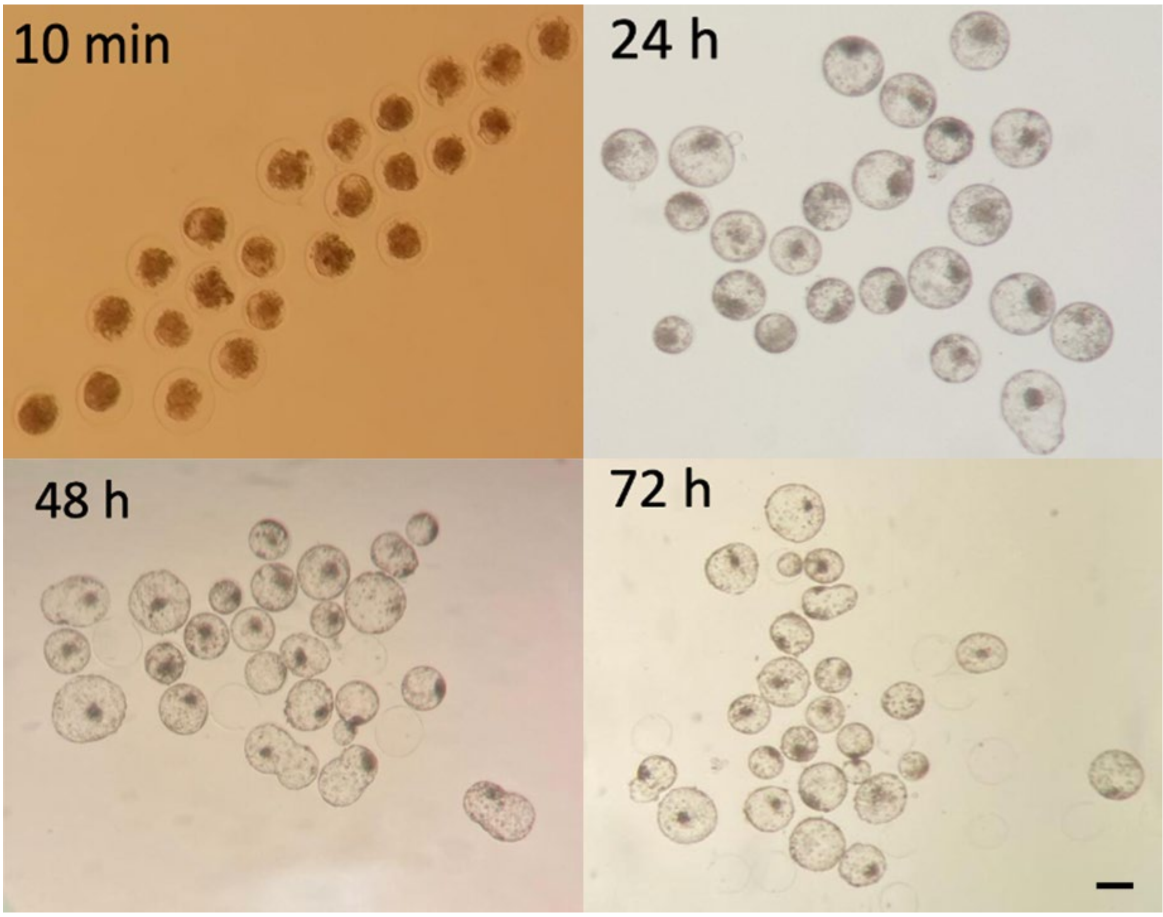


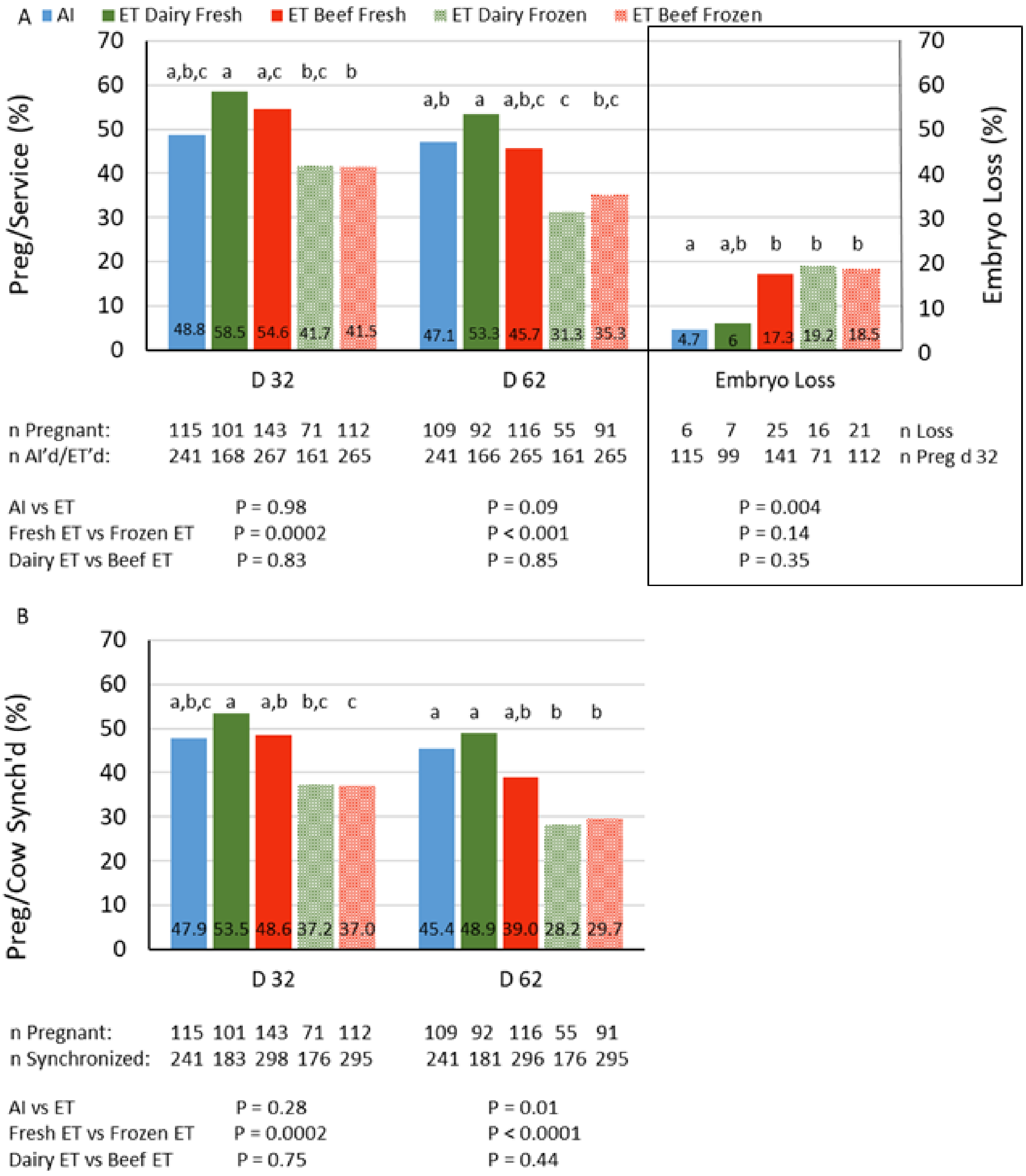
Figure 2.5: Representative images of blastocyst survival and hatching post-freezing/thawing. Frozen blastocysts (n = 107, 3 replicates) were thawed and cultured in vitro for 72 h. Survival (re-expansion) and hatching were recorded at 24, 48 and 72 h post thawing. Representative images of blastocysts were captured at 10 min, 24 h, 48 h and 72 h post thawing. For more details, see text and Table 2.3. Scale bar represents 100 µm.

2.5.3 Factors affecting the probability of pregnancy

Pregnancy results (P/S and P/Sync) are presented in Figure 4.6. Overall, the predicted probability (95% CI) of P/S at d 32 was not different (P = 0.983) between AI (48.8%; 42.1, 55.6) and ET (48.9%; 44.9, 52.9) and did not differ between ET-DAIRY and ET-BEEF (Dairy: 50.3%; 44.3, 56.2 vs Beef: 48.1%; 43.2, 53.0; P = 0.827). Overall, P/S was less (P = 0.0002) on d 32 following transfer of frozen embryos (41.6%; 36.6, 46.7) compared with fresh embryos (56.1%; 51.0, 61.1), and the magnitude of the difference between frozen embryos and fresh embryos was similar for ET-DAIRY [41.7% (33.9, 49.8) vs 58.5% (50.4, 66.1), respectively; P = 0.027] and ET-BEEF [41.5% (35.3, 48.1) vs 54.6% (48.2,60.9), respectively; P = 0.029] (Figure 2.6A).

Overall, the predicted probability (95% CI) of P/S at d 62 tended to be greater (P = 0.092) following AI compared with ET (AI: 47.0% (40.4, 53.7) vs. ET: 40.6% (36.8, 44.6)) and did not differ between ET-DAIRY and ET-BEEF: (42.1% (35.7, 48.8) vs. 39.7% (34.8, 44.9) for Dairy vs. Beef, respectively; P = 0.85). Overall, P/S was greater (P < 0.001) on d 62 following transfer of fresh embryos (48.4%; 43.3, 53.4) compared with frozen embryos (32.9%; 28.3, 37.8). The magnitude of the difference between fresh embryos and frozen embryos was larger for ET-DAIRY (53.3% vs 31.3%; P = 0.0009) than for ET-BEEF (45.7% vs 35.3%; P = 0.122; Figure 2.6A).

To compare the efficiency of AI (i.e., all synchronized cows inseminated) versus ET (9.6% of synchronized cows deemed unsuitable for ET), data were analyzed to determine P/Sync for each treatment. The predicted probability of P/Sync at d 32 was not different between AI and ET (47.9% vs 43.8%; P = 0.279). P/Sync was not different between ET-DAIRY and ET-BEEF at d 32 (45.4% vs. 42.8%; P = 0.746) and was greater following transfer of a fresh embryo than following transfer of a frozen embryo (50.5% vs 37.1%; P = 0.0002). The magnitude of the difference between fresh embryos and frozen embryos was larger for ET-DAIRY (53.5% vs. 37.2%; P = 0.024) than ET-BEEF (48.6% vs. 37.0%; P = 0.0498) (Figure 2.6B), primarily driven by the greater percentage of cows that became pregnant in the ET-DAIRY treatment after transfer of a fresh embryo. By d 62, P/Sync was less (P = 0.01) for cows assigned to ET (35.9%) compared with cows assigned to AI (45.4%). P/Sync was not different between ET-DAIRY and ET-BEEF at d 62 (38.5% vs. 34.3%; P = 0.438) and was greater for fresh embryos than frozen embryos (42.8% vs 29.1%; P < 0.0001). The magnitude of the difference between fresh embryos and frozen embryos was larger for ET-DAIRY (48.9% vs. 28.2%; P = 0.0009) than for ET-BEEF (39.0% vs. 29.7%; P = 0.141; Figure 2.6B).



A

	D 32	D 62	Embryo Loss	
n Pregnant:	115 101 143 71 112	109 92 116 55 91	6 7 25 16 21	n Loss
n AI'd/ET'd:	241 168 267 161 265	241 166 265 161 265	115 99 141 71 112	n Preg d 32
AI vs ET	P = 0.98	P = 0.09	P = 0.004	
Fresh ET vs Frozen ET	P = 0.0002	P < 0.001	P = 0.14	
Dairy ET vs Beef ET	P = 0.83	P = 0.85	P = 0.35	

B

	D 32	D 62
n Pregnant:	115 101 143 71 112	109 92 116 55 91
n Synchronized:	241 183 298 176 295	241 181 296 176 295
AI vs ET	P = 0.28	P = 0.01
Fresh ET vs Frozen ET	P = 0.0002	P < 0.0001
Dairy ET vs Beef ET	P = 0.75	P = 0.44

Figure 2.6: (A) Predicted probability of pregnancy/service event (P/S) (%) depending on service type at d 32 and d 62, and predicted probability of embryo loss (%) between d 32 and d 62 depending on service type. (B) Predicted probability of pregnancy/cows synchronized (P/Sync) (%) depending on service type at d 32 and d 62. P/Sync includes cows that were synchronized for ET but were deemed unsuitable to receive ET on the day of timed-ET. Values not sharing a common letter (a, b, c) differ ($P < 0.05$). Numbers under each bar are actual numbers of cows that were pregnant, received AI or ET and were synchronized.

2.5.4 Factors affecting the probability of embryonic loss between d 32 and d 62

Across all cows that were diagnosed pregnant on d 32, the predicted probability of pregnancy loss between d 32 and 62 was greater (P = 0.004) for ET (15.1%; 11.0, 20.5) compared with AI (4.7%; 2.0, 10.4) (Figure 2.6A). There was no overall effect of embryo type (fresh, 12.2% vs. frozen, 18.8%; P = 0.141 or dairy vs beef; P = 0.347) on probability of embryo loss; frozen beef (18.5%), fresh beef (17.3%), frozen dairy (19.2%) and fresh dairy (6.0%). Embryo loss following the transfer of fresh dairy embryos tended to be less than the transfer of frozen dairy embryos (P = 0.06). There was no association between P4 concentration (P = 0.934) or days in milk (P = 0.431) on embryonic loss between d 32 and 62, but parity tended to be associated with embryo loss across all treatments (P = 0.071). Embryonic loss between d 32 and d 62 occurred in 14.9% of parity 1 cows compared to 6.3% of parity 2, 11.2% of parity 3 and 15.6% of parity 4 cows.

Day of culture when blastocysts were cryopreserved, based on when the embryo reached the blastocyst stage, tended to be associated with P/S on d 32 (P = 0.078) and on d 62 (P = 0.052), but was not associated with embryo loss between d 32 and d 62 (P = 0.346). These data are summarized in Table 2.4.

Table 2.4: Effect of blastocyst age at freezing on pregnancy/embryo transfer at d 32 and d 62. Grade 1 blastocysts and expanded blastocysts were removed from culture on d 6 pm, d 7 am, d 7 pm or d 8 am.

Day (d) of blastocyst cryopreservation[1]	Number of transfers	n Pregnant d 32 (%)	n Pregnant d 62 (%)	n Embryo Loss d 32-d 62 (%)[2]
d 6 pm	87	43 (49.4)	33 (37.9)	10 (23.2)
d 7 am	219	98 (44.7)	82 (37.4)	16 (16.3)
d 7 pm	42	15 (35.7)	10 (23.8)	5 (33.3)
d 8 am	49	14 (28.6)	10 (20.4)	4 (28.6)
Total	397	170 (42.8)	135 (34.0)	35 (20.6)

[1] Day of blastocyst cryopreservation tended to be associated with P/S on d 32 (P = 0.078) and on d 62 (P = 0.052), but was not associated with embryo loss between d 32 and d 62 (P = 0.346).

[2] Embryo loss was calculated as n pregnant d 32 minus n pregnant d 62 divided by n pregnant d 32.

2.5.5 Serum P4 concentration on d 7

Serum P4 concentration on d 7 was associated with P/S at both d 32 and d 62 (P < 0.0001; Figure 2.7). Mean serum P4 concentration (and 95% CI) was less (P = 0.04) in cows assigned to AI (7.22 ng/ml; 6.60, 7.85) compared with cows that received ET (7.76 ng/ml; 7.20, 8.31). This occurred because cows that were deemed unsuitable as recipients on the day of scheduled ET had the least (P < 0.0001) P4 concentrations (3.59 ng/ml; 3.46, 5.04) (Figure 2.7A). Cows with greater serum concentrations of P4 on d 7 had greater P/S at both d 32 (Figure 2.7B) and d 62 (Figure 2.7C, Table 2.5). The distribution of d 7 serum P4 concentrations in lactating dairy cows that were pregnant or non-pregnant on d 32 is displayed in Figure 2.7D. Cows in Q1 (i.e., least P4 concentration) had lesser probability (P < 0.0001) of being pregnant on d 32 (33.4%) and d 62 (30.1%) than cows in Q2 (d 32 = 45.7%; d 62 = 42.2%), Q3 (d 32 = 55.6%; d 62 = 49.2%) or Q4 (d 32 = 61.2%; d 62 = 54.6%). Cows in Q2 had similar probability of pregnancy (P = 0.102) as cows in Q3, and had lesser probability of becoming pregnant than cows in Q4 (P = 0.002). There was no interaction (P = 0.71) between P4 quartile and service treatment on P/S.

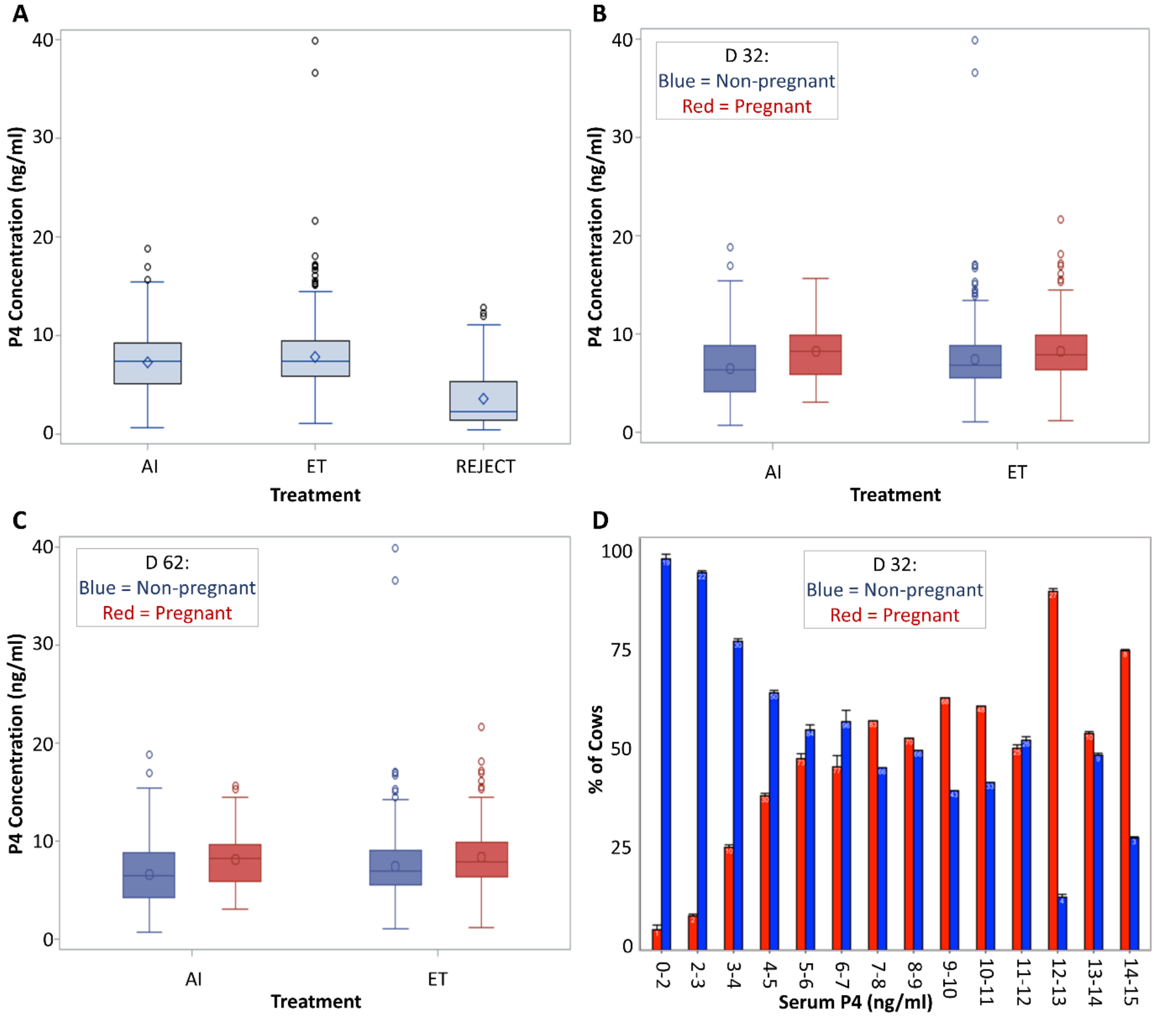


Figure 2.7: Serum progesterone (P4) concentrations on d 7 after synchronized ovulation for all cows that were assigned to receive AI or ET. (A) Box and whisker plot indicating variation in serum P4 concentration on d 7 for cows that received AI or ET, and for cows that were rejected for ET following transrectal ultrasound examination on d 7. Progesterone concentration differed between cows that were rejected and cows that received AI or ET ($P < 0.0001$). The ends of the whiskers represent the maximum (Quartile 3 + 1.5*Inter-quartile range) and minimum (Quartile 1 - 1.5*Inter-quartile range) P4 concentration for each group. The upper and lower quartiles make up the boundaries of the box. The height of the box represents the interquartile range, and the median is indicated by the horizontal line within the box. The arithmetic mean serum P4 concentration for each group is indicated by the circle within the box and outliers are identified by the circles outside of the ends of the whiskers. (B) Interquartile ranges in d 7 serum P4 concentrations in cows that received AI or ET that were non-pregnant (blue) or pregnant (red) 32 d after estrus. Mean serum P4 concentration did not differ between cows that were diagnosed pregnant or non-pregnant on d 32 after the service with AI or ET ($P = 0.113$). (C) Interquartile ranges in d 7 serum P4 concentration in cows serviced using AI or ET that were non-pregnant (blue) or pregnant (red) 62 days after estrus. Mean serum P4 concentration did not differ between pregnant and non-pregnant (d 62) cows serviced using AI or ET ($P = 0.262$). (D) Distribution of d 7 serum P4 concentrations in lactating dairy cows that were pregnant (red) or non-pregnant (blue) on d 32 after AI or ET service events.

Table 2.5: Predicted probability values for both pregnancy/service event (P/S) and embryo loss in lactating dairy cows separated into quartiles based on serum progesterone (P4) concentration on d 7. Values in parentheses represent 95% CI.

			P/S Day 32	P/S Day 62	Embryo Loss
Quartile	P4 ng/ml	n	Mean (95% CI)	Mean (95% CI)	Mean
1	< 5.79	274	33.4%^{a} (27.7-39.7)	30.1%^{a} (24.5-36.4)	11.7%
2	5.79 - 7.36	274	45.7%^{b} (39.1-52.4)	42.2%^{b} (35.7-49.0)	9.9%
3	7.37 - 9.42	274	55.6%^{bc} (49.1-61.9)	49.2%^{bc} (42.8-55.7)	12.0%
4	> 9.42	275	61.2%^{c} (54.7-67.4)	54.6%^{c} (48.0-61.1)	11.3%

$^{a\text{-}c}$Mean values in the same column with different superscripts differ ($P < 0.025$).

2.5.6 Sex ratio

Overall, the sex ratio (M:F) of fetuses scanned on d 62 differed (P = 0.001) for AI (43.2:56.8; n = 109) and ET (61.1:38.9; n = 331). For cows that received ET, there was no difference in sex ratio between beef embryos vs. dairy embryos (60.5:39.5 vs. 62.1:37.9, respectively; P = 0.96), fresh embryos vs. frozen embryos (61.4:38.6 vs. 60.6:39.4, respectively; P = 0.988). The sex ratio determined in IVP blastocysts (n = 103) was 61.2:38.8 (M:F), and was not affected by day of culture (d 7 vs. d 8) when the blastocyst stage was reached (61.2:38.8 vs. 65.4:34.6, respectively; P = 0.685).

2.6 Discussion

In vitro embryo production is now an established technology in the toolbox of assisted reproductive technologies available to farmers and breeding companies. Despite greater fixed costs, it offers significant advantages over traditional superovulation and embryo transfer (MOET) including increased numbers of embryos produced per donor per unit of time and greater flexibility in sire usage. In addition, IVF facilitates more efficient use of rare or high-cost semen straws, and thanks to the predictability of a donor’s performance (once a donor has been collected previously) in terms of oocyte and embryo yields, IVF can simplify the logistics of recipient synchronization and management. All of these factors have contributed to the marked increase in the use of IVF compared with MOET as a method of generating embryos for transfer worldwide (Viana, 2022).

The mean number of oocytes recovered (Dairy: 16.9, Beef: 15.8) and mean yield of transferable embryos (Dairy: 4.0, Beef: 4.5) per OPU session was highly variable between donors, with means ranging from 4.3 to 36.3 oocytes per donor per OPU session and zero to 15 transferable embryos per donor per IVF session. This observation is consistent with published commercial data (Demetrio et al., 2020), where dairy donors produced an average of 15.6 oocytes and 3.6 viable embryos per OPU session and beef donors produced an average of 19.2 oocytes and 5.2 viable embryos per OPU session. Similar to superovulation, yield per session was repeatable within donor. In agreement with other studies (e.g., Ortega et al., 2018), significant variation was noted between sires used in IVF, with blastocyst yield ranging from 14.3% to 45.5%. Such sire variation is not unusual; for example, significant variation in field fertility exists amongst bulls used in AI, despite rigorous assessments of sperm quality before semen is released (Fair and Lonergan, 2018). Furthermore, sire field fertility does not correlate well with IVF success (Al Naib et al., 2011).

Over the past decade, the success of commercial IVP has been reflected in the number of IVP embryos transferred annually now superseding that of in vivo-derived embryos (Crowe et al., 2021, Viana, 2022). Nevertheless, embryos generated in vitro still differ from their in vivo-produced counterparts, particularly in terms of cryotolerance, P/S and embryo loss after freeze-thawing (Pontes et al., 2009, Sartori et al., 2018). Given that ET bypasses potential issues in the recipient cow related to oocyte quality, sperm transport and fertilization, one would expect P/S to be greater than that achieved with AI or natural service; however, this potential is typically not observed in practice (Hansen, 2020). It is well accepted that the quality of the oocyte at the start of the process is the key factor determining the proportion of oocytes developing to the blastocyst stage (Rizos et al., 2002, Lonergan and Fair, 2016). Culture conditions throughout IVP, particularly during post-fertilization culture, influence the quality, including cryotolerance, and developmental potential of the early embryo (Rizos et al., 2002, Gad et al., 2012). In the current study, P/ET was similar to P/AI when embryos were transferred fresh, demonstrating the potential for fresh ET to be used effectively in a seasonal dairy production system without compromising subsequent calving pattern for recipient dams. It is important to note, however, that across all cows assigned to be recipients, 9.6 % were deemed unsuitable on the day of scheduled ET. Hence, the proportion of synchronized cows that could become pregnant following ET would be less than reported for cows assigned to be artificially inseminated. One benefit of removing unsuitable cows was that they could be re-bred, receive veterinary treatment for any problems identified, or be assigned to be culled without further expenditure on reproductive interventions or insemination costs. The financial cost and loss of days in milk that arises following synchronization of cows that are subsequently unsuitable for transfer also need to be considered. Nonetheless, the results of this study illustrate the feasibility of using IVF technology in a seasonal system.

Pregnancies per ET have generally been reported to be less for IVP embryos compared with in vivo-derived embryos (Pontes et al., 2009, Carrenho-Sala et al., 2016, Pereira et al., 2016, Sartori et al., 2018). In addition, pregnancy loss has been reported to be greater for IVP embryos than for either in vivo-derived embryos or pregnancies from AI, further reducing reproductive efficiency achieved with IVP embryos (Carrenho-Sala et al., 2016, Pereira et al., 2016, Sartori et al., 2018). Thus, strategies to improve P/ET and reduce pregnancy loss are needed to maximize the efficiency of IVP-ET programs in cattle.

The majority of cryopreserved embryos that were later transferred to recipient cows were frozen on the morning of d 7. Day of culture when blastocysts were cryopreserved, based on when the embryo reached the blastocyst stage, tended to affect P/S on d 32 or d 62. Failure to find a significant association in this study was likely due to the relatively small number of embryos in each age category. It is interesting to note that P/ET appeared to diminish and embryo loss appeared to increase as embryo age at cryopreservation increased. This is consistent with previous observations that the timing of blastocyst formation affects blastocyst quality (Dinnyes et al., 1999). This association between embryo age at cryopreservation and P/S merits further investigation in future studies that are adequately powered to specifically address this issue.

In the current study, pregnancy loss between d 32 and d 62 was significantly greater following ET, particularly following transfer of frozen-thawed embryos (12.2% vs 18.8% loss in fresh vs frozen embryos respectively). Seasonal production systems require excellent herd reproductive performance, and hence any management strategy that reduces P/S and increases embryonic losses will reduce profitability (Shalloo et al., 2014). Therefore, uptake of IVP and ET in seasonal dairy production systems will largely focus on use of fresh ET. Further investigation is necessary to improve the cryopreservation of IVP embryos.

The relationship between circulating P4 and uterine receptivity has been well described (Lonergan and Sanchez, 2020). Elevated P4 concentrations in the first week after conception have been associated with accelerated post-hatching conceptus elongation, mediated through advancement in the regular temporal changes in the uterine endometrial transcriptome (Forde et al., 2009) and alterations in the uterine lumen fluid (histotroph) composition (Simintiras et al., 2019). Consistent with other studies that have shown positive linear (Herlihy et al., 2013) and quadratic relationships (Diskin et al., 2006), there was a positive, quadratic relationship between serum P4 on d 7 and likelihood of pregnancy establishment, irrespective of method of breeding (AI or ET); cows with P4 concentrations in the quartile with the least P4 concentrations were almost half as likely to become pregnant than those in the upper quartile. Furthermore, Wallace et al. (2011) reported that administration of hCG at the same time as ET increased incidence of accessory CL formation, serum P4 in pregnant recipients, and P/S and reduced early embryonic losses after transfer. Treatment of cows with GnRH on d 5 after estrus was reported to also reduce embryo loss between d 33 and d 60 in heifers that received fresh IVP embryos (Garcia-Guerra et al., 2020). In the current study, P/S increased quadratically as P4 concentration on d 7 increased, plateauing at around 5-8 ng/ml.

To determine whether the deviation in sex ratio was due to inadvertent preferential selection of male embryos for transfer or was due to preferential survival of male embryos post transfer, a representative number of embryos (n = 103) was sexed post-thawing. Our observations indicate that the bias towards male fetuses on d 62-65 in recipient cows was mirrored by a similar sex bias in IVP blastocysts on d 7 and d 8, indicating similar survival of male and female embryos after transfer to recipients on d 7. A deviation in sex ratio towards more males following IVF in cattle was first described over 30 years ago (Avery et al., 1991, 1992). Subsequently, several studies reported that this bias was due to impaired imprinted X chromosome inactivation (Gutierrez-Adan et al., 2001, Wrenzycki et al., 2002, Tan et al., 2016). A similar phenomenon of greater male birth rate following IVF has been reported in humans (Dean et al., 2010, Maalouf et al., 2014).

The difference in developmental rates between male and female embryos in vitro (Gutierrez-Adan et al., 1996) has been attributed to differences in metabolic activity of X-linked enzymes involved in energy metabolism (Tiffin et al., 1991). The X-linked gene glucose-6-phosphate dehydrogenase (G6PD) has been considered a likely candidate for involvement in sex differences due to impaired imprinted X chromosome inactivation in IVF female bovine embryos (Wrenzycki et al., 2002). Furthermore, glucose concentration > 2.5 mM during embryo culture impairs bovine embryo development and increases the sex ratio towards males, most likely as a result of increased pentose-phosphate pathway activity in female embryos (Kimura et al., 2008). Irrespective of the cause of the deviation in sex ratio in IVP embryos, this can be overcome through the use of sexed semen in IVF (Bermejo-Alvarez et al., 2010).

2.7 Conclusion

In conclusion, P/S was similar for AI and ET on d 32 post-estrus, but subsequent pregnancy loss was greater for ET than for AI. Transfer of frozen embryos resulted in fewer pregnancies and tended to be associated with greater embryo loss between d 32 and d 62 compared with fresh ET or AI. Nonetheless, our results demonstrate the potential for using OPU/IVF to produce embryos from high genetic merit donors within the calendar constraints of the seasonal-calving system. In the present era where beef on dairy is very topical, IVP and ET provides a clear route accelerating genetic gain in both dairy breeds and beef breeds suitable for crossing with dairy dams, and also producing beef breed embryos using abattoir-derived ovaries to allow premium quality beef to be produced from dairy dams. Additional research is required to improve fertility performance and reduce embryo loss of IVP embryos.

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Chapter 3: Incidence and timing of pregnancy loss following timed artificial insemination or timed embryo transfer with a fresh or frozen in vitro-produced embryo

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3.1 Abstract

The aim of this study was to characterize the incidence and timing of pregnancy loss from service event (timed artificial insemination or timed embryo transfer) to parturition. Lactating Holstein Friesian cows were randomly assigned to receive either artificial insemination (AI, n = 243) or embryo transfer (ET, n = 863) with a fresh or frozen in vitro-produced blastocyst derived from oocytes collected from the ovaries of elite dairy donors (n = 14 Holstein Friesian and n = 8 Jersey) and elite beef donors (n = 21 Angus) using transvaginal ovum pick-up. In addition, oocytes were collected from the ovaries of beef heifers of known pedigree following slaughter at a commercial abattoir (n = 119). Blood samples were collected on d 7 from all cows to determine progesterone concentration and from subsets of cows on d 18 (n = 524) and d 25 (n = 378) to determine mRNA abundance of interferon-stimulated gene-15 and pregnancy-specific protein B concentration, respectively, to provide an early pregnancy diagnosis. Transrectal ultrasonography was conducted to determine pregnancy status on d 32, d 62 and d 125 after synchronized ovulation. Parturition date was recorded for all cows that reached a term delivery. The predicted probability of pregnancy (%) varied at each time point (d7, 18, 25, 32, 62, 125, parturition) depending on treatment (AI: 77.0, 60.2, 52.3, 48.8, 47.0, 44.6, 44.0; fresh ET: 100.0, 69.5, 60.3, 56.1, 48.4, 46.8, 45.5; frozen ET: 100.0, 61.7, 52.2, 41.6, 32.9, 31.8, 30.2). Irrespective of treatment, the largest proportion of pregnancy loss occurred in the period from service event (AI on d 0 or ET on d 7) to d 18, with minimal loss occurring between d 62 and parturition (AI: 1.8%, fresh ET: 1.9%, frozen ET: 3.5%). Treatment differences in the predicted probability of pregnancy per service event were detected between fresh ET vs frozen ET on d 32 and both AI and fresh ET vs frozen ET on d 62, 125 and at parturition. There was greater probability of pregnancy loss between d 32 and 62 following ET (Fresh: 11.3%, Frozen: 18.0%) than AI (4.0%). The percentage of cows that calved following transfer of a fresh embryo (45.5%) was similar to AI (44.0%), but less when a frozen embryo was transferred (30.2%). In conclusion, AI and fresh ET led to a greater probability of a cow becoming pregnant and maintaining the pregnancy to term than frozen ET. Cows that were still pregnant on d 62 had a very strong likelihood of maintaining the pregnancy to full-term parturition, irrespective of treatment. Further work is required to improve the likelihood of pregnancy establishment and reduce embryonic and fetal mortality following transfer of a cryopreserved in vitro-produced embryo.

3.2 Introduction

Pregnancy loss in dairy cows is a major contributor to reproductive inefficiency at herd level. This is particularly true in seasonal, pasture-based systems of production with a short, well-defined, breeding season. The causes and timing of pregnancy loss in dairy cattle have been the subject of several comprehensive reviews (Wiltbank et al., 2016, Ealy and Seekford, 2019). Sartori et al. (2010) reported that up to 50% of embryos in single-ovulating dairy cows were no longer viable by d 7. More recently, Berg et al. (2022) used a combination of morphological examination of embryos on d 7 and 15 and pregnancy diagnosis by ultrasonography on d 28, 35 and 70 and reported pregnancy per AI (P/AI) success rates of 70.9%, 59.1%, 63.8%, 62.3%, and 56.7% at d 7, 15, 28, 35, and 70, respectively, in seasonal-calving dairy cows managed under a pasture-based system. Fertilization failure (15.8%) and embryonic arrest before the morula stage (10.3%) were identified as the major developmental events that contributed to pregnancy losses during the first week after estrus, and conceptus elongation failure (7%) contributed to pregnancy failure during the second week after estrus (Berg et al., 2022).

Non-pregnant dams are typically identified when they return to estrus, using transrectal ultrasonography to confirm absence of a viable conceptus (i.e., embryo and associated extraembryonic membranes) or using chemical tests on blood or milk samples (i.e., pregnancy-associated glycoproteins, PAGs) from approximately d 28 of pregnancy onwards. Until relatively recently, the period from artificial insemination (AI) or embryo transfer (ET) to this first pregnancy diagnosis represented a ‘black box’ due to the inability to either visualize or detect the conceptus. For successful pregnancy establishment, the developing conceptus must signal its presence by secreting sufficient interferon-tau (IFNT) for maternal recognition of pregnancy to occur. The maternal response to this conceptus-derived IFNT can be measured by quantifying the mRNA abundance of interferon-stimulated genes (ISGs) in circulating blood as early as d 18 after estrus (Stevenson et al., 2007, Gifford et al., 2008, Green et al., 2010, Dalmaso de Melo et al., 2020). Similarly, concentrations of PAGs secreted from the binucleate trophoblast giant cells of the bovine conceptus can be determined in blood or milk samples collected from the gestating dam and used to assess pregnancy status from d 25 after AI (Green et al., 2000). These developments allow earlier determination of pregnancy status and characterization of the timing of early pregnancy losses.

Pregnancy losses are greater in females impregnated with in vitro-produced (IVP) embryos compared with those impregnated with in vivo-derived embryos or served by AI at estrus (Hansen, 2020). This greater embryo mortality presents an obstacle to more widespread use of IVP embryos, particularly in seasonal systems of production with a compact breeding season. Previous studies have estimated that 27% of cattle receiving IVP embryos will produce a live calf (although few studies actually follow the recipient females to parturition), with approximately 60% of the animals that received an IVP embryo undergoing pregnancy loss during the first 6 wk of gestation (Ealy et al., 2019). We recently reported that the proportion of cows diagnosed pregnant on d 32 was greater in lactating dairy cows following timed ET (TET) with a fresh IVP embryo compared with cows that received a frozen IVP embryo, and that embryonic loss between d 32 and 62 was greater following TET compared with timed AI (TAI) (Crowe et al., 2024a). The temporal pattern of pregnancy losses in cows that were bred using AI or IVP embryos is currently poorly resolved, and the impact of embryo status (fresh vs. frozen) and breed (dairy vs. beef) on the timing of pregnancy loss remains to be fully elucidated. The aim of the current study was to characterize the incidence and timing of pregnancy loss from initial service event to parturition following TAI or TET with fresh or frozen IVP embryos by using a combination of detection of cows returning to estrus, chemical assays for ISGs and PAGs and transrectal ultrasonography. Specifically, we tested the hypothesis that lactating dairy cows that received TAI would have similar probability of pregnancy per service event (P/S) and incidence and timing of pregnancy loss to cows that received TET with fresh IVP embryos and greater P/S and less pregnancy loss than cows that received TET with a frozen IVP embryo.

3.3 Materials and Methods

All experimental procedures involving animals were approved by the Teagasc Animal Ethics Committee and authorized by the Health Products Regulatory Authority in Ireland, in accordance with Statutory Instrument No. 543 of 2012 under European Union legislation (Directive 2010/63/EU) for the Protection of Animals used for Scientific Purposes. Experimental procedures with animals were conducted between March 2021 and March 2022.

The experimental design was previously described in detail in Crowe et al. (2024a), and the timeline of events for the current study is summarized in Figure 3.1. The industry target for pregnancy to first service in seasonal-calving systems in 60%. Based on previous publications, the P/S for cows following transfer of a frozen IVP embryo has been reported to be 15 to 20 percentage points less than P/S for cows following either AI or transfer of a fresh IVP embryo (Carrenho-Sala et al., 2016, Pereira et al., 2016, Hansen, 2020). For the current study, a power test indicated that 150 cows per treatment were required to have an 80% chance of detecting, as significant at the 5% level, a decrease in the pregnancy per service event from 60% (target for AI and fresh ET treatments) to 45% in the frozen ET treatments. To mitigate against cows being dropped from the study for various reasons, a greater number of cows was initially enrolled. Briefly, lactating dairy cows in 16 herdlets were synchronized using a progesterone (P4)-Ovsynch protocol. Cows were blocked based on parity, calving date and Economic Breeding Index (**EBI**) and randomly assigned (within each herdlet) to each treatment. Of these, 243 (22.0%) received TAI 16 h after the second GnRH and 863 (78.0%) received TET on d 7 after presumptive estrus. Cows enrolled on this study only received TAI or TET once at the first postpartum service event. The embryos were either fresh or frozen grade 1 in vitro-produced blastocysts derived from oocytes collected from the ovaries of elite dairy donors (n = 14 Holstein Friesian and n = 8 Jersey) and elite beef donors (n = 21 Angus) using transvaginal ovum pick-up. In addition, oocytes were collected from the ovaries of beef heifers of known pedigree following slaughter at a commercial abattoir (n = 119). As previously described by Crowe et al. (2024a), the oocytes collected were matured in vitro for approximately 24 h before in vitro-fertilization, and presumptive zygotes were then cultured in-vitro until reaching blastocyst stage when they were either cryoprezerved or transported to one of the farms for direct transfer. Vytelle LLC (Lenexa, KS) conducted all activities related to transvaginal ovum pick-up, oocyte maturation, fertilization, in vitro culture to the blastocyst stage and embryo cryopreservation. Pregnancy per service event was diagnosed in cows that had not been detected returning to estrus by quantification of the mRNA abundance of interferon-stimulated gene-15 (ISG15) in maternal peripheral blood on d 18, concentration of pregnancy specific protein B (PSPB) in maternal serum on d 25, and transrectal ultrasonography on d 32, d 62 and d 125 after synchronized ovulation and finally by recording a parturition event at full-term.

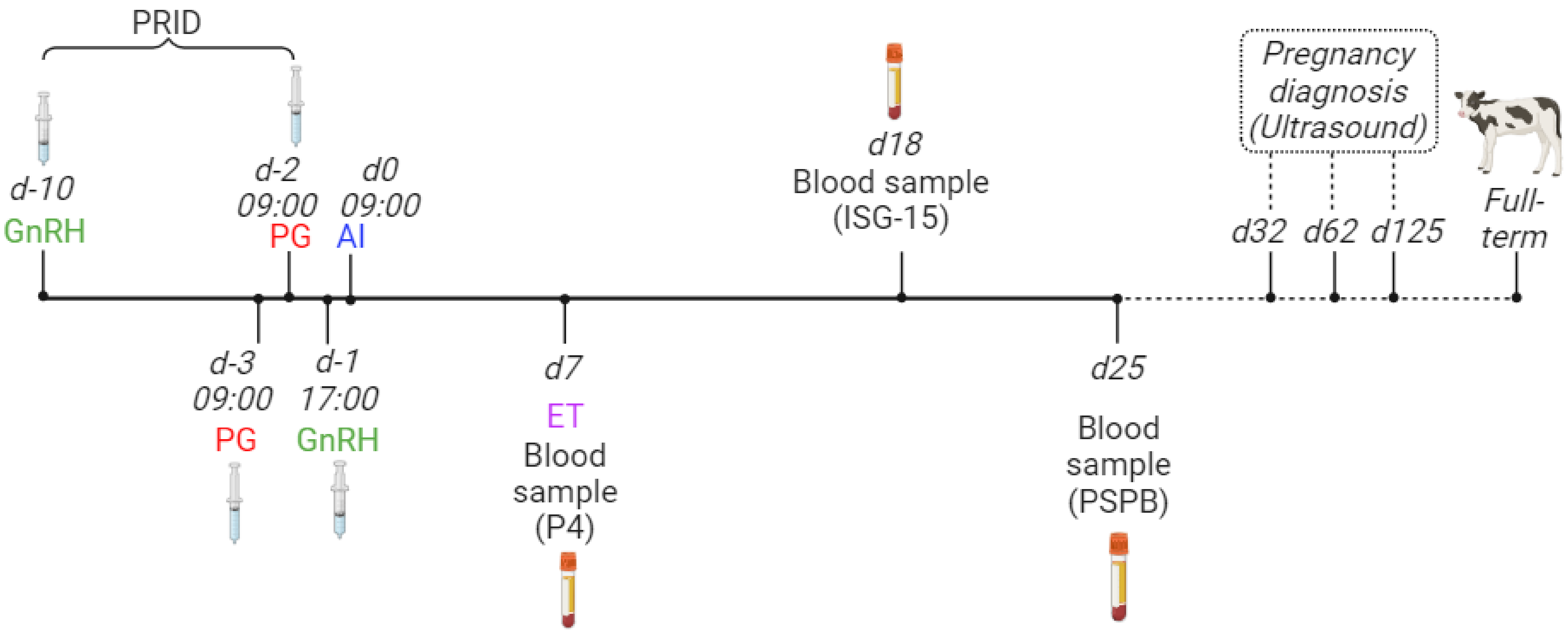


Figure 3.1 Schematic outline of the synchronization, service event (AI or ET), blood sample collection, ultrasound diagnosis of pregnancy, and recording of calf delivery. Cows received an intramuscular (IM) injection of GnRH (Ovarelin, CEVA Santé, France) and had a progesterone-releasing intravaginal device (PRID Delta) inserted into the vagina on d -10. Cows received IM injections of prostaglandin-F2α (Enzaprost, CEVA Santé, France) on d -3 and -2, with PRIDs removed on d -2. Cows received GnRH again on d -1. Cows were assigned to receive either TAI on d 0 or TET on d 7. Blood samples were collected on d 7 for determination of serum progesterone (P4), on d 18 for determination of peripheral blood mRNA abundance of interferon-stimulated gene-15 (ISG15) and on d 25 for determination of pregnancy specific protein B (PSPB). Pregnancy diagnosis was conducted using transrectal ultrasound on d 32, d 62 and d 125. Parturition date was recorded for all cows (mean = d 280).

3.3.1 Blood sample collection and ultrasound examination

Blood samples were collected from the coccygeal vessels of all cows on the study on d 7 (P4) and then from a subset of cows that had not returned to estrus from 6 herdlets on d 18 (ISG15; n = 524) and d 25 (PSPB; n = 378) after synchronized ovulation. On d 18, blood samples (3 mL) were collected into Tempus Blood RNA Tubes (Applied Biosystems, Foster City, CA, USA) containing 6 mL of RNA stabilization solution. Immediately following collection, blood and RNA stabilization solution were thoroughly mixed by shaking the Tempus tubes vigorously by hand for 20 s. The blood tube was stored at -20 °C for 24 h and then transferred to storage at -80 °C until RNA extraction. On d 7 and d 25, blood samples (10 mL) were collected from the coccygeal vessels into serum tubes (BD Vacutainer, BD, Plymouth, UK). Blood samples were stored at 4 °C for 24 h before centrifugation at 1922 x g at 4 °C for 15 min. Serum was separated using a Pasteur pipette and stored at -20 °C until analysis (d 7 P4 analysis described by Crowe et al., 2024a). The detection limit of the P4 solid-phase radioimmunoassay (PROG-RIA-CT) kit (DIAsource ImmunoAssays S.A., Louvain-la-Neuve, Belgium) was 0.05 ng/ml. All cows that received either AI (d 0) or ET (d 7) had serum P4 concentrations greater than the minimum detection limit of the assay on d 7; the least serum P4 concentration measured in a sample was 0.48 ng/ml. All cows that had not returned to estrus by d 32 were examined by transrectal ultrasonography to determine their pregnancy status. Cows that were pregnant on d 32 were re-examined at d 62 and d 125 and a successful full-term pregnancy was confirmed following parturition.

3.3.2 RNA Extraction and cDNA Synthesis

Whole blood in the Tempus tubes was thawed and RNA was extracted from each sample using the Tempus Spin RNA Isolation Reagent Kit (Applied Biosystems) according to the manufacturer's instructions and including the optional DNase step. A NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) was used to quantify the concentration (ng/µl) of RNA and to determine the absorbance ratios 260/280 and 260/230 in order to ascertain the purity of the RNA. RNA integrity was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies Ireland Ltd., Dublin, Ireland). With the exception of one sample with an RNA integrity number of 6.1, all samples had an RNA integrity number between 7.7 and 10.0. The cDNA was synthesized in a 20 µL reaction, according to the manufacturer's instructions (from 1 µg of total RNA per sample for samples with an RNA concentration ≥75.5 ng/µL (n=511), and 1 µg of total RNA per sample for samples with an RNA concentration of <75.5 ng/µL (n=28), synthesized in a 40 µL reaction) using the High Capacity cDNA Reverse Transcription Kit with the optional RNase inhibitor included in the reaction (Applied Biosystems). The cDNA was then stored at −80 °C.

3.3.3 Quantitative PCR

Primer sequences for candidate genes were obtained from the literature (Fricke et al., 2016) (Table 3.1) and were commercially synthesized (Sigma-Aldrich Ireland Ltd., Dublin, Ireland). As described by Fricke et al. (2016), serial dilutions of pooled cDNA samples were used to determine qPCR amplification efficiencies using the equation $E = -1 + 10(-1/slope)$. The slope was calculated by plotting a linear curve of quantitative cycle (Cq) values against the log dilutions (Pfaffl, 2001). Primers had PCR amplification efficiencies of between 90% and 110%. Expression of ISG15 was normalized against the expression of the reference gene β-actin (ACTβ) previously shown to have stable expression in bovine peripheral blood samples (Fricke et al., 2016). One microliter of the optimal concentration of cDNA (determined when calculating efficiencies using serial dilutions) was added to 19 µL of master mix (10 µL of Fast SYBR Green 1 master mix; Applied Biosystems), 8 µL of nuclease-free water, and 0.5 µL each of forward and reverse primers at individually optimized concentrations. Quantitative PCR (qPCR) was conducted on a 7500 FAST RT-PCR v2.0.1 machine (Applied Biosystems) using the following cycling conditions:1 cycle of 95 °C for 20 s; 40 cycles of 95 °C for 3 s and 60 °C for 30 s; 1 cycle of amplicon dissociation (95 °C for 15 s, 60 °C for 1 min, increasing 1 °C per cycle until 95 °C was reached for 15 s, and then 60 °C for 15 s) to generate a melt curve. Resulting Cq values were used to measure gene expression of the reference gene and the target gene (Table 3.1) according to Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009).

Table 3.1 Primer sequences for interferon-stimulated gene-15 (ISG15) mRNA expression analysis.

Gene	Accession No.	Primer sequence
Target gene		
ISG15	NM_174366	Forward: 5'-GGTATGAGCTGAAGCAGTT-3'
		Reverse: 5'-ACCTCCCTGCTGTCAAGGT-3'
Reference gene		
ACTβ	AY141970	Forward: 5'-CTGGACTTCGAGCAGGAGAT-3'
		Reverse: 5'-GGATGTCGACGTCACACTTC-3'

3.3.4 Analysis of Pregnancy-Specific Protein B (PSPB)

Serum concentrations of PSPB were determined using the quantitative BioPRYN-research assay (BioTracking, Moscow, ID, USA) according to the manufacturer’s instructions and included the standard curve provided, as previously described by Sasser et al. (1986). When serum samples had a PSPB concentration that was greater than the concentration of the greatest standard, samples were diluted with phosphate-buffered saline (PBS) as required to bring their concentration onto the standard curve and allow PSPB concentration to be quantified. The d 25 serum samples collected from 9 cows had a PSPB concentration less than this threshold and were therefore assigned a value of 0.15 ng/ml PSPB, the minimum detectable limit of the assay. These cows were recorded as non-pregnant on d 25, and this diagnosis was subsequently confirmed by transrectal ultrasound scanning of the reproductive tract on d 32.

3.3.5 Calculation of pregnancy loss

An estimate of the percentage of cows that were pregnant was determined for d 7, d 18, d 25, d 32, d 62, d 125 and, ultimately, calving data were recorded. Pregnancy loss was calculated as the percentage of those cows pregnant at each time point that were no longer pregnant at the subsequent pregnancy diagnosis. On d 7, all cows in the TET treatments were assumed pregnant as they had received a viable d 7 embryo. For the frozen ET embryos, we have previously reported that 96.3% re-expanded after thawing and culture in vitro (Crowe et al., 2024a). As a guide to estimate P/AI on d 7, we used Receiver Operating Characteristic curves to identify the optimum cut-off values for P4 on d 7 in order to give an estimate of the potential pregnancy outcome for cows that received AI, deeming 77.0% were pregnant on d 7. Furthermore, in the same cohort of cows as used in the current study, we previously reported that 9.6% of the cows that were synchronized to be embryo recipients were deemed unsuitable on the day of TET (lack of, or small, corpus luteum, CL) (Crowe et al., 2024a).

3.3.6 Statistical Analysis

All statistical analyses were conducted using SAS v. 9.4 (SAS Institute, Cary, NC, USA). As previously described by Crowe et al. (2024a), 1197 cows were enrolled in the study and synchronized for either AI or ET. A total of n = 18 cows were subsequently removed at varying time points throughout the study, due to culling (n = 15), death (n = 2) and sale off farm (n = 1), all of which were unrelated to treatment. The number of animals available for inclusion in the analysis on each day of pregnancy diagnosis is summarized in Table 3.2.

Table 3.2 Number of cows used to calculate the probability of pregnancy or probability of pregnancy loss on each day of pregnancy diagnosis. Day 0 represents the number of cows that were initially synchronized for each treatment and includes animals rejected for ET on d 7. A subset of cows (6 of the 16 herdlets in the study) was used on on d 18 for determination of peripheral blood mRNA abundance of interferon-stimulated gene-15 and on d 25 for determination of pregnancy specific protein B.

n cows included in analysis

Day of pregnancy diagnosis	0	7	18	25	32	62	125	280
AI	243	243	119	119	241	241	241	239
Beef Fresh	298	267	106	106	298	296	293	294
Beef Frozen	295	265	99	99	295	295	292	290
Dairy Fresh	184	169	107	107	183	181	181	180
Dairy Frozen	177	162	105	105	176	176	176	176
Total	1197	1106	536	536	1193	1189	1183	1179

Pregnancy status on d 18 and d 25 were predicted based on the mRNA abundance of ISG15 in peripheral blood (d 18) and the serum concentration of PSPB on d 25 for each cow. Receiver Operating Characteristic curves were used to identify the optimum cut-off values for ISG15 on d 18 and serum PSPB on d 25 to predict pregnancy status diagnosed by ultrasound on d 32. Receiver Operating Characteristic curves were also used to identify the optimum cut-off values for P4 on d 7 in order to give an estimate of the potential pregnancy outcome for cows that received AI. The greatest Youden Index (J) values were selected, and where more than one cut-off value had the same J value (within 0.01), the cut-off value with the greatest sensitivity was selected. The sensitivity and specificity of the selected cut-offs values were 62.9% and 57.9% for P4 on d 7 (AI cows only), 80.5% and 73.7% for ISG15 on d 18, and 95.8% and 72.0% for PSPB on d 25. When a cow was predicted to be non-pregnant on d 18 and subsequently predicted to be pregnant by PSPB on d 25 (n = 58) or predicted non-pregnant on d 25, and subsequently diagnosed pregnant by ultrasound on d 32 (n = 11), the earlier diagnosis of pregnancy status was corrected for the purpose of these analyses. The upper and lower cut-off values used to assign cows to quartiles for serum P4 concentration, mRNA abundance ISG15 in peripheral blood and serum PSPB concentrations are summarized in Table 3.3.

Table 3.3 Summary of the upper and lower quartile cut-off values for serum P4 concentration, mRNA abundance of ISG15 in peripheral blood and serum PSPB concentrations (n = number of cows in each quartile).

	P4 (ng/ml) [n]	*ISG15* relative abundance [n]	PSPB (ng/ml) [n]
Q 1	< 5.496 [299]	$< 6.51 \times 10^{-4}$ [130]	< 4.4 [95]
Q 2	5.496 - 7.152 [299]	$6.51 \times 10^{-4} - 1.434 \times 10^{-3}$ [131]	4.4 - 10.03 [95]
Q 3	7.153 - 9.23 [299]	$1.435 \times 10^{-3} - 2.81 \times 10^{-3}$ [131]	10.04 - 17.9 [94]
Q 4	> 9.23 [300]	$> 2.81 \times 10^{-3}$ [132]	> 17.9 [95]

Pregnancy per service event data for each time point were analyzed using generalized linear mixed models, with a binary distribution specified. Multiple variables and interactions were included as fixed effects and retained in the final models when $P \leq 0.25$. Several models were constructed to compare the type of service event on each day of pregnancy diagnosis: (1) AI vs. ET, (2) AI vs. Beef ET vs. Dairy ET and (3) AI vs. Fresh ET vs. Frozen ET. The models included treatment, P4 concentration (as a continuous or categorical variable), days in milk (DIM) on mating start date (MSD; ≤75, 76 to 82, ≥83) and parity (1, 2, 3 and ≥4). For each model, only significant effects were retained. In all models, herdlet was included as a random effect. The GLIMMIX model for testing the effect of type of service event on the incidence of embryonic loss included treatment and parity, with herdlet included as a random effect. For all binary outcome variables analyzed, the GLIMMIX model output values for treatment means are reported, which equate to the predicted probability of that event.

For the analysis of effects on pregnancy loss, similar models were constructed to those used for P/S. All models contained herdlet as a random effect.

The associations between serum P4 on day 7, peripheral blood mRNA abundance of ISG15 on d 18 and serum PSPB concentrations on d 25 were first explored using correlation and simple linear regression analysis, and plots were generated to illustrate the overall pattern and variability in the relationships. Subsequently, box-cox transformations to normalize the data were determined using PROC TRANSREG. The associations between serum P4 concentration on d 7, peripheral blood mRNA abundance of ISG15 on d 18 and serum PSPB concentrations on d 25 with binary outcome variables for pregnancy per service event (d 32, d 63, d 125 and term delivery) and pregnancy loss were then explored using the GLIMMIX procedure.

3.4 Results

3.4.1 Factors affecting the probability of pregnancy

Crowe et al. (2024a) previously reported the treatment effects on the predicted probability of pregnancy for d 32 and d 62. These results, along with the P/S on d 7, d 18 and 25 (in the subset of cows selected for blood sample collection on d 18 and 25) and all cows on d 125 and at full-term (mean gestation length ± SD was 280.3 ± 5.7 d), are summarized in Table 3.4 and Figure 3.2. Of the n = 58 cows whose pregnancy diagnosis was corrected on d 18, n = 17 were in the first quartile (ISG-Q1) and n = 41 in ISG-Q2 for mRNA abundance of ISG15. Of the n = 17 cows that were in ISG-Q1, n = 3 underwent pregnancy loss between d 25 and d 32, n = 4 underwent pregnancy loss and n = 1 was removed from the trial for culling between d 32 and d 62, n = 1 underwent pregnancy loss after d 125 and n = 8 reached parturition. Of the n = 41 cows in ISG-Q2, n = 9 underwent pregnancy loss between d 25 and d 32, n = 4 underwent pregnancy loss between d 32 and d 62 and n = 28 reached parturition. Of the n = 11 cows whose pregnancy diagnosis was corrected on d 25, all were in the first quartile (PSPB-Q1) for circulating PSPB concentration, n = 6 underwent pregnancy loss between d 32 and d 62, n = 1 underwent pregnancy loss between d 125 and d 280 and n = 4 reached parturition. The predicted P/S and probability of pregnancy loss did not differ between beef and dairy ET at any of the time points (all P > 0.05), and hence the results are reported for the AI, fresh ET and frozen ET treatments. Overall, the predicted P/S did not differ between AI and ET on any of the days of pregnancy diagnosis except d 7 (AI - potential P/S: 77.0%, ET: 100.0%). Cows that received AI, however, tended to be more likely to be pregnant on d 62 and d 125 (47.0% and 44.6%) than cows that received ET (40.6% and 38.2%; P = 0.092 and P = 0.087). The predicted probability of pregnancy was greater following AI than frozen ET on d 62 (47.0% vs 32.9%; P = 0.002), d 125 (44.6% vs 31.8%; P = 0.001) and at full-term (44.0% vs 30.2%; P = 0.0004). Similarly, the predicted probability of pregnancy was greater following fresh ET than frozen ET on d 32 (56.1% vs. 41.6%; P = 0.0002), d 62 (48.4% vs. 32.9%; P < 0.0001), d 125 (46.8% vs 31.8%; P < 0.0001), and at full-term (45.5% vs. 30.2%; P < 0.0001).

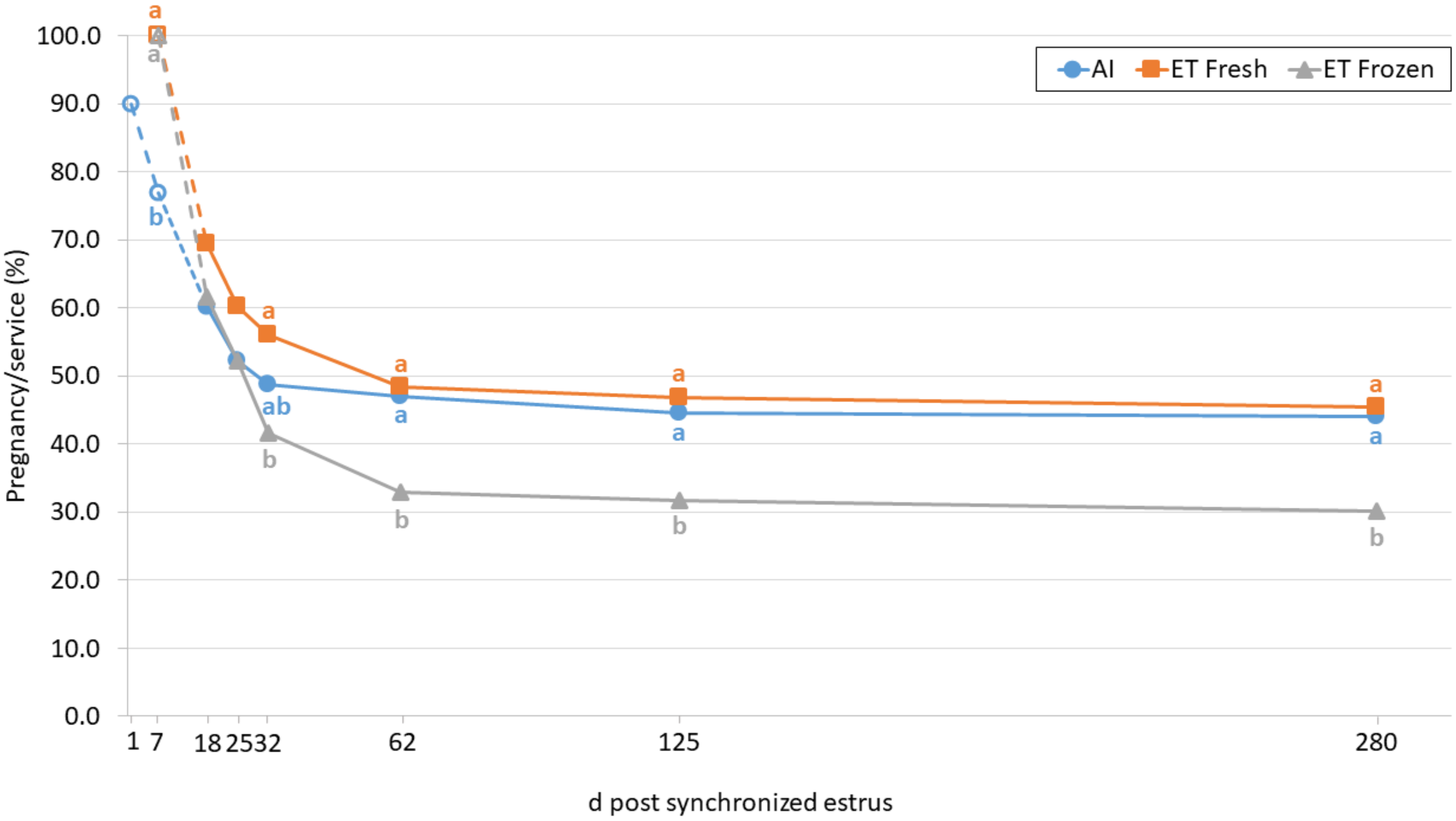


Figure 3.2 Incidence and timing of embryonic loss following timed artificial insemination (AI) or timed embryo transfer (ET) with fresh or frozen in vitro produced embryos. The data-points indicate the predicted probability of pregnancy/service event at d 7, 18, 25, 32, 62, 125 and 280 (parturition). Values not sharing a common letter (a–b) differ (P < 0.05). Open shapes and dashed lines indicate 'assumed' pregnancy results (see text for further details). The probability of pregnancy and 95% CI values are included in Table 3.4.

Table 3.4 Predicted probability of pregnancy (± 95% CI) for lactating dairy cows following timed artificial insemination (AI) or timed embryo transfer (ET) with a fresh or frozen in vitro produced embryo.

Day of Gestation

	d 7	d 18	d 25	d 32*	d 62*	d 125	Full-term
AI	77.0%^{a}	60.2%	52.3%	48.8%^{ab}	47.0%^{a}	44.6 %^{a}	44.0%^{a}
	(72.8 - 81.2)	(49.3 - 71.2)	(42.3 - 62.3)	(42.1 - 55.6)	(40.4 - 53.7)	(38.0 - 51.3)	(37.3 - 50.9)
ET Fresh	100% ^{b}	69.5%	60.3%	56.1%^{a}	48.4%^{a}	46.8%^{a}	45.5%^{a}
		(61.3 - 77.8)	(52.2 - 68.4)	(51.0 - 61.1)	(43.3 - 53.4)	(42.1 - 51.4)	(40.9 - 50.1)
ET Frozen	100% ^{b}	61.7%	52.2%	41.6%^{b}	32.9%^{b}	31.8%^{b}	30.2%^{b}
		(53.3 - 70.1)	(44.0 - 60.4)	(36.6 - 46.7)	(28.3 - 37.8)	(27.2 - 36.5)	(25.5 - 34.8)

$^{a-c}$Values in the same column with different superscripts differ significantly ($P < 0.05$).

* Probability of pregnancy on d 32 and d 62 was previously reported by Crowe et al. (2024a).

We previously reported that serum P4 concentration on d 7 was associated with P/S (Crowe et al., 2024a). The association between serum P4 quartile and P/S at each time point of pregnancy diagnosis is summarized in Table 3.5. Cows in P4-Q1 had lesser P/S at all time-points from d 32 to full-term compared with the cows in P4-Q2, P4-Q3 and P4-Q4 (all $P < 0.05$). Using the same P4 concentration quartile cut-offs to determine the relationship between d 7 serum P4 concentration and predicted pregnancy status in the subsets of cows that also had data for peripheral blood mRNA abundance of ISG15 on d 18 ($n = 524$), PSPB data on d 25 ($n = 379$) and observed pregnancy failure by return to estrous (up to and including d 18, $n = 12$ and from d 18 to d 25, $n = 145$), cows in P4-Q1 had lesser P/S than cows in P4-Q2 on all days of pregnancy diagnosis from d 32 onwards and lesser than cows in P4-Q3 and P4-Q4 (all $P < 0.05$) on all days from d 18 onwards. Cows in P4-Q2 had lesser P/S than cows in P4-Q3 on d 25 only ($P = 0.044$) and lesser P/S than cows in P4-Q4 at all times of pregnancy diagnosis from d 25 onwards ($P < 0.05$) apart from a tendency ($P = 0.057$) to have lesser P/S at full-term. There was no difference in P/S for cows in P4-Q3 vs P4-Q4 at any time-point.

Cows that had the longest interval from calving to MSD (≥83 DIM) tended to have greater P/S than cows with a shorter interval (≤75 DIM) on d 18 (70.5% vs. 58.5%; $P = 0.073$) and had greater P/S on d 62 (49.4% vs. 40.0%; $P = 0.04$), d 125 (46.8% vs. 37.8%; $P = 0.047$) and at full-term (45.3% vs. 35.6%; $P = 0.027$). Parity did not affect P/S at any time-point ($P > 0.05$).

The serum concentration of P4 on d 7 ($P = 0.0004$) and mRNA abundance of ISG15 in peripheral blood on d 18 ($P < 0.0001$) were associated with P/S on d 25. Greater P4 concentration on d 7 and greater mRNA abundance of ISG15 on d 18 were associated with greater P/S on d 25 ($P < 0.0001$). Cows with greater mRNA abundance of ISG15 on d 18 ($P < 0.0001$) and greater concentration of PSPB on d 25 ($P < 0.0001$) had a greater P/S on all subsequent days of pregnancy diagnosis, d 32, d 62, d 125 and at parturition (all $P < 0.0001$, Figure 3.5).

Table 3.5 Predicted probability of pregnancy (± 95% CI) in lactating dairy cows by quartile of serum progesterone concentration on d 7 for all cows that received TAI or TET.

Quartile	d 18	d 25	d 32*	d 62*	d 125	Full-term
1	50.3%^{a}	35.8%^{a}	33.4%^{a}	30.1%^{a}	25.0%^{a}	22.9%^{a}
	(39.3 - 61.3)	(25.8 - 45.9)	(27.7 - 39.7)	(24.5 - 36.4)	(19.5 - 31.4)	(17.7 - 29.0)
2	60.3%^{ab}	50.1%^{a}	45.7%^{b}	42.2%^{b}	41.5%^{b}	40.6%^{b}
	(49.6 - 71.1)	(40.4 - 59.9)	(39.1 - 52.4)	(35.7 - 49.0)	(35.2 - 48.1)	(34.7 - 46.8)
3	71.7%^{b}	65.1%^{b}	55.6%^{bc}	49.2%^{bc}	48.2%^{bc}	45.7%^{b}
	(60.9 - 82.4)	(55.3 - 74.9)	(49.1 - 61.9)	(42.8 - 55.7)	(41.9 - 54.5)	(39.9 - 51.7)
4	69.9%^{b}	66.1%^{b}	61.2%^{c}	54.6%^{c}	53.0%^{c}	51.4%^{b}
	(58.6 - 81.2)	(55.7% - 76.5)	(54.7 - 67.4)	(48.0 - 61.1)	(46.4 - 59.4)	(45.3 - 57.4)

$^{a-c}$ Values in the same column with different superscripts different significantly ($P < 0.05$).

*Probability of pregnancy on d 32 and d 62 was previously reported by Crowe et al. (2024a).

3.4.2 Factors affecting the probability of pregnancy loss

The overall effect of treatment on the timing and incidence of pregnancy loss is summarized in Table 3.6 and Figure 3.3. Treatment affected pregnancy loss between d 7 and d 18; 20.3% of cows that received TAI and 34.3% of cows that received TET (P = 0.01) underwent pregnancy loss between d 7 and d 18 based on relative mRNA abundance of ISG15 on d 18. This difference, however, was largely driven by the assumption of pregnancy being 100% on d 7 for all cows assigned to ET compared with 77.0% for cows assigned to AI. There was no difference (P = 0.159) in the incidence of embryo loss between fresh and frozen ET during the same interval and there was no difference in the predicted pregnancy loss between cows that received ET with beef or dairy embryos at any time-point (all P > 0.05). Cows that received AI had a lesser chance of pregnancy loss between d 7 and d 18 than cows that received frozen ET (20.3% vs. 38.5%; P = 0.006). Cumulative pregnancy loss after d 7 was calculated for each treatment. Cows that received AI had a 42.0% probability of undergoing pregnancy loss. This was less than cows that received fresh ET (54.7%; P = 0.011) or frozen ET (69.8%; P < 0.0001). The predicted probability of a cow undergoing pregnancy loss before full-term parturition was also greater following frozen ET than fresh ET (P < 0.0001).

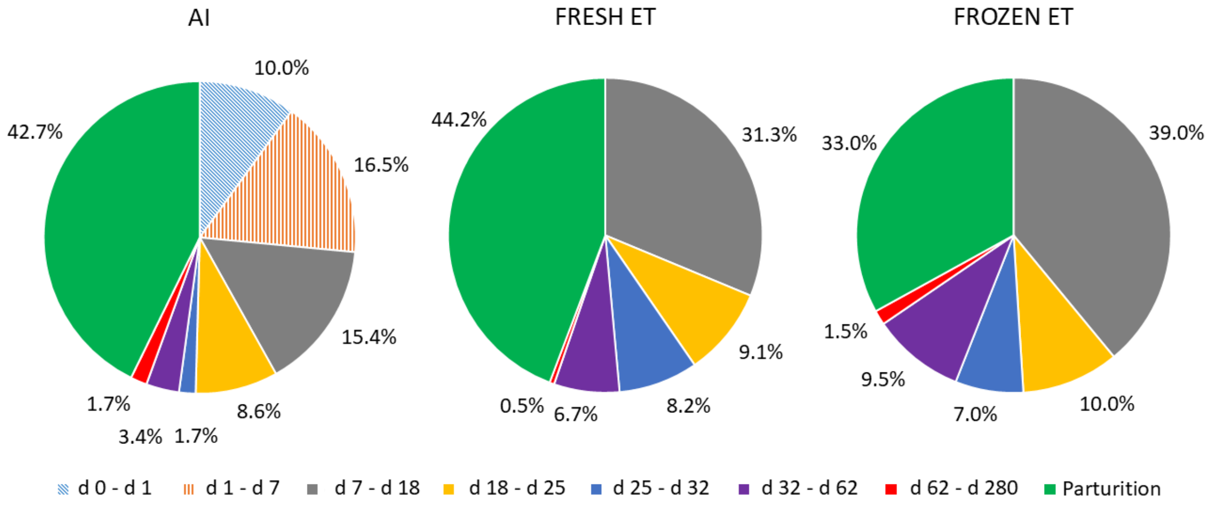


Figure 3.3 Incidence and timing of pregnancy loss following timed artificial insemination (AI) or timed embryo transfer (ET) with fresh or frozen in vitro-produced embryos. The pie charts illustrate raw data from the subset of cows (n = 536) for which d 18 and d 25 pregnancy data were available. Percentage pregnancy loss within the windows of d 0 - d 1 (AI only), d 1 - d 7 (AI only), d 7 - d 18, d 18 - d 25, d 25 - d 32, d 32 - d 62, d 62 - d 280 and the percentage of cows that reached parturition at full-term are indicated. Note that these raw values differ slightly from the predicted probability of pregnancy loss included in Table 3.4. Pregnancy was diagnosed by measurement of mRNA abundance of interferon-stimulated gene 15 in peripheral blood on d 18, serum pregnancy specific protein B concentration on d 25 and ultrasound scanning on d 32, d 62 and d 125 (see text for details). For cows that received AI, the hatched quadrants are based on 'assumed' pregnancy on d 0, d 1 and d 7.

Table 3.6 Predicted probability of pregnancy loss (± 95% CI) in lactating dairy cows following timed artificial insemination (AI) or timed embryo transfer (ET) with a fresh or frozen in vitro produced embryo.

	d 7 - d 18	d 18 - d 25	d 25 - d 32*	d 32 - d 62**	d 62 - Full-term
AI	27.8%	11.3%	3.3%	$4.0\%^{a}$	1.8%
	(16.9-42.1)	(0.1-93.5)	(0.8-12.5)	(1.6-9.7)	(0.4-7.3)
ET Fresh	27.1%	12.8%	11.8%	$11.3\%^{b}$	1.9%
	(18.1-38.5)	(0.5-80.1)	(0.4-81.5)	(6.5-19.0)	(0.7-5.4)
ET Frozen	32.8%	13.0%	14.0%	$18.0\%^{b}$	3.5%
	(21.8-46.0)	(0.4-83.7)	(0.5-84.9)	(10.8-28.4)	(1.3-8.7)

$^{a\text{-}b}$Values in the same column with different superscripts differ significantly ($P < 0.05$).

*Cows that received AI tended to have lesser probability of pregnancy loss between d 25 and d 32 than the mean probability of pregnancy loss of all cows that received ET (Fresh and Frozen combined); $P = 0.053$

**Cows that received AI had lesser probability of pregnancy loss between d 32 and d 62 than the mean probability of pregnancy loss of all cows that received ET (Fresh and Frozen combined); $P = 0.002$. Probability of pregnancy loss between d 32 and d 62 was previously reported by Crowe et al. (2024a).

Treatment had no effect on the predicted pregnancy loss between d 18 and d 25 (AI: 14.6%, ET fresh: 14.2% and ET frozen 16.9%; P = 0.795). Cows assigned to AI had less pregnancy loss between d 25 and d 32 compared with cows assigned to ET (3.3% vs. 13.2%, respectively; P = 0.031). The effect of treatment on the predicted pregnancy loss between d 32 and d 62 were previously reported by Crowe et al. (2024a). The probability of pregnancy loss (%, 95% CI) was small in all treatments after d 62 and did not differ between AI (1.8%; 0.4, 7.3), fresh ET (1.9%; 0.7 - 5.4) or frozen ET (3.5%; 1.3 - 8.7).

Serum P4 concentration on d 7 was associated with the predicted pregnancy loss between d 7 and d 18; cows in P4-Q1 had a greater probability of undergoing pregnancy loss between d 7 and d 18 than cows in P4-Q3 and P4-Q4 (P = 0.018 and P = 0.051, respectively). Day 7 serum P4 concentration was not associated with pregnancy loss at any point during gestation after d 18.

The peripheral blood mRNA abundance of ISG15 was associated with the probability of a cow undergoing pregnancy loss at some time between d 18 and parturition (P = 0.0139). The predicted probability of pregnancy loss (%, 95% CI) was calculated for each quartile of peripheral blood mRNA abundance of ISG15: ISG-Q1 = 47.2% (24.9, 69.4), ISG-Q2 = 39.3% (26.8, 51.9), ISG-Q3 = 43.6% (34.5, 52.7) and ISG-Q4 = 22.4% (13.6, 31.2). Cows in ISG-Q3 had greater pregnancy loss than cows in ISG-Q4 (P = 0.002). The number of cows that were predicted to be pregnant on d 18 and therefore included in this analysis was less in both ISG-Q1 (n = 18) and ISG-Q2 (n = 61) than ISG-Q3 (n = 126) or ISG-Q4 (n = 127). Of cows diagnosed pregnant by ultrasound on d 32 and in the least quartile for ISG15 on d 18 (n = 15), 66.7% (n = 10) were still pregnant at the d 62 scan and 60.0% (n = 9) reached parturition. Conversely, of those in the greatest quartile for ISG15 on d 18 (n = 105), 93.3% (n = 98) were still pregnant at d 62 and 91.4% (n = 96) reached parturition.

Serum concentration of PSPB on d 25 was associated with the probability of pregnancy loss (%, 95% CI) after d 25 (P < 0.0001). Cows in the first quartile (PSPB-Q1; 58.1%, 32.5 - 83.8) had greater pregnancy loss than cows in PSPB-Q3 (19.2%, 9.9 - 28.6; P = 0.023) and PSPB-Q4 (9.6%, 0.2 - 19.0; P = 0.002). Cows in PSPB-Q2 (35.5%, 25.8 - 45.3) had greater pregnancy loss than cows in PSPB-Q4 (P = 0.0003) and tended to have greater probability of loss than PSPB-Q3 (P = 0.051). Of cows diagnosed pregnant by ultrasound on d 32 and in the least quartile for PSPB on d 25 (n = 11), 45.5% (n = 5) were still pregnant at the d 62 scan and 36.4% (n = 4) reached parturition.

Conversely, of those in the greatest quartile for PSPB on d 25 (n = 86), 93.0% (n = 80) were still pregnant at d 62 and 90.7% (n = 78) reached parturition.

3.4.3 Factors associated with the abundance of ISG15 on d 18 and serum PSPB concentrations on d 25

The serum concentration of P4 on d 7 was associated with both the peripheral blood mRNA abundance of ISG15 on d 18 ($P < 0.0001$) and serum concentration of PSPB on d 25 ($P = 0.002$). The mRNA abundance of ISG15 on d 18 was also associated with the serum concentration of PSPB on d 25 ($P < 0.0001$). Cows with greater serum P4 concentrations on d 7 had greater peripheral blood mRNA abundance of ISG15 on d 18 and greater serum concentrations of PSPB on d 25. The mean blood mRNA abundance of ISG15 on d 18 and mean serum concentration of PSPB on d 25 are displayed based on quartile of P4 in Figure 3.4. The relationships between P4 vs ISG15, P4 vs PSPB and ISG15 vs PSPB are displayed in Figure 3.5. Treatment did not affect the relative mRNA abundance of ISG15 of cows that were deemed pregnant on d 18 ($P = 0.158$); expression of ISG15 following AI vs Frozen ET didn't differ ($P = 0.1108$) and there was only a tendency for cows that received fresh ET to have a greater mRNA abundance of ISG15 than cows that received frozen ET ($P = 0.097$). By contrast, treatment affected the serum concentrations of PSPB in cows that were deemed pregnant on d 25 ($P = 0.0002$). In this cohort, cows that received AI and fresh ET had greater serum concentrations of PSPB than cows that received frozen ET ($P = 0.006$ and $P < 0.0001$ respectively). The effects of treatment on mRNA abundance of ISG15 on d 18 and serum concentrations of PSPB on d 25 are displayed in Figure 3.6.

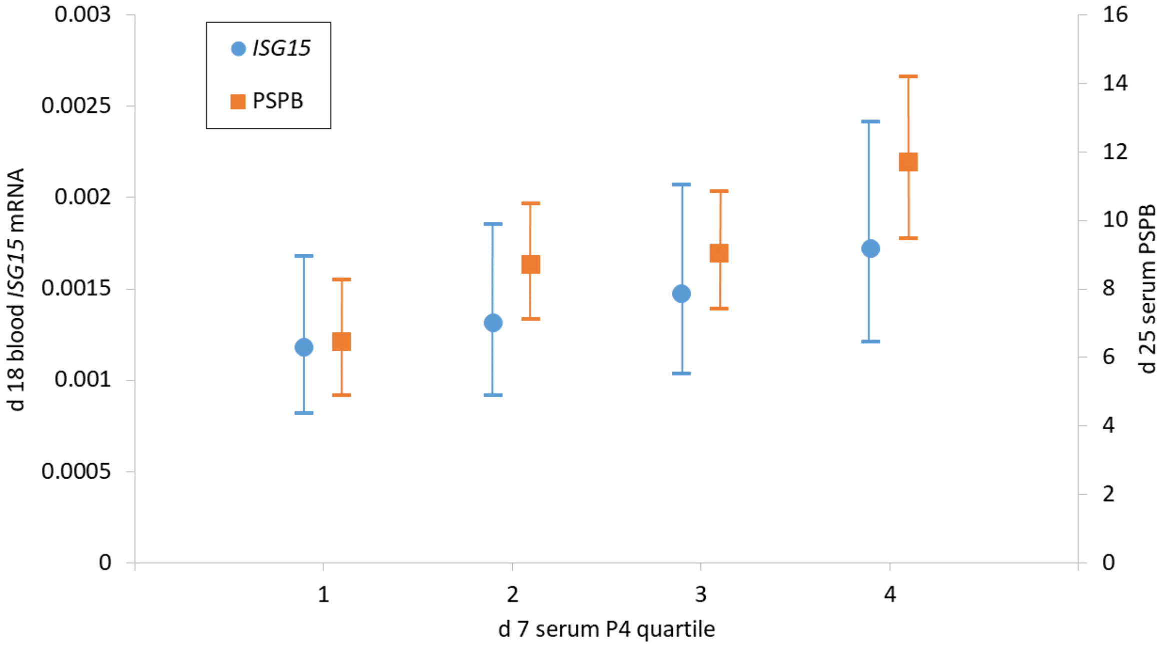
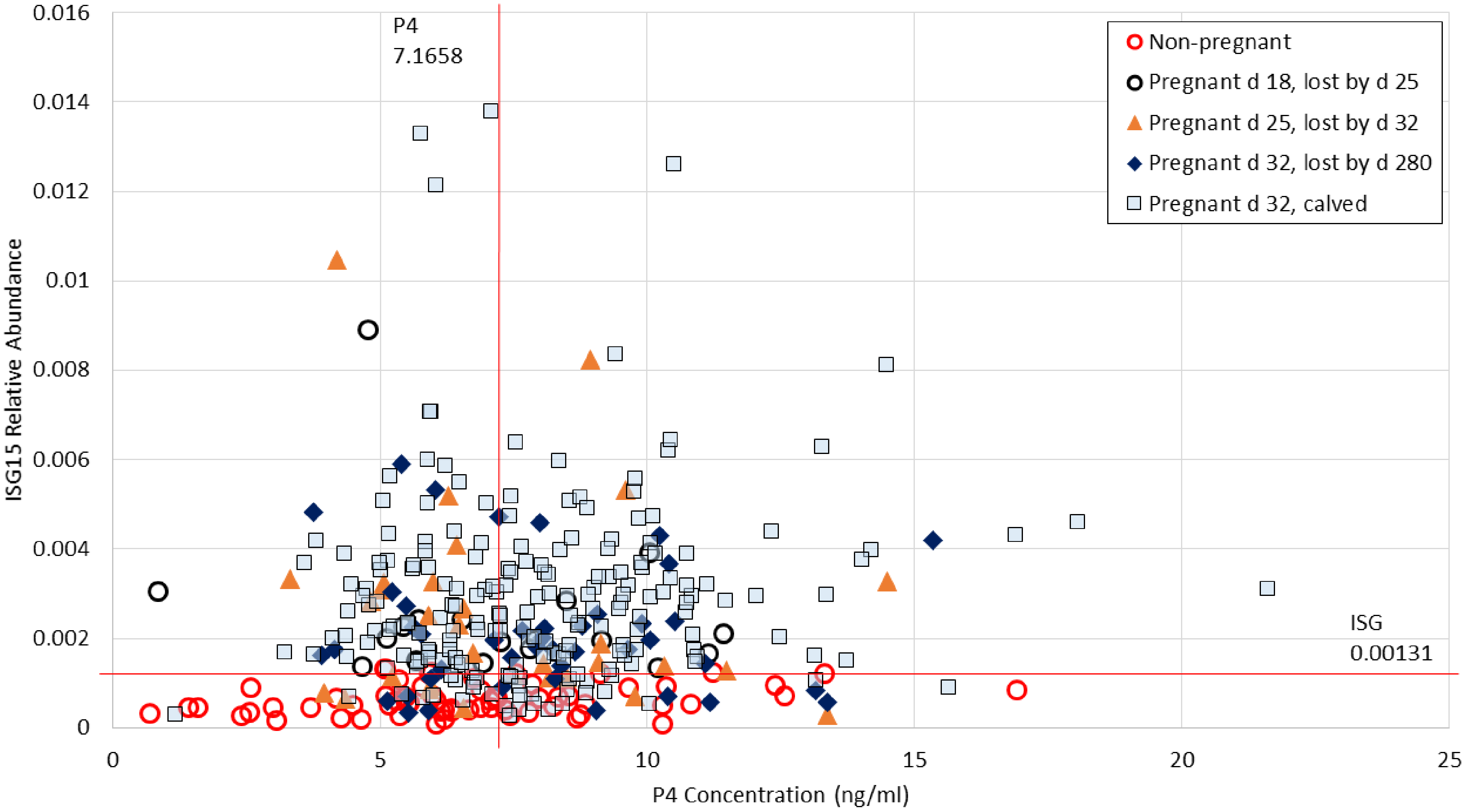
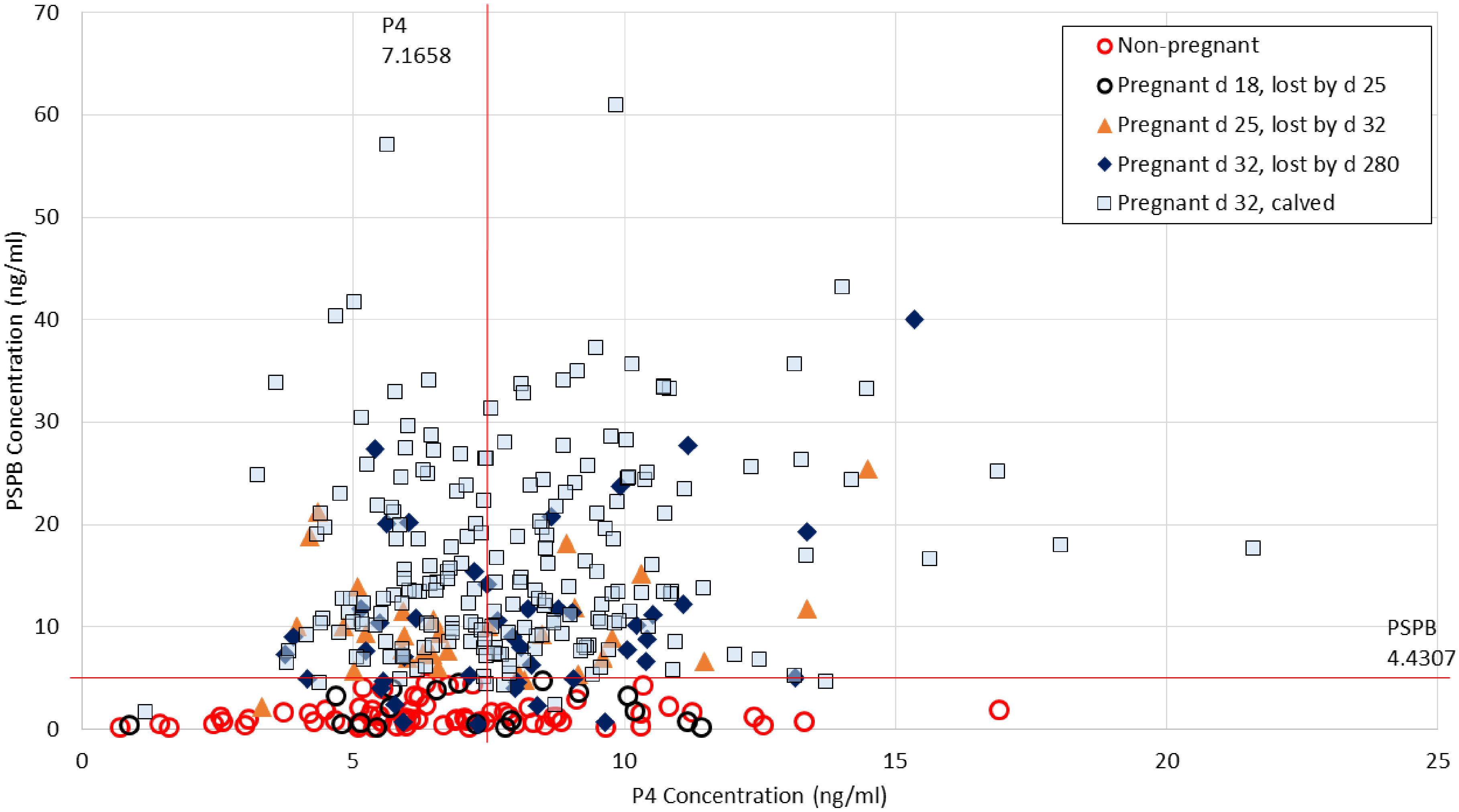


Figure 3.4 Relationships between d 7 serum progesterone (P4) concentration (by quartile) with mean d 18 interferon-stimulated gene-15 (ISG15) mRNA abundance in peripheral blood and mean d 25 serum pregnancy-specific protein B (PSPB) concentration . The whiskers around each mean indicate the 95% CI. Progesterone quartile was associated with mean d 18 mRNA abundance of ISG15 in peripheral blood (P = 0.01) and the mean d 25 serum PSPB concentration (P < 0.0001).

A

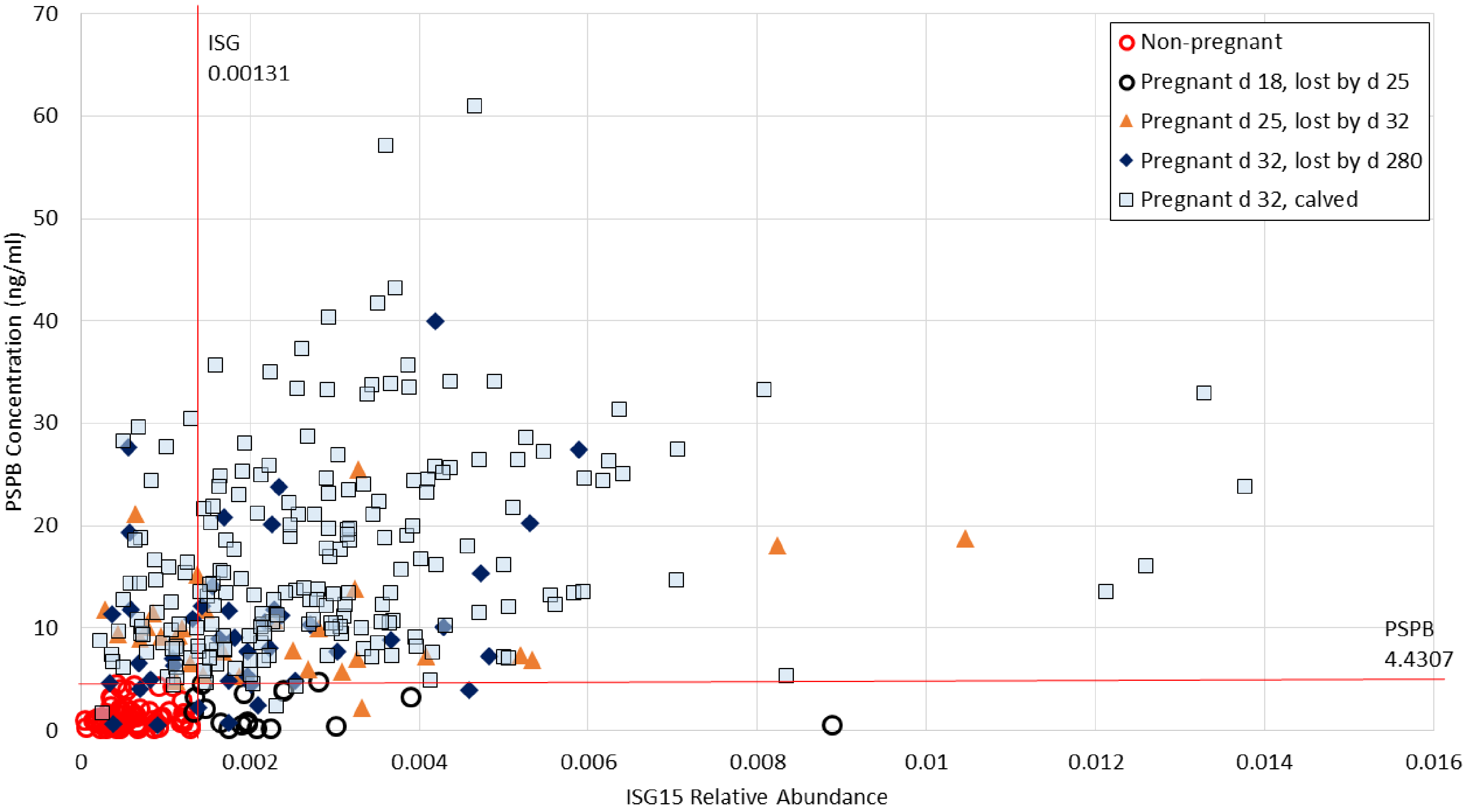

0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
ISG15 Relative Abundance
0
5
10
15
20
25
P4 Concentration (ng/ml)
P4
7.1658
ISG
0.00131
Non-pregnant
Pregnant d 18, lost by d 25
Pregnant d 25, lost by d 32
Pregnant d 32, lost by d 280
Pregnant d 32, calved

B



P4
7.1658
PSPB
4.4307
○ Non-pregnant
○ Pregnant d 18, lost by d 25
▲ Pregnant d 25, lost by d 32
◆ Pregnant d 32, lost by d 280
□ Pregnant d 32, calved
PSPB Concentration (ng/ml)
P4 Concentration (ng/ml)
0
10
20
30
40
50
60
70
0
5
10
15
20
25

c



ISG
0.00131
PSPB
4.4307
○ Non-pregnant
○ Pregnant d 18, lost by d 25
▲ Pregnant d 25, lost by d 32
◆ Pregnant d 32, lost by d 280
☐ Pregnant d 32, calved
PSPB Concentration (ng/ml)
ISG15 Relative Abundance
0
10
20
30
40
50
60
70
0
0.002
0.004
0.006
0.008
0.01
0.012
0.014
0.016

Figure 3.5 Scatterplots of the relationship between (A) d 7 serum progesterone (P4) concentration and d 18 interferon-stimulated gene-15 (ISG15) expression in peripheral blood, (B) d 7 serum P4 concentration and d 25 serum pregnancy-specific protein B (PSPB) concentration, and (C) d 18 ISG15 expression in peripheral blood and d 25 serum PSPB concentration. Varying pregnancy outcomes are displayed in separate sets of data points: Non-pregnant cows, cows deemed pregnant on d 18 based on ISG-15 but had undergone pregnancy loss by d 25, cows deemed pregnant on d 25 based on PSPB concentration but had undergone pregnancy loss by d 32, cows pregnant on d 32 but had undergone pregnancy loss before parturition and cows that reached full-term parturition. The solid red vertical and horizontal lines indicates the cut-off values for P4, ISG15 and PSPB, respectively, that were used for pregnancy determined by ROC curve analysis (see text for details). The relationships between serum P4, peripheral blood mRNA abundance of ISG15 and serum PSPB were explored, but the R2 was <0.1 in all cases with the exception of the relationship between P4 and PSPB (R2 = 0.13) and the relationship between ISG15 and PSPB (R2 = 0.12).

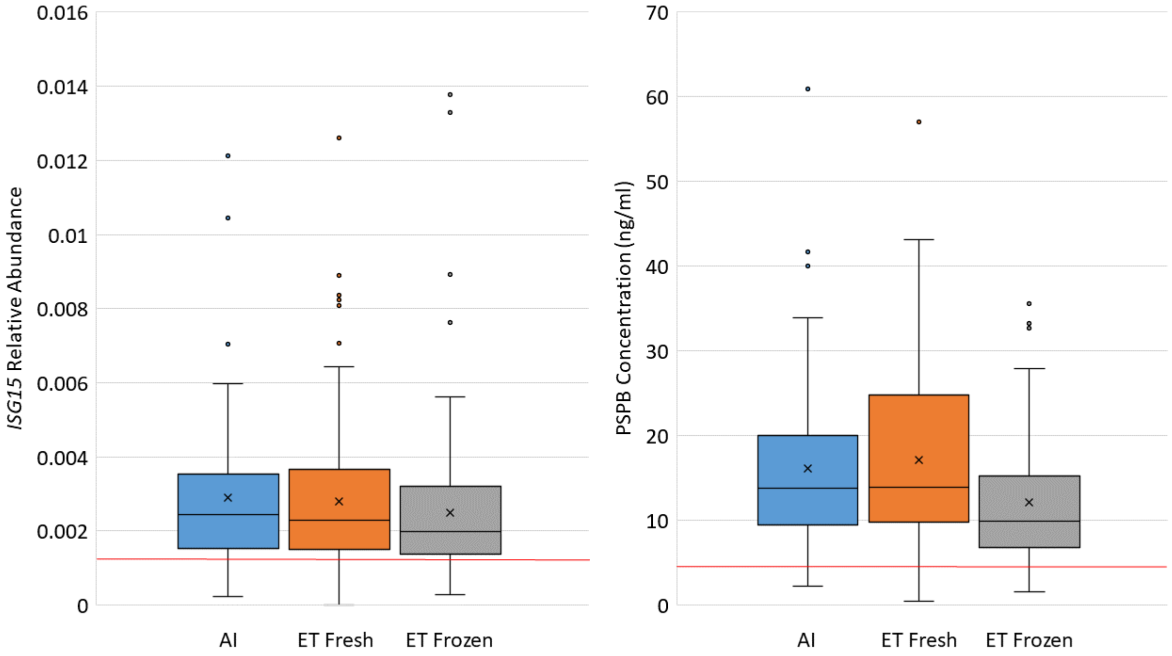


Figure 3.6 Day 18 interferon-stimulated gene-15 (ISG15) expression in cows predicted pregnant on d 18 (left) and d 25 pregnancy-specific protein B (PSPB) concentration in cows predicted pregnant on d 25 (right) for each treatment. Cows that received fresh ET tended to have a greater mRNA abundance of ISG15 than cows that received frozen ET ($P = 0.097$). Cows that received AI and fresh ET had greater serum concentrations of PSPB than cows that received frozen ET ($P = 0.006$ and $P < 0.0001$ respectively). The solid red horizontal lines indicates the cut-off values for ISG15 (0.00131) and PSPB (4.4307 ng/mL), respectively, that were used for pregnancy determined by ROC curve analysis (see text for details). When a cow was predicted to be non-pregnant on d 18 and subsequently predicted to be pregnant by PSPB on d 25 (n = 58) or predicted non-pregnant on d 25, and subsequently diagnosed pregnant by ultrasound on d 32 (n = 11), the earlier diagnosis of pregnancy status was corrected for the purpose of the data analyses.

3.5 Discussion

Studies that have reported the timing of pregnancy loss from conception to parturition in lactating dairy cows are scarce, and controlled studies comparing pregnancy and pregnancy loss outcomes in dairy cows bred using AI, fresh ET and frozen ET appear to be non-existent. Using data from 1106 lactating dairy cows, this study builds on data previously reported (Crowe et al., 2024a) by characterizing the incidence and timing of pregnancy loss from service event (AI or ET) to calving following TAI or TET with a fresh or frozen IVP embryo. The main findings reported by Crowe et al. (2024a) were that the transfer of fresh IVP embryos led to similar P/S to that achieved with AI, and both fresh IVP ET and AI had greater P/S than transfer of frozen IVP embryos. These findings have been extended here by adding additional days of pregnancy diagnosis by measuring peripheral blood mRNA ISG15 expression on day 18 and serum PSPB concentration on d 25, transrectal ultrasound pregnancy diagnosis at d 125 and recording of parturition date. This has provided a greater insight into the timing of when pregnancy loss occurred. As expected, greater P/S on d 32 and d 63 previously reported by Crowe et al. (2024a) translated into a greater probability of the recipient dam subsequently achieving a full-term pregnancy and parturition. Measurement of peripheral blood ISG15 mRNA abundance and serum PSPB concentrations have provided evidence that cows that fail to achieve critical threshold levels of these markers of pregnancy have markedly reduced likelihood of maintaining a pregnancy to full-term.

In the discussion that follows, the findings related to pregnancy establishment and pregnancy loss in the current study are examined for each time-period (d 0 to d 7, d 7 to d 18, d 18 to d 25, d 25 to d 32, d 32 to d 62, d 62 to d 125 and d 125 to parturition). Wiltbank et al. (2016) summarized four pivotal periods of pregnancy loss during the first trimester of gestation in high-producing lactating dairy cows: d 0 to d 7, d 8 to d 27, d 28 to d 60, and d 61 to d 90. Berg et al. (2022) reported estimates of pregnancy success at d 7, 15, 28, 35, and 70 in seasonal-calving dairy cows managed under a pasture-based system. These studies have provided useful estimates of the timing of pregnancy loss to compare against the results of the current study.

d 0 to d 7:

Several major developmental events occur in the first week after insemination and ovulation, including fertilization, the first mitotic divisions of the embryo, the timing of which is associated with developmental competence (Lonergan et al., 1999), activation of the embryonic genome at the 8- to 16-cell stage (Rabaglino et al., 2023), morula compaction and blastocyst formation. Fertilization and the first 3 to 5 days of embryonic development occur in the oviduct, which plays a central role in early embryo development. The quality of embryos cultured in the oviduct is better than embryos cultured in vitro in terms of morphology, gene expression and cryotolerance (Rizos et al., 2002a,b,c, Lonergan et al., 2003). Maillo et al. (2012) conducted endoscopic transfer of IVP embryos into the oviduct of either lactating or non-lactating cows on d 2 and recovered embryos from the uterus on d 7 by non-surgical flushing. The results provided strong evidence that the environment for embryo development is compromised by lactation-induced metabolic stress (greater concentrations of non-esterified fatty acids and β-hydroxybutyrate and lower concentrations of glucose, insulin, and insulin-like growth factor-1 than non-lactating cows) with fewer embryos developing to the blastocyst stage in lactating cows compared with non-lactating cows (Maillo et al., 2012).

Earlier studies indicated that heifers, beef cattle and dairy cows with moderate milk production exhibited >90% fertilization rate (Diskin and Sreenan, 1980, Diskin and Morris, 2008). Sartori et al. (2010) reported that the mean fertilization rate in seven studies that captured data from single-ovulating high producing dairy cows was 83%. More recently, Berg et al. (2022) estimated that the incidence of fertilization failure was 15.8% in pasture-based dairy cows in New Zealand that had been inseminated following detected estrus. Fertilization rate was not recorded in the current study in the cows assigned to be bred using AI; however, 9.6% of synchronized ET recipients (similar to the cows bred using AI) were deemed unsuitable for transfer on d 7, mostly due to absence of a CL or a small CL. This could reflect some level of synchronization protocol non-compliance, but more likely reflects individual animals exhibiting a sub-optimal response to one or more of the protocol interventions, resulting in cows either failing to ovulate or having a delay in the timing of ovulation. Absence of a CL is not compatible with pregnancy, while a small CL is associated with reduced P4 production, an altered endometrial transcriptome and delayed conceptus elongation (Lonergan and Sánchez, 2020). Fertilization is unlikely to have occurred in these animals if they had been inseminated. For this reason, along with the literature cited here, for the purposes of discussion we have assumed that the (potential) percentage of cows with successful fertilization in the AI group on d 1 after synchronized estrus was 90%.

The incidence of embryo loss during the first week post-breeding can be captured by recording the proportion of non-viable embryos recovered following non-surgical uterine flushing on d 6 to d 7 post-breeding; this has been estimated to be as high as 50% in some reports (Sartori et al., 2010, Wiltbank et al., 2016). On d 7 post-breeding, Berg et al. (2022) estimated that the incidence of early embryo death/arrest before the morula stage was 10.3%, and an additional 5.5% were classified as non-viable early morulae. The causes of early embryo loss before d 7 likely reflect compromised oocyte quality due to a variety of factors including postpartum negative energy balance and body condition loss (Maillo et al., 2012), postpartum disease (Ribeiro et al., 2016a), heat stress (Hansen et al., 2001), and inadequate circulating P4 concentrations (Forde et al., 2012). For the purposes of estimating pregnancy on d 7 for the AI treatment in the current study, we created a similar model using the d 7 serum P4 concentration values as we did with ISG15 and PSPB. This gave rise to a similar (potential) P/S following AI on d 7 (77.0%) as Berg et al. (2022) had previously reported (70.9%).

For the ET treatments, we assumed a pregnancy/ET of 100% on d 7. For the fresh embryos, only grade 1 blastocysts were transferred. Post-thaw in vitro survival of frozen grade 1 blastocysts was assessed in a subset of embryos produced during this study by Crowe et al. (2024a). Assessments were made at 24 h, 48 h and 72 h after thawing. 94.4% of blastocysts had re-expanded and 19.6% had hatched at 24 h, 96.3% had re-expanded and 51.4% had hatched at 48 h and 72 h after thawing, 96.3% had re-expanded and 72.0% had hatched. Based on this assessment, with > 95% survival post-thaw, we have assumed 100% pregnancy for both fresh and frozen ET treatments on d 7.

Transfer of a grade 1 blastocyst to the uterus bypasses any problems or deficiencies associated with the follicle, oocyte and oviduct in the recipient dam, and therefore, could potentially contribute to improved pregnancy outcomes (Hansen, 2020). Despite this, pregnancy per ET with an IVP embryo is not better than cows bred with AI because pregnancy losses are greater in cows bred using IVP embryos compared with AI. The temporal pattern of this pregnancy loss is discussed in the following sections.

d 7 to d 18:

This window of time represented the period with the greatest proportion of pregnancy loss in the current study. The losses were broadly similar for all treatments, ranging from 27.1% to 32.8%. Following hatching from the zona pellucida on around d 8 to 9, the blastocyst undergoes a change in morphology from a spherical to ovoid shape during a transitory phase preceding the elongation or outgrowth of the trophectoderm to a tubular, then filamentous form that usually begins between d 12 and 14. Around this time, the trophectoderm cells of the conceptus begin to secrete significant amounts of IFNT, the maternal pregnancy recognition factor in cattle, which ultimately blocks the uterine luteolytic mechanism to ensure maintenance of a functional CL and production of P4 (Bazer and Thatcher, 2017). Conceptus elongation during the second week after fertilization is driven by increasing circulating P4 concentrations that induce changes in the endometrial transcriptome (Forde et al., 2009, Spencer et al., 2016), which are reflected in changes in the uterine lumen fluid composition (Simintiras et al., 2019a, Simintiras et al., 2019b). Inadequate concentrations of P4 have been associated with delayed conceptus elongation (Forde et al., 2011) and failure of the conceptus to elicit an appropriate transcriptomic response from the endometrium (Forde et al., 2012, Sanchez et al., 2019). In support of these previous studies, there was strong evidence of an association between serum P4 concentration on d 7 and peripheral blood mRNA abundance of ISG15 on d 18 and serum concentration of PSPB on d 25 in the current study, reinforcing the central role of P4 in regulating early embryo development. We have previously reported that 72.0% of frozen-thawed blastocysts had hatched in vitro by 72 h post-thawing (Crowe et al., 2024a). The hatching rate in vivo after transfer is not known; however, embryos that fail to hatch cannot elongate and attach and hence would not establish a pregnancy. Reasons for hatching failure could be related to differences in zona pellucida ultrastructure and hardness between oocytes and embryos developed in vivo and those cultured in vitro (Duby et al., 1997).

According to Wiltbank et al. (2016), pregnancy loss from d 8 to d 27 averaged approximately 30%, and failed or delayed trophoblast elongation accounted for 7% of pregnancy loss in the second week after estrus. On d 15 after detected estrus and insemination, Berg et al. (2022) recovered conceptuses and uterine luminal fluid from 406 lactating dairy cows, and estimated pregnancy success to be 59.1%. Of 259 conceptuses recovered, 19 (7.3%) had failed to elongate beyond the spherical or ovoid stage and were deemed non-viable, contributing to pregnancy failure during the second week after estrus. Sánchez et al. (2019) transferred multiple IVP Grade 1 blastocysts into recipient heifers on d 7 and conducted post-mortem uterine flushing on d 15, and reported heterogeneous conceptus morphology with 18% ovoid, 16% tubular, and 66% filamentous. This variation in conceptus length and morphology was similar to that observed by Ribeiro et al. (2016b), who reported the length of recovered conceptuses on d 15 after AI in non-superstimulated dairy cows. Delayed or failed conceptus elongation during this window is associated with reduced IFNT secretion and a failure to elicit an adequate response from the endometrium for maternal recognition of pregnancy (Sánchez et al., 2019). In addition, inadequate conceptus IFNT secretion will lead to reduced expression of ISGs in maternal peripheral blood, which was associated with pregnancy failure in the current study.

d 18 to d 25:

Conceptus attachment to the endometrium typically first occurs around d 20-21 post-fertilization (Wathes and Wooding, 1980). During this period, trophoblast giant binucleate cells develop within the chorion to migrate and fuse with the uterine surface epithelium to form syncytial plaques. These binucleate cells produce PSPB, which migrates from the conceptus, across the newly forming placenta, into maternal circulation. Recent studies have highlighted that the timing of conceptus attachment, as assessed by increasing concentrations of PSPB in maternal circulation, is strongly associated with subsequent pregnancy loss in lactating dairy cows (Middleton and Pursley, 2019, Middleton et al., 2022, Santos et al., 2023). In cows that had conceptus attachment later than d 21 post-ovulation, the likelihood of pregnancy loss was four times greater compared with cows that had conceptus attachment on d 20 or 21 (Santos et al., 2023). Interestingly, preliminary analysis from a subsequent study (Crowe et al. 2024b) suggests that conceptus attachment is, on average, delayed following the transfer of frozen IVP embryos compared with AI.

Berg et al. (2022) reported that total pregnancy losses in lactating dairy cows after d 15 were minimal, with pregnancy success on d 15 not differing from that at d 70. Wiltbank et al. (2016) reported that 20% of pregnancies were lost during the window between d 8 and 28, citing delayed elongation, inadequate circulating P4 concentrations after AI (a potential cause of the compromised elongation) and histotroph deficiencies as the main causes of loss in this window. In the current study, pregnancy loss during the interval between d 18 and d 25 varied between 11.3% and 13.0% depending on treatment. Based on the previously cited studies by Berg et al (2022) and Wiltbank et al. (2016), it is likely that these losses were related to the failure of the embryo to attach (or weak/late attachment) to the uterine endometrium.

d 25 to d 32:

The estimated incidence of pregnancy loss between d 25 to d 32 in the current study ranged from 3.3 to 14.0% (AI: 3.3%; ET fresh: 11.8%; ET frozen: 14.0%). Due to the limited size of the subset of cows sampled on d 18 and d 25, statistical power was limited, and treatment differences were not detected. Cows that received AI, however, did tend to have a lesser incidence of pregnancy loss in this window of time than cows that received ET.

During this window, the conceptus should be attached, the fetal heartbeat is detectable and the initiation of limb and organ development is evident (Valadão et al., 2018). From approximately d 26, the conceptus becomes identifiable by transrectal ultrasound (Fricke, 2002, Romano et al., 2006). Inadequate placentation is a potential explanation for embryonic mortality after d 28 of gestation, since placentome formation occurs between d 25 and 50 of gestation in cattle (Seo et al., 2023).

In a meta-analysis of the incidence of pregnancy losses in dairy cows at different stages to d 90 of gestation, Albaaj et al. (2023) report that pregnancy losses averaged 27% during the early embryonic stage between d 19 and d 32 of gestation. During this window, the early estimates of pregnancy status included in the meta-analysis relied on measures of blood mRNA abundance of ISG15 to predict pregnancy. Wiltbank et al. (2016) suggested that this method of predicting pregnancy may produce approximately 15% false-negative results. In the current study, we observed that using mRNA abundance of ISG15 in peripheral blood on d 18 to predict pregnancy status resulted in 10.7% (58/541) false negatives (i.e., cows deemed not pregnant on d 18 but subsequently detected as pregnant on d 25 or later).

d 32 to d 62:

Similar to the d 25 to d 32 window of time between pregnancy diagnoses, poor placentome development increases the risk of pregnancy loss up to d 62. We previously reported treatment differences in pregnancy loss during this interval between AI (4.0%), ET fresh (11.3%) and ET frozen (18.0%), indicating that pregnancies derived from IVP embryos had a greater risk of compromised placentome formation and vascular development. Wiltbank et al. (2016) reported that the main causes of pregnancy loss between d 28 and d 60 were defects in placentome and/or vascular development (losses of ~12%). Domingues et al. (2023) reported that approximately 50% of pregnancy loss is initiated by embryonic death and 50% by luteal regression. Their review estimated that the typical incidence of pregnancy loss between d 28 and d 60 is between 5 and 20%. These values are broadly in agreement with Albaaj et al. (2023), who reported pregnancy loss during the late embryonic period (d 30 to d 45) of approximately 13% and an additional 7% during the early fetal period (d 45 to d 60), resulting in a cumulative 20% pregnancy loss between d 30 to d 60. Our results are largely in agreement, suggesting that a conceptus derived from IVP may have a greater risk of poor placentome formation and vascularity compared with a conceptus derived from AI. Conversely, Berg et al. (2022) observed a similar pregnancy success on d 15 as they did on d 28, d 35 and d 70, suggesting little to no pregnancy losses between d 15 and d 70 in cows that received AI after detected estrus, agreeing with the minimal pregnancy losses reported by Diskin et al. (2006). We hypothesize that the risks associated with these IVP embryos are compounded by cryopreservation and thawing of the embryo for transfer.

d 62 to parturition:

Pregnancy losses were minimal from d 62 to term (AI: 1.9%, ET fresh: 2.9% and ET frozen: 4.3%) and no differences were observed between treatments. This period begins during the first trimester and ends at parturition. Fetal development continues through the second trimester, and formation of internal organs is largely complete by 180 days (Valadão et al., 2018). The majority of fetal growth occurs in the last trimester, particularly during the final month when it will reach full size (Prior and Laster, 1979). Pregnancy losses after d 62 are generally small in lactating dairy cows. Previous studies have estimated the incidence of pregnancy loss during the interval from d 60 to d 90 to be approximately 2% (Wiltbank et al., 2016, Albaaj et al., 2023). Pregnancy losses can be greater in specific categories of cows, particularly those carrying twins in the same uterine horn (Garcia-Ispierto and López-Gatius, 2019). In the current study, none of the pregnancy losses were associated with twin pregnancies; however, a small number of late term abortions did occur during months 7 and 8 of gestation (n = 3 for ET fresh; n = 4 for ET frozen). As this is clearly a low frequency event, much larger datasets will be required to determine whether the likelihood of a cow aborting is affected by the origin of the embryo.

In agreement with Wiltbank et al. (2016), Berg et al. (2022) and Albaaj et al. (2023), the majority of the embryonic mortality in the current study occurred early in gestation (before d 25), and was broadly similar for each treatment. After d 25, the incidence of pregnancy loss began to differ between treatments (no major loss was recorded in the AI treatment after d 25; however, there was a further 23.1% and 32.0% loss from fresh ET and frozen ET, respectively, between d 25 and d 62.

Overall:

The underlying causes of greater pregnancy loss following ET are unclear but likely multifactorial, related to oocyte quality and, in particular, suboptimal in vitro conditions impacting embryo survival after transfer, as well the adverse effects of cryopreservation of IVP embryos. Despite high fertilization success in vitro, typically only 30 to 40% of immature oocytes progress to the blastocyst stage (Rizos et al., 2002c). A proportion of this loss may be related to a greater incidence of chromosomal abnormalities in IVP embryos (Viuff et al., 1999, Lonergan et al., 2004). Domingues et al. (2023) investigated the effect of increased plasma concentrations of P4 on embryonic attachment and concentrations of PAG, and also examined relationships among luteal regression, pregnancy loss, and plasma PAG concentrations in cows undergoing pregnancy loss by d 33 of pregnancy. Approximately 50% of pregnancy loss was due to luteal regression and the remaining 50% was due to conceptus failure (i.e., a decrease in PAG in the absence of luteal regression). They concluded that increased P4 concentrations did not accelerate embryonic attachment or early placental development, but did increase PAG concentrations on d 47 and d 61 of gestation. In the current study, P4 concentration on d 7 was not associated with pregnancy loss after d 25 of gestation. The cows in this study had similar distributions of serum P4 concentrations on d 7 in all treatments (Crowe et al., 2024a). The major factors that contributed to embryo mortality after maternal recognition of pregnancy likely reflect inherent differences in embryo quality and developmental competence that already existed on d 7 in cows that received TET vs TAI, and additional complications arose from embryo cryopreservation. Further examination of media usage in IVP, alternative media or methods for the cryopreservation of blastocysts may be of merit in future studies.

3.6 Conclusion

In conclusion, cows assigned to both AI and fresh ET had greater probability of becoming pregnant, maintaining the pregnancy and ultimately reaching full-term parturition compared with cows assigned to frozen ET. Cows that had greater serum concentration of P4 on d 7 had greater peripheral blood mRNA abundance of ISG15 on d 18, indicating a stronger maternal response to embryo derived IFNT. These cows also tended to have a greater serum concentration of PSPB on d 25, which in turn was associated with greater likelihood of reaching full-term parturition. Further work is required to optimize the in vitro production and cryopreservation of embryos to increase the probability of pregnancy establishment and reduce the incidence of early embryonic mortality.

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Chapter 4: Time to conceptus attachment and subsequent pregnancy loss in seasonal-calving pasture-based lactating dairy cows following timed artificial insemination with conventional or X-sorted semen or timed embryo transfer with frozen-thawed in vitro produced embryos

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4.1 Abstract

The objective of this study was to determine the timing of presumptive conceptus attachment (pCA) and its relationship with subsequent pregnancy loss in lactating dairy cows following timed artificial insemination with conventional (TAI-C) or X-sorted (TAI-S) semen or timed embryo transfer (TET) with frozen-thawed in vitro-produced (IVP) embryos. Lactating cows (n = 417, predominantly Holstein Friesian) located in 3 herds were blocked based on parity, calving date and economic breeding index and randomly assigned to receive TAI-C (n = 136), TAI-S (n = 136) or TET with a single, grade 1 frozen-thawed IVP blastocyst (n = 145). Cows were synchronized with a 10-d Progesterone-Ovsynch protocol, and were either inseminated (TAI-C, TAI-S) 16 h after the second GnRH or received TET on d 7 after synchronized ovulation. Serum progesterone was measured in all cows on d 7 after expected ovulation. For all cows that had not returned to estrus, serum pregnancy-specific protein B (PSPB) was measured on d 17 and daily from d 19 through 28 after expected ovulation to characterize the timing of pCA. The d of pCA was defined as the first day of an increase in PSPB of ≥ 12.5% from baseline (d 17) followed by two more consecutive d of ≥ 12.5% increase from the previous d. Pregnancy was diagnosed in cows that had not returned to estrus via ultrasound examination on d 32, 62, and 120 post-ovulation, and calving data were recorded. Day of pCA (mean; 95% CI) was earlier for TAI-C (20.0; 19.7, 20.3) compared with TET (20.6; 20.3, 20.9), and TAI-S (20.3; 19.9, 20.6) was not different from the other two treatments. Calving/service event (C/SE) was greater (83.2% vs 54.4%) and pregnancy loss during the interval from pCA to expected calving date was less (16.8% vs 45.6%) for cows with early pCA (≤ d 20, 23/137) compared with cows that had late pCA (≥ d 21, 36/79). The incidence of pregnancy loss was greater for cows assigned to TAI-S (30.7%) and TET (33.8%) than TAI-C (16.4%). Consistent with previous studies, measuring time to pCA provides new insights to understanding early pregnancy establishment and pregnancy loss in lactating dairy cows. The timing of pCA was later for TET compared with TAI-C and may be associated with increased risk of pregnancy loss. Further research into the regulatory mechanisms underlying conceptus attachment is warranted.

4.2 Introduction

The use of assisted reproductive technologies, such as in vitro embryo production (IVP) and embryo transfer (ET), are of significant interest to the dairy sector as a means of propagating superior genetics (Crowe et al., 2021). The increased use of sex-sorted semen (SS) to breed replacements (Berodier et al., 2019) and beef semen to improve the value of non-replacement calves (Ettema et al., 2017) has markedly reduced the pool of male dairy calves available as future potential AI sires. Targeted use of IVP with elite breeding stock is now widely used to facilitate the production of future generations of elite bulls.

The number of IVP embryos transferred annually has markedly increased in the past decade and now supersedes the number of in vivo embryos transferred (Viana, 2023). Some challenges remain to be resolved before use of IVP embryos becomes more widespread, however, including poor cryotolerance and greater incidence of embryo loss (Crowe et al., 2024a, Crowe et al., 2024b). The underlying mechanisms responsible for such losses are not clear, but are likely related to the consequences of suboptimal post-fertilization culture conditions on blastocyst quality (Rizos et al., 2002b, Lonergan et al., 2003). Pregnancy loss in dairy cows leads to major reproductive inefficiency at herd level. The increased risk of pregnancy loss observed following ET with IVP blastocysts (Crowe et al., 2024b) may hamper the uptake of this technology due to the financial implications of reduced herd fertility. These costs are exacerbated in seasonal, pasture-based systems of production with a compact breeding season (Shalloo et al., 2014). Comprehensive reviews published by Sartori et al. (2010), Wiltbank et al. (2016) and Ealy and Seekford (2019) have examined the perceived causes and timing of pregnancy loss in dairy cattle. Recently, Berg et al. (2022) and Crowe et al. (2024a) reported new data on the timing of pregnancy losses in lactating dairy cows, with Crowe et al. (2024a) reporting the timing and incidence of pregnancy loss following timed artificial insemination (TAI) or timed ET (TET; fresh and frozen-thawed IVP embryo), from service event through to parturition. In that study, the largest proportion of pregnancy loss occurred before d 18 (TAI and TET). Pregnancy loss from d 32 to d 62 was greater following ET compared with AI, particularly with frozen embryos. Losses after d 62 were small (≤ 3.5%). The percentage of cows that calved following fresh ET was similar to AI (both greater than frozen ET).

Conceptus attachment in cattle is estimated to begin between d 18 and 22 after artificial insemination (AI) (Wathes and Wooding, 1980, Guillomot and Guay, 1982). Recent studies indicated that measurement of circulating concentrations of pregnancy-specific protein B (PSPB) in individual cows during the expected time of conceptus attachment could accurately detect non-pregnant cows; failure to detect a 10% or greater increase in serum PSPB relative to a pre-attachment baseline concentration on d 17, accurately diagnosed non-pregnancy at 24 d post-AI (Middleton and Pursley, 2019). Stangaferro et al. (2021) used a combination of plasma PSPB measurements on d 22, d 25, d 29 and d 32 and transrectal ultrasound to estimate pregnancy and pregnancy loss following TAI. Compared with cows that lost their pregnancy by d 32 after TAI, cows that remained pregnant had greater circulating concentrations of PSPB on days 25, 29 and 32 after TAI. Middleton et al. (2022) and Santos et al. (2023) reported that daily serum PSPB measurements could pinpoint the specific day when presumptive conceptus attachment (pCA) occurred. Interestingly, both studies reported that nulliparous heifers had earlier pCA compared with both primiparous and multiparous cows. Importantly, these data support the time to pCA as a direct determinant of subsequent pregnancy loss in lactating dairy cows; cows that had a delayed increase in PSPB (on d 22 or after) exhibited a marked increase in the likelihood of embryonic mortality (Santos et al., 2023).

The use of SS has increased in many countries during the past decade (Crowe et al., 2021). Pregnancy/AI is less with SS compared with conventional unsorted semen (Maicas et al., 2019, Drake et al., 2020, Maicas et al., 2020). Whether this is due to poorer fertilization rates, increased pregnancy loss or a combination of both is not clear. There are few published reports from strongly powered controlled studies that examined the effect of semen type used for AI (i.e., conventional or SS) on the incidence and timing of pregnancy loss. In one relatively large study that used Holstein heifers to examine the timing of AI with SS or conventional semen (approximately 400 heifers per treatment), there was no effect of semen type on pregnancy loss from d 30 through to parturition (Chebel and Cunha, 2020).

The objective of the current study was to determine the timing of pCA and subsequent incidence of pregnancy loss in seasonal-calving pasture-based lactating dairy cows following TAI with conventional (TAI-C) or X-sorted (TAI-S) semen or TET with a frozen-thawed IVP embryo. We tested the hypothesis that the increased incidence of embryo loss following the transfer of frozen-thawed IVP embryos is due, at least in part, to delayed conceptus attachment.

4.3 Materials and Methods

All experimental procedures involving animals were approved by the Teagasc Animal Ethics Committee and authorized by the Health Products Regulatory Authority in Ireland, in accordance with Statutory Instrument No. 543 of 2012 under European Union legislation (Directive 2010/63/EU) for the Protection of Animals used for Scientific Purposes. Experimental procedures with animals were conducted between April 2022 and February 2023.

4.3.1 Synchronization for TAI and TET

Lactating cows (n=417, predominantly Holstein Friesian) located in 3 Teagasc research herds at Moorepark Animal and Grassland Research and Innovation Centre were blocked based on parity, calving date and Economic Breeding Index and randomly assigned to receive TAI-C (n=136), TAI-S (n=136) or TET with a single, grade 1 frozen-thawed IVP blastocyst (n=145). Blastocysts were produced from oocytes collected from the ovaries of elite Holstein Friesian donors using transvaginal oocyte pick-up, as previously described (Crowe et al., 2024b). Cows were synchronized with a 10-d Progesterone-Ovsynch protocol, as described by Drake et al. (2020), and were either inseminated (TAI-C, TAI-S) 16 h after the second GnRH or received TET on d 7 after synchronized ovulation. On the day of scheduled TET, recipient reproductive tracts were examined by transrectal ultrasonography and 9 cows (5.8%) were removed from the study (absent or poor quality corpus luteum, uterine infection). Thus, 136 recipients received TET. The experimental design is illustrated in Figure 4.1.

Across all treatments on all 3 farms, 36 elite dairy bulls and 3 beef bulls (only used in TAI-C) were used. Of these 22, 6 and 2 were used only for TAI-C, TAI-S and TET, respectively. Four bulls were used for both TAI-C and TAI-S, 2 bulls were used for both TAI-C and TET, and 1 bull was used for both TAI-S and TET. Two bulls were used across all three treatments.

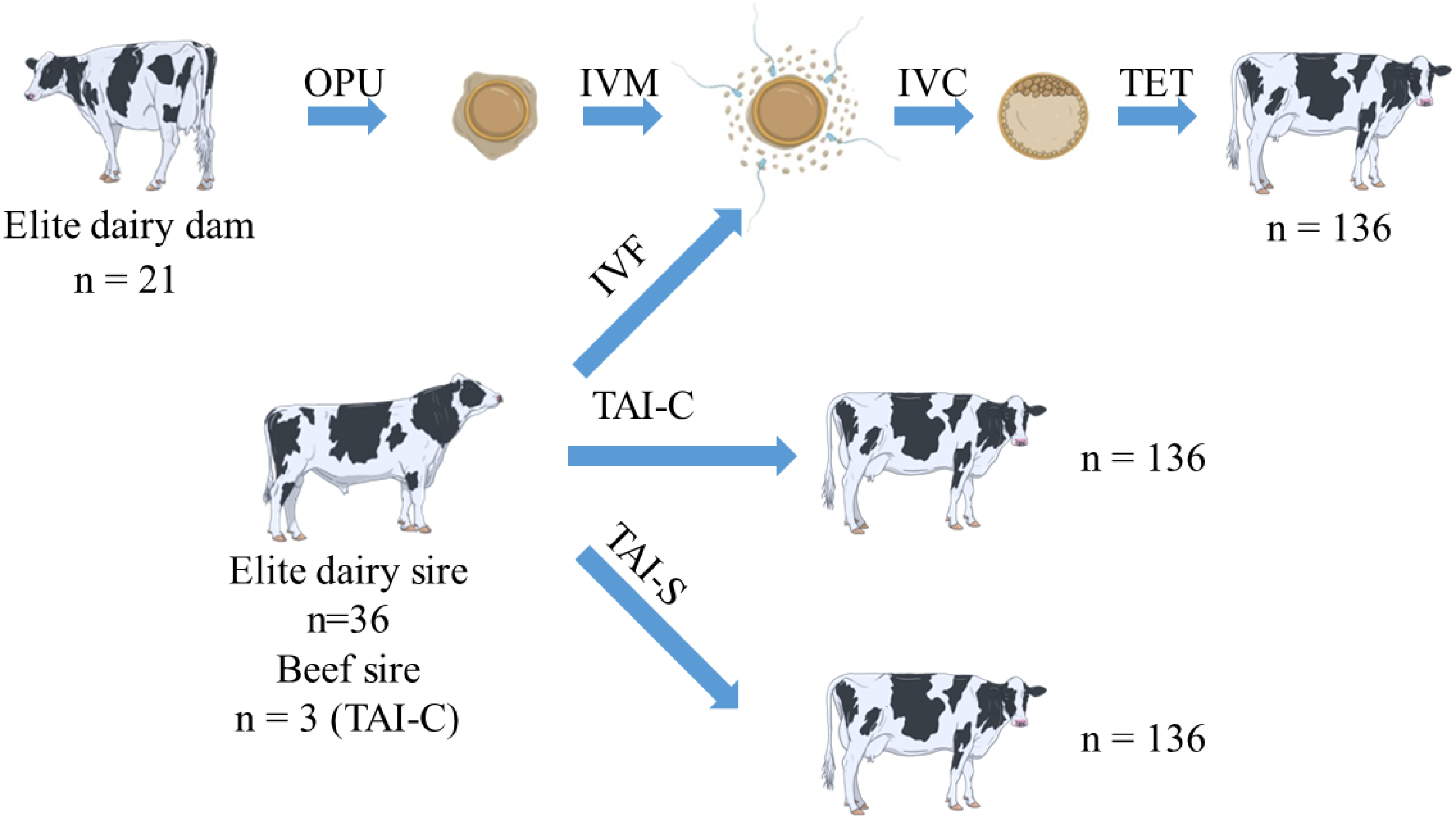
4.3.2 Blood sample collection

Blood samples were collected into serum tubes (BD Vacutainer, BD, Plymouth, UK) from the coccygeal vessels of 408 cows that were available for daily blood sample collection on d 7, and subsequently from all cows that had not returned to estrus on d 17 and daily from d 19 through d 28 after expected ovulation. Blood samples were stored at 4 °C for 24 h before centrifugation at 1,922 x g at 4 °C for 15 min. Aliquots of serum were separated using a Pasteur pipette, and transferred into 3.5 mL, labelled microtubes and stored at -20 °C until analysis. The timeline of events and schedule for sample collection is illustrated in Figure 4.1. The number of cows in each treatment that had serum PSPB concentration determined on each day of sample collection is reported in Table 4.1.

Table 4.1 Number of cows with pregnancy-specific protein B concentrations on each d of blood sample collection following timed artificial insemination with conventional semen (TAI-C), X-sorted semen (TAI-S) or timed transfer of a frozen-thawed in vitro-produced embryo (TET). As cows were observed returning to estrus, blood sample collection was ceased.

d of sample	TAI-C	TAI-S	TET	Total
17	132	135	136	403
19	121	126	124	371
20	102	113	106	321
21	95	106	99	300
22	91	103	93	287
23	89	101	88	278
24	88	91	84	263
25	86	88	82	256
26	85	88	82	255
27	85	87	82	254
28	85	87	82	254

A


OPU
IVM
IVC
TET
Elite dairy dam
n = 21
n = 136
IVF
TAI-C
n = 136
TAI-S
n = 136
Elite dairy sire
n=36
Beef sire
n = 3 (TAI-C)

B

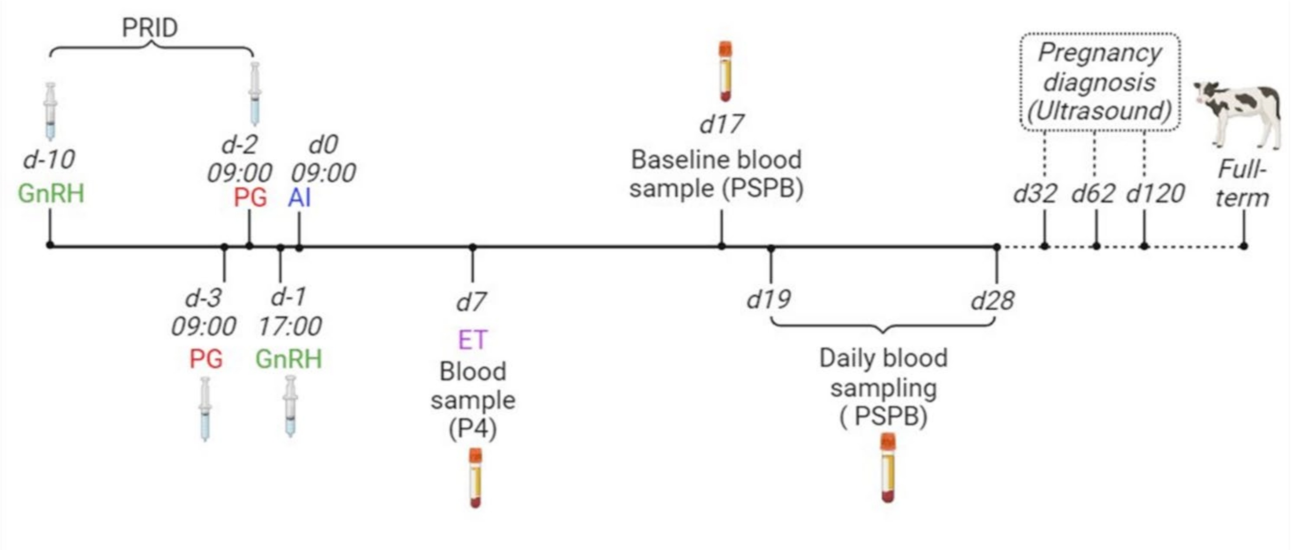


Figure 4.1 (A) Experimental design. Lactating dairy cows were bred by timed artificial insemination with conventional (TAI-C) or X-sorted (TAI-S) semen or by timed embryo transfer (TET) of a frozen-thawed in vitro-produced embryo. Oocytes were collected weekly from the ovaries of elite dairy donors using transvaginal ovum pick-up (OPU). Following OPU, cumulus-oocyte-complexes were transported to the laboratory where they completed in vitro maturation (IVM), approximately 24 h after the time of OPU. Following in vitro fertilization (IVF), presumptive zygotes were cultured in vitro (IVC) and resulting grade 1 frozen-thawed blastocysts were transferred by direct transfer on d 7 (d 0 – d of TAI). (B) Experimental timeline of the synchronization, service event (AI or ET), blood sample collection, ultrasound diagnosis of pregnancy, and recording of calf delivery. Cows received an intramuscular (IM) injection of GnRH (Ovarelin, CEVA Santé Animal, France) and had a progesterone-releasing intravaginal device (PRID Delta, CEVA Santé Animal) inserted into the vagina on d -10. Cows received IM injections of prostaglandin-F2α (Enzaprost, CEVA Santé Animal) on d -3 and -2, with PRIDs removed on d -2. Cows received a second GnRH injection on d -1. Cows were assigned to receive either TAI on d 0 or TET on d 7. Blood samples were collected on d 7 for determination of serum progesterone (P4), on d 17 for determination of a baseline concentration of pregnancy-specific protein-B (PSPB) and on d 19 - d 28 for determination of PSPB concentration. Pregnancy diagnosis was conducted using transrectal ultrasound on d 32, d 62 and d 120. Full-term parturition date was recorded for all cows (mean ± std. dev. = d 278.1 ± 5.5).

4.3.3 Measurement of progesterone concentration on d 7

Analysis of serum progesterone (**P4**) concentration was conducted using solid-phase radioimmunoassay with PROG-RIA-CT kits (DIAsource ImmunoAssays S.A., Louvain-la-Neuve, Belgium) according to the manufacturer´s instructions. The sensitivity of the assay was 0.05 ng/mL. The inter-assay coefficients of variation for quality control samples were 17.5% (low), 7.3% (medium), and 7.4% (high), respectively. The intra-assay coefficients of variation were 13.6% (low), 15.9% (medium), and 12.7% (high).

4.3.4 Analysis of Pregnancy-Specific Protein B (PSPB)

Frozen blood serum samples were shipped on dry-ice to the BioTracking facility, where concentrations of PSPB were determined using the quantitative BioPRYN-research assay (BioTracking, Moscow, ID, USA) according to the manufacturer's instructions and included a standard curve, as previously described by (Sasser et al., 1986). The PSPB standards (0, 0.125, 0.25, 0.5, 1, 2, 4, 8 and 16 ng/mL) were assayed in duplicate to generate the standard curve. The source of the PSPB used in the standards was native PSPB harvested from placental tissue and purified using an anti-PSPB antibody column (Branen et al., 2011). When serum samples had a PSPB concentration that was greater than the concentration of the greatest standard, samples were diluted with phosphate-buffered saline (PBS) as required to bring their concentration onto the standard curve and allow PSPB concentration to be quantified. Based on the model described by Santos et al. (2023), we tested several magnitudes of serum PSPB increase (10%, 12.5% and 15%) and different baseline PSPB values (d 7, d 17 and the mean of d 7 and 17). Ultimately, the model with the greatest sensitivity (100.0%) and specificity (83.5%) to determine the d of pCA (based on pregnancy on d 32) was based on the first day with an increase in serum PSPB of ≥12.5% relative to the baseline value (d 17), followed by two more consecutive d of ≥12.5% increase from the previous d. All samples from an individual cow were assayed in duplicate on the same plate along with positive and negative controls. Overall inter- and intra-assay CV were calculated using low and high controls on 57 plates. Inter- and intra-assay CV were 7.5% and 6.7% respectively. Based on previous findings reported by Santos et al. (2023) on the timing of pCA in lactating dairy cows, cows in the current study were classified as having early pCA (d ≤ 20) or late pCA (d ≥ 21).

4.3.5 Transrectal ultrasound examination

Pregnancy was diagnosed in cows that had not returned to estrus via transrectal ultrasound examination on d 32, 62, and 120 post-ovulation, and calving data were recorded. Fetal sex was determined based on the location of the genital tubercle during the ultrasound examination on d 62 and was confirmed at calving.

4.3.6 Power analysis

Recently, Santos et al. (2023) reported that mean (± SD) day of pCA was later for multiparous cows (21.1 ± 1.48 d) compared with primiparous cows (d 20.5 ± 0.79). For the current study, a power test indicated that to have an 80% chance of detecting, as significant at the 5% level, a difference in the day of pCA between treatments (TAI-C, TAI-S and TET) of 0.5 d (SD = 1.4 d) would require 123 cows per treatment. To mitigate against cows being removed from the study for various reasons, an additional 13 cows (10%) were enrolled for TAI-C and TAI-S and an additional 22 cows were enrolled for TET due to expected rejection of cows (5 to 10%) on the day of ET following examination of the reproductive tract.

4.3.7 Statistical Analysis

All statistical analyses were conducted using SAS v. 9.4 (SAS Institute, Cary, NC, USA).

The day of pCA and classification of pCA (early or late) were analyzed using generalized linear mixed models. Service treatment (TAI-C vs. TAI-S vs. TET), recipient breed and d 7 serum P4 concentration (initially as a continuous variable, and subsequently as a categorical variable) were included as fixed effects, and herd was included as a random effect. If the P-value for a variable was > 0.1, it was removed from that model. Treatment effects on binary outcome variables (pregnancy, pregnancy loss) were analyzed using Chi-square and Fisher's exact tests.

The dataset for daily PSPB was not normally distributed; d 19 data were removed (all basal concentrations causing a skew) and an appropriate Box-Cox transformation identified. Treatment effects on daily measures of PSPB were analyzed using generalized linear mixed models with repeated measures in 3 separate analyses: (1) cows that had pCA versus cows with no pCA; (2) cows that had pCA and reached full term parturition; and (3) cows that had pCA and were diagnosed pregnant on d 32 but subsequently underwent pregnancy loss. Fixed effects included treatment, day and their interaction, and the effects of farm, parity, days in milk and bodyweight were tested and retained if P < 0.1. Cow nested within treatment was included as a random effect. Evidence for an association between fetal sex and daily PSPB was also tested using repeated measures.

4.4 Results

The number and percentage of cows with pCA on each d and the number and percentage of cows classified as having had early pCA or late pCA are summarized in Table 4.2. Based on daily serum PSPB concentration data, 54.7% (223/408) of cows exhibited evidence of pCA. Of those, 63.2% (141/223) had pCA on or before d 20 (early pCA) and 36.8% (83/223) had pCA on or after d 21 (late pCA). The latest d on which pCA was observed for any cows on this study was d 25 (n = 2 cows). Day of pCA (mean; 95% CI) differed between TAI-C (20.0; 19.7, 20.3) and TET (20.6; 20.3, 20.9; P = 0.006). The mean d of pCA for TAI-S (20.3; 19.9, 20.6) was intermediate, and not different from either TAI-C (P = 0.393) or TET (P = 0.167).

Table 4.2 Number and percentage of cows with presumptive conceptus attachment (pCA) on each day of measurement (19 - 26) and classification as early (on or before d 20) or late (on or after d 21) pCA in lactating dairy cows following timed artificial insemination with conventional (TAI-C) or X-sorted semen (TAI-S) or timed embryo transfer (TET) with a frozen/thawed in vitro-produced embryo.

d of pCA	TAI-C n (%)	TAI-S n (%)	TET n (%)	
19	21 (30.0)	20 (26.0)	17 (22.4)	
20	33 (47.1)	27 (35.0)	23 (30.3)	
21	10 (14.4)	18 (23.4)	22 (28.9)	
22	5 (7.1)	11 (14.3)	8 (10.5)	
23	1 (1.4)	1 (1.3)	4 (5.3)	
24	0 (0.0)	0 (0.0)	0 (0.0)	
25	0 (0.0)	0 (0.0)	2 (2.6)	
Total n	70	77	76	
Timing of pCA^1				P-value
Early pCA	54 (77.1)	47 (61.0)	40 (52.6)	0.0001
Late pCA	16 (22.9)	30 (39.0)	36 (47.4)	
Total n	70	77	76	

^{1}The values reported are the raw figures for the total number of cows with early pCA or late pCA across all treatments.

4.4.1 Factors affecting the probability of pCA and the d of pCA

There was no effect of treatment (P = 0.655) on the incidence of pCA during the interval between d 19 and 28 post-ovulation (51.5%, 56.6% and 55.9% for TAI-C, TAI-S and TET, respectively). For cows that did have pCA, the timing of pCA was affected by treatment (P = 0.031). The mean d of pCA was earlier in cows assigned to TAI-C compared with cows assigned to TET, but cows assigned to TAI-S were not different from either TAI-C or TET (Figure 4.2). Compared with cows assigned to TAI-C, the incidence of early pCA was less for cows assigned TET (-24.7%; P = 0.007) and tended to be less for cows assigned to TAI-S (-16.0%; P = 0.095). The incidence of early pCA was not different between cows that received TET compared with cows that received TAI-S (P = 0.54).

Fetal sex was not associated with the d of pCA; cows gestating a female fetus had similar mean d (95% CI) of pCA (20.1; 19.8, 20.4) compared with cows gestating a male fetus (20.0; 19.7, 20.3).

Of those cows with early pCA, 93.6%, 87.2%, 85.8% and 83.2% were pregnant on d 32, d 62, d 120 and reached full-term, respectively. Pregnancy success was less (P < 0.0001) at each corresponding time point for cows with late pCA (65.9%, 57.3%, 57.3%, and 54.4%, respectively; Table 3.3). Seven cows (4 early pCA, 3 late pCA) were culled or sold, for reasons unrelated to the study, between diagnosis of pregnancy on d 120 and expected parturition. Calving/service event (**C/SE**) was greater (P < 0.0001) and pregnancy loss from pCA to expected parturition date was less (P < 0.0001) for cows with early pCA compared with cows that had late pCA (Figure 4.3). There was an overall effect (P = 0.019) of treatment on cumulative pregnancy loss from pCA to expected parturition; compared with cows assigned to TAI-C, cumulative pregnancy loss was greater for cows assigned to TET (+17.4%) and TAI-S (+14.3%). Presumptive conceptus attachment, pregnancy per service event and pregnancy loss data are reported for each treatment in Table 3.3 and for cows classified as having early pCA or late pCA in Table 4.4.

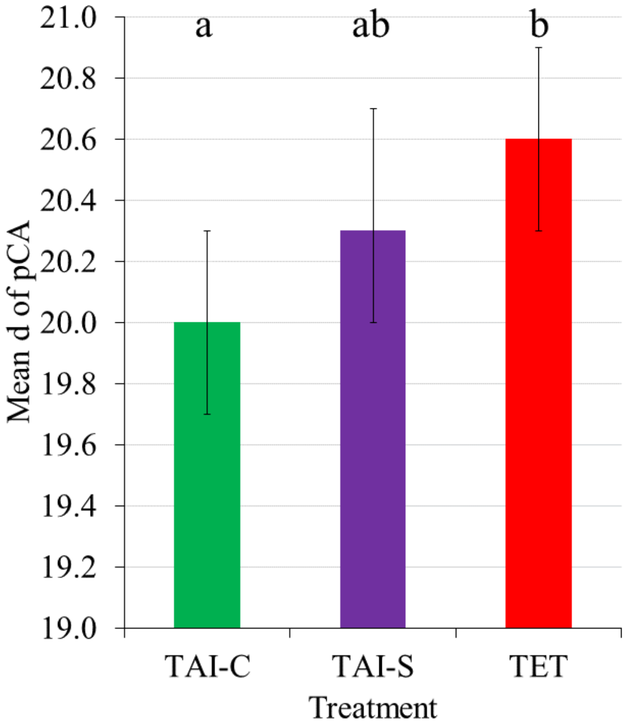


Figure 4.2 Mean d of presumptive conceptus attachment (pCA) following timed artificial insemination with conventional (TAI-C), n = 70, or X-sorted (TAI-S), n = 77, semen or timed embryo transfer (TET), n = 76, with a frozen-thawed in vitro-produced embryo. Treatment affected timing of pCA (P = 0.031). Cows that received TAI-C had earlier

pCA than cows that received TET (P = 0.027). Bars with different letters indicate a difference between treatments (P < 0.05).

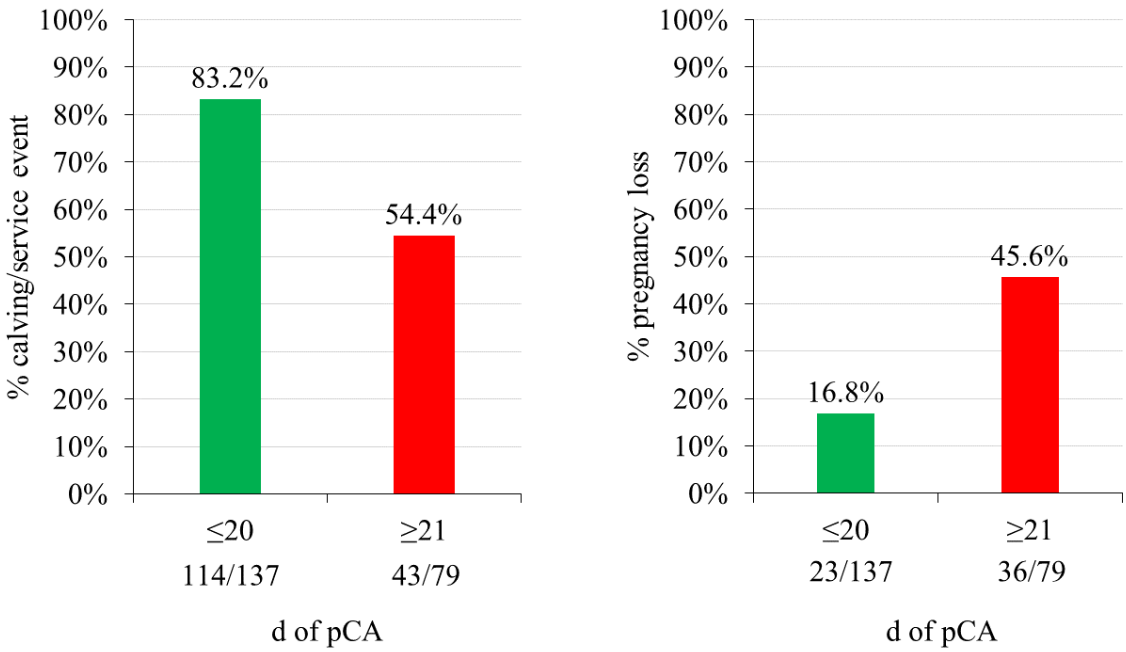


Figure 4.3 Calving/service event (left) and pregnancy loss from time of presumptive conceptus attachment (pCA) to calving (right) for cows with early pCA (≤ d 20, n = 137) compared with cows that had late pCA (≥ d 21, n = 79). Both calving/service event and pregnancy loss differed between cows with early and late pCA (P < 0.0001).

Table 4.3 Pregnancy/service event (P/SE) and pregnancy loss (PL) by each d of pregnancy diagnosis and at parturition (mean = d 278) in lactating dairy cows with early (on or before d 20) or late (on or after d 21) presumptive conceptus attachment (pCA).

Day of pregnancy diagnosis	Early pCA n/n (%)	Late pCA n/n (%)	P-value**
P/SE d 32	132/141 (93.6)	54/82 (65.9)	<0.0001
P/SE d 62	123/141 (87.2)	47/82 (57.3)	<0.0001
P/SE d 120	121/141 (85.8)	47/82 (57.3)	<0.0001
Term parturition*	114/137 (83.2)	43/79 (54.4)	<0.0001
Timing of pregnancy loss			
PL from pCA to d 32	9/141 (6.4)	28/82 (34.2)	<0.0001
PL from d 32 to d 62	9/132 (6.8)	7/54 (13.0)	0.089
PL from d 62 to d 120	2/123 (1.6)	0/47 (0.0)	0.522
PL after d 120*	3/117 (2.6)	1/44 (2.3)	0.425
Cumulative PL from pCA to Parturition *	23/137 (16.8)	36/79 (45.6)	<0.0001

* n = 4 cows with early pCA and n = 3 cows with late pCA, that had been pregnant on d 120, were removed from the study (culled or sold) between d 120 and parturition, for reasons unrelated to the study.

** P-values determined by Chi-square and Fisher's exact test.

Table 4.4 Presumptive conceptus attachment (pCA), pregnancy/service event (P/SE) and pregnancy loss (PL) for each d of pregnancy diagnosis and at parturition in lactating dairy cows following timed artificial insemination with conventional (TAI-C) or sex-sorted (TAI-S) semen or timed embryo transfer (TET) with a frozen-thawed in vitro-produced embryo.

Day of pregnancy diagnosis	TAI-C n/n (%)	TAI-S n/n (%)	TET n/n (%)
pCA	70/136 (51.5)^{c}	77/136 (56.6)^{d}	76/136 (55.9)^{e}
P/SE d 32	64/136 (47.1)^{c}	62/136 (45.6)^{d}	60/136 (44.1)^{e}
P/SE d 62	62/136 (45.5)^{c}	55/136 (40.4)^{d}	53/136 (39.0)^{e}
P/SE d 120	60/136 (44.1)^{c}	55/136 (40.4)^{d}	53/136 (39.0)^{e}
Term parturition*	56/133 (42.1)^{c}	52/134 (38.8)^{d}	49/134 (36.6)^{e}
Timing of pregnancy loss			
PL from pCA to d 32	6/70 (8.6)^{a}	15/77 (19.5)^{b}	16/76 (21.1)^{b}
PL d 32 to d 62	2/64 (3.1)^{c}	7/62 (11.3)^{d}	7/60 (11.7)^{d}
PL d 62 to d 120	2/62 (3.2)	0/55 (0.0)	0/53 (0.0)
PL d 120 to Parturition (%)*	1/57 (1.8)	1/53 (1.9)	2/51 (3.9)
Cumulative PL from pCA to Parturition*	11/67 (16.4)^{a}	23/75 (30.7)^{b}	25/74 (33.8)^{b}

* Of cows that were diagnosed pregnant on d 120, n = 3, 2 and 2 cows from the TAI-C, TAI-S and TET treatments were removed from the study (culled or sold) before parturition for reasons unrelated to the study.

$^{a-b}$ Values within a row with different superscripts differ ($P < 0.05$)**.

$^{c-e}$ Values within a row with different superscripts tend to differ ($0.05 < P < 0.1$)**.

** P-values determined by Chi-square and Fisher's exact test.

4.4.2 Factors affecting the daily concentration of PSPB

Across all treatments, cows with pCA had greater mean daily serum PSPB concentration than cows that had no evidence of pCA ($P < 0.0001$; Figure 4.4). Treatment effects on mean daily concentrations of PSPB (ng/mL) for cows that had pCA and were diagnosed pregnant on d 32, but subsequently either reached full-term parturition or underwent pregnancy loss are illustrated in Figure 4.5 and summarized Table 4.5. For cows that reached full-term parturition, there was no difference in mean daily serum PSPB concentration between treatments (treatment effect $P = 0.50$; treatment × time effect $P = 0.099$). The tendency for a treatment × time interaction arose because serum PSPB concentrations in the cows assigned to the TAI-C treatment diverged from the other treatments in the final two days of the sample collection period, but significant differences between treatments were not detected on any of the days. For cows that had pCA and were diagnosed pregnant on d 32 but subsequently underwent pregnancy loss before reaching full-term parturition, mean daily serum PSPB concentration were different between treatments (treatment effect $P = 0.053$; treatment × time effect $P = 0.38$). Cows assigned to TAI-C had greater mean serum PSPB concentration than cows that received TET ($P = 0.053$), whereas serum PSPB concentration in cows assigned to TAI-S was intermediate and did not differ from either TAI-C or TET ($P = 0.90$ and $P = 0.41$, respectively). Although the interaction between treatment and time was not significant, post-hoc analysis revealed that cows assigned to TET had lesser mean serum PSPB concentrations than cows assigned on TAI-C days 20, 21, 22 and 23 (all $P < 0.05$), and tended to have lesser serum PSPB concentrations than cows assigned to TAI-S on days 21, 22 and 23 (all $P < 0.1$). Mean daily serum PSPB concentration for cows gestating a male or a female fetus is illustrated in Figure 4.6 (fetus sex effect $P = 0.63$; fetus sex × time effect $P = 0.026$). Although the interaction between fetus sex and time was significant, post-hoc analysis revealed that serum PSPB concentrations were not different between cows gestating a male or a female fetus on any individual day (all $P > 0.2$).

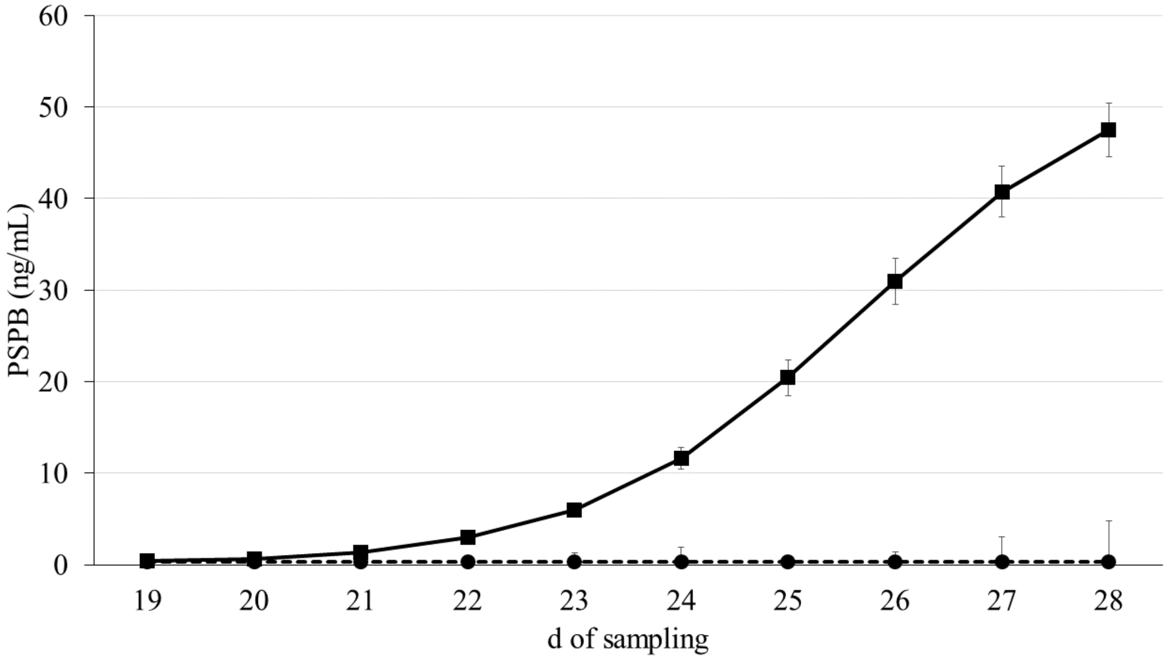
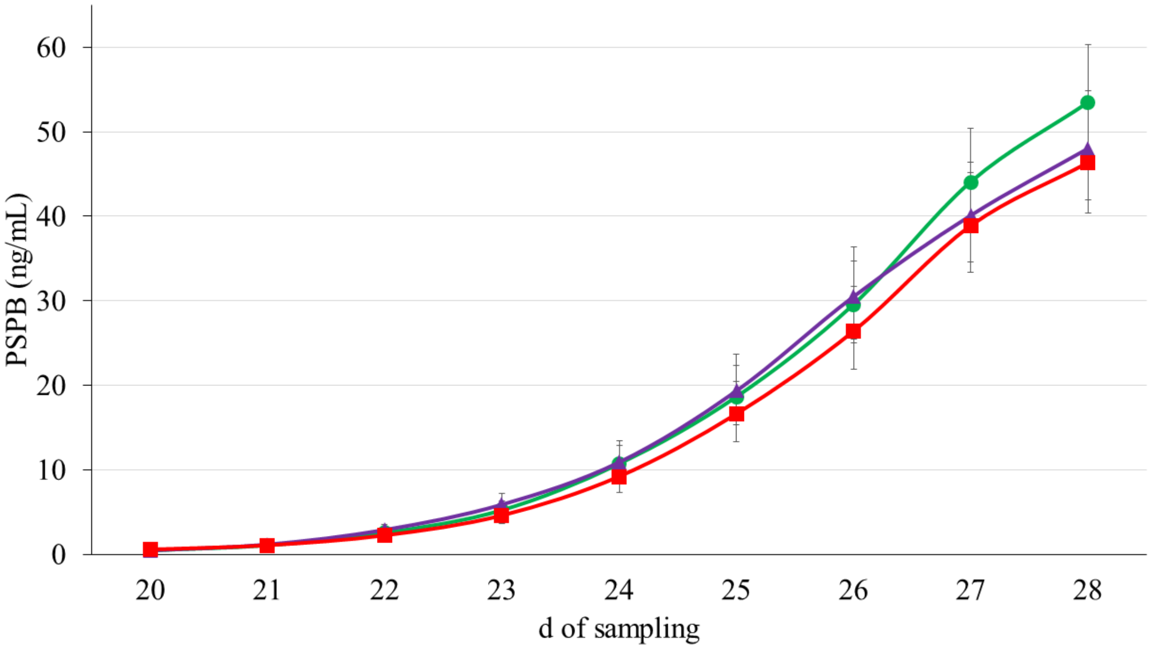


Figure 4.4 Mean daily serum pregnancy-specific protein B (PSPB) concentrations categorized by cows that had presumptive conceptus attachment (pCA) (■) and cows that had no evidence of pCA (-●-). The whiskers around each mean indicate the 95% CI. The first day with an increase in serum PSPB of ≥12.5% relative to the baseline value (d 17) followed by two more consecutive day of ≥12.5% increase from the previous day was defined as the day of pCA.

A



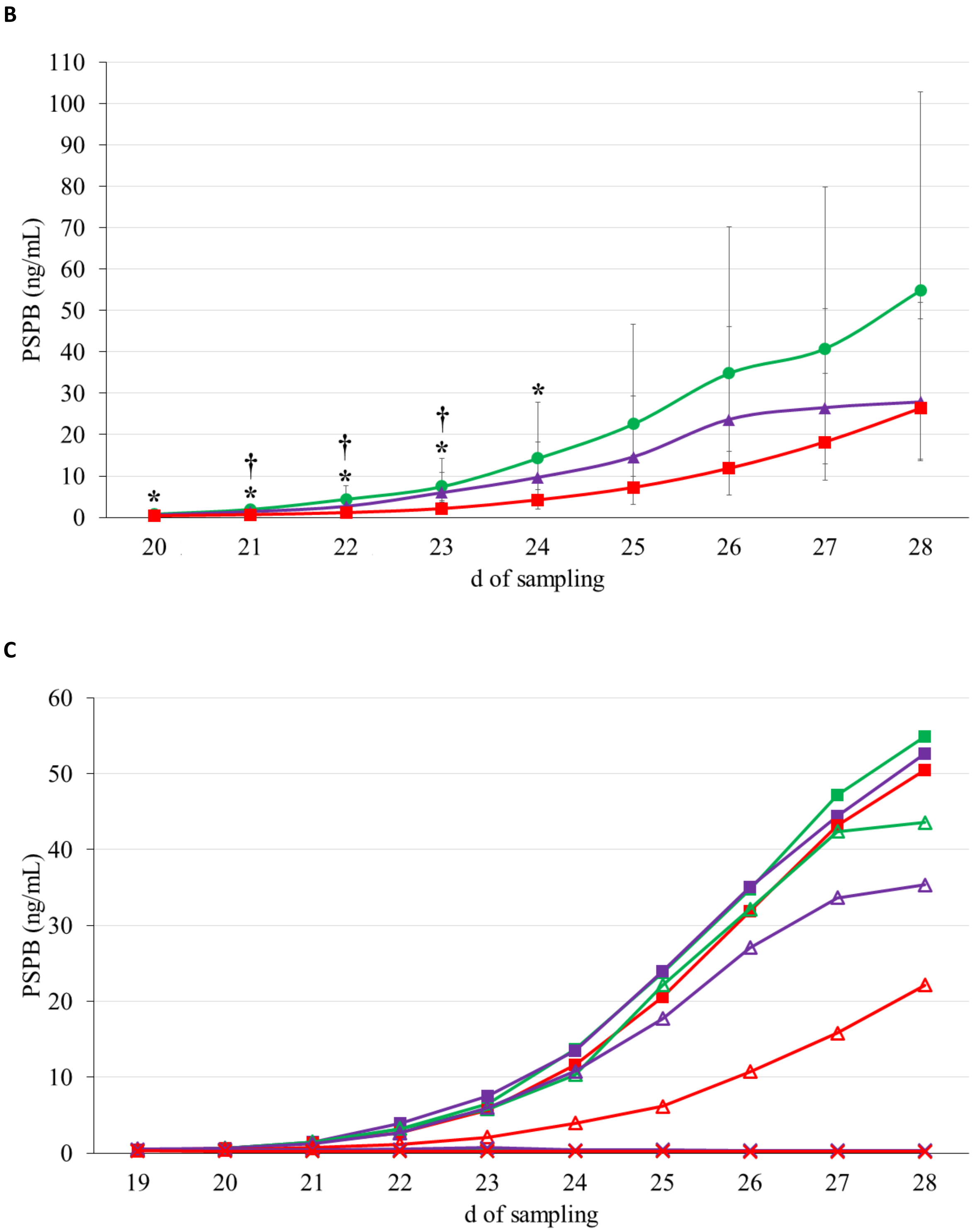


Figure 4.5 Mean daily serum pregnancy-specific protein B (PSPB) concentrations categorized by pregnancy status in lactating dairy cows following timed artificial insemination with conventional (TAI-C) or X-sorted (TAI-S) semen or timed embryo transfer (TET) with a frozen-thawed in vitro-produced embryo. The whiskers around each mean indicate the 95% CI. (A) Cows that had presumptive conceptus attachment (pCA) and reached full-term parturition (-●- - TAI-C, n = 56, -▲- TAI-S, n = 52, -■- TET, n = 49). The values presented are back-transformed model-adjusted least square means. Mean daily serum PSPB concentration did not differ between treatments (treatment effect P = 0.50; treatment × time effect P = 0.099). (B) Cows that had pCA but underwent pregnancy loss between pCA and reaching full-term parturition

(-●- - TAI-C, n = 11, -▲- TAI-S, n = 23, -■- TET, n = 25). The values presented are back-transformed model-adjusted least square means. Mean daily serum PSPB concentration was affected by treatment (treatment effect P = 0.053; treatment × time effect P = 0.38). * indicates greater serum PSPB concentration for cows that received TAI-C than cows that received TET (all P < 0.05) and † indicates a tendency for greater serum PSPB concentration for cows that received TAI-S than cows that received TET (0.05<P<0.1). (C) Raw data for serum PSPB concentrations for all cows across each of the three treatments TAI-C (—), TAI-S, (—) or TET (—) that had evidence of presumptive conceptus attachment (pCA) and reached full-term parturition (-■, -■, -■), evidence of pCA and underwent pregnancy loss between pCA and full-term (-△-, -△-, -△-) or had no evidence of attachment (-✶-, -✶-, -✶-), n = 66, 59 and 60 respectively.

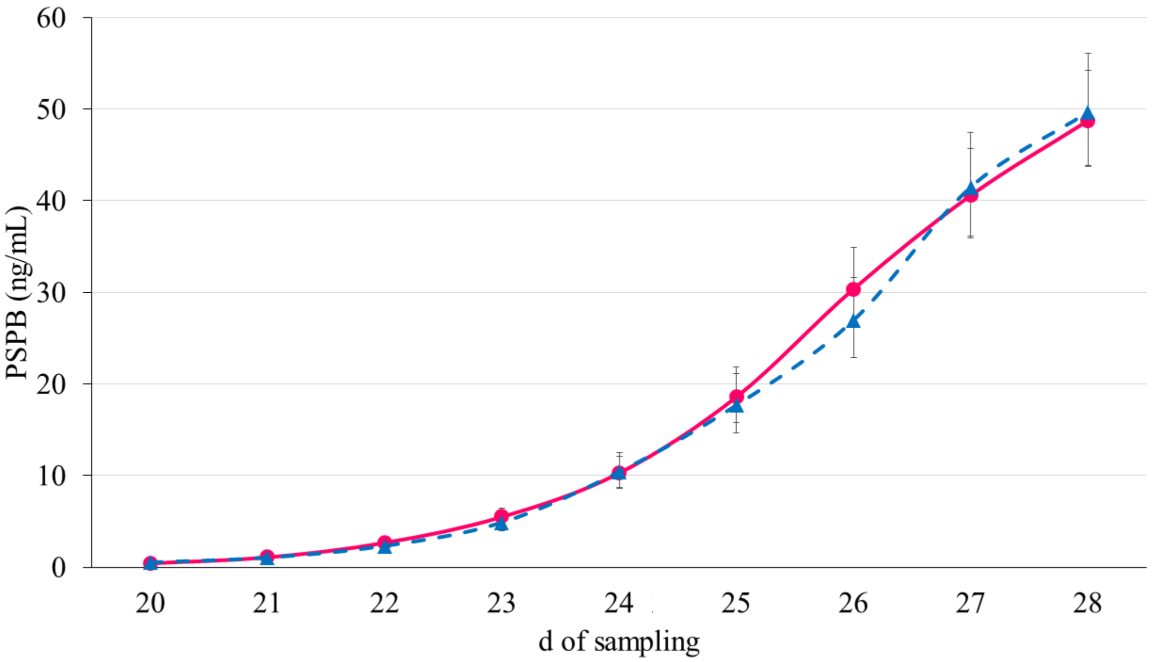


Figure 4.6 Mean daily serum serum pregnancy-specific protein B (PSPB) concentration for cows gestating male, n = 66, (-▲) and female, n = 90, (-●-) fetuses. The values presented are back-transformed model-adjusted least square means. The whiskers around each mean indicate the 95% CI. There was no difference in serum PSPB concentration observed on any day of PSPB sampling between cows gestating male and female fetuses (P = 0.63), but an interaction between fetus sex and day was detected (P = 0.026).

Table 4.5 Mean daily serum concentration (raw data) of pregnancy-specific protein B (ng/mL) for cows that reached full-term parturition, underwent pregnancy loss between presumptive conceptus attachment (pCA) and full-term parturition (Pregnancy Loss) or had no evidence of pCA or pregnancy (Non-pregnant) following timed artificial insemination with conventional (TAI-C) or X-sorted (TAI-S) semen or timed embryo transfer (TET) with a frozen-thawed in vitro-produced embryo.

	Full-term Parturition			Pregnancy Loss			Non-pregnant		
d of Sampling	TAI-C	TAI-S	TET	TAI-C	TAI-S	TET	TAI-C	TAI-S	TET
17	0.362	0.327	0.412	0.239	0.549	0.390	0.414	0.418	0.368
19	0.348	0.341	0.432	0.331	0.560	0.373	0.407	0.428	0.283
20	0.578	0.600	0.646	0.660	0.612	0.432	0.378	0.463	0.263
21	1.362	1.469	1.318	1.494	1.208	0.687	0.311	0.459	0.228
22	3.168	3.869	2.725	3.206	2.665	1.172	0.298	0.511	0.252
23	6.450	7.528	5.697	5.809	6.001	2.093	0.302	0.712	0.262
24	13.709	13.518	11.644	10.313	10.768	3.931	0.315	0.404	0.235
25	23.752	23.970	20.609	22.119	17.740	6.144	0.308	0.426	0.224
26	34.794	35.069	31.851	32.169	27.051	10.705	0.301	0.368	0.206
27	47.241	44.424	43.206	42.383	33.624	15.830	0.304	0.366	0.200
28	54.853	52.589	50.441	43.551	35.352	22.124	0.284	0.330	0.192

4.4.3 Effect of P4 on pCA and d of pCA

Serum P4 concentration on d 7 was associated with the probability of a cow having pCA; cows that had pCA had greater mean serum P4 concentration than those that did not have pCA (6.6 ng/mL vs 5.8 ng/mL, respectively; P = 0.002). Of the cows that had pCA, serum P4 concentration on d 7 was not associated with the d of pCA (P = 0.694) or with the probability of a cow having early pCA or late pCA (6.6 ng/mL for both groups; P = 0.87).

4.5 Discussion

Most pregnancy loss in cattle occurs during early embryogenesis or during conceptus attachment and the initial phases of placental development (Wiltbank et al., 2016, Berg et al., 2022). Inadequate placentation, including failure of binucleate cell differentiation or placentome formation, is a potential explanation for embryonic mortality after Day 30 of gestation, since placentome formation occurs between d 25 and 50 of gestation in cattle (Seo et al., 2023). Recent studies have clearly demonstrated that the precise timing of the initial increase in maternal serum PSPB concentration, and using this as a proxy for conceptus attachment, is a direct determinant of subsequent pregnancy loss in lactating dairy cows (Santos et al., 2023). It is widely acknowledged that the use of assisted reproductive technologies, in particular frozen IVP-ET, and to a lesser extent AI with sex-sorted semen, are associated with poorer pregnancy success compared with AI using conventional semen (Chebel and Cunha, 2020, Crowe et al., 2024b). To date, no studies have compared the timing of pCA in cows that were served with conventional semen, sex-sorted semen or frozen IVP-ET. In the current study, we assigned cows to each of these 3 breeding strategies, measured daily PSPB concentration to pinpoint the day when pCA occurred, and then used these data to examine associations with pregnancy and pregnancy loss outcomes at subsequent time points up to and including parturition. Consistent with previous studies (Santos et al., 2023), time to pCA was an important milestone, and helped to explain much of the pregnancy loss in lactating dairy cows associated with service type.

We hypothesized that the timing of pCA would be later for cows that received TET compared with those receiving TAI-C. This builds on data reported in our previous study, where the incidence of pregnancy loss was greater and the likelihood of reaching successful term parturition was less in lactating cows assigned to frozen IVP-ET compared with cows assigned to conventional AI (Crowe et al., 2024b). Delayed pCA may explain, at least partly, the increased pregnancy loss associated with IVP embryos. Other factors such as chromosomal abnormalities (Viuff et al., 1999, Lonergan et al., 2004), increased lipid content (Lonergan et al., 2006) and poorer morphology (Rizos et al., 2002a, Rizos et al., 2002b) induced by the IVF process may also contribute to pregnancy loss. Interestingly, there was some evidence from the current study that pCA was also delayed following AI with SS. Many studies have reported that pregnancy per AI is less following insemination with sex-sorted semen compared with conventional semen. It is generally assumed that this reflects greater incidence of fertilization failure due to a combination of fewer sperm per insemination and sperm damage during the sorting process (Maicas et al., 2019, Chebel and Cunha, 2020, Drake et al., 2020, Maicas et al., 2020). Controlled studies that evaluated the fertility performance of sex-sorted semen were not specifically designed to examine pregnancy loss. Additional studies to elucidate the effect of sex-sorted semen on early conceptus development and timing of attachment are warranted.

Delayed pCA observed in cows assigned to TET in the current study impacted pregnancy loss up to d 62. After that time point, pregnancy losses were minimal, and were not associated with timing of pCA, regardless of the service treatment. It should be noted, however, that all cows pregnant on d 62 had pCA between d 19 to d 23 inclusive. It is well described that serum P4 concentrations during the first two weeks of pregnancy, through its effects on the endometrium, are critical for conceptus elongation and therefore, by association, the ability of the conceptus to have early attachment (Forde et al., 2009, Spencer et al., 2016). In our previous study, cows with greater serum concentrations of P4 on d 7 had greater P/S at both d 32 and d 62 (Crowe et al., 2024b). Interestingly, while d 7 P4 concentration was associated with the likelihood of a cow having pCA in this study, it was not associated with the timing of pCA. Previously, Forde et al. (2011) reported that heifers with lesser serum concentrations of P4 had retarded embryo elongation on d 14 compared with heifers with normal serum P4. We would expect that delayed elongation would subsequently delay the timing of pCA, and perhaps with a larger sample size, or measuring P4 later in pregnancy, we may have observed an association between serum P4 concentrations and the timing of pCA.

By determining the absolute serum concentrations of PSPB, we were able to evaluate the associations between true PSPB concentration with pregnancy and pregnancy loss. When treatment effects on PSPB concentration were compared in cows with different pregnancy outcomes (pregnant, initially pregnant with subsequent loss, or pCA never detected), no treatment difference were detected in mean concentration or the mean daily PSPB concentration in cows that reached full-term parturition. For cows that initially had pCA but subsequently underwent pregnancy loss, however, differences in the daily mean PSPB concentration between treatments were apparent. Cows in the TET treatment that had evidence of pCA but subsequently underwent pregnancy loss had lesser serum PSPB compared with cows that received TAI-C or TAI-S, and underwent pregnancy loss following initial pCA, during the interval from d 20 to 24. This indicates that delayed and/or weaker pCA following TET with frozen IVP embryos has a significant impact on pregnancy loss. It is clear from these data that an early increase of PSPB (and hence, early pCA) was a key milestone to achieve pregnancy establishment and maintenance to parturition.

Histological studies have reported that attachment to the endometrium occurs on d 20-21 (Wathes and Wooding, 1980), which is consistent with the timing of pCA based on an increase in serum PSPB concentration in the current study. In previous publications, pCA was reported to occur approximately one d earlier in North American Holstein heifers (d 20) than Holstein cows (d 21) (Middleton et al., 2022). The current study enrolled only lactating dairy cows, but mean d of pCA occurred approximately one day earlier than previously reported in lactating Holstein cows in North America (on d 21) (Middleton et al., 2022, Santos et al., 2023). This difference may reflect differences in cow type and milk yield between the two systems of production (pasture-based vs. total mixed ration diet). During the last 25 years, fertility traits have been included in selection indices worldwide. The emphasis placed on genetic selection for superior daughter fertility traits has been most intense in seasonal-calving systems, however, due to the imperative for excellent herd reproductive performance during a short breeding period. Cows that are managed under a seasonal-calving pasture-based system produce less milk per cow compared with cows managed under confinement systems, and this may also have contributed to earlier pCA.

We have previously reported a deviation in sex ratio towards males amongst pregnancies derived from the transfer of IVP embryos compared with those derived from AI (Crowe et al., 2024b). Our observations in that study indicated that the bias toward male fetuses on d 62 to 65 in recipient cows was mirrored by a similar sex bias in IVP blastocysts on d 7, indicating similar survival of male and female embryos after transfer to recipients on d 7. Berry and Cromie (2007) reported that cows that received AI (both dairy and beef breeds) had a 51:49 male:female calf sex ratio, and the corresponding values for Holstein Friesian sires only was 52:48. In the current study, of the cows that reached d 62 of pregnancy and had fetal sex determined, no differences in subsequent losses beyond d 62 were observed between male or female fetuses, indicating that fetal sex was not associated with maintenance of pregnancy after that time. This is, however, limited to cows that reached d 62 of pregnancy, and does not give any indication of associations between fetal sex and pregnancy losses before d 62. Nevertheless, daily PSPB profiles and timing of pCA were largely similar for pregnancies with a male and female conceptus in the current study, and no difference was observed in the timing of pCA between TAI-C (presumed 50% female) and TAI-S (presumed 90% female). This may be evidence of the lack of an effect of embryo/fetal sex on pregnancy loss prior to d 62, but this study was not designed to address that specific question.

This study did have some limitations. As previously reported, post-thaw survival in vitro of frozen-thawed grade 1 IVP blastocysts was >95% (Crowe et al., 2024b). It is not possible to know what percentage of the embryos that were transferred in the current study successfully re-expanded or hatched. For those cows that did not have pCA, both the timing and causes of pregnancy loss are unknown. Another limitation was inability to use exactly the same team of bulls for TAI-C, TAI-S and TET. Hence, it was not possible to determine if sire had an effect on pCA and other pregnancy variables. Individual sires can have a strong effect on fertility performance, with significant variation in fertility after AI and after in vitro fertilization, despite passing quality control testing (Fair and Lonergan, 2018, Ortega et al., 2018).

Daily measurement of serum PSPB concentrations are a valuable aid to identifying the timing of pCA, even though unequivocal evidence that the increase in PSPB represents actual conceptus attachment is lacking. The filamentous bovine embryo is weakly attached to the epithelial surface until approximately Day 23 of pregnancy. During this time, the trophoblast binucleate cells migrate to the endometrial luminal epithelium and syncytial plaques that produce pregnancy associated glycoproteins begin to form. Additional research is needed to confirm that the timing of conceptus attachment to the endometrial epithelium is coincident with the initial onset of a sustained increase in PSPB.

4.6 Conclusion

Daily measurement of maternal serum PSPB concentration to determine the time of pCA is an important advancement that has allowed us to undertake detailed studies on pregnancy establishment and pregnancy losses in cows assigned to different reproductive technologies for breeding. Cows that received frozen-thawed IVP-ET had later pCA than cows that received TAI-C. This may explain, at least in part, the greater pregnancy losses observed in cows that received TET. Further work is required to optimize the in vitro environment and the cryopreservation techniques used for IVP to improve the likelihood of successful pregnancy establishment, advance the timing of pCA and increase the probability of pregnancy maintenance through to parturition.

4.7 Acknowledgements

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Chapter 5: Gestation length, calf birth weight, calving difficulty and calf health following timed artificial insemination (TAI) or timed embryo transfer (TET) with fresh or frozen in vitro produced (IVP) embryos

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In preparation

5.1 Abstract

The aim of this study was to determine the effect of embryo origin (artificial insemination (AI) vs in vitro embryo production (IVP)), calf breed, and calf sex on gestation length (GL), birthweight (BW), and calving difficulty (CD) score. Lactating dairy cows were randomly assigned to be bred by timed AI (frozen-thawed semen) or timed embryo transfer with fresh or frozen IVP embryos. The embryos were either dairy (Holstein Friesian (HF) or Jersey (JE)) or beef breed (Angus (AA) or Limousin (LM)). Data were subsequently obtained from 442 calves derived from these breeding events. For all calves, calving difficulty was scored on a scale of 1 to 4: 1 = unassisted calving, 2 = minor assistance, 3 = considerable difficulty or 4 = veterinary assistance/caesarean. For 281 calves, weight was recorded immediately after birth and for 108 of these calves, blood samples were collected at birth for biochemical and hematological analysis. The effects of the independent variables (origin, breed and sex) on GL, BW and CD score, and the association between these dependent variables was determined through a multiple regression model. There was a positive correlation between GL and BW (R2=0.11) and between and BW and CD score (R2=0.30). Overall, origin of the calf affected GL (d); 95% CI (AI: 278.9; 277.0, 280.8; ET-Fresh: 281.8; 279.8, 283.7; ET-Frozen: 282.0; 280.0, 284.0) and BW (kg), for HF calves only; 95% CI (AI: 36.1 kg; 34.6, 37.6; ET-Fresh: 40.6 kg; 38.9, 42.5; ET-Frozen: 39.0 kg; 37.2, 40.8). Breed affected BW, GL and CD; amongst calves born from ET, AA calves were heavier than HF or JE. Considering HF calves only, calves born from ET (fresh or frozen) were heavier than those born from AI. Overall, male calves were heavier (kg; 95% CI) than female calves (33.7 kg; 31.0, 36.6 vs. 32.5 kg; 29.9, 35.3, respectively). Results were within the normal parameters for healthy calves and treatment did not adversely affect calf health. In conclusion, calves originating from IVP/ET were heavier at birth, had longer GL and a greater incidence of CD than calves born from AI. Also, AA calves were heavier at birth than HF or JE.

5.2 Introduction

Assisted reproductive technologies (ARTs) such as sex-sorted semen, multiple ovulation and embryo transfer (MOET) and in vitro embryo production (IVP) are now well established as breeding tools available to dairy and beef producers and cattle breeding companies. The widespread use of artificial insemination (AI) using semen collected from elite genetic merit dairy sires has enabled dairy producers to improve the genetic merit of their herd. In recent years, there has been a marked increase in the usage of sex-sorted dairy semen to generate replacement heifers in many countries, which has facilitated greater use of beef semen to generate beef-cross calves of greater economic value from cows not required to generate replacements (Bittante et al., 2020, Pahmeyer and Britz, 2020, Cabrera, 2022).

Calves derived from dairy dams with 50% beef breed genetics following AI or natural service with a beef sire have greater economic value than male calves with 100% dairy breed genetics (Berry et al., 2019, Berry and Ring, 2020). Previously, we reported that pregnancy per ET was similar for dairy and beef breed IVP embryos transferred into lactating dairy cow recipients (Crowe et al., 2024). As dairy farmers continue to prioritize usage of sex-sorted semen on the best genetic merit dams to generate replacement heifers, the decline in number of male dairy calf births will be proportionally greatest for calves with elite genetic merit. There is great potential, therefore, to employ IVP-ET to produce future generations of elite AI bulls.

Since 2017, the number of IVP embryos transferred globally has surpassed the number derived by traditional superovulation (Crowe et al., 2021), and accounted for approximately 80% of all bovine embryos produced and transferred in 2022 (Viana, 2023). Indeed, a new milestone was reached in 2021 with over one million transfers of IVP embryos worldwide (Viana, 2022). Although issues with embryo cryotolerance, embryo mortality, and calf birth weight remain to be fully resolved, IVP embryos are likely to remain a key tool for genetic improvement in dairy herds (Sanchez et al., 2019), providing greater numbers of embryos per unit time, and greater capacity to generate multiple pregnancies from elite dam-bull combinations compared with traditional superovulation.

The birth of healthy offspring following the use of ARTs is essential to promote greater usage and acceptance. Since the birth of the first calf derived from IVP-ET in 1981 (Brackett et al., 1982), there have been numerous reports of Large Offspring Syndrome (LOS) in calves that were derived from IVP-ET, the first of which was reported by Behboodi et al. (1995). These calves were larger at birth, had greater incidence of dystocia and greater mortality than those derived from AI. Further reports of LOS and investigations into contributing factors were recently reviewed by Nava-Trujillo and Rivera (2023). Despite the marked increase in the use of IVP embryos in the last decade, reports of LOS in recent years have become scarce, possibly a reflection of improvements in culture media (Demetrio et al., 2020) but also due to a lack of follow-up studies. More recently, research has been undertaken to identify causes of LOS during early stages of pregnancy (Rivera et al., 2022). Large offspring syndrome is also associated with other symptoms including macrosomia (overgrowth), macroglossia (enlarged tongue) and abdominal wall defects (e.g., umbilical hernia and visceromegaly; Chen et al., 2013).

Although the exact causes of LOS remain to be fully resolved, links have been made to the in vitro culture techniques used to generate the embryos. The use of serum and oviductal cells in the media as well as incubator oxygen concentrations have been linked with occurrence of LOS (Farin et al., 2001, Fischer-Brown et al., 2005). Nevertheless, LOS has not yet been eliminated and remains a risk with IVP-ET calves (Bonilla et al., 2014, Siqueira et al., 2017b, Tribulo et al., 2017).

Separate from the incidence of LOS, IVP-ET calves were also reported to have poorer neonatal health compared with calves derived from AI or MOET. A greater proportion of IVP-ET calves were stillborn or died within 20 d of birth (Bonilla et al., 2014, Siqueira et al., 2017b, Tribulo et al., 2017), developed congenital abnormalities (van Wagtendonk-de Leeuw et al., 1998) and exhibited developmental abnormalities compared with calves derived from AI. Additionally, Siqueira et al. (2017b) reported that female calves derived from IVP embryos that had been fertilized using reverse X-sorted semen had increased risk of mortality and reduced milk yield during their first lactation. A recent study however, has shown in a large cohort of calves (born between 2009 and 2019) derived from IVP-ET (n = 2736) and multiple-ovulation embryo transfer (n = 3436) that there were no differences between the performance of the two cohorts of calves in terms of health and fertility parameters (Mullaart et al., 2021).

Dystocia has an adverse effect on the subsequent fertility of dairy cows (Zaborski et al., 2009, Bonilla et al., 2014). Any increase in calf birthweight, dystocia or incidence of LOS (and associated increase in incidence of morbidity and mortality) would reduce the attractiveness of IVP-ET. Following on from a large study examining the fertility of lactating dairy cows following timed AI or timed ET (Crowe et al., 2024), the objective of this study was to compare the gestation length, calf birth weight, calving difficulty and overall health status of calves derived from either AI or ET, with ET treatments comprising of fresh and frozen IVP embryos of both beef and dairy breeds.

5.3 Materials and Methods

All experimental procedures involving animals were approved by the Teagasc Animal Ethics Committee and authorized by the Health Products Regulatory Authority in Ireland, in accordance with Statutory Instrument No. 543 of 2012 under European Union legislation (Directive 2010/63/EU) for the Protection of Animals used for Scientific Purposes. All calves were born between January and March 2022.

The experimental design (illustrated in Figure 5.1), the animals used to produce the IVP embryos and the synchronization of the recipient lactating dairy cows were described in detail in (Crowe et al., 2024). All IVP blastocysts were produced using oocytes collected by transvaginal ultrasound-guided ovum pick-up (OPU) from the ovaries of elite dairy donors (n = 40 Holstein Friesian (**HF**) and Jersey (**JE**); ET-DAIRY), elite beef donors (n = 21 Angus (**AA**); ET-ELITE-BEEF) and by aspiration of follicles from ovaries that were collected from beef heifers (with at least 50% beef breed genetics) of known pedigree following slaughter at a commercial abattoir (COMM-BEEF, n = 119 donors, comprised of AA (n = 92), Limousin, (n = 18) and other (n = 9)). Lactating HF cows were synchronized and assigned to receive either AI (n = 243) or ET with IVP blastocysts (n = 863). In six herds, recipient cows were randomly assigned to receive AI, fresh ET-DAIRY, frozen ET-DAIRY, fresh ET-ELITE-BEEF or frozen ET-ELITE-BEEF. In one herd, recipient cows were randomly assigned to receive AI, fresh ET-ELITE-BEEF or frozen ET-ELITE-BEEF. In the remaining three herds, recipient cows were randomly assigned to receive AI, fresh COMM-BEEF or frozen COMM-BEEF. Grade 1 blastocysts were either transferred fresh (n = 436) or frozen (n = 427) for on-farm thawing and direct transfer. As described by Crowe et al. (2024), all synchronized cows (AI and ET) had blood serum samples collected for progesterone (**P4**) concentration analysis on d 7 after synchronized ovulation. Analysis of serum P4 concentration was conducted using solid-phase radioimmunoassay with PROG-RIA-CT kits (DIAsource ImmunoAssays S.A., Louvain-la-Neuve, Belgium) according to the manufacturer´s instructions. Pregnancy outcomes were previously reported by (Crowe et al., 2024). Gestation length, calf mortality and calving difficulty/dystocia data were recorded for all calves born (n = 442) on all of the herds enrolled in the original study (n = 9 herds). A subset of 281 calves born in five of these herds were also weighed within one hour of birth. These were calves from cows bred by timed AI with frozen-thawed semen (n = 65 calves; n = 2 cows had twins), timed ET with fresh IVP embryos (n = 123) and timed ET with frozen IVP embryos (n = 93). The ET calves were either dairy breed (HF, n = 71, JE, n = 38, or HF × JE crossbred, n = 10) or beef breed (AA, n = 97). In addition, blood samples were collected within one hour of birth from a subset of calves in three of these herds for blood biochemistry (n = 108) and blood hematology (n = 101) analyses.

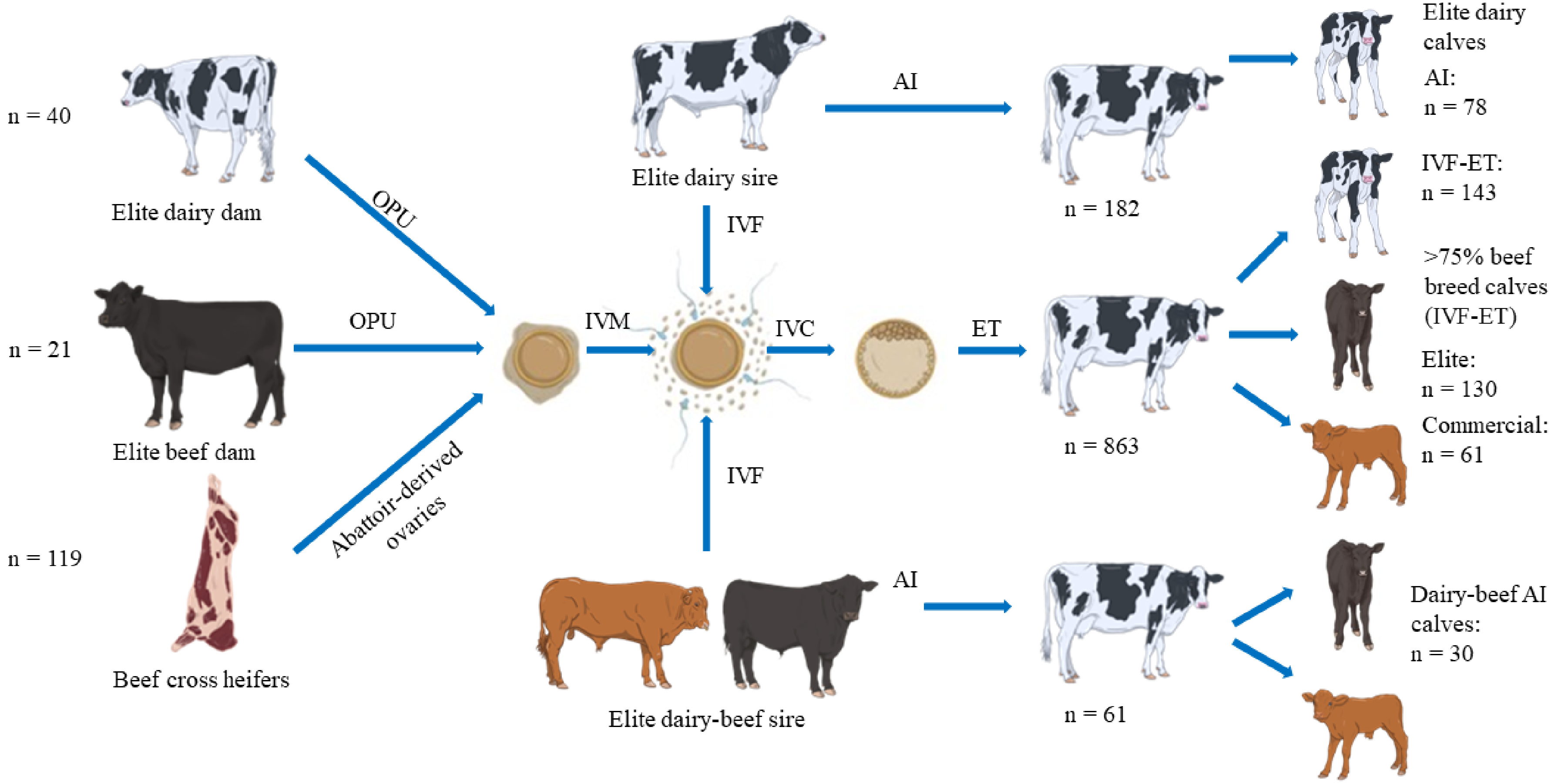

n = 40
Elite dairy dam
OPU
n = 21
OPU
Elite beef dam
n = 119
Abattoir-derived ovaries
Beef cross heifers
Elite dairy sire
IVF
IVM
IVC
IVF
Elite dairy-beef sire
AI
n = 182
ET
n = 863
AI
n = 61
Elite dairy calves
AI:
n = 78
IVF-ET:
n = 143
>75% beef breed calves (IVF-ET)
Elite:
n = 130
Commercial:
n = 61
Dairy-beef AI calves:
n = 30

Figure 5.1. Experimental design. Oocytes collected using oocyte pick-up (OPU) from the ovaries of elite dairy donors (n = 40 Holstein Friesian (**HF**) and Jersey (**JE**); ET-DAIRY), elite beef donors (n = 21 Angus (**AA**); ET-ELITE-BEEF) and by aspiration of follicles from ovaries that were harvested from beef heifers (with at least 50% beef breed genetics) of known pedigree following slaughter at a commercial abattoir (COMM-BEEF, n = 119 donors, comprised of AA (n = 92), Limousin, (n = 18) and other (n = 9)). Following OPU/oocyte aspiration, COCs from each donor were transported to the IVF laboratory where they completed maturation, approximately 24 h after the time of OPU. Following IVF, presumptive zygotes were cultured in vitro (IVC). The resulting grade 1 blastocysts were either transferred fresh (all d 7) or frozen (d 6, d 7 or d 8) for on-farm thawing and direct transfer (ET). Lactating HF cows were synchronized using an 8-day P4 Ovsynch protocol, and timed AI was carried out for 243 control cows and ET occurred on d 7 after synchronized estrus for 863 cows. In six herds, recipient cows were randomly assigned to receive AI, fresh ET-DAIRY, frozen ET-DAIRY, fresh ET-ELITE-BEEF or frozen ET-ELITE-BEEF. In one herd, recipient cows were randomly assigned to receive AI, fresh ET-ELITE-BEEF or frozen ET-ELITE-BEEF. In the remaining three herds, recipient cows were randomly assigned to receive AI, fresh COMM-BEEF or frozen COMM-BEEF. Gestation length, calf mortality and calving difficulty/dystocia data were recorded for all calves born (n = 442) on all of the herds enrolled in the original study (n = 9).

5.3.1 Dry-period management of cows

Dairy cows that had established a pregnancy following either AI or IVP-ET were located in four Teagasc herds in Co. Cork (Moorepark, Dairygold, Curtin's and Clonakilty) and one herd in Co. Cavan (Ballyhaise). These cows were dried-off on average (± SD) 70.9 ± 13.1 days before expected parturition. Cows had ad libitum access to perennial ryegrass silage (either ensiled in silos or bales) and received dry-cow minerals (either top-dressed on silage, mixed with the silage using a diet feeder or automatically dispensed in the drinking water). Cows on each farm were housed in free-stall cubicle sheds. As parturition approached, cows were moved to straw bedded pens until after parturition.

5.3.2 Birthweight and dystocia

In the five herds that recorded birth weight, calves were weighed immediately after birth (within one hour) and before they had consumed colostrum using a calibrated cattle weigh scales. Calving difficulty was scored by staff on each farm using a 1 to 4 scale: 1 = unassisted calving, 2 = minor assistance, 3 = considerable difficulty or 4 = veterinary assistance/caesarean.

5.3.3 Gestation Length

Gestation length was calculated (in days) for each calf as the difference between date of birth and the date of synchronized ovulation for cows assigned to both AI and ET treatments.

5.3.4 Determination of Blood Hematological and Biochemical Properties

At birth, blood samples were collected into serum and EDTA tubes (BD Vacutainer, BD, Plymouth, UK) by jugular venipuncture from a subset of calves (n = 108). Blood samples were stored at 4 °C until they were transported to a commercial veterinary diagnostics laboratory (Veterinary Pathology Group, Cork, Ireland) for biochemical and hematological analysis. Before laboratory analysis, 7 EDTA tube samples had clotted and were unsuitable for hematological analysis, hence the total number of samples processed for hematological analysis was 101.

5.3.5 Statistical Analysis

All Statistical analyses were conducted using SAS v. 9.4 (SAS Institute, Cary, NC). Before analysis, data were assessed for normality and transformed using optimum Box-Cox transformations where necessary (TRANSREG).

A total of 442 calves had CD and mortality data recorded. Of these calves, 281 were weighed at birth and were included in the analysis for BW. The three herds that used AI and COMM-BEEF embryos and the one herd that used AI and only ELITE-BEEF embryos did not weigh calves at birth, and hence birthweight was only recorded in the herds that used all of the following treatments: AI (all dairy), fresh and frozen ET-ELITE-BEEF (AA) and fresh and frozen ET-DAIRY (HF, JE and HF × JE). Data were analyzed using generalized linear mixed models (GLIMMIX) with repeated measures. Several models were constructed to determine treatment effects on the dependent variables BW, GL, CD and calf mortality. For BW analysis, treatment (AI vs ET (including fresh vs frozen ET), breed (AA vs dairy breeds, HF and JE), sire, GL and the sex of the calf were included as fixed effects, with farm included as a random effect. There were no two-way interactions between any of the variables. Gestation length was calculated and CD scored for all 439 calving events. For GL analysis, treatment (AI vs ET; including fresh vs frozen ET), breed (beef breeds, AA and LMX vs dairy breeds, HF and JE) and sire were included as fixed effects, with farm included as a random effect. For CD analysis, treatment (AI vs ET, including fresh vs frozen ET), sire, sex of the calf and the duration (d) of the dry period were included as fixed effects, with farm included as a random effect. For analysis of perinatal mortality, treatment (AI vs ET, including fresh vs frozen ET) and calf breed were included as fixed effects, with farm included as a random effect.

Blood biochemistry and hematology data were first assessed for normality, with n = 21 variables having normal distributions. Creatinine, Aspartate transferase (**AST**), gamma-glutamyl transpeptidase **(Gamma-GT)**, Creatine kinase (**CK**), Reticulocyte count, hematocrit test (**HTC)**, Platelet count and white blood cell (**WBC**) counts were not normally distributed and were transformed using optimum Box-Cox transformations (TRANSREG) for analysis, and least square means were subsequently back transformed. Data were analyzed using generalized linear mixed models (GLIMMIX) with repeated measures. Several models were constructed to determine treatment effects on each blood analyte. Fixed effects included treatment (AI vs ET, including fresh vs frozen ET and dairy vs beef breed), calf sex, recipient dam parity, recipient dam blood serum P4 concentration on d 7, CD, GL and farm as fixed effects, with calf included as a random effect. Two-way interactions were included in the models when $P < 0.25$.

5.4 Results

5.4.1 Factors affecting calf birthweight

The effect of treatment on BW and associations between BW and other independent variables are summarized in Table 5.1 and Figure 5.2. Treatment (AI vs ET) affected BW ($P < 0.0001$). The mean BW (kg; 95% CI) for calves derived from AI was less (31.1 kg; 28.4, 34.0) than that of calves derived from ET (35.3 kg; 32.5, 38.3). Calf breed affected BW ($P < 0.0001$); AA calves had a mean BW (± 95% CI) of 37.0 kg (32.1, 42.7), HF calves 35.6 kg (31.3, 40.3) and JE calves 25.5 kg (22.4, 28.8). Within HF breed calves, treatment affected calf BW ($P = 0.0002$), with calves derived from AI having lighter mean (95% CI) BW (36.1 kg; 34.6, 37.6) than HF calves derived from fresh ET (40.6 kg; 38.9, 42.5; $P = 0.0002$) and frozen ET (39.0 kg; 37.2, 40.8; $P = 0.032$). Gestation length was positively associated with calf BW ($P < 0.0001$), whereas shorter gestation length was associated with lighter BW (Table 5.1).

Across all breeds, calf sex affected BW ($P = 0.042$) with male calves being heavier at birth (33.7 kg; 31.0, 36.6) than female calves (32.5 kg; 29.9, 35.3). Calf BW is summarized by breed and treatment in Table 5.1. Within HF breed calves, (the largest cohort for analysis in this study), IVP-ET calves had greater BW ($P < 0.05$).

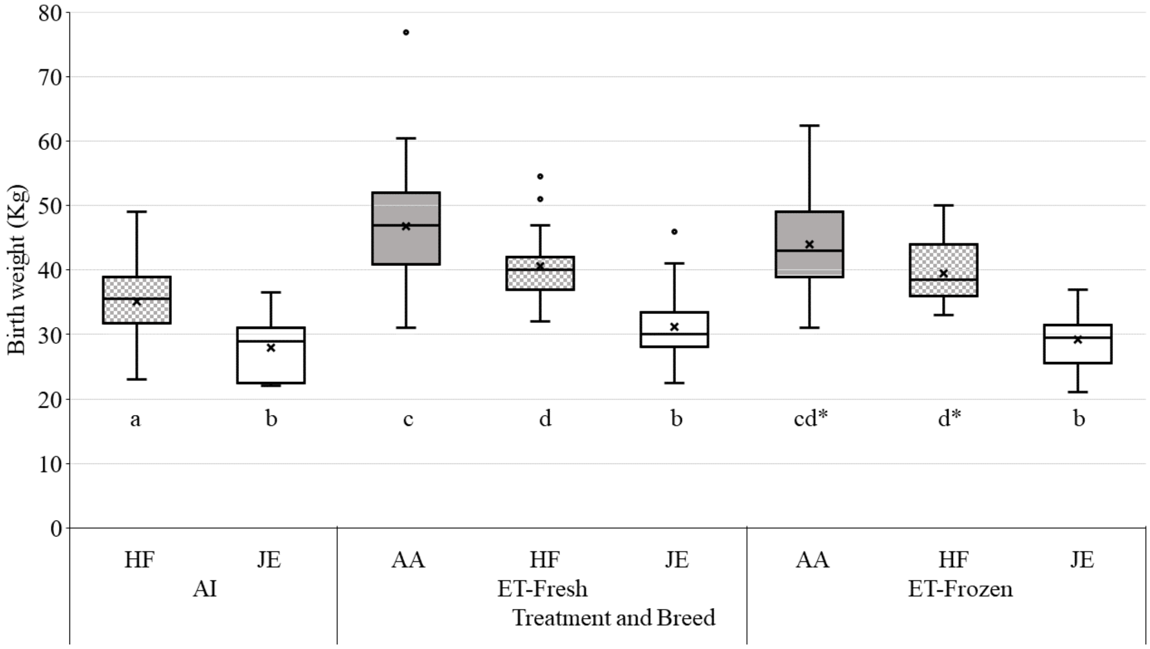


Figure 5.2 Variation between birth weights (BW) of Angus (AA), Holstein Friesian (HF) or Jersey (JE) calves born following the timed artificial insemination (AI) or timed embryo transfer (ET) with fresh or frozen in vitro-produced blastocysts. For each treatment and breed, × indicates the mean BW, the line intersecting the box indicates the median BW, the box indicates the interquartile range of BW and the whiskers indicate the 5th and 95th percentiles of BW.

The mean birth weights included are raw BW for all calves in each breed and treatment for which BW was recorded.

a-b Different superscripts indicate different mean BW between treatments and breeds ($P < 0.05$).

* Indicates a tendency for mean BW to differ between treatments and breeds ($0.05 < P < 0.1$).

Number of calves per treatment and breed:

AI: HF n = 53. JE n = 11.

ET-Fresh: AA n = 54. HF n = 37. JE n = 32.

ET-Frozen: AA n = 43. HF n = 34. JE n = 16.

Table 5.1 Mean (± 95% CI) birth weight (BW), gestation length (GL), calving difficulty score (CD) and perinatal mortality (within 14 d of birth) following timed artificial insemination (AI) or embryo transfer (ET) with fresh or frozen in vitro-produced embryos. HF: Holstein Friesian, JE: Jersey, AA: Angus.

Origin	AI		ET-Fresh			ET-Frozen		
Breed	HF	JE	AA	HF	JE	AA	HF	JE
n calves	67	11	110	54	35	74	38	16
BW (kg)	34.8^{a}	27.8^{b}	46.2^{c}	40.3^{d}	30.8^{b}	43.5^{cd*}	39.3^{d*}	28.9^{b}
95% CI	33.2 - 36.5	25.1 - 30.7	44.2 - 48.3	38.2 - 42.4	28.9 - 32.7	41.4 - 45.6	37.2 - 41.5	26.6 - 31.4
GL (d)	276.4^{a*}	277.6^{ab}	281.9^{b}	280.0^{b}	281.1^{b}	281.4^{b}	279.9^{ab*}	280.6^{ab}
95% CI	275.1 - 277.7	274.4 - 280.7	280.9 - 282.9	278.6 - 281.4	279.4 - 282.9	280.2 - 282.6	278.2 - 281.6	278.0 - 283.2
CD	1.27^{a}	1.09^{a}	1.85^{b}	1.33^{a}	1.20^{a}	1.57^{ab}	1.55^{ab}	1.31^{ab}
95% CI	1.10 - 1.43	0.68 - 1.50	1.73 - 1.98	1.15 - 1.52	0.97 - 1.43	1.41 - 1.72	1.33 - 1.77	0.97 - 1.65
Mortality (%)	0.03	0.09	0.05	0.06	0.06	0.08	0.0	0.06
95% CI	-0.02 - 0.08	-0.03 - 0.22	0.01 - 0.10	0.0 - 0.11	-0.02 - 0.13	0.03 - 0.13	-0.07 - 0.07	-0.04 - 0.17

5.4.2 Factors affecting gestation length

Gestation length data are summarized in Table 5.1 and displayed in Figure 5.3. Treatment (P = 0.0002) and sire (P < 0.0001) affected GL. Calves derived from AI had shorter GL (278.9 d; 277.0, 280.8) than calves derived from ET-Fresh (281.8 d; 279.8, 283.7; P = 0.0005) and ET-Frozen (282.0; 280.0, 284.0; P = 0.0003). Calves derived from fresh ET did not differ from calves derived from frozen ET (P = 1.0). Calf breed also affected GL (P = 0.002). Across all breeds, the COMM-BEEF calves that were sired by a Limousin bull had a longer GL (290.9 d (286.4, 295.4)) than AA; 282.0 d (280.6, 283.3); P = 0.0006), HF; 280.5 d (278.9, 282.1); P < 0.0001) and JE calves; 282.0 d (280.0, 284.0; P = 0.002). Gestation length was not different between AA, HF and JE calves (all P > 0.05).

There was a positive correlation between GL and BW (R2=0.11; P<0.0001) and between BW and CD score (R2=0.3; P<0.0001). The relationship between GL and BW is summarized in Table 5.2.

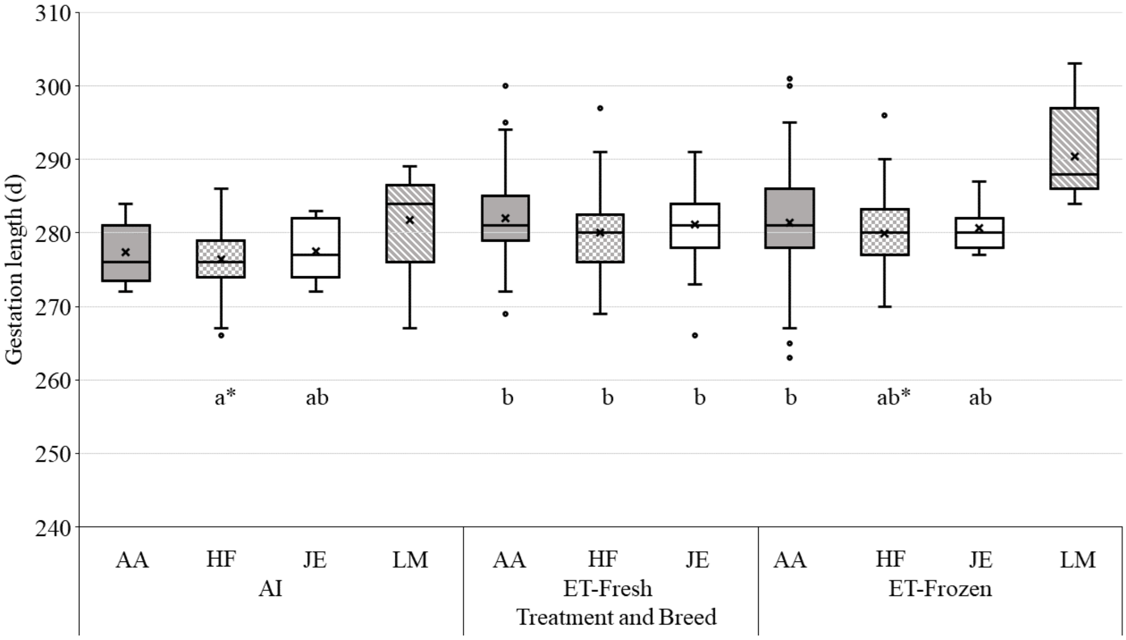


Figure 5.3 Variation between gestation length (GL) of Angus (AA), Holstein Friesian (FR), Jersey (JE) or Limousin (LM) calves born following the timed artificial insemination (AI) or timed embryo transfer (ET) with fresh or frozen in vitro-produced blastocysts. For each treatment and breed, × indicates the mean GL, the line intersecting the box indicates the median GL, the box indicates the interquartile range of GL's and the whiskers indicate the 5th and 95th percentiles of GL's.

The GL's included are raw GL's for all calves in each breed and treatment. The n for each of the groups AI-AA, AI-LM and ET-Frozen-LM was too small to analyze as independent variables within treatments. However, across breeds, LM calves had longer GL than all other breeds ($P < 0.05$).

a-b Different superscripts indicate different mean GL between treatments and breeds ($P < 0.05$).

* Indicates a tendency for mean GL to differ between treatments and breeds ($0.05 < P < 0.1$).

Number of calves per treatment and breed:

AI: AA n = 17. HF n = 67. JE n = 11. LM n = 13.

ET-Fresh: AA n = 110. HF n = 54. JE n = 35.

ET-Frozen: AA n = 74. HF n = 38. JE n = 16. LM n = 7.

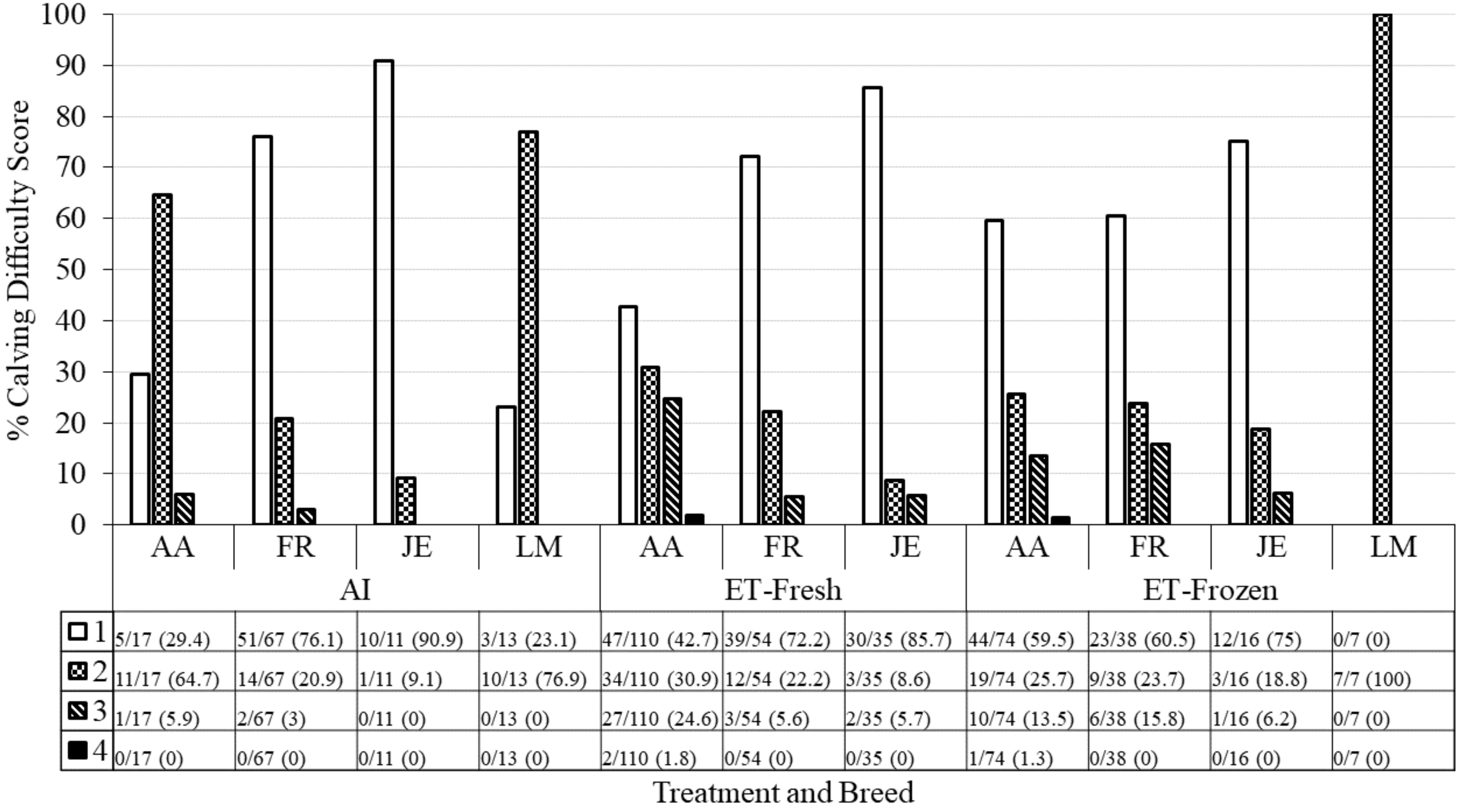
Table 5.2 Mean (± 95% CI) birth weight (BW) for calves within each quartile of gestation length (GL) following timed artificial insemination or embryo transfer with fresh or frozen in vitro-produced embryos.

GL Quartile	Q1: 263 - 276 d	Q2: 277 - 279 d	Q3: 280 - 283 d	Q4: 284 - 303 d
n calves	107	93	121	121
BW (kg)	30.3^{a}	33.1^{b}	34.3^{b}	33.4^{ab}
(95% CI)	(27.7 - 33.0)	(30.2 - 36.2)	(31.3 - 37.4)	(29.7 - 37.4)

$^{a\text{-}b}$ Different superscripts indicate differences in amounts of metabolites between treatments ($P < 0.05$).

5.4.3 Factors affecting calving difficulty score

Calving difficulty score was affected by treatment (P = 0.05), with calves derived from AI tending to have lesser incidence of dystocia (mean CD score; 95% CI = 1.38; 1.21, 1.55) than calves derived from fresh ET (1.62; 1.44, 1.81), and calves derived from frozen ET were intermediate (1.52; 1.33, 1.72). The main driver of increased calving difficulty, however, was calf breed. AA calves from fresh ET had greater incidence of CD than HF calves from both AI and fresh ET (P < 0.05). Among calves derived from frozen ET, there was no difference in CD between breeds. Mean CD is summarized by treatment and breed in Table 5.1 and illustrated in Figure 5.4.



	AI AA	AI FR	AI JE	AI LM	ET-Fresh AA	ET-Fresh FR	ET-Fresh JE	ET-Frozen AA	ET-Frozen FR	ET-Frozen JE	ET-Frozen LM
□ 1	5/17 (29.4)	51/67 (76.1)	10/11 (90.9)	3/13 (23.1)	47/110 (42.7)	39/54 (72.2)	30/35 (85.7)	44/74 (59.5)	23/38 (60.5)	12/16 (75)	0/7 (0)
▩ 2	11/17 (64.7)	14/67 (20.9)	1/11 (9.1)	10/13 (76.9)	34/110 (30.9)	12/54 (22.2)	3/35 (8.6)	19/74 (25.7)	9/38 (23.7)	3/16 (18.8)	7/7 (100)
▧ 3	1/17 (5.9)	2/67 (3)	0/11 (0)	0/13 (0)	27/110 (24.6)	3/54 (5.6)	2/35 (5.7)	10/74 (13.5)	6/38 (15.8)	1/16 (6.2)	0/7 (0)
■ 4	0/17 (0)	0/67 (0)	0/11 (0)	0/13 (0)	2/110 (1.8)	0/54 (0)	0/35 (0)	1/74 (1.3)	0/38 (0)	0/16 (0)	0/7 (0)

Figure 5.4 Percentage of calves within each breed and treatment that fell into each of the four points on the calving difficulty score scale (1 = unassisted calving, 2 = minor assistance, 3 = considerable difficulty or 4 = veterinary assistance/caesarean section). Calving difficulty score was affected by treatment (P = 0.05).

The data included are raw numbers and percentages included to illustrate the breakdown of dystocia within each breed and treatment. The n in each group is included to aid the interpretation of the figure.

5.4.4 Factors affecting perinatal mortality

Mortality within the first 14 d after birth was not affected by treatment or calf breed. The overall incidence of mortality within the first 14 d was less than 5%.

5.4.5 Factors affecting calf blood biochemistry and hematology

Twenty-eight blood variables were measured during analysis of calf blood serum for biochemical analysis (n = 14 metabolites) and whole blood for hematological analysis (n = 14 variables). Calf blood biochemistry results are summarized in Table 5.3 and displayed in Figure 5. There was an overall treatment effect on 6 of the biochemistry metabolites, including total protein ($P < 0.0001$), albumin ($P < 0.0001$), globulin ($P = 0.003$), cholesterol ($P = 0.0006$), creatinine ($P = 0.005$) and Gamma-GT ($P < 0.0001$). Dairy AI and dairy ET derived calves had greater total protein and albumin than beef ET calves ($P < 0.05$). Dairy calves derived from fresh ET had greater globulin than dairy calves from AI and beef ET calves ($P < 0.05$). Beef calves derived from fresh ET had greater creatinine than dairy calves from AI and fresh ET ($P < 0.05$). Dairy calves from fresh ET had greater Gamma GT than beef ET calves and dairy AI calves, beef frozen ET calves had greater Gamma GT than dairy fresh ET calves ($P < 0.05$). Blood cholesterol levels were greater in dairy fresh ET calves than beef calves and dairy AI calves ($P < 0.05$). Potassium tended to be affected by treatment ($P = 0.063$) with frozen dairy ET calves tending to have greater potassium than beef frozen ET calves. Aspartate transferase tended to be affected by treatment ($P = 0.062$) with frozen dairy ET calves tending to have greater AST than beef frozen ET calves.

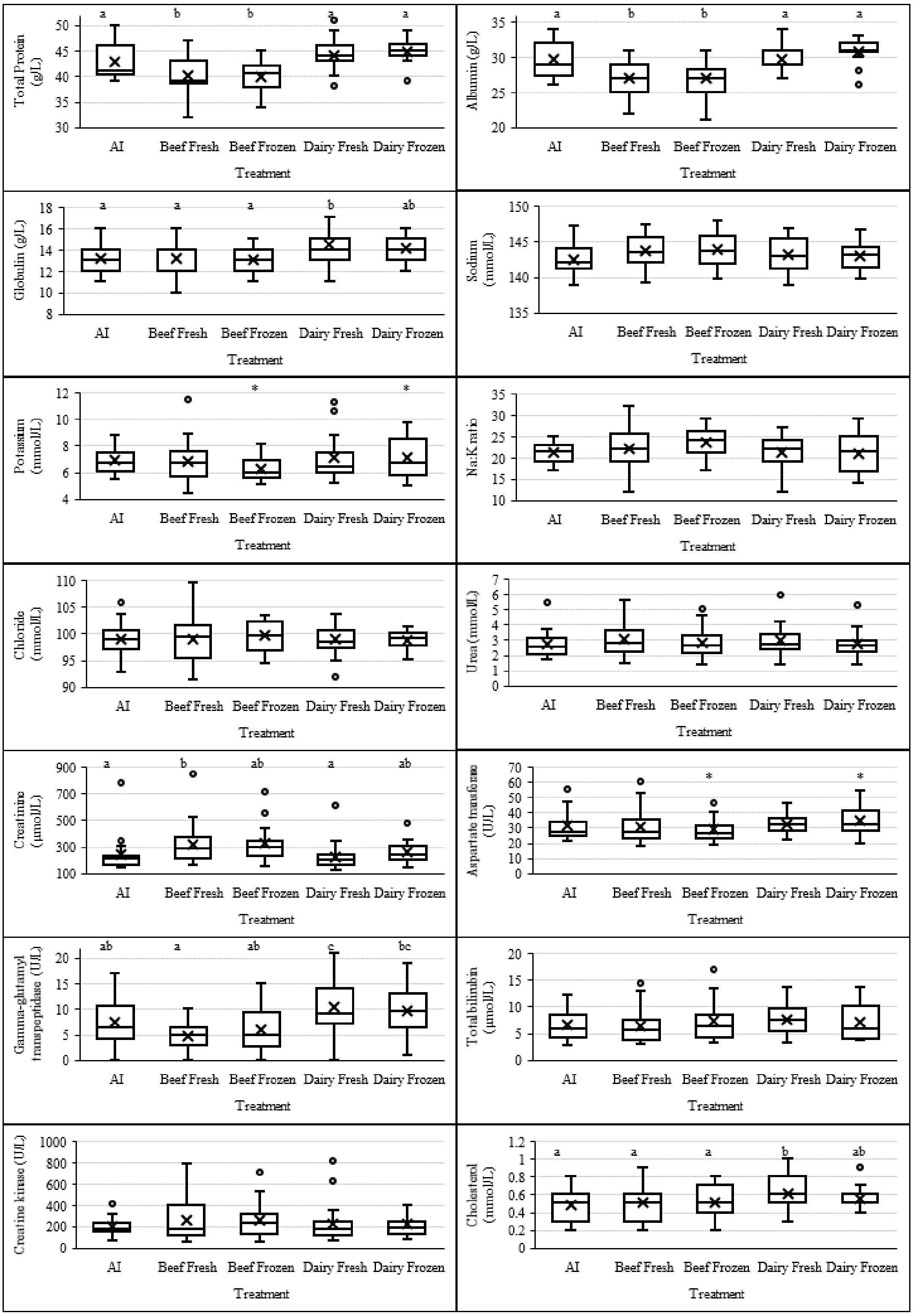


Figure 5.5 Blood biochemistry results for calves of beef (Angus) and dairy (Holstein Friesian and Jersey) breeds that were derived from timed artificial insemination (dairy only) and timed embryo transfer with fresh and frozen in vitro-produced embryos.

a-d Different superscripts indicate differences in amounts of metabolites between treatments ($P < 0.05$).

* Indicates a tendency for amounts of metabolites to differ between treatments ($0.05 < P < 0.1$).

Table 5.3 Blood biochemistry results for calves of beef (Angus) and dairy (Holstein Friesian and Jersey) breeds that were derived from timed artificial insemination (dairy only) and timed embryo transfer with fresh and frozen in vitro-produced embryos.

Blood Biochemistry	AI	Beef Fresh	Beef Frozen	Dairy Fresh	Dairy Frozen
n calves	20	25	18	27	18
Total Protein (g/L)	42.03^{a}	39.35^{b}	39.17^{b}	43.97^{a}	44.18^{a}
	40.39 - 43.68	37.81 - 40.89	37.47 - 40.88	42.83 - 45.10	42.60 - 45.76
Albumin (g/L)	29.60^{a}	26.92^{b}	26.94^{b}	29.67^{a}	30.72^{a}
	28.63 - 30.57	26.05 - 27.89	25.92 - 27.97	28.83 - 30.50	29.70 - 31.74
Globulin (g/L)	12.92^{a}	12.90^{a}	12.71^{a}	14.17^{b}	13.73^{ab}
	11.59 - 14.24	11.47 - 14.32	11.37 - 14.05	12.91 - 15.44	12.24 - 15.23
Sodium (mmol/L)	143.57	144.76	144.94	144.42	144.37
	142.08 - 145.06	143.20 - 146.31	143.35 - 146.54	143.01 - 145.83	142.71 - 146.03
Potassium (mmol/L)	6.54	6.53	5.51*	6.69	6.83*
	5.50 - 7.59	5.67 - 7.39	4.49 - 6.54	5.70 - 7.68	5.80 - 7.86
Na:K ratio	21.73	22.81	23.63	21.72	21.71
	17.80 - 25.66	18.44 - 27.17	19.67 27.60	17.90 - 25.55	17.21 - 26.22
Chloride (mmol/L)	102.58	102.92	98.23	102.45	102.93
	95.83 - 109.32	96.09 - 109.75	91.30 - 105.16	96.22 - 108.68	95.46 - 110.39
Urea (mmol/L)	3.67	4.08	3.61	3.9	3.84
	3.13 - 4.21	3.54 - 4.61	3.06 - 4.17	3.41 - 4.39	3.25 - 4.44
Creatinine (µmol/L)	211.43^{a}	303.80^{b}	287.59^{ab}	219.5^{a}	254.14^{ab}
	186.63 - 243.33	256.51 - 370.95	236.85 - 363.89	194.23 - 251.89	213.75 - 312.00

Aspartate transferase (AST) (U/L)	30.6	28.18	26.75*	32.65	34.4*
	27.27 - 34.59	25.54 - 31.29	23.99 - 30.01	29.00 - 37.15	29.86 - 40.08
Gamma-glutamyl transpeptidase (Gamma GT) (U/L)	4.51^{ab}	3.63^{a}	5.21^{ab}	9.07^{c}	8.19^{bc}
	2.32 - 7.55	1.69 - 6.44	2.68 - 8.72	5.88 - 13.06	4.64 - 12.93
Total bilirubin (µmol/L)	6.07	6.15	7.09	7.19	6.8
	1.80 - 10.34	-12.52	2.85 - 11.33	2.26 - 12.12	1.20 - 12.40
Creatine kinase (CK) (U/L)	171.31	185.48	202.65	177.58	183.33
	131.80 - 226.62	145.36 - 240.30	152.15 - 275.67	140.85 - 226.97	138.75 - 247.13
Cholesterol (mmol/L)	0.39^{a}	0.37^{a}	0.4^{a}	0.57^{b}	0.5^{ab}
	0.27 - 0.50	0.25 - 0.48	0.28 - 0.51	0.47 - 0.68	0.38 - 0.63

$^{a\text{-}d}$ Different superscripts indicate differences in amounts of metabolites between treatments ($P < 0.05$).

* Indicates a tendency for amounts of metabolites to differ between treatments ($0.05 < P < 0.1$).

Calf blood hematology results are summarized in Table 5.4 and displayed in Figure 5.6. There was an overall treatment effect on five of the hematology variables, including mean corpuscular volume (**MCV**) (P < 0.0001), mean corpuscular hemoglobin (**MCH**) (P = 0.005), neutrophils (P < 0.0001), lymphocytes (P = 0.013) and WBC count (P < 0.0001). Dairy calves derived from fresh ET had lesser MCV and MCH than beef calves (P < 0.05), and MCV tended to be lesser in dairy fresh ET calves than dairy AI calves (P = 0.097). Dairy calves derived from fresh ET had greater mean corpuscular hemoglobin concentration (**MCHC**) than dairy AI and dairy frozen ET calves (P < 0.05). WBC counts were greater for calves from the beef fresh ET group than dairy AI or dairy frozen ET calves. Dairy fresh ET calves also tended to have greater WBC counts than dairy AI calves (P = 0.096). Beef calves had more neutrophils than dairy AI or dairy fresh ET calves (P < 0.05). Beef fresh ET calves had more blood lymphocytes than beef frozen ET calves (P < 0.05) and tended to have greater lymphocytes than dairy fresh ET calves (P = 0.076). Treatment tended to effect Reticulocyte % (P = 0.069), with beef fresh ET calves tending to have a greater percentage than dairy frozen ET calves (P = 0.052). Platelet count also tended to be affected by treatment (P = 0.083).

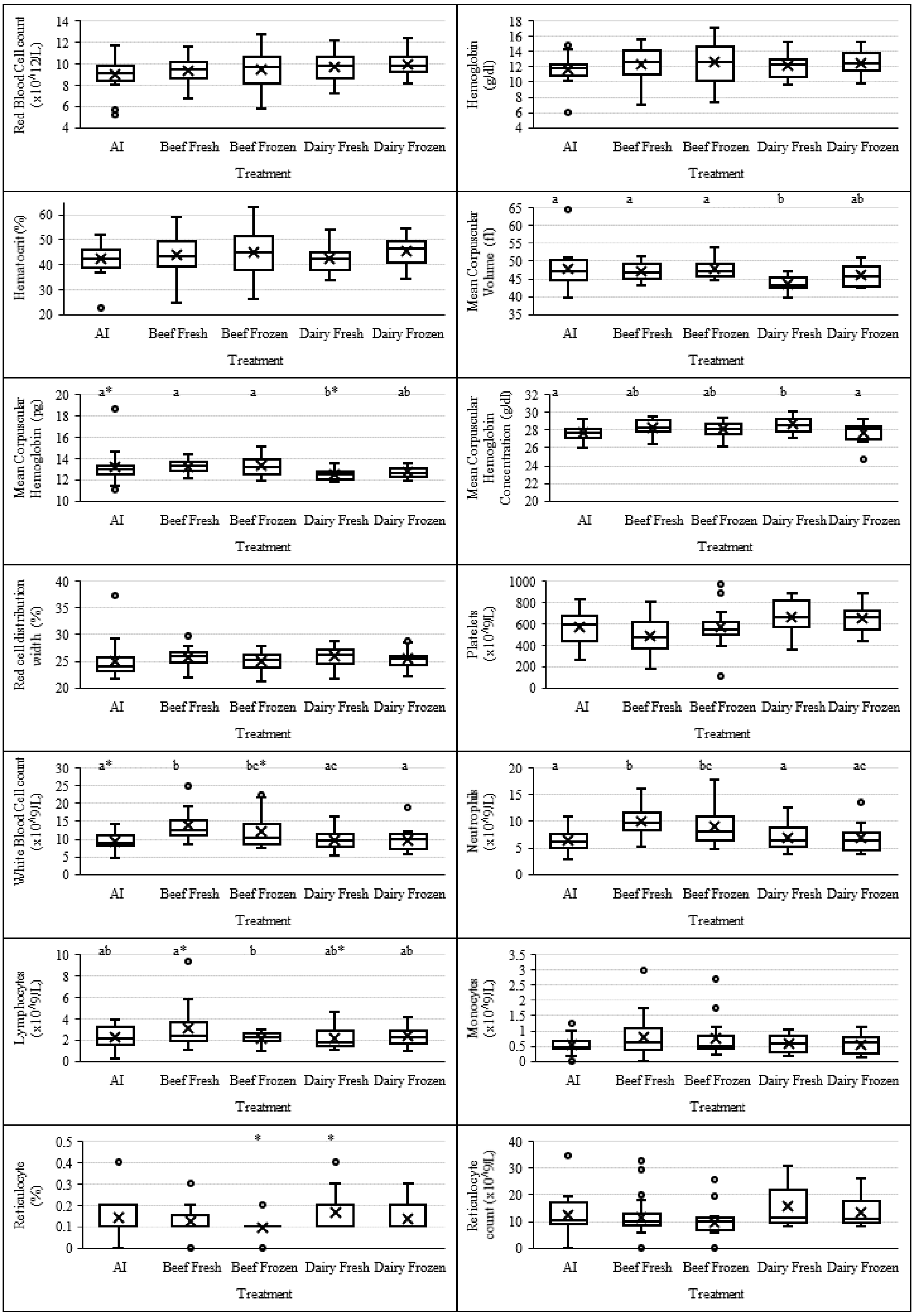


Figure 5.6 Blood hematology results for calves of beef (Angus) and dairy (Holstein Friesian and Jersey) breeds that were derived from timed artificial insemination (dairy only) and timed embryo transfer with fresh and frozen in vitro-produced embryos.

a-d Different superscripts indicate differences in amounts of metabolites between treatments ($P < 0.05$).

* Indicates a tendency for amounts of metabolites to differ between treatments ($0.05 < P < 0.1$).

Table 5.4 Blood hematology results for calves of beef (Angus) and dairy (Holstein Friesian and Jersey) breeds that were derived from timed artificial insemination (dairy only) and timed embryo transfer with fresh and frozen in vitro-produced embryos.

Blood Haematology	AI	Beef Fresh	Beef Frozen	Dairy Fresh	Dairy Frozen
n calves	18	25	17	24	17
Red Blood Cell count (x10^12/L)	8.31	8.6	8.43	8.72	8.91
	7.28 - 9.34	7.74 - 9.46	7.39 - 9.47	7.71 - 9.73	7.88 - 9.94
Hemoglobin (g/dl)	11.64	12.17	12.58	11.9	12.42
	10.70 - 12.58	11.35 - 12.99	11.61 - 13.54	11.10 - 12.71	11.46 - 13.37
Hematocrit (HCT) (%)	35.04	39.27	37.43	34.09	36.67
	30.95 - 40.94	34.76 - 45.72	32.71 - 44.51	30.12 - 39.84	32.09 - 43.53
Mean Corpuscular Volume (MCV) (fl)	47.83^{a}	47.31^{a}	47.61^{a}	43.64^{b}	45.98^{ab}
	46.45 - 49.20	46.12 - 48.49	46.14 - 49.08	42.33 - 44.94	44.57 - 47.40
Mean Corpuscular Hemoglobin (MCH) (pg)	13.13^{a*}	13.24^{a}	13.27^{a}	12.39^{b*}	12.65^{ab}
	10.77 - 15.48	7.43 - 19.04	11.35 - 15.20	10.28 - 14.50	9.08 - 16.23
Mean Corpuscular Hemoglobin Concentration (MCHC) (g/dl)	27.56^{a}	28.18^{ab}	27.98^{ab}	28.56^{b}	27.66^{a}
	27.15 - 27.96	27.84 - 28.53	27.57 - 28.40	28.21 - 28.91	27.24 - 28.07
	24.82	25.51	25.1	25.6	25.1

Red cell distribution width (RDW) (%)	23.81 - 25.83	24.62 - 26.40	24.07 - 26.13	24.74 - 26.46	24.06 - 26.15
Platelets (x10^9/L)	477.91	400.78	412.97	566.76	567.66
	372.39 - 682.57	331.34 - 513.44	331.90 - 556.18	435.89 - 832.01	417.07 - 928.49
White Blood Cell count (WBC) (x10^9/L)	7.67^{a*}	12.12^{b}	9.9^{bc*}	8.56^{ac}	7.36^{a}
	6.43 - 9.19	9.81 - 15.09	8.09 - 12.19	7.14 - 10.32	6.02 - 9.06
Neutrophils (x10^9/L)	6.78^{a}	10.25^{b}	9.23^{bc}	7.06^{a}	7.24^{ac}
	5.60 - 7.96	9.24 - 11.26	8.02 - 10.43	6.04 - 8.07	6.01 - 8.47
Lymphocytes (x10^9/L)	1.98^{ab}	2.79^{a*}	1.68^{b}	1.91^{ab*}	1.86^{ab}
	1.01 - 2.95	1.79 - 3.79	0.64 - 2.71	1.05 - 2.78	0.80 - 2.92
Monocytes (x10^9/L)	0.52	0.85	0.78	0.63	0.57
	0.31 - 0.74	0.66 - 1.05	0.56 - 1.00	0.44 - 0.82	0.34 - 0.79
Reticulocyte %	0.14	0.12	0.09*	0.16*	0.14
	0.10 - 0.17	0.09 - 0.15	0.06 - 0.13	0.13 - 0.19	0.10 - 0.17
Reticulocyte count	9.84	10.24	9.37	11.93	10.76
	8.24 - 12.18	8.64 - 12.54	7.83 - 11.65	10.17 - 14.40	8.94 - 13.44

$^{a-d}$ Different superscripts indicate differences in amounts of metabolites between treatments ($P < 0.05$).

* Indicates a tendency for amounts of metabolites to differ between treatments ($0.05 < P < 0.1$).

5.5 Discussion

This study characterized the phenotypic differences at birth between calves derived from AI or ET with fresh or frozen IVP-ET embryos of beef and dairy origin. The main findings of this study are that calves originating from IVP-ET had moderately increased BW and GL than calves born from AI, which led in some cases to increased calving difficulty/dystocia. In particular, AA calves had greater BW and CD than dairy breeds. Calves derived from IVP-ET had similar health status and results from blood biochemistry and hematology analysis and had similar survival post-partum to calves derived from AI. It compared differences between breeds and treatments on calf BW, GL, CD and mortality as well as comparing the levels of metabolites in blood biochemistry and hematology at birth. The use of IVP-ET (along with other assisted reproductive technologies) has been linked with LOS or "Abnormal Offspring" (**AOS**) since it was first reported nearly 30 years ago (Behboodi et al., 1995, Walker et al., 1996). Although much work has been done to remove some of the causes of LOS (such as addition of serum or bovine serum albumin to embryo culture media; Lazzari et al., 2002, Soto-Moreno et al., 2021), isolated incidences of LOS/AOS are still being reported (Siqueira et al., 2017a, Weiller et al., 2021). There have also been studies that reported incidences of LOS where serum was not used in media, indicating that LOS is a complex syndrome and that the processes of IVP, including small variations in composition of serum-free media, or manipulations experienced by the gametes/embryos during IVP procedures, can epigenetically alter both imprinted and non-imprinted genes, potentially leading to LOS (Nava-Trujillo et al., 2005, Siqueira et al., 2017a, Nava-Trujillo and Rivera, 2023). It should be noted that LOS has been known to occur spontaneously in cases where ART such as IVP or cloning had not been used (Li et al., 2019, Li et al., 2022). Other previous research linked increased birth weight with perinatal mortality in calves (Johanson and Berger, 2003, Bonilla et al., 2014), which in turn will have an adverse effect on the economics of a dairy herd (Meyer et al., 2001). The main findings from this study are that calf BW was affected by both breed and treatment, with beef calves having greater BW but also calves derived from ET treatments having greater BW than AI. Treatment also affected GL, with AI calves having shorter GL than ET. Calving difficulty, however, was mainly affected by breed, with AA calves from fresh ET having the greatest incidence of CD. Perinatal mortality was not affected by treatment or breed, and mortality was < 5% across all treatments. While variation was observed for several blood biochemistry and hematology analytes, values were generally within the normal expected ranges (Santos et al., 2023).

Birthweight:

Both treatment and calf breed affected BW. In order to directly compare the effect of IVP-ET on BW within breed, data from HF calves from each treatment were compared (AI, ET-Fresh and ET-Frozen). Treatment differences of 3 to 4 kg were observed between AI- and ET-derived calves. While this was statistically significant, the increased BW did not lead to a notable increase in incidence of dystocia or perinatal mortality. In the 12 studies reviewed by Nava-Trujillo and Rivera (2023), 10 studies used a minimum of > 50 kg as the indicator of LOS, with numerous being closer to 60 kg and upwards. The breeds used in these studies were HF (n = 5), other dairy breeds (n = 2) and beef (Simmental, AA, LM, Wagyu) calves (n = 5). In these studies, incidence of LOS was usually <1% or not observed (0%), with only Kruip and denDaas (1997) reporting an incidence > 1 % (7.4 - 10%). In this study, out of n = 281 birthweights recorded, 25 calves weighed > 50 kg (n = 23 of these were AA calves) and 4 of those calves weighed > 60 kg (all AA). In Ireland, ICBF (2014) reported the mean of (n = 9,576 calves) birthweight for AA sired calves (irrespective of dam breed) to be 40 kg.

Gestation Length

Longer GL was previously reported to have been associated with greater BW, and increased incidence of dystocia and stillbirths in cattle (Hansen et al., 2004, Norman et al., 2009). Shorter GL is desirable in seasonal calving herds in order to maintain a short calving season and maximize pasture utilization (Shalloo et al., 2014). In the current study, GL was affected by treatment with AI-derived calves having 2-3 d shorter gestations than ET-derived calves. The breed effect on GL was predominantly driven by the longer GL of calves with a Limousin sire, which is typically associated with longer gestation than AA, HF and JE when used in conventional AI. This is consistent with work from Basiel et al. (2024) who reported that calves from dairy dams that were sired by LM bulls had longer GL than those sired by HF bulls. The longer GL observed in ET calves in this study (derived from both fresh and frozen blastocysts), suggests that the IVP process, rather than the cryopreservation and thawing procedures, increases GL. In their study, Bonilla et al. (2014), observed longer GL for cows that had received vitrified embryos compared to fresh ET or AI, but they also observed a greater incidence of induced parturition (cows with GL > 280 d were induced) in all ET pregnancies compared with AI. The breed-specific differences in GL further emphasize the importance of considering the breeds and even genetics of sire and dam combinations when using IVF-ET.

Calving Difficulty

An increased incidence of CD is a significant concern to farmers using IVP-ET. Greater BW, prolonged GL, calf abnormalities and increased incidence of stillbirth can exacerbate dystocia (Bellows et al., 1971, Meyer et al., 2000, Norman et al., 2011, Jukna et al., 2024). This study reported a higher incidence of dystocia in ET calves compared to AI calves, particularly among those derived from fresh embryos. This finding is consistent with earlier studies that have linked the use of IVP embryos with increased calving difficulties, often due to the larger size of the calves at birth (Behboodi et al., 1995, van Wagtendonk-de Leeuw et al., 1998). While the use of frozen embryos did not significantly differ from fresh embryos in terms of CD, the overall trend suggests that the use of IVP embryos, both fresh and frozen, requires careful management to mitigate the risk of dystocia.

Perinatal Mortality

Mortality rates were low and below the accepted level on the farms utilized in the study. Despite the increased BW and CD associated with IVP-ET derived calves, no difference in perinatal mortality rate was observed between AI and ET calves. This is an encouraging outcome, indicating that their survival was not adversely affected in the particular cohort of cows on this study. Perhaps advancements in techniques and culture media in recent years may have helped to reduce the risk of increased incidence of stillbirth. Most evidence that stillbirths can be increased following IVP-ET is from older studies (Kruip and denDaas, 1997, van Wagtendonk-de Leeuw et al., 1998), with limited recent evidence of increased stillbirth incidence following IVP-ET.

Blood biochemistry and hematology

For the most part, among calves on this study, no differences were observed solely between AI and IVP-ET derived calves for any of the 28 blood biochemistry and hematology variables. All differences observed in biochemistry variables appear to have been driven by breed differences between beef and dairy breeds. Beef ET calves had lesser total protein and albumin concentration than dairy calves (both AI and ET), while dairy calves from fresh ET had greater globulin and cholesterol concentrations. In terms of hematological differences, breed caused most of the differences between groups of calves. Beef calves had greater WBC counts than AI- and dairy-ET derived calves, while red blood cell count (**RBC**) was the same across all groups. Agerholm et al. (2023) compared blood biochemistry and hematology between calves derived from IVP-ET and a control group of calves from AI. They reported that IVP-ET calves were more heterogeneous than the AI group but also that they had raised Gamma GT levels. In the current study, only calves in the dairy fresh ET group had greater Gamma GT than the dairy AI calves, again indicating that differences in this study were not entirely due to IVP-ET.

5.6 Conclusion

In conclusion, calves originating from IVP-ET had moderately increased BW and GL than calves born from AI. In some instances, larger BW led to calving difficulty/dystocia underpinning the need for careful selection of genetics for an IVP-ET program in dairy cows. While ET produced calves with a moderately increased BW among HF calves, breed played a bigger factor than treatment and care must be exercised when selecting beef sires and dams for use in IVP. Calves derived from IVP-ET, although heavier at birth, particularly where beef embryos were used, were as healthy and had similar survival post-partum as calves derived from AI. This indicates that once care is taken to use easy calving sires IVP-ET derived calves do not pose a markedly increased risk of poor health or survivability compared with AI derived calves. The findings suggest that there is still room for further research in order to optimize IVP protocols, particularly in terms of embryo culture conditions and criteria for selection of donors and sires, to further reduce complications arising at parturition.

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Chapter 6: General Discussion

‘Beef on dairy’ is currently a hot topic. Traditionally, in dairy herds, all cows and heifers were mated with dairy breed bulls, with the required number of heifer calves kept as replacements and the remaining surplus (mostly male) dairy calves being sold for a low value. With the introduction of genomic evaluations and technologies such as sex-sorted semen, it is now possible to target only the top elite females in the herd to generate replacements, allowing new approaches to increase the value of the non-replacement calves for beef production. On many farms, this has facilitated the use of high genetic merit beef bulls that are suitable for use on dairy cows, in order to develop greater value dairy × beef calves. Genetic gain in this particular cohort of suitable beef bulls is slow, given the various goals within the pedigree beef breeding industry. Assisted reproductive technologies such as in-vitro production (IVP) and embryo transfer (ET) provide opportunities to generate elite beef bulls and heifers, as well as a new avenue for producing high genetic merit dairy bulls in a world where X-sorted semen dominates the dairy artificial insemination (AI) industry.

Fertility remains a critical metric in dairy production, particularly in seasonal calving systems (Shalloo et al., 2014). In order for IVP-ET to be viable in a seasonal, pasture-based system, as is operated on Irish dairy farms, fertility performance must reach close to the same levels as conventional and sex-sorted AI. Achieving target conception to first service (AI or ET) is key to achieving good fertility, but of particular interest and importance is reducing pregnancy loss.

The aims of this thesis were: (i) Chapter 2; to compare pregnancy outcomes in lactating dairy cows following timed AI or timed ET using either fresh or frozen IVP embryos from dairy or beef breeds. Specifically, we tested the hypotheses that cows that received a fresh embryo would have similar pregnancy per service event (P/S) as cows artificially inseminated with conventional semen, and that both AI and ET with fresh embryos would achieve better P/S than that achieved with frozen embryos. (ii) Chapter 3; to characterize the incidence and timing of pregnancy loss from initial service event to parturition following timed-AI or timed-ET with fresh or frozen IVP embryos by using a combination of detection of cows returning to estrus, chemical assays for interferon-stimulated genes (ISGs) and pregnancy associated glycoproteins (PAGs) and transrectal ultrasonography. Specifically, we tested the hypothesis that lactating dairy cows that received TAI would have similar P/S and incidence and timing of pregnancy loss to cows that received timed-ET with fresh IVP embryos and greater pregnancy per service event and less pregnancy loss than cows that received timed-ET with a frozen IVP embryo. (iii) Chapter 4; to determine the timing of presumptive conceptus attachment and subsequent incidence of pregnancy loss in seasonal-calving pasture-based lactating dairy cows following timed-AI with conventional (TAI-C) or X-sorted (TAI-S) semen or timed-ET with a frozen-thawed IVP embryo. We tested the hypothesis that the increased incidence of embryo loss following the transfer of frozen-thawed IVP embryos is due, at least in part, to delayed conceptus attachment. (iv) Chapter 5; to compare the gestation length, calf birth weight, calving difficulty and overall health status of calves derived from AI or ET with fresh or frozen IVP embryos of both beef and dairy breeds.

In vitro embryo production is now an established technology in the toolbox of assisted reproductive technologies available to farmers and breeding companies. Despite greater fixed costs, it offers significant advantages over traditional superovulation and embryo transfer (MOET) including increased numbers of embryos produced per donor per unit of time, greater flexibility in sire usage and the opportunity to reduce the generation interval by recovering oocytes from prepubertal calves. In addition, IVF facilitates more efficient use of low availability or high-cost semen straws, and thanks to the predictability of a donor’s performance and IVP yield (once a donor has been collected previously) in terms of oocyte and embryo yields, IVF can simplify the logistics of recipient synchronization and management. All of these factors have contributed to the marked increase in the use of IVF compared with MOET as a method of generating embryos for transfer worldwide (Viana, 2023).

In Chapter 2, the mean number of oocytes recovered (Dairy: 16.9, Beef: 15.8) and mean yield of transferable embryos (Dairy: 4.0, Beef: 4.5) per OPU session was highly variable between donors, with means ranging from 4.3 to 36.3 oocytes per donor per OPU session and zero to 15 transferrable embryos per donor per IVF session. This observation is consistent with published commercial data (Demetrio et al., 2020), where dairy donors produced an average of 15.6 oocytes and 3.6 viable embryos per OPU session and beef donors produced an average of 19.2 oocytes and 5.2 viable embryos per OPU session. The P/ET was similar to P/AI when embryos were transferred fresh, demonstrating the potential for fresh ET to be used effectively in a seasonal dairy production system without compromising subsequent calving pattern for recipient dams. It is important to note, however, that across all cows assigned to be recipients, 9.6 % were deemed unsuitable on the day of scheduled ET. Hence, the proportion of synchronized cows that could become pregnant following ET would be less than reported for cows assigned to be artificially inseminated. Pregnancies per ET have generally been reported to be less for IVP embryos compared with in vivo-derived embryos (Pontes et al., 2009, Carrenho-Sala et al., 2016, Pereira et al., 2016, Sartori et al., 2018). In addition, pregnancy loss has been reported to be greater for IVP embryos than for either in vivo-derived embryos or pregnancies from AI, further reducing reproductive efficiency achieved with IVP embryos (Carrenho-Sala et al., 2016, Pereira et al., 2016, Sartori et al., 2018). Thus, strategies to improve P/ET and reduce pregnancy loss are needed to maximize the efficiency of IVP-ET programs in cattle.

The majority of cryopreserved embryos that were later transferred to recipient cows were frozen on the morning of d 7. Day of culture when blastocysts were cryopreserved, based on when the embryo reached the blastocyst stage, tended to affect P/S on d 32 or d 62. Failure to find a significant association in this study was likely due to the relatively small number of embryos in each age category. It is interesting to note that P/ET appeared to diminish and embryo loss appeared to increase as embryo age at cryopreservation increased. This is consistent with previous observations that the timing of blastocyst formation affects blastocyst quality (Dinnyes et al., 1999). This association between embryo age at cryopreservation and P/S merits further investigation in future studies that are adequately powered to specifically address this issue.

In Chapter 2, pregnancy loss between d 32 and d 62 was significantly greater following ET, particularly following transfer of frozen-thawed embryos (12.2% vs 18.8% loss in fresh vs frozen embryos respectively). Seasonal production systems require excellent herd reproductive performance, and hence any management strategy that reduces P/S and increases embryonic losses will reduce profitability (Shalloo et al., 2014). Therefore, uptake of IVP and ET in seasonal dairy production systems will largely focus on use of fresh ET. Further investigation is necessary to improve the cryopreservation of IVP embryos.

To determine whether the deviation in sex ratio was due to inadvertent preferential selection of male embryos for transfer or was due to preferential survival of male embryos post transfer, a representative number of embryos (n = 103) was sexed post-thawing. Our observations indicate that the bias towards male fetuses on d 62-65 in recipient cows was mirrored by a similar sex bias in IVP blastocysts on d 7 and d 8, indicating similar survival of male and female embryos after transfer to recipients on d 7. A deviation in sex ratio towards more males following IVF in cattle was first described over 30 years ago (Avery et al., 1991, Avery et al., 1992). Subsequently, several studies reported that this bias was due to impaired imprinted X-chromosome inactivation (Gutierrez-Adan et al., 2001, Wrenzycki et al., 2002, Tan et al., 2016). Irrespective of the cause of the deviation in sex ratio in IVP embryos, this can be overcome through the use of sexed semen in IVF (Bermejo-Alvarez et al., 2010).

Chapter 3 builds on data reported in Chapter 2 (Crowe et al., 2024) by characterizing the incidence and timing of pregnancy loss from service event (AI or ET) to calving following TAI or TET with a fresh or frozen IVP embryo. In Chapter 3, additional days of pregnancy diagnosis were added by measuring peripheral blood mRNA ISG15 expression on d 18 and serum PSPB concentration on d 25, performing transrectal ultrasound pregnancy diagnosis at d 125 and recording of parturition date. This has provided a greater insight into the timing of when pregnancy loss occurred. As expected, greater P/S on d 32 and d 63 reported in Chapter 2 (Crowe et al., 2024) translated into a greater probability of the recipient dam subsequently achieving a full-term pregnancy and parturition. Measurement of peripheral blood ISG15 mRNA abundance and serum PSPB concentrations provided evidence that cows that fail to achieve critical threshold levels of these markers of pregnancy have markedly reduced likelihood of maintaining a pregnancy to full-term.

Based on 96.3% re-expansion of embryos post-thaw, we have assumed 100% pregnancy for both fresh and frozen ET treatments on d 7. Transfer of a grade 1 blastocyst to the uterus bypasses any problems or deficiencies associated with the follicle, oocyte and oviduct in the recipient dam, and therefore, could potentially contribute to improved pregnancy outcomes (Hansen, 2020). Despite this, pregnancy per ET with an IVP embryo is not better than cows bred with AI because pregnancy losses are greater in cows bred using IVP embryos compared with AI. The temporal pattern of this pregnancy loss is discussed in the following sections.

In Chapter 4 it is hypothesized that the timing of conceptus attachment would be later for cows that received TET compared with those receiving TAI-C. This builds on data reported in Chapters 2 and 3, where the incidence of pregnancy loss was greater and the likelihood of reaching successful term parturition was less in lactating cows assigned to frozen IVP-ET compared with cows assigned to conventional AI (Crowe et al., 2024).

Delayed pCA observed in cows assigned to TET in Chapter 4 impacted pregnancy loss up to d 62. After that time point, pregnancy losses were minimal, and were not associated with timing of pCA, regardless of the service treatment. It should be noted, however, that all cows pregnant on d 62 had pCA between d 19 to d 23 inclusive.

The study reported in Chapter 5 characterized the phenotypic differences at birth between calves derived from AI or ET with fresh or frozen IVP-ET embryos of beef and dairy origin. It compared differences between breeds and treatments on calf BW, GL, CD and mortality as well as comparing the levels of metabolites in blood biochemistry and hematology at birth. The main findings in Chapter 5 are that calves originating from IVP-ET had moderately increased BW and GL compared with calves born from AI, which led, in some cases, to increased calving difficulty/dystocia. In particular, AA calves had greater BW and CD than dairy breeds. Calves derived from IVP had similar blood biochemistry and hematology results, and had similar health status and survival post-partum to calves derived from AI.

Chapter 2 concluded that P/S was similar for AI and ET on d 32 post-estrus, but subsequent pregnancy loss was greater for ET than for AI. Transfer of frozen embryos resulted in fewer pregnancies and tended to be associated with greater embryo loss between d 32 and d 62 compared with fresh ET or AI. Nonetheless, our results demonstrate the potential for using OPU/IVF to produce embryos from high genetic merit donors within the calendar constraints of the seasonal-calving system. In the present era where beef on dairy is very topical, IVP and ET provides a clear route to accelerating genetic gain in both dairy breeds and beef breeds suitable for crossing with dairy dams. In particular, AI companies have now the potential to select genetically elite donors, and through the use of IVP-ET, produce the next generation of dairy and beef AI bulls. These donors can still be bred during the breeding season and remain productive in the dairy herd.

In Chapter 3, early pregnancy losses were broadly similar for all treatments, ranging from 27.1% to 32.8% between d 7 and d 18. This is largely in agreement with data from Wiltbank et al. (2016), who claim that pregnancy loss from d 8 to d 27 averaged approximately 30%. Delayed or failed conceptus elongation during this window is associated with reduced IFNT secretion and a failure to elicit an adequate response from the endometrium for maternal recognition of pregnancy (Sanchez et al., 2019). In addition, inadequate conceptus IFNT secretion will lead to reduced expression of ISGs in maternal peripheral blood, which was associated with pregnancy failure in the current study.

Berg et al. (2022) reported that total pregnancy losses in lactating dairy cows after d 15 were minimal, with pregnancy success on d 15 not differing from that at d 70. Wiltbank et al. (2016) reported that 20% of pregnancies were lost during the window between d 8 and 28, citing delayed elongation, inadequate circulating P4 concentrations after AI (a potential cause of the compromised elongation) and histotroph deficiencies as the main causes of loss in this window. It is important to note that Berg et al. (2022) looked largely at pasture-based cows, while Wiltbank et al. (2016) dealt predominantly with high input systems and high yielding Holstein cows. In the current study, pregnancy loss during the interval between d 18 and d 25 varied between 11.3% and 13.0% depending on treatment. Based on the previously cited studies by Berg et al. (2022) and Wiltbank et al. (2016), it is likely that the losses observed between d 18 and d 25 were related to the failure of the embryo to attach (or weak/late attachment) to the uterine endometrium or inadequate IFNτ production.

The estimated incidence of pregnancy loss between d 25 to d 32 in the current study ranged from 3.3 to 14.0% (AI: 3.3%; ET fresh: 11.8%; ET frozen: 14.0%). Due to the limited size of the subset of cows sampled on d 18 and d 25, statistical power was limited, and treatment differences were not detected. Cows that received AI, however, did tend to have a lesser incidence of pregnancy loss in this window of time than cows that received ET. We observed that using mRNA abundance of ISG15 in peripheral blood on d 18 to predict pregnancy status resulted in 10.7% (58/541) false negatives (i.e., cows deemed not pregnant on d 18 but subsequently detected as pregnant on d 25 or later).

We previously reported treatment differences in pregnancy loss during the interval from d 32 to d 62 between AI (4.0%), ET fresh (11.3%) and ET frozen (18.0%), indicating that pregnancies derived from IVP embryos had a greater risk of compromised placentome formation and vascular development. Wiltbank et al. (2016) reported that the main causes of pregnancy loss between d 28 and d 60 were defects in placentome and/or vascular development (losses of ~12%). Domingues et al. (2023) reported that approximately 50% of pregnancy loss is initiated by embryonic death and 50% by luteal regression. Their review estimated that the typical incidence of pregnancy loss between d 28 and d 60 is between 5 and 20%. Our results are largely in agreement, suggesting that a conceptus derived from IVP may have a greater risk of poor placentome formation and vascularity compared with a conceptus derived from AI.

Pregnancy losses were minimal from d 62 to full-term (AI: 1.9%, ET fresh: 2.9% and ET frozen: 4.3%) and no differences were observed between treatments. In the current study, none of the pregnancy losses were associated with twin pregnancies; however, a small number of late term abortions did occur during months 7 and 8 of gestation (n = 3 for ET fresh; n = 4 for ET frozen). As this is clearly a low frequency event, much larger datasets will be required to determine whether the likelihood of a cow aborting is affected by the origin of the embryo.

In agreement with Wiltbank et al. (2016), the majority of the embryonic mortality in Chapter 2 occurred early in gestation (before d 25), and was broadly similar for each treatment. After d 25, the incidence of pregnancy loss began to differ between treatments (no major loss was recorded in the AI treatment after d 25; however, there was a further 23.1% and 32.0% loss from fresh ET and frozen ET, respectively, between d 25 and d 62.

The underlying causes of greater pregnancy loss following ET are unclear but likely multifactorial, related to oocyte quality and, in particular, suboptimal in vitro conditions impacting embryo survival after transfer, as well the adverse effects of cryopreservation of IVP embryos. Despite high fertilization success in vitro, typically only 30 to 40% of immature oocytes progress to the blastocyst stage (Rizos et al., 2002). A proportion of this loss may be related to a greater incidence of chromosomal abnormalities in IVP embryos (Viuff et al., 1999, Lonergan et al., 2004). Domingues et al. (2023) investigated the effect of increased plasma concentrations of P4 on embryonic attachment and concentrations of PAG, and also examined relationships among luteal regression, pregnancy loss, and plasma PAG concentrations in cows undergoing pregnancy loss by d 33 of pregnancy. Approximately 50% of pregnancy loss was due to luteal regression and the remaining 50% was due to conceptus failure (i.e., a decrease in PAG in the absence of luteal regression). They concluded that increased P4 concentrations did not accelerate embryonic attachment or early placental development, but did increase PAG concentrations on d 47 and d 61 of gestation. In the current study, P4 concentration on d 7 was not associated with pregnancy loss after d 25 of gestation. The cows in this study had similar distributions of serum P4 concentrations on d 7 in all treatments (Crowe et al., 2024). The major factors that contributed to embryo mortality after maternal recognition of pregnancy likely reflect inherent differences in embryo quality and developmental competence that already existed on d 7 in cows that received TET vs TAI, and additional complications arose from embryo cryopreservation. Further examination of media usage in IVP, alternative media or methods for the cryopreservation of blastocysts may be of merit in future studies.

Chapter 3 concludes that cows assigned to both AI and fresh ET had greater probability of becoming pregnant, maintaining the pregnancy and ultimately reaching full-term parturition compared with cows assigned to frozen ET. Cows that had greater serum concentration of P4 on d 7 had greater peripheral blood mRNA abundance of ISG15 on d 18, indicating a stronger maternal response to embryo derived IFNT. These cows also tended to have a greater serum concentration of PSPB on d 25, which in turn was associated with greater likelihood of reaching full-term parturition. Further work is required to optimize the in vitro production and cryopreservation of embryos to increase the probability of pregnancy establishment and reduce the incidence of early embryonic mortality.

It is well described that serum P4 concentrations during the first two weeks of pregnancy, through its effects on the endometrium, are critical for conceptus elongation and therefore, by association, the ability of the conceptus to have early attachment (Forde et al., 2009, Spencer et al., 2016). In Chapter 2, cows with greater serum concentrations of P4 on d 7 had greater P/S at both d 32 and d 62 (Crowe et al., 2024). Interestingly, while d 7 P4 concentration was associated with the likelihood of a cow having pCA in Chapter 4, it was not associated with the timing of pCA. Previously, Forde et al. (2011) reported that heifers with lesser serum concentrations of P4 had retarded embryo elongation on d 14 compared with heifers with normal serum P4. We would expect that delayed elongation would subsequently delay the timing of pCA, and perhaps with a larger sample size, we may have observed an association between serum P4 concentrations and the timing of pCA.

By determining the absolute serum concentrations of PSPB, we were able to evaluate the associations between true PSPB concentration with pregnancy and pregnancy loss. When treatment effects on PSPB concentration were compared in cows with different pregnancy outcomes (pregnant, initially pregnant with subsequent loss, or pCA never detected), no treatment difference were detected in mean concentration or the mean daily PSPB concentration in cows that reached full-term parturition. For cows that initially had pCA but subsequently underwent pregnancy loss, however, differences in the daily mean PSPB concentration between treatments were apparent. Cows in the TET treatment that had evidence of pCA but subsequently underwent pregnancy loss had lesser serum PSPB compared with cows that received TAI-C or TAI-

S, and underwent pregnancy loss following initial pCA, during the interval from d 20 to 24. This indicates that delayed and/or weaker pCA following TET with frozen IVP embryos has a significant association with subsequent pregnancy loss. It is clear from these data that an early increase of PSPB (and hence, early pCA) was a key milestone to achieve pregnancy establishment and maintenance to parturition.

Chapter 4 concludes that daily measurement of maternal serum PSPB concentration to determine the time of pCA is an important advancement that has allowed us to undertake detailed studies on pregnancy establishment and pregnancy losses in cows assigned to different reproductive technologies for breeding. Cows that received frozen-thawed IVP-ET had later pCA than cows that received TAI-C. This may explain, at least in part, the greater pregnancy losses observed in cows that received TET. Further work is required to optimize the in vitro environment and the cryopreservation techniques used for IVP to improve the likelihood of successful pregnancy establishment, advance the timing of pCA and increase the probability of pregnancy maintenance through to parturition.

The use of IVP-ET (along with other assisted reproductive technologies) has been linked with LOS or “Abnormal Offspring” (AOS) since it was first reported nearly 30 years ago (Behboodi et al., 1995, Walker et al., 1996) and isolated incidences are still being reported (Siqueira et al., 2017, Weiller et al., 2021). The main findings from Chapter 5 are that calf BW was affected by both breed and treatment, with beef calves having greater BW but also calves derived from ET treatments having greater BW than AI. Treatment also affected GL, with AI calves having shorter GL than ET. Calving difficulty, however, was mainly affected by breed, with AA calves from fresh ET having the greatest incidence of CD. Perinatal mortality was not affected by treatment or breed, and mortality was < 5% across all treatments. While variation was observed for several blood biochemistry and hematology analytes, values were generally within the normal expected ranges (Santos et al., 2023).

Both treatment and calf breed affected BW, with only a small difference (3-4 kg) in BW between treatments within HF calves, which did not lead to a notable increase in incidence of dystocia or perinatal mortality. Gestation length was affected by treatment with AI-derived calves having 2-3 d shorter gestations than ET-derived calves. The breed effect on GL was predominantly driven by the longer GL of calves with a Limousin sire, which is typically associated with longer gestation than AA, HF and JE when used in conventional AI. The longer GL observed in ET calves in this study (derived from both fresh and frozen blastocysts), suggests that the IVP process, rather than the cryopreservation and thawing procedures, increases GL. There was a higher incidence of dystocia in ET calves compared to AI calves, particularly among those derived from fresh embryos. While the use of frozen embryos did not significantly differ from fresh embryos in terms of CD, the overall trend suggests that the use of IVP embryos, both fresh and frozen, requires careful management to mitigate the risk of dystocia, particularly where beef breeds are being used as sires and donors. No difference in perinatal mortality rate was observed between AI and ET calves.

For the most part, among calves on this study, no differences were observed solely between AI and IVP-ET derived calves for any of the 28 blood biochemistry and hematology variables. All differences observed in in biochemistry variables appear to have been driven by breed differences between beef and dairy breeds.

Chapter 5 concluded that calves originating from IVP-ET had moderately increased BW and GL than calves born from AI. In some instances, larger BW led to calving difficulty/dystocia underpinning the need for careful selection of genetics for an IVP-ET program in dairy cows. While ET produced calves with a moderately increased BW among HF calves, breed played a bigger factor than treatment and care must be exercised when selecting beef sires and dams for use in IVP. Calves derived from IVP-ET, although heavier at birth, particularly where beef embryos were used, were as healthy and had similar survival post-partum as calves derived from AI. This indicates that once care is taken to use easy calving sires IVP-ET derived calves do not pose a markedly increased risk of poor health or survivability compared with AI derived calves. The findings suggest that there is still room for further research in order to optimize IVP protocols, particularly in terms of embryo culture conditions and criteria for selection of donors and sires, to further reduce complications arising at parturition.

Overall conclusion:

Given the importance of fertility in seasonal, pasture-based dairy systems, use of IVP-ET will be used on a small proportion of herds as part of their annual breeding programme. Limitations in fertility and increased pregnancy loss following frozen ET and the potential for greater birthweight using beef breed embryos mean it is a high risk strategy for farmers to aim to produce 100% beef breed calves in the near future. However, with the use of fresh ET, and careful selection of sires and dams, IVP-ET has great potential to transform the way high genetic merit AI sires and dams are produced in Ireland in the future. Fresh ET allows for similar P/S rates to AI, though with some variability in early pregnancy losses. In contrast, frozen ET presents higher rates of pregnancy loss, potentially impacted by delayed conceptus attachment, which can impact pregnancy establishment and maintenance. Additionally, the birth characteristics of ET-derived calves, including increased BW and GL, highlight the need for careful donor and sire considerations in IVP-ET use. While both fresh and frozen ET hold promise, the findings suggest that the current cryopreservation methods used require improvement in order to increase the viability of embryos, improve pregnancy success and reduce early pregnancy loss.

Future Implications

1. Improvement in Embryo Cryopreservation Techniques

The increased pregnancy loss observed in frozen ET underscores the need for improved or enhanced cryopreservation techniques and media. Enhancing embryo survival post-thaw, in order to generate faster developing and attaching embryos that will reach parturition would allow broader adoption of frozen ET. If improved, frozen IVP-ET could be a more flexible option for seasonal dairy farms, allowing for embryo production outside of the breeding season. Future research should focus on improving the resilience of IVP embryos during the cryopreservation process to reduce losses related to delayed attachment and early embryonic/fetal mortality.

2. Optimizing Genetic Selection and Reproductive Efficiency

The use of IVP embryos allows for targeted genetic selection, which can increase the value of both dairy and beef crosses. By selecting high genetic merit sires and dams for fresh ET, farmers can accelerate genetic improvement. However, given the increased risk of dystocia with beef embryos, careful selection of easy-calving sires and donors remains essential. Optimizing donor and recipient selection criteria could further improve reproductive outcomes, ensuring a balance between genetic gain and calf viability.

3. Directions for Future Research

Several avenues for future research are evident:

Embryo Culture and Cryopreservation: Further studies should investigate embryo culture media and alternative cryopreservation methods to improve IVP embryo quality and pregnancy success and reduce pregnancy loss.

Breed-Specific Management: With the increasing trend toward beef-dairy crosses, breed-specific strategies should be developed to select for easier calving and shorter gestation genetics along with improved beef characteristics, that would be suitable for use in the dairy herd.

This research represents a step toward optimizing the use of IVP-ET in seasonal calving, pasture-based dairy systems.

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Full-length peer-reviewed manuscripts:

Crowe AD, Lonergan P, Butler ST. 2021. Invited review: Use of assisted reproduction techniques to accelerate genetic gain and increase value of beef production in dairy herds. Journal of Dairy Science, Volume 104, Issue 12, December 2021, Pages 12189-12206.

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Crowe AD, Sánchez JM, Moore SG, McDonald M, Rodrigues R, Morales MF, de Freitas LO, Randi F, Furlong J, Browne JA, Rabaglino MB, Lonergan P, Butler ST. 2024. Fertility in seasonal-calving pasture-based lactating dairy cows following timed artificial insemination or timed embryo transfer with fresh or frozen in vitro produced embryos.

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Crowe AD, Sánchez JM, Moore SG, McDonald M, McCabe MS, Randi F, Lonergan P, Butler ST. Concentration of progesterone, relative mRNA abundance of Interferon-Stimulated Gene-15 and concentration of Pregnancy-Specific Protein B in maternal circulation following timed artificial insemination or embryo transfer in lactating dairy cows. AETE Annual Meeting, September 4th-6th 2024, Brescia, Italy.

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