The first week following insemination is the period of major pregnancy failure in pasture-grazed dairy cows

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ABSTRACT

A 60% pregnancy success for inseminations is targeted to optimize production efficiency for dairy cows within a seasonal, pasture-grazed system. Routine measures of pregnancy success are widely available but are limited, in practice, to a gestation stage beyond the first 28 d. Although some historical data exist on embryonic mortality before this stage, productivity of dairy systems and genetics of the cows have advanced significantly in recent decades. Accordingly, the aim was to construct an updated estimate of pregnancy success at key developmental stages during the first 70 d after insemination. Blood samples were collected for progesterone concentrations on d 0 and 7. A temporal series of 4 groups spanning fertilization through d 70 were conducted on 4 seasonal, pasture-grazed dairy farms (n = 1,467 cows) during the first 21 d of the seasonal breeding period. Morphological examination was undertaken on embryos collected on d 7 (group E7) and 15 (group E15), and pregnancy was diagnosed via ultrasonography on approximately d 28 and 35 (group E35) as well as d 70 (group E70). Fertilization, embryo, and fetal evaluation for viability established a pregnancy success pattern. Additionally, cow and on-farm risk factor variables associated with pregnancy success were evaluated. We estimated pregnancy success rates of 70.9%, 59.1%, 63.8%, 62.3%, and 56.7% at d 7, 15, 28, 35, and 70, respectively. Fertilization failure (15.8%) and embryonic arrest before the morula stage (10.3%) were the major developmental events contributing to first-week pregnancy failures. Embryo elongation failure of 7% contributed to pregnancy failure during the second week. The risk factors for pregnancy success that were related to the cows included interval between calving and insemination, and d-7 plasma progesterone concentrations, whereas insemination sire was associated with pregnancy outcome. Most pregnancy failure occurs during the first week among seasonal-calving pasture-grazed dairy cows.

Key words: pasture-grazed dairy cows, pregnancy success, embryonic loss, bovine reproduction

INTRODUCTION

Reproductive performance of dairy cows remains an important driver of dairy production efficiency, particularly in a seasonal, pasture-based system that is constrained to maintaining a 365-d calving interval to align the increased energy demand of early lactation with the seasonal peak in pasture growth (Sreenan and Diskin, 1986; Dillon et al., 1995; Roche et al., 2011). In addition to being efficient convertors of pasture to milk, efficient cows in a seasonal system are those with fertile estrous cycles within 6 to 9 wk after calving and a 60% chance of conceiving to AI within the first 21 d of the breeding period. Industry statistics of reproductive performance in New Zealand dairy cows are published annually. Current levels of performance for AI within the first 21 d and conception success to AI are 80% and 54%, respectively (DairyNZ and Livestock Improvement Corporation, 2020). First-service conception rates are influenced by many factors, including estrous cyclicity status, inaccurate estrus detection, insemination of cows early in the postpartum period (late-calving cows), low body condition score, and late embryonic loss (Roche et al., 2009; Santos et al., 2009; Burke et al., 2012; Cummins et al., 2012).

The bovine embryonic period spans from fertilization to approximately d 45 of gestation, and the fetal period extends from d 45 to parturition (Hubbert et al., 1972). A developmental cascade of embryonic and fetal competence, as well as a receptive maternal tract environment, are required to ensure a pregnancy proceeds.

The first step in embryonic development is fertilization of the newly ovulated oocyte. This is quickly fol-
lowed by the crucial events of correct embryonic genome activation (Graf et al., 2014), followed by compaction and cavitation that are required for establishing the first lineage decision at d 7. A morphologically distinct blastocyst results, which comprises 2 cell types: the inner cell mass and the trophectoderm (TE; Pfeffer, 2018). During the second week, the inner cell mass differentiates into the epiblast and the hypoblast lineages, the embryo sheds the zona pellucida and transitions from the spherical to the ovoid stage and elongates, and maternal embryonic signaling necessary for maternal recognition of pregnancy is initiated. During this time, the epiblast transforms from the sphere of cells to a flat embryonic disc and gastrulation begins (Artus et al., 2020). Pregnancy recognition and implantation (Bazer, 2013; Spencer and Hansen, 2015; Imakawa et al., 2017), complete with extraembryonic membranes and rudimentary organs, occurs during the third and fourth week of gestation (Pedersen et al., 2020). Failure at any of these developmental milestones will result in the termination of the pregnancy.

Pasture-grazed dairy cows are reported to have a combined embryonic and fetal loss of 40%, largely during the embryonic period between d 8 and 16 (Diskin et al., 2006). This is followed by a 7% fetal loss beyond d 42 of gestation (McDougall et al., 2005; Diskin et al., 2006). In contrast, the North American (NA) Holstein-Friesian (HF) cows experience greater embryonic and fetal losses of approximately 60%, mostly during the embryonic period, with the majority of loss occurring from fertilization to d 7 (Wiebold, 1988; Sartori et al., 2002; Wiltbank et al., 2016).

Pasture-grazed dairy cow pregnancy loss patterns are based on 35-yr-old data (Sreenan and Diskin, 1986). Dairy cow genetic selection indices have changed since these data were collected. In New Zealand, for example, genetic selection for increased milk production resulted in a greater influence of the NAHF genetics in the 1990s (Harris and Kolver, 2001), followed by a greater emphasis on crossbreeding (Harris, 2005) and, more recently, increased genetic selection for functional traits, including fertility (Harris et al., 2006). These changes could have altered the previously reported temporal pattern of early embryonic survival, and current research efforts to improve conception success may not be targeting the most vulnerable stage of development.

Accordingly, our study aimed to reconstruct a temporal estimate of pregnancy success (pregnancy per AI or viable embryo per AI) to first insemination in seasonal, pasture-grazed dairy cows, representative of the current population genetics. Secondary aims were to identify and quantify the developmental events most likely contributing to pregnancy failure between fertilization and d 35 of gestation; and, finally, to identify factors associated with pregnancy success to d 35 by examining farm and industry data records for the enrolled cows.

**MATERIALS AND METHODS**

Experimental procedures were carried out in accordance with the 1999 Animal Protection (Codes of Ethical Conduct) Regulations of New Zealand, and with prior approval of the Ruakura Animal Ethics Committee (AE13285 and AE13549).

**Farms and Animals**

Four commercial dairy farms participated in the study, with 2 farms participating for 2 consecutive years, 2014 and 2015. Cows ranged in age from 2 to 14 yr. All farms were located on the North Island of New Zealand and were seasonal-grazing systems, predominantly managed on ryegrass and white clover pasture. A total of 2,433 dairy cows were available for enrollment from the 4 farms over the 2 breeding seasons.

The farms used in this study were considered representative of average New Zealand dairy farms. General information about the farms and animals submitted for insemination at each farm is presented in Supplemental Table S1 (https://figshare.com/s/4339b73009acdb1aeea54; Berg et al., 2022).

**Experimental Design**

The study began at the time of AI; therefore, all farm management practices and decisions were specific for each farm, including the sires used for AI. Cows that participated in a pre-mating synchrony program were excluded. As part of the farms’ standard breeding practice, cows detected in estrus by farm staff were inseminated once per day, using Long Last liquid semen (Livestock Improvement Corporation), after the morning milking on all farms. In general, cows on the small farms (T2 and W) were inseminated with the same bull on a single day. The larger farms (N and T1), with more cows to inseminate on a single day, tended to use 2 to 3 bulls. Therefore, bulls were randomly distributed across the 4 collection groups in the blocks for each day (see below). Insemination sires were used across the farms and years. Retrospective analysis of d-0 plasma progesterone identified those cows inseminated on estrus, as described further in the Methods section. The bull identity codes and dates of AI were recorded for each cow, including return inseminations, for the entire AI period.

Four groups comprised 5 different sampling time points after AI (d 0). The first group of cows (E7) was used to assess fertilization success and early cleavage
and embryo quality, which together established an estimate of pregnancy success during the first 7 d. Embryos were recovered from the second group of cows (E15) 15 d after AI, allowing the estimation of embryonic survival from d 8 to 15. This collection period included the developmental events of conceptus elongation and peri-gastrulation up to the critical stage of pregnancy recognition that occurs around d 16 (Northey and French, 1980). At d 15, good recovery rates are achievable (Berg et al., 2010) and the interferon-tau (IFN-τ) protein can be identified in uterine flushing (Ribeiro et al., 2016b).

For the remaining time points, we used ultrasonography to diagnose pregnancy success. This was achieved by scanning uterine horns using a portable Easy-Scan scanner (BCF Ultrasound) with a 128-element, broadband linear probe operating on a frequency of 4.5 to 8.5 MHz. The third experimental group of cows (E35) were scanned twice: first between d 28 and 32, with pregnancy diagnosed by visual confirmation of a fetal heartbeat, followed by a second ultrasonography between d 35 and 38 to verify results from the first scan.

The fourth group of cows (E70) underwent pregnancy diagnosis and fetal aging between d 68 and 74 after AI. These cows were considered a herd control group, with no intervention other than a blood sample at the time of AI.

A randomized block design was used to allocate cows inseminated within the first 21 d of the planned start of mating. The block consisted of 9 sample collection times, with the 4 collection groups represented twice, plus an additional E7 collection group. This extra collection group was added to account for E7 cows that could not be identified. Using the blood samples taken. Therefore, this group (n = 368) was not included in the IE calculations, because anestrous cows that were inseminated could not be identified. Using the data from the E7, E15, and E35 collection groups, IE was calculated using the following formula: (Number of cows submitted for AI with d-0 progesterone >1.0 ng/mL or d-0 and d-7 progesterone <1.0 ng/mL)/(number of cows inseminated ≥30 DIM)/(number of cows inseminated ≥30 DIM) = 89/1,413.

**Blood Sampling and Progesterone Analysis**

Blood samples were taken at AI from all enrolled cows via coccygeal venipuncture into a lithium heparin tube (Vacutainer, Becton Dickinson). A second blood sample was taken at d 7, except for the E70 group, and a third blood sample was taken from the E15 group at embryo recovery. Blood samples were immediately placed on ice and then centrifuged at 2,000 × g for 10 min at 4°C. The resulting plasma was stored at −20°C.

The progesterone concentration in blood plasma was measured with an electrochemiluminescence detection system (Elecsys Progesterone II Kit, Roche Diagnostics GmbH) using an automated clinical immunology analyzer (Roche Modular E170, Hoffmann-La Roche Ltd.) at New Zealand Veterinary Pathology Ltd. (Hamilton, New Zealand). The detection limit of the assay was 0.03 ng/mL. The average coefficient of variation for the controls was 4.33% for the low control (target concentration 0.711 ng/mL) and 1.90% for the high control (target concentration 9.98 ng/mL).

**Submission to Insemination Errors**

We identified incorrect timing of AI (IE) by retrospective analysis of plasma progesterone concentrations. A progesterone cut point of 1.0 ng/mL (Geisert et al., 1992) was used to identify cows that were inseminated while in a luteal phase of the estrous cycle (i.e., progesterone >1.0 ng/mL). Plasma progesterone values of <1.0 ng/mL on both d 0 and 7 were considered to indicate that these cows failed to ovulate after displaying estrus.

The E70 collection group did not have a d-7 blood sample taken. Therefore, this group (n = 368) was not included in the IE calculations, because anestrous cows that were inseminated could not be identified. Using the formula: (Number of cows submitted for AI with d-0 progesterone >1.0 ng/mL or d-0 and d-7 progesterone <1.0 ng/mL)/(number of cows inseminated ≥30 DIM) = 89/1,413.

**Body Condition Score**

Body condition score of individual cows was assessed by experienced operators using a 1 to 10 scale (1 =
Embryo Recovery

In total, 6 flushing technicians, across 4 commercial companies specializing in embryo transfer, participated during the 2-yr study. To recover embryos, cows from the E7 and E15 groups underwent a standard nonsurgical uterine flush technique (Berg et al., 2010) with the following modifications. The embryo flushing medium was 1-L infusion packs of sodium lactate (Hartmann’s solution, Baxter, Viaflex) supplemented with 1 mL of 1 M 3-morpholinopropanone-1-sulfonic acid (MOPS) titrated with sodium salt to a pH of 7.4, and 1 mL of liquid BSA (20% wt/vol; ICPbio Reproduction). The final concentration of the flushing medium was 1 mM MOPS and 0.02% wt/vol BSA, pH 7.4.

For the E7 group, the uterine horn ipsilateral to the ovary bearing a corpus luteum (CL) was flushed; however, both uterine horns were flushed when a CL was present on both ovaries. Commercial flushing operators used up to 500 mL per uterine horn. Volume varied among flushing operators and was not recorded. The flushing medium containing the uterine contents was passed through an E-Z Way Filter (SPI Mfg) to capture both flushing debris and the embryo. Material captured by the filter was immediately searched for the E7 embryo.

For the E15 group, the horn ipsilateral to the CL was flushed using a 2-step procedure, an initial saline flush to capture the uterine luminal fluid (ULF), and a second flush with embryo flushing medium. For the initial flush, a catheter was placed into the ipsilateral uterine horn, and 30 mL of isotonic sterile saline (0.9% sodium chloride intravenous infusion BP, Baxter) was introduced using a 35-mL syringe as described by Ledgard et al. (2011). If the embryo was recovered in the first 30-mL collection, the flushing procedure was terminated. If an embryo was not present, or only a fragment of an embryo was collected, a second flushing was undertaken using 300 to 600 mL of embryo flushing medium per uterine horn.

Embryo Evaluation of the E7 Group

The filter dish containing the uterine flushing from each cow was searched independently by 2 experienced embryologists using a Nikon SMZ1500 stereo microscope. The filter dish was searched up to 3 times, and if no embryo was located, the cow was excluded from the data set (n = 204).

Recovered embryos were immediately placed in an EmCare Holding Solution (ICPbio Reproduction) to evaluate the stage and grade according to the International Embryo Transfer Society scoring system (Stringfellow and Seidel, 2007).

All recovered embryos were graded and photographed (Nikon Digital Sight DS-Fi1) at 80- to 100× magnification. To ensure quality control, the embryo photographs were then “blind” evaluated by 2 independent embryologists for grade and stage. Disagreement of embryo stage and quality was resolved by a third embryologist, and, as a result, 1.8% of recovered embryos (11/614) were re-evaluated. Embryos recovered at d 7 were considered viable if they were at the compact morula or blastocyst stage, as those stages do not differ in survival rates after transfer (Hasler, 2001). Grades 1 and 2 were transferrable quality, whereas grade-3 compact morula or blastocyst embryos were classified as poor quality, given their lower probability of pregnancy establishment (Hasler, 2001; Bo and Mapletoft, 2013). Nine cows had multiple embryos; however, only the most advanced stage and highest-quality embryo or conceptus was included for pregnancy success results. In cases where only a single cell was recovered, these were processed for fertilization analysis as described subsequently.

Fertilization and Analysis of Single-Cell Ova

Single-cell ova recovered at d 7 were subjected to DNA parentage to ascertain absence or presence of sperm penetration. At the time of flushing, a hair sample was taken from all E7 cows and stored for future DNA extraction. For all single-cell samples, the zona pellucida was removed using 0.5% Pronase (Sigma P-881, Sigma-Aldrich) in HEPES-buffered synthetic oviduct fluid containing 0.1% polyvinyl alcohol (Sigma P-8136, Sigma-Aldrich) at the time of recovery. The zona-free ova were washed twice in EmCare Holding Solution (ICPbio Reproduction), followed by a final wash in PBS-0.1% polyvinyl alcohol, and transferred with a small volume of medium to a 0.6-mL Eppendorf tube and stored at −20°C.

The ova were thawed and lysed, and then their DNA was amplified using the Illustra GenomiPhi V2 DNA Amplification Kit (Global Science & Technology). Genomnz (AgResearch Ltd., Mosgiel, New Zealand) genotyped the amplified samples using the StockMarks for Cattle Genotyping Kit (Applied Biosystems) plus an additional 8 Genomnz proprietary markers. Genotypes of each sample were compared with those of the
cow and recorded sire. Sire genotype profiles not already stored on the Genomnz databases were obtained from DNA extracted from a frozen-thawed sperm sample. Those ova that possessed one or more alleles that could only have been inherited from the sire were defined as fertilized. Those that possessed only maternal alleles were classified as unfertilized. Fertilization status could not be determined for all samples due to genotyping failure or when the genotypes were not informative.

**Embryo Evaluation in the E15 Group**

Conceptuses were considered viable if they were ≥5 mm in length. Spherical and ovoid embryos (<5 mm) were considered delayed in development and were scored as nonviable. Spherical and ovoid stages are normally recovered from single ovulating cows at d 10 to 11 and d 12 to 13, respectively (Degrelle et al., 2005). Although no standardized criteria exist for conceptus evaluation at d 15, the most frequent stages observed at this time point were used for viability criteria. These were tubular (5–30 mm in length), early filamentous (31–100 mm), and filamentous stages (>100 mm). In cases where several TE fragments were found, the length of each fragment was measured. The fragment lengths were added together for an estimation of conceptus length, but these measurements were not included in the length data, because not all fragments may have been recovered. If the sum of the fragments was 5 mm or greater, the conceptus was considered viable. Conceptuses with obvious TE degeneration (black and fragmenting) were considered nonviable.

All conceptuses, including those recovered in fragments, were further examined to record the presence or absence of an embryonic disc. Tubular through filamentous-stage conceptuses without an embryonic disc were considered viable at d 15. Our rationale was that a trophoblastic vesicle produces IFN-τ and will initiate pregnancy signaling, even if such pregnancies are likely to fail between d 21 and d 37 of gestation (Heyman et al., 1984). Microdissection techniques (ultra-sharp splitting blades; Bioniche Animal Health) were used to separate the embryonic disc from the conceptus. Once the embryonic disc was removed, its length and width were measured using an eyepiece micrometer, and the shape of the disc was recorded as circular (stage 2), ellipse, or pear-shaped (stage 3; van Leeuwen et al., 2015), and photographed at 100× magnification. A circular shape indicated that the length and width of the embryonic disc were similar in size. An ellipse shape indicated that the length of the disc was greater than its width, and a pear-shaped disc indicated that the cranial portion of the disc is wider than the caudal end. Embryonic disc measurements from 8 conceptuses were not recorded.

**IFN-τ Analysis**

From the ULF samples collected at d 15, the cellular debris was pelleted by centrifugation at 1,000 × g for 10 min at 4°C, and the supernatant was recovered. The remaining supernatant was divided into two 5-mL aliquots. Protease inhibitors (Complete, Roche) were added to each aliquot, as recommended by the manufacturer, and then rapidly frozen on dry ice and stored at −80°C until analysis.

The ULF from cows that did not contain an embryo was analyzed for the presence of IFN-τ, which determined the technical flushing error for the E15 recoveries (i.e., cases where the cow was pregnant but an embryo was not recovered). Positive controls were ULF from cows that had an ovoid (n = 3), tubular (n = 29), or early and filamentous (n = 19) embryo recovered. To verify that the fragmented embryos produced IFN-τ, a subset of fragmented embryos was analyzed for IFN-τ. These samples contained IFN-τ, and thus fragmented embryos were considered viable at d 15 if the sum of the fragments was 5 mm or greater.

Total protein concentration in the E15 ULF was measured in duplicate using a Direct Detect Spectrometer (Merck Millipore). This technique uses infrared spectroscopy to detect amide (peptide) bonds that are compared by the analyzer to an internal standard curve to calculate the sample protein concentration in milligrams per milliliter. The presence of IFN-τ was determined by Western blotting as described by Ledgard et al. (2011). The primary antibody used was rabbit anti-bovine IFN-τ (1:2,000; gift from R. Roberts, University of Missouri, Columbia, USA), and the secondary antibody was horseradish peroxidase conjugated goat anti-rabbit (1:20,000; Sigma-Aldrich). Bands were quantified using Quantity One software (version 4.6.9; Bio-Rad) and expressed as optical density units per 20 μg of total ULF protein (Ledgard et al., 2012).

**Embryo Developmental Potential and Projected Pregnancy Success**

The developmental fate of the preimplantation embryo was assessed by morphology (embryo stages and quality) to understand when pregnancy failure had or would occur, rather than assigning loss to the day of recovery. The concept of predicting future pregnancy outcomes at d 7 has been previously reported using a candidate-gene approach associated with pregnancy outcomes (El-Sayed et al., 2006; Zolini et al., 2020; Salilew-Wondim et al., 2021).
Table 1. Descriptive statistics of ova and embryos recovered from uterine flushes performed 7 d after AI (i.e., E7 group) in pasture-grazed, seasonally managed dairy cows¹

| Embryo stage          | Number of ova and embryos recovered | Percentage of ova and embryos recovered | 95% CI          | Percentage of embryos only recovered | 95% CI          |
|-----------------------|--------------------------------------|----------------------------------------|-----------------|--------------------------------------|-----------------|
| Nonviable ova or embryos (total no.) | 107                                   | 29.1<sup>a</sup>                       | 24.5–34.0       | 15.8<sup>a</sup>                     | 11.9–20.4       |
| Single                | 58                                    | 15.8<sup>b</sup>                       | 12.2–19.9       |                                      |                 |
| 2–16 cells            | 32                                    | 8.7<sup>c</sup>                        | 6.0–12.1        |                                      |                 |
| Early morula<sup>2</sup> | 17                                    | 4.6<sup>d</sup>                        | 2.7–7.3         |                                      |                 |
| Viable embryos (total no.) | 261                                   | 70.9<sup>e</sup>                       | 66.0–75.5       | 84.2<sup>e</sup>                     | 79.6–88.1       |
| Morulae               | 114                                   | 31.0<sup>f</sup>                       | 26.3–36.0       | 36.8<sup>f</sup>                     | 31.4–42.4       |
| Grade 1               | 27                                    | 7.3<sup>g</sup>                        | 4.9–10.5        | 8.7<sup>g</sup>                      | 5.8–12.4        |
| Grade 2               | 55                                    | 14.9<sup>h</sup>                       | 11.5–19.0       | 17.7<sup>h</sup>                     | 13.7–22.5       |
| Grade 3               | 32                                    | 8.7<sup>i</sup>                        | 6.0–12.1        | 10.3<sup>i</sup>                     | 7.2–14.3        |
| Blastocysts           | 147                                   | 39.9<sup>j</sup>                       | 34.9–45.2       | 47.4<sup>j</sup>                     | 41.7–53.1       |
| Grade 1               | 68                                    | 18.5<sup>k</sup>                       | 14.6–22.8       | 21.9<sup>k</sup>                     | 17.5–27.0       |
| Grade 2               | 66                                    | 17.9<sup>l</sup>                       | 14.2–22.2       | 21.3<sup>l</sup>                     | 16.9–26.3       |
| Grade 3               | 13                                    | 3.5<sup>m</sup>                        | 1.9–6.0         | 4.2<sup>m</sup>                      | 2.3–7.1         |
| Total nonviable and viable ova/embryos | 368                                   |                                        |                 |                                      |                 |

¹Presented are the number and percentage of ova and embryos classified as nonviable and viable, as well as the developmental stages within these classifications. Also presented is the percentage of embryos recovered, excluding single-cell, unfertilized ova. Confidence intervals (95% CI) are included for each of these percentage-recovered metrics.

<sup>a</sup>Within a column, percentages for viable and nonviable classification not sharing a common letter are different (P < 0.001), Fisher’s exact test.

<sup>b</sup>Within a column, percentages of ova and embryo main stages (single cells, 2–16 cells, early morula, morulae, and blastocyst) not sharing a common letter are different (P < 0.05), Fisher’s exact test.

<sup>c</sup>Within blastocyst grade, percentages not sharing a common letter are different (P < 0.01), Fisher’s exact test.

<sup>d</sup>Within morula grade, percentages not sharing a common letter are different (P < 0.01), Fisher’s exact test.

<sup>e</sup>Within embryo stage, percentages not sharing a common letter are different (P < 0.001), Fisher’s exact test.

Embryo developmental potential was morphologically based (Van Soom et al., 2003) using microscopy at 80× to 100× magnification. Arrested embryos were assigned to the approximate gestation day that a particular embryo stage was normally recovered. The E7 arrested embryos were based upon the timing of embryo cell cycles (Van Soom et al., 1992) and whether the arrested embryos would have been viable at d 2 and 5. Fertilization occurs quickly after AI (Hunter, 1985); therefore, for the E7 group, the estimated time of ovulation was considered to define the time of fertilization. The percentage of fertilized embryos (2-cell to blastocyst stage) defined pregnancy success at d 2. Day-5 pregnancy success was calculated using the number of embryos that had developed beyond the 16-cell stage. Arrested d-15 conceptus stages determined pregnancy success at d 11 through d 15 and were based on stages described by Degrelle et al. (2005). The earliest-stage conceptus recovered at d 15 was at the spherical stage. This stage of embryo is normally recovered by d 10. Therefore, cows in which an embryo or conceptus was not recovered and the corresponding uterine flush was negative for IFN-τ were specified as undergoing fertilization failure or embryonic loss by d 10. Therefore, pregnancy success at d 10 was the number of cows that had a spherical-stage embryo or greater recovered, divided by the number of E15 cows inseminated. Pregnancy success for d 13 was calculated as the number of cows that had ovoid or greater stage conceptus recovered, divided by the number of E15 cows inseminated (calculations are detailed in Supplemental Table S3; https://doi.org/10.6084/m9.figshare.20001635; Berg et al., 2022).
Animal Records

Data from reproductive and nonreproductive outcomes were obtained from a commercial database using data-linking software (MINDA; LIC). These data included individualized information such as calving date, AI dates, sire used for AI, age, and breed, as well as herd test data sampled at the beginning of the breeding season, including milk volume and composition (LIC, 2019). These data, along with information about embryo recovery, embryo viability, and pregnancy diagnosis, were combined into a single database.

Statistical Analysis

Statistical analyses were performed using Genstat (Genstat for Windows, 18th edition; VSN International). Associations between maternal and non-maternal factors and pregnancy success were analyzed using logistic mixed models with generalized linear mixed models. Nonsignificant ($P > 0.05$) variables were removed from the initial model by a manual backward stepwise elimination procedure. Pregnancy success and fertilization success were separately fitted to logistic mixed models using different data sets. Fertilization success could only be assessed using E7 data. Pregnancy success included E7, E15, and E35 but excluded the E70 group, as d-7 progesterone was not available. The BCS at calving was also excluded, because these data were missing for farm N. Both models included fixed effects: farm, year, cow age (coded as 2, 3, 4, 5, 6, 7, or 8+ years old), breed of embryo and cow (the proportion of HF breed composition out of a 16th; i.e., n/16), BCS at AI, herd test milk volume, percentage of milk fat and protein sampled at the beginning of the breeding season, and d-7 progesterone (log10 transformed) fitted as a linear and a quadratic term. Random effects were AI sire and cow for pregnancy success, and cow was the random effect for fertilization success. The cow effect took account of the 347 cows that participated in both years of the study on farms T1 and W.

Data for embryo length (log10 transformed), and embryonic disc length and width (log10 transformed) were analyzed using regression analysis. Differences between E15 conceptus stages and d-15 circulating progesterone values (log10 transformed) were compared with farm, year, DIM, and embryo stages as fixed effects, and d-7 progesterone as a covariate. Least significant differences at the 5% level determined progesterone differences among the farms, years, and embryo stages.

Percentage data were analyzed using Fisher’s exact test and presented as percentages with 95% confidence intervals (CI).

RESULTS

Percentage of Incorrect Submission to AI

On average, 6.3% [89/1,413 (95% CI: 5.1–7.7%)] of cows in the E7 (n = 42), E15 (n = 21), and E35 (n = 26) groups had plasma progesterone >1 ng/mL (n = 58) on d 0 or <1 ng/mL on d 0 and d 7 (n = 31), indicating luteal activity inconsistent with coincident ovulation timing relative to AI. This assessment of incorrect AI timing ranged from 1.4 to 11% across the farms, and all such cases were excluded from further pregnancy success calculations.

To minimize underestimation of E70 pregnancy successes, we excluded the 15 cows that were identified as having IE from pregnancy success calculations.

Ova and Embryo Recovery Rate in E7 and E15 Groups

The overall ova and embryo recovery rates for E7 and E15 cows were 64.3% [368/572 (95% CI: 60.3–68.3%)] and 63.8% [259/406 (95% CI: 58.9–68.5%)], respectively. These recovery rates were not different between flushing technicians ($P = 0.91$), years ($P = 0.73$), or farms ($P = 0.13$).

Pregnancy Success by d 7

Pregnancy success at d 7 for the E7 group of cows inseminated at the correct time was estimated to be 70.9% [261/368 (95% CI: 66.0–75.5%)] based on the number of embryos that had developed to the correct stage by d 7 (compact morula or blastocyst stage) among the 368 ova and embryos recovered. Fertilization failure and arrested development of fertilized ova were the sources of loss by d 7.

Fertilization

The overall fertilization rate was 84.2% (i.e., 310 of the 368 ova or embryos recovered on d 7; Table 1).

Embryo Quality

The stages and qualities of ova and embryos at d 7 recovery are presented in Table 1. Of fertilized embryos recovered at d 7, 15.8% (49/310) were arrested at the 2-cell to early morula stage (Table 1). The percentage of grade-1 and grade-2 compact morulae and blastocysts from fertilized ova was 69.7% [216/310 (CI:64.2–74.7%)].

A higher percentage of blastocyst-stage embryos were recovered (56.3%, 147/261, $P = 0.01$) than compact
morulae (43.7%, 114/261), although either compact morula or blastocysts are appropriate for d-7 recovery. Of all compact morula and blastocyst-stage embryos recovered, a higher percentage of grade-2 embryos (fair quality) were recovered (46.4%; 121/261), compared with grade 1 (excellent or good; 36.4%, 95/261, \( P = 0.03 \)) and grade 3 (poor-quality; 17.2%, 45/261, \( P < 0.001 \)). A lower proportion of grade-3 compact morula and blastocyst-stage embryos (17.2%) were recovered compared with grades 1 (36.4%, \( P < 0.005 \); Table 1). In contrast, among compact morula recovered, half were grade 2 (48.2%) compared with grade 1 (23.7%, \( P < 0.003 \)).

Among blastocysts recovered, a similar percentage of grades 1 and 2 were observed (46.3% and 44.9%, respectively), both of which were greater than the proportion of grade-3 blastocysts (8.8%, \( P < 0.005 \); Table 1). In contrast, among compact morula recovered, half were grade 2 (48.2%) compared with grade 1 (23.7%, \( P < 0.003 \)).

### Pregnancy Success by d 15

A total of 406 cows were inseminated at the correct time in the E15 group. In all, 147 cows had no conceptuses recovered and ULF tested negative for IFN-τ. These cows were classified as having pregnancy failure before d 15.

Pregnancy success at d 15 was estimated to be 59.1% [240/406 (95% CI: 54.2–63.9%)]. This was based on the number of conceptuses at the tubular, early filamentous, or filamentous stages (n = 240) of all conceptuses recovered (n = 259; Table 2). Overall, 81.5% (211/259) of the conceptuses were recovered intact, and 80.4% (193/240) of viable embryos were recovered intact (Table 2). The mean ± standard deviation (SD) length of the tubular, early, and filamentous conceptuses was 42.7 ± 44.98 mm (back-transformed from log10) and tended to be longer than the recovered fragments (33.07 ± 31.95 mm, \( P = 0.08 \), back-transformed from log10). Plasma concentrations of progesterone tended to be greater at d 7 (\( P = 0.09 \)) and d 15 (\( P = 0.09 \)) when intact conceptuses were recovered, as opposed to when fragmented conceptuses were recovered: 6.3 ± 2.11 versus 5.8 ± 1.78 at d 7, and 12.8 ± 3.95 versus 11.8 ± 3.22 at d 15 (mean ± SD, back-transformed from log10). The interval from calving to insemination (71.2 ± 16.4 and 72.0 ± 15.9 d, mean ± SD) was similar between cows with intact or fragmented conceptuses.

An embryonic disc was observed in 94.8% (200/211) of the recovered intact conceptuses and 97.4% (188/193) of viable conceptuses (Table 2). A total of 94.5% (180/193) of conceptuses exhibited normal embryonic disc morphology. Abnormal embryonic disc morphology was observed in 11 intact conceptuses: 3 at the ovoid

### Table 2. Descriptive statistics of conceptuses recovered from uterine flushes performed 15 d after AI (i.e., E15 group) in pasture-grazed, seasonally managed dairy cows

| Conceptus stage | Number of conceptuses recovered (%), length (mm), mean (95% CI) |
|-----------------|---------------------------------------------------------------|
| Spherical      | 13.0 (3.0–5.8)c                                               |
| Ovoid           | 10.6 (6.3–17.9)a                                              |
| Tubular         | 12.6 (12.0–13.3)                                              |
| Early filamentous| 12.7 (12.0–13.5)                                              |
| Filamentous     | 6.7 (6.0–7.5)                                                 |
| Total           | 259                                                           |

- For progesterone at d 7 and 15, within a column, values not sharing a common letter are significantly different (least square difference; \( P < 0.05 \)).
- Data are presented as back-transformed from log10, geometric mean with 95% CI.
(<5 mm in length), 7 at the tubular, and 1 at the early filamentous stage.

Embryonic disc length increased with increasing conceptus length (log10 transformed; \( R^2 = 0.49, P < 0.001 \), Figure 1). Embryonic disc width (log10 transformed) also increased with increasing embryonic disc length (log10 transformed; \( R^2 = 0.67, P < 0.001 \)) and was correlated with neither d-7 nor d-15 progesterone concentrations.

**Embryo Developmental Potential and Projected Pregnancy Success**

The pattern of developmental potential and projected pregnancy success is presented in Figure 2B. At insemination (d 0), all cows that were inseminated at the correct time were assumed to be pregnant at that time. Pregnancy success at d 2 was defined by fertilized embryos and was 84.2% [95% CI: 80.1–87.8% (\( P = 0.003 \))]. This was greater than pregnancy success at d 5, 74.2% [95% CI: 69.4–78.6% (\( P = 0.003 \))], which included all embryos that developed to at least the noncompacted morula stage. Whereas d 5 and d 7 pregnancy successes were similar (\( P = 0.18 \)), pregnancy success at d 10 decreased to 63.8% [95% CI: 58.9–68.5% (\( P = 0.04 \))], and this was similar to the d-13 to d-15 pregnancy success rate (\( P = 0.31 \)).

Developmental potential of the grade-3 compact morula to d 15 (n = 32) was estimated to be approximately 20%; thus 6 of the 32 embryos would still be viable on d 15. The E7 group’s projected pregnancy success on d 15 was 63.9% [235/368 (95% CI: 58.7–68.8%)] and was similar to the observed d-15 pregnancy success of the E15 group at 59.1% [95% CI: 54.2–62.5%] (\( P = 0.20 \)). At d 35, a 40% developmental potential of the grade-3 blastocysts projected that 5 of 13 would survive to d 35. The E7 group’s projected pregnancy success was 61.7% [228/368 (95% CI: 56.5–66.7%)] and, again, was similar to the observed d-35 pregnancy success rates (62.3%, \( P = 0.60 \)).

Developmental potential of the conceptuses on d 15 was based on the presence or absence of an embryonic disc. Five viable conceptuses were intact at d 15, with
Figure 2. Pregnancy success pattern for pasture-grazed cows following the first insemination. (A) Pregnancy success (percentage and 95% CI) on the day of embryo collection or pregnancy diagnosis. At d 0, the day of insemination, all cows (100%) were considered pregnant, as only cows with d 0 progesterone <1 ng/mL were retained in the data set. E7 = the percentage (95% CI) of viable embryos recovered on d 7 from 368 cows with an ova or embryo. There were 261 morulae and blastocysts (grades 1-3) that were viable upon embryo collection. E15 = the percentage (95% CI) of viable conceptus recovered on d 15. Viable conceptuses (n = 240) ≥5 mm in length were recovered from 406 cows inseminated. E35 = the percentage (95% CI) of cows pregnant on d 28 and 35. A total of 215 cows were confirmed pregnant on d 28, and 210 cows were confirmed pregnant on d 35, from 337 cows inseminated. E70 = the percentage (95% CI) of cows pregnant on d 70. A total of 202 cows were confirmed pregnant on d 70 from 356 cows inseminated. Percentages for embryo collection groups followed by a common letter are not significantly different at the 5% level of significance, using Fisher’s exact test (P < 0.05). *E35 group: pregnancy on d 35 is significantly lower than on d 28 (P < 0.05, McNemar test for paired data, d 28 and 35). Days 28, 35, and 70: pregnancy success was the visual confirmation of a fetal heartbeat using ultrasonography. Blue square = viable embryos at d 7; green circle = viable conceptuses at d 15; gray diamond = successful pregnancy at d 28 and 35; red triangle = successful pregnancy at d 70. (B) Projected pregnancy success (percentage and 95% CI) based on embryo and conceptus stage and morphology recovered from E7 and E15 groups. Filled symbols represent observed pregnancy success as presented in panel A. Open markers are projected pregnancy success based on the recovered E7 and E15 embryo stage and quality. Day 0, the day of insemination, all cows (100%) were considered pregnant, as only cows with d-0 progesterone <1 ng/mL were retained in the data set. Day 2, all animals with a single-cell structure were considered a pregnancy failure, and this value was calculated using the observations from the E7 group. Day 5, pregnancy success was projected using data obtained from the E7 group, with embryos not reaching at least the early morula stage considered not viable. Day 10, pregnancy success was projected using data from the E15 group, with embryos not reaching at least the spherical stage considered not viable. Day 13, pregnancy success was projected from data of the E15 group, with embryos not reaching at least the ovoid stage considered not viable. Day 15, in addition to the observed pregnancy success from the E15 group, observations of the E7 group were used to predict pregnancy success based upon survival of all grade-1 and grade-2 morulae and blastocysts, grade-3 blastocysts, and 20% of grade-3 morulae. Day 35, in addition to the observed pregnancy success from the E35 group, survival was also predicted from the observations of the E7 and E15 groups. From the E7 group, additional loss from d 15 to 35 was projected based upon the estimated 60% embryo mortality of grade 3. From the E15 group, the additional loss to d 35 was projected based on the projected pregnancy failure of conceptuses without an embryonic disc. Dotted lines connect the actual and projected pregnancy success. Note the broken x-axis between d 16 and 35. A–D Percentages for pregnancy success at specified days after insemination not sharing a common letter are different (P < 0.05, Fisher’s exact test). §Within similar days after insemination, no differences were observed between projected and observed pregnancy success (P > 0.19). ‡Within similar days after insemination, no differences were observed between projected and observed pregnancy success (P > 0.60). TM = compact morula; Bl = blastocyst-stage embryo (includes early, mid, and expanded blastocysts).

Maternal and Nonmaternal Factors Associated with Pregnancy Success from Insemination to d 35

The cow and on-farm risk factors and their association with pregnancy success are presented in Table 3. Neither farm and year (P = 0.19) nor the cow’s age (P = 0.63) were associated with pregnancy success. The BCS at insemination was also not associated with pregnancy success (P = 0.49). Further, neither cow breed...
Both the cow and the sire used for AI had a significant effect \( (P < 0.001) \) on pregnancy success. A total of 40 sires (21 HF and 19 HF × Jersey) used over the farms during the 2-yr study. The probability of a successful pregnancy ranged from 0.47 to 0.82, depending upon sire used for AI (Figure 3).

We found a positive linear relationship between pregnancy success and \( \log_{10} \) d-7 progesterone concentrations \( (\text{log}_{10} \text{ transformed}; \ P = 0.002; \text{Table 3}) \). The odds of a successful pregnancy increased by a factor of 4.9 \( (95\% \text{ CI: 1.7 to 13.8}) \) when d-7 progesterone increased by 1-unit \( \log_{10} \) ng/mL \( (\text{d-7 progesterone increased by a factor of 10}) \). For example, when d-7 progesterone increased from 3.5 to 6 ng/mL, the probability of a successful pregnancy of 0.2 would increase to 0.22, 0.5 to 0.53, and 0.8 to 0.82. Progesterone concentration at d 7 was a predictor of the \( \text{d-15} \) progesterone concentrations \( (P < 0.001) \), but \( \text{DIM} \) was not \( (P = 0.76) \). Progesterone concentrations at both d 7 and 15 were similar among the farms and year.

We found a positive linear relationship between pregnancy success and \( \text{DIM} \) \( (P < 0.001; \text{Table 3}) \). The odds of a successful pregnancy outcome increase by a factor of 1.12 \( [95\% \text{ CI: 1.05–1.20}] \) for every additional week postpartum before insemination.

Fertilization rate increased with increasing \( \text{DIM} \) \( (P = 0.02) \). The odds of successful fertilization increased by a factor of 1.17 \( [95\% \text{ CI: 1.02–1.33}] \) for every additional week postpartum before insemination. Neither farm and year \( (P = 0.80) \) nor the cow’s age \( (P = 0.24) \) were associated with fertilization. The BCS at insemination was also not associated with fertilization \( (P = 0.91) \). Further, neither cow breed (Holstein-Friesian, Holstein-Friesian × Jersey, or Jersey; \( P = 0.98 \)) nor sire (expressed as a proportion of HF, \( P = 0.74 \)) had any association with fertilization success. Milk yield and composition were not found to be associated with fertilization success \( (P > 0.37) \).

### Table 3. Slope and probability estimates from the final logistic regression model of cow and on-farm risk factor variables on pregnancy success to first AI in pasture-grazed, seasonally managed dairy cows

| Variable                          | Slope   | SE     | Odds ratio (95% CI) | \( P \)-value |
|-----------------------------------|---------|--------|---------------------|---------------|
| DIM                               | 0.017   | 0.005  | 1.02 (1.01,1.03)    | <0.001        |
| d-7 progesterone (log\text{_{10}} \text{ ng/mL}) | 1.58    | 0.52   | 4.85 (1.71,13.77)   | 0.002         |
| Milk yield\(^{2}\) (kg)           | −0.036  | 0.023  | 0.97 (0.92,1.01)    | 0.13          |
| Milk fat (%)                      | 0.16    | 0.14   | 1.18 (0.89,1.55)    | 0.24          |
| Milk protein (%)                  | −0.026  | 0.34   | 0.97 (0.49,1.93)    | 0.94          |
| BCS at insemination               | 0.14    | 0.19   | 1.15 (0.78,1.69)    | 0.49          |
| Farm and year\(^{3}\)             | 0.03    | 0.30   | 0.61 (0.29,1.29)    | 0.19          |
| N 2014                            |         |        | 0.47 (0.18,1.26)    |               |
| T2 2014                           |         |        | 0.88 (0.38,2.02)    |               |
| T1 2014                           |         |        | 0.59 (0.28,1.24)    |               |
| T1 2015                           |         |        | 1.12 (0.44,2.87)    |               |
| W 2015                            |         |        | 0.61 (0.29,1.29)    |               |
| Cow age\(^{4}\) (yr)              |         | 0.25   |                    | 0.63          |
| 3                                 |         | 1.54   | (0.83,2.87)        |               |
| 4                                 |         | 1.66   | (0.87,3.20)        |               |
| 5                                 |         | 1.71   | (0.87,3.37)        |               |
| 6                                 |         | 1.70   | (0.84,3.42)        |               |
| 7                                 |         | 2.09   | (0.97,4.48)        |               |
| +8                                |         | 1.44   | (0.73,2.84)        |               |
| Cow proportion HF\(^{5}\)         | −0.57   | 0.75   | 0.57 (0.13,2.56)    | 0.45          |
| Embryo or fetus proportion HF\(^{5}\) | 0.98   | 1.21   | 2.66 (0.24,30.0)   | 0.42          |

\(^{1}\)Back-transformed proportions pregnant were as follows: E7 0.75, E15 0.58, E35 0.66. The E70 group is omitted from the model because d-7 blood sampling was not done for this group. Treatment groups: morphological examination of embryos collected on d 7 (E7) and 15 (E15); pregnancy diagnosed by ultrasonography on d 28 and 35 (E35) and d 70 (E70).

\(^{2}\)Kilograms of milk harvested during a single-day herd test.

\(^{3}\)Farm and year are compared with year 2014 for farm W. SE is the average SE, on the logit scale, of all farm years.

\(^{4}\)Cow age is compared with 2-yr-old cows. SE is the average SE, on the logit scale, of all ages.

\(^{5}\)Proportion HF is the proportion of Holstein-Friesian breed composition out of a 16th (i.e., n/16).
Our study presents pregnancy success of lactating cows submitted for AI in the first 21 d of the breeding season. The study began at insemination and determined pregnancy success at d 7, 15, 28, 35, and 70 over 4 commercial dairy herds with data from 1,467 lactating cows. Our overall d-70 pregnancy success of 54.4% (including IE cows) was similar to the mean pregnancy success of 54% calculated from over 4,000 herds that have done fetal-aged pregnancy testing in recent years (DairyNZ and Livestock Improvement Corporation, 2020). This provides confidence that our study herds are representative of industry reproduction performance in seasonal-calving pasture-grazed dairy cows.

The key finding was that 29% of cows experienced pregnancy failure by d 7, and the majority of this loss occurred between insemination and d 5. Inseminating cows at increasing DIM improved fertilization rates and was associated with increased pregnancy success to d 35. Progesterone concentrations at d 7 and insemination sire were associated with pregnancy success to d 35. The percentage of excellent and good-quality morula and blastocyst-stage embryos recovered at d 7 were similar to the pregnancy rate at d 35, indicating that gastrulation, maternal recognition of pregnancy, and implantation are not major developmental events associated with embryonic loss in seasonal-calving pasture-based dairy cows. This may be an important difference between the dairying systems of seasonal-calving pasture-grazed dairy cows and the intensive indoor, year-round calving, high-production systems.

**Pregnancy Success Profile from Insemination to d 70**

A pregnancy success pattern was established using a temporal series of embryo and conceptus recoveries and pregnancy diagnosis from d 7 through d 70 of gestation, spanning the embryonic (fertilization to d 42) and early fetal development periods. Embryo survival data previously reported for grazing dairy cattle, which excluded fertilization failure, reported a 28% embryo loss to d 28, with the majority of this loss, 22%, occurring between d 8 and 16 (Diskin et al., 2006). In our study, 24% of embryo loss occurred by d 28, which was similar in magnitude to what Diskin observed; however, the embryo loss pattern differed: 15% embryo mortality occurred by d 7, with an additional of 8% loss from d 8 to 28. Approximately half of this loss, 10%, had occurred by d

![Figure 3. Effect of insemination sire on the probability of pregnancy success to d 35. A total of 40 sires inseminated 1,111 cows over the 2 yr, 4 farms, and all collection groups. Sires are plotted by decreasing probability (± SEM) of pregnancy success and ranged from 0.82 to 0.47 probability. Insemination sire had an effect on the probability of pregnancy success ($P < 0.001$) when fitted as a random factor to a logistic mixed model (generalized linear mixed models, Genstat for Windows, 18th edition; VSN International). Fixed effects included farm, year, cow age, DIM, d-7 progesterone, breed of cow, breed of embryo or fetus, BCS at calving and insemination, milk yield (kg), and milk fat and protein (%). Blue circles represent a Holstein-Friesian bull (n = 21). Holstein-Friesian sires were categorized as having >0.75 proportion of Holstein-Friesian genetics (ranged from 0.87 to 1.0). Red circles represent a crossbred sire (n = 19). Crossbred sires’ proportion of Holstein-Friesian genetics ranged from 0.31 to 0.75, with Jersey breed as the remainder. The number of cows inseminated per sire is reflected in the value of the SEM. The SEM ranging from 0.02 to 0.05 represents 71 to 163 cows inseminated per sire; 0.06 to 0.10 SEM range represents 17 to 63 cows inseminated per sire; SEM 0.11 to 0.21 represents 5 to 21 cows inseminated per sire.]

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**Figure 3.** Effect of insemination sire on the probability of pregnancy success to d 35. A total of 40 sires inseminated 1,111 cows over the 2 yr, 4 farms, and all collection groups. Sires are plotted by decreasing probability (± SEM) of pregnancy success and ranged from 0.82 to 0.47 probability. Insemination sire had an effect on the probability of pregnancy success ($P < 0.001$) when fitted as a random factor to a logistic mixed model (generalized linear mixed models, Genstat for Windows, 18th edition; VSN International). Fixed effects included farm, year, cow age, DIM, d-7 progesterone, breed of cow, breed of embryo or fetus, BCS at calving and insemination, milk yield (kg), and milk fat and protein (%). Blue circles represent a Holstein-Friesian bull (n = 21). Holstein-Friesian sires were categorized as having >0.75 proportion of Holstein-Friesian genetics (ranged from 0.87 to 1.0). Red circles represent a crossbred sire (n = 19). Crossbred sires’ proportion of Holstein-Friesian genetics ranged from 0.31 to 0.75, with Jersey breed as the remainder. The number of cows inseminated per sire is reflected in the value of the SEM. The SEM ranging from 0.02 to 0.05 represents 71 to 163 cows inseminated per sire; 0.06 to 0.10 SEM range represents 17 to 63 cows inseminated per sire; SEM 0.11 to 0.21 represents 5 to 21 cows inseminated per sire.
5. A similar embryo mortality pattern to d 7 has been reported for NAHF (Cerri et al., 2009a, b, c; Wilthbank et al., 2016), although the magnitude of loss is greater for NAHF. A meta-analysis of pregnancy failure in beef animals reported an overall failure (including fertilization failure) of 47.9% to d 32. Failures to d 7 were 28%, and 23% of the losses occurred during fertilization and initial embryo cleavage stages (Reese et al., 2020). In our study, pregnancy failure to d 7 was similar to that reported for beef cows, but our pregnancy success to d 35 was greater. In general, the observed percentages of pregnancy failures and embryo mortality differ between cows managed under different production systems, yet a commonality is that fertilization and initial embryo cleavage stages account for a major portion of pregnancy failures.

The pregnancy success at d 15 was not different than at d 28, 35 and 70, and this sample collection may have overestimated the pregnancy failure between d 7 and 15, as the observed pregnancy success was lower than at d 35. We reported a pregnancy failure of 8.6% from d 7 to d 35, compared with the 11.8% failure between d 7 and 15, but values were not statistically different. A perceived pregnancy success gain has been previously reported using a similar multiple-sample collection design for comparisons between gestation days (Ryan et al., 1993). We analyzed all uterine flush for IFN-α from cows from which an embryo was not recovered. However, our method only picked up ovoid-stage embryos and greater. It is conceivable that the E15 group of cows, by random chance, included an over-representation of cows with greater fertilization failure or embryo mortality to d 10, compared with the other 3 groups of cows. Evaluating the recovered conceptus stage embryos at d 15, we observed that 7% of the recovered conceptus were not viable, and this figure is similar to the 3% reported in beef cattle (Reese et al., 2020), but lower than the 22% reported for NAHF (Ribeiro et al., 2016b).

The d-28, d-35, and d-70 pregnancy successes were measured using ultrasound, which is a more robust measurement of pregnancy success than d-15 conceptus evaluation. Our d-70 pregnancy success was similar to the overall industry average, as previously noted. A 7% late embryonic and fetal loss to calving is reported in seasonal-calving pasture-grazed cows (Diskin et al., 2006), with a 4% loss reported for New Zealand cows from d 42 to 70 (McDougall et al., 2005), a similar figure to our reported losses from d 35 to 70, again suggesting that our d-15 collection point may overestimate pregnancy failure. Our study set criteria for d-15 conceptus embryos was based on the expected lengths and stages for conceptuses recovered at d 15 (Betteridge and Fléchon, 1988; Degrelle et al., 2005; Richard et al., 2015) as opposed to treating all recovered conceptus or embryos as viable. Pregnancy success would have increased to 62.8% (255/406) if we had included ovoid-stage embryos, although we are confident that these embryos would not be viable at d 15. To experimentally confirm this, retransfer of d-15 ovoid-stage conceptus with pregnancy diagnosis at d 35 would be required (Kimura and Matsuyama, 2014; Richard et al., 2015).

**Embryo Developmental Potential and Projected Pregnancy Success**

Assessing the developmental potential of the recovered embryos provided us with a means to predict when pregnancies might fail rather than assigning failure to the day of recovery or pregnancy diagnosis or, in cases of serial recoveries, the gestation interval between the recoveries or diagnosis. This is an important distinction when identifying developmental events associated with reproductive outcomes. This is not a new concept, as predicting future pregnancy outcomes at d 7 has been previously reported using a candidate-gene approach and associating those with pregnancy outcomes (El-Sayed et al., 2006; Zolini et al., 2020; Salilew-Wondim et al., 2021). The difference is that we used a morphologically based evaluation system of the obvious poor-quality embryos rather than predicting pregnancy outcome of excellent and good blastocyst-stage embryos. Our data suggested that embryo developmental potential is determined by d 7, as the projected pregnancy success was similar to the observed pregnancy success at the comparative collection time points. This potential was solely based on the embryo, independent of the assessment of the maternal tract. The newly fertilized zygote possesses all the information for development to the blastocyst stage, and development to the blastocyst stage can occur in vitro. However, the embryo and the maternal tract are intimately involved (Sánchez et al., 2018; Sponchiado et al., 2019; Talukder et al., 2020), and embryo development beyond d 8 is dependent on the uterine environment.

**Fertilization Success**

Our fertilization success rate of 84.2% was similar to the average fertilization rate of 83% for the NAHF lactating cows inseminated under thermoneutral conditions (Sartori et al., 2010). This fertilization rate is lower than previously reported in lactating dairy cows (i.e., 90%; Diskin et al., 2006), as well as in nonlactating beef cows, beef breeds, and dairy heifers (Sartori et al., 2002). Our study was not designed to optimize fertilization rates but to represent actual industry conditions, where fertilization failure accounts for half...
of the losses during the first week. Using many commercial sires may have inflated our fertilization failure rate, although the sires used were represented across all farms and collection groups and were included as a random effect in the model. Fertility variation exists among commercial sires, and phenotypic fertility is available as sire conception rate and semen fertility value traits (Kuhn and Hutchison, 2008; Rezende et al., 2020). In our study, we could not determine any sire effect on fertilization rates or early embryo survival (Ortega et al., 2018; O’Callaghan et al., 2021), due to low number of cows inseminated per sire (i.e., the median sire usage was 7 cows inseminated per sire for the E7 group data set). Given that fertilization failure was a significant cause of pregnancy failure, understanding sire effects on fertilization is an area that could potentially improve pregnancy successes following the first insemination in pasture-grazed dairy cows.

Recovered single cells are usually defined as fertilization failure, the failure of sperm to reach or penetrate the oocyte. Failure can also occur after the sperm has penetrated the oocyte, resulting in a paternal and maternal set of chromosomes (Yeste et al., 2016). We chose parental microsatellite genotyping to distinguish fertilized (the paternal DNA) and non-fertilized (maternal DNA). Quality of DNA was a technical issue we encountered when single cells were recovered 7 d after insemination. One-third of the single cells were able to be genotyped, and, assuming the remaining single cells followed a similar pattern, 10% of the single cells were penetrated by sperm. The additional 6 ova classified as fertilized would not have significantly changed the fertilization rate reported here. These preliminary data indicate that sperm failed to reach the site of fertilization or failed to penetrate the cumulus oocyte complex.

It is well accepted that the calving interval to insemination has an effect on fertility, but our results demonstrated that this relationship might be explained by an effect on fertilization success. After calving, a rapid inflammatory response clears the bacterial population from the uterus. Polymorphonuclear neutrophils (PMN) infiltrate the uterus for a short time, followed by a return to a non-inflamed state to prepare for the embryo (Gilbert and Santos, 2016; Chastant and Saint-Dizier, 2019). A reduction in fertilization was observed in cows that experienced uterine disease before insemination, with an inverse relationship between fertilization rate and increasing PMN (Carvalho et al., 2013; Ribeiro et al., 2016a). Inflammation of the oviducts (salpingitis) also interferes with sperm storage and transport, which decreases fertilization rates (Owhor et al., 2019). We omitted any cows that had uterine infection when presented for flushing, any cows whose uterine flush was turbid at collection (PMN in the flushing), and cows inseminated before 30 DIM. Perhaps cows expressing estrus on a shorter interval from calving to insemination may experience a milder form of these disease inflammatory mechanisms, negatively affecting fertilization. This finding is especially important for seasonal pasture-based production systems, where a 365-d calving interval must be maintained. Regardless of calving-to-insemination interval, cows observed in estrus during the breeding period are inseminated. In our data set, 164 cows were inseminated that expressed estrus between d 30 and 42 d DIM, representing approximately 10% of the cows. Delaying insemination (i.e., applying a voluntary waiting period policy) would save insemination costs and increase the probability of pregnancy success to first AI, but also would reduce the overall reproductive performance of the herd when constrained to a limited breeding period.

**Pregnancy Success After Fertilization**

Beyond fertilization failure, the remaining losses (i.e., 15 percentage points) during the first week were due to embryonic mortality. These losses are higher than the 6% embryonic mortality reported 35 years ago (Sreenan and Diskin, 1986), lower than the 50% losses for NAHF (Wiltbank et al., 2016), and similar to losses among beef animals (Reese et al., 2020). Our study used a randomized sample collection design, with 4 different collection times, using similar types of cows under similar management systems, and 4 farms, rather than a compilation of several studies. Cow numbers in our study were sufficient for 5% confidence errors, a larger single data set than has been previously reported in seasonal-calving pasture-grazed dairy cows. Embryonic mortality from earlier years may have underestimated d-7 losses, as the data were a mixture of dairy, beef, and heifer studies.

The first 2 cell cycles in embryonic development are regulated by mRNA stored during oocyte growth, and a gradual transition of control occurs beginning with the 4-cell embryo (with active transcription; Memili and First, 1999). The major embryonic genome activation (EGA), however, occurs at the 8- to 16-cell stage, and continues to the blastocyst stage (Graf et al., 2014). Combining the arrested embryos in our study into minor (4-cell) and major (8- and 16-cell) EGA categories revealed that 46.9% (23/49) of the arrested embryos failed during the embryo stages associated with the major EGA developmental event. This is the most critical development period, as the basic requirements of the cells need to be maintained while degradation and activation of new genetic material occur simultaneously (Pfeffer, 2018).
Embryo arrest after the major EGA stages was low, with only 4% of embryos failing to compact. This agrees with previous embryo survival data in pasture-grazed dairy cows (Sreenan and Diskin, 1986) and is additionally supported when comparing strains of HF. New Zealand HF produced more blastocyst- and morulae-stage embryos compared with NAHF-strain cows (de Feu et al., 2008), indicating that compaction and cavitation is a minor developmental hurdle for pasture-grazed dairy cows.

**Pregnancy Success by d 15**

Previous work has identified the period of embryo elongation and pregnancy recognition, d 8 to 16, as a key period of embryonic mortality in seasonal-grazed dairy cows, accounting for 70 to 80% of losses (excluding fertilization failure; Sreenan and Diskin, 1986; Diskin et al., 2006). The TE secretes IFN-τ required for maternal recognition of pregnancy, and is detected in uterine flushings by approximately d 13. The amount of IFN-τ secretion is related to the conceptus size. Therefore, a shorter conceptus would produce inadequate concentrations of IFN-τ to prevent CL lysis, progesterone would not be maintained, and the pregnancy would fail (Shorten et al., 2010). We observed 2 different progesterone patterns associated with pre-elongation-stage embryos: spherical-stage embryos were recovered from cows with low d-7 progesterone, and ovoid-stage conceptuses were recovered from cows with d-7 and d-15 progesterone concentrations that were similar to the elongation stages. It is well established that early progesterone concentrations indirectly influence conceptus length by exerting effects on the uterine endometrium, and artificially lowered progesterone concentrations are associated with shorter conceptuses (reviewed by Loneragan and Sánchez, 2020). Others have demonstrated that d-7 progesterone concentrations are also associated with embryonic length (Ledgard et al., 2012; Randi et al., 2016; Shorten et al., 2018). Therefore, it was not unexpected that the spherical-stage embryos were recovered from cows with significantly lower d-7 progesterone concentrations.

Decreased progesterone concentrations would be expected to delay conceptus elongation, potentially resulting in recovery of ovoid embryos on d 15; however, progesterone concentrations on d 7 and 15 in these cows with ovoid conceptuses were similar to those with elongated conceptuses. Thus, the ovoid-stage embryos observed in our study likely failed the transition to the elongation stage due to factors independent of progesterone concentrations. These results differ from the those of Ribeiro et al. (2016b), who reported lower d-15 progesterone concentrations for ovoid-stage conceptuses recovered from NAHF lactating cows at d 15. The higher clearance rate of circulating progesterone associated with the increased feed intake required for milk production in NAHF (Sangsritavong et al., 2002) may explain the increased rate of ovoid embryos (22%) recovered at d 15 and their association with progesterone compared with the 5.8% ovoid embryo recovery observed in this study.

**Pregnancy Success by d 28, 35, and 70**

The d-15 pregnancy success was similar to the d-35 and d-70 pregnancy success, indicating negligible pregnancy failures after d 15, which agrees with the minor pregnancy failures found after d 16 for pasture-grazed dairy cows (Diskin et al., 2006), but differs from the 15.6% observed in beef animals (Reese et al., 2020) and 31.5% observed in NAHF (Wiltbank et al., 2016). Maternal recognition of pregnancy, which occurs around d 16 to 17, was not a developmental milestone that contributed to pregnancy failure in this study, nor was implantation. Embryo survival at d 35, when corrected for fertilization failure, was 74.3% and was similar to d-7 and d-15 embryo survival. This agrees with previously reported data for pasture-grazed dairy cows (Diskin et al., 2006) and beef heifers (Diskin and Sreenan, 1980; Roche et al., 1981), and contrasts with data from NAHF reviewed by Wiltbank et al. (2016) and recent data on beef heifers (Geary et al., 2016). Further losses associated with the early fetal period to d 70 were similar to those previously reported (McDougall et al., 2005; Diskin et al., 2006), and the pregnancy rate to first AI at d 70 is similar to the industry-reported conception rate (DairyNZ and Livestock Improvement Corporation, 2020).

**CONCLUSIONS**

Pregnancy success in seasonal pasture-grazed dairy cows is determined within the first week of gestation, rather than the second and third weeks. Increasing the calving-to-insemination interval was associated with fertilization success. Our results imply that failures with maternal recognition of pregnancy, gastrulation events, and implantation are not major impediments for pregnancy success in dairy cows that have evolved and are managed within a seasonal, pasture-based system. Rather, future work in this type of dairy cow should focus on the critical developmental events of oocyte development and maturation, fertilization, embryonic genome activation, elongation, and factors including the maternal reproductive tract, affecting the quality of the morula and blastocyst.
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