

Development of a novel timed rebreeding program to optimize bovine reproductive performance

By

João Paulo Nascimento Andrade

A dissertation submitted in partial fulfillment of

The requirements for the degree of

Doctor of Philosophy

(Animal & Dairy Sciences)

at the

UNIVERSITY OF WISCONSIN-MADISON

2024

Date of oral examination: 9/25/2024

The dissertation is approved by the following members of the Final Oral Committee:

Milo C Wiltbank, Professor, Animal and Dairy Sciences

Laura L Hernandez, Professor, Animal and Dairy Sciences

Francisco Peñagaricano, Professor, Animal and Dairy Sciences

Chad Vezina, Professor, Pathobiological Sciences

Dedication

This dissertation is a testament to the unwavering support and inspiration I have received from my lovely Fabiana, my children, and my parents. I also dedicate this work to hardworking farmers worldwide, especially my old man Zenildão, whose passion for nature and commitment to feeding the world are truly inspiring.

Acknowledgments

To my lovely wife, Fabiana, for all the support and encouragement we needed to overcome the barriers we have had since leaving Brazil to work at UW. To my kids (Mateus, Maria, Teresa, the new baby, and the others to come) for enlightening our lives with the amazingness, craziness, and beauty of parenthood.

To my father, Zenildo (in Memoriam), thank you for inspiring me throughout my whole life as a man, father, brother, and colleague with all he taught me, primarily through his examples and morals. To my mother, Cidinha, for all her hard work to afford my studies and for believing in me when few did. I love you, mom! To my brother, Joao Luiz, for all his support, all the memories we have from our childhood, and his genuine way of loving others. To my in-laws, thanks for the love and support you all gave to my family.

To my mentor and friend, Dr. Milo Wiltbank, whom I was blessed to work with. For all the hours he dedicated to my ideas and all the support he gave me to pursue my goals of improving bovine performance as a scientist. Most of all, for his unique style of training students, firing up our passion for science, and challenging us to our limits to be a better version of ourselves. To my committee members, Dr. Laura Hernandez, Dr. Francisco Peñagaricano, and Dr. Chad Vezina, thanks for being a part of my committee that challenged my concepts in reproduction to a broader level, from bovine to women reproductive assisted biotechniques (BabyInSynch!). For the commercial herds and companies that allowed us to perform all of our trials (Dairy Forage Research Center, STGenetics, Crave Brothers Farm, Fazenda Roncador, Fazenda Sussego, GobalGen Vet Sciences, Parnell Vet Pharmaceuticals, and P4Gold). For the support from Dr. Sartori's Lab in the experiments in Brazil. To my lab mates and friends in the

Animal and Dairy Sciences Department, especially the 8th-floor family and those who helped in my beginning of grad school (Victor, Pedro, and Raf). A special thanks to Laura and Faye for keeping us in line.

To my previous mentors and friends in Brazil who trusted and inspired me to go beyond what I could imagine. Dr. Marco Mello was the first to make all the effort to start my career as a scientist, and I am grateful for his exemplification of a Teacher and Mentor whose lessons I look forward to instilling in my students. To Dr. Bruno, for the friendship and all his support in experiments in Brazil. To all the farmers I worked with while practicing, thanks for entrusting me with their own business and giving me the opportunity to be part of your lives. To the Vets students I trained while practicing, whom I gladly see succeeding in their careers as Vets.

Lastly and most importantly, I praise and dedicate this dissertation to God, whose creations I have come to know and appreciate through science, which is one of the ways He called me to have a close relationship with Him. *“And you shall know the truth, and the truth shall set you free.” Jonh 8:32*

Abstract

This thesis pursued the development/optimization of ReBreed21 for Artificial Insemination (AI) and Embryo Transfer (ET) every 21 d in cattle. We proposed a model for developing a high-performance bovine reproductive program by assembling: (1) No decrease in fertility to first service, (2) Accurate pregnancy diagnosis, and (3) Adequate fertility to rebreeding. This model was tested in a series of experiments using cows from different types of operations and productive purposes (beef, dairy, and ET recipients) under different physiological disruptions from the optimal reproductive hormonal milieu. In a physiology study, we demonstrated in non-bred beef heifers how the intervention of ReBreed21 matches key steps for potential early timed rebreeding 21 d after a previous Timed AI (TAI). In a second large study (n = 2,085), the best ReBreed21 approach was evaluated against an efficiency-established reproductive program for beef cattle in commercial cow/calf operations and our results supported the hypothesis that ReBreed21 would improve reproductive performance. For primiparous, the modification that included estradiol cypionate treatment in the ReBreed21 program was essential to achieve adequate P/AI but was not needed in nulliparous heifers. There was a decrease in P/AI for nonpregnant multiparous cows bred with the ReBreed21 programs. Pregnancies at 21 d of the breeding season were increased in parities when using ReBreed21: 45 % more pregnancies for heifers, 26 % increase in primiparous, and 15 % increase in multiparous cows. In a third experiment, Holstein ET recipients were used to test the modified program, termed ReBreed21-ET, that allowed timed ET every 21 d vs. a standard, highly-efficient Resynch28 for ETs every 36 d. The ReBreed21-ET increased pregnancies by about 10 percentage points at the end of the 105-d timed ET (TET) season. The improvement was driven by a combination of increased P/ET in the first TET, probably due to the use of the progesterone (P4) vaginal implant from Day 14 to

19 of pregnancy, combined with an earlier selection of nonpregnant recipients, and adequate P/ET in the second and later TET. Thus, all three points of our rebreed model were met by the ReBreed21-ET program producing a high-performance reproductive program for ET. In another analysis, we reported that the beneficial effects of P4 supplementation during the embryo elongation stage (Days 14 to 19) primarily benefited heifers with suboptimal placentation. In a series of three experiments using high-producing lactating dairy cows, we developed a method of synchronization of luteolysis using oxytocin treatments. Using a minimum of two oxytocin treatments, it was possible to synchronize luteolysis by Day 20 in about 80% of nonpregnant cows after TAI compared with only 50% of nonpregnant cows undergoing luteolysis when oxytocin was not used. In addition, we evaluated on-farm tools to select nonpregnant cows with luteolysis. Commercial on-farm rapid milk tests for P4 were validated to be more efficient in detecting lactating dairy cows having luteolysis 20 d after induced ovulation compared with corpus luteum blood perfusion evaluated by Doppler ultrasound. Nevertheless, the third study comparing ReBreed21-Dairy with the state-of-the-art reproductive program for lactating dairy cows (Resynch25) showed a poorer overall reproductive performance for ReBreed21. The oxytocin treatments decreased fertility to the first TAI using the Double Ovsynch program for multiparous cows, and there was also reduced P/AI for cows that were bred at 21 d, even when additional GnRH treatments were used to improve follicular synchrony. Thus, improvements are still needed in ReBreed21-Dairy due to decreased fertility at the first TAI and poor P/AI at the second and later TAI, although rebreedings were done at an earlier time. In summary, the work in this thesis expands the frontiers in bovine timed rebreeding programs. The ReBreed21 concept significantly advances bovine reproductive performance while bringing new insights into the challenges of developing aggressive timed rebreeding programs in lactating dairy cows.

Contents

Dedication.....	i
Acknowledgments	ii
Abstract	iv
Contents.....	vi
List of Tables.....	ix
List of Figures.....	xii
Chapter 1: Literature review – State-of-the-art in bovine reproductive management: challenges and opportunities to optimize performance in beef and dairy cows	1
Abstract.....	2
Introduction.....	4
Beef cattle reproductive programs	7
The optimal time for pregnancy: “translated” into kg of calf	7
Describing the reproductive programs for beef cattle:.....	9
Beef section conclusion and take-away	14
Dairy cattle reproductive programs	15
Measuring reproductive performance in dairy cows.....	15
The optimal time for pregnancy: “translated” into milk yield	16
Describing the reproductive programs for dairy cows:.....	18
Dairy section conclusion and take-away	21
Conclusion	22
Appendix - Data processing and analysis	23
Beef section	23
Dairy section.....	24
References.....	29
Chapter 2: Optimizing ReBreed21 I: Evaluation of endocrine and ovarian dynamics in non-bred <i>Bos indicus</i> heifers.....	48
Abstract.....	49
Introduction.....	51
Materials and Methods	53
Results.....	55
Discussion	57

Conclusions	61
References	71
Chapter 3: Optimizing ReBreed21 II: Fertility and reproductive efficiency in different parities during a shortened breeding season in beef cattle.....	77
Abstract	78
Introduction	80
Materials and Methods	82
Results.....	86
Discussion	89
Conclusions	95
References	105
Chapter 4: Identification of nonpregnant beef cows based on CL size vs. luteal blood perfusion at 21 days after timed artificial insemination	112
Abstract	113
Introduction	115
Materials and Methods	117
Results.....	120
Discussion	121
Conclusion	126
References	131
Chapter 5: ReBreed21-Dairy: Use of oxytocin to synchronize luteolysis in development of a novel reproductive program for dairy cows with fixed-time rebreeding every 21 days.....	138
Abstract	139
Introduction	141
Materials and Methods	143
Experiment 1. Synchronization of luteolysis using oxytocin	143
Experiment 2. Dose effect of oxytocin in previously bred cows.....	145
Experiment 3. Reproductive performance of timed rebreeding programs.....	147
Results.....	150
Experiment 1. Synchronization of luteolysis using oxytocin	150
Experiment 2. Dose effect of oxytocin in previously bred cows.....	150
Experiment 3. Reproductive performance of timed rebreeding programs.....	151
Discussion	153

Conclusions	159
References	172
Chapter 6: ReBreed21-ET: Evaluation of a rapid resynchronization program that allows timed embryo transfer every 21 days	182
Abstract	183
Introduction	185
Materials and Methods	187
Results	191
Discussion	193
Conclusion	198
References	208
Chapter 7: Delayed embryonic attachment and profile of pregnancy-associated glycoproteins are associated with pregnancy loss at different stages of gestation in Holstein embryo recipients	213
Abstract	214
Introduction	216
Materials and Methods	219
Results	222
Discussion	224
Conclusion	228
References	239
Chapter 8: General Conclusions and Future Directions	247
Challenges and opportunities in developing ReBreed21: where does this concept come from?	247
Challenges and opportunities in developing ReBreed21: why target an intense reproductive program with a shorter breeding period?	248
Challenges and opportunities in developing ReBreed21: a program built at the intersection of basic biology and applied biotechnology	249
Future directions	253
Conclusion	255

List of Tables

Chapter 2

Table 1. Percentage distribution % (n) of timing of each physiologic event in non-inseminated Nelore heifers submitted to different resynchronization strategies. d0 represents the time of final GnRH in previous protocol, with no heifers receiving TAI.....62

Chapter 3

Table 1. Results for pregnancy/AI at each timed artificial insemination for all cows and for each parity during a 42-day breeding season using ReBreed21 or a traditional reinsemination program (Resynch33).....97

Table 2. Results from all early pregnancy diagnoses with use or not of estradiol cypionate (EC) in ReBreed21 (pregnancy diagnosis based on corpus luteum blood flow on D21 after artificial insemination).....98

Chapter 4

Table 1. Incidence of different true (correct) and false (incorrect) diagnoses for an early pregnancy diagnosis using CL morphology based on B-mode ultrasound or CL blood perfusion based on Doppler ultrasound.....127

Chapter 5

Table 1. PGFM responsiveness and luteolysis synchronization dynamics for lactating dairy cows treated or not with oxytocin in Experiment 1.....160

Table 2. Pregnancy outcomes and luteolysis synchronization for lactating dairy cows treated with different frequencies of oxytocin in Experiment 2.....161

Table 3. Pregnancy outcomes of the first and rebreeding TAI for lactating dairy cows managed in timed rebreeding programs for AI every 21 or 35 days.....162

Table 4. Synchronization dynamics and fertility outcomes of nonpregnant cows with low P4 based on P4Gold that received TAI on day 21 since the last TAI.....163

Table 5. Time to pregnancy and reproductive performance for lactating dairy cows managed in timed rebreeding programs for AI every 21 or 35 days.....164

Chapter 6

Table 1. Pregnancy loss of Holsteins embryo recipients resynchronized with two different resynchronization programs.....199

Table 2. Dynamics of ReBreed21 synchronization from D19 to D21 after the previous TET in nonpregnant embryo recipients on D28.....200

Chapter 7

Table 1. Circulating PAG in Holstein embryo recipient heifers with pregnancy losses in different periods during the first two months of gestation.....230

Table 2. Pregnancy outcomes in Holstein embryo recipient heifers according to circulating PAG on day 21.....231

Table 3. Pregnancy outcomes in Holstein embryo recipient heifers according to circulating progesterone on day 21.....232

List of Figures

Chapter 1

Figure 1. Panel A Body weight of calves at calving and the adjusted weaning weight at 240 d according to the season of the year at calving. Panel B Hypothetical performance of body weight of calves born in the first three 21-d periods of the calving season and weaned at 180 d after the onset of calving season. Panel C Predicted change in calf body weight weaned at 180 d after the onset of calving season relative to a calf born on the first day. Panel D Performance of different reproductive programs in beef cattle (primiparous and multiparous) from published manuscripts evaluating the long-term AI strategies. (Bull) natural mating breeding season; (TAI) timed artificial insemination for the first AI and clean-up bulls; (R30) TAI for first AI with resynchronization onset 30 d days after TAI; (R22) TAI for first AI with resynchronization onset 22 d days after TAI; (R14) TAI for first AI with resynchronization onset 14 d days after TAI; and (RB21) TAI every 21 d. Included manuscripts: (Baruselli et al., 2017, Pessoa et al., 2018, Sa Filho et al., 2013, Gutierrez et al., 2014, Marques et al., 2015, Junior et al., 2021, Pugliesi et al., 2019, Palhao et al., 2020, da Silva et al., 2022, Faleiro et al., 2019, de Oliveira et al., 2018, Rodrigues et al., 2018, Andrade et al., 2024, Sa filho et al., 2014).....25

Figure 2. Panel A Partial budget analysis for the optimal time to pregnancy based on income over feed cost (IOFC) with three different levels of lactation curves. Panel B Lactation curve profiles for commercial herds in WI with herd average daily production lbs/cow/day of < 75 lbs (Botton), ≥ 75 to < 95.0 lbs (Middle), and ≥ 95 lbs (Top). Panel C Cumulative pregnancies after the voluntary waiting period prioritizing AI after detection of estrus or Timed-AI from published manuscripts evaluating the long-term AI strategies (primiparous and multiparous). Included

manuscripts: (Pursley et al., 1997, Galvao et al., 2007, Fricke et al., 2014, Burnett et al., 2017, Machado et al., 2017, Denis-Robichaud et al., 2018, Stangaferro et al., 2018, Rial et al., 2022, Gonzalez et al., 2023, Sitko et al., 2023, Laplacette et al., 2024). Panel D Percentage of nonpregnant cows inseminated after detection of estrus before the nonpregnant diagnosis. NDP = day of nonpregnant diagnosis.....26

Figure 3. Model of optimal hormonal milieu to achieve high pregnancy per service in beef or dairy cows. High-producing dairy cow. Hormonal disruption: high clearance of circulating steroid hormones (progesterone and estradiol) due to high metabolism increases GnRH/LH frequency, resulting in prolonged follicular development, delayed and double ovulation, low-quality oocytes, and lower fertility per AI and high pregnancy loss. Exogenous hormonal intervention: Resynchronization (PreSynch) aims to concentrate the number of cows at day seven of the estrous cycle at the initiation of the synchronization protocol. Thus, most cows will have a mature corpus luteum (CL), assuring high progesterone to decrease the GnRH/LH frequency by negative feedback in the hypothalamus, decreasing follicular growth and double ovulation. Bos indicus beef cattle. Hormonal disruption: negative feedback at hypothalamus on GnRH/LH release (calf presence, low body condition score, and/or low energy intake) triggers a sequence of negative effects (reduced follicular growth, lower circulating estradiol during the follicular dominance phase, lack of estradiol-positive GnRH feedback, lower amplitude/surge of LH, anovulatory condition, and/or smaller ovulatory follicle which results in smaller corpus luteum [lower circulating progesterone in diestrus]) that culminates in lower fertility per AI. Exogenous hormonal intervention: Synchronization of a new follicular wave emergence with eCG treatment

that binds to LH/FSH receptors, increases follicular growth, and indirectly mitigates the negative feedback effect of GnRH release.....27

Figure 4. Description and schedule organization of the most commonly used Resynch programs for dairy and beef cows, with performance validated in scientifically published manuscripts.

Abbreviations: TAI = timed artificial insemination, NDP = nonpregnancy diagnosis, G = GnRH analogs, P = prostaglandin F2α analogs, EB = estradiol benzoate, ECP = estradiol cypionate, eCG = equine chorionic gonadotrophin.....28

Chapter 2

Figure 1. Experimental design for strategies of resynchronization of ovulation programs (ReBreed21 and Rebreed219+G) and Control. All heifers were initially synchronized with an ovulation synchronization protocol but with no breeding (time of GnRH = d0). On d7, heifers were evaluated by ultrasound and heifers that ovulated to the initial protocol were randomized to the three experimental groups and treated as shown. The design shows the timing of intravaginal P4 device, estradiol benzoate (EB), estradiol cypionate (EC), equine chorionic gonadotropin (eCG), cloprostenol (PGF2α), and buserelin (GnRH) treatments.....65

Figure 2. Incidence of heifers with synchronized wave emergence between d13 and d15 of the estrous cycle (d0 = final GnRH of initial protocol). Additional analyses were also done for heifers that were enrolled in ReBreed21+G based on whether they ovulated or not to the GnRH treatment on d12. Lowercase letters indicate differences at P ≤ 0.05.....66

Figure 3. Day of emergence of the last follicular wave and number of follicular waves during the estrous cycle (two waves = triangle, three waves = circle, and four waves = squares) for heifers that ovulated (black/filled shapes) or did not ovulate (open shapes) at the end of the estrous cycle. Different lowercase letters indicate $P \leq 0.05$ and uppercase letters indicate $0.05 < P \leq 0.10$67

Figure 4. Dominant follicle size and growth rate in different protocols. Panel A, average size (mm) of the largest follicle at d19 and d21. Panel B, growth rate (mm/2 days) of the largest follicle from d17 to d19 and d19 to d21 of the estrous cycle. Different lowercase letters indicate $P \leq 0.05$68

Figure 5. Distribution of physiologic events (CL regression, decrease in circulating P4, and ovulation) by days after GnRH from the previous synchronization protocol. Each circle represents an individual heifer, whereas mean + SEM for Control, ReBreed21, and ReBreed21+G are provided at the bottom of each panel. Panel A, Distribution, and average day of structural luteolysis. Panel B, Distribution of day when P4 was < 1ng/mL. Panel C, Distribution of day of ovulation for individual heifers that ovulated at the end of the estrous cycle.....69

Figure 6. Physiological model on ReBreed21, a reinsemination program in Nelore heifers. The model includes letters that highlight the key physiologic events: (a) The intravaginal P4 device

provides better endocrine milieu for development of the ovulatory follicle and, of prime importance, maintains P4 concentrations greater than 1 ng/mL until d19 to prevent premature estrus and ovulation (82.1% of heifers reached P4 <1ng/mL between d20 and d21). (b) The eCG treatment increases follicle growth after d19 (two-fold increase compared to control) resulting in a larger preovulatory follicle on d21. (c) The GnRH treatment induces synchronized ovulation of the dominant follicle with 77.8% of heifers (42/54) having ovulation on d22 or d23 compared to 26.9% (7/26) of Control heifers.....70

Chapter 3

Figure 1. Illustration of the treatments for ReBreed21 and Resynch33 including timing of intravaginal progesterone (P4) device (*kept for 7, 8, or 9 days [previously shown to produce similar outcomes [32]]), estradiol benzoate (EB), estradiol cypionate (EC), equine chorionic gonadotropin (eCG), cloprostenol (PGF), licerelin acetate (GnRH), pregnancy diagnosis (PD) by Doppler (D21 and 42) or B-mode ultrasonography (D33, D54, and 74), nonpregnant (NP) heifers and cows. All cows had a recheck by ultrasound on Day 33.....100

Figure 2. Survival analysis of the nonpregnant heifers and cows in 42d breeding season with reproductive programs that allow TAIs at every 21 or 42 d. The right side of survival curve the cumulative pregnancy at d 0, 21, and 42 of the breeding season with the average day to pregnancy (\pm SEM) according to survival analysis.....101

Figure 3. Cumulative pregnancy during 42d breeding season according to the fertility of the first TAI (moderate fertility P/AI < median - high fertility P/AI > median) for cows enrolled in 2 different reproductive programs Resynch33 and ReBreed21+EC.....102

Figure 4. Cumulative pregnancy at 21d of the breeding season for each of 8 pastures (Past. in Figure) of cow/ heifers (2 heifers, 3 primiparous, and 3 multiparous) enrolled in Resynch33 and ReBreed21with and without EC.....103

Figure 5. ReBreed21 work schedule, different shapes (Circle, square, triangle, and rhombus) means different groups of cows exposing to a breeding season and one future breeding season. US = Doppler ultrasonography exam to evaluate whether cow is “non-pregnant” and ready for second AI based on CL blood perfusion. "P4" with an arrow to top “↑” means progesterone device insert, and arrow to down “↓” device removed. EC = estradiol cypionate, eCG = equine chorionic gonadotrophin. All cows have to be rechecked by ultrasound on day 33 to determine if there are any false diagnosis and to enroll any false positives for resynchronization and a second TAI at D42.....104

Chapter 4

Figure 1. Illustration of the experimental design. On D21 after TAI the early pregnancy diagnosis was performed using B mode ultrasound based on estimation of CL size using as a reference two vertical lines fixed in ultrasound screen 10mm apart from each other. After CL size score estimation, the Doppler mode was turned on for estimation of CL blood perfusion based on

subjective scores. All results were compared to the gold standard method of B mode ultrasound on D33 by visualization of embryonic heartbeat.....128

Figure 2. Panel A. Receiver operating characteristics comparing the area under the curve for early methods of pregnancy diagnosis by B mode vs. Doppler ultrasound. Assessing the differences in sensitivity and specificity generates a curve; an area under the curve closest to 1 means more accuracy in the test. Panel B. Accuracy, Sensitivity, Specificity, NPV (negative predictive value), and PPV (positive predictive value) obtained by 2x2 contingency approach analysis. The dashed line in the pentagon indicates 80% to 100%. Individual values of each test are indicated; values closest to 100% indicate more precision for each test.....129

Chapter 5

Figure 1. Schematic timeline of the series of experiments. In experiment 1, lactating dairy cows were induced to ovulate with Double Ovsynch (DO) but were not bred. In experiment 2, after TAI, lactating dairy cows were randomized into one of four groups receiving 50 IU of oxytocin or saline. Experiment 3 lactating dairy cows after the first TAI from DO were randomized into one of three timed rebreeding programs. BS = blood sample, PD = pregnancy diagnosis, NPD = nonpregnancy diagnosis, PG = cloprostenol sodium, G = gonadorelin.....167

Figure 2. Circulating PGFM dynamics after treatments with oxytocin on days 17, 18, and 19 of the estrous cycle after induced ovulation. The dashed red line means two standard deviations from the average distribution of day 17 before treatment in both groups.....168

Figure 2. Luteolysis distribution after oxytocin treatments on days 17, 18, and 19 of the estrous cycle after induced ovulation. Panel A onset of luteolysis (the day before when P4 decreased >50% compared to the average of the 3 high values). Panel B final luteolysis (first day when P4 decreased < 1ng/mL). μ = average day of onset or final luteolysis and σ^2 = variance of onset or final luteolysis. * In the Control group, one cow had not onset luteolysis until 30 d and two had not complete luteolysis until 30 d.....169

Figure 4. Percentage of nonpregnant lactating dairy cows with low P4 on day 20 of the estrous cycle after induced ovulation using different methods. Radioimmunoassay (RIA) cut-off P4 <1ng/mL; ELISA lateral flow assay (P4Gold) low P4 when test-line darker than control-line; and color Doppler ultrasound, low P4 when total area of the CL with less than 25% of blood perfusion.....170

Figure 5. Timed rebreeding season performance of Resynch25 and ReBreed21. Survival percentage of nonpregnant lactating cows and time to pregnancy during a 105-day timed rebreeding season relative to days in milk (Panel A) for Resynch25 (day 112.7 A [\pm 3]), ReBreed21 (day 122.2 B [\pm 3]), and ReBreed21+G (day 121.5 AB [\pm 3]) P = 0.09; or relative to days since the first TAI Panel (B) for Resynch25 (day 27.7 A [\pm 3]), ReBreed21 B (day 37.4 [\pm 3]), and ReBreed21+G (day 35.8 AB [\pm 3]) P = 0.1.....171

Chapter 6

Figure 1. Schematic timeline of the experimental groups with two resynchronization of ovulation programs for TET ReBreed21-ET and Resynch28. ET = embryo transfer, NP = nonpregnant, PGF = PGF2 α analog, G = GnRH analog, CL = nonpregnant heifer on D28 with CL \geq 18 mm diameter, w/o CL = nonpregnant heifer on D28 without CL or CL <18mm diameter. Other examinations: BS = Blood sampling for P4; US indicates ovarian ultrasound; PD = Pregnancy diagnosis by ultrasound.....203

Figure 2. Pregnancy/ET for the first TET in the trial (Panel A) and pregnancy loss (Panel B) between pregnancy diagnosis by ultrasound from day 28 to 63.....204

Figure 3. Pregnancy/ET for the second and later TET in the trial (Panel A) and pregnancy loss (Panel B) between pregnancy diagnosis by ultrasound from D28 to D63 for second and later TET.....205

Figure 4. Reproductive performance at different times in a 105-d TET season using Resynch28 or ReBreed21-ET. Panel (A) cumulative pregnancies by each 21-d period. Panel (B) Survival percentage of nonpregnant heifers during the 105-d TET season and time to pregnancy for Resynch28 (day 53.3 [\pm 3]) and ReBreed21-ET (day 44.9 [\pm 3]) P<0.01.....206

Figure 5. Practical schedule for implementing a TET season using ReBreed21-ET and Resynch28, showing days relative to first Timed-ET (TET), pregnancy diagnosis (PD) by ultrasound to evaluate embryonic heartbeat and hormonal treatments (GnRH analogs [G],

intravaginal progesterone insert [P4 insert], PGF2 α analogs [PGF]). In ReBreed21-ET, after PD on D28, nonpregnant (NP) heifers with corpus luteum (CL) received a new TET on D28, and NP heifers without CL or CL<18mm, received a modified 5-d CoSynch initiated on D28 for a potential second TET 42d after first TET.....207

Chapter 7

Figure 1. Experiment 1. Observational retrospective cohort experimental design to explore circulating pregnancy-associate glycoproteins with pregnancy loss (PL) from 28 d to 33 d (n = 32), 33 d to 47 d (n = 21), and 47 d to 63 d (n = 15) after 1003 timed embryo transfer (TET). Each heifer that had a pregnancy loss event was matched with a heifer in the same cohort that was nonpregnant (NP on 28 d, and the P4 was <1ng/mL [Low P4] on 19 d) and Pregnant in all pregnancy diagnoses (PD). In addition, for both pregnant and nonpregnant groups, each heifer must match with a heifer in the pregnancy loss group for TET date, embryo quality, embryo development, embryo source, and number of previous TET. BS = blood samples.

Experiment 2. Experimental design for the progesterone supplementation (Plus P4) using an intravaginal P4 insert (CIDR) from 14 d to 19 d after induced ovulation using a modified 5-d CoSynch for TET.....235

Figure 2. Individuals circulating PAGs on day 21 and 28 of the gestation for Holstein embryo recipients with pregnancy loss in three periods compared with pregnant and nonpregnant. For the period from day 28 to 33: (Nonpregnant [n = 32], Pregnancy loss [n = 33], and Pregnant [n = 32]; period from day 33 to 47: Nonpregnant [n = 27], Pregnancy loss [n = 23], and Pregnant [n =

27]; period from day 47 to 63: Nonpregnant [n = 16], Pregnancy loss [n = 15], and Pregnant [n = 16]. Different lowercase letters mean $P \leq 0.05$236

Figure 3. Percentage of embryonic attachment on day 21 of the gestation for Holstein embryo recipients with pregnancy loss in three periods. Different lowercase letters mean $P \leq 0.05$ and different uppercase letters means $P > 0.05$ and < 0.1237

Figure 4. Percentage of embryonic attachment and pregnancy per embryo transfer for Holstein embryo recipients that receive a P4 insert (Plus P4) from days 14 to 19 compared with no P4 supplementation (Control). Different lowercase letters mean $P \leq 0.05$ and different uppercase letters means $P > 0.05$ and < 0.1238

Chapter 8

Figure 1. Model for the ReBreed21 strategy. Three fundamental precepts for development of the ReBreed21 approach: (1) Maximize or No decrease in fertility to previous 1st service, (2) Accurate early pregnancy diagnosis, (3) Adequate fertility to rebreeding services.....256

Figure 2. Model of optimal hormonal milieu to achieve high pregnancy per service in beef or dairy cows. High-producing dairy cow. Hormonal disruption: high clearance of circulating steroid hormones (progesterone and estradiol) due to high metabolism increases GnRH/LH frequency, resulting in prolonged follicular development, delayed and double ovulation, low-quality oocytes, and lower fertility per AI and high pregnancy loss. Exogenous hormonal

intervention: Resynchronization (PreSynch) aims to concentrate the number of cows at day seven of the estrous cycle at the initiation of the synchronization protocol. Thus, most cows will have a mature corpus luteum (CL), assuring high progesterone to decrease the GnRH/LH frequency by negative feedback in the hypothalamus, decreasing follicular growth and double ovulation. Bos indicus beef cattle. Hormonal disruption: negative feedback hypothalamus on GnRH/LH release (calf presence, low body condition score, and/or low energy intake) triggers a sequence of negative effects (reduced follicular growth, lower circulating estradiol during the follicular dominance phase, lack of estradiol-positive GnRH feedback, lower amplitude/surge of LH, anovulatory condition, and/or smaller ovulatory follicle which results in smaller corpus luteum [lower circulating progesterone in diestrus]) that culminates in lower fertility per AI. Exogenous hormonal intervention: Synchronization of a new follicular wave emergence with eCG treatment that binds to LH/FSH receptors, increases follicular growth, and indirectly mitigates the negative feedback effect of GnRH release.....257

Figure 3. Graphical abstract of the main results of ReBreed21 in beef cattle, ET recipients, and dairy cows.....258

**Chapter 1: Literature review – State-of-the-art in bovine reproductive management:
challenges and opportunities to optimize performance in beef and dairy cows**

João Paulo N. Andrade^a; Victor Gomez-Leon^b, Milo C. Wiltbank^{a*}

^a Department of Animal and Dairy Sciences, University of Wisconsin-Madison, Wisconsin
53706, USA

^b Department of Animal Sciences and Industry, Kansas State University, Manhattan, Kansas
66506, USA.

Corresponding Author: Milo C. Wiltbank

E-mail: wiltbank@wisc.edu

Abstract

Reproductive management is one of the pillars of bovine operations, but physiological changes after parturition indirectly or directly alter the GnRH/LH pulse frequency, thereby decreasing female fertility. The development and optimization of timed AI (TAI) programs that focus on achieving the optimal hormonal milieu near TAI have overcome the poor fertility in post-partum dairy and beef cows. Despite the advancements, in the best scenario only about 50% of eligible cows will be pregnant after the first TAI and a greater number of pregnancies are necessary to keep a herd inventory aligned with each herd's goals (~80% of cows within cohort's post-partum) preestablished by veterinarians and producers. Additionally, in either dairy or beef cattle, there is an optimal time for pregnancy that leads to high animal performance and farm profitability. In beef cattle operations, calves born in the first two 21-d periods of the calving season are heavier and will be the high-performance males and females in their cohort. Thus, increasing the number of pregnancies in the first 42-d of the breeding season is essential to optimize cow/calf operations performance. The use of the TAI on the first day of the breeding season increases the pregnancies in the first 21-d period compared with natural mating and said increases can be even more pronounced when TAI is used multiple times with resynchronization of ovulation programs. Conversely, the optimal time to pregnancy in lactating dairy cows is closely related to the herd's milk production (cow/day) and the herd's lactation curve profile. Thus, to optimize performance cows should become pregnant near the optimal time of pregnancy at a herd level. Reproductive programs available for lactating cows lead to a breeding period as short as 70d in length when TAI is prioritized and increases to 100d when AI based on detection of estrus is the priority. Several reports have suggested the optimal timing for breeding cows and different beef and dairy cattle reproductive programs; herein, we address the multiple aspects of

the optimal time for pregnancy in cows and dissect the differences between beef and dairy cows in their physiology and apply their physiology to on-farm state-of-the-art bovine reproductive programs.

Key words: reproduction efficiency, cattle, rebreeding.

Introduction

Bovine reproductive performance is one of the critical aspects for livestock operations in beef and dairy alike since calving marks the productive stage of the female bovine (a calf for a beef cow and milk production for a dairy cow). Reproductive efficiency involves multiple processes, but can be summarized with a few measures, such as the 21-day pregnancy rate for dairy cows, the percentage of pregnancies at the end of the breeding season, and the length of the breeding season for beef [1, 2]. On the other hand, interpreting and understanding how to optimize those parameters requires a holistic view of bovine biology associated with a solid knowledge of real-life livestock operations. For example, understanding the physiology of the estrous cycle in cattle allowed innovative technical scientists to develop synchronization of ovulation programs for timed artificial insemination (TAI) [3-5]. Use of TAI eliminates the requirement of expression/detection of estrus, which is the major challenge of bovine reproduction for performing AI with reasonable fertility. Using TAI, regardless of cyclicity and puberty status, 100% of eligible cows can receive an AI on a programmed day without estrus detection. The development of TAI not only facilitated the execution of AI but also opened new avenues for scientists worldwide to optimize this technique and design further modifications precisely aligned with cows in different physiologic phases (lactating dairy cows, beef cows, and ET recipients) [6-9]. The further optimization of TAI has dramatically improved bovine reproductive performance, but from a herd perspective, use of TAI strategies are only one part of the reproductive program. The central goal of veterinarians and producers is to reach the number of pregnancies necessary to keep a herd inventory aligned with each herd's goals. Additionally, it is desirable to target the pregnancies close to the optimal time for pregnancy to optimize bovine performance. Whereas TAI significantly improved the potential to optimize reproductive

performance, it further complicated reproductive management as it has to be aligned with the herd capability to execute the protocol treatment precisely. This underscores the importance of strategic planning in bovine reproductive management.

In general, if it is desired to target the first calving for either beef or dairy cattle at 24 months of age, then the optimal time to initiate pregnancy is about 14 months old, assuming said heifers have already reached roughly 55 to 65% of what would be their expected mature body weight [10, 11]. On the other hand, after calving, cows first undergo a voluntary waiting period (VWP) for uterine involution and return to cyclicity. The length of the VWP varies among herds and is decided jointly by veterinarians and producers. For a beef herd, the breeding season usually starts on a fixed day of the year, and all cows < 40 d postpartum (i.e., < 40-d VWP) are eligible to onset the breeding period because the goal is to have a shorter calving period. Conversely, in dairy herds in which cows calve continuously throughout the year, VWP varies significantly from 50 to 90 d in milk (DIM) to start the breeding period. This variation is associated with cows that eventually become pregnant sooner after calving (around 50 DIM), will be dried off with a reasonable daily milk yield. Therefore, driven by instinct, producers are willing to extend VWP in lactating dairy cows to capitalize on high-producing cows. An economic model suggests that, to an extent, later VWPs could be more profitable for high-producing lactating dairy cows [12]. Nevertheless, it is still difficult to consider the optimal VWP or, in other words, the optimal time for pregnancy at a herd/cow level due to milk yield variation observed among commercial herds and for individual cows. Regardless of when the onset of the breeding period will be initiated in beef and dairy herds, the length of the breeding period implies how long a given herd takes to reach the goal of the proportion of cows to become pregnant. A combination of strategies (biotechnologies and management decisions) will dictate

how efficient a reproductive program will be and how it will economically impact bovine operations. Thus, designing bovine reproductive programs requires understanding the breeding period dynamics and the differences in bovine physiology between beef and dairy. This demonstrates the value of technical decisions for optimizing reproductive performance in bovine livestock operations.

To optimize bovine reproductive performance, a program must combine three features: (1) maximizing pregnancies from the first AI, (2) early nonpregnant diagnosis, and (3) attaining reasonable fertility in the rebreeding. For the first AI, developing fertility programs in lactating dairy cows using TAI overcame the negative effect of high metabolism and standardized fertility among herds. In contrast, on the beef cattle side, integrating eCG into the TAI synchronization overcame the negative impact of low body condition score and suckling calves on fertility [13, 14]. Nevertheless, not all cows will eventually become pregnant in the first AI; in the best scenario, about half of eligible cows are expected to be pregnant. Thus, a common strategy for reinseminating nonpregnant cows is by detecting cows that return to estrus. Even so, in well-managed conditions in beef and dairy cattle, only about 50% of the remaining nonpregnant cows are detected in estrus and receive insemination during the first estrous cycle after the first AI (Figures 1 and 2).

Another strategy is using a subsequent TAI after a nonpregnancy diagnosis, termed the Resynch program [15, 16]. For nonpregnant identification, the most traditional method is via transrectal palpation or ultrasound 30 d to 40 d after AI [17]. Promising indirect methods for nonpregnancy diagnosis, evaluating corpus luteum blood perfusion, and detecting hormones in blood or milk have been under development to be integrated into bovine reproductive programs [18, 19]. Nevertheless, even with the best performance choice reproductive program, only about

60% of eligible cows will be pregnant by 42 days of the breeding period (Dairy 55% [n = 6,905 Figure 2] and Beef 65% [7,248 Figure 1]). Thus, the purpose of this review is to revisit and bring new insight into the challenges and opportunities to optimize bovine reproductive performance in beef and dairy cows according to their physiology and profitable purpose/goal. Specifically, the aim of each section (beef or dairy) is to (1) discuss the critical aspects of what should be an optimal time for pregnancy within a bovine productive purpose and (2) describe and summarize the performance of bovine reproductive programs (first service as well as strategies for rebreeding).

Beef cattle reproductive programs

The optimal time for pregnancy: “translated” into kg of calf

In cow/calf operation, one of the main goals is to reduce the calving interval to achieve a one-year calving interval to maximize productivity [20, 21]. This has been desirable because cows will deliver one calf/year in the strategic climatic season, which is critical for calf development and abundance of pasture during the breeding season, guaranteeing nutritional support for dams during reproduction [22]. Therefore, the starting point is to achieve a calving interval of ≤ 1 year in a successful pregnancy establishment of ≤ 80 d postpartum. For instance, considering a 40-d voluntary VWP, the entire breeding period must happen ~ 40 days (i.e., from 40 to 80 days postpartum) [23]. Conversely, the long-term impact of a shorter breeding season (≤ 42 d) for heifers leads to earlier calving in their first calving season, allowing females to restore cyclicity before the next calving season begins [24, 25]. Accordingly, simulation models comparing different lengths of breeding periods suggested that the earlier heifers and cows become pregnant in the breeding season, is associated with an increase in animal productivity

and dams are more likely to remain in the herd and produce heavier calves [26, 27]. Furthermore, studies have explored performance beyond the dam effect and showed that the calves born in the first weeks of the calving season had enhanced performance compared with calves born after the third week of the calving season [28, 29]. An elegant study by Funston et al. (2012) evaluating the performance of calves born in the first three 21-d periods reported that calves born in the first period were heavier at weaning compared to those born in the third period (+34kg for steers [238 vs. 204kg] and +22kg for heifers [219 vs. 197kg]) [29]. The greater performance in kg for steers born in the first 21-d period remained even after the feedlot. Additionally, the heifers born in the first two periods had a greater proportion of pubertal heifers, resulting in more pregnancies at the end of the breeding season. Taken together, it is highly recommended to increase pregnancies in the first 21-d period of the breeding season to maximize the number of calves born in the first 21-d period of the calving season.

The season of the year and the length of the breeding season are vital aspects that greatly change the kg of calves at weaning. In North American beef herds, the best season of the year to target calving depends upon the interaction between environmental conditions and marketing opportunities [22]. Nevertheless, calves born in the transition of winter/spring are expected to be the heavier ones [30]. In addition, beef operations in tropical conditions also show a remarkable association with the season and kg of calf at weaning [31, 32]. Indeed, the present manuscript using data from commercial beef operations in Brazil illustrated similar results as found in previous reports (Figure 1, Panel B). Calves born in the transition of Winter/Spring were 16kg heavier at weaning adjusted for 205 days (199kg [n=36,590]) than calves born in the transition of Spring/Summer (183kg [n=39,516]). Additionally, within the group of calves born in the transition of Winter/Spring, we performed a further analysis comparing performance among

calves born in the first three 21-d periods of the calving season. Using the daily gain information from calving to adjusted 205 d BW, a hypothetical BW for weaning at 180 days after the onset of the calving season was calculated. Similar to reported data by Funston et al. (2013) using *bos taurus*, male and female calves born in the third period were lighter than those born in the first two periods (Figure 1, Panel B). Taken together, to achieve a calf/year interval, cows must become pregnant before 80 days after parturition, but the high-performance animals are those that become pregnant in the first 21-d period of the breeding season and calve earlier in the next calving season. This aspect is central to beef herd performance due to an increase in voluntary culling rate, which gives greater opportunity to direct herd growth or increase pressure in genetic selection. The optimal time for pregnancy in beef cattle is the first day of the breeding season, which will yield heavier calves and more fertile replacements. Therefore, the reproductive programs should be designed and chosen to increase the number of pregnancies at the beginning of the breeding season and focus on increasing the pregnancies in the first 21 d.

Describing the reproductive programs for beef cattle:

First Service programs for beef cattle

The percentage of cycling cows or heifers at the beginning of the breeding season is pivotal for fertility in the first and all services in the breeding season [21, 33]. Nevertheless, few cows and heifers are cycling at the beginning of the breeding season [34-37]. Because natural mating is still the most used strategy for reproduction worldwide in beef cattle operations, the overall performance of such a strategy is diminished due to the low percentage of cows and heifers, not cycling (not coming into estrus) [21, 38, 39]. The biological reason for prolonged anestrus is the negative feedback effects of estradiol on the hypothalamus decreasing GnRH/LH

release, which can be attributed to calf presence, low body condition score, and/or low energy intake (Figure 3) [40-44]. Those factors described above, trigger a sequence of negative effects (reduced follicular growth, lower circulating estradiol during the follicular dominance phase, lack of estradiol-positive GnRH feedback, lower amplitude/surge of LH, anovulatory condition, and/or smaller ovulatory follicle which results in smaller corpus luteum [lower circulating progesterone in diestrus]), that culminates in lower fertility per service (i.e. pregnancy/AI [P/AI]) [21, 33, 45-47]. Management strategies such as calf removal and energy nutritional supplementation can improve GnRH/LH pulsatility contributing to increased follicular growth, cyclicity, and fertility [44, 48-54].

Another option is to induce ovulation through TAI programs [5]. A recent review of TAI in beef cattle from our laboratory using 228 manuscripts containing 272,668 TAIs reported about 50% of P/AI involved the usage of reproductive hormones to induce ovulation [7]. Nevertheless, treatment with equine chorionic gonadotrophin (eCG) has a major impact on increasing beef cattle fertility. The eCG binds to LH/FSH receptors, increases follicular growth, and indirectly mitigates the negative feedback effect of GnRH release (Figure 3) [14, 55-59]. Using the previous reports that composed our review, the gonadotrophin supplementation by eCG positively increases the pregnancy/AI in beef cattle by 19% (49.9% (6682) vs. 41.7% [4904]; $P < 0.01$), which is represented by a 36% increase in *Bos indicus* (48.7% [3844] vs. 36.1% [2379]; $P < 0.01$) and 7% in *Bos taurus* (51.6% [2838] vs. 46.9% [2525]; $P < 0.01$), only for manuscripts in which there were direct comparisons for cows treated with or without eCG (eCG data not shown in the published review) [7]. In the present manuscript, a comparison of studies that directly compared starting the breeding season with TAI or natural service showed a substantial increase of 157% in pregnancies in the first 21 days of the breeding season when synchronization

of ovulation was used for TAI (59% [615/1,043]) vs. natural mating (23% [188/817]) (Figure 1, Panel D). Assembling TAI with eCG supplementation has been a vital strategy for cow/calf operations, allowing about 50% of eligible cows to become pregnant in the first days of the breeding season regardless of cyclicity status. Therefore, TAI is the optimal strategy to increase the percentage of pregnant cows on the first day of the breeding season in beef cows. However, since the producer typically focuses on achieving 85% of cows pregnant, rebreeding strategies must also be utilized to achieve the desired overall number of pregnancies.

Rebreeding strategies for beef cattle

In beef cattle operations, use of cleanup bulls is the most common approach after a TAI through to the end of the breeding season [60-62]. Use of TAI followed by natural service yields 157% more pregnancies in the first 21 days of the breeding season compared to when only natural mating was used (Figure 1, Panel D). However, the day that nonpregnant cycling beef cows return to estrus accumulated about 21 d after TAI, making the number of bulls required to breed these cows in a short time period somewhat similar to what was calculated if the first service was using natural mating. Interestingly, a recent publication has shown that specifically in *Bos indicus* beef cattle, only 30% of nonpregnant cows remained cycling after the first TAI [63]. Therefore, the cleanup bull strategy had diminished efficiency in such conditions, requiring about 90 days of natural mating to allow cows to resume cyclicity [64]. On the other hand, for *Bos taurus* beef cattle with good nutritional management, cyclicity is restored earlier, allowing a shorter 60-day breeding season [60, 65]. Nevertheless, *Bos taurus* in the winter breeding season also results in low fertility, requiring a 90-day breeding season, likely due to poor availability/quality of dry matter [61]. Regardless of the strategy, the goal is to reach around 85% of pregnancies sooner in the breeding season [26, 66]. Therefore, to overcome the issue of low

cyclicity and achieve a tighter breeding season, consecutive synchronization of ovulation strategies (Resynch) have been used with the aim of optimizing reproductive/ productive performance [27, 67].

Resynch programs prepare nonpregnant animals for a second or subsequent service using TAI. The obvious benefit of this strategy is that even noncycling cows will receive AI, having a chance to become pregnant due to the induction of ovulation, with the additional advantage of using high-merit genetic sires through AI instead of clean-up bulls. A key step to designing a resynch program is the identification of nonpregnant cows, commonly carried out either by ultrasound (~32 days after AI) or palpation (~40 days after AI) [68]. Usually, resynch is labeled according to the day of onset or the resynchronization protocol after the previous AI. For instance, Resynch30 is a resynchronization program that starts 30 d after the previous breeding, leading to a potential AI around 40 d after the first TAI. Replicating this approach will result in an 80-d breeding season length with 3 TAI 40 days apart. Usually, after three services, with reasonable P/AI (~ 45%), more than 80% of eligible beef cows should become pregnant at the end of the breeding season. Studies on Resynch30 has generally reported ~85% of cows pregnant at the end of the 80-d breeding season [61, 69]. Nevertheless, this Resynch30 has not improved the number of pregnancies in the first 21-d period of the breeding season compared to using TAI only at the first service because of the 40d interval between TAI. In addition, even with a low percentage of cows returning to estrus, using clean-up bulls will increase the percentage of cows pregnant by 5 to 10% in the first 21-d period compared with Resynch30 (Figure 1, Panel D).

The new generation of rebreeding programs for beef cows

Targeting the development of more Resynch approaches for TAI <40 d apart, scientists proposed the onset of the hormonal treatments before the nonpregnancy diagnosis, regardless of

gestation status [70]. Then, after diagnosing the nonpregnant animals, the treatment is completed for a second TAI ~ 2 d later. The first developed program was Resynch22, in which eight days after the initial resynch treatment, a pregnancy diagnosis is performed using ultrasound (presence of embryonic heartbeat by ~30 d after AI), nonpregnant cows receive the further synchronization treatments (primarily PGF2 α analogs, eCG, and estradiol analogs), and a TAI is then performed two days later (~32 days after previous AI) [71]. With this design, Resynch22 allows 3 TAIs in about 64 d, with about 85% of eligible animals pregnant during a restricted breeding season using AI [2, 61]. A subsequent generation of Resynchs programs have been developed based on the indirect strategy to identify nonpregnant cows evaluating corpus luteum function using Color Doppler ultrasound around 21 d after AI [72]. The Doppler ultrasound allows the identification of ~90% of nonpregnant cows on day 22 since the previous breeding, and when combined with a Resynch14 strategy, leads to a potential TAI 24 days after the previous AI [73-76]. The evident strength of Resynch14 is in the possibility of a 48-d breeding season with 3 TAIs. Nevertheless, there are some critical inconveniences/limitations for Resynch22 and Resynch14. First, with such designs, many cows are unnecessarily treated since the treatment starts without a determination of pregnancy status. Second, the calendar for TAI at 24-d or 32-d intervals results in a complex schedule to handle and organize the weekday tasks, usually requiring weekend labor. Lastly, there are a few false negatives, specifically for the Resynch programs that use Doppler ultrasound to select nonpregnant cows [18]. However, since PGF2 α analogs are used, it will eventually lead to an iatrogenic abortion [77, 78]. Despite the difficulties, both programs Resynch22 or Resynch14 increase pregnancies in the first two months of the breeding season compared with Resynch30 or clean-up bulls [2, 27]. However, neither of those rebreeding

strategies is able to consistently increase the pregnancies in the first 21-d period of the breeding season.

ReBreed21 is one of the new generation of Resynchs, built with a unique design that proposes to rebreed nonpregnant cows every 21 d, in a user-friendly schedule with the TAIs at the same time as the pregnancy diagnosis using Doppler ultrasound [79, 80]. The first two synchronization steps are performed without knowing the pregnancy status (on 12 d and 19 d after TAI). The selection of nonpregnant animals is determined using Doppler ultrasound on day 21 to assess the functionality of the CL by blood perfusion, and finally, the nonpregnant animals bred on day 21 after the ultrasound exam [80, 81]. The hormonal treatments were developed to avoid iatrogenic abortion (no estradiol at the initiation of the Resynch and no PGF2 α analogs). Evaluating ReBreed21 in 2,085 *Bos indicus* beef cattle, there was an increased number of pregnancies at 21 d of the breeding season (71.1%) compared with a Resynch33 (55.5%) [82]. In addition, ReBreed21 at the end of a 42-d breeding season, there were 73% of heifers pregnant, providing sufficient pregnancies to reach the replacement requirement (~20%). The combination of a user-friendly schedule and an increase in performance in heifers makes ReBreed21 the most aggressive validated program that is available nowadays for *Bos indicus* heifers. Noteworthy, improvements are needed in ReBreed21 for multiparous beef cows due to the lack of fertility found in multiparous cows that received timed rebreeding using ReBreed21.

Beef section conclusion and take-away

The advancements in the understanding of bovine reproductive biology have facilitated the development of strategies to overcome anestrus postpartum, increase P/AI, and allow for about 50% of exposed nonpregnant cows to become pregnant on the first day of the breeding

season [7]. The incorporation of TAI in commercial cow/calf operations has greatly improved performance on beef farms, especially in tropical countries [83]. Resynch programs allowed an entire breeding season using only TAI in large operations, taking advantage of sires with high genetic merit and increasing weaning weight of calves [27, 84]. Nevertheless, efficient reproductive programs strive to increase pregnancies during the first 42 days of the breeding season to optimize bovine performance (kg of calf produced/exposed cow) [26, 29]. Even with all the advances in reproductive programs, only ReBreed21 is a program that has increased pregnancies during the first 21 days of the breeding season using TAI. Further improvements are needed to produce a program that achieves optimal fertility in multiparous animals.

Dairy cattle reproductive programs

Measuring reproductive performance in dairy cows

Unlike what was discussed for beef cattle, most dairy farms have no fixed breeding season during the year. Therefore, there are a cohort of cows that could potentially begin their breeding period daily in a dynamic process that requires different approaches to measure reproduction [85, 86]. Generally, three services will be enough to reach 70 to 85% of pregnant lactating cows, if the herd has reasonable fertility (>40% in Preg/AI) [1]. Nevertheless, herds will vary according to individual goals (targeting to increase or stabilize the herd mature cows inventory), culling rates (voluntary or involuntary), and pregnancy per service [87-89]. For this reason, the primary reproductive efficiency measurement for most herds is the 21-day pregnancy rate, which is the ratio between (number of pregnant cows) / (number of eligible cows) during a 21-d period [90]. The 21-d pregnancy rate is affected directly by two other values: P/AI (conception rate [% of bred cows that become pregnant]) and 21-d insemination risk (service rate

[% of eligible cows that were bred during a 21-d period]). In summary, the 21-d pregnancy rate is comprised of the “fertility of bred cows” (conception rate) and “how efficiently a herd breeds eligible cows” (service rate). Conversely, due to the complexity of handling this measurement in a controlled trial, studies that compare reproductive programs often use the survival analyses of nonpregnant cows after VWP, highlighting the percentage of cows that become pregnant by 150 to 180 days in milk DIM [15, 91-93]. Nevertheless, a given reproductive program that leads to high 21-d pregnancy rates implies that in this particular herd, cows are being bred rapidly with high fertility and becoming pregnant faster after the VWP.

The optimal time for pregnancy: “translated” into milk yield

For decades, veterinarians have debated the optimal VWP for lactating dairy cows. Interestingly, researchers have also arrived at conflicting conclusions in trying to address the same question. One of the main arguments is that extending the VWP would increase profitability for high-producing cows with more persistent lactations [94]. For example, extending the VWP in lactating cows that had lactation persistence induced with bST increased the profitability in the current and subsequent lactation [95-97]. On the other hand, others have argued that a decrease in profitability was observed when the VWP was extended, which could be related to different profiles of the lactation curve (peak and persistence) [12, 98]. Interestingly, another aspect is that extending the VWP can increase fertility to the first AI even under heat stress conditions due to allowing more time for cows to return to cyclicity [99-101]. Yet, similar profitability was found during extended or shorter VWP. Conversely, recent research by Dr. Giordano’s laboratory at Cornell University showed a parity effect on VWP duration, such that primiparous cows showed increased profitability with an extending VWP, while multiparous cows had a decrease in profitability with extended VWP [102, 103]. The

authors argued that the persistent lactation curve in first-lactation cows favors an extended VWP. On the other hand, an extended VWP would cause a herd distribution change, decreasing the percentage of the herd represented by the highly profitable multiparous cows. The contradiction in the literature potentially can be explained by the variability of milk yields and management strategies, such as rBST [104].

Despite the contradictions on whether the VWP should or should not be extended for dairy cows, another way to explore the same dilemma is by evaluating what would be the optimal time for pregnancy. For instance, the present review uses dynamic partial budgeting analysis developed in the laboratory of Dr. Victor Cabrera at University of Wisconsin-Madison to explore the income over feed cost (IOFC) using data from commercial herds in Wisconsin (235 herds [total n of cows = 240,617]) as an illustration to bring insights for this discussion. The three levels of milk production (bottom, middle, and top) show a change in optimal time to pregnancy according to herd profiles of the lactation curve (peak and persistence). Similar to what was discussed in the previous paragraph, the optimal time of pregnancy is greatly affected by milk yield. Intuitively, it suggests that each herd would have a unique optimal time to pregnancy, or more accurately, each cow would have an optimal time to pregnancy driven by the herd/cow lactation curve level of production. Hence, this result may help explain the conflict related to determining an optimal VWP found in the literature, particularly the potential bias that arises from the observed milk yield that is included in the model. In summary, the main aspect is not simply when to start breeding cows after calving (i.e., VWP length) but also to get more cows pregnant near the optimal time of pregnancy. Nevertheless, while an accurate model associated with decision-making tools must still be developed to predict the optimal time to pregnancy at a herd level, an ideal reproductive program should have a tighter breeding period

length. This could allow precision in programming when the cows become pregnant to decrease variability and potentially increase profitability.

Describing the reproductive programs for dairy cows:

First Service programs for dairy cows

A critical step for achieving high reproductive performance is fertility at the first AI after VWP. Increasing the number of pregnancies in the first service in lactating dairy cows increased both conception and service rates, potentially leading to a ~70-d breeding period, achieving over 80% of eligible cows pregnant [1]. Improvements in TAI programs for high-producing lactating dairy cows have focused on optimizing the hormonal milieu during follicle development and proestrus to mitigate the negative impact of high metabolism in the clearance of circulating steroid hormones (progesterone and estradiol) (Figure 3) [9, 105-109]. Strategies for pre-synchronization in lactating dairy cows are termed “Fertility programs” due to a consistently improved P/AI across herds compared to cows bred by a standard Ovsynch or AI after detection of estrus [6, 110-113]. Interestingly, a recent publication considering the genetic merit for fertility in first-lactation dairy cows showed that regardless of the genetic merit, fertility programs still improve P/AI by about ten percentage points compared to animals bred mostly after detection of estrus [92]. In the present review, a total of 17 studies evaluated the long-term effect of starting a breeding period with TAI or AI based on detection of estrus (n = 25,216). The pooled average considering studies (11 studies n = 14,465) that directly compared TAI for the first AI vs. detection of estrus shows an increase of 87% (Figure 2, Panel C) in pregnancies in the first 21 d after VWP (TAI = 43% [2,964/6,905] vs. AI based on detection of estrus = 23% [1,733/7,559]). Interestingly, only including studies that used fertility programs, the pooled

cumulative pregnancies at 21 d after VWP was 43.1% (2,906/6,738). Conversely, it has been reported from other reviews that a fertility program should achieve ~50% of eligible cows pregnant on the first day after VWP [6, 114]. In addition, considering the cumulative pregnancies during the 21 d after VWP also includes some cows actually becoming pregnant from a rebreeding returning to estrus, and thus may overestimate the P/AI from the first TAI. One factor to help explain those differences is to account for pregnancy losses that generally happen from the first to the third month of gestation in lactating dairy cows [115]. Studies reporting the performance of fertility programs often use P/AI results from the pregnancy diagnosis ~30 days after TAI while when cumulative pregnancies were evaluated, animals that underwent pregnancy loss are generally considered nonpregnant in the analysis. Nevertheless, the utilization of TAI with fertility programs is the best strategy for increasing the proportion of cows becoming pregnant on the first day of the breeding period. Nevertheless, the rebreeding strategy is also fundamental in achieving an efficient reproductive management program for dairy cattle that provides the number of pregnancies required for each particular herd.

Rebreeding strategies for dairy cows

After the first AI, the focus moved toward identifying cows that eventually did not become pregnant to rapidly assign them to a rebreeding program [17]. The traditional worldwide method to identify nonpregnant dairy cows is visually monitoring for their return in estrus or, alternatively, detect estrus by using tail chalk or an activity monitor. However, only 59% (14,841/25,310) of nonpregnant cows were reported to be detected in estrus and bred before the nonpregnancy diagnosis (pooled average including 11 studies; Figure 2, Panel D). On the other hand, for herds with poor detection of estrus or lack of full-time AI technicians, the resynchronization of ovulation programs (Resynchs) can be an effective option [15, 116]. An

important feature of the Resynch program is to schedule a pregnancy diagnosis, allocating the Resynch hormonal treatments before or after the pregnancy diagnosis [117-119]. The most common approaches to evaluating the pregnancy status of a cow are by transrectal palpation (~40 d after AI), transrectal ultrasound to identify the presence of an embryonic heartbeat (~30 d after AI), or by blood or milk markers for pregnancy (pregnancy-associated glycoproteins ~28 d after AI) [68, 120-122]. Usually, the program is named based on the day after the previous AI when the resynchronization program begins. For instance, Resynch23 and Resynch25 are programs initiated before pregnancy diagnosis 23 d and 25 after AI, respectively (see Figure 4A). Using a Resynch protocol allows a dairy farm to operate on a fixed workday routine, avoid reproductive management during the weekend, and focus the majority of the management procedures on a few days of the week when well-trained technicians are available [15]. Therefore, farmers can select one of the various rebreeding program based on factors that are distinct for each operation, aiming to optimize productive performance through an efficient rebreeding program.

Researchers have been exploring methods to optimize reproductive performance through several modifications in the Resynch protocols greatly increasing P/AI. Modifications included testing different days after TAI to start the Resynch [116, 117], using Resynch with resynchronization treatments [123, 124], Resynch with intravaginal P4 inserts [125-127], and using ovarian structures after nonpregnancy diagnosis to select appropriate synchronization strategies [118, 128]. In many herds, the most effective rebreeding strategy is a hybrid version, prioritizing AI in cows returning to estrus combined with a Resynch for a reinsemination no later than 35 d after the first TAI [118, 128, 129]. Noteworthy, if a GnRH treatment or an intravaginal P4 insert is used before the nonpregnant diagnosis this can delay the pattern of nonpregnant cows

returning to estrus [129-132]. Interestingly, regardless of the rebreeding strategy, most reproductive program results are about 80% of eligible animals pregnant by 150 DIM [15, 91-93, 128, 129, 133, 134]. To achieve $\geq 80\%$ of cows pregnant, reproductive program that prioritize TAI for all services typically requires at least 70 d in the breeding period, while programs that prioritize detection of estrus for first AI, must start breeding cows about one month earlier, resulting in a breeding period of ~ 100 d [92, 93, 135-138]. Therefore, the strategy for first AI are likely to affect the length of the breeding season more than the rebreeding strategies. In summary, the best available reproductive programs for dairy cattle generally result in a wider breeding period of 70 d to 100 d, producing fewer cows pregnant near the optimal time for pregnancy, whenever that may be for a given herd. Thus, developing a reproductive program with a tighter breeding period can increase the proportion of cows becoming pregnant at the proper time to optimize profitability.

Dairy section conclusion and take-away

Reproductive performance in lactating dairy cows in the United States has greatly improved through the development and practical utilization of management strategies such as TAI and fertility programs [139]. Interestingly, these management strategies can improve reproduction even in cows with divergent genetics for reproductive efficiency [92]. Genetic selection for reproduction, use of sexed semen, and management strategies to improve fertility led dairy farmers to overproduce replacement heifers, and has led to dramatic changes in the dairy industry such as greatly increased production of beef calves in dairy cattle [140]. These factors have led some people to assume that there is little room for improvement in reproduction and that current programs have reached an optimal state-of-the-art performance. However, there

is still substantially variability between dairy herds and within dairy herds for when cows become pregnant [93]. Therefore, the present review proposes a different question to challenge the current and future generations of bovine scientists; instead of when to start breeding dairy cows, we are questioning when most of the cows should become pregnant and how to develop a reproductive program with a more narrow breeding period. While accurate models for the optimal time of pregnancy in on-farm decision-making tools are still to be developed, reproductive programs that will produce a more narrow breeding period must also be explored. As successfully developed for beef heifers, ReBreed21 for TAI every 21 d could lead to a breeding period of 42-d with a potential of 3 AI to produce over 80% of eligible cows pregnant in a 42-d window [82]. Thus, an aggressive reproductive program for a more narrow breeding period (~42 days) would allow more cows to become pregnant near the optimal time, optimizing lactating dairy cows' performance and herd profitability.

Conclusion

In summary, maximizing pregnancies at the first service is the most important aspect of any given bovine reproductive program. The second step is the rapid identification of nonpregnant cows for rebreeding with reasonable fertility. The use of TAI can mitigate the negative effect on fertility due to the physiological status of beef and dairy cows, making it a unique strategy to improve pregnancies at first and later AIs. Resynch strategies can shorten the breeding period in beef or dairy herds and allow producers to adjust the length of the breeding period according to herd goal, optimizing bovine performance (kg of calf weaned or milk yield). Nevertheless, only beef cattle programs for short intervals between AIs (21 to 24 days) are available, allowing a 42 to 48-day breeding season. Novel reproductive programs for dairy cows are still to be developed to reduce the breeding period length to 42 d or less.

Appendix - Data processing and analysis

Beef section

Data analyses for the optimal time to pregnancy were performed using data from eleven commercial cow-calf operations in Brazil (ANCP courtesy [Brazilian Producers and Researchers Beef Association, Ribeirão Preto-SP]). The information used was birth-weight and weaning-weight (adjusted for 240 days of life; $n = 198,597$). Calves with missing data were excluded from the analysis; the final number was 88,881 calves. From the calves born from August to October, a further analysis was performed using their daily weight gain and simulating their predicted body weight at 180 d after the onset of the calving season. Similar to what was published by Funston et al. (2012) [29], the performance of calves was evaluated by dividing them into groups for the first three 21-d periods of the calving season for steers (male calf) and heifers (female calf). The optimal time for pregnancy was extrapolated using the prediction of weaning weight at 180 d after the onset of the calving season by plotting the change in profitability (8 R\$/kg) and weaning weight relative to a calf born on the first day of the calving season (Figure 1C). Data were analyzed using the PROC MIXED procedure for linear models. Assumptions (linearity, independence, and normality) were evaluated by plotting the residuals, QQ plot, and the Shapiro-Wilk test. If case deviations from assumptions were found, data were guided by BoxCox transformation to select the best transformation: natural logarithms, square root, or ranks. The pooled average for pregnancies among different studies that evaluated different reproductive programs in beef cattle ($n = 18$) was combined using a Bayesian inference using the PROC MCMC procedure of SAS for Markov Chain Monte Carlo analysis (Figure 1D).

Dairy section

Data analyses for the optimal time to pregnancy were performed using a partial budgeting analysis model developed by the Dr. Victor Cabrera laboratory to predict income over the feed cost (IOFC) cow/year according to time to pregnancy from the first to third lactation (UW-Madison Animal and Dairy Sciences Department). To simulate the model, data from commercial dairy herds in Wisconsin were retrieved ($n = 734$) to determine the milk yield for first, second, and third lactation cows at the herd level from March 2022 to March 2023 (© AgSource 2023-URUS, Madison-WI). Herds with missing values or smaller than 200 head/herd were excluded to avoid bias. The final number of herds was 235 herds (total n of cows = 240,617). According to total milk yield production (lbs/cow/day), herds were split into three distinct profiles: Top (≥ 95 lbs/cow/day, $n = 44$), Middle (< 95 and ≥ 75 lbs/cow/day $n = 161$), and Bottom (< 75 lbs/cow/day $n = 30$); Figure 2B). The average values of monthly milk production of the lactation curve of each level of output for first to third-lactation cows were input into an online management tool (dairymgt.cals.wisc.edu/tools/mcf/index.php; UW Dairy Management Tool, Milk Curve Fitter & Pregnancy Timing). To calculate IOFC, the model included the predicted equation values (scale, ramp, offset, decay) of the level of herd production and parity as well the following assumptions: dry matter cost (0.15 \$/lb.), milk price (0.2 \$/lb.), dry period (60 d), Gestation length (280 d), and a constant herd inventory for three years. The optimal time to pregnancy was predicted using the relative change of IOFC compared to a cow that became pregnant at 50 d in milk within a production level (Top, Middle, and Bottom, Figure 2A). The pooled average for pregnancies among different studies that evaluated different reproductive programs in lactating dairy cows (Figure 2C, $n = 11$; Figure 2D, $n = 11$) was combined using a Bayesian inference using the PROC MCMC procedure of SAS for Markov Chain Monte Carlo analysis.

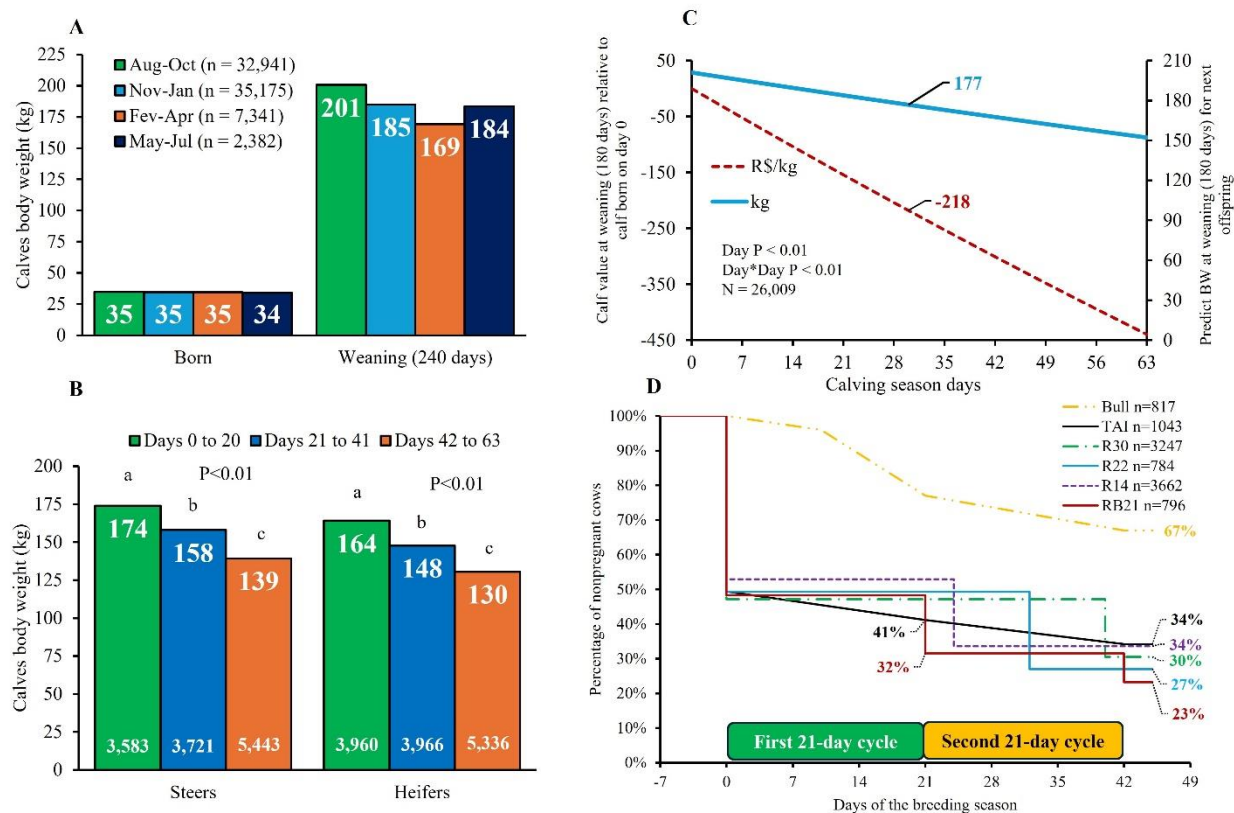


Figure 1

Figure 1. Panel A Body weight of calves at calving and the adjusted weaning weight at 240 d according to the season of the year at calving. **Panel B** Hypothetical performance of body weight of calves born in the first three 21-d periods of the calving season and weaned at 180 d after the onset of calving season relative to a calf born on the first day. **Panel C** Predicted change in calf body weight weaned at 180 d after the onset of calving season relative to a calf born on the first day. **Panel D** Performance of different reproductive programs in beef cattle (primiparous and multiparous) from published manuscripts evaluating the long-term AI strategies. (Bull) natural mating breeding season; (TAI) timed artificial insemination for the first AI and clean-up bulls; (R30) TAI for first AI with resynchronization onset 30 d days after TAI; (R22) TAI for first AI with resynchronization onset 22 d days after TAI; (R14) TAI for first AI with resynchronization onset 14 d days after TAI; and (RB21) TAI every 21 d. Included manuscripts: (Baruselli et al., 2017, Pessoa et al., 2018, Sa Filho et al., 2013, Gutierrez et al., 2014, Marques et al., 2015, Junior et al., 2021, Pugliesi et al., 2019, Palhao et al., 2020, da Silva et al., 2022, Faleiro et al., 2019, de Oliveira et al., 2018, Rodrigues et al., 2018, Andrade et al., 2024, Sa filho et al., 2014).

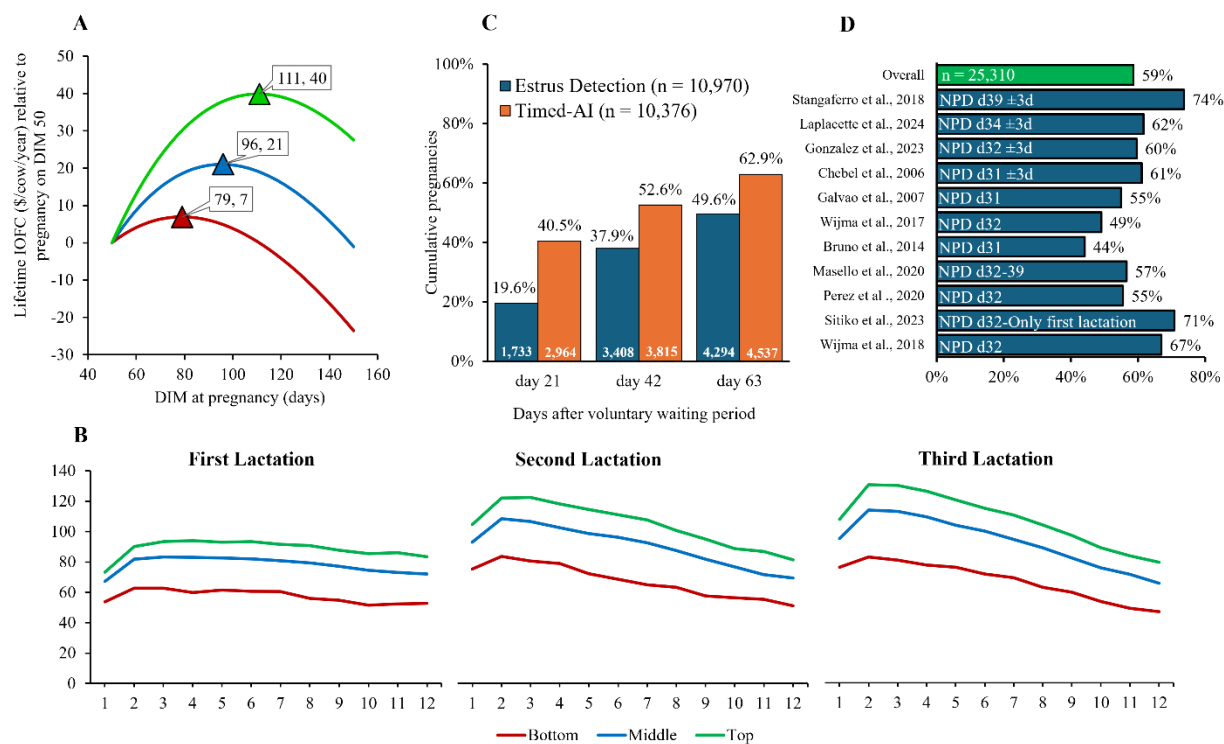


Figure 2

Figure 2. Panel A Partial budget analysis for the optimal time to pregnancy based on income over feed cost (IOFC) with three different levels of lactation curves. **Panel B** Lactation curve profiles for commercial herds in WI with herd average daily production lbs/cow/day of < 75 lbs (Bottom), ≥ 75 to < 95.0 lbs (Middle), and ≥ 95 lbs (Top). **Panel C** Cumulative pregnancies after the voluntary waiting period prioritizing AI after detection of estrus or Timed-AI from published manuscripts evaluating the long-term AI strategies (primiparous and multiparous). Included manuscripts: (Pursley et al., 1997, Galvao et al., 2007, Fricke et al., 2014, Burnett et al., 2017, Machado et al., 2017, Denis-Robichaud et al., 2018, Stangaferro et al., 2018, Rial et al., 2022, Gonzalez et al., 2023, Sitko et al., 2023, Laplacette et al., 2024). **Panel D** Percentage of nonpregnant cows inseminated after detection of estrus before the nonpregnant diagnosis. NDP = day of nonpregnant diagnosis.

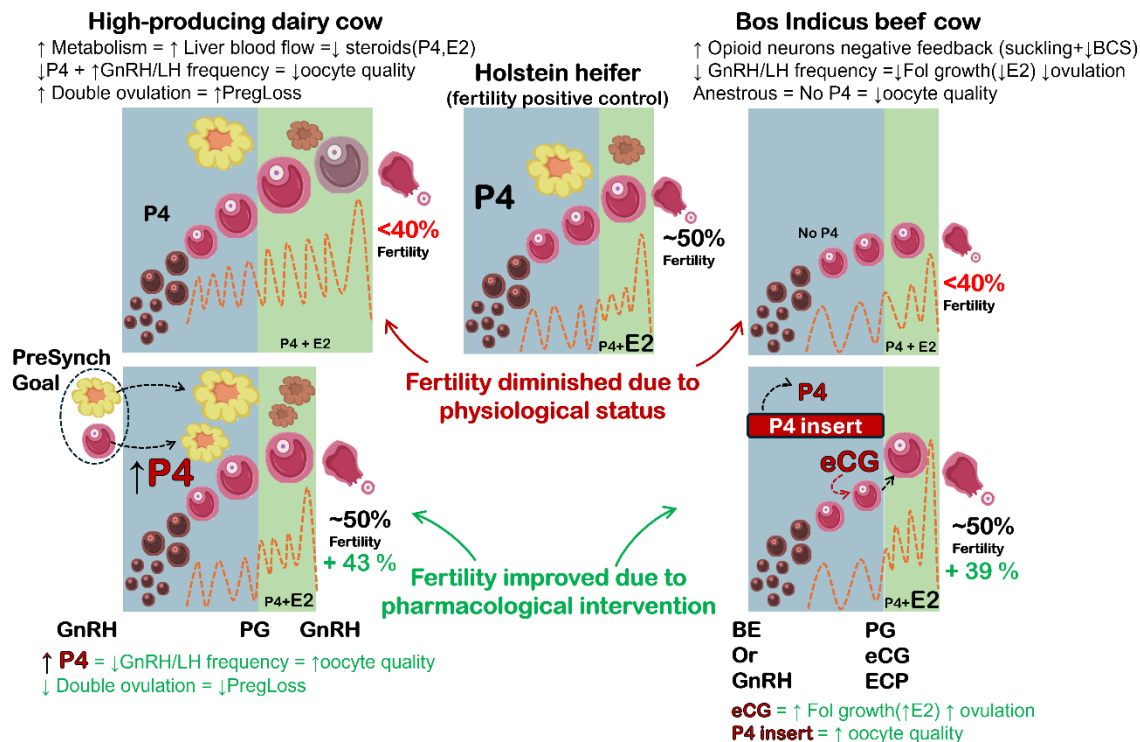


Figure 3

Figure 3. Model of optimal hormonal milieu to achieve high pregnancy per service in beef or dairy cows. **High-producing dairy cow. Hormonal disruption:** high clearance of circulating steroid hormones (progesterone and estradiol) due to high metabolism increases GnRH/LH frequency, resulting in prolonged follicular development, delayed and double ovulation, low-quality oocytes, and lower fertility per AI and high pregnancy loss. **Exogenous hormonal intervention:** Resynchronization (PreSynch) aims to concentrate the number of cows at day seven of the estrous cycle at the initiation of the synchronization protocol. Thus, most cows will have a mature corpus luteum (CL), assuring high progesterone to decrease the GnRH/LH frequency by negative feedback in the hypothalamus, decreasing follicular growth and double ovulation. **Bos indicus beef cattle. Hormonal disruption:** negative feedback at hypothalamus on GnRH/LH release (calf presence, low body condition score, and/or low energy intake) triggers a sequence of negative effects (reduced follicular growth, lower circulating estradiol during the follicular dominance phase, lack of estradiol-positive GnRH feedback, lower amplitude/surge of LH, anovulatory condition, and/or smaller ovulatory follicle which results in smaller corpus luteum [lower circulating progesterone in diestrus]) that culminates in lower fertility per AI. **Exogenous hormonal intervention:** Synchronization of a new follicular wave emergence with eCG treatment that binds to LH/FSH receptors, increases follicular growth, and indirectly mitigates the negative feedback effect of GnRH release.

A - Resynch programs in dairy cows

Resynch32-NPD d39							Resynch32-NPD d32							Resynch25						
Sun	Mon	Tue	Wed	Thurs	Fri	Sat	Sun	Mon	Tue	Wed	Thurs	Fri	Sat	Sun	Mon	Tue	Wed	Thurs	Fri	Sat
				1 st TAI							1 st TAI							1 st TAI		
				d7							d7							d7		
				d14							d14							d14		
				d21							d21							d21		
				d28							d28							d28		
				d35							d35							d35		
				G							G							G		
				NPD+P							NPD+G							NPD+P		
				d39							d32							d32		
				P							P							P		
				d40							d40							d40		
				G							G							G		
				d41							d41							d41		
				2 nd TAI							2 nd TAI							2 nd TAI		
				d42							d42							d42		
				d35							d35							d35		
				NPD+EB							NPD+EB							NPD+EB		
				d30							d30							d30		
				ECR+eCG							ECR+eCG							ECR+eCG		
				d38							d38							d38		
				2 nd TAI							2 nd TAI							2 nd TAI		
				d40							d40							d40		

B - Resynch programs in beef cattle

Resynch30							Resynch22							Resynch14							ReBreed21						
Sun	Mon	Tue	Wed	Thurs	Fri	Sat	Sun	Mon	Tue	Wed	Thurs	Fri	Sat	Sun	Mon	Tue	Wed	Thurs	Fri	Sat	Sun	Mon	Tue	Wed	Thurs	Fri	Sat
				1 st TAI							1 st TAI							1 st TAI							1 st TAI		
				d7							d7							d7							d7		
				d14							d14							d14							d14		
				d21							d21							d21							d21		
				d28							d28							d28							d28		
				d35							d35							d35							d35		
				NPD+EB							NPD+EB							NPD+EB							NPD+EB		
				d30							d30							d30							d30		
				ECR+eCG							ECR+eCG							ECR+eCG							ECR+eCG		
				d38							d38							d38							d38		
				2 nd TAI							2 nd TAI							2 nd TAI							2 nd TAI		
				d40							d40							d40							d40		
				PA Insemt.							PA Insemt.							PA Insemt.							PA Insemt.		
				d35							d35							d35							d35		
				EB							EB							EB							EB		
				d22							d22							d22							d22		
				PA Insemt.							PA Insemt.							PA Insemt.							PA Insemt.		
				d28							d28							d28							d28		
				NPD+CG							NPD+CG							NPD+CG							NPD+CG		
				d21							d21							d21							d21		
				ECR+eCG							ECR+eCG							ECR+eCG							ECR+eCG		
				d19							d19							d19							d19		
				2 nd TAI							2 nd TAI							2 nd TAI							2 nd TAI		
				d24							d24							d24							d24		
				EB							EB							EB							EB		
				d14							d14							d14							d14		
				PA Insemt.							PA Insemt.							PA Insemt.							PA Insemt.		
				d21							d21							d21							d21		
				ECR+eCG							ECR+eCG							ECR+eCG							ECR+eCG		
				d22							d22							d22							d22		
				NPD+CG							NPD+CG							NPD+CG							NPD+CG		
				d21							d21							d21							d21		
				ECR+eCG							ECR+eCG							ECR+eCG							ECR+eCG		
				d22							d22							d22							d22		
				2 nd TAI							2 nd TAI							2 nd TAI							2 nd TAI		
				d24							d24							d24							d24		

Figure 4

Figure 4. Description and schedule organization of the most used Resynch programs for dairy and beef cows, with performance validated in scientifically published manuscripts. Abbreviations: TAI = timed artificial insemination, NDP = nonpregnancy diagnosis, G = GnRH analogs, P = prostaglandin F2α analogs, EB = estradiol benzoate, ECP = estradiol cypionate, eCG = equine chorionic gonadotrophin.

References

- [1] Carvalho PD, Santos VG, Giordano JO, Wiltbank MC, Fricke PM. Development of fertility programs to achieve high 21-day pregnancy rates in high-producing dairy cows. *Theriogenology*. 2018;114:165-72.
- [2] Baruselli PS, Ferreira RM, Colli MHA, Elliff FM, Sa MF, Vieira L, et al. Timed artificial insemination: current challenges and recent advances in reproductive efficiency in beef and dairy herds in Brazil. *Anim Reprod*. 2017;14:558-71.
- [3] Ginther OJ, Kot K, Kulick LJ, Wiltbank MC. Emergence and deviation of follicles during the development of follicular waves in cattle. *Theriogenology*. 1997;48:75-87.
- [4] Ginther OJ, Wiltbank MC, Fricke PM, Gibbons JR, Kot K. Selection of the dominant follicle in cattle. *Biol Reprod*. 1996;55:1187-94.
- [5] Pursley JR, Mee MO, Wiltbank MC. Synchronization of ovulation in dairy cows using PGF2alpha and GnRH. *Theriogenology*. 1995;44:915-23.
- [6] Consentini CEC, Wiltbank MC, Sartori R. Factors That Optimize Reproductive Efficiency in Dairy Herds with an Emphasis on Timed Artificial Insemination Programs. *Animals*. 2021;11:301.
- [7] Monteiro PL, Consentini CEC, Andrade JPN, Beard AD, Garcia-Guerra A, Sartori R, et al. Research on timed AI in beef cattle: Past, present and future, a 27-year perspective. *Theriogenology*. 2023; 2011:161-71
- [8] Sala RV, Melo LF, Motta JCL, Leffers-Neto L, Carrenho-Sala LC, Fosado M, et al. Optimization of a 5-day fixed-time embryo transfer (FTET) protocol in heifers I. Manipulation of circulating progesterone through reutilization of intravaginal progesterone devices during FTET. *Theriogenology*. 2020;156:171-80.

- [9] Wiltbank MC, Baez GM, Vasconcelos JLM, Pereira M, Souza AH, Sartori R, et al. The physiology and impact on fertility of the period of proestrus in lactating dairy cows. *Anim Reprod.* 2014;11:225-36.
- [10] Lauber M, Fricke P. The association between insemination eligibility and reproductive performance of nulliparous heifers on subsequent body weight and milk production of primiparous Holstein cows. *JDS communications.* 2023;4:428-32.
- [11] Funston R, Deutscher G. Comparison of target breeding weight and breeding date for replacement beef heifers and effects on subsequent reproduction and calf performance. *J Anim Sci.* 2004;82:3094-99.
- [12] Inchaisri C, Jorritsma R, Vos P, van der Weijden GC, Hogeveen H. Analysis of the economically optimal voluntary waiting period for first insemination. *J Dairy Sci.* 2011;94:3811-23.
- [13] Fricke P, Wiltbank M, Pursley J. Mini-Review: The high fertility cycle. *JDS Communications.* 2022; 4:127-31.
- [14] Baruselli PS, Reis EL, Marques MO, Nasser LF, Bo GA. The use of hormonal treatments to improve reproductive performance of anestrus beef cattle in tropical climates. *Anim Reprod Sci.* 2004;82-83:479-86.
- [15] Pursley JR, Kosorok MR, Wiltbank MC. Reproductive management of lactating dairy cows using synchronization of ovulation. *J Dairy Sci.* 1997;80:301-06.
- [16] Stevenson JS, Johnson SK, Medina-Britos MA, Richardson-Adams AM, Lamb GC. Resynchronization of estrus in cattle of unknown pregnancy status using estrogen, progesterone, or both. *J Anim Sci.* 2003;81:1681-92.

[17] Fricke PM, Ricci A, Giordano JO, Carvalho PD. Methods for and Implementation of Pregnancy Diagnosis in Dairy Cows. *Vet Clin North Am Food Anim Pract.* 2016;32:165-80.

[18] Pugliesi G, Miagawa BT, Paiva YN, Franca MR, Silva LA, Binelli M. Conceptus-induced changes in the gene expression of blood immune cells and the ultrasound-accessed luteal function in beef cattle: how early can we detect pregnancy? *Biol Reprod.* 2014;91:95.

[19] Reese ST, Pereira MC, Vasconcelos JLM, Smith MF, Green JA, Geary TW, et al. Markers of pregnancy: how early can we detect pregnancies in cattle using pregnancy-associated glycoproteins (PAGs) and microRNAs? *Anim Reprod.* 2016;13:200-08.

[20] Wiltbank JN, Priode BM, Vernon EH, Warwick EJ. Factors affecting net calf crop in beef cattle. *J Anim Sci.* 1961;20:409.

[21] Fontes PL, Oosthuizen N, Lamb GC. Reproductive management of beef cattle. *Anim Agric.* 2020; 57-73.

[22] Funston R, Grings E, Roberts A, Tibbitts B. Invited Review: Choosing a calving date. *The Professional Animal Scientist.* 2016;32:145-53.

[23] Burriss MJ, Priode BM. Effect of calving date on subsequent calving performance. *J Anim Sci.* 1958;17:527-33.

[24] Wiltbank JN, Ingalls JE, Gregory KE, Koch RM, Rowden WW. Effect of energy level on reproductive phenomena of mature hereford cows. *J Anim Sci.* 1962;21:219.

[25] Cooke RF, Lamb GC, Vasconcelos JLM, Pohler KG. Effects of body condition score at initiation of the breeding season on reproductive performance and overall productivity of *Bos taurus* and *B. indicus* beef cows. *Anim Reprod Sci.* 2021;232.

- [26] Shane DD, Larson RL, Sanderson MW, Miesner M, White BJ. A deterministic, dynamic systems model of cow-calf production: The effects of the duration of postpartum anestrus on production parameters over a 10-year horizon. *J Anim Sci.* 2017;95:1680-95.
- [27] Ojeda-Rojas OA, Gonella-Diaza AM, Bustos-Coral D, Sartorello GL, Reijers T, Pugliesi G, et al. An agent-based simulation model to compare different reproductive strategies in cow-calf operations: Technical performance. *Theriogenology.* 2021;160:102-15.
- [28] Paloma JAG, Alberio R, Miquel MC, Grondona MO, Carrillo J, Schiersmann G. Effect of calving date on lifetime productivity of cows in a winter-calving aberdeen angus herd. *Animal Production.* 1992;55:177-84.
- [29] Funston RN, Musgrave JA, Meyer TL, Larson DM. Effect of calving distribution on beef cattle progeny performance. *J Anim Sci.* 2012;90:5118-21.
- [30] Grings EE, Short RE, Klement KD, Geary TW, MacNeil MD, Haferkamp MR, et al. Calving system and weaning age effects on cow and preweaning calf performance in the Northern Great Plains. *J Anim Sci.* 2005;83:2671-83.
- [31] Bocchi AL, Teixeira RA, de Albuquerque LG. Idade da vaca e mês de nascimento sobre o peso ao desmame de bezerros nelore nas diferentes regiões brasileiras. *Acta Scientiarum Animal Sciences.* 2004;26:475-82.
- [32] Paz CCPd, Albuquerque LGd. Efeitos ambientais sobre ganho de peso no período do nascimento ao desmame em bovinos da raça Nelore. *Revista Brasileira de Zootecnia.* 1999;28:55-64.
- [33] Montiel F, Ahuja C. Body condition and suckling as factors influencing the duration of postpartum anestrus in cattle: a review. *Anim Reprod Sci.* 2005;85:1-26.

- [34] Wheaton JE, Lamb GC. Induction of cyclicity in postpartum anestrous beef cows using progesterone, GnRH and estradiol cypionate (ECP). *Anim Reprod Sci.* 2007;102:208-16.
- [35] Nishimura TK, Martins T, da Silva MI, Lafuente BS, Maio JRD, Binelli M, et al. Importance of body condition score and ovarian activity on determining the fertility in beef cows supplemented with long-acting progesterone after timed-AI. *Anim Reprod Sci.* 2018;198:27-36.
- [36] Yavas Y, Walton JS. Postpartum acyclicity in suckled beef cows: a review. *Theriogenology.* 2000;54:25-55.
- [37] Kasimanickam RK, Kasimanickam VR, Oldham J, Whitmore M. Cyclicity, estrus expression and pregnancy rates in beef heifers with different reproductive tract scores following progesterone supplementation. *Theriogenology.* 2020;145:39-47.
- [38] Baruselli PS, de Abreu LA, de Paula VR, Carvalho B, Gricio EA, Mori FK, et al. Applying assisted reproductive technology and reproductive management to reduce CO₂-equivalent emission in dairy and beef cattle: a review. *Anim Reprod.* 2023;20.
- [39] Fordyce G, Williams P, Corbet NJ, Costilla R, McGowan MR. Pregnancy rate per cycle is heritable and reduces with cycle in naturally mated tropically adapted beef cows. *Reprod Domest Anim.* 2021;56:1286-92.
- [40] Griffith MK, Williams GL. Roles of maternal vision and olfaction in suckling-mediated inhibition of luteinizing hormone secretion, expression of maternal selectivity, and lactational performance of beef cows. *Biol Reprod.* 1996;54:761-68.
- [41] Walters DL, Kaltenbach CC, Dunn TG, Short RE. Pituitary and ovarian-function in postpartum beef-cows .1. Effect of suckling on serum and follicular-fluid hormones and follicular gonadotropin receptors. *Biol Reprod.* 1982;26:640-46.

- [42] Walters DL, Short RE, Convey EM, Staigmiller RB, Dunn TG, Kaltenbach CC. Pituitary and ovarian-function in postpartum beef-cows .2. Endocrine changes prior to ovulation in suckled and non-suckled postpartum cows compared to cycling cows. *Biol Reprod.* 1982;26:647-54.
- [43] Grimard B, Humblot P, Ponter AA, Mialot JP, Sauvant D, Thibier M. Influence of postpartum energy restriction on energy status, plasma-lh and estradiol secretion and follicular development in suckled beef-cows. *J Reprod Fertil.* 1995;104:173-79.
- [44] Wiltbank JN, Cook AC. The comparative reproductive performance of nursed cows and milked cows. *J Anim Sci.* 1958;17:640-48.
- [45] Fontes PLP, Oosthuizen N, Ciriaco FM, Sanford CD, Canal LB, Cooke RF, et al. Effects of nutrient restriction on the metabolic profile of *Bos indicus*-influenced and *B. taurus* suckled beef cows. *Animal.* 2021;15.
- [46] Sartori R, Gimenes LU, Monteiro PLJ, Melo LF, Baruselli PS, Basto MR. Metabolic and endocrine differences between *Bos taurus* and *Bos indicus* females that impact the interaction of nutrition with reproduction. *Theriogenology.* 2016;86:32-40.
- [47] Smith MF, Lishman AW, Lewis GS, Harms PG, Ellersieck MR, Inskoop EK, et al. Pituitary and ovarian responses to gonadotropin-releasing hormone, calf removal and progesterone in anestrous beef-cows. *J Anim Sci.* 1983;57:418-24.
- [48] Waltens D, Smith M, Harms P, Wiltbank J. Effect of steroids and/or 48 HR calf removal on serum luteinizing hormone concentrations in anestrous beef cows. *Theriogenology.* 1982;18:349-56.

- [49] Mondragón V, Galina CS, Rubio I, Corro M, Salmerón F. Effect of restricted suckling on the onset of follicular dynamics and body condition score in Brahman cattle raised under tropical conditions. *Anim Reprod Sci.* 2016;167:89-95.
- [50] Wright IA, Rhind SM, Whyte TK, Smith AJ. Effects of body condition at calving and feeding level after calving on lh profiles and the duration of the postpartum anestrous period in beef-cows. *Animal Production.* 1992;55:41-46.
- [51] D'Occhio MJ, Baruselli PS, Campanile G. Influence of nutrition, body condition, and metabolic status on reproduction in female beef cattle: A review. *Theriogenology.* 2019;125:277-84.
- [52] Lopes CN, Scarpa AB, Cappellozza BI, Cooke RF, Vasconcelos JLM. Effects of rumen-protected polyunsaturated fatty acid supplementation on reproductive performance of *Bos indicus* beef cows. *J Anim Sci.* 2009;87:3935-43.
- [53] Lopes CN, Cooke RF, Reis MM, Peres RFG, Vasconcelos JLM. Strategic supplementation of calcium salts of polyunsaturated fatty acids to enhance reproductive performance of *Bos indicus* beef cows. *J Anim Sci.* 2011;89:3116-24.
- [54] Cordeiro MB, Peres MS, de Souza JM, Gaspar P, Barbieri F, Sá MF, et al. Supplementation with sunflower seed increases circulating cholesterol concentrations and potentially impacts on the pregnancy rates in *Bos indicus* beef cattle. *Theriogenology.* 2015;83:1461-68.
- [55] Perez GC, Vasconcelos JLM, Santos RM, Pereira ETN, Sa OG. Effect of eCG and/or calf removal after CRESTAR protocol in Nellore cows. *J Anim Sci.* 2004;82:371-71.
- [56] Sa Filho MF, Ayres H, Ferreira RM, Marques MO, Reis EL, Silva RCP, et al. Equine chorionic gonadotropin and gonadotropin-releasing hormone enhance fertility in a

norgestomet-based, timed artificial insemination protocol in suckled Nelore (*Bos indicus*) cows. *Theriogenology*. 2010;73:651-58.

[57] Sa Filho MF, Crespilho AM, Santos JE, Perry GA, Baruselli PS. Ovarian follicle diameter at timed insemination and estrous response influence likelihood of ovulation and pregnancy after estrous synchronization with progesterone or progestin-based protocols in suckled *Bos indicus* cows. *Anim Reprod Sci*. 2010;120:23-30.

[58] Sa Filho MF, Torres JRS, Penteadó L, Gimenes LU, Ferreira RM, Ayres H, et al. Equine chorionic gonadotropin improves the efficacy of a progestin-based fixed-time artificial insemination protocol in Nelore (*Bos indicus*) heifers. *Anim Reprod Sci*. 2010;118:182-87.

[59] Sales JNS, Bottino MP, Silva L, Giroto RW, Massoneto JPM, Souza JC, et al. Effects of eCG are more pronounced in primiparous than multiparous *Bos indicus* cows submitted to a timed artificial insemination protocol. *Theriogenology*. 2016;86:2290-95.

[60] Kasimanickam R, Kasimanickam V, Kappes A. Timed artificial insemination strategies with or without short-term natural service and pregnancy success in beef heifers. *Theriogenology*. 2021;166:97-103.

[61] Pessoa GA, Martini AP, Sa MF, Rubin MI. Resynchronization improves reproductive efficiency of suckled *Bos taurus* beef cows subjected to spring-summer or autumn-winter breeding season in South Brazil. *Theriogenology*. 2018;122:14-22.

[62] Rodgers JC, Bird SL, Larson JE, Dilorenzo N, Dahlen CR, Dicostanzo A, et al. An economic evaluation of estrous synchronization and timed artificial insemination in suckled beef cows. *J Anim Sci*. 2012;90:4055-62.

- [63] Alves R, Silva MA, Consentini CEC, Silva LOE, Folchini NP, Oliva AL, et al. Hormonal combinations aiming to improve reproductive outcomes of *Bos indicus* cows submitted to estradiol/progesterone-based timed AI protocols. *Theriogenology*. 2021;169:89-99.
- [64] Sa Filho MF, Penteadó L, Reis EL, Reis T, Galvão KN, Baruselli PS. Timed artificial insemination early in the breeding season improves the reproductive performance of suckled beef cows. *Theriogenology*. 2013;79:625-32.
- [65] Gutierrez K, Kasimanickam R, Tibary A, Gay JM, Kastelic JP, Hall JB, et al. Effect of reproductive tract scoring on reproductive efficiency in beef heifers bred by timed insemination and natural service versus only natural service. *Theriogenology*. 2014;81:918-24.
- [66] Ramsey R, Doye D, Ward C, McGrann J, Falconer L, Bevers S. Factors affecting beef cow-herd costs, production, and profits. *J Agric Appl Econ*. 2005;37:91-99.
- [67] Ojeda-Rojas OA, Bustos-Coral D, Sartorello GL, Reijers TSSS, Pugliesi G, Mercadante MEZ, et al. An agent-based simulation model to compare different reproductive strategies in cow-calf operations: Economic performance. *Theriogenology*. 2022;189:11-19.
- [68] Fricke PM. Scanning the future - Ultrasonography as a reproductive management tool for dairy cattle. *J Dairy Sci*. 2002;85:1918-26.
- [69] Marques MD, Morotti F, da Silva CB, Ribeiro M, da Silva RCP, Baruselli PS, et al. Influence of category - heifers, primiparous and multiparous lactating cows - in a large-scale resynchronization fixed-time artificial insemination program. *J Vet Sci*. 2015;16:367-71.
- [70] Baruselli PS, Ferreira RM, Sá MF, Bó GA. Review: Using artificial insemination vs.. natural service in beef herds. *Animal*. 2018;12:S45-S52.

- [71] Sa Filho M, Marques M, Girotto R, Santos F, Sala R, Barbuio J, et al. Resynchronization with unknown pregnancy status using progestin-based timed artificial insemination protocol in beef cattle. *Theriogenology*. 2014;81:284-90.
- [72] Pugliesi G, Oliveria ML, Scolari SC, Lopes E, Pinaffi FV, Miagawa BT, et al. Corpus luteum development and function after supplementation of long-acting progesterone during the early luteal phase in beef cattle. *Reprod Domest Anim*. 2014;49:85-91.
- [73] Pugliesi G, Bisinotto DZ, Mello BP, Lahr FC, Ferreira CA, Melo GD, et al. A novel strategy for resynchronization of ovulation in Nelore cows using injectable progesterone (P4) and P4 releasing devices to perform two timed inseminations within 22 days. *Reprod Domest Anim*. 2019;54:1149-54.
- [74] da Silva AG, Nishimura TK, Rocha CC, Motta IG, Neto AL, Ferraz PA, et al. Comparison of estradiol benzoate doses for resynchronization of ovulation at 14 days after timed-AI in suckled beef cows. *Theriogenology*. 2022;184:41-50.
- [75] Motta I, Rocha C, Bisinotto D, Melo G, Júnior GA, Silva A, et al. Increased pregnancy rate in beef heifers resynchronized with estradiol at 14 days after TAI. *Theriogenology*. 2020;147:62-70.
- [76] Vieira CC, Pinto HF, Buss V, de Freitas BG, Guerreiro BM, Leivas FG, et al. Resynchronization of follicular wave using long-acting injectable progesterone or estradiol benzoate at 14 days post-timed AI in *Bos taurus x Bos indicus*. *Theriogenology*. 2021;176:194-99.
- [77] Youngquist RS, Bierschwal CJ, Elmore RG, Jenkins AL, Schultz RH, Widmer JH. Induction of abortion in feedlot Heifers with Cloprostenol (ICI 80,996). *Theriogenology*. 1977;7:305-12.

[78] Copeland DD, Schultz RH, Kemtrup ME. Induction of Abortion in Feedlot Heifers with Cloprostenol (A Synthetic Analog of Prostaglandin-F2alpha) - Dose-Response Study. *Can Vet J-Rev Vet Can.* 1978;19:29-32.

[79] Andrade JPN, Andrade FS, Guerson YB, Domingues RR, Gomez-Leon VE, Cunha TO, et al. Early pregnancy diagnosis at 21 days post artificial insemination using corpus luteum vascular perfusion compared to corpus luteum diameter and/or echogenicity in Nelore heifers. *Anim Reprod Sci.* 2019;209:106144.

[80] Andrade JPN, Gomez-Leon VE, Andrade FS, Carvalho BP, Lacouth KL, Garcia FZ, et al. Development of a novel 21-day reinsemination program, ReBreed21, in *Bos indicus* heifers. *Theriogenology.* 2020;155:125-31.

[81] Andrade JPN, Domingues RR, Carvalho BP, Gomez-Leon V, Prata AB, Sartori R, et al. Optimizing ReBreed21 I: Evaluation of endocrine and ovarian dynamics in non-bred *Bos indicus* heifers. *Theriogenology.* 2024;220:77-83.

[82] Andrade JPN, Monteiro PJJ, Prata AB, Robl AJ, Neto J, Lippe B, et al. Optimizing ReBreed21 II: Fertility and reproductive efficiency in different parities during a shortened breeding season in beef cattle. *Theriogenology.* 2024;224:41-49.

[83] Baruselli PS, de Souza AH, de Sa MF, Marques MO, Sales JND. Genetic market in cattle (Bull, AI, FTAI, MOET and IVP): financial payback based on reproductive efficiency in beef and dairy herds in Brazil. *Anim Reprod.* 2018;15:247-55.

[84] Bo GA, de la Mata JJ, Baruselli PS, Menchaca A. Alternative programs for synchronizing and resynchronizing ovulation in beef cattle. *Theriogenology.* 2016;86:388-96.

[85] Giordano JO, Fricke M, Wiltbank MC, Cabrera VE. An economic decision-making model for comparing reproductive management programs in dairy herds. *J Dairy Sci.* 2010;93:2301-01.

[86] Giordano JO, Fricke PM, Wiltbank MC, Cabrera VE. A stochastic evaluation of reproductive management programs for dairy herds. *J Dairy Sci.* 2010;93:752-52.

[87] Souza AH, Carvahlo PA, Shaver RD, Wiltbank MC, Cabrera V. Effect of timed AI use on reproductive performance and culling rate in Wisconsin dairy herds. *J Dairy Sci.* 2013;96:390.

[88] Schefers JM, Weigel KA, Rawson CL, Zwald NR, Cook NB. Management practices associated with conception rate and service rate of lactating Holstein cows in large, commercial dairy herds. *J Dairy Sci.* 2010;93:1459-67.

[89] De Vries A, Marcondes MI. Review: Overview of factors affecting productive lifespan of dairy cows. *Animal.* 2020;14:S155-S64.

[90] Cabrera VE. The economic value of changes in 21-day pregnancy rate and what controls this value. 21st American Dairy Science Association Discover Conference: Improving Reproductive Efficiency of Lactating Dairy Cows 2011.

[91] Galvao KN, Sa MF, Santos JEP. Reducing the interval from presynchronization to initiation of timed artificial insemination improves fertility in dairy cows. *J Dairy Sci.* 2007;90:4212-18.

[92] Sitko EM, Perez MM, Granados GE, Masello M, Hernandez S, Cabrera EM, et al. Effect of reproductive management programs that prioritized artificial insemination at detected estrus or timed artificial insemination on the reproductive performance of primiparous Holstein cows of different genetic merit for fertility. *J Dairy Sci.* 2023;106:6476-94.

[93] Rial C, Laplacette A, Giordano J. Effect of a targeted reproductive management program designed to prioritize insemination at detected estrus and optimize time to insemination on the reproductive performance of lactating dairy cows. *J Dairy Sci.* 2022;105:8411-25.

[94] Arbel R, Bigun Y, Ezra E, Sturman H, Hojman D. The effect of extended calving intervals in high-yielding lactating cows on milk production and profitability. *J Dairy Sci.* 2001;84:600-08.

[95] vanAmburgh ME, Galton DM, Bauman DE, Everett RW. Management and economics of extended calving intervals with use of bovine somatotropin. *Livestock Production Science.* 1997;50:15-28.

[96] Bauman DE, Vernon RG. Effects of exogenous bovine somatotropin on lactation. *Annual Review of Nutrition.* 1993;13:437-61.

[97] Chalupa W, Galligan DT. Nutritional implications of somatotropin for lactating cows. *J Dairy Sci.* 1989;72:2510-24.

[98] Sorensen JT, Ostergaard S. Economic consequences of postponed first insemination of cows in a dairy cattle herd. *Livestock Production Science.* 2003;79:145-53.

[99] Gobikrushanth M, De Vries A, Santos JEP, Risco CA, Galvao KN. Effect of delayed breeding during the summer on profitability of dairy cows. *J Dairy Sci.* 2014;97:4236-46.

[100] Niozas G, Tsousis G, Steinhofel I, Brozos C, Römer A, Wiedemann S, et al. Extended lactation in high-yielding dairy cows. I. Effects on reproductive measurements. *J Dairy Sci.* 2019;102:799-810.

[101] Niozas G, Tsousis G, Malesios C, Steinhofel I, Boscós C, Bollwein H, et al. Extended lactation in high-yielding dairy cows. II. Effects on milk production, udder health, and body measurements. *J Dairy Sci.* 2019;102:811-23.

[102] Stangaferro ML, Wijma R, Masello M, Giordano JO. Reproductive performance and herd exit dynamics of lactating dairy cows managed for first service with the Presynch-Ovsynch or Double-Ovsynch protocol and different duration of the voluntary waiting period. *J Dairy Sci.* 2018;101:1673-86.

[103] Stangaferro ML, Wijma RW, Giordano JO. Profitability of dairy cows submitted to the first service with the Presynch-Ovsynch or Double-Ovsynch protocol and different duration of the voluntary waiting period. *J Dairy Sci.* 2019;102:4546-62.

[104] De Vries A. Ranking dairy cows for optimal breeding decisions. *Proceedings 43rd Florida Dairy Production Conference 2006.* p. 49-66.

[105] Wiltbank MC, Sartori R, Herlihy MM, Vasconcelos JL, Nascimento AB, Souza AH, et al. Managing the dominant follicle in lactating dairy cows. *Theriogenology.* 2011;76:1568-82.

[106] Bisinotto RS, Lean IJ, Thatcher WW, Santos JEP. Meta-analysis of progesterone supplementation during timed artificial insemination programs in dairy cows. *J Dairy Sci.* 2015;98:2472-87.

[107] Sangsritavong S, Combs DK, Sartori R, Armentano LE, Wiltbank MC. High feed intake increases liver blood flow and metabolism of progesterone and estradiol-17 beta in dairy cattle. *J Dairy Sci.* 2002;85:2831-42.

[108] Wiltbank M, Lopez H, Sartori R, Sangsritavong S, Gumen A. Changes in reproductive physiology of lactating dairy cows due to elevated steroid metabolism. *Theriogenology.* 2006;65:17-29.

[109] Lopez H, Satter LD, Wiltbank MC. Relationship between level of milk production and estrous behavior of lactating dairy cows. *Anim Reprod Sci.* 2004;81:209-23.

[110] Souza AH, Ayres H, Ferreira RM, Wiltbank MC. A new presynchronization system (Double-Ovsynch) increases fertility at first postpartum timed AI in lactating dairy cows.

Theriogenology. 2008;70:208-15.

[111] Moreira F, Orlandi C, Risco CA, Mattos R, Lopes F, Thatcher WW. Effects of presynchronization and bovine somatotropin on pregnancy rates to a timed artificial insemination protocol in lactating dairy cows. J Dairy Sci. 2001;84:1646-59.

[112] Ayres H, Ferreira RM, Cunha AP, Araujo RR, Wiltbank MC. Double-Ovsynch in high-producing dairy cows: Effects on progesterone concentrations and ovulation to GnRH treatments. Theriogenology. 2013;79:159-64.

[113] Santos VG, Carvalho PD, Maia C, Carneiro B, Valenza A, Fricke PM. Fertility of lactating Holstein cows submitted to a Double-Ovsynch protocol and timed artificial insemination versus artificial insemination after synchronization of estrus at a similar day in milk range. J Dairy Sci. 2017;100:8507-17.

[114] Fricke PM, Wiltbank MC. *Symposium review*: The implications of spontaneous versus synchronized ovulations on the reproductive performance of lactating dairy cows. J Dairy Sci. 2022;105:4679-89.

[115] Wiltbank MC, Baez GM, Garcia-Guerra A, Toledo MZ, Monteiro PLJ, Melo LF, et al. Pivotal periods for pregnancy loss during the first trimester of gestation in lactating dairy cows. Theriogenology. 2016;86:239-53.

[116] Fricke PM, Caraviello DZ, Weigel KA, Welle ML. Fertility of dairy cows after resynchronization of ovulation at three intervals following first timed insemination. J Dairy Sci. 2003;86:3941-50.

[117] Lopes G, Jr., Giordano JO, Valenza A, Herlihy MM, Guenther JN, Wiltbank MC, et al. Effect of timing of initiation of resynchronization and presynchronization with gonadotropin-releasing hormone on fertility of resynchronized inseminations in lactating dairy cows. *J Dairy Sci.* 2013;96:3788-98.

[118] Giordano JO, Thomas MJ, Catucuamba G, Curler MD, Masello M, Stangaferro ML, et al. Reproductive management strategies to improve the fertility of cows with a suboptimal response to resynchronization of ovulation. *J Dairy Sci.* 2016;99:2967-78.

[119] Bartolome JA, Silvestre FT, Kamimura S, Arteché AC, Melendez P, Kelbert D, et al. Resynchronization of ovulation and timed insemination in lactating dairy cows I: use of the Ovsynch and Heatsynch protocols after non-pregnancy diagnosis by ultrasonography. *Theriogenology.* 2005;63:1617-27.

[120] Wisnicky W, Casida LE. A manual method for the diagnosis of pregnancy in cattle. *J Am Vet Med Assoc.* 1948;113:451-52.

[121] Mayo LM, Moore SG, Poock SE, Silvia WJ, Lucy MC. *Technical note:* Validation of a chemical pregnancy test in dairy cows that uses whole blood, shortened incubation times, and visual readout. *J Dairy Sci.* 2016;99:7634-41.

[122] Zoli AP, Guilbault LA, Delahaut P, Ortiz WB, Beckers JF. Radioimmunoassay of a bovine pregnancy-associated glycoprotein in serum - its application for pregnancy diagnosis. *Biol Reprod.* 1992;46:83-92.

[123] Giordano JO, Wiltbank MC, Guenther JN, Ares MS, Lopes G, Jr., Herlihy MM, et al. Effect of presynchronization with human chorionic gonadotropin or gonadotropin-releasing hormone 7 days before resynchronization of ovulation on fertility in lactating dairy cows. *J Dairy Sci.* 2012;95:5612-25.

[124] Giordano JO, Wiltbank MC, Guenther JN, Pawlisch R, Bas S, Cunha AP, et al. Increased fertility in lactating dairy cows resynchronized with Double-Ovsynch compared with Ovsynch initiated 32 d after timed artificial insemination. *J Dairy Sci* 2012;95:639-53.

[125] Bilby TR, Bruno RG, Lager KJ, Chebel RC, Moraes JG, Fricke PM, et al. Supplemental progesterone and timing of resynchronization on pregnancy outcomes in lactating dairy cows. *J Dairy Sci*. 2013;96:7032-42.

[126] Dewey ST, Mendonca LG, Lopes G, Jr., Rivera FA, Guagnini F, Chebel RC, et al. Resynchronization strategies to improve fertility in lactating dairy cows utilizing a presynchronization injection of GnRH or supplemental progesterone: I. Pregnancy rates and ovarian responses. *J Dairy Sci*. 2010;93:4086-95.

[127] Motavalli T, Dirandeh E, Deldar H, Colazo MG. Evaluation of shortened timed-AI protocols for resynchronization of ovulation in multiparous Holstein dairy cows. *Theriogenology*. 2017;95:187-92.

[128] Wijma R, Pérez MM, Masello M, Stangaferro ML, Giordano JO. A resynchronization of ovulation program based on ovarian structures present at nonpregnancy diagnosis reduced time to pregnancy in lactating dairy cows. *J Dairy Sci*. 2018;101:1697-707.

[129] Pérez MM, Wijma R, Scarbolo M, Cabrera E, Sosa F, Sitko EM, et al. Lactating dairy cows managed for second and greater artificial insemination services with the Short-Resynch or Day 25 Resynch program had similar reproductive performance. *J Dairy Sci*. 2020;103:10769-83.

[130] Stevenson JS, Sauls-Hiesterman JA. Resynchronizing the first eligible estrus in dairy cattle after a prior insemination and fertility of the prior insemination after gonadotropin-releasing hormone and progesterone treatments. *Theriogenology*. 2021;170:54-66.

[131] Wijma R, Stangaferro M, Masello M, Granados G, Giordano J. Resynchronization of ovulation protocols for dairy cows including or not including gonadotropin-releasing hormone to induce a new follicular wave: Effects on re-insemination pattern, ovarian responses, and pregnancy outcomes. *J Dairy Sci.* 2017;100:7613-25.

[132] Ribeiro ES, Cerri RL, Bisinotto RS, Lima FS, Silvestre FT, Greco LF, et al. Reproductive performance of grazing dairy cows following presynchronization and resynchronization protocols. *J Dairy Sci.* 2011;94:4984-96.

[133] Masello M, Ceglowski B, Thomas MJ, Giordano JO. A reproductive management program aimed at increasing reinsemination of nonpregnant dairy cows at detected estrus resulted in similar reproductive performance to a program that favored timed artificial insemination. *J Dairy Sci.* 2020;103:3719-29.

[134] Sinedino LDP, Lima FS, Bisinotto RS, Cerri RLA, Santos JEP. Effect of early or late resynchronization based on different methods of pregnancy diagnosis on reproductive performance of dairy cows. *J Dairy Sci.* 2014;97:4932-41.

[135] Fricke PM, Giordano JO, Valenza A, Lopes G, Jr., Amundson MC, Carvalho PD. Reproductive performance of lactating dairy cows managed for first service using timed artificial insemination with or without detection of estrus using an activity-monitoring system. *J Dairy Sci.* 2014;97:2771-81.

[136] Denis-Robichaud J, Cerri RLA, Jones-Bitton A, LeBlanc SJ. Performance of automated activity monitoring systems used in combination with timed artificial insemination compared to timed artificial insemination only in early lactation in dairy cows. *J Dairy Sci.* 2018;101:624-36.

[137] Machado VS, Neves RC, Lima FS, Bicalho RC. The effect of Presynch-Ovsynch protocol with or without estrus detection on reproductive performance by parity, and the long-term effect of these different management strategies on milk production, reproduction, health and survivability of dairy cows. *Theriogenology*. 2017;93:84-92.

[138] Gonzalez TD, Factor L, Mirzaei A, Montevecchio AB, Casaro S, Merenda VR, et al. Targeted reproductive management for lactating Holstein cows: Reducing the reliance on exogenous reproductive hormones. *J Dairy Sci*. 2023;106:5788-804.

[139] Fricke PM, Wiltbank MC. The implications of spontaneous versus synchronized ovulations on the reproductive performance of lactating dairy cows. *J Dairy Sci*. 2022;105:4679-89.

[140] Lauber MR, Peñagaricano F, Fourdraine RH, Clay JS, Fricke PM. Characterization of semen type prevalence and allocation in Holstein and Jersey females in the United States. *J Dairy Sci*. 2023;106:3748-60.

**Chapter 2: Optimizing ReBreed21 I: Evaluation of endocrine and ovarian dynamics in
non-bred *Bos indicus* heifers**

João Paulo N. Andrade^a; Rafael R. Domingues^{a,b}; Bruno P. Carvalho^c; Victor Gomez-Leon^{a,d};
Alexandre B. Prata^e; Roberto Sartori^f; Milo C. Wiltbank^{a*}

^a Department of Animal and Dairy Sciences, University of Wisconsin-Madison, Wisconsin
53706, USA

^b Current address: Department of Animal Sciences, The Ohio State University, Columbus, Ohio,
43210, USA.

^c Empresa Brasileira de Pesquisa Agropecuária-Rio Branco, Acre, 69900-970, Brazil

^d Current address: Department of Animal Sciences and Industry, Kansas State University,
Manhattan, Kansas 66506, USA.

^e GlobalGen Vet Science, Jaboticabal, SP, 14887-360, Brazil

^f Department of Animal Science, Luiz de Queiroz College of Agriculture (ESALQ), University
of São Paulo, Av. Pádua Dias, 11, Piracicaba, SP, 13418-900, Brazil

Corresponding Author: Milo C. Wiltbank

E-mail: wiltbank@wisc.edu

Published in Theriogenology

Andrade JPN, Domingues RR, Carvalho BP, Gomez-Leon V, Prata AB, Sartori R, et al.

Optimizing ReBreed21 I: Evaluation of endocrine and ovarian dynamics in non-bred *Bos indicus*
heifers. Theriogenology. 2024;220:77-83.

Abstract

The present study evaluated follicular and endocrine dynamics during ReBreed21, a reproductive strategy that allows resynchronization of ovulation every 21 days in *Bos indicus* (Nelore) heifers. A synchronized estrous cycle was induced using a standard timed ovulation protocol (d -10: P4 implant inserted + 2 mg estradiol benzoate; d -2: P4 removed+ 0.5 mg cloprostenol + 0.6 mg estradiol cypionate + 200 IU equine chorionic gonadotropin (eCG); d0: 8.4 µg buserelin) without AI to ensure nonpregnancy in heifers. Day of GnRH was designated d0 of estrous cycle. On d12, heifers (n=80) were randomized into three experimental groups: (1) ReBreed21 (n=28) d12 P4 device inserted, d19 P4 device withdrawal plus 200 IU eCG, and d21 8.4 µg buserelin (GnRH); (2) ReBreed21+G (n=26) same as ReBreed21 plus GnRH (16.8 µg) treatment on d12; and (3) Control (n=26) no treatment. ReBreed21+G increased two-fold (62.9%; 18/26) percentage of heifers with synchronized follicular wave emergence compared to Control (34.6%; 9/26) whereas ReBreed21 (53.6%; 15/28) was intermediate. The ReBreed21 groups (eCG on d19) increased ($P<0.01$) follicular growth between d19 and d21 in ReBreed21 (2.3 ± 0.2 mm) and ReBreed21+G (3.4 ± 0.2 mm) compared with Control (1.2 ± 0.3 mm), resulting in greater ($P<0.01$) follicle diameter on d21 for ReBreed21 (10.7 ± 0.4 mm) and ReBreed21+G (10.8 ± 0.4 mm) compared with Control (9.1 ± 0.5 mm). Structural luteolysis was similar among groups ($P=0.51$), although the average day when P4 was $<1\text{ng/mL}$ was later ($P<0.01$) for ReBreed21 (20.5 ± 0.2) and ReBreed21+G (20.7 ± 0.2) compared to Control (19.2 ± 0.4). Overall ovulation at the end of the estrous cycle was increased ($P=0.03$) for ReBreed21 groups (83.3%; 45/54) compared with Control (57.7%; 15/26). Synchronized ovulation on day 22 to 23 was greater ($P<0.01$) for ReBreed21 (78.6%; 22/28) and ReBreed21+G (76.9%; 20/26) compared with Control (30.8%; 8/26). Thus, the ReBreed21 resynchronization program produced acceptable endocrine and

follicular dynamics, including synchronized ovulation at the end of the protocol in nonpregnant heifers providing good rationale for testing the fertility and practical implementation of this protocol under field conditions.

Keywords: *Bos indicus*, reproductive efficiency, resynchronization, TAI.

Introduction

Implementation of timed artificial insemination (TAI) programs in beef cattle increases beef reproductive and production efficiency, and improves profitability [1, 2]. Heifers or cows that become pregnant in the first 42 days of the breeding season produce heavier calves at weaning and have more time to become pregnant in the next breeding season resulting in greater longevity in the herd and more calves produced during productive life [3, 4]. In beef cattle, TAI allows ~50% of cows to become pregnant on the first day of the breeding season [5]. After first TAI, cleanup bulls have been used to increase the number of heifers/cows that are pregnant before 42 days of the breeding season. In one study, however, more than 60% of nonpregnant cows returned to an anovular condition after induction of ovulation during the first TAI protocol [6]. To overcome these negative impacts of anovulation on beef cattle operations, serial use of TAI programs can be used to induce and synchronize ovulation during second and later TAI [7-9]. Use of TAI at first and later AI can increase the number of pregnant cows during the breeding season and increase the percentage of cows that are pregnant early in the breeding season.

In the past decades, multiple resynchronization (Resynch) strategies have been reported with interbreeding intervals of 24 to 40 days [10-12]. These protocols have used different approaches for pregnancy diagnosis and Resynch treatments, often initiating the protocol before pregnancy diagnosis, i.e. in all cows without knowledge of gestational status of the cow. Two features of Resynch protocols that have limited their widespread use are: (1) animal management for each TAI is on different days of the week with schedules that limit the number of groups of cows that receive first AI and Resynch AI during the breeding season and (2) most Resynch protocols only allow a maximum of two TAIs by day 42 of the breeding season, a critical time for efficiency of beef operations.

The ReBreed21 resynchronization program was developed to improve reproductive efficiency on beef production operations [13] by assuring that all TAIs occur on the same day of the week which optimizes its implementation and applicability. Furthermore, the ReBreed21 protocol allows three TAI by day 42 of the breeding season increasing the number of cows that are pregnant early in the breeding season. In our first report of ReBreed21, heifers had larger ovulatory follicle size at the time of reinsemination compared to another Resynch strategy. However, the follicle dynamics and endocrine profiles of animals submitted to the ReBreed21 protocol were not evaluated.

We developed a series of studies to understand physiological and reproductive outcomes using the ReBreed21 program in large beef production operations. This particular manuscript reports the results of a study designed to assess the follicular dynamics and endocrine profiles of *Bos indicus* Nelore beef heifers during the ReBreed21 program. In addition, a further modification to the original protocol is tested endeavoring to optimize the endocrine and follicular profile leading to reinsemination. We hypothesized that (1) GnRH treatment at the start of ReBreed21 will increase synchronization of follicular wave emergence (within 1 to 3 days); (2) ReBreed21 protocol does not alter the timing of structural luteolysis but synchronizes ovulation by maintaining circulating concentrations of P4 ($P4 \geq 1$ ng/ml) until after removal of P4 device; (3) The ReBreed21 protocol, including eCG treatment on d19, will increase follicle growth rate (d19 to d21) and increase follicle size on d21; and (4) The ReBreed21 protocol, including GnRH treatment on d21 (expected time of AI), will increase synchronized (d22 and d23) and overall ovulation compared to Control non-pregnant heifers.

Materials and Methods

Animals, location, and reproductive management

All experimental procedures and animal management were approved by the Animal Research Ethics Committee of “Luiz de Queiroz” College of Agriculture (ESALQ/USP) (Protocol #2018-18). Nulliparous *Bos-indicus* Nelore beef heifers (n = 96) with body weight of ≥ 280 kg and 14 to 20 months of age were used in the current study. The experiment was performed during the breeding season of 2018/2019 at the Sussego Farm, located in Rio Branco, state of Acre, Brazil. Heifers were kept on pasture (*Brachiaria brizantha*) supplemented with mineral salt and had *ad libitum* access to water.

An estrous cycle was induced using a synchronization of ovulation program that is regularly used for TAI but, in the current study, heifers were not bred to allow analysis of follicular and hormonal dynamics of nonpregnant heifers (Figure 1). On random days of the estrous cycle, all heifers received one intravaginal P4 device (0.5 g; Repro one, GlobalGen vet science) and 2.0 mg estradiol benzoate (EB; Syncrogen, GlobalGen vet science) administered i.m (d –10). The P4 device was left for 8 days. On day –2, heifers were treated with 0.5 mg PGF, 0.6 mg estradiol cypionate (EC; Cipion, GlobalGen vet science), and 200 IU of equine chorionic gonadotropin (eCG; ECGen, GlobalGen vet science). Finally, on d0 heifers received 8.4 μ g busarelin acetate im (GnRH; Maxrelin, GlobalGen vet science) to induce ovulation. No AI was performed to ensure nonpregnancy in all heifers. Ovulation was confirmed 7 d later (83.3% [80/96]) by the presence of a corpus luteum (CL) of at least 13 mm in diameter as measured with an ultrasound machine.

Experimental treatments: Resynchronization protocols

On d7 of the estrous cycle, i.e., seven days after GnRH, all heifers that ovulated to the first protocol were randomized into three Groups: ReBreed21 (n = 28), ReBreed21+G (n = 26), and Control (n = 26) (Figure 1). On d12, heifers in ReBreed21 received an intravaginal P4 device, 7 d later (d19) the P4 device was removed, and heifers received 200 IU of eCG. On d21, ovulation was induced with GnRH (8.4 µg buserelin). For the ReBreed21+G, heifers received the same treatments as for ReBreed21 heifers but with the addition of a double dose of GnRH (16.8 µg buserelin) at the time of P4 device insertion on d12. Heifers in the Control group did not receive any hormonal treatment.

Ultrasonography and blood sample collections

A Mindray DP 2200 Vet (Shenzhen, China) equipped with a linear multi-frequency transducer set at 7.5 MHz was used to record daily (24 h interval) videos of both ovaries from d7 to d25 (d0 = GnRH from previous synchronization). Follicles (> 3 mm) and CL data were analyzed with a 2D video motion analysis software (Kinovea version 0.9.1, <https://kinovea.org>) to evaluate the luteal and follicular dynamics. Structural luteolysis was defined for each heifer as done previously [14] as the day before a 50% decrease from the average volume of the five largest CL observations. Ovulation was determined by disappearance of a dominant follicle. For example, when a dominant follicle was observed on d21 but not on d22, ovulation was reported as occurring on d22. Day of wave emergence was defined as the day when the dominant follicle of the wave was 3 - 4 mm in diameter, based on retrospective evaluation of the entire follicular wave. The last wave of the estrous cycle was defined as the wave with a growing dominant follicle at the time of luteolysis [15]. Synchronization of wave emergence was defined as a wave that emerged within 1 to 3d after beginning the ReBreed21 protocol (d13 to d15).

Daily blood samples from d7 until d25 were collected into evacuated tubes (Vacutainer, Dickinson, Franklin Lakes, NJ) by puncture of the tail coccygeal vein. Immediately after collection, tubes were placed on ice and kept refrigerated until centrifuged at 1700 x g for 20 min. Serum was recovered, aliquoted into duplicate vials, and stored frozen at -20°C until assayed for P4.

Circulating P4 was determined using a solid-phase RIA kit containing antibody-coated tubes and I^{125} -labeled P4 (ImmuChem Coated Tube P4 125I RIA Kit, MP Biomedicals, CA, USA) as described and validated for bovine plasma [16]. Intra- and inter-assay CVs were 1.1 and 1.8 % and sensitivity was 0.07 ng/mL.

Statistical analyses

Binary variables were analyzed using the GLIMMIX procedure of SAS (Version 9.4; SAS Institute). Continuous variables were analyzed using the MIXED procedure of SAS. Studentized residuals with deviations from assumptions of normality (based on Shapiro-Wilk test) and/or homogeneity of variance were transformed to natural logarithms or ranks. Significant differences between treatment groups were considered for $P \leq 0.05$, whereas differences between $P > 0.05$ and $P \leq 0.10$ were considered as a tendency. Data are presented as means \pm SEM and as percentages for continuous and binary outcomes, respectively.

Results

The endocrine and follicular profile of heifers are summarized in Table 1. The ReBreed21 and ReBreed21+G had an increased percentage of heifers at the optimal time for

each event: day of wave emergence, day when P4 was $<1\text{ng/mL}$, day of ovulation, and percentage of heifers with the largest follicle $\geq 11\text{ mm}$ on d21.

The addition of GnRH on d12 of the estrous cycle (ReBreed21+G) doubled ($P = 0.05$) the percentage of heifers with synchronized follicular wave emergence compared to the Control (Figure 2). The GnRH on d12 in the ReBreed21+G group induced ovulation in 9 of 26 heifers; however, synchronization of wave emergence was not different for heifers that ovulated or did not ovulate. The ReBreed21 group had an intermediate percentage of heifers with synchronized wave emergence (53.6%) that was not different from either Control or ReBreed21+G groups. The day of emergence of the last follicular wave is shown (Figure 3). There were no significant differences in the incidence of heifers with 2-wave patterns among Control, ReBreed21, and ReBreed21+G. However, ReBreed21 and ReBreed21+G had greater incidence of heifers with 3-wave patterns and fewer 4-wave patterns compared with the Control.

Mean follicle size on d19 and follicle growth between d17 and d19 were not different among groups (Figure 4). The ReBreed21 protocols (including eCG treatment on d19) increased ($P < 0.01$) follicle size on d 21, either expressed in mm diameter (Figure 4A) or as volume in both ReBreed21 (360.6mm^3) and ReBreed21+G (370.8 mm^3) groups compared with the Control (221.8 mm^3). The follicular growth was also greater, expressed as mm growth for the 2d (Figure 4B) or as volume growth (mm^3) for the 2 d, which represented an increase in follicular volume growth of 110% (from 174.5 to 360.6 mm^3) and 210% (from 119.3 to 370.8 mm^3), respectively, for ReBreed21 and ReBreed21+G compared with Control (from 156.4 to 221.8 mm^3 [40% volume growth]).

The day of structural luteolysis was not different ($P = 0.51$) among the three treatment groups (Figure 5A). In contrast, the average day when P4 concentrations were $<1\text{ ng/mL}$ was one

day later for the ReBreed21 and ReBreed21+G groups ($P < 0.01$; Figure 5B). The percentage of heifers with $P4 < 0.5$ ng/mL on d21 was not different ($P = 0.71$) among groups (Table 1).

Only 57.7% of heifers in the Control group ovulated at the end of the estrous cycle, which tended to be lower ($P = 0.06$) compared with ReBreed21 (82.1%) and ReBreed21+G (84.6%). For ReBreed21 and ReBreed21+G, ovulation at the end of estrous cycle was 44 and 48% greater, respectively, compared to the Control group. Synchronized ovulation on days 22 to 23 increased ($P < 0.01$) to 53.6% for ReBreed21 and 69.2% for ReBreed21+G groups compared with Controls 34.6% (Table 1 and Figure 5C).

Discussion

The development of new protocols for resynchronization of ovulation in beef heifers or cows is essential to improve productivity and profitability of beef cattle operations. To enhance the efficiency and applicability of Resynch protocols, rational manipulation of physiological processes must align with practical on-farm management. Although previous studies have attempted to synchronize estrus with the use of P4 implants during the time of normal luteolysis [17, 18], only two studies from our group have reported a synchronized ovulation protocol, termed ReBreed21, that allows pregnancy diagnosis and TAI in non-pregnant heifers on d21 after previous AI [13, 19]. Herein, we report follicular dynamics and endocrine data for non-pregnant (non-bred), synchronized heifers submitted to ReBreed21 [13].

The first hypothesis was partially supported, that GnRH added at the time of P4 device insertion promotes greater synchronization of follicular wave emergence (within 1 to 3 days). In the present study, most heifers had three wave patterns during the estrous cycle, the third follicular wave emerged in a range of d10 to d17 and the interval between emergence of second

and third follicular waves was ~5 days. Treatment with GnRH would be expected to cause an LH surge triggering ovulation of dominant follicles that are present at time of treatment. In addition, the GnRH-induced FSH surge and the FSH surge following disappearance of the dominant follicle can cause emergence of a new follicular wave [20]. Other studies also report earlier emergence of a new follicular wave in response to GnRH, even in the absence of ovulation, likely due to the GnRH-induced FSH surge [20-22]. Interestingly, insertion of a P4 device alone resulted in an intermediate proportion of heifers with wave emergence within three days. Insertion of a P4 device increases circulating concentrations of P4 which would be expected to decrease LH pulses and earlier senescence of the dominant follicle [25]. Elimination of LH pulses with a GnRH antagonist results in interruption of dominant follicle growth at about the time of expected follicle diameter deviation and produces emergence of a new follicular wave within a few days [24, 25]. Likewise, follicular waves under greater circulating P4 are shorter and the dominant follicle reaches smaller maximal diameter [23, 26]. Taken together, either strategy, ReBreed21 or ReBreed21+G, results in >53% of heifers or cows with wave emergence between d13 and d15 which favors the development of a large dominant follicle with ovulatory capacity (>8.5mm) by d21.

The second hypothesis was supported, in which the timing of structural luteolysis would not be altered in ReBreed21, but the presence of the P4 device maintains $P4 \geq 1$ ng/mL until after its removal on d19. In cattle, structural (CL demise) and functional (P4 decrease) luteolysis occur at about the same time [27]. Indeed, heifers in the Control group had structural luteolysis and circulating concentrations of P4 <1 ng/mL on d19, on average. In the present study, insertion of a P4 device on d12 did not alter the timing of structural luteolysis. Nevertheless, removal of the P4 device on d19 resulted in an increased proportion of heifers or cows reaching P4

concentrations <1 ng/mL on d20 but not before [28]. Maintenance of P4 concentrations above 1 ng/mL until d20 promotes a better environment for follicle development preventing ovulation before d21 and optimizing the timing of ovulation, potentially improving fertility [22, 29].

The third hypothesis, that the ReBreed21 protocol, including eCG treatment on d19, will increase follicle growth rate (d19 to d21) and increase follicle size on d21, was also supported. Both ReBreed21 groups had increased follicle growth from d19 to d21 and increased follicle size on d21 compared with the Control group. Stimulation of follicle growth with eCG has been extensively used for TAI programs in *Bos indicus* beef cattle to increase follicle size at ovulation with a positive relationship with fertility [30-33]. A larger follicle provides an adequate estradiol environment during proestrus and forms a larger CL with greater P4 production, all potentially positive effects for fertility [34]. Future studies are needed to definitively show that eCG is required to optimize fertility in the ReBreed21 protocol since no ReBreed21 trial has been done without eCG treatment.

Finally, the last step of any synchronization of ovulation protocol is to induce synchronized ovulation to allow optimal TAI and this was done with GnRH on d21 in ReBreed21. The fourth hypothesis was supported that the ReBreed21 protocol, including GnRH treatment on d21 (expected time of AI), will increase synchronized (d22 and d23) and overall ovulation compared to Control non-pregnant heifers. Induction of synchronized ovulation was the first strategy used in TAI programs and is still successfully used in beef cattle TAI protocols [6, 22, 35-37]. Treatment with GnRH produces a satisfactory ovulation rate for follicles >8.5 mm and greater than 90% ovulation for growing follicles >10 mm [38]. In addition, stimulation of a larger follicle size using eCG treatment is likely a key determinant of the high GnRH-induced ovulation rate in ReBreed21 and ReBreed21+G protocols. In contrast, the Control group had less

than 60% of heifers that ovulated at the end of the experimental period (d25 of the estrous cycle) suggesting that more than 40% had returned to an anovular state after a single estrous cycle was induced by the first TAI protocol. In a previous report, only about 30% of non-pregnant *Bos indicus* cows had CL present at 30 days after TAI [6]. Therefore, it seems likely that Resynch programs, including ReBreed21, increase the percentage of heifers that continue cycling after a TAI program, increasing the likelihood of pregnancy establishment early in the breeding season.

Noteworthy, the proportion of heifers with three follicular waves was increased in both Rebreed21 and ReBreed21+G, which may be potentially explained by two physiologic mechanisms. First, no wave emergence before d13 and no two-wave patterns were observed in ReBreed21+G, likely related to the GnRH treatment on d12, since eight of nine heifers that ovulated to GnRH treatment on d12 had a dominant follicle from wave-2. On the other hand, the eCG treatment on d19 potentially prevented some dominant follicles from wave 3 from turning over and regressing, keeping these follicles growing with subsequent ovulation in response to the GnRH on d21. Interestingly, the number of follicular waves was altered without affecting the timing of structural luteolysis, in contrast to the later luteolysis in Holstein heifers with three vs two follicular waves [26].

We developed a physiological model to illustrate the key aspects of the ReBreed21 program that were observed in this study (Figure 6). An intravaginal P4 device inserted on d12 provides a better endocrine milieu for follicle development and prevents spontaneous ovulations before d21 with removal of the P4 device on d19 allowing a synchronized decrease in circulating P4 that is completed by the time of GnRH treatment on d21. In addition, treatment with eCG on d19 stimulates follicle growth so that a larger dominant follicle is present and ovulates in response to GnRH treatment, likely improving fertility. Collectively, the efficiency of Rebreed21

and ReBreed21+G for the key end points measured in this physiologic study were similar. The fewer hormonal treatments (one GnRH on d12) and therefore reduced cost of Rebreed21 is likely to favor this program compared to ReBreed21+G for on-farm implementation, although no comparison of fertility was made in the current trial.

Conclusions

In conclusion, the ReBreed21 program synchronized the endocrine and follicular profiles of *Bos indicus* heifers allowing synchronized ovulation of a large preovulatory follicle near d21 in a high percentage of heifers. Hormonal and follicular profiles during ReBreed21 indicate a protocol with good synchronization of ovulation after a previous TAI and provide strong rationale for testing this protocol in a large number of beef cattle in commercial, field conditions.

Acknowledgments

The authors thank the Carvalho's family, owners of Sussego farm and Mr. Edilson owner of Batista Farm (Acre-Brazil) for their contribution with the heifers and facilities, and GlobalGen vet science for the hormone donation. This research supported by Hatch Project WIS01240 to MCW and USDA-NIFA project 2019-67015-29704 to MCW.

Table 1. Percentage distribution % (n) of timing of each physiologic event in non-inseminated Nelore heifers submitted to different resynchronization strategies. d0 represents the time of final GnRH in previous protocol, with no heifers receiving TAI.

End points	Control n=26	ReBreed21 n=28	ReBreed21+G n=26	P
Wave emergence (day)				
≤ d12	30.8 (8)	17.8 (5)	0	0.55
13 to 15*	34.6 ^b (9)	53.6 ^{ab} (15)	69.2 ^a (18)	0.05
≥ d16	34.6 (9)	28.6 (8)	30.8 (8)	0.89
P4 < 1ng/mL (day)				
≤ d19	61.6 (16)	0	0	N/A
20 to 21*	26.9 ^b (7)	82.1 ^a (23)	73.1 ^a (19)	<0.01
≥ 22	11.5 (3)	17.9 (5)	26.9 (7)	0.38
Circulating P4 at d21 (ng/mL)				
≤ 0.5*	80.8 (21)	71.4 (20)	69.2 (18)	0.60
0.6 to 0.9	7.7 (2)	10.7 (3)	3.9 (1)	0.65
≥ 1	11.5 (3)	17.9 (5)	26.9 (7)	0.56
Ovulation (day)				
≤ 21	3.7 (1)	0	0	N/A
22 to 23*	26.9 ^b (7)	78.6 ^a (22)	76.9 ^a (20)	<0.01
≥ 23	26.9 ^A (7)	3.6 ^B (1)	7.7 ^B (2)	0.06
Largest follicle (mm)				
≤ 8.5	38.4 (9)	10.7 (3)	19.2 (5)	0.11
8.6 to 10.9	38.5 (10)	42.9 (12)	19.2 (5)	0.17
≥ 11*	23.1 ^b (6)	46.4 ^a (13)	61.6 ^a (16)	0.03

“*” indicates what is considered as the optimum for each event.

N/A: Not applicable.

Figure Legends

Figure 1. Experimental design for strategies of resynchronization of ovulation programs (ReBreed21 and Rebreed219+G) and Control. All heifers were initially synchronized with an ovulation synchronization protocol but with no breeding (time of GnRH = d0). On d7, heifers were evaluated by ultrasound and heifers that ovulated to the initial protocol were randomized to the three experimental groups and treated as shown. The design shows the timing of intravaginal P4 device, estradiol benzoate (EB), estradiol cypionate (EC), equine chorionic gonadotropin (eCG), cloprostenol (PGF2 α), and buserelin (GnRH) treatments.

Figure 2. Incidence of heifers with synchronized wave emergence between d13 and d15 of the estrous cycle (d0 = final GnRH of initial protocol). Additional analyses were also done for heifers that were enrolled in ReBreed21+G based on whether they ovulated or not to the GnRH treatment on d12. Lowercase letters indicate differences at $P \leq 0.05$.

Figure 3. Day of emergence of the last follicular wave and number of follicular waves during the estrous cycle (two waves = triangle, three waves = circle, and four waves = squares) for heifers that ovulated (black/filled shapes) or did not ovulate (open shapes) at the end of the estrous cycle. Different lowercase letters indicate $P \leq 0.05$ and uppercase letters indicate $0.05 < P \leq 0.10$.

Figure 4. Dominant follicle size and growth rate in different protocols. Panel A, average size (mm) of the largest follicle at d19 and d21. Panel B, growth rate (mm/2 days) of the largest follicle from d17 to d19 and d19 to d21 of the estrous cycle. Different lowercase letters indicate $P \leq 0.05$.

Figure 5. Distribution of physiologic events (CL regression, decrease in circulating P4, and ovulation) by days after GnRH from the previous synchronization protocol. Each circle represents an individual heifer, whereas mean \pm SEM for Control, ReBreed21, and ReBreed21+G are provided at the bottom of each panel. Panel A, Distribution, and average day of structural luteolysis. Panel B, Distribution of day when P4 was $< 1\text{ng/mL}$. Panel C, Distribution of day of ovulation for individual heifers that ovulated at the end of the estrous cycle.

Figure 6. Physiological model on ReBreed21, a reinsemination program in Nelore heifers. The model includes letters that highlight the key physiologic events: (a) The intravaginal P4 device provides better endocrine milieu for development of the ovulatory follicle and, of prime importance, maintains P4 concentrations greater than 1 ng/mL until d19 to prevent premature estrus and ovulation (82.1% of heifers reached P4 $< 1\text{ng/mL}$ between d20 and d21). (b) The eCG treatment increases follicle growth after d19 (two-fold increase compared to control) resulting in a larger preovulatory follicle on d21. (c) The GnRH treatment induces synchronized ovulation of the dominant follicle with 77.8% of heifers (42/54) having ovulation on d22 or d23 compared to 26.9% (7/26) of Control heifers.

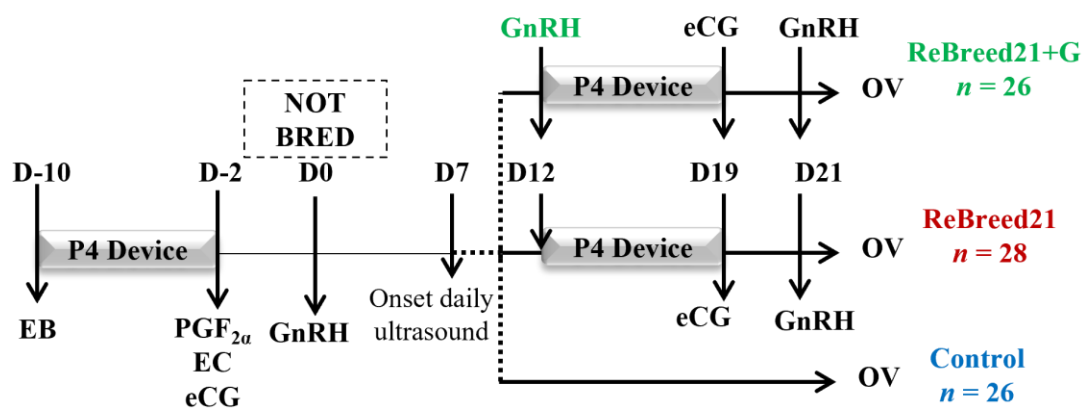


Figure 1

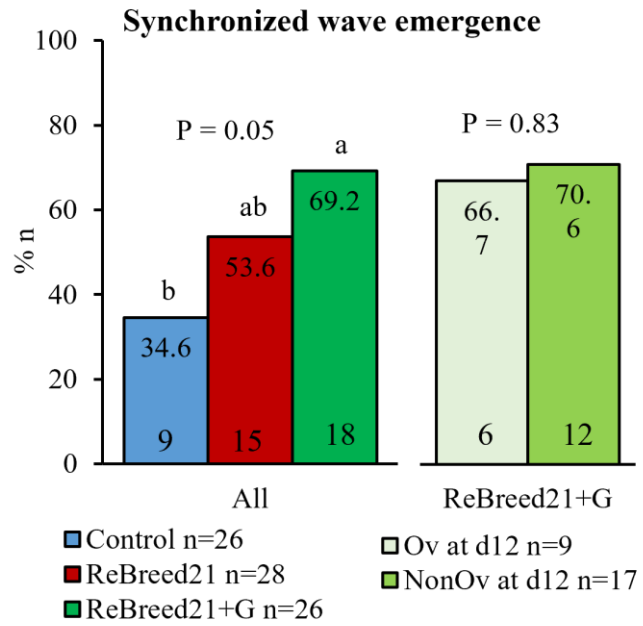


Figure 2

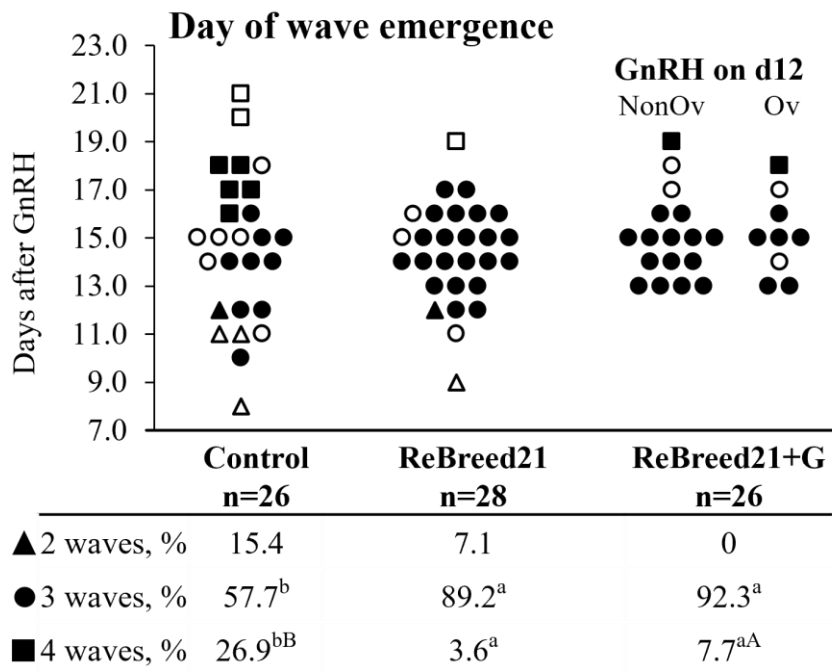


Figure 3

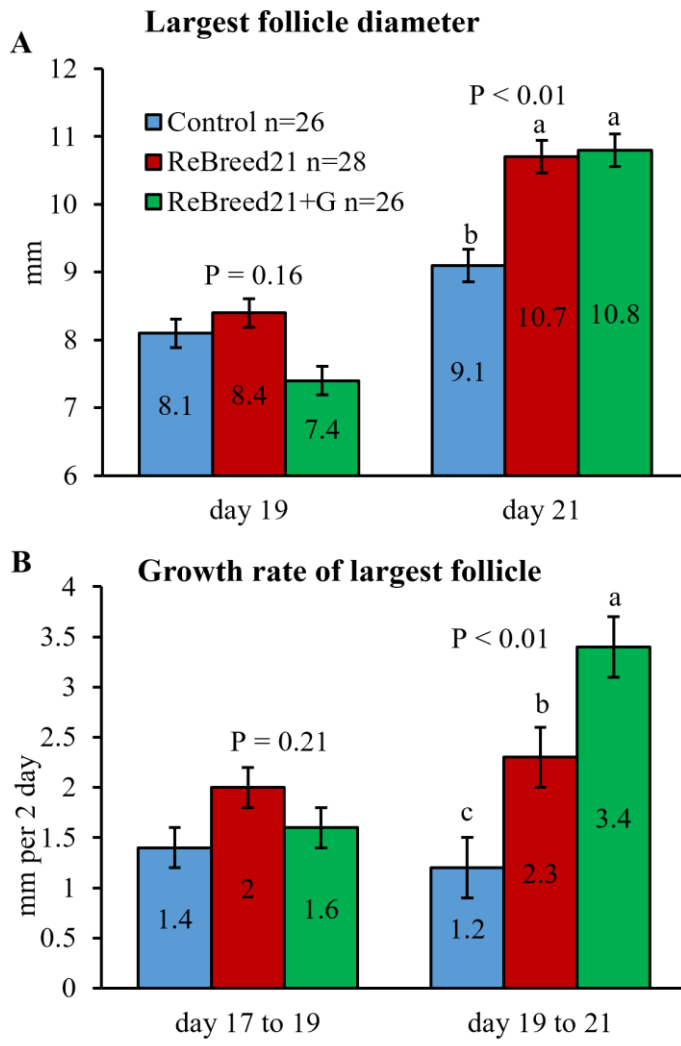


Figure 4

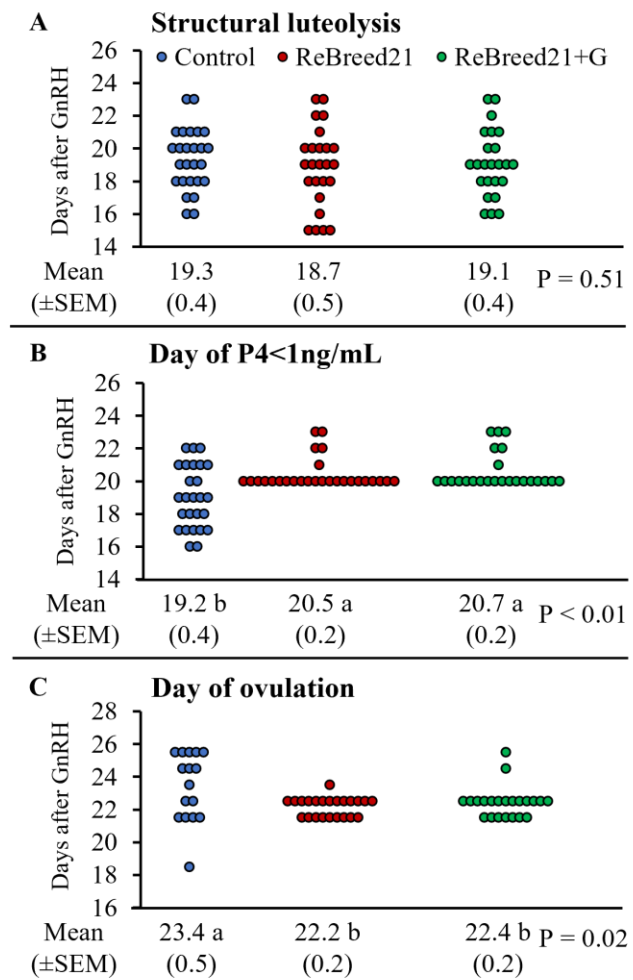


Figure 5

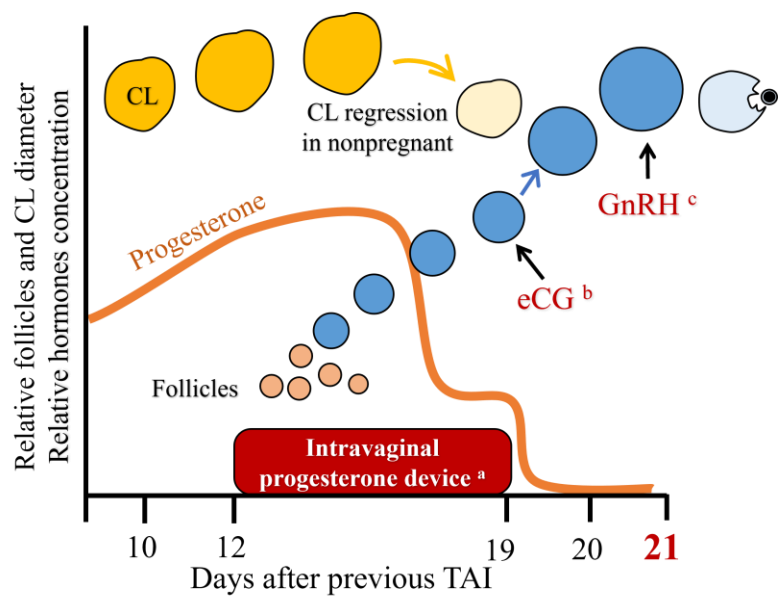


Figure 6

References

- [1] Baruselli PS, de Souza AH, de Sa MF, Marques MO, Sales JND. Genetic market in cattle (Bull, AI, FTAI, MOET and IVP): financial payback based on reproductive efficiency in beef and dairy herds in Brazil. *Anim Reprod.* 2018;15:247-55.
- [2] Vasconcelos JLM, Pereira MHC, Wiltbank MC, Guida TG, Lopes Jr. FR, Sanches Jr. CP, et al. Evolution of fixed-time AI in Brazil. *Anim Reprod.* 2018;15 (Suppl. 1): 940-51.
- [3] Funston RN, Musgrave JA, Meyer TL, Larson DM. Effect of calving distribution on beef cattle progeny performance. *J Anim Sci.* 2012;90:5118-21.
- [4] Shane DD, Larson RL, Sanderson MW, Miesner M, White BJ. A deterministic, dynamic systems model of cow-calf production: The effects of the duration of postpartum anestrus on production parameters over a 10-year horizon. *J Anim Sci.* 2017;95:1680-95.
- [5] Baruselli PS, Ferreira RM, Colli MHA, Elliff FM, Sa MF, Vieira L, et al. Timed artificial insemination: current challenges and recent advances in reproductive efficiency in beef and dairy herds in Brazil. *Anim Reprod.* 2017;14:558-71.
- [6] Alves R, Silva MA, Consentini CEC, Silva LOE, Folchini NP, Oliva AL, et al. Hormonal combinations aiming to improve reproductive outcomes of *Bos indicus* cows submitted to estradiol/progesterone-based timed AI protocols. *Theriogenology.* 2021;169:89-99.
- [7] Rodrigues WB, Jara JD, Borges JC, de Oliveira LOF, de Abreu UPG, Anache NA, et al. Efficiency of mating, artificial insemination or resynchronisation at different times after first timed artificial insemination in postpartum Nellore cows to produce crossbred calves. *Anim Reprod Sci.* 2019;59:225-31.

- [8] Bo GA, de la Mata JJ, Baruselli PS, Menchaca A. Alternative programs for synchronizing and resynchronizing ovulation in beef cattle. *Theriogenology*. 2016;86:388-96.
- [9] Marques MD, Morotti F, da Silva CB, Ribeiro M, da Silva RCP, Baruselli PS, et al. Influence of category - heifers, primiparous and multiparous lactating cows - in a large-scale resynchronization fixed-time artificial insemination program. *J Vet Sci*. 2015;16:367-71.
- [10] Pugliesi G, Bisinotto DZ, Mello BP, Lahr FC, Ferreira CA, Melo GD, et al. A novel strategy for resynchronization of ovulation in Nelore cows using injectable progesterone (P4) and P4 releasing devices to perform two timed inseminations within 22 days. *Reprod Domest Anim*. 2019;54:1149-54.
- [11] Pessoa GA, Martini AP, Sa MF, Rubin MI. Resynchronization improves reproductive efficiency of suckled *Bos taurus* beef cows subjected to spring-summer or autumn-winter breeding season in South Brazil. *Theriogenology*. 2018;122:14-22.
- [12] Sa Filho M, Marques M, Giroto R, Santos F, Sala R, Barbuio J, et al. Resynchronization with unknown pregnancy status using progestin-based timed artificial insemination protocol in beef cattle. *Theriogenology*. 2014;81:284-90.
- [13] Andrade JPN, Gomez-Leon VE, Andrade FS, Carvalho BP, Lacouth KL, Garcia FZ, et al. Development of a novel 21-day reinsemination program, ReBreed21, in *Bos indicus* heifers. *Theriogenology*. 2020;155:125-31.
- [14] Domingues RR, Ginther OJ, Gomez-Leon VE, Wiltbank MC. Up-regulation of endometrial oxytocin receptor is associated with the timing of luteolysis in heifers with two and three follicular waves. *Biol Reprod*. 2020;102:316-26.

- [15] Domingues RR, Ginther O, Toledo MZ, Wiltbank MC. Increased dietary energy alters follicle dynamics and wave patterns in heifers. *Reproduction*. 2020;160:943-53.
- [16] Domingues RR, Ginther O, Gomez-Leon V, Castro T, Wiltbank MC. Endometrial and luteal responses to a prostaglandin F2alpha pulse: A comparison between heifers and mares. *Biol Reprod*. 2022;106:979-91.
- [17] Colazo MG, Dias FC, Lightfoot K, Dochi O, Kastelic JP, Mapletoft RJ. Pregnancy rate following timed AI in beef heifers treated with Cue-Mate and pLH or GnRH. *Repro Fertil Dev*. 2007;19:122.
- [18] Colazo MG, Kastelic JP, Mainar-Jaime RC, Gavaga QA, Whittaker PR, Small JA, et al. Resynchronization of previously timed-inseminated beef heifers with progestins. *Theriogenology*. 2006;65:557-72.
- [19] Andrade JPN, Andrade FS, Guerson YB, Domingues RR, Gomez-Leon VE, Cunha TO, et al. Early pregnancy diagnosis at 21 days post artificial insemination using corpus luteum vascular perfusion compared to corpus luteum diameter and/or echogenicity in Nelore heifers. *Anim Reprod Sci*. 2019;209:106144.
- [20] Motta JCL, Madureira G, Silva LO, Alves R, Silvestri M, Drum JN, et al. Interactions of circulating estradiol and progesterone on changes in endometrial area and pituitary responsiveness to GnRH. *Biol Reprod*. 2020;103:643-53.
- [21] Adams GP, Matteri RL, Kastelic JP, Ko JC, Ginther OJ. Association between surges of follicle-stimulating hormone and the emergence of the follicular wave in heifers. *J Reprod Fertil*. 1992;94:177-88.

[22] Madureira G, Motta JC, Drum JN, Consentini CE, Prata AB, Monteiro Jr PL, et al. Progesterone-based timed AI protocols for *Bos indicus* cattle I: Evaluation of ovarian function. *Theriogenology*. 2020;145:126-37.

[23] Gomez-León V, Ginther O, Guimarães J, Wiltbank M. Hormonal mechanisms regulating follicular wave dynamics II: Progesterone decreases diameter at follicle selection regardless of whether circulating FSH or LH are decreased or elevated. *Theriogenology*. 2020;143:148-56.

[24] Gomez-Leon VE, Ginther OJ, Domingues RR, Guimaraes JD, Wiltbank MC. Necessity for LH in selection and continued growth of the bovine dominant follicle. *Reproduction*. 2020;159:559-69.

[25] Ginther OJ, Khan FA, Hannan MA, Rodriguez MB, Pugliesi G, Beg MA. Role of LH in luteolysis and growth of the ovulatory follicle and estradiol regulation of LH secretion in heifers. *Theriogenology*. 2012;77:1442-52.

[26] Domingues RR, Ginther OJ, Toledo MZ, Wiltbank MC. Increased dietary energy alters follicle dynamics and wave patterns in heifers. *Reproduction*. 2020;160:943-53.

[27] Domingues RR, Ginther O, Gomez-León VE, Wiltbank MC. Up-regulation of endometrial oxytocin receptor is associated with the timing of luteolysis in heifers with two and three follicular waves. *Biol Reprod*. 2020;102:316-26.

[28] Silva LOE, Valenza A, Alves R, Silvestri M, Contini MC, Motta JCL, et al. Progesterone release profile and follicular development in Nelore cows receiving intravaginal progesterone devices. *Theriogenology*. 2022;178:77-84.

- [29] Denicol AC, Lopes G, Jr., Mendonca LG, Rivera FA, Guagnini F, Perez RV, et al. Low progesterone concentration during the development of the first follicular wave reduces pregnancy per insemination of lactating dairy cows. *J Dairy Sci.* 2012;95:1794-806.
- [30] Peres RFG, Claro I, Sa OG, Nogueira GP, Vasconcelos JLM. Strategies to improve fertility in *Bos indicus* postpubertal heifers and nonlactating cows submitted to fixed-time artificial insemination. *Theriogenology.* 2009;72:681-9.
- [31] Sales JNS, Bottino MP, Silva L, Giroto RW, Massoneto JPM, Souza JC, et al. Effects of eCG are more pronounced in primiparous than multiparous *Bos indicus* cows submitted to a timed artificial insemination protocol. *Theriogenology.* 2016;86:2290-5.
- [32] Sa Filho MF, Ayres H, Ferreira RM, Marques MO, Reis EL, Silva RCP, et al. Equine chorionic gonadotropin and gonadotropin-releasing hormone enhance fertility in a norgestomet-based, timed artificial insemination protocol in suckled Nelore (*Bos indicus*) cows. *Theriogenology.* 2010;73:651-8.
- [33] Sa Filho MF, Torres JRS, Penteadó L, Gimenes LU, Ferreira RM, Ayres H, et al. Equine chorionic gonadotropin improves the efficacy of a progestin-based fixed-time artificial insemination protocol in Nelore (*Bos indicus*) heifers. *Anim Reprod Sci.* 2010;118:182-7.
- [34] Sa Filho MF, Crespilho AM, Santos JE, Perry GA, Baruselli PS. Ovarian follicle diameter at timed insemination and estrous response influence likelihood of ovulation and pregnancy after estrous synchronization with progesterone or progestin-based protocols in suckled *Bos indicus* cows. *Anim Reprod Sci.* 2010;120:23-30.
- [35] Pursley JR, Mee MO, Wiltbank MC. Synchronization of ovulation in dairy cows using PGF₂α and GnRH. *Theriogenology.* 1995;44:915-23.

- [36] Prata AB, Madureira G, Robl AJ, Ribeiro HS, Sagae M, Elias MCV, et al. Progesterone-based timed AI protocols for *Bos indicus* cattle III: Comparison of protocol lengths. *Theriogenology*. 2020;152:29-35.
- [37] Monteiro PL, Consentini CEC, Andrade JPN, Beard AD, Garcia-Guerra A, Sartori R, et al. Research on timed AI in beef cattle: Past, present and future, a 27-year perspective. *Theriogenology*. 2023;211:161-71.
- [38] Gimenes LU, Sa MF, Carvalho NAT, Torres JRS, Souza AH, Madureira EH, et al. Follicle deviation and ovulatory capacity in *Bos indicus* heifers. *Theriogenology*. 2008;69:852-8.

Chapter 3: Optimizing ReBreed21 II: Fertility and reproductive efficiency in different parities during a shortened breeding season in beef cattle

João Paulo. N. Andrade^a; Pedro J. L. Monteiro^a; Alexandre B. Prata^b; Adelino J. Robl^c José Neto^c; Beatriz Lippe^d; Heuller S. Ribeiro^c; Diego Hartmman^c; Roberto Sartori^d; Milo C. Wiltbank^{a*}

^a Department of Animal and Dairy Sciences, University of Wisconsin-Madison, WI 53706, USA

^b GlobalGen Vet Science, Jaboticabal, SP, 14887-360, Brazil

^c Agropecuária Roncador, Querência, MT, 78643-000, Brazil

^d Department of Animal Science, Luiz de Queiroz College of Agriculture (ESALQ), University of São Paulo, Piracicaba, SP, 13418-900, Brazil

*Corresponding Author: wiltbank@wisc.edu

Published in Theriogenology

Andrade JPN, Monteiro PJL, Prata AB, Robl AJ, Neto J, Lippe B, et al. Optimizing ReBreed21 II: Fertility and reproductive efficiency in different parities during a shortened breeding season in beef cattle. Theriogenology. 2024;224:41-49.

Abstract

This study evaluated the efficiency of a rapid reinsemination program allowing timed AI (TAI) every 21d (ReBreed21) in a commercial beef cow-calf operation. Nelore females from different parities (n=2,085) were synchronized for first TAI (D0=TAI) using an estradiol/progesterone (E2/P4) protocol and assigned to one of three reinsemination programs: Resynch33 (n=753), traditional resynch program with second TAI at D42 after first TAI; ReBreed21 (n=687); or ReBreed21+EC (n=670). The ReBreed females (n = 1,357) received intravaginal P4 insert on D12, on D19 P4 was removed, and a dose of equine chorionic gonadotropin (eCG) was administered, then, ReBreed21 females received 0.6mg of EC (ReBreed21+EC) or nothing (ReBreed21) and on D21, nonpregnancy (NP) was determined using Doppler ultrasound to detect corpus luteum (CL) blood flow (BF) (NP: <25% BF pixels of total CL area) and NP cows received immediate TAI and GnRH to induce ovulation. Pregnancy diagnosis was performed at D33 after TAI following all TAIs. Cows considered pregnant at D21, based on CL BF, but NP on D33 were designated False-Positives (FP) and false negatives (FN) were number of nonpregnant cows/heifers on d21 based on the CL BF found to subsequently be pregnant on D33 divided by the total number pregnant. Pregnancy/AI (P/AI) did not differ for the first TAI (55.1%) among the treatments. Heifers had similar P/AI at the second AI in all groups and similar to the first AI. Primiparous had greater P/AI in ReBreed21+EC and Resynch33 at second TAI compared to ReBreed21, 51.7%, 55.8%, 34.2%, respectively. Multiparous had greater P/AI at second TAI in Resynch33 (60.9%) than ReBreed21 programs (34.7%). The percentage FP and FN among ReBreed21 programs did not differ, 13.8 and 0.2%, respectively. Overall accumulative pregnancies on D21 of the breeding season were greater for ReBreed21 and ReBreed21+EC than Resynch33 (69.7%, 71.6%, and 55.5%, respectively). However, on D42 of

the breeding season, only heifers had greater pregnancies in ReBreed21 programs than Resynch33 (73.3%, 74.3.6%, and 63.2%, respectively). Average days to pregnancy were less ($P=0.01$) for ReBreed21 and ReBreed21+EC than Resynch33. Thus, the ReBreed21 strategy can improve the efficiency of TAI programs in beef cattle. Of interest, ReBreed21 was particularly effective in nulliparous, somewhat effective in primiparous when EC was added to the program, but relatively ineffective in multiparous beef cattle.

Keywords: *Bos indicus*, breeding season, Doppler ultrasound, timed AI.

Introduction

Timed artificial insemination (TAI) is a biotechnology that allows cows to receive AI without detection of estrus [1]. It has been particularly useful in beef cattle because it allows the entire herd to potentially receive AI near the start of the breeding season with a minimal number of animal handlings [2]. Protocols that allow TAI also induce cyclicity in non-cycling cattle [3]. In addition, TAI allows the use of high genetic sires from anywhere in the world to produce higher-quality F1 offspring or improve the herd's genetics. Using TAI, a beef cattle operation can have 50% of cows pregnant on the first day of the breeding season [4-6]. Thus, the use of TAI reduces the length of the breeding season, improving the productivity and profitability of beef cattle operations [7]. In all beef cattle systems, the most productive and profitable cows are those that become pregnant during the first 21d of the breeding season, followed by those that become pregnant during the second 21d cycle, followed by a decrease in economic value and efficiency (lighter steers and lower fertility heifers) in cows pregnant later in the breeding season [8-10]. Nevertheless, modeling of the breeding season shows that, even in high efficiency operations, more than 40% of heifers and cows become pregnant after the first 21d from the start of the breeding season, reducing profitability and increasing the risk of involuntary culling of females [5].

To increase the number of cows pregnant in the early breeding season and during a shortened breeding season, strategies have been developed, termed Resynch strategies, that seek to reduce the interval between TAIs [11]. The method of pregnancy diagnosis is a key determinant of the type of Resynch strategy that can be utilized in a herd. The gold standard for pregnancy diagnosis is the use of transrectal ultrasound, utilizing the B mode, to visualize the embryonic heartbeat around 30d after TAI. Utilizing this method of pregnancy diagnosis, a

Resynch strategy can be initiated in nonpregnant cows allowing TAI at about 40d after the first TAI. Alternatively, a Resynch strategy can begin at D22 after TAI, without knowing the pregnancy status of the cow, with the pregnancy diagnosis at about 30d and TAI two days later [12-16]. In addition, research has utilized Doppler ultrasound to indirectly determine the pregnancy status based on CL blood flow around 21d post-TAI [17, 18]. Based on this technology, an early Resynch can be initiated that will allow TAI two days after the Doppler pregnancy diagnosis, i.e. ~23d after previous TAI [19, 20]. Despite potential improvements with these different Resynch programs, all these strategies produce a second TAI that is after the first 21d of the breeding season.

One additional complication is that early Resynch strategies have been difficult to practically implement on commercial beef cattle operations due to: 1) Treatments occurring on different days of the week than the typical days used for the first TAI and may be required to be performed on weekends, 2) Early pregnancy evaluations can produce false positive diagnoses that need to be accounted for in the breeding program, 3) Doppler ultrasound can be expensive and technically challenging to perform. Our research group has recently been working with a program termed ReBreed21 that allows TAI every 21d and that matches a similar daily schedule as used for the first TAI [21]. The pregnancy diagnosis and TAI are both performed on D21 after the previous TAI to optimize the labor schedule. Finally, unlike other early Resynch strategies, there is no treatment with prostaglandin $F_{2\alpha}$ (PGF) during the Resynch protocol, thus reducing the risk of iatrogenic abortions in false-negative cows. [14, 22, 23]. In the ReBreed21 protocol, the intravaginal P4 implant is removed two days before the early pregnancy diagnosis and treatment with equine chorionic gonadotropin (eCG) is used to stimulate preovulatory follicle growth from d19 to 21. Another potential improvement to the protocol may be the addition of

estradiol cypionate (EC) to increase circulating estradiol-17 β (E2) during the proestrus period and potentially increase synchrony of luteolysis in nonpregnant cows. Previous research has shown that E2 treatment during early pregnancy (d18 to 21) will only induce luteolysis in nonpregnant cows, due to inhibition of endometrial ESR1 expression by embryonic interferon- τ in early pregnant cows [24, 25]. Hence, fertility may be optimized and potentially false positives reduced by increasing circulating E2 during the period of proestrus [26-30].

Thus, this study had practical objectives focused on evaluating the impact of using ReBreed21 and a modified version of ReBreed21 during the breeding season in a large, commercial cow-calf operation using cows of different parities. Specifically, we evaluated the fertility and the technical implementation of this program compared with a traditional Resynch program that allowed TAI every 42d. We hypothesized that: 1) ReBreed21 would increase the reproductive performance during a shortened breeding season (42d) in all parities, 2) pregnancy per AI (P/AI) after the first TAI would be similar with or without the EC treatment during the ReBreed21 program, and 3) use of EC would increase P/AI during the ReBreed21 program, at second and third AI, and decrease the incidence of false-positives during the program.

Materials and Methods

The Animal Research Ethics Committee of “Luiz de Queiroz” College of Agriculture of the University of São Paulo (ESALQ/USP) approved all animal procedures.

Animals, location, and reproductive management

Nelore *Bos-indicus* beef cattle (n = 2,163) at Roncador Farm, located in Querencia, MT, Brazil were used in the present study. Animals were kept on pasture condition (*Brachiaria brizantha*) supplemented with mineral salt and had ad libitum access to water. The experiment

was done with a total of 844 heifers, maintained in two different pastures, a total of 638 primiparous cows maintained in three different pastures, and a total of 603 multiparous cows maintained in three different pastures. Animals within a pasture were randomized to treatments so that all treatments were represented in each group. The treatments began when multiparous and primiparous cows were approximately 35d after calving, and heifers were at 14 to 20 months of age with ≥ 280 kg of body weight.

To synchronize ovulation for the first TAI, on random days of the estrous cycle all cows/heifers received a traditional synchronization program which consists of: start, insertion of an intravaginal P4 device (containing either 0.5 g P4 [Repro one, GlobalGen Vet Science, Jaboticabal, SP, Brazil] or 1.0 g P4 [Repro neo; new or once-used; [31]] GlobalGen Vet Science) together with 2.0 mg estradiol benzoate (EB; Syncrogen, GlobalGen Vet Science), and either 7, 8, or 9 days later (previously shown to produce similar outcomes [32]) the P4 implant was removed and animals were treated i.m. with 0.5 mg cloprostenol sodium (PGF; Induscio, GlobalGen Vet Science), 0.6 mg estradiol cypionate (EC; Cipion, GlobalGen Vet Science), and equine chorionic gonadotropin (eCG; 200 IU for heifers and 300 IU for cows; ECGen, GlobalGen Vet Science). Two days later all cattle received TAI (D0). All TAI were performed by one of five experienced technicians using 20×10^6 frozen/thawed sperm using semen from one of five Rubia Gallega or one of two Nelore sires of proven fertility.

For management reasons (missing in the pasture or escape into another pasture) 78 cows/heifers were not found on the day of pregnancy diagnosis and these cows were excluded from further analyses, making 2,085 as the final number of cows and heifers in the study.

Reinsemination protocols and pregnancy diagnoses (PD)

As summarized in Figure 1, twelve days after the first TAI cows/heifers were randomized into one to three treatments: ReBreed21 (n = 687), heifers/cows received an intravaginal P4 implant (either new or used) on D12. Seven days later (D19), the implant was removed, and eCG (cows - 300 IU; heifers - 200 IU) was administered; ReBreed21+EC (n = 670), cows/ heifers received an intravaginal P4 implant on D12. Seven d later (D19), the implant was removed, eCG (cows - 300 IU; heifers - 200 IU) and 0.6 mg of EC were administered. In both ReBreed21 groups, heifers/cows had ovaries evaluated by an experienced technician to determine pregnancy status two days later (D21) using subjective CL blood flow scores [33]. Heifers/cows considered to be nonpregnant, based on the Doppler ultrasound, received immediate TAI together with i.m. treatment with 25 µg of *lecirelin acetate*, a GnRH agonist (TecRelin®, Uniao Quimica, São Paulo, SP, Brazil). This procedure was replicated one more time using the same treatments in each heifer/cow to replicate the ReBreed21 program, allowing a 42-day breeding season with three potential TAIs. Pregnancy diagnoses were evaluated at D33 after the first TAI in all cows/heifers (ReBreed21 and Resynch33) by B mode ultrasound. The presence of an amniotic vesicle containing an embryo with a heartbeat was the determinant of pregnancy. In the Resynch33 group (n = 728), nonpregnant cows/heifers were enrolled in a traditional Resynch breeding program: Insertion of an intravaginal P4 implant together with 2 mg EB (D33 after previous AI), 7d later, removal of P4 and treatment with PGF, EC, and eCG (200 IU – heifers; 300 IU – cows) and 2d later TAI (D42 after previous AI). Heifers/cows enrolled in ReBreed21 or ReBreed21+EC received the D33 pregnancy diagnosis to confirm the diagnosis on D21. Heifers/cows that were diagnosed pregnant on D21 by CL blood flow but were not pregnant on D33 were considered false positives. These heifers/cows were enrolled in the synchronization program using the same protocol as the Resynch33 group. Thus, they received a second TAI on D42 of the breeding season.

Regardless of the treatments and the TAI number, all cows/heifers had pregnancy diagnosed on D33 after AI. The P/AI was determined by the number of heifers/cows pregnant on D33 after AI divided by the total number of cows/heifers that received TAI. The final number in each treatment and parity were heifers (Resynch33 [n = 283], ReBreed21 [n =281], and ReBreed21+EC [n =280]); primiparous (Resynch33 [n =240], ReBreed21 [n =203], and ReBreed21+EC [n =195]); and multiparous (Resynch33 [n = 205], ReBreed21 [n =203], and ReBreed21+EC [n =195]).

Statistical analyses

Binomial variables including P/AI, cumulative pregnancies during first 21d of breeding season, overall pregnancies by the end of the breeding season, false positives, and false negatives were evaluated by the GLIMMIX procedure of SAS (Version 9.4; SAS Institute). Models included the fixed effect of treatment, AI technician, pasture, and sire. In addition, orthogonal contrasts were analyzed, Resynch33 vs. both ReBreed21 groups, and ReBreed21 vs. ReBreed21+EC for all binomial variables.

To analyze the relationship of fertility at first AI with percentage increase caused by the rebreeding program (ReBreed21 plus ReBreed21 + EC), each replicate (pasture of cows kept in same pasture and bred on the same day) was separated (< or >) based on the average P/AI at first TAI (55.1%; 1149/2085 – Moderate fertility < 55.1% and High fertility >55.1%). The replicates were then analyzed for the improvement caused by using ReBreed21 + EC (with estradiol cypionate) vs. Resynch33.

Days to pregnancy was analyzed by survival curves using the PROC LIFETEST procedure of SAS using the Peto-Prentice test that gives more emphasis to the earlier event times [34].

Significant differences between treatment groups were considered for $P \leq 0.05$, whereas differences between $P > 0.05$ and $P \leq 0.10$ were considered a tendency.

Results

Pregnancy/AI for each parity and each TAI of the breeding season are shown in Table 1. Overall pregnancy per AI (P/AI) at the first TAI (55.1%; 1149/2085) did not differ between treatments Resynch33, ReBreed21, ReBreed21+EC and all contrasts). There were differences in P/AI by parity ($P < 0.01$) with 43.0% (361/844) in heifers, 61.3% (391/638) in primiparous, and 66.0% (397/603) in multiparous. For the second TAI, there was no difference between treatments in P/AI in heifers (39.3%; 171/435) and this was not different from overall P/AI at first AI in heifers ($P = 0.23$). However, the ReBreed21+EC group increased P/AI by 51.2% (34.2 vs. 51.7%; $P < 0.01$) in primiparous compared with ReBreed21. In addition, no difference was detected in P/AI for primiparous at second TAI between Resynch33 and ReBreed21+EC (55.8 and 51.7%) and these two groups, combined, were not different than first TAI in primiparous cows ($P = 0.09$). In contrast, multiparous cows at second TAI had greater P/AI in Resynch33 (60.9%, $P < 0.01$) compared with ReBreed21 groups (34.7%; 42/121) regardless of EC treatment. For the third TAI, there were no differences between ReBreed21 and ReBreed21+EC overall and for each parity.

The overall pregnancies at D21 of the breeding season was 24% greater ($P < 0.01$) for cows enrolled in the ReBreed21 programs (70.7%; 959/1357) compared to Resynch33 (55.5%;

404/728) (Figure 2). The most dramatic effect of the ReBreed21 programs compared with Resynch33 was on pregnancies at D21: 45% more pregnancies for heifers (62.9% [353/561] vs. 43.6% [123/283]; $P < 0.01$), followed by a 26% increase in primiparous (76.1% [303/398] vs. 60.4% [145/240]; $P < 0.01$), and 15% for multiparous (76.1% [303/398] vs. 66.3% [136/205]; $P = 0.01$). Thus, the magnitude of the increase in pregnancy at D21 for ReBreed21 groups vs Resynch33 was greatest for heifers (45%), less for primiparous (26%), and least for multiparous (15%), consistent with the differences in fertility at first TAI (highest in multiparous, least in heifers) and the reduction in fertility at second TAI in multiparous but not in heifers (Figure 3).

The percentage of heifers pregnant at the end of the D42 breeding season (Figure 2) was greater ($P < 0.01$) in the ReBreed21 groups compared to Resynch33. However, the percentage of primiparous and multiparous cows that were pregnant at the end of the 42d breeding season was not different for the two ReBreed21 groups compared to Resynch33. Similarly, the overall number of cows pregnant at the end of the breeding season was not different ($P = 0.14$) for the two ReBreed21 groups (79.4%; 1077/1357) compared to Resynch33 (76.2% (555/728).

Survival analysis of nonpregnant cows was used to evaluate the average day to pregnancy during the 42d breeding season (Figure 2). In ReBreed21 and ReBreed21+EC groups the days to pregnancy were earlier than Resynch33 for all cows ($P < 0.01$) and for heifers ($P < 0.01$), whereas there was a tendency in primiparous ($P = 0.06$), and no difference in multiparous ($P = 0.79$) cows (Figure 2). Based on the increase in P/AI in primiparous using EC in the ReBreed21 protocol, a direct analysis compared Resynch33 vs. ReBreed21+EC. An earlier day to pregnancy was found for ReBreed21+EC compared with Resynch33 ($P < 0.01$).

Animals were kept in eight different pastures in this experiment, within each pasture all three treatment groups represented, bred, and pregnancy diagnosed on the same day. Figure 4

shows the results for each individual pasture at 21d and 42d of the breeding season. At 21d of the breeding season, all parities had increased pregnancy with ReBreed21 programs. Nevertheless, at 42d of the breeding season, only the heifers pastures were increased with no difference in cumulative pregnancy in primiparous and multiparous cows (Figure 4).

Based on the fertility at first TAI, pastures were determined to be either high ($n = 4$) or moderate ($n = 4$) fertility pastures (Figure 3). At 21d of the breeding season, the moderate fertility groups had 44.9% (154/343) of cows pregnant at 21d in the Resynch33 group (only first TAI), whereas the percentage pregnant was increased ($P < 0.01$) by using ReBreed21+EC to 64.3% (437/680) producing a relative increase of 43.2%. In high fertility groups, the Resynch33 groups had 64.9% (250/385) pregnant at D21 and ReBreed21+EC increased ($P < 0.01$) percentage pregnant to 77.1% (522/677) for a relative increase of 18.8%. At 42 d of the breeding season, the moderate fertility pastures continued to have an increase in percentage pregnant (66.5 vs 75.7%; $P < 0.01$), whereas the high fertility pastures did not have a difference in overall percentage pregnant (84.9 vs 85.1%; $P = 0.98$).

In the ReBreed21 groups, false negatives could be determined as the percentage of cows that were detected nonpregnant on D21 but were detected pregnant on D33 (Table 2). False negatives were extremely low at 0.2% (2/965) in this study and not affected by using EC ($P = 0.99$). Alternatively, false positives were calculated as the percentage of cows detected pregnant on D21 (by CL blood flow with Doppler) and then were subsequently found to be nonpregnant on D33. Overall, false positives were 13.8% (128/930) and were affected by parity: heifers had more ($P = 0.04$) incidence of false positives (15.7%) compared with cows (primiparous and multiparous = 11.4%). Conversely there was no effect of EC on false positives ($P = 0.36$) in any parity, and there was no interaction of parity and treatment ($P = 0.41$).

Discussion

Our first study with ReBreed21 introduced the basic concept for the rapid reinsemination program [21] and a companion paper (Andrade et. al, submitted) evaluates the physiology associated with this program, however these studies were done in only in a limited number of heifers. In the present study, the ReBreed21 program was evaluated in more than 2000 animals of different parities in a shortened breeding season. Overall, doing a second TAI at 21d after first TAI: 1) increased the percentage of heifers/cows pregnant at 21d of the breeding season, 2) increased the percentage of heifers pregnant at the end of the breeding season, and 3) shortened the day to pregnancy, based on survival analysis. Thus, this large, randomized, controlled trial on a commercial cow-calf operation provides critical information on parity and fertility at first TAI to consider in determining whether to utilize ReBreed21 in a reproductive management program.

The important physiological and fertility differences between parities make it critical to analyze all results by individual parity and, importantly, the number of animals used in this study allowed this analysis. Heifers had the lowest P/AI at first TAI and relative P/AI increased by 43.3% for primiparous (42.8 vs 61.3%, respectively) and increased 53.9% comparing heifers to multiparous cows (42.8% vs 65.8%). For heifers, pregnancy early in the breeding season is critical for productive life, subsequent reproductive performance of the heifer, and for overall economic outcomes in a commercial cow-calf operation. Our results in this study (43.3%) were somewhat lower than the average results in the scientific literature during the last 27 years with P/TAI in *Bos indicus* heifers of 47.0% (7,443/15,841) and 55.1% (30,333/55,012) for *Bos taurus* heifers [35]. Most of the reported *Bos indicus* results are from older heifers (~20 months of age), whereas our study had many younger Nelore heifers (most less than 18 months of age) with many heifers likely

to be prepubertal, based on other reports [36-38]. The ReBreed21 strategy was developed and optimized in Nelore beef heifers and, comparable to our previous results [21], the P/TAI was similar for heifers at second ReBreed21 TAI as at first TAI (41.8 vs 42.4%, respectively). The P/AI was also similar to heifers bred to the Resynch33 strategy (35.0%) but with the obvious advantage that ReBreed21 heifers were bred at 21d after previous TAI compared to 42d after AI for Resynch33 heifers. Accordingly, on the D21st of the breeding season there were 45% more heifers pregnant in ReBreed21 than Resynch33 (62.9% vs. 43.6%). Most non-pregnant heifers in the ReBreed21 programs also had the chance for 2 TAIs by the end of the breeding season (42d) and, accordingly, there were 17% more heifers pregnant by the end of the breeding season in ReBreed21 programs compared to Resynch33 (73.8 vs 63.2%). Of particular importance, the synchronization produced with ReBreed21 allows a synchronized ovulation soon after the previously induced cycle, thus reducing the likelihood that heifers will return to an anovular state after TAI. In herds that use natural service after first TAI, heifers that do not become pregnant to first TAI and return to an anovular state would not be rebred, thus reducing the efficiency of reproductive programs in young non-cycling heifers. The efficiency produced by ReBreed21 in heifers is well-illustrated in the survival curves with, not only more heifers becoming pregnant during the breeding season, but heifers also having earlier pregnancy compared to Resynch33 heifers. Thus, the results with ReBreed21 in heifers are exceptional and warrant continued testing of this protocol in this parity group.

Primiparous beef cattle also face substantial challenges with energy demands of first lactation competing with the metabolic demands of continuing development of the structural frame of the animal [38, 39]. Our review of over 275,000 TAI in beef cattle indicated that primiparous *Bos indicus* had lower P/TAI (39.2%) in published scientific manuscripts than either

heifers or multiparous cows [35]. In contrast, the primiparous cows in our study had much greater P/TAI than heifers (42.6% higher P/TAI; 61.3 vs 43.0%) and only slightly lower fertility than multiparous (7.7% higher). Thus, this particular *Bos indicus* herd did not appear to exhibit the typical low fertility problems in primiparous cows. Arguably the most interesting result with primiparous cows was the improvement in the ReBreed21 program by adding EC to the protocol, resulting in 51.2% more pregnancies compared to the ReBreed21 without exogenous estradiol (51.7 vs 34.2%). This was consistent with our third hypothesis, that EC would increase fertility in the ReBreed21 program, but this hypothesis was only supported in primiparous and not in heifers or multiparous cows. This indicates that primiparous cows may have insufficient endogenous estradiol production during the ReBreed21 protocol used in this study. Other studies are consistent with increasing estradiol during the proestrus period being associated with an increase in fertility [15, 40, 41]. The ReBreed21 program with EC increased (32%) the percentage of primiparous cows pregnant by the 21st day of the breeding season to 79.5% (155/195), compared with Resynch33 (60.4% [145/240]), leading to pregnancy 4.7d earlier during the breeding season. Nevertheless, there was no difference in percentage of primiparous cows pregnant at D42 of the breeding season comparing ReBreed21 programs to Resynch33, likely due to the high fertility in primiparous cows in this herd allowing excellent results with Resynch33 using only two TAI during the 42d breeding season.

In multiparous cows, the results with the present ReBreed21 program, even with the addition of EC, did not indicate an advantage in using this intensive reinsemination program. This herd was clearly well-managed in a way that optimized reproductive performance with about two-thirds (65.8%) of multiparous cows pregnant after the first TAI. This was much greater than what we summarized from previous TAI studies with multiparous *Bos indicus* beef

cattle showing only 50.9% (22,649/44,463) pregnant to first TAI [35]. After only two TAI, a total of 86.8% of multiparous cows were pregnant in our study, a value that would be difficult to surmount, even using a reinsemination program that was optimized for multiparous cows. The current ReBreed21 program was unmistakably not optimal for multiparous cows as P/AI was only about half the fertility observed at first TAI or after the Resynch33 program.

The dramatically improved reproductive performance of heifers in this study highlights the value of using ReBreed21 during the first breeding season in *Bos indicus* females. The first 21d of the breeding season are critical for obtaining high performance in beef operations [10]. Heifers/cows that become pregnant in this period will wean heavier calves (21 kg heavier than dams calving in the second 21d [9]). In addition, heifers that were born in the first 21d of the calving season had greater fertility during their first breeding season [9]. Models on the length of breeding seasons, showed an increase in profitability when heifers and cows become pregnant during the first 21d of the breeding season, due to greater longevity in the herd and more kg of calf weaned during the whole productive life [8, 42]. In this study, 77.4% of cows were pregnant by 21d of the breeding season, indicating a high percentage of cows (77.4% represents 91% of the total pregnant cows) will be eligible for the first TAI and all subsequent TAIs during the next breeding season. This is particularly important for heifers that are entering their primiparous breeding season, resulting in an outstanding beginning to a productive herd life for these animals. Early pregnancy in replacement heifers should result in: production of sufficient replacement cows, increased voluntary culling, earlier parturition and shorter calving season, and calving in more optimal seasonal conditions with greater likelihood of pregnancy as primiparous cow [8, 43, 44]. Potential return on investment (ROI) of 11% increase in pregnancies in heifers for ReBreed21 compared with Resynch33 in a hypothetical breeding season for 200 head is ~21

more calves, thus an increase of ~4,620 kg of calf at weaning (R\$36,960.00 in gross profit at R\$8/kg; Brazilian Real). Assuming R\$942.00 in extra synchronization costs and R\$2,824 extra veterinary fees (2d), this results in a net profit of R\$33,194.00 and an ROI of 881.4%. This value does not include the increase in kg of calf due to earlier pregnancy.

In addition to the major parity effects that were observed with ReBreed21, other observations provided noteworthy physiologic information on the ReBreed21 protocol that may be useful for modification of this protocol or development of more optimized protocols in the future. For example, our second hypothesis, that EC treatment would not reduce fertility to the first TAI, was supported by our results in all three parities with no reduction in P/AI to first TAI by EC treatment in ReBreed21+EC. On D19 after breeding, cows/ heifers should be in the interferon-tau period of pregnancy, which should inhibit any upregulation by estradiol of endometrial oxytocin receptors [24, 28, 45]. In another study, treatment with estradiol benzoate on D22 after TAI was also reported to not decrease P/AI to previous TAI [15].

Doppler ultrasound was found to be a practical tool for detection of nonpregnant heifers/cows [46] with accuracy >90% and few false-negatives (0.2% [2/965]; cows detected non-pregnant by Doppler CL blood flow but found to be pregnant at D33). Previous studies have reported an occurrence of 0% for false negatives [19, 20, 47], although use of PGF in those studies in females detected non-pregnant would likely induce iatrogenic pregnancy loss and reduce/eliminate detection of false negatives [23, 48, 49]. Additionally, in our study, we had a 13.8% overall false positive rate with greater false positives detected in heifers than primiparous and multiparous cows. Our false positive rate is consistent with previous studies [19, 47, 50]. False positives may be due to later CL regression in some heifers/cows or pregnancy loss. Our recent study with non-bred heifers indicates that 22.2% (12/54) of unquestionably non-pregnant

heifers did not have CL regression (circulating progesterone $<1\text{ng/mL}$) by D21, suggesting that later CL regression may account for at least half of false positives (Andrade et. al, submitted). Importantly, the ReBreed21 strategy includes an ultrasound pregnancy diagnosis at D33 that will allow detection of both false negatives (minimal numbers) and false positives, allowing appropriate management decisions to deal with these animals. Evaluation of pregnancy on D33 allows any non-pregnant animals to be resynchronized and receive their second TAI at d42 after previous AI. Thus, the use of the complete ReBreed21 program will allow a minimum of 2 TAIs in non-pregnant females during a 42d breeding season with most non-pregnant animals having the opportunity for 3 TAIs in 42d.

Finally, the practical implementation of the ReBreed21 strategy on commercial beef cattle operations is illustrated by the calendar that can be used to implement a 42d breeding season with 2 ReBreed21 protocols in 4 pastures of cows (Figure 5). First, the priority on this farm is to have no labor on Sundays. The first TAIs are done on Friday and Saturday and the next week on Wednesday and Thursday. The ReBreed21 strategy is implemented and completed during the next 2 weeks with the Doppler pregnancy diagnosis and second TAI done on the same d of the week but 21d later, thus ReBreed21. A critical practical aspect of earlier reinsemination programs is the intense work schedule that can overlap with other treatment days or weekends, according to the chosen strategy [6, 11]. Using the ReBreed21 schedule allows execution of an entire breeding season (3 TAIs) in only 2 months in four groups of cows without work on Sundays, without overlapping workdays, and optimizing the work in the middle of the week. This allows cows that become pregnant to the first and second TAI (ReBreed21) to receive the first and all potentials breeding in the next breeding season. This can be particularly important

for heifers to optimize the number and timing of primiparous calving and the productive life of the cows.

Conclusions

In summary, intensifying the breeding season with ReBreed21: (1) increased the cumulative pregnancy in the first 21d in all parities and at the end of 42d breeding season in heifers; (2) advanced the day to pregnancy in heifers and primiparous females; (3) addition of EC increased the P/AI and tended to increase pregnancies at the end of the breeding season in primiparous cows. Thus, evidence is provided that ReBreed21 can be a strategy to increase reproductive efficiency in *Bos indicus* heifers. Future research should focus on optimizing and testing the ReBreed21 strategy in other physiologic conditions and environments.

Acknowledgments

The authors thank José Ricardo Dias, Cesar Pimenta, Afonso S. Guimaraes, Leuton Barbosa, Sami Merhi, the owners, and staff of the farm Roncador (Mato Grosso state) for their contribution with the heifers and facilities. Support was provided by Hatch Project WIS01240 to MCW and JPNA and USDA-NIFA project 2019-67015-29704 to MCW.

Table 1. Results for pregnancy/AI at each timed artificial insemination for all cows and for each parity during a 42-day breeding season using ReBreed21 or a traditional reinsemination program (Resynch33).

	TAI	Resynch33	ReBreed21	ReBreed21 + EC	¹ FP breeding	P-value	² Resynch33 vs. RBs	³ RB vs. RB+EC
Heifers	1st	43.5% (123/283)	42.3% (119/281)	42.5% (119/280)	.	0.95	0.77	0.97
	2nd	35.0% (56/160)	40.0% (56/140)	43.7% (59/135)	45.8% (22/48)	0.37	0.16	0.53
	3rd	.	32.8% (22/67)	27.0% (17/63)	.	0.46	.	.
	*Preg	63.2% b (179/283)	73.3% a (206/281)	74.3% a (208/280)	.	< 0.01	< 0.01	0.57
Primiparous	1st	60.4% (145/240)	60.1% (122/203)	63.6% (124/195)	.	0.72	0.71	0.47
	2nd	55.8 % a (53/95)	34.2% b (26/76)	51.7% a (31/61)	12.5% b (2/16)	< 0.01	0.05	0.04
	3rd	.	28.9% (13/45)	38.5% (10/26)	.	0.41	.	.
	*Preg	82.5% (198/240)	79.8% (162/203)	85.1% (166/195)	.	0.46	0.94	0.22
Multiparous	1st	66.3% (136/205)	66.5% (135/203)	64.6% (126/195)	.	0.91	0.85	0.69
	2nd	60.9% a (42/69)	34.4% b (21/61)	35.0% b (21/60)	50.0%ab (8/16)	< 0.01	< 0.01	0.95
	3rd	.	21.2% (7/33)	38.2% (13/34)	.	0.14	.	.
	*Preg	86.8% (178/205)	81.8% (166/203)	84.1% (164/195)	.	0.52	0.85	0.32

*Pregnancies at the end of the breeding season.

¹FP breeding is defined as a cow that had a functional CL on D21 but was nonpregnant on D33 and had second TAI at D42 using the Resynch33 program.

²Contrast Resynch33 vs. ReBreed21 + ReBreed21+ECP.

³Contrast ReBreed21 vs. ReBreed21+ECP.

Lowercase letters indicate differences ($p \leq 0.05$) among the pregnancy/AI in each endpoint and

Uppercase letters indicate tendency ($p > 0.05 \leq 0.1$).

Table 2. Results from all early pregnancy diagnoses with use or not of estradiol cypionate (EC) in ReBreed21 (pregnancy diagnosis based on corpus luteum blood flow on D21 after artificial insemination).

		ReBreed21	ReBreed21 + EC	P-value
False Positive¹	Overall	13.0% (62/485)	14.6% (65/445)	0.36
	Heifers	15.8% (39/246)	15.6% (37/237)	0.86
	Primiparous	7.0% (9/131)	14.0% (14/100)	0.12
	Multiparous	13.0% (14/108)	13.0% (14/108)	1.00
	1st TAI	10.6% (33/311)	15.0% (45/301)	0.11
	2nd TAI	16.7% (29/174)	14.6% (21/144)	0.61
	False Negative²	0.2% (1/479)	0.2% (1/480)	0.99

¹False-positives were calculated as the ratio of the number of cows/heifers with CL blood flow on d21 that were not pregnant on d33 divided by the total number of cows/heifers nonpregnant on d33.

²False-negatives were calculated as the ratio of the number of not pregnant cows/heifers without CL blood flow on d21 divided by the total number of cows/heifers pregnant on d33.

Figure Legends

Figure 1. Illustration of the treatments for ReBreed21 and Resynch33 including timing of intravaginal progesterone (P4) device (*kept for 7, 8, or 9 days [previously shown to produce similar outcomes [32]]), estradiol benzoate (EB), estradiol cypionate (EC), equine chorionic gonadotropin (eCG), cloprostenol (PGF), *licerelin acetate* (GnRH), pregnancy

diagnosis (PD) by Doppler (D21 and 42) or B-mode ultrasonography (D33, D54, and 74), nonpregnant (NP) heifers and cows. All cows had a recheck by ultrasound on Day 33.

Figure 2. Survival analysis of the nonpregnant heifers and cows in 42d breeding season with reproductive programs that allow TAIs at every 21 or 42 d. The right side of survival curve the cumulative pregnancy at d 0, 21, and 42 of the breeding season with the average day to pregnancy (\pm SEM) according to survival analysis.

Figure 3. Cumulative pregnancy during 42d breeding season according to the fertility of the first TAI (moderate fertility $P/AI < \text{median}$ - high fertility $P/AI > \text{median}$) for cows enrolled in 2 different reproductive programs Resynch33 and ReBreed21+EC.

Figure 4. Cumulative pregnancy at 21d of the breeding season for each of 8 pastures (Past. in Figure) of cow/ heifers (2 heifers, 3 primiparous, and 3 multiparous) enrolled in Resynch33 and ReBreed21 with and without EC.

Figure 5. ReBreed21 work schedule, different shapes (Circle, square, triangle, and rhombus) means different groups of cows exposing to a breeding season and one future breeding season. US = Doppler ultrasonography exam to evaluate whether cow is “non-pregnant” and ready for second AI based on CL blood perfusion. "P4" with an arrow to top “ \uparrow ” means progesterone device insert, and arrow to down “ \downarrow ” device removed. EC = estradiol cypionate, eCG = equine chorionic gonadotrophin. All cows have to be rechecked by ultrasound on day 33 to determine if there are any false diagnosis and to enroll any false positives for resynchronization and a second TAI at D42.

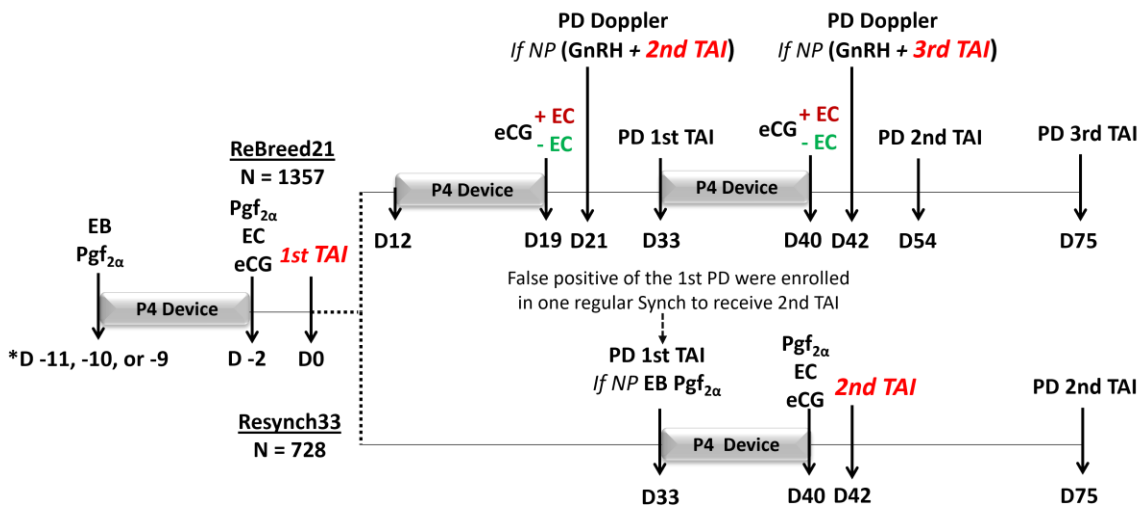


Figure 1

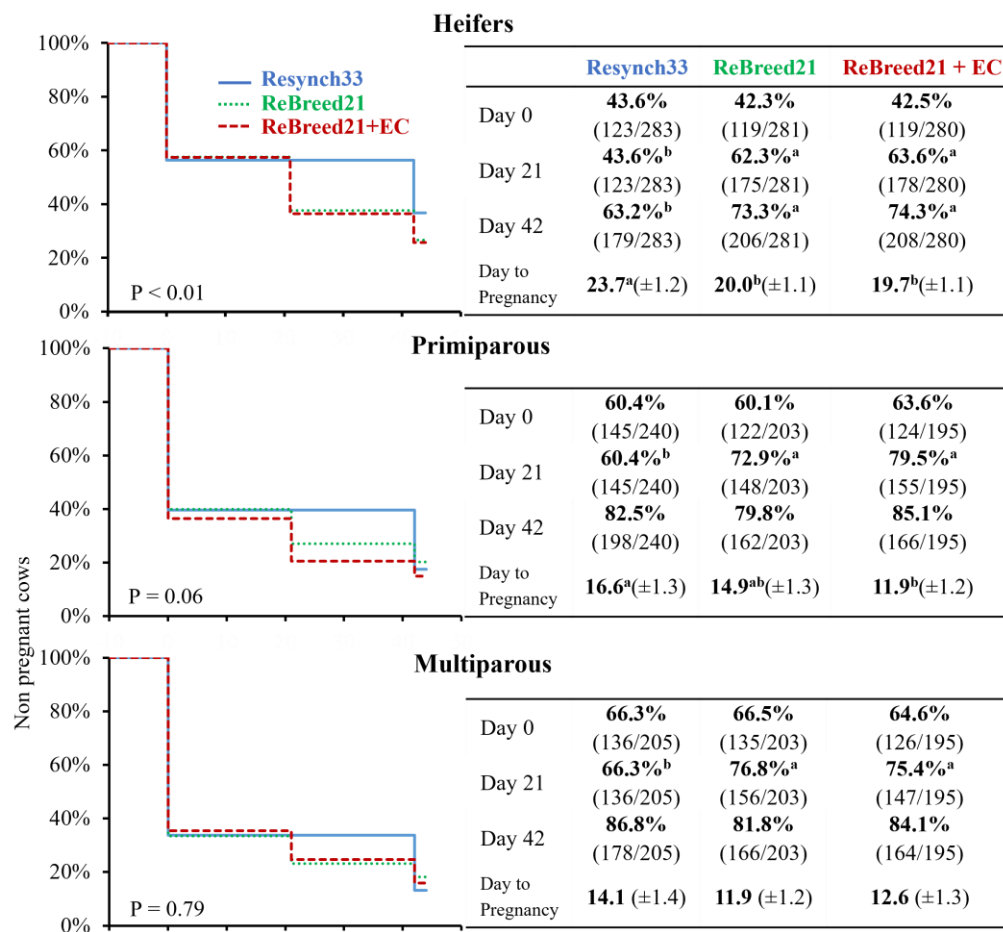
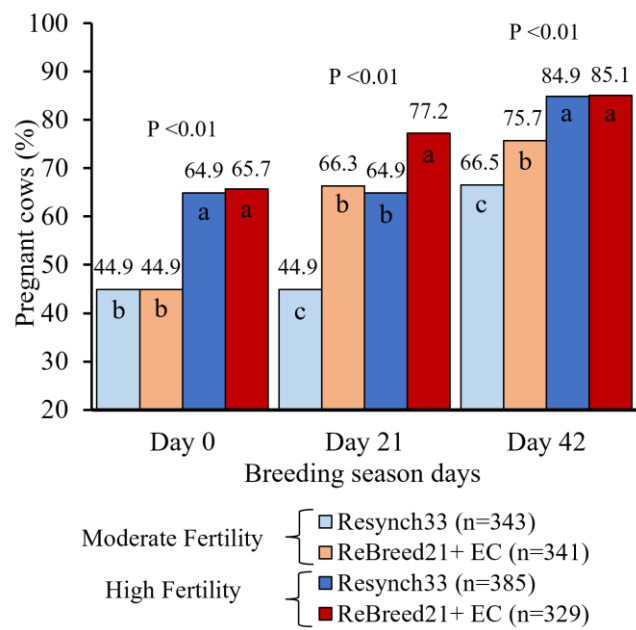


Figure 2



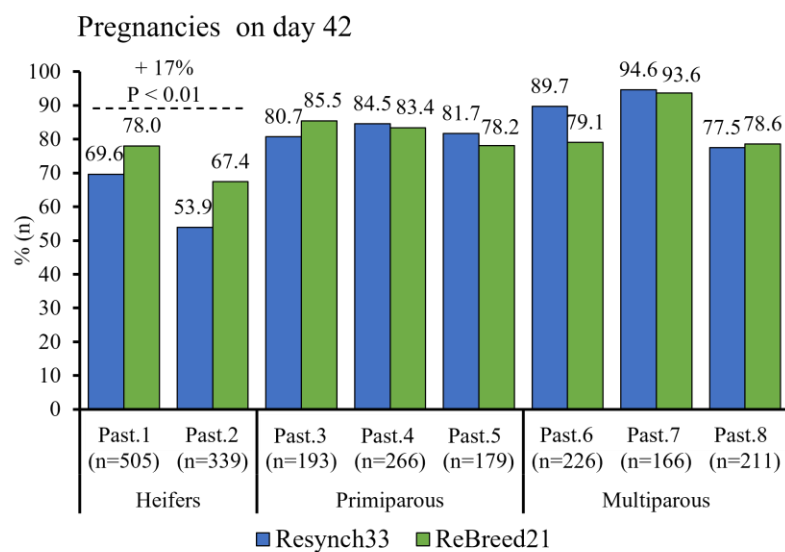
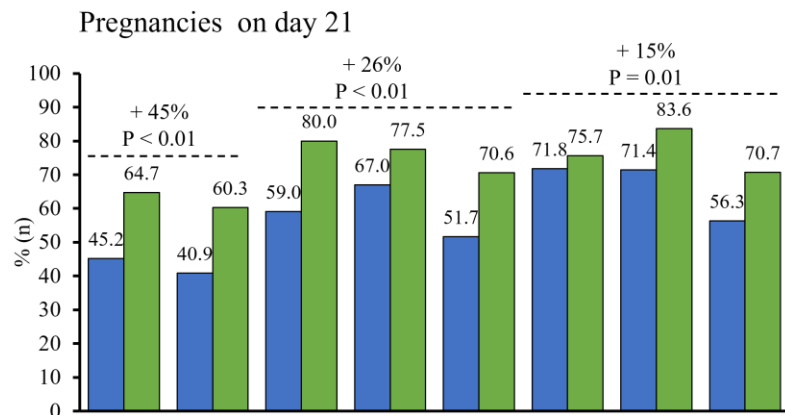


Figure 4

	Sun	Mon	Tus	Wed	Thurs	Fri	Sat
1 st month	X					● 1 st TAI	■ 1 st TAI
	X			▲ 1 st TAI	◆ 1 st TAI		
	X			● P4↑	■ P4↑		
	X	▲ P4↑	◆ P4↑	● P4↓+eCG+EC	■ P4↓+eCG+EC	● US+2 nd TAI	■ US+2 nd TAI
2 nd month	X	▲ P4↓+eCG+EC	◆ P4↓+eCG+EC	▲ US+2 nd TAI	◆ US+2 nd TAI		
	X			● P4↑	■ P4↑		
	X	▲ P4↑	◆ P4↑	● P4↓+eCG+EC	■ P4↓+eCG+EC	● US+3 rd TAI	■ US+3 rd TAI
	X	▲ P4↓+eCG+EC	◆ P4↓+eCG+EC	▲ US+3 rd TAI	◆ US+3 rd TAI		

Figure 5

References

- [1] Pursley JR, Mee MO, Wiltbank MC. Synchronization of ovulation in dairy cows using PGF₂alpha and GnRH. *Theriogenology*. 1995;44:915-23.
- [2] Sa Filho MF, Penteadó L, Reis EL, Reis T, Galvao KN, Baruselli PS. Timed artificial insemination early in the breeding season improves the reproductive performance of suckled beef cows. *Theriogenology*. 2013;79:625-32.
- [3] Baruselli PS, Reis EL, Marques MO, Nasser LF, Bo GA. The use of hormonal treatments to improve reproductive performance of anestrous beef cattle in tropical climates. *Anim Reprod Sci*. 2004;82-83:479-86.
- [4] Vasconcelos JLM, Pereira MHC, Wiltbank MC, Guida TG, Lopes Jr. FR, Sanches Jr. CP, et al. Evolution of fixed-time AI in Brazil. *Anim Reprod*. 2018;15 (Suppl. 1): 940-51.
- [5] Baruselli PS, de Souza AH, de Sa MF, Marques MO, Sales JND. Genetic market in cattle (Bull, AI, FTAI, MOET and IVP): financial payback based on reproductive efficiency in beef and dairy herds in Brazil. *Anim Reprod*. 2018;15:247-55.
- [6] Bo GA, de la Mata JJ, Baruselli PS, Menchaca A. Alternative programs for synchronizing and resynchronizing ovulation in beef cattle. *Theriogenology*. 2016;86:388-96.
- [7] Ojeda-Rojas OA, Gonella-Díaz AM, Bustos-Coral D, Sartorello GL, Reijers T, Pugliesi G, et al. An agent-based simulation model to compare different reproductive strategies in cow-calf operations: Technical performance. *Theriogenology*. 2021;160:102-15.
- [8] Shane DD, Larson RL, Sanderson MW, Miesner M, White BJ. A deterministic, dynamic systems model of cow-calf production: The effects of the duration of postpartum anestrus on production parameters over a 10-year horizon. *J Anim Sci*. 2017;95:1680-95.

- [9] Funston RN, Musgrave JA, Meyer TL, Larson DM. Effect of calving distribution on beef cattle progeny performance. *J Anim Sci.* 2012;90:5118-21.
- [10] Wiltbank J. Challenges for improving calf crop. In: Fields MJ SR, editor. *Factors affecting calf crop*: CRC Press; 1994. p. 1-22.
- [11] Baruselli PS, Ferreira RM, Colli MHA, Elliff FM, Sa MF, Vieira L, et al. Timed artificial insemination: current challenges and recent advances in reproductive efficiency in beef and dairy herds in Brazil. *Anim Reprod.* 2017;14:558-71.
- [12] Pessoa GA, Martini AP, Sa MF, Rubin MI. Resynchronization improves reproductive efficiency of suckled *Bos taurus* beef cows subjected to spring-summer or autumn-winter breeding season in South Brazil. *Theriogenology.* 2018;122:14-22.
- [13] Colazo MG, Kastelic JP, Mainar-Jaime RC, Gavaga QA, Whittaker PR, Small JA, et al. Resynchronization of previously timed-inseminated beef heifers with progestins. *Theriogenology.* 2006;65:557-72.
- [14] Fricke PM. Scanning the future - Ultrasonography as a reproductive management tool for dairy cattle. *J Dairy Sci.* 2002;85:1918-26.
- [15] Sa Filho M, Marques M, Girotto R, Santos F, Sala R, Barbuio J, et al. Resynchronization with unknown pregnancy status using progestin-based timed artificial insemination protocol in beef cattle. *Theriogenology.* 2014;81:284-90.
- [16] Fricke PM, Ricci A, Giordano JO, Carvalho PD. Methods for and Implementation of Pregnancy Diagnosis in Dairy Cows. *Vet Clin North Am Food Anim Pract.* 2016;32:165-80.
- [17] Pugliesi G, Miagawa BT, Paiva YN, Franca MR, Silva LA, Binelli M. Conceptus-induced changes in the gene expression of blood immune cells and the ultrasound-accessed luteal function in beef cattle: how early can we detect pregnancy? *Biol Reprod.* 2014;91:95.

- [18] Scully S, Butler ST, Kelly AK, Evans ACO, Lonergan P, Crowe MA. Early pregnancy diagnosis on days 18 to 21 postinsemination using high-resolution imaging in lactating dairy cows. *J Dairy Sci.* 2014;97:3542-57.
- [19] Palhão M, Ribeiro A, Martins A, Guimarães C, Alvarez R, Seber M, et al. Early resynchronization of non-pregnant beef cows based in corpus luteum blood flow evaluation 21 days after Timed-AI. *Theriogenology.* 2020;146:26-30.
- [20] Motta I, Rocha C, Bisinotto D, Melo G, Júnior GA, Silva A, et al. Increased pregnancy rate in beef heifers resynchronized with estradiol at 14 days after TAI. *Theriogenology.* 2020;147:62-70.
- [21] Andrade JPN, Gomez-Leon VE, Andrade FS, Carvalho BP, Lacouth KL, Garcia FZ, et al. Development of a novel 21-day reinsemination program, ReBreed21, in *Bos indicus* heifers. *Theriogenology.* 2020;155:125-31.
- [22] Copeland DD, Schultz RH, Kemtrup ME. Induction of Abortion in Feedlot Heifers with Cloprostenol (A Synthetic Analog of Prostaglandin-F2alpha) - Dose-Response Study. *Can Vet J.* 1978;19:29-32.
- [23] Colak A, Oral H, Pancarci SM, Hayirli A. Comparison of the Efficacy of the Administration Route of D-Cloprostenol to Induce Abortion in Undesirable Pregnancy. *J Vet Adv.* 2008;7:903-6.
- [24] Domingues RR, Ginther O, Gomez-León VE, Wiltbank MC. Up-regulation of endometrial oxytocin receptor is associated with the timing of luteolysis in heifers with two and three follicular waves. *Biol Reprod.* 2020;102:316-26.

- [25] Drum JN, Wiltbank MC, Monteiro PLJ, Prata AB, Gennari RS, Gamarra CA, et al. Oxytocin-induced prostaglandin F2-alpha release is low in early bovine pregnancy but increases during the second month of pregnancy. *Biol Reprod.* 2020;102:412-23.
- [26] Sa Filho MF, Gonella-Diaza AM, Sponchiado M, Mendanha MF, Pugliesi G, Ramos RDS, et al. Impact of hormonal modulation at proestrus on ovarian responses and uterine gene expression of suckled anestrous beef cows. *J Anim Sci Biotechnol.* 2017;8:79.
- [27] Bridges GA, Mussard ML, Pate JL, Ott TL, Hansen TR, Day ML. Impact of preovulatory estradiol concentrations on conceptus development and uterine gene expression. *Anim Reprod Sci.* 2012;133:16-26.
- [28] Araujo RR, Ginther OJ, Ferreira JC, Palhao MM, Beg MA, Wiltbank MC. Role of follicular estradiol-17beta in timing of luteolysis in heifers. *Biol Reprod.* 2009;81:426-37.
- [29] Arosh JA, Lee J, Stephen SD, Stanley JA, Yang B, Nithy TK, et al. Intrauterine Infusion of Interferon Tau Selectively Directs Intraluteal Prostaglandin Biosynthesis Towards PGE2 and Activates EP2 and EP4-Mediated Signaling in the Corpus Luteum at the Time of Establishment of Pregnancy in Ruminants. *Biol Reprod.* 2011;85:373.
- [30] Sa MF, Marques MO, Giroto R, Santos FA, Sala RV, Barbuio JP, et al. Resynchronization with unknown pregnancy status using progestin-based timed artificial insemination protocol in beef cattle. *Theriogenology.* 2014;81:284-90.
- [31] Meneghetti M, Sa OG, Peres RFG, Lamb GC, Vasconcelos JLM. Fixed-time artificial insemination with estradiol and progesterone for *Bos indicus* cows I: Basis for development of protocols. *Theriogenology.* 2009;72:179-89.

- [32] Prata AB, Madureira G, Robl AJ, Ribeiro HS, Sagae M, Elias MCV, et al. Progesterone-based timed AI protocols for *Bos indicus* cattle III: Comparison of protocol lengths. *Theriogenology*. 2020;152:29-35.
- [33] Andrade JPN, Andrade FS, Guerson YB, Domingues RR, Gomez-Leon VE, Cunha TO, et al. Early pregnancy diagnosis at 21 days post artificial insemination using corpus luteum vascular perfusion compared to corpus luteum diameter and/or echogenicity in Nelore heifers. *Anim Reprod Sci*. 2019;209:106144.
- [34] Karadeniz PG, Ercan I. Examining tests for comparing survival curves with right censored data. *Stat Transit*. 2017;18:311-28.
- [35] Monteiro PL, Consentini CEC, Andrade JPN, Beard AD, Garcia-Guerra A, Sartori R, et al. Research on timed AI in beef cattle: Past, present and future, a 27-year perspective. *Theriogenology*. 2023;211:161-71.
- [36] Sa MF, Nasser LFT, Penteadó L, Prestes R, Marques MO, Freitas BG, et al. Impact of progesterone and estradiol treatment before the onset of the breeding period on reproductive performance of *Bos indicus* beef heifers. *Anim Reprod Sci*. 2015;160:30-9.
- [37] de Lima R, Martins T, Lemes K, Binelli M, Madureira E. Effect of a puberty induction protocol based on injectable long acting progesterone on pregnancy success of beef heifers serviced by TAI. *Theriogenology*. 2020;154:128-34.
- [38] Marques MD, Morotti F, da Silva CB, Ribeiro M, da Silva RCP, Baruselli PS, et al. Influence of category - heifers, primiparous and multiparous lactating cows - in a large-scale resynchronization fixed-time artificial insemination program. *J Vet Sci*. 2015;16:367-71.

[39] Cooke RF, Lamb GC, Vasconcelos JLM, Pohler KG. Effects of body condition score at initiation of the breeding season on reproductive performance and overall productivity of *Bos taurus* and *B. indicus* beef cows. *Anim Reprod Sci.* 2021;232.

[40] Sa Filho MF, Santos JE, Ferreira RM, Sales JN, Baruselli PS. Importance of estrus on pregnancy per insemination in suckled *Bos indicus* cows submitted to estradiol/progesterone-based timed insemination protocols. *Theriogenology.* 2011;76:455-63.

[41] Pessoa GA, Martini AP, Sá Filho MF, Rubin MIB. Resynchronization improves reproductive efficiency of suckled *Bos taurus* beef cows subjected to spring-summer or autumn-winter breeding season in South Brazil. *Theriogenology.* 2018;122:14-22.

[42] Cushman RA, Kill LK, Funston RN, Mousel EM, Perry GA. Heifer calving date positively influences calf weaning weights through six parturitions. *J Anim Sci.* 2013;91:4486-91.

[43] Sa Filho OG, Meneghetti M, Peres RFG, Lamb GC, Vasconcelos JLM. Fixed-time artificial insemination with estradiol and progesterone for *Bos indicus* cows II: Strategies and factors affecting fertility. *Theriogenology.* 2009;72:210-8.

[44] Sa Filho OG, Thatcher WW, Vasconcelos JL. Effect of progesterone and/or estradiol treatments prior to induction of ovulation on subsequent luteal lifespan in anestrus Nelore cows. *Anim Reprod Sci.* 2009;112:95-106.

[45] Arosh JA, Banu SK, Kimmins S, Chapdelaine P, MacLaren LA, Fortier MA. Effect of interferon- π on prostaglandin biosynthesis, transport, and signaling at the time of maternal recognition of pregnancy in cattle: Evidence of polycrine actions of prostaglandin E-2. *Endocrinology.* 2004;145:5280-93.

[46] Pugliesi G, Rezende RG, Silva Jd, Lopes E, Nishimura TK, Baruselli PS, et al. Uso da ultrassonografia Doppler em programas de IATF e TETF em bovinos. *Rev Bras Reprod Anim.* 2017;41:140-50.

[47] Pugliesi G, Bisinotto DZ, Mello BP, Lahr FC, Ferreira CA, Melo GD, et al. A novel strategy for resynchronization of ovulation in Nelore cows using injectable progesterone (P4) and P4 releasing devices to perform two timed inseminations within 22 days. *Reprod Domest Anim.* 2019;54:1149-54.

[48] Youngquist RS, Bierschwal CJ, Elmore RG, Jenkins AL, Schultz RH, Widmer JH. Induction of Abortion in Feedlot Heifers with Cloprostenol (ICI 80,996). *Theriogenology.* 1977;7:305-12.

[49] Lobago F, Gustafsson H, Bekana M, Beckers JF, Kindahl H. Clinical features and hormonal profiles of cloprostenol-induced early abortions in heifers monitored by ultrasonography. *Acta Veterinaria Scandinavica.* 2006;48.

[50] da Silva AG, Nishimura TK, Rocha CC, Motta IG, Neto AL, Ferraz PA, et al. Comparison of estradiol benzoate doses for resynchronization of ovulation at 14 days after timed-AI in suckled beef cows. *Theriogenology.* 2022;184:41-50.

Chapter 4: Identification of nonpregnant beef cows based on CL size vs. luteal blood perfusion at 21 days after timed artificial insemination

João Paulo. N. Andrade^a; Rafael R. Domingues^{a,b}; Pedro L. J. Monteiro^{a,c}; José Ricardo Dias^d; Cesar Pimenta^d; Afonso S. Guimarães^e; Leuton Barbosa^f; Sami Merhi^d; Roberto Sartori^e; Milo C. Wiltbank^{a*}

^a Department of Animal and Dairy Sciences, University of Wisconsin-Madison, Wisconsin 53706, USA

^b Department of Animal Sciences, The Ohio State University, Columbus, Ohio, 43210, USA

^c Department of Large Animal Clinical Sciences, University of Florida, Gainesville, FL 32608, USA

^d Agropecuária Roncador, Av. Rio Grande do Sul, 387, Querência, MT, 78643-000, Brazil

^e Department of Animal Science, Luiz de Queiroz College of Agriculture (ESALQ), University of São Paulo, Av. Pádua Dias, 11, Piracicaba, SP, 13418-900, Brazil

^f Agrobeef Ltda, Avenida Senador Valdon Varjão, 4025. Barra do Garças, MT, 78605-970.

Corresponding Author: Milo C. Wiltbank E-mail: wiltbank@wisc.edu

Published in Theriogenology

Andrade JPN, Domingues RR, Monteiro PLJ, et al. Identification of nonpregnant beef cows based on CL size vs. luteal blood perfusion at 21 days after timed artificial insemination.

Theriogenology. 2024;230:15-20.

Abstract

The aim was to evaluate the efficiency of two different ultrasonographic systems, Doppler mode vs. Two-dimensional mode (B mode), to identify the pregnancy status of beef cows and heifers on day 21 (D21) after Timed Artificial Insemination (TAI). The experiment was performed on a commercial beef farm in central-west region of Brazil using 1,895 Nelore heifers and cows. All females had ovulation synchronized for a TAI that was performed on D0. Twenty-one days after the TAI, all animals had their ovaries evaluated by ultrasound for pregnancy diagnosis based on the size of the corpus luteum (CL). Using B mode ultrasonography, females without a CL or with a CL ≤ 10 mm in diameter were considered nonpregnant, whereas females with a CL >10 mm in diameter were considered potentially pregnant. After the B mode examination, the Doppler mode was turned on, and the CL was evaluated by the subjective percentage of blood perfusion in the total area of the CL. Using Doppler mode, females were considered nonpregnant if they had no CL or the CL had $\leq 25\%$ of the total area with detectable blood perfusion, whereas animals with $>25\%$ blood perfusion in the CL were considered potentially pregnant. The results for each method (potentially pregnant or nonpregnant) were later compared with the gold standard technique, which was a pregnancy diagnosis on D33 after TAI using ultrasound with visualization of an embryonic heartbeat. The accuracy was determined using the 2x2 contingency table approach. The area under the curve using the receiver operating characteristic curve for Doppler mode and B mode were 0.929 and 0.902 ($P<0.01$), respectively. There were almost no false negatives (designated non-pregnant but later pregnant at D33) with either technique (0.2% vs. 0.3%; $P=0.65$ for Doppler mode vs. B mode, respectively). False positives (designated pregnant but non-pregnant on D33) were greater for B mode compared to Doppler (19.1% vs. 14.0%; $P<0.01$). This resulted in Doppler mode having similar high values as B

mode for Negative Predictive Value (99.9 vs. 99.6%; $P=0.85$) and Sensitivity (99.8 vs. 99.7%; $P=0.86$) but there were differences in Specificity (86 vs. 80.9%; $P<0.01$), Positive Predictive Value (88 vs. 84.3%; $P<0.01$), and Accuracy (93.0 vs. 90.4%; $P<0.01$). In conclusion, evaluation of CL blood perfusion by Doppler produced greater accuracy in the early identification of nonpregnant heifers and cows on D21 after TAI than measurement of CL diameter with B mode ultrasound; although both had over 90% accuracy in identifying pregnant and nonpregnant females.

Keywords: *Bos indicus*, reproductive efficiency, resynchronization, corpus luteum, ultrasonography.

Introduction

Use of rectal palpation for pregnancy diagnosis after the end of the breeding season with subsequent culling of non-pregnant cows was one of the first strategies used to improve the economics and reproductive performance of beef cattle operations. Veterinarians could perform a pregnancy diagnosis using rectal palpation with high accuracy (> 95%); however, only after ~40 days in gestation [1]. As new reproductive biotechniques, such as Timed Artificial Insemination (TAI), were incorporated into cow-calf operations, it became desirable to have earlier identification of pregnancy status in order to have more intensive programs that produced greater reproductive efficiency [2]. Rectal ultrasonography became the gold standard technique to determine pregnancy status with high accuracy at ~30 days after TAI [3]. The ultrasound in the two-dimensional mode (B mode) provides visualization of the embryo so that pregnancy diagnosis is determined by direct detection of an embryo with a positive heartbeat and selection of appropriate approaches for rebreeding any nonpregnant cows during the breeding season [4-6]. Nevertheless, to further optimize reproductive efficiency, nonpregnant cows should ideally be bred before D30 after the previous TAI, which is earlier than typically done using classical pregnancy diagnosis with B mode ultrasonography. Therefore, indirect methods for earlier pregnancy diagnosis emerged as a possibility to develop reproductive programs with shorter interbreeding intervals.

Indirect methods for pregnancy diagnosis have utilized laboratory assays that target the presence or absence of molecules (hormones, proteins, or mRNA) that are associated with a specific stage of the female reproductive cycle and/or the pregnancy status of the female with some degree of accuracy [7-9]. The accuracy of each technique relies on its ability to precisely detect pregnant (specificity) or nonpregnant (sensitivity) cows [10]. For indirect methods of

pregnancy diagnosis, false negative results must be avoided as this misdiagnosis may lead to pregnancy loss in pregnant cows incorrectly treated with prostaglandin (PG) F_{2α}. On the other hand, high incidence of false positives decreases the utilization rate of nonpregnant cows in the subsequent TAI program [11]. The most common molecules that are used for pregnancy diagnosis in cattle are progesterone (P₄; in blood or milk samples), pregnancy-associated glycoproteins (PAGs; in blood or milk samples), and mRNA for interferon-stimulated genes (ISGs; in cells from blood, vagina and/or cervix) [12-17]. These techniques require collection of a biological sample (blood, milk, or vaginal/cervical epithelial cells) and processing/analyzing either in a laboratory or at the farm. In practical terms, however, immediate results (such as in ultrasonography or rectal palpation) are critical in beef cattle operations to optimize decision-making strategies that minimize animal handling and reduce intervals between breeding. Thus, although P₄ tests are generally impractical on commercial beef cattle operations, identification of cows with high P₄ can be immediately determined indirectly by assessing corpus luteum (CL) blood perfusion using color Doppler ultrasound (Doppler mode) [18]. This has recently been used as an indirect method for early pregnancy diagnosis in cattle [16, 19-21].

Early reports showed dramatic changes in ovarian and uterine blood perfusion during the reproductive cycle and pregnancy of cattle using Doppler ultrasound [22, 23] and strategies were proposed to use CL blood perfusion as a technique for early pregnancy diagnosis [21, 24]. To produce a practical tool, researchers proposed a classification system for luteal blood perfusion using subjective scores of the total CL area containing blood perfusion, reporting a similar or better accuracy compared to circulating P₄ or ISGs in beef cattle [16, 22-25]. Using this technique for early pregnancy diagnosis, novel reproductive programs were proposed that allowed resynchronization of ovulation and shorter intervals between breeding of 24, 23, or even

21 days between TAIs [21, 24, 26, 27]. Importantly, there were few or no false negative diagnoses reported when using the Doppler mode as a technique for earlier nonpregnancy diagnosis. However, assessing CL blood perfusion with Doppler mode requires technical skill under field conditions to accurately classify cows as pregnant or nonpregnant. In addition, ultrasound machines equipped with Doppler are more expensive than a B mode ultrasound, again making them less practical in the field. Interestingly, research prior to the studies exploring Doppler for pregnancy diagnosis, evaluated the use of CL size by B mode ultrasound for early identification of nonpregnant dairy cows but the researchers reported low accuracy, discouraging further development of the technique [28]. Nevertheless, our recent study and a previous study from another laboratory comparing CL ultrasonography images using Doppler mode vs. B mode in Nelore heifers or cows showed similar accuracy, but a limited number of females were used in both these experiments (n = 113 [29]; n = 110 [16]).

Thus, the present study was designed to compare the use of Doppler-mode (CL blood perfusion) vs. B-mode (CL size) to determine the precision of these techniques for early nonpregnancy diagnosis on D21 in a large cow/calf operation. We hypothesized that both techniques would have: (1) similar accuracy, (2) similar precision to detect pregnant cows (specificity and positive predictive value), and (3) similar precision to detect nonpregnant cows (sensitivity and negative predictive value).

Materials and Methods

The experiments were performed at Roncador Farm, located in Querência, MT, Brazil. Animals were kept on pasture (*Brachiaria brizantha*) supplemented with mineral salt and had ad libitum access to water. Nelore *Bos-indicus* beef cattle (n = 1,895) from different parities

(Nulliparous [n = 836] kept in two different pastures, Primiparous [n = 534] kept in three different pastures, and Multiparous [n = 525] kept in three different pastures) were used in the study. The Animal Research Ethics Committee of “Luiz de Queiroz” College of Agriculture of the University of São Paulo (ESALQ/USP) approved all animal procedures. Heifers/cows were enrolled in an estradiol/P4-based protocol that allowed first TAI (designated D0) [30]. The treatments began when multiparous and primiparous cows were approximately 35d after calving, and heifers were at 14 to 20 months of age with ≥ 280 kg of body weight. All TAI were performed by five experienced technicians using frozen/thawed semen from sires of proven fertility (five Rubia Gallega or two Nelore).

A commercial ultrasound machine (SonoScape E2 PRO) B mode (6.8-10.1 MHz frequency probe, settings: 8 cm depth, 170 gain, and 140 dynamic range) and color Doppler-mode (5.7 MHz frequency, settings: 80 color gain, 130 gain, 130 color filter, and 1.2 kHz pulse repetition frequency) was used for ultrasonography exams. All the ultrasound exams were performed by one experienced technician. The B mode evaluation of ovaries utilized subjective structural features of the CL. To aid in estimating the CL size, images were compared to two vertical white lines that were fixed on the ultrasound screen at 10 mm apart (Figure 1).

On D21 (21 days after TAI), the B mode ultrasonography of the CL was performed with Doppler mode off. Heifers/cows were classified into two subjective categories and information on B mode was recorded for later analysis: nonpregnant (no CL or CL ≤ 10 mm of diameter) and potentially pregnant (at least one CL > 10 mm of diameter). After the ovaries were examined by B mode, the Doppler mode was then turned on, and CL were evaluated using subjective scores of blood perfusion [29] and classified into two categories: nonpregnant (no CL or CL with $\leq 25\%$ of

the total area with color blood perfusion signals) and potentially pregnant (at least one CL with >25% of the total area with color blood perfusion signals).

The gold standard diagnosis for pregnancy was the ultrasonography exam on D33 after TAI. Heifers/cows with a positive embryonic heartbeat were considered pregnant. False positives were cows/heifers that were classified as potentially pregnant by the early nonpregnancy diagnosis (on D21) but were not pregnant on D33 divided by the number of all nonpregnant heifers/cows on D33. False negatives were heifers/cows that were nonpregnant by the early pregnancy diagnosis (on D21) but were pregnant on D33 divided by the number of all pregnant females on D33.

Statistical analyses

The precision of each method was evaluated using the 2x2 contingency table approach [29]. Data were analyzed with SAS (Version 9.4; SAS Institute) using PROC GLIMMIX for binomial data (negative predictive value [NPV], positive predictive value [PPV], specificity, sensitivity, accuracy, false negative, and false positive). The statistical model includes as fixed effects Sire, Parity and US mode as a last predictor to estimate extra sum of squares from US mode. Each pasture has unique parity distribution, thus pasture was not included in overall model to avoid multicollinearity when two or more explanatory variables are moderately or highly correlated. But, within parity analysis for false positive Pasture was include in the model instead of Parity. Receiver operating characteristics analysis was performed using PROC LOGISTIC. Significant differences between techniques were considered for $P \leq 0.05$, whereas differences between $P > 0.05$ and $P \leq 0.10$ were considered a tendency.

Results

Table 1 shows the frequency of different diagnoses in *Bos indicus* Nelore heifers and cows on D21 after TAI using ultrasound on either B mode to evaluate CL size vs. Doppler mode to evaluate CL blood perfusion. The true positive rate was 86.0% for Doppler and 80.9% for B mode with an effect of ultrasound mode ($P < 0.01$) and Parity ($P < 0.0001$). Accordingly, false positives were greater in B mode than Doppler ($P < 0.01$) and greater for heifers than for primiparous or multiparous ($P < 0.0001$) cows (Table 1). In contrast, there were very few false negatives in either group ($\leq 0.3\%$) with no differences between ultrasound method ($P = 0.65$) resulting in true negatives in both groups of $>99\%$. Since the incidence of false negatives was almost zero in both groups, these data were not analyzed by parity. The overall accuracy or true diagnoses were greater ($P < 0.01$) for Doppler at 93.0% (1763/1895) than for B mode 90.4% (1714/1895).

Receiver operating characteristics (ROC) and efficiency of Doppler mode and B mode ultrasonography for early identification of nonpregnancy status of beef cows/heifers are shown in Figure 2. For the ROC curve analysis, Doppler mode had a greater area under the curve compared to B mode (0.9291 vs. 0.9027; $P < 0.0001$). The overall accuracy of pregnancy/nonpregnancy diagnoses performed by either Doppler or B mode ultrasound was greater than 90%; yet Doppler mode was slightly (93.0 vs 90.4%; 2.6% absolute difference or 2.9% relative difference) but significantly ($P < 0.01$) more accurate than B mode ultrasonography (Figure 2B).

Since the frequency of false negatives was so low for both groups, both techniques were similar and had NPV and sensitivity above 99%, indicating that both techniques were excellent in not classifying pregnant females as nonpregnant (Figure 2). On the other hand, the PPV and specificity were different ($P < 0.01$) between techniques showing that B-mode designated more of

the non-pregnant cows as pregnant compared to Doppler. For example, the PPV for Doppler was 88.0% compared to only 84.3% with B mode (3.7% absolute difference and 4.2% relative difference) ($P < 0.01$; Figure 2B).

Discussion

Although methods of early pregnancy/nonpregnancy diagnoses have been previously compared [14, 17, 31], including comparisons of B mode vs. Doppler [16, 29, 32], the present study did these important comparisons in a commercial operation, under field conditions with a large number of females. Using the B mode ultrasound for subjective estimation of the CL size produced almost no false negatives, similar to the Doppler method using estimation of CL blood perfusion. Nevertheless, in contrast to previous studies [16, 29] with limited numbers of females in the study, the present study using almost 2,000 females detected a small (about 3%) but significant increase in accuracy along with a lower incidence of false positives utilizing the Doppler mode compared to B mode [29]. Yet, both methods gave an overall accuracy above 90%, showing that either method can be reliably used for nonpregnancy diagnoses at D21 after breeding. Importantly, our results demonstrate that these methods can be used to support current resynchronization practices by identifying nonpregnant heifers/cows under field conditions. Combining these early pregnancy diagnosis techniques with a rapid resynchronization program, such as ReBreed21 [33, 34], can decrease the interval between TAI, potentially decreasing time to pregnancy and thereby increasing reproductive and productive efficiency on beef cattle operations. In this discussion we will focus on the factors affecting accuracy of the two methods, particularly the physiology behind the incidence of false positives and how these methods can be practically applied in beef cattle reproductive programs.

The overall accuracy of any diagnostic test is calculated using a gold standard which, in this trial, was direct ultrasound of the embryo on D33 after TAI including detection of an embryonic heartbeat. In contrast to our first hypothesis and to previous studies [16, 29], the accuracy differed between these two techniques due to greater percentage of false positives with the B mode than with Doppler but both had an accuracy > 90%, which is generally considered acceptable [10].

For an earlier pregnancy diagnosis in cattle, the error that must be avoided is the false negative as this may lead to treatment with PGF2a and an iatrogenic pregnancy loss [35]. In other studies and reproductive programs, nonpregnant heifers/cows received treatment with an analog of PGF2 α that could cause loss of the pregnancy [24, 26, 36]. Therefore, most early resynchronization programs would not have false negatives [35]. In this study no PGF2 α was given, as is typical for ReBreed21 [33], to nonpregnant females and both techniques (B mode and Doppler) showed a low incidence of false negative diagnoses. By comparison, measurement of mRNA for ISGs in peripheral blood can have 10-30% false negatives, limiting this technique to mainly research purposes [16, 25, 37]. Recently, measurement of ISGs in cervical or vaginal epithelial cells improves accuracy (Domingues et al., 2023) but the costs and need for a complex laboratory analysis still limits the usefulness of ISGs in commercial operations. The less complex pregnancy assays for circulating PAGs have a low incidence of false negatives when used after D27 [14, 15, 17, 38]. A recent study in lactating dairy cows showed that an increase of at least 10% in optical density from D17 to D24 after TAI had 100% sensitivity, although this approach would likely not be practical on beef cattle operations due to the requirement to collect multiple samples [39]. Thus, based on infrequent false negatives, measurement of either CL diameter or CL blood perfusion can be effectively used for nonpregnancy diagnosis, although under field

conditions there are a few false negatives, probably due to technical issues such as miscommunication during the ultrasound exam. These rare mistakes highlight the importance of avoiding treatment with a $\text{PGF}_{2\alpha}$ after early pregnancy diagnosis either by Doppler or B mode ultrasound.

The other side of accuracy for an early pregnancy diagnosis technique is the positive predictive ability of the method. Our second hypothesis, based on previous results [16, 29], was that B mode and Doppler mode would have similar precision in detecting pregnant cows; although we knew that false positive diagnoses have been frequently reported with any technique, including CL blood perfusion, used near D21 of bovine pregnancy [21, 24]. Our results did not support our second hypothesis since the false positives were greater using B mode than Doppler in all parities. Thus, about 5.1% of cows that were diagnosed nonpregnant based on CL blood perfusion were diagnosed pregnant by CL size using B mode ultrasound. This difference is likely due to differences in the timing among measurable CL functions. For example, a 50% decrease in circulating P4 precedes the 50% decrease in CL blood perfusion by about 0.5 days, and this is followed by the decrease in CL diameter 0.5 to 1d later [40-42]. That is, during luteolysis, CL blood perfusion decreases earlier than the decrease in CL size. Therefore, the greater false positives based on CL size are likely due to heifers/cows that are in the earlier stages of luteolysis when CL blood perfusion had decreased but there was not yet a large enough change in CL size to be designated nonpregnant [43, 44]. Nevertheless, there were also a substantial number of females that were false positives, even with the Doppler technique. These are likely due to either early pregnancy loss between D21 and D33 or later CL regression in nonpregnant females. A high incidence of pregnancy loss between D21 and D33 has been reported in dairy cows [37, 45, 46] but this is likely lower in beef cattle than dairy cattle [24, 26,

36]. Several studies have labelled false positives as pregnancy losses [24, 26]. However, nonpregnant females that have CL regression later than 21 days would also be false positives. In other studies using nonbred Nelore heifers and cows some animals had luteolysis later than D21 [47], for example, we observed 22.2% (12/54) of nonbred Nelore heifers had luteolysis after D21, suggesting that many false positives may be due to later luteolysis [48]. Of special interest, heifers had greater incidence of false positives than cows perhaps due to a combination of greater pregnancy losses, as would be expected [36], and greater incidence of four follicular wave patterns (luteolysis after D21). Thus, false positives are a problem with either method of early pregnancy diagnosis and this study has provided a reliable estimate of this problem in *Bos indicus* cattle using the two ultrasound methods and evaluating almost 2000 cows/heifers.

Finally, the practical role for a method that precisely identifies nonpregnant females at earlier stages of pregnancy is primarily related to potential incorporation in a reproductive program that includes a rapid rebreeding programs [10]. The use of ultrasonography either by Doppler mode or B mode conveniently provides the information in real time at cow side, thus providing on-farm applicability. For example, combining ReBreed21 with either ultrasound technique would allow 80% or more of the nonpregnant cows that would be identified on D33, to be bred at D21 after previous TAI permitting completion of an entire breeding season with three potential TAIs in 42 days. This strategy can be particularly effective in heifers due to the inherent lower fertility at first AI and relatively high fertility to the ReBreed21 program [34]. Several studies have reported that a shorter, more intensive breeding season with shorter postpartum anestrus can increase herd reproductive performance consequently increasing the profitability of the operation by producing more calves and heavier calves at weaning and producing more fertile replacement heifers [49-51]. An important practical finding of this large

study was that either B mode or Doppler can be used with low risk for iatrogenic pregnancy losses, due to their exceptionally low frequency of false negatives, using the 10 mm diameter criteria applied in this study. On the other hand, using B mode produced greater false positives than Doppler, thus decreasing the number of nonpregnant cows receiving a new earlier TAI. Of importance, there are several limitations for our study. First, only a single very conservative cutoff was utilized to determine non-pregnancy. Other studies that have utilized a larger CL size (CL area $<2 \text{ cm}^2$; $\sim 16 \text{ mm}$ diameter) report a much greater number of false negative results [32]. Second, future practical economic studies are needed to model and investigate the investments/returns using different lengths of breeding season, methods of early pregnancy diagnosis, and rebreeding strategies, particularly comparing whether the economics differ by parity. These studies should focus on one of the prime endpoints for profitability in beef cattle, the weight of calves at weaning. Some studies have used simulated computer models to predict the production and economic performance of beef cattle herds under different reproductive management programs (10 different scenarios) that included resynchronization programs that used Doppler ultrasonography to determine pregnancy [50, 51]. Use of TAI and use of more rapid resynchronization programs (24 days between AIs in their most aggressive scenario) were associated with weaning of more calves and heavier calves [51] and generally better economics than use of natural mating or less aggressive programs [50]. Nevertheless, further studies are needed that directly compare programs in beef cattle herds, rather than just with computer simulations, to validate the concept that more aggressive TAI programs using rapid resynchronization programs, such as ReBreed21, can improve production and economics of commercial beef cattle operations. A third limitation of our study is that a single, well-experienced ultrasound technician performed all examinations in this study and studies are

needed to compare the variability in results obtained with other ultrasound technicians under field conditions.

Conclusion

In summary, using B mode ultrasonography as an early (D21) pregnancy diagnosis procedure in comparison to Doppler mode ultrasound had similar and low incidence of false negatives but greater number of false positives, resulting in lower accuracy. Hence, the present results demonstrate that B mode can be used as a fairly accurate method to select nonpregnant heifers/cows at an earlier stage of pregnancy (D21) but the slight but significant increase in false positives is likely to reduce efficiency of the rebreeding program. Future studies should evaluate the economics and efficiency of using these two techniques within practical rebreeding programs during a realistic breeding season on commercial beef cattle operations.

Acknowledgments

The authors thank Alexandre B. Prata, Adelino J. Robl, José Neto, Beatriz Lipped, Heuller S. Ribeiro, Diego Hartmman, the owners, and staff of the farm Roncador (Mato Grosso state) for their contribution with the heifers and facilities. Support was provided by Hatch Project WIS01240 to MCW and JPNA and USDA-NIFA project 2019-67015-29704 to MCW.

Table 1. Incidence of different true (correct) and false (incorrect) diagnoses for an early pregnancy diagnosis using CL morphology based on B-mode ultrasound or CL blood perfusion based on Doppler ultrasound.

	Ultrasound mode			P-value	
	Doppler	B mode	Mode	Parity	Mode*Parity
True Positives (930)	86.0% (800)	80.9% (752)	<0.01		
True Negatives (965)	99.8% (963)	99.7% (962)	0.99		
True Diagnoses (1,895)	93.0% (1,763)	90.4% (1,714)	<0.01		
False Positives (930)	14% (130) a	19.1% (178) b	<0.01	<0.0001	0.89
Heifers (483) a	16.1% (78)	21.3% (103)	0.05		
Primiparous (231) b	10.4% (24)	16.0% (37)	0.07		
Multiparous (216) b	13.0% (28)	17.6% (38)	0.17		
False Negatives (965)	0.2% (2)	0.3% (3)	0.65		

The false-positive rate was calculated based on the total number of non-pregnant cows at day 33 of gestation, and the false negative rate was calculated based on pregnant cows at day 33 of gestation. Lowercase different letters indicate $P < 0.01$.

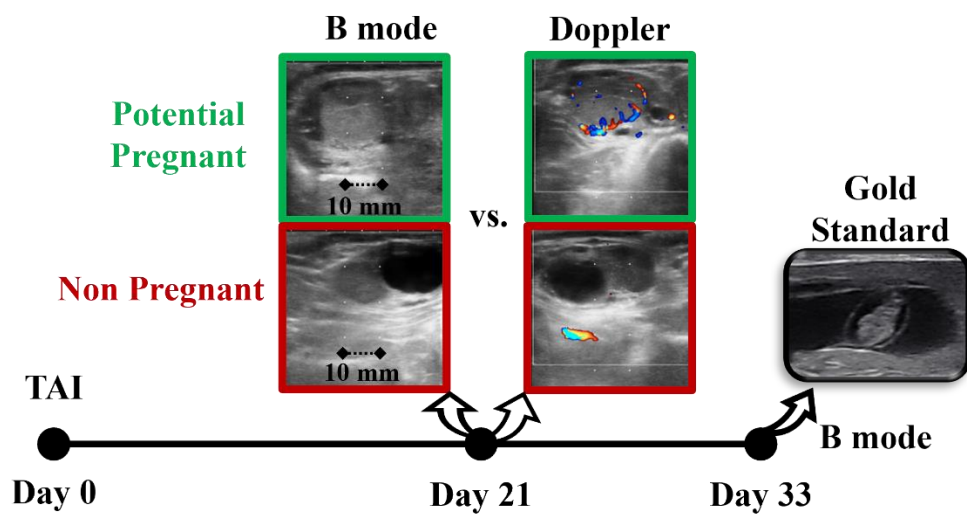
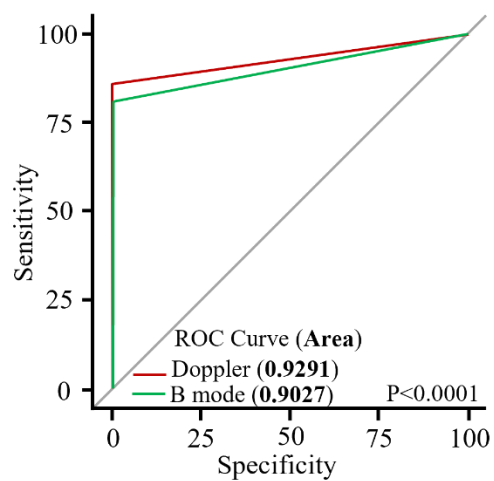


Figure 1

A.

Receiver operating characteristic



B.

Diagnosis efficiency

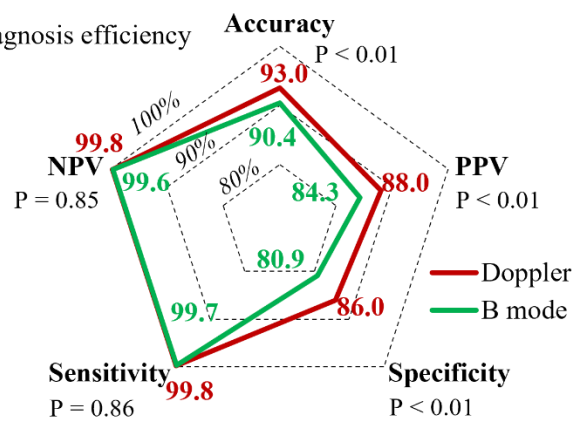


Figure 2

Figure Legends

Figure 1. Illustration of the experimental design. On D21 after TAI the early pregnancy diagnosis was performed using B mode ultrasound based on estimation of CL size using as a reference two vertical lines fixed in ultrasound screen 10mm apart from each other. After CL size score estimation, the Doppler mode was turned on for estimation of CL blood perfusion based on subjective scores. All results were compared to the gold standard method of B mode ultrasound on D33 by visualization of embryonic heartbeat.

Figure 2. Panel A. Receiver operating characteristics comparing the area under the curve for early methods of pregnancy diagnosis by B mode vs. Doppler ultrasound. Assessing the differences in sensitivity and specificity generates a curve; an area under the curve closest to 1 means more accuracy in the test. **Panel B.** Accuracy, Sensitivity, Specificity, NPV (negative predictive value), and PPV (positive predictive value) obtained by 2x2 contingency approach analysis. The dashed line in the pentagon indicates 80% to 100%. Individual values of each test are indicated; values closest to 100% indicate more precision for each test.

References

- [1] Bonafos LD, Kot K, Ginther OJ. Physical Characteristics of the Uterus during the Bovine Estrous-Cycle and Early-Pregnancy. *Theriogenology*. 1995;43:713-21.
- [2] Monteiro PL, Consentini CEC, Andrade JPN, Beard AD, Garcia-Guerra A, Sartori R, et al. Research on timed AI in beef cattle: Past, present and future, a 27-year perspective. *Theriogenology*. 2023;211:161-71.
- [3] Fricke PM. Scanning the future - Ultrasonography as a reproductive management tool for dairy cattle. *J Dairy Sci*. 2002;85:1918-26.
- [4] Baruselli PS, Ferreira RM, Colli MHA, Elliff FM, Sa MF, Vieira L, et al. Timed artificial insemination: current challenges and recent advances in reproductive efficiency in beef and dairy herds in Brazil. *Anim Reprod*. 2017;14:558-71.
- [5] Vasconcelos JLM, Pereira MHC, Wiltbank MC, Guida TG, Lopes Jr. FR, Sanches Jr. CP, et al. Evolution of fixed-time AI in Brazil. *Anim Reprod*. 2018;15 (Suppl. 1): 940-51.
- [6] Baruselli PS, Catussi BLC, Abreu LÂd, Elliff FM, Silva LGd, Batista EdOS. Challenges to increase the AI and ET markets in Brazil. *Anim Reprod*. 2019;16:364-75.
- [7] Green JA, Parks TE, Avalle MP, Telugu BP, McLain AL, Peterson AJ, et al. The establishment of an ELISA for the detection of pregnancy-associated glycoproteins (PAGs) in the serum of pregnant cows and heifers. *Theriogenology*. 2005;63:1481-503.
- [8] Cordoba MC, Sartori R, Fricke PM. Assessment of a commercially available early conception factor (ECF) test for determining pregnancy status of dairy cattle. *J Dairy Sci*. 2001;84:1884-9.
- [9] Han HC, Austin KJ, Rempel LA, Hansen TR. Low blood ISG15 mRNA and progesterone levels are predictive of non-pregnant dairy cows. *J Endocrinol*. 2006;191:505-12.

[10] Fricke PM, Ricci A, Giordano JO, Carvalho PD. Methods for and Implementation of Pregnancy Diagnosis in Dairy Cows. *Vet Clin North Am Food Anim Pract.* 2016;32:165-80.

[11] Silva E, Sterry RA, Kolb D, Mathialagan N, McGrath MF, Ballam JM, et al. Accuracy of a pregnancy-associated glycoprotein ELISA to determine pregnancy status of lactating dairy cows twenty-seven days after timed artificial insemination. *J Dairy Sci.* 2007;90:4612-22.

[12] Ricci A, Carvalho PD, Amundson MC, Fourdraine RH, Vincenti L, Fricke PM. Factors associated with pregnancy-associated glycoprotein (PAG) levels in plasma and milk of Holstein cows during early pregnancy and their effect on the accuracy of pregnancy diagnosis. *J Dairy Sci.* 2015;98:2502-14.

[13] Bazer FW, Spencer TE, Ott TL. Interferon tau: a novel pregnancy recognition signal. *Am J Reprod Immunol.* 1997;37:412-20.

[14] Ealy AD, Seekford ZK. Symposium review: Predicting pregnancy loss in dairy cattle. *J Dairy Sci.* 2019;102:11798-804.

[15] Pohler KG, Reese ST, Franco GA, Oliveira Filho RV, Paiva R, Fernandez L, et al. New approaches to diagnose and target reproductive failure in cattle. *Anim Reprod.* 2020;17.

[16] Pugliesi G, Miagawa BT, Paiva YN, Franca MR, Silva LA, Binelli M. Conceptus-induced changes in the gene expression of blood immune cells and the ultrasound-accessed luteal function in beef cattle: how early can we detect pregnancy? *Biol Reprod.* 2014;91:95.

[17] Holton MP, de Melo GD, Dias NW, Pancini S, Lamb GC, Pohler KG, et al. Evaluating the use of luteal color Doppler ultrasonography and pregnancy-associated glycoproteins to diagnose pregnancy and predict pregnancy loss in *Bos taurus* beef replacement heifers. *J Anim Sci.* 2022;100.

[18] Couto SRBd, Guerson YB, Morais RdCL, Grillo GF, Andrade JPN, Jacob JCF, et al. Relationships between follicle and corpus luteum size and vascularization with ovulation, progesterone production, and pregnancy in Nelore beef cattle. *Rev Bras Zootecn.* 2023;52:148.

[19] Miyamoto A, Shirasuna K, Hayashi KG, Kamada D, Awashima C, Kaneko E, et al. A potential use of color ultrasound as a tool for reproductive management: New observations using color ultrasound scanning that were not possible with imaging only in black and white. *J Reprod Dev.* 2006;52:153-60.

[20] Araujo RR, Ginther OJ, Ferreira JC, Siqueira LGB, Beg MA, Wiltbank MC. Color-Doppler ultrasonography: a non-invasive method to assess luteolysis in cattle. *Reprod Domest Anim.* 2008;43:30.

[21] Pugliesi G, da Silva AG, Viana JHM, Siqueira LGB. Review: Current status of corpus luteum assessment by Doppler ultrasonography to diagnose non-pregnancy and select embryo recipients in cattle. *Animal.* 2023;17.

[22] Herzog K, Bollwein H. Application of doppler ultrasonography in cattle reproduction. *Reprod Domest Anim.* 2007;42:51-8.

[23] Pinaffi FLV, Araujo ER, Silva LA, Ginther OJ. Color-Doppler signals of blood flow in the corpus luteum and vascular perfusion index for ovarian and uterine arteries during expansion of the allantochorion in *Bos taurus* heifers. *Theriogenology.* 2017;102:35-43.

[24] Pugliesi G, Bisinotto DZ, Mello BP, Lahr FC, Ferreira CA, Melo GD, et al. A novel strategy for resynchronization of ovulation in Nelore cows using injectable progesterone (P4) and P4 releasing devices to perform two timed inseminations within 22 days. *Reprod Domest Anim.* 2019;54:1149-54.

- [25] de Melo GD, Mello BP, Ferreira CA, Godoy CAS, Rocha CC, Silva AG, et al. Applied use of interferon-tau stimulated genes expression in polymorphonuclear cells to detect pregnancy compared to other early predictors in beef cattle. *Theriogenology*. 2020;152:94-105.
- [26] Palhão M, Ribeiro A, Martins A, Guimarães C, Alvarez R, Seber M, et al. Early resynchronization of non-pregnant beef cows based in corpus luteum blood flow evaluation 21 days after Timed-AI. *Theriogenology*. 2020;146:26-30.
- [27] Siqueira LGB, Areas VS, Ghetti AM, Fonseca JF, Palhao MP, Fernandes CAC, et al. Color Doppler flow imaging for the early detection of nonpregnant cattle at 20 days after timed artificial insemination. *J Dairy Sci*. 2013;96:6461-72.
- [28] Bicalho RC, Galvao KN, Guard CL, Santos JEP. Optimizing the accuracy of detecting a functional corpus luteum in dairy cows. *Theriogenology*. 2008;70:199-207.
- [29] Andrade JPN, Andrade FS, Guerson YB, Domingues RR, Gomez-Leon VE, Cunha TO, et al. Early pregnancy diagnosis at 21 days post artificial insemination using corpus luteum vascular perfusion compared to corpus luteum diameter and/or echogenicity in Nelore heifers. *Anim Reprod Sci*. 2019;209:106.
- [30] Prata AB, Madureira G, Robl AJ, Ribeiro HS, Sagae M, Elias MCV, et al. Progesterone-based timed AI protocols for *Bos indicus* cattle III: Comparison of protocol lengths. *Theriogenology*. 2020;152:29-35.
- [31] Motta JCL, Madureira G, Silva LO, Alves R, Silvestri M, Drum JN, et al. Interactions of circulating estradiol and progesterone on changes in endometrial area and pituitary responsiveness to GnRH. *Biol Reprod*. 2020;103:643-53.

- [32] Pugliesi G, de Melo GD, Ataide GA, Pellegrino CAG, Silva JB, Rocha CC, et al. Use of Doppler ultrasonography in embryo transfer programs: feasibility and field results. *Anim Reprod.* 2018;15:239-46.
- [33] Andrade JPN, Gomez-Leon VE, Andrade FS, Carvalho BP, Lacouth KL, Garcia FZ, et al. Development of a novel 21-day reinsemination program, ReBreed21, in *Bos indicus* heifers. *Theriogenology.* 2020;155:125-31.
- [34] Andrade JPN, Monteiro PJL, Prata AB, Robl AJ, Neto J, Lippe B, et al. Optimizing ReBreed21 II: Fertility and reproductive efficiency in different parities during a shortened breeding season in beef cattle. *Theriogenology.* 2024;224:41-9.
- [35] Youngquist RS, Bierschwal CJ, Elmore RG, Jenkins AL, Schultz RH, Widmer JH. Induction of Abortion in Feedlot Heifers with Cloprostenol (ICI 80,996). *Theriogenology.* 1977;7:305-12.
- [36] Reese ST, Franco GA, Poole RK, Hood R, Montero LF, Oliveira RV, et al. Pregnancy loss in beef cattle: A meta-analysis. *Anim Reprod Sci.* 2020;212.
- [37] Wiltbank MC, Baez GM, Garcia-Guerra A, Toledo MZ, Monteiro PLJ, Melo LF, et al. Pivotal periods for pregnancy loss during the first trimester of gestation in lactating dairy cows. *Theriogenology.* 2016;86:239-53.
- [38] Mercadante PM, Ribeiro ES, Risco C, Ealy AD. Associations between pregnancy-associated glycoproteins and pregnancy outcomes, milk yield, parity, and clinical diseases in high-producing dairy cows. *J Dairy Sci.* 2016;99:3031-40.
- [39] Middleton EL, Pursley JR. Short communication: Blood samples before and after embryonic attachment accurately determine non-pregnant lactating dairy cows at 24 d post-

artificial insemination using a commercially available assay for pregnancy-specific protein B. *J Dairy Sci.* 2019;102:7570-5.

[40] Ginther OJ, Silva LA, Araujo RR, Beg MA. Temporal associations among pulses of 13,14-dihydro-15-keto-PGF(2 α), luteal blood flow, and luteolysis in cattle. *Biol Reprod.* 2007;76:506-13.

[41] Araujo RR, Ginther OJ, Ferreira JC, Palhao MM, Beg MA, Wiltbank MC. Role of follicular estradiol-17 β in timing of luteolysis in heifers. *Biol Reprod.* 2009;81:426-37.

[42] Siqueira LG, Torres CA, Amorim LS, Souza ED, Camargo LS, Fernandes CA, et al. Interrelationships among morphology, echotexture, and function of the bovine corpus luteum during the estrous cycle. *Anim Reprod Sci.* 2009;115:18-28.

[43] Acosta TJ, Miyamoto A. Vascular control of ovarian function: ovulation, corpus luteum formation and regression. *Anim Reprod Sci.* 2004;82:127-40.

[44] Rocha CC, Martins T, Cardoso BO, Silva LA, Binelli M, Pugliesi G. Ultrasonography-accessed luteal size endpoint that most closely associates with circulating progesterone during the estrous cycle and early pregnancy in beef cows. *Anim Reprod Sci.* 2019;201:12-21.

[45] Wijma R, Stangaferro ML, Kamat MM, Vasudevan S, Ott TL, Giordano JO. Embryo mortality around the period of maintenance of the corpus luteum causes alterations to the ovarian function of lactating dairy cows. *Biol Reprod.* 2016;95:112.

[46] Domingues RR, Andrade JPN, Cunha TO, Madureira G, Moallem U, Gomez-Leon V, et al. Is pregnancy loss initiated by embryonic death or luteal regression? Profiles of pregnancy-associated glycoproteins during elevated progesterone and pregnancy loss. *JDS Commun.* 2023;4:149-54.

- [47] Ataide GA, Kloster A, de Moraes ÉG, Motta IG, Claro I, Vasconcelos JLM, et al. Early resynchronization of follicular wave emergence among Nelore cattle using injectable and intravaginal progesterone for three timed artificial inseminations. *Anim Reprod Sci.* 2021;229.
- [48] Andrade JPN, Domingues RR, Carvalho BP, Gomez-Leon V, Prata AB, Sartori R, et al. Optimizing ReBreed21 I: Evaluation of endocrine and ovarian dynamics in non-bred *Bos indicus* heifers. *Theriogenology.* 2024;220:77-83.
- [49] Shane DD, Larson RL, Sanderson MW, Miesner M, White BJ. A deterministic, dynamic systems model of cow-calf production: The effects of the duration of postpartum anestrus on production parameters over a 10-year horizon. *J Anim Sci.* 2017;95:1680-95.
- [50] Ojeda-Rojas OA, Bustos-Coral D, Sartorello GL, Reijers TSSS, Pugliesi G, Mercadante MEZ, et al. An agent-based simulation model to compare different reproductive strategies in cow-calf operations: Economic performance. *Theriogenology.* 2022;189:11-9.
- [51] Ojeda-Rojas OA, Gonella-Diaza AM, Bustos-Coral D, Sartorello GL, Reijers TSSS, Pugliesi G, et al. An agent-based simulation model to compare different reproductive strategies in cow-calf operations: Technical performance. *Theriogenology.* 2021;160:102-15.

Chapter 5: ReBreed21-Dairy: Use of oxytocin to synchronize luteolysis in development of a novel reproductive program for dairy cows with fixed-time rebreeding every 21 days

J.P. Andrade ¹, C. Consentini ^{1,2}, N.Teixeira ¹, T. Silva ¹, J. Gille ¹, M. Lavagnoli ¹, S. Moghbeli ¹, A. Beard ¹, R. Domingues ^{1,3}, P. Monteiro ^{1,4}, E. Cabrera ¹, Milo C. Wiltbank ^a

¹Department of Animal and Dairy Sciences, University of Wisconsin-Madison, 1675 Observatory Drive, Madison, WI 53706, USA

²GlobalGen Vet Science, Av. Italo Poli, 40, Jaboticabal, SP, 14887-360, Brazil

³Department of Animal Sciences, The Ohio State University, Columbus, Ohio, 43210, USA

⁴Department of Large Animal Clinical Sciences, University of Florida, Gainesville, FL 32608, USA

Corresponding Author: Milo C. Wiltbank

E-mail: wiltbank@wisc.edu

Abstract

Our goal in this series of experiments was to develop a rapid rebreeding program for high-producing lactating dairy cows for timed AI (TAI) every 21d. In experiment 1, lactating Holstein cows were induced to ovulate with Double-Ovsynch (DO, last GnRH = -1 d) but were not bred to ensure nonpregnant synchronized cows to assess the impact of treatment on the timing of luteolysis and subsequent ovulation. Cows were randomized to receive either oxytocin (n = 39, Oxt-50 IU IM) or control (n = 37, saline 2.5 mL i.m.) on 17d, 18d, and 19d after the induced ovulation. Blood samples were collected daily from 14d to 30d, and an extra blood sample was collected 2 hours after the treatments. Circulating PGFM (pg/mL) was greater for Oxt than Sal two h after treatment: 17d = 136 vs. 25.6; 18d = 121.1 vs. 24; and 19d = 112.2 vs. 29.2. The day to luteolysis was earlier for Oxt ($19.6d \pm 0.3$) compared to control (21.3 ± 0.5), with 76.9% of Oxt treated cows having complete luteolysis by 20d, as opposed to 43.2% for control. In experiment 2, lactating Holstein cows were randomized to receive 3 (3Oxt; 17d, 18d, 19d; n = 129), 2 (2Oxt; 18d, 19d; n = 130), 1 (1Oxt; 18d; n = 129), or 0 (CON; n = 129) oxytocin treatments after TAI (DO or Resynch25). Luteolysis was evaluated ($P4 < 1\text{ng/mL}$) by P4 radioimmunoassay and pregnancy diagnoses (PD) were performed after TAI on 25d (PAG) and 32d (ultrasound). Pregnancy/AI (P/AI) was similar among groups, but the percentage of nonpregnant cows (5d PD) with luteolysis by 20d was greater in cows treated with 2Oxt (78%) or 3Oxt (79.6%) than CON (58.9%) or 1Oxt (64.3%). Thus, 2Oxt promoted synchronized luteolysis without reducing P/AI. In experiment 3, after the first TAI with DO, cows were assigned to one of 3 rebreeding programs: Resynch25 (R25 [n = 204]; GnRH 25d, PD 32d, NP PGF [32d and 33d], GnRH 34d, and AI 35d), ReBreed21 (RB21 [n = 203]; Oxt on 18d and 19d, rapid MilkP4 test on 20d, LowP4 GnRH on 20d p.m., AI 21d a.m.), and ReBreed21G (RB21G [n

= 204]; similar to RB21 but with GnRH treatments on 5d and 11d]). The P/AI for the first breeding was greater for R25 (53.4%) than RB21 (42.9%) and intermediate for RB21G (44.1%) with a parity effect. The percentage of cows with luteolysis by 20d was greater for RB21 (74%) than for R25 (51.4%) and RB21G (62.4%). The P/AI during rebreeding was greater for R25 (43.2%) than RB21 (24.6%) and RB21G (24.3%). Pregnancies at 180 DIM were greater for R25 (86.2%) than RB21 (76.5%) and intermediate for RB21G (82.2%) with a tendency for an earlier time to pregnancy for R25 (112.7) than RB21 (122.2), and intermediate for RB21G (121.5). In summary, the 2Oxt ReBreed21 program promoted synchronized luteolysis by 20d but reduced fertility to first TAI in multiparous lactating dairy cows. Thus, ReBreed21 allows an early timed rebreeding but requires further optimization to improve fertility outcomes.

Keywords: Resynchronization, timed-AI, reproduction, dairy cattle

Introduction

The reproductive performance of lactating dairy cows in the USA has increased in the last 25 years, driven by changes in management, genetic, and nutrition but with a major impact coming from introduction of timed artificial insemination (TAI) [1]. Newer versions of TAI programs, termed “fertility programs”, consistently achieve values of ~50% pregnancy per AI (P/AI) at first TAI in well-managed, high-producing dairy cows [2, 3]. These programs produce more efficient reproduction, both because of greater service rate due to all cows receiving TAI, and because of increased fertility to the AI compared to cows bred after detection of estrus [4-6]. Thus, herds that prioritize a fertility program for the first AI had ~80% of cattle pregnant by 150 days in milk (DIM), resulting in ~70-d breeding period compared to ~100-d breeding period when detection of estrus is prioritized for first service [7, 8].

Nevertheless, there are still non-pregnant cows after use of any program for first TAI, thus requiring use of pregnancy diagnosis and a rebreeding strategy to achieve a successful reproductive management program. The most common approach is daily detection of estrus after the first breeding, either visual, with tail chalk, or using activity monitors, but these approaches require daily labor to detect and breed cows in estrus [9, 10]. Another option is to replicate a fertility program, such as Double-Ovsynch, for rebreeding and while this achieves good fertility [11], excessive time is required between breedings. Other, more common, rebreeding TAI strategies are to replicate the Ovsynch program allowing for standardization of tasks and breeding usually on specific days of the week that match first TAI, termed resynchronization of ovulation protocols (Resynch) [12-14]. Many different Resynch strategies have to be used, testing different days to start the first GnRH treatment [12, 15], using a modified Ovsynch with progesterone (P4) insert [16-18], applying the concept of pre-synchronization into Resynchs [19-

21], and assigning cows to Resynch strategies based on ovarian structures [22, 23]. However, one of the most common is termed Resynch25, with the first GnRH of Ovsynch given at 25d after previous TAI seven d prior to the pregnancy diagnosis, and the Ovsynch program is continued in nonpregnant cows [24]. This program allows a potential TAI every 35d, with three TAIs in 70d, with reported results of ~80% of cows pregnant [7, 25]. This allows cows to become pregnant before 130 DIM, mitigating the excessive gain in BCS that occurs in later lactation and achieving what has been termed the “high fertility cycle” [26, 27]. Therefore, producing pregnancies near the optimal time requires timely and effective first AI with a short interval to a high fertility rebreeding. Hence, rebreeding programs that allow short intervals between TAI with a schedule that matches labor for first TAI could allow good reproductive performance with efficiency of farm labor utilization, potentially improving profitability.

Three fundamental precepts for development of practical rebreeding programs are: (1) No decrease in fertility to previous AI, (2) Accurate pregnancy diagnosis, (3) Adequate fertility to rebreeding AI. An efficient program has been developed and validated for beef cattle, termed ReBreed21, that allows TAI every 21d thus producing a 42d breeding season with potentially 3 TAIs [28-30]. One of the major barriers to accomplishing such an ambitious timed rebreeding protocol (TAI every 21d) in lactating dairy cows is the greater variation in time of luteolysis and greater length of the estrous cycle in lactating cows compared to heifers or beef cattle [31-33]. In spite of these physiologic challenges, the potential increase in reproductive performance and farm efficiency that could be achieved with the shorter breeding period encourages an effort to achieve this ambitious goal.

To design a strategy to synchronize luteolysis before a nonpregnancy diagnosis without causing luteolysis in pregnant cows necessitates a solid understanding of the physiology of

corpus luteum (CL) regression in cattle. The time of luteolysis is associated with upregulation of oxytocin receptors in the uterine endometrium [34] and an increase in oxytocin-induced prostaglandin F₂ α (PGF) secretion from the uterus [35, 36]. During early pregnancy (D16 to D21, the response to oxytocin, as measured by an increase in PGF metabolite (PGFM) after oxytocin treatment, is suppressed/ prevented due to the actions of embryonic interferon-tau [37-39]. Thus, we postulated that it may be possible to synchronize and perhaps shorten the time to luteolysis in non-pregnant cows by treating with oxytocin. We also postulated that oxytocin treatment would not cause luteolysis in pregnant cows due a lack of uterine oxytocin receptors.

The three experiments reported in this manuscript demonstrate both the challenges and opportunities for using oxytocin to synchronize luteolysis in the development of a novel ReBreed21 program for dairy cattle. We specifically hypothesized that: (1) Oxytocin-induced PGF₂ α treatment would synchronize luteolysis about 20 d after the previous TAI; (2) P/AI to the previous TAI would be similar for cows treated or not treated with oxytocin; (3) P/AI for nonpregnant cows rebred using ReBreed21 and Resynch25 would be similar; and (4) ReBreed21 would lead to an earlier time to pregnancy compared to Resynch25.

Materials and Methods

Experiment 1. Synchronization of luteolysis using oxytocin

This experiment was performed from April 2021 to August 2021 at the USDA Dairy Forage Research Center located in Prairie du Sac-WI, with an average of 312 Holstein milking cattle, with a yearly average reproductive performance of 76.4% insemination risk, 50.2% conception rate, and 38% 21-d pregnancy rate (BoviSynch LLC Software - Fond du Lac WI). All experimental procedures and animal management were approved by the Animal Care and Use Committee of the College of Agriculture and Life Sciences at the University of Wisconsin-

Madison (Protocol ID: A005653). The experiment was a completely randomized design, assigning cows after an induced ovulation protocol (Double-Ovsynch [40]). No artificial insemination was performed. After the last GnRH treatment (-1 d) of the Double-Ovsynch protocol, all cows had their ovaries evaluated via ultrasound (5-9 MHz; Ibex Evo II; E. I. Medical Imaging) to determine the disappearance of the pre-ovulatory follicle. Only cows that ovulated were randomized into two treatments: Oxytocin (50IU, n = 39) on 17 d, 18 d, and 19 d or Control (2mL of saline, n = 39) on 17 d, 18 d, and 19 d relative to induced ovulation. Two cows in the Control group underwent luteolysis before day 12 d relative to induced ovulation and were removed from further analysis (Control final n = 37).

Blood samples were collected daily from 7 d to 32 d after induced ovulation to evaluate circulating P4. During the treatment periods (17, 18, and 19), blood samples were collected before the treatments and 2 h after treatment to evaluate PGFM. Blood was collected into tubes without anticoagulant (BD, Franklin Lakes, NJ) by venipuncture of coccygeal vessels and kept on ice until centrifugation. Serum was isolated using centrifugation at 2,000× g, decanted, and stored at -20°C until assayed for P4 and PGFM. Serum circulating P4 was determined by a solid-phase RIA kit containing antibody-coated tubes and 125I-labeled P4 (ImmuChem Coated Tube P4 125I RIA Kit, MP Biomedicals) following the commercial recommendation for a short incubation time of 120 min in a water bath (set at 37 °C) followed by aspiration of tubes. The sensitivity, intraassay coefficient of variation (CV), and interassay CV were 0.1 ng/mL, 5.5%, and 6.1%, respectively. Circulating PGFM from 17 d to 19 d (0 h and 2 h for each day) was determined by ELISA as described and validated in our laboratory (Mezera et al., 2019). The sensitivity, intraassay CV, and interassay CV were 8.4 pg/mL, 11.5%, and 5.2%, respectively. Luteolysis was defined as the first day in which P4 was < 1ng/mL. The cutoff to determine the

increases in PGFM after treatment was based on three standard deviations from the circulating mean PGFM on 17 d before the treatment.

The statistical model used to assess the binary variable (percentage of cows with luteolysis and percentage of cows with an increase in PGFM after oxytocin treatment) was performed with SAS (version 9.4; SAS Institute Inc.) using PROC GLIMIX procedure for generalized linear model analyses. Continuous variables (day of luteolysis, circulating PGFM, and delta increase in PGFM) were analyzed using the PROC MIXED procedure for linear models. Assumptions (linearity, independence, and normality) were assessed by plotting the residuals, quantile–quantile plot, and by the Shapiro-Wilk test. If case deviations from assumptions were found, data were transformed guided by BoxCox transformation to select the best transformation: natural logarithms, square root, or ranks. The variances in day of luteolysis were tested using Leves's test.

Experiment 2. Dose effect of oxytocin in previously bred cows

The experiment was performed from February 2022 to April 2022 in a commercial dairy farm located in Waterloo-WI, with an average of 2,010 milking Holstein cows with a yearly average reproductive performance of 72.3% insemination risk, 47.3% conception rate, and 34.2% 21-d pregnancy rate (BoviSynch software). All experimental procedures and animal management were approved by the Animal Care and Use Committee of the College of Agriculture and Life Sciences at the University of Wisconsin-Madison (Protocol ID: A005653). The experiment was a completely randomized block design, assigning cows after TAI. On the fifth day after the last TAI, cows had their ovaries evaluated by ultrasound, and only cows with CL > 15 mm of diameter were used. Enrolled cows were blocked by parity (first lactation and \geq two lactations) and synchronization protocol (Double-Ovsynch or Resynch25 [Ovsynch] [25,

40]). Cows were then assigned to one of four groups: No treatment (Control n = 120), oxytocin treatment (50 IU i.m.) on D18 since the last TAI (1Oxt [n = 125]), oxytocin treatment on D18 and D19 since the last TAI (2Oxt [n = 122]), and oxytocin treatment on D17, D18, and D19 since the last TAI (3Oxt [n = 121]).

Blood samples were collected on 7 d, 20 d, and 27 d after TAI for circulating P4 and pregnancy-associated glycoproteins (PAGs) assessments. Blood was collected into tubes without anticoagulant (BD, Franklin Lakes, NJ) by venipuncture of coccygeal vessels, put on ice, centrifuged at 2000xg to isolate serum, and serum stored at -20°C until assayed for P4 (as described for experiment 1) and PAGs. The PAGs were determined by ELISA (BioPRYN Flex; BioTracking LLC) using the commercial recommendations for assay procedures.

Pregnancy diagnosis on D27 d after TAI was performed based on the optical density (OD) results from the final step of the quantitative sandwich PAGs assay. The commercial assay provides two standards: “High” and “Low” (not used in these assays). In each of the plates (96 well plates), two wells had the “High” standard and two wells had serum from a virgin Holstein heifer as a negative control. Therefore, using samples from D27, all cows with OD higher than the “High” standard were considered pregnant. Additionally, cows with $OD \leq \text{“High”}$ and $>$ negative controls were re-assayed, including serum samples from D17 for that cow. Cows with at least a 50% increase from D17 to D27 were considered pregnant. Rectal ultrasonography was performed on D32 and D60 for pregnancy diagnosis based on presence of embryonic heartbeat (32 d and 60 d after TAI).

The percentage of nonpregnant cows (pregnancy diagnosis based on PAGs on D27) with luteolysis by D20 was determined using direct and indirect methods: (1) Radioimmunoassay (RIA

; Luteolysis = $P4 < 1\text{ng/mL}$), (2) On-farm commercial rapid qualitative lateral flow assay for milk P4 (P4Gold; luteolysis = test line darker than the control line), and (3) CL Doppler ultrasonography to determine blood perfusion using subjective scores (1 to 4) in which cows without a CL or a CL with less than 25% of the total area with blood flow (score 1) were considered in the process of luteolysis [41].

The statistical model used to evaluate the binary variables (P/AI, percentage nonpregnant with luteolysis, and pregnancy loss) included the two blocks (parity and protocol) and sire. Statistical analyses were performed with SAS (version 9.4; SAS Institute Inc). PROC GLIMIX procedure was used for generalized linear model analyses. Interaction between parity and treatment was evaluated in the model and kept in the model if significant. Differences among groups' means were also analyzed using a set of three orthogonal contrasts (C1: Control vs. [1Oxt, 2Oxt, and 3Oxt]; C2: 1Oxt vs. [2Oxt and 3Oxt]; and C3: 2Oxt vs. 3Oxt).

Experiment 3. Reproductive performance of timed rebreeding programs

The experiment was performed from April 2022 to November 2022 at the same commercial dairy farm as Experiment 2. All experimental procedures and animal management were approved by the Animal Care and Use Committee of the College of Agriculture and Life Sciences at the University of Wisconsin-Madison (Protocol ID: A005653). Cows were inseminated on their first day of the breeding period using Double-Ovsynch program [40], after a VWP of 72 ± 3 DIM for the first lactation and 86 ± 3 DIM for \geq two lactations. The experiment was a completely randomized block design (blocked by parity: first lactation and \geq two lactations), and five days after the first insemination, cows were assigned to one of three timed rebreeding programs, remaining in the same program once enrolled for 120 d. About one-third of

the cows (n = 204) were assigned to Resynch25, which begins with GnRH treatment (gonadorelin 100µg im) on D25 since last breeding in all cows with an undetermined pregnancy status. On D32, a pregnancy diagnosis was performed by transrectal ultrasound with pregnancy determined by presence of an embryonic heartbeat. Nonpregnant cows had their ovaries evaluated by ultrasound, and those with at least one CL >15mm diameter received a PGF2α analog (cloprostenol sodium 500 µg) on D32 and 33 from previous AI, followed by a GnRH treatment on D34 pm and a subsequent TAI on D35 am. Nonpregnant cows without CL or CL <15mm diameter were enrolled in an Ovsynch protocol and bred the following week (rebred 42 d after the previous TAI).

Two-thirds of the cows were assigned to a timed rebreeding program for potential rebreed TAI on the 21st d since the last breeding. The ReBreed21 (n = 203) consisted of intramuscular injection of oxytocin on D18 and D19 followed by a rapid milk P4 test on D20 AM; cows considered with low P4 received 100µg of gonadorelin on D20 pm and were bred on D21 AM. A modification of ReBreed21 using GnRH to start the follicular wave was tested consisting of 200µg of gonadorelin on D5 and D11 since the last TAI (ReBreed21+G [n = 204]). Afterward, the protocol and procedures were the same as ReBreed21 (oxytocin, milk sample, and rebreeding). In both ReBreed21 groups, cows with high P4 followed the same protocol and procedures as in Resynch25 (GnRH on D25, nonpregnant with CL on D32 then rebred on D35, and nonpregnant without CL rebred on D42). Regardless of the rebreeding protocol, all cows were kept within the same resynchronization program (Resynch25, ReBreed21, or ReBreed21+G) for at least 120 d after enrollment.

Blood samples were collected in a subsample of animals (n = 629) on D18, 20, and 27 for evaluating circulating P4 and PAGs for P4 and PAGs as described in experiments 1 and 2. The

percentage of nonpregnant cows (based on PAGs on D27) with luteolysis by D20 after the last TAI was determined by $P4 < 1\text{ng/mL}$ using RIA and a commercial qualitative milk P4 rapid test (P4Gold). Pregnancy diagnosis on d27 d was performed based on the optical density (OD) results as described in Experiment 2. Transrectal ultrasound was performed on D32 and D60 for pregnancy diagnosis based on presence of an embryonic heartbeat. The synchronization rate at TAI on D21 was based on the combination of $P4 > 1\text{ng/mL}$ on D18, $P4 < 0.4$ on D20, and at least one CL $> 15\text{mm}$ diameter on D27. A subset of 407 cows of ReBreed21+G had their ovaries evaluated using ultrasound to check for ovulation (CL $> 15\text{ mm}$ diameter) five days after each GnRH treatment (5 d and 11 d after TAI). Eleven nonpregnant cows (Control [$n=1$], ReBreed21 [$n = 3$], and ReBreed21+G [$n = 7$]) were culled before completing 120 d in the experiment and were removed from the survival analysis.

The statistical model used to access the binaries variable included: parity (block factor), sire, and incidence of diseases during first 60 DIM. Statistical analyses were performed with SAS (version 9.4; SAS Institute Inc.) using the PROC GLIMIX procedure to analyze the generalized linear model. Additionally, the interaction between parity and the reproductive program was tested and used if significant. Differences among group means were evaluated using the Tukey honestly significant difference as a post hoc test. Additionally, differences among group means were analyzed using a set of two orthogonal contrasts (C1: Resynch25 vs. (ReBreed21 + ReBreed21+G) and C2: ReBreed21 vs. ReBreed21+G. Time to pregnancy was analyzed using the PROCLIFETEST procedure, using the Wilcoxon test. In all experiments, probability ≤ 0.05 indicated that the difference was significant and considered a tendency when the probability was between >0.05 and ≤ 0.1 .

Results

Experiment 1. Synchronization of luteolysis using oxytocin

The circulating PGFM profile after cows were treated or not treated with oxytocin is shown in Figure 2. Two hours after the treatments, cows that received oxytocin had an average 3.7-fold increase in circulating PGFM (123.1 ng/mL) compared to Controls on 17 d, 18 d, and 19 d (26.3 ng/mL). The dynamics of PGFM and luteolysis after treatments are described in Table 1. The percentage of cows that had an increase in PGFM 2 hours after the treatment was greater for cows treated with oxytocin vs. Control on 17 d (66.7% vs. 3% $P < 0.01$), 18 d (51.3% vs. 0% $P < 0.01$), and 19 d (56.4% vs. 8.1% $P < 0.01$). Overall, 87.2% of cows treated with oxytocin had at least one increase in PGFM 2 hours after the treatment combining the three days, compared to the Control group (8.1%; $P < 0.01$). The luteolysis distribution is shown in Figure 3. The percentage of cows with luteolysis by Day 20 after the induced ovulation was 78% greater for the groups of cows treated with oxytocin (76.9%) compared with the Control (43.2%; $P < 0.01$). The onset of luteolysis happened one day earlier ($P < 0.01$) for cows treated with oxytocin (18.2 $[\pm 0.2]$) with less variation compared with Control cows (20.1 $[\pm 0.5]$). The average day to luteolysis (first day of $P_4 < 1$ ng/mL) was about 2 d earlier for cows treated with oxytocin (19.6 $[\pm 0.3]$) compared with Control (21.5 $[\pm 0.5]$; $P < 0.01$), with a narrow variance for oxytocin 2.4 compared with Control 8.6 ($P = 0.01$).

Experiment 2. Dose effect of oxytocin in previously bred cows

Table 2 shows the effect of oxytocin treatment frequency on the fertility of the previous TAI and luteolysis synchronization in nonpregnant cows. There were similar fertility outcomes to the previous TAI for cows treated with oxytocin three times (17 d, 18 d, and 19 d), two times (day 18 d and 19 d), one time (only 18 d), or not treated with oxytocin. Nevertheless, the contrast

analysis one (C1: $P = 0.02$) shows that there was an increase in the percentage of nonpregnant cows with luteolysis synchronization on 20 d after TAI for cows treated with oxytocin (73.5% [114/155]) compared to cows that were not treated (58.9% [33/56]). In addition, cows treated two or three times with oxytocin (78.8% [78/99]) had an even greater percentage of luteolysis synchronization compared with cows treated only one time (64.3% [36/56] C2: $P = 0.05$). The percentage of luteolysis synchronization was similar between groups of cows that received two (78% [39/50]) or three Oxytocin treatments (79.6% [39/49] C:3 $P = 0.82$). The performance of the on-farm method to select cows with low P4 at 20 d after TAI is shown in Figure 3. The P4Gold (lateral flow assay for milk P4) was able to detect 96.2% more nonpregnant cows with luteolysis at 20 d (71.6%) compared to Doppler ultrasound (36.5%; $P < 0.01$). Nonetheless, the gold standard method RIA (70%) and P4Gold (71.6%) had similar performance in detecting nonpregnant cows with luteolysis ($P = 0.9$).

Experiment 3. Reproductive performance of timed rebreeding programs

First TAI fertility outcomes

The fertility outcomes among timed rebreeding programs are shown in Table 3. The P/AI at the first TAI was decreased by 18.5% for cows enrolled in ReBreed21 programs (ReBreed21 + ReBreed21+G) compared to cows enrolled in Resynch25 based on pregnancy diagnosis on 32 d (C1 = 0.04) and 20.6% in pregnancy diagnosis on 60 d (C1 = 0.03). In addition, there was a similar P/AI in the ReBreed21 compared to ReBreed21+G on 32 d (C2 = 0.8) and on 60 d (C2 = 0.87). Nevertheless, there was a tendency for interaction between Lactation and synchronization programs for the P/AI on 32 d ($P = 0.07$) and on 60 d ($P = 0.09$). The P/AI for multiparous cows on 32 d ($P = 0.02$) and 60 d ($P = 0.01$) was greater for Control (53.6% and 51.7% [n = 151])

compared to ReBreed21 (37.1% and 35.1% [n = 151]) and ReBreed21+G (44% and 41.3% [n = 150]). On the other hand, there are similar P/AI for first-lactation cows either on 32 d (P = 0.81) or 60 (P = 0.99) comparing Control (50.9% and 50.9% [n = 55] to ReBreed21 (58.5% and 56.6% [n = 53]) and ReBreed21+G (44.4% and 42.6% [n = 54]). However, there were similar pregnancy losses from 32 d to 60 d among timed rebreeding programs (Table 3).

Timed rebreeding synchronization dynamics and fertility outcomes

The GnRH treatment after TAI in ReBreed21+G induced ovulation in 88% (358/407) on 5 d and 76.4% (311/407) on 11 d. Oxytocin treatments on 18 d and 19 d synchronized luteolysis in 62.4% (131/210) of nonpregnant cows on 20 d in ReBreed21+G, which was significantly greater (P < 0.01) compared to Resynch25 (51.4% [91/177]), but lower (P < 0.01) compared with ReBreed21 (74% [179/242]). In addition, using P4Gold as a tool to select cows with low P4, ReBreed21 also had a greater (P = 0.02) percentage of nonpregnant cows with luteolysis on 20 d (73.1% [177/242]) compared with ReBreed21+G (62.4% [131/210]). The synchronization for nonpregnant cows rebred at 21 d since the last TAI ReBreed21 and ReBreed21+G had similar performance except by the percentage of cows with P4 < 0.8ng/mL on D20 (98.6% ReBreed21 vs. 92.5% ReBreed21+G; P = 0.04). Nevertheless, the synchronization rate was less than half in both ReBreed21 (47.2%) and ReBreed21+G (42.5%) groups. Only 61.2% (153/248) of cows in both ReBreed21 protocols had P4 > 1ng/mL on day 18, and those animals had greater (P<0.01) P/AI on 27 d (36.6% [56/153]) compared with cows with P4 < 1ng/mL (21.1% [20/95]). Overall, the P/AI for synchronized vs. non-synchronized cows bred on 21 d was greater by pregnancy diagnosis on 27 d after TAI (42% [47/112] vs. 21.3% [29/136]; P <0.01) and pregnancy diagnosis on 32 d after TAI (32.1% [36/112] vs. 14.7% [20/136]; P <0.01).

Timed rebreeding fertility outcomes and time to pregnancy

The P/AI and pregnancy loss for each timed rebreeding are shown in Table 3. There was a greater P/AI on D32 (C1: $P < 0.01$) and D60 (C1: $P < 0.01$) for cows receiving their second and subsequent TAI in Resynch25 (43.2% and 42.7%; timed rebreed 35 d since the last TAI) compared to cows rebred in ReBreed21 groups (24.4% and 22.6% [n=389]; timed rebreed 21 d since the last TAI). Nevertheless, the P/AI was similar among groups within the comparison of animals timed rebred 35 and 42 d since the last TAI. The pregnancy loss was similar among groups in all comparisons of P/AI in different timed rebreeding protocols (rebred on 21, 35, or 42 d since the last TAI). The overall performance of each reproductive program is shown in Table 5 and Figure 4 with a survival curve analysis. The percentage of pregnancies was similar among timed rebreeding programs by 120 and 150 DIM. However, on 180 DIM, there was a 12.7% ($P = 0.04$) increase in the percentage of pregnancies for Resynch25 (86.2%) compared to ReBreed21 (76.5%) and an intermediate percentage for ReBreed21+G (82.2%). There was a tendency for ten days earlier time to pregnancy for Resynch25 compared to ReBreed21, either analyzing relative to DIM or relative to the first TAI. On the other hand, ReBreed21+G had an intermediary time to pregnancy compared to Resynch25 and ReBreed21.

Discussion

The development of new reproductive programs for lactating dairy cows that allow a narrow breeding period is crucial for producers who desire to achieve a more precise time for pregnancy with the aim to optimize profitability. Our laboratory had already reported the development of a reproductive program for beef cattle that allows potential TAI every 21 d, successfully accomplishing a 42-d breeding season [28-30]. Now, we are attempting to adapt this

success to the unique reproductive physiology of lactating dairy cows, aiming to achieve a shortened interval between TAIs with high fertility in a timed rebreeding protocol. Compared to beef cattle, lactating dairy cows have a longer and wider window for luteolysis [31, 32]. In addition, a key aspect for fertility in high producing dairy cows is increasing the P4 concentrations during growth of the preovulatory follicle which is generally suboptimal due to high steroid metabolism [42-44]. Finally, P4 needs to be very low near the time of AI to allow optimal gamete transport [45-47], however, PGF2 α analogs cannot be utilized in earlier resynchronization programs to avoid iatrogenic abortion [48]. The results from three experiments reported in this manuscript demonstrate both the challenges and opportunities in the development of a new timed rebreeding program for lactating dairy cows using oxytocin to synchronize luteolysis both from a physiologic perspective and with practical on-farm fertility trials.

The first hypothesis, that oxytocin treatment would synchronize luteolysis at 20 d after the previous TAI, was supported. The binding of oxytocin to the endometrial oxytocin receptor is a key aspect of the bovine luteolytic cascade, releasing endometrial PGF2 α that triggers luteolysis including the amplification of luteal PGF2 α production [36, 49, 50]. Treatment with oxytocin would be expected to trigger and synchronize luteolysis in non-bred or nonpregnant cows due to the presence of upregulated oxytocin receptors in the endometrium around 18 d of the estrous cycle [51, 52] and we evaluated this physiology by monitoring oxytocin-induced PGFM [37, 53, 54]. The rationale that multiple oxytocin treatments could synchronize luteolysis was validated in both Experiment 1 in non-bred cows and in Experiment 2 in bred cows. Interestingly, Experiment 2 of the present manuscript shows that a minimum of two oxytocin treatments on 18 d and 19 d are needed to optimize the percentage of cows with luteolysis by 20 d since the last TAI. On the other hand, exogenous oxytocin treatments are not expected to

induce PGF2 α secretion in pregnant cows due to the presence of interferon tau, which prevents the upregulation of ESR1, a key transcription factor in induction of oxytocin receptors. Consequently, there is no up-regulation of endometrium oxytocin receptor preventing the production of PGF2 α for about 8 to 10 d [38, 39]. Thus, it was our assumption in these experiments that oxytocin treatments could be an ideal strategy to use in early-timed rebreeding programs since it could synchronize luteolysis in non-pregnant cows without negatively affecting pregnant cows. However, conflicting results were found related to this idea.

Our second hypothesis was that P/AI to the previous AI would not be altered by the resynchronization treatments with oxytocin and this hypothesis was supported by the results of Experiment 2 but, confusingly, not supported by the results from Experiment 3. The P/AI in Experiment 2, testing different numbers of oxytocin treatments, were similar among all groups, with not even numerical evidence to suggest a negative effect of oxytocin in fertility. Paradoxically in Experiment 3, two oxytocin treatment decreased the P/AI to the first TAI by 11 percentage points compared with cows not treated with oxytocin. Considering the total number of animals used in Experiment 2 ($n = 488$), if there was a decrease in fertility of this magnitude it would have been statistically detected (82% power). Interestingly, in Experiment 3 there was an interaction between parity and the resynchronization protocol that suggested that the negative effect was specifically in multiparous cows, with a decrease of more than 15 % in P/AI to first TAI in multiparous but with no decrease in primiparous cows. The reasons for the differences between experiments and between parities require speculation since both experiments were done in the same commercial dairy with roughly the same population of cows. One possibility relates to the later time for presumed embryo attachment in older lactating cows [55] possibly due to lower P4 concentrations post-TAI after Double-Ovsynch [42, 56, 57]. Thus, the signaling

process to down-regulate oxytocin receptors may also be delayed, and in some of the cows, the oxytocin receptors may be activated by exogenous oxytocin, triggering luteolysis in multiparous pregnant cows. To partially evaluate this potential explanation, we analyzed the circulating P4 in late diestrus (17 d for Exp2 and 18 d for Exp3) in 570 pregnant cows. There was an effect of parity (Multiparous $[4.3 \pm 0.09]$ vs. Primiparous $[4.9 \pm 0.15]$, $P = 0.0005$) and Protocol (DoubleOvsynch $[4.3 \pm 0.11]$ vs. Resynch25 $[4.9 \pm 0.14]$, $P = 0.0007$), without interaction ($P = 0.15$) between Parity and Protocol. Thus, it seems likely that the lower P4 in multiparous cows, combined with lower P4 for the DoubleOvsynch protocol may delay interferon-tau secretion and the resistance to oxytocin treatments. This effect may differ by season of the year, since much of Experiment 3 was done during summer (April to Nov) whereas, Experiment 2 was done primarily in cooler times (Feb to April). Thus, we have plausible speculations for the differing results between the two experiments that could be tested in the future. Regardless of the mechanism, a decrease in fertility to the first TAI is a major negative in a reproductive program [3].

The third hypothesis was that nonpregnant cows rebred using ReBreed21 (bred 21 d after the last TAI) would have similar P/AI compared to cows rebred using Resynch25 (bred 35 d after the last TAI) but this hypothesis was also not supported by the results from Experiment 3. Many different modifications to Resynch programs have been tested in an attempt to optimize rebreeding fertility. For example, different timing to start Resynch after previous TAI [12, 13, 15], applying the pre-synch concept into Resynchs [19, 21], modifications using intravaginal P4 insert [16, 17], and assigning cows to protocols according to ovarian structures after a nonpregnancy diagnosis [22, 23] have been tested. Despite the various modifications, all of them shared the main goal, which is to accomplish the hormonal milieu that results in high fertility in

lactating dairy cows (High P4 before AI, Low P4 near AI, and High P4 after AI) [58]. In addition to the hormonal protocol strategy, another feature of the timed rebreeding programs is the organization of treatments throughout the week to fit a logical schedule. Taking into consideration those aspects, one of the most used timed rebreeding programs is the Resynch25 because the treatments are set in a way that fits what is already used for the first TAI; additionally, the pregnancy diagnosis from the previous TAI is programmed on the same day in which the nonpregnant cows would be treated with PGF2 α . Thus, after the pregnancy diagnosis, nonpregnant cows have their ovaries scanned, and only cows with the presence of at least one CL continue the synchronization protocol with TAI 3 d later. Furthermore, another optimization is to resynchronize nonpregnant cows without a CL by using an intravaginal P4 insert and rebreeding them one week later [24]. Interestingly, by precisely selecting nonpregnant cows with CL prior to the PGF2 α , one study reported that GnRH treatment seven days before pregnancy diagnosis can even be removed without change in fertility [25]. These results highlight the importance of accomplishing the key markers for high fertility in TAI programs (High P4 before AI, Low P4 near AI, and High P4 after AI) and explain why cows rebred with ReBreed21 on 21 d since the last TAI had low P/AI. Only about 60% of nonpregnant cows in ReBreed21 had High P4 before TAI, considering that almost all cows rebred in Resynch25 had High P4 before TAI (all cows were confirmed to have CL >15 mm diameter after the nonpregnancy diagnosis). Overall, less than half of the cows inseminated with ReBreed21 were successfully synchronized with the P/AI for cows that were synchronized based on the three P4 measurements were double (42%) compared to non-synchronized cows (21.5%). Nevertheless, nonpregnant cows that were in the ReBreed21 group but were bred at 35 or 42 d after previous AI had similar P/AI as in Resynch25 group, likely due to a greater percentage of cows being well synchronized.

Our fourth hypothesis that ReBreed21 would lead to an earlier time of pregnancy was not supported. Cows enrolled in ReBreed21 had a tendency to become pregnant at a later time than Resynch25 (112.7 DIM vs. 122.2 DIM) and have fewer pregnancies by 180 DIM (76.5% vs. 86.2%). Thus, ReBreed21 was unsuccessful at two key parts of an optimal reproductive program, since P/AI was lower to first TAI and also lower at second and later TAI. Interestingly, the time to pregnancy and the percentage of pregnancies were similar for ReBreed21+G and Resynch25. Partially this is due to the more rapid rebreeding in cows detected non-pregnant at 21 d. Further, we speculate that the multiple accessory CL in ReBreed21+G may somewhat mitigate the negative effect of oxytocin in decreased fertility, but multiple ovarian scans were not performed to properly assess this potential effect [59, 60]. The ultimate goal of optimized reproductive programs is to connect the strategies for the first, second, and later AIs to reach ~80% of cows pregnant in a relatively short period of time [61]. Thus, an optimal formula for designing an optimal reproductive program is to combine the following steps: (1) high Preg/AI in the first AI, (2) earlier and accurate nonpregnancy diagnosis, (3) high P/AI in the second and later AIs. This has been achieved with some programs including the Resynch25 program [3, 7, 62]. To achieve shorter intervals between TAIs, potentially leading to a breeding period of less than 70 d is the ability to accurately perform an early diagnose of nonpregnancy.

The present manuscript reports a comparison of on-farm methods to select cows with low P4 20 d since the last TAI. The commercial lateral flow rapid assay (P4Gold) to qualitatively select cows with low P4 had similar performance as a gold standard RIA (low P4 = circulating P4 <1ng/mL), while Doppler ultrasonography was not as successful at identifying cows with low P4 by evaluating the CL blood perfusion. Interestingly, the blood perfusion of CL has been reported as a highly accurate tool (accuracy >90%) to identify nonpregnant beef cows in early

Resynch programs [41, 63-65]. Physiologically, the decrease in P4 (<1ng/mL) occurs 12 to 24 h earlier than the decrease in CL blood perfusion (<25% of the total area with color pixels). In addition, with a later and more variable time of luteolysis in lactating dairy cows, it seems likely that many dairy cows undergo luteolysis near 20 d after previous TAI [32, 66]. Thus, some cows may still have some CL blood perfusion but low P4 production, making Doppler not as accurate for early pregnancy diagnosis in dairy cows compared to beef cows and heifers. In addition, there were 30% of nonpregnant cows at 32 d that had elevated P4 on 20 d. These cows may either have a longer estrous cycle or underwent pregnancy loss before pregnancy diagnosis, which has been reported to be about 20% from elongation to attachment [33, 67]. Nevertheless, in the groups of cows that received two or three oxytocin treatments to synchronize luteolysis, only ~21% of nonpregnant cows still had high P4 at 20 d after TAI compared to non-treated cows that had 41% of nonpregnant cows with elevated P4 at 20 d.

Conclusions

In summary, oxytocin treatment successfully synchronized luteolysis in nonpregnant cows, but there was an indication of a negative effect of oxytocin in multiparous cows on the fertility to the first TAI. In addition, likely due to an inadequate synchronization rate, the timed rebreeding on 21 d since the last TAI culminates in poor fertility. Thus, ReBreed21 did not improve reproductive performance in lactating dairy cows, especially when no GnRH treatment was used, leading to a tendency for later pregnancy. Improvements are needed to optimize ReBreed21 in lactating dairy cows. Future studies may be considered to delay the time to rebreed cows (from 21 d to ~23 d), to add a P4 implant during the protocol, and/or test lower doses of oxytocin to synchronize luteolysis.

Table 1. PGFM responsiveness and luteolysis synchronization dynamics for lactating dairy cows

treated or not with oxytocin in Experiment 1

Item	Control	Oxytocin	P value
Day 17			
Delta increase (ng/mL)	-5.7 (3.6)	99.4 (12.0)	<0.0001
Response (%)	3 (1/37)	66.7 (26/39)	<0.0001
Day 18			
Delta increase (ng/mL)	-10.4 (5.1)	84.4 (14.8)	<0.0001
Response (%)	0 (0/37)	51.3 (20/39)	<0.0001
Day 19			
Delta increase (ng/mL)	-2.8 (5.9)	70.5(12.5)	<0.0001
Response (%)	8.1 (3/37)	56.4 (22/39)	<0.0001
Overall response			
At least one response	8.1% (3/37)	87.2% (34/39)	<0.0001
Luteolysis up to 20 d	43.2% (16/37)	76.9% (30/39)	0.003
Luteolysis from 18 d to 20 d	40.0% (15/37)	69.2% (27/39)	0.008

Table 2. Pregnancy outcomes and luteolysis synchronization for lactating dairy cows treated with different frequencies of oxytocin in Experiment 2

Item	Oxytocin treatments				Contrast		
	Control	1Oxt	2Oxt	3Oxt	C1	C2	C3
Preg/AI on 27 d	53.3% (64/120)	55.2% (69/125)	59% (72/122)	59.5% (72/121)	0.39	0.37	0.86
Preg/AI on 32 d	42.5% (51/120)	43.2% (54/125)	43.4% (53/122)	47.1% (57/121)	0.66	0.60	0.51
Preg/AI on 60 d	41.7% (50/120)	40% (50/125)	41.8% (51/122)	46.3% (56/121)	0.80	0.38	0.44
PregLoss 27 d to 32 d	20.3% (13/64)	21.7% (15/69)	26.4% (19/72)	20.8% (15/72)	0.62	0.69	0.51
PregLoss 27 d to 32 d	2% (1/51)	7.4% (4/54)	3.8% (2/53)	1.8% (1/57)	0.54	0.21	0.59
Luteolysis on 20 d	58.9% (33/56)	64.3% (36/56)	78% (39/50)	79.6% (39/49)	0.02	0.05	0.82

Control = not treated, 1Oxt = (Oxt 18 d), 2Oxt = (Oxt 18 d + 19 d), and 3Oxt (Oxt 17 d + 18 d + 19 d); C1: Control vs. (1Oxt, 2Oxt, and 3Oxt); C2: 1Oxt vs. (2Oxt and 3Oxt); and C3: 2Oxt vs. 3Oxt.

Table 3. Pregnancy outcomes of the first and rebreeding TAI for lactating dairy cows managed in timed rebreeding programs for AI every 21 or 35 days

Item	Resynch25	ReBreed21	ReBreed21+G	C1	C2
<i>First TAI</i>					
Preg/AI 32 d	53.4% (109/204)	42.9% (87/203)	44.1% (90/204)	0.04	0.8
Preg/AI 60 d	52% (106/204)	40.9% (83/203)	41.7% (85/204)	0.03	0.87
PregLoss	2.8% (3/109)	4.6% (4/87)	5.6% (5/90)	0.4	0.91
<i>*Second \geq TAI</i>					
Preg/AI 32 d	43.2% (92/213)	24.6% (54/220)	24.3% (41/169)	<.0001	0.98
Preg/AI 60 d	42.7% (91/213)	22.3% (49/220)	23.1% (39/169)	<.0001	0.83
PregLoss	1.2% (1/91)	9.3% (5/54)	4.9% (2/41)	0.11	0.44
<i>Bred 35 days since the last TAI</i>					
Preg/AI 32 d	43.2% (92/213)	37.3% (47/126)	44.3% (62/140)	0.60	0.21
Preg/AI 60 d	42.7% (91/213)	34.9% (44/203)	42.1% (59/140)	0.33	0.19
PregLoss	1.2% (1/91)	6.4% (3/47)	4.8% (3/62)	0.16	0.95
<i>Bred 42 days since the last TAI</i>					
Preg/AI 32 d	38.2% (21/55)	40% (16/40)	40.5% (15/37)	0.91	0.76
Preg/AI 60 d	32.7% (18/55)	37.5% (15/40)	40.5% (15/37)	0.70	0.59
PregLoss	14.3% (3/21)	6.2% (1/16)	0% (0/15)	0.97	0.98

*Preg/AI for second \geq TAI for cows rebred 35 days since the last TAI in Resynch25 and 21 days

since the last TAI in ReBreed21 groups. Contrasts = C1: Resynch25 vs. (ReBreed21 and

ReBreed21+G); C2: ReBreed21 vs. ReBreed21+G.

Table 4. Synchronization dynamics and fertility outcomes of nonpregnant cows with low P4

based on P4Gold that received TAI on day 21 since the last TAI

Item	By protocol			P/AI PD 27d			P/AI PD 32d		
	RB21	RB21+G	P	Sync	NoSync	P	Sync	NoSync	P
P4 >1ng/mL D18	62% (88/142)	61.3% (65/106)	0.83	36.6% (56/153)	21.1% (20/95)	0.01	25.5% (39/153)	17.9% (17/95)	0.18
P4 < 0.8ng/mL D20	98.6% (140/142)	92.5% (98/106)	0.04	31.1% (74/238)	20.0% (2/10)	0.43	23.1% (55/238)	10.0% (1/10)	0.33
P4 < 0.4ng/mL D20	91.6% (130/142)	84% (89/106)	0.08	32.0% (70/219)	7.0% (2/29)	0.21	24.7% (54/219)	6.9% (2/29)	0.04
¹ Ov after day 21	92.3% (131/142)	89.6% (95/106)	0.54	32.3% (73/226)	13.6% (3/22)	0.08	24.3% (55/226)	4.5% (1/22)	0.07
² Synchronization rate	47.2% (67/142)	42.5% (45/106)	0.46	42.0% (47/112)	21.3% (29/136)	<0.01	32.1% (36/112)	14.7% (20/136)	<0.01

¹Ov after day 21: percentage of cows with at least one CL > 15mm diameter on D27²Synchronization rate: Percentage of cows that had a combination of high P4 (P4 >1ng/mL) on

D18, low P4 on D20 (P4 <0.04ng/mL) and ovulated after TAI on day 21. P/AI = pregnancy per

AI, PD = pregnancy diagnosis on days 27 or 32 after ReBreed21-TAI.

Table 5. Time to pregnancy and reproductive performance for lactating dairy cows managed in timed rebreeding programs for AI every 21 or 35 days

Item	Resynch25	ReBreed21	ReBreed21+G	P
Day to pregnancy Relative to DIM	112.7A (\pm 3)	122.2B (\pm 3)	121.5AB (\pm 3)	0.09
Day to pregnancy Relative to 1 st AI	27.7A (\pm 3)	37.4B (\pm 3)	35.8AB (\pm 3)	0.1
<i>Pregnancy at</i>				
DIM 120	60.6% (123/203)	55.0% (110/200)	55.3% (109/197)	0.52
DIM 150	75.9% (154/203)	66.5% (133/200)	69.5% (137/197)	0.14
DIM 180	86.2% ^a (175/203)	76.5% ^b (153/200)	82.2% ^{ab} (162/197)	0.04

Figure Legends

Figure 1. Schematic timeline of the series of experiments. In experiment 1, lactating dairy cows were induced to ovulate with Double Ovsynch (DO) but were not bred. In experiment 2, after TAI, lactating dairy cows were randomized into one of four groups receiving 50 IU of oxytocin or saline. Experiment 3 lactating dairy cows after the first TAI from DO were randomized into one of three timed rebreeding programs. BS = blood sample, PD = pregnancy diagnosis, NPD = nonpregnancy diagnosis, PG = cloprostenol sodium, G = gonadorelin.

Figure 2. Circulating PGFM dynamics after treatments with oxytocin on days 17, 18, and 19 of the estrous cycle after induced ovulation. The dashed red line means two standard deviations from the average distribution of day 17 before treatment in both groups.

Figure 2. Luteolysis distribution after oxytocin treatments on days 17, 18, and 19 of the estrous cycle after induced ovulation. Panel A onset of luteolysis (the day before when P4 decreased >50% compared to the average of the 3 high values). Panel B final luteolysis (first day when P4 decreased < 1ng/mL). μ = average day of onset or final luteolysis and σ^2 = variance of onset or final luteolysis. * In the Control group, one cow did not have onset luteolysis until 30 d and two had not complete luteolysis until 30 d.

Figure 4. Percentage of nonpregnant lactating dairy cows with low P4 on day 20 of the estrous cycle after induced ovulation using different methods. Radioimmunoassay (RIA) cut-off P4 <1ng/mL; ELISA lateral flow assay (P4Gold) low P4 when test-line darker than control-line; and

color Doppler ultrasound, low P4 when total area of the CL with less than 25% of blood perfusium.

Figure 5. Timed rebreeding season performance of Resynch25 and ReBreed21. Survival percentage of nonpregnant lactating cows and time to pregnancy during a 105-day timed rebreeding season relative to days in milk (**Panel A**) for Resynch25 (day 112.7 A [\pm 3]), ReBreed21 (day 122.2 B [\pm 3]), and ReBreed21+G (day 121.5 AB [\pm 3]) P = 0.09; or relative to days since the first TAI **Panel (B)** for Resynch25 (day 27.7 A [\pm 3]), ReBreed21 B (day 37.4 [\pm 3]), and ReBreed21+G (day 35.8 AB [\pm 3]) P = 0.1.

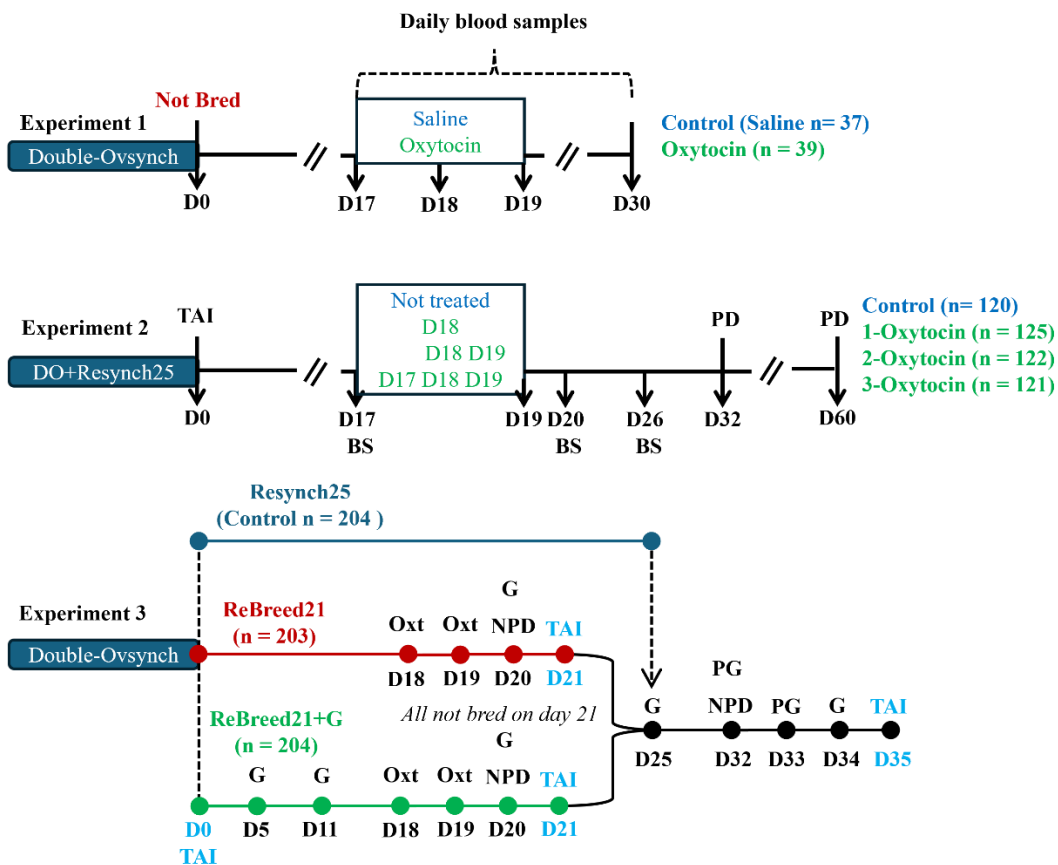


Figure 1

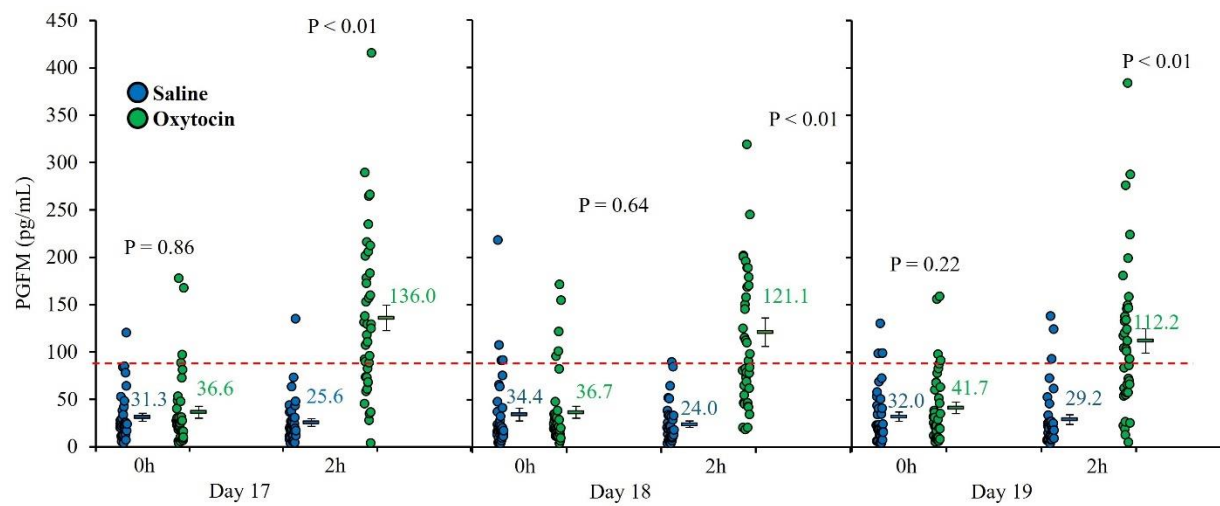


Figure 2

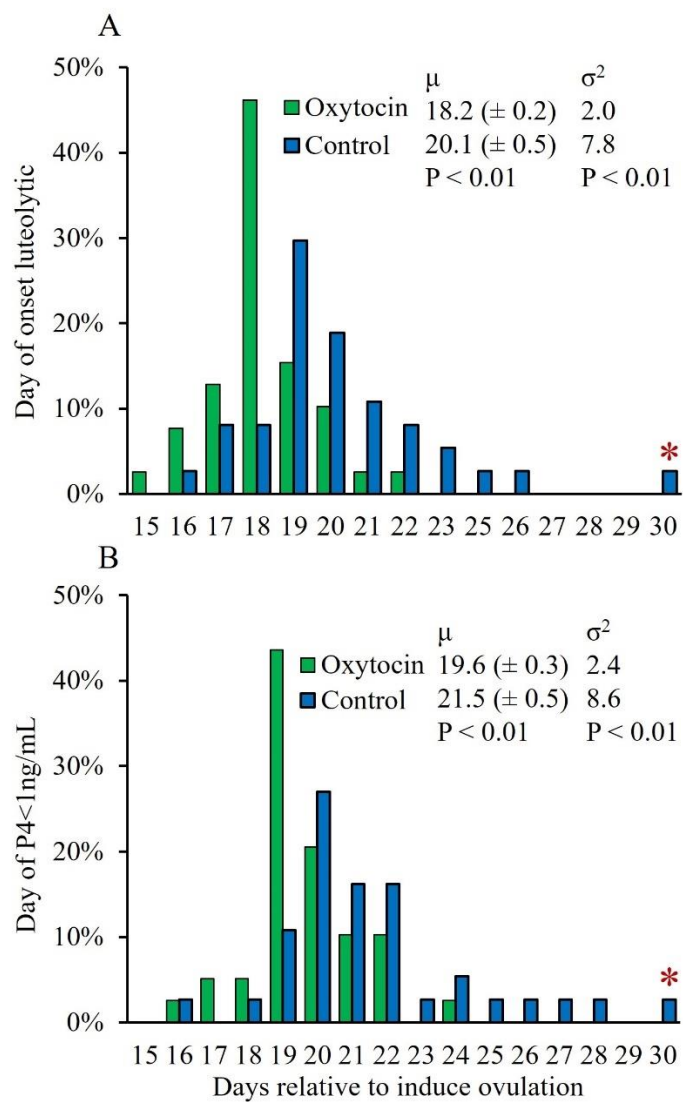


Figure 3

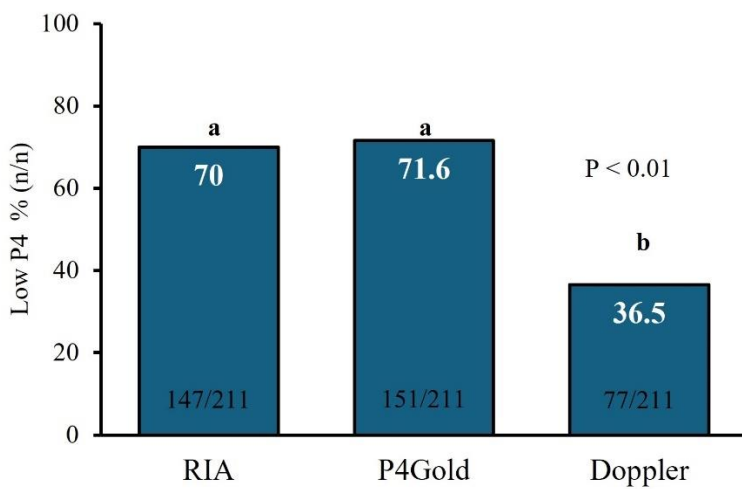


Figure 4

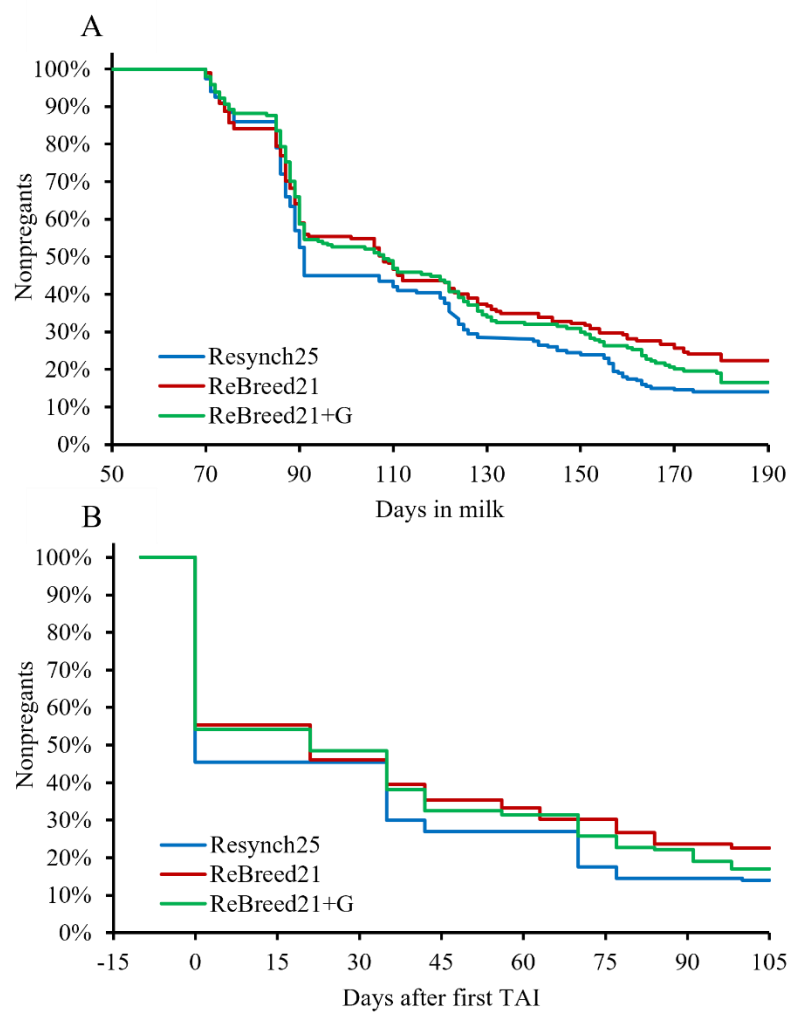


Figure 5

References

- [1] Fricke PM, Wiltbank MC. The implications of spontaneous versus synchronized ovulations on the reproductive performance of lactating dairy cows. *J Dairy Sci.* 2022;105:4679-89.
- [2] Consentini CEC, Wiltbank MC, Sartori R. Factors That Optimize Reproductive Efficiency in Dairy Herds with an Emphasis on Timed Artificial Insemination Programs. *Animals.* 2021;11:301.
- [3] Carvalho PD, Santos VG, Giordano JO, Wiltbank MC, Fricke PM. Development of fertility programs to achieve high 21-day pregnancy rates in high-producing dairy cows. *Theriogenology.* 2018;114:165-72.
- [4] Laplacette AL, Rial C, Baños GSM, Escalera JAG, Torres S, Kerwin A, et al. Effect of a targeted reproductive management program based on automated detection of estrus during the voluntary waiting period on reproductive performance of lactating dairy cows. *Theriogenology.* 2024;225:130-41.
- [5] Fricke PM, Giordano JO, Valenza A, Lopes G, Jr., Amundson MC, Carvalho PD. Reproductive performance of lactating dairy cows managed for first service using timed artificial insemination with or without detection of estrus using an activity-monitoring system. *J Dairy Sci.* 2014;97:2771-81.
- [6] Santos VG, Carvalho PD, Maia C, Carneiro B, Valenza A, Fricke PM. Fertility of lactating Holstein cows submitted to a Double-Ovsynch protocol and timed artificial insemination versus artificial insemination after synchronization of estrus at a similar day in milk range. *J Dairy Sci.* 2017;100:8507-17.

[7] Sitko EM, Perez MM, Granados GE, Masello M, Hernandez S, Cabrera EM, et al. Effect of reproductive management programs that prioritized artificial insemination at detected estrus or timed artificial insemination on the reproductive performance of primiparous Holstein cows of different genetic merit for fertility. *J Dairy Sci.* 2023;106:6476-94.

[8] Gonzalez TD, Factor L, Mirzaei A, Montevecchio AB, Casaro S, Merenda VR, et al. Targeted reproductive management for lactating Holstein cows: Reducing the reliance on exogenous reproductive hormones. *J Dairy Sci.* 2023;106:5788-804.

[9] Denis-Robichaud J, Cerri RLA, Jones-Bitton A, LeBlanc SJ. Performance of automated activity monitoring systems used in combination with timed artificial insemination compared to timed artificial insemination only in early lactation in dairy cows. *J Dairy Sci.* 2018;101:624-36.

[10] Fricke PM, Carvalho PD, Giordano JO, Valenza A, Lopes G, Jr., Amundson MC. Expression and detection of estrus in dairy cows: the role of new technologies. *Animal.* 2014;8 Suppl 1:134-43.

[11] Giordano JO, Wiltbank MC, Guenther JN, Pawlisch R, Bas S, Cunha AP, et al. Increased fertility in lactating dairy cows resynchronized with Double-Ovsynch compared with Ovsynch initiated 32 d after timed artificial insemination. *J Dairy Sci.* 2012;95:639-53.

[12] Fricke PM, Caraviello DZ, Weigel KA, Welle ML. Fertility of dairy cows after resynchronization of ovulation at three intervals following first timed insemination. *J Dairy Sci.* 2003;86:3941-50.

[13] Pursley JR, Kosorok MR, Wiltbank MC. Reproductive management of lactating dairy cows using synchronization of ovulation. *J Dairy Sci.* 1997;80:301-06.

[14] Chebel RC, Santos JE, Cerri RL, Galvao KN, Juchem SO, Thatcher WW. Effect of resynchronization with GnRH on day 21 after artificial insemination on pregnancy rate and pregnancy loss in lactating dairy cows. *Theriogenology*. 2003;60:1389-99.

[15] Lopes G, Jr., Giordano JO, Valenza A, Herlihy MM, Guenther JN, Wiltbank MC, et al. Effect of timing of initiation of resynchronization and presynchronization with gonadotropin-releasing hormone on fertility of resynchronized inseminations in lactating dairy cows. *J Dairy Sci*. 2013;96:3788-98.

[16] Dewey ST, Mendonca LG, Lopes G, Jr., Rivera FA, Guagnini F, Chebel RC, et al. Resynchronization strategies to improve fertility in lactating dairy cows utilizing a presynchronization injection of GnRH or supplemental progesterone: I. Pregnancy rates and ovarian responses. *J Dairy Sci*. 2010;93:4086-95.

[17] Bilby TR, Bruno RGS, Lager KJ, Chebel RC, Moraes JGN, Fricke PM, et al. Supplemental progesterone and timing of resynchronization on pregnancy outcomes in lactating dairy cows. *J Dairy Sci*. 2013;96:7032-42.

[18] Sani RN, Farzaneh N, Moezifar M, Seifi HA, Tabatabaei AA. Evaluation of five resynchronization methods using different combinations of PGF₂alpha, GnRH, estradiol and an intravaginal progesterone device for insemination in Holstein cows. *Anim Reprod Sci*. 2011;124:1-6.

[19] Giordano JO, Wiltbank MC, Guenther JN, Pawlisch R, Bas S, Cunha AP, et al. Increased fertility in lactating dairy cows resynchronized with Double-Ovsynch compared with Ovsynch initiated 32 d after timed artificial insemination. *J Dairy Sci* 2012;95:639-53.

[20] Mendonca LGD, Dewey ST, Lopes G, Rivera FA, Guagnini FS, Fetrow JP, et al. Effects of resynchronization strategies for lactating Holstein cows on pattern of reinsemination, fertility, and economic outcome. *Theriogenology*. 2012;77:1151-58.

[21] Giordano JO, Wiltbank MC, Guenther JN, Ares MS, Lopes G, Jr., Herlihy MM, et al. Effect of presynchronization with human chorionic gonadotropin or gonadotropin-releasing hormone 7 days before resynchronization of ovulation on fertility in lactating dairy cows. *J Dairy Sci*. 2012;95:5612-25.

[22] Wijma R, Pérez MM, Masello M, Stangaferro ML, Giordano JO. A resynchronization of ovulation program based on ovarian structures present at nonpregnancy diagnosis reduced time to pregnancy in lactating dairy cows. *J Dairy Sci*. 2018;101:1697-707.

[23] Giordano JO, Thomas MJ, Catucuamba G, Curler MD, Masello M, Stangaferro ML, et al. Reproductive management strategies to improve the fertility of cows with a suboptimal response to resynchronization of ovulation. *J Dairy Sci*. 2016;99:2967-78.

[24] Wijma R, Stangaferro M, Masello M, Granados G, Giordano J. Resynchronization of ovulation protocols for dairy cows including or not including gonadotropin-releasing hormone to induce a new follicular wave: Effects on re-insemination pattern, ovarian responses, and pregnancy outcomes. *J Dairy Sci*. 2017;100:7613-25.

[25] Pérez MM, Wijma R, Scarbolo M, Cabrera E, Sosa F, Sitko EM, et al. Lactating dairy cows managed for second and greater artificial insemination services with the Short-Resynch or Day 25 Resynch program had similar reproductive performance. *J Dairy Sci*. 2020;103:10769-83.

- [26] Fricke PM, Wiltbank MC, Pursley JR. The high fertility cycle. *JDS Commun.* 2023;4:127-31.
- [27] Middleton EL, Minela T, Pursley JR. The high-fertility cycle: How timely pregnancies in one lactation may lead to less body condition loss, fewer health issues, greater fertility, and reduced early pregnancy losses in the next lactation. *J Dairy Sci.* 2019;102:5577-87.
- [28] Andrade JPN, Domingues RR, Carvalho BP, Gomez-Leon V, Prata AB, Sartori R, et al. Optimizing ReBreed21 I: Evaluation of endocrine and ovarian dynamics in non-bred *Bos indicus* heifers. *Theriogenology.* 2024;220:77-83.
- [29] Andrade JPN, Monteiro PJJ, Prata AB, Robl AJ, Neto J, Lippe B, et al. Optimizing ReBreed21 II: Fertility and reproductive efficiency in different parities during a shortened breeding season in beef cattle. *Theriogenology.* 2024;224:41-49.
- [30] Andrade JPN, Gomez-Leon VE, Andrade FS, Carvalho BP, Lacouth KL, Garcia FZ, et al. Development of a novel 21-day reinsemination program, ReBreed21, in *Bos indicus* heifers. *Theriogenology.* 2020;155:125-31.
- [31] Sartori R, Haughian JM, Shaver RD, Rosa GJ, Wiltbank MC. Comparison of ovarian function and circulating steroids in estrous cycles of Holstein heifers and lactating cows. *J Dairy Sci.* 2004;87:905-20.
- [32] Ricci A, Carvalho PD, Amundson MC, Fricke PM. Characterization of luteal dynamics in lactating Holstein cows for 32 days after synchronization of ovulation and timed artificial insemination. *J Dairy Sci.* 2017;100:9851-60.
- [33] Denis-Robichaud J, Oliveira A, Sica A, Soriano S, Araújo R, Pereira M, et al. Is prolonged luteal phase a problem in lactating Holstein cows? *J Dairy Sci.* 2024.

[34] Domingues RR, Ginther OJ, Gomez-Leon VE, Wiltbank MC. Up-regulation of endometrial oxytocin receptor is associated with the timing of luteolysis in heifers with two and three follicular waves. *Biol Reprod.* 2020;102:316-26.

[35] Mann GE, Lamming GE. Timing of prostaglandin F-2 alpha release episodes and oxytocin receptor development during luteolysis in the cow. *Anim Reprod Sci.* 2006;93:328-36.

[36] Mezera MA, Hamm CS, Gamarra CA, Gennari RS, Prata AB, Sartori R, et al. Profiles of prostaglandin F metabolite in dairy cattle during luteal regression and pregnancy: implications for corpus luteum maintenance. *Biol Reprod.* 2019;101:76-90..

[37] Drum JN, Wiltbank MC, Monteiro PLJ, Prata AB, Gennari RS, Gamarra CA, et al. Oxytocin-induced prostaglandin F2-alpha release is low in early bovine pregnancy but increases during the second month of pregnancy. *Biol Reprod.* 2020;102:412-23.

[38] Bazer FW, Ott TL, Spencer TE. Interferon tau: An antiluteolytic hormone for pregnancy recognition in ruminants. *Biol Reprod.* 1997;56.

[39] Hansen TR, Sinedino LDP, Spencer TE. Paracrine and endocrine actions of interferon tau (IFNT). *Reproduction.* 2017;154:F45-F59.

[40] Souza AH, Ayres H, Ferreira RM, Wiltbank MC. A new presynchronization system (Double-Ovsynch) increases fertility at first postpartum timed AI in lactating dairy cows. *Theriogenology.* 2008;70:208-15.

[41] Andrade JPN, Andrade FS, Guerson YB, Domingues RR, Gomez-Leon VE, Cunha TO, et al. Early pregnancy diagnosis at 21 days post artificial insemination using corpus luteum vascular perfusion compared to corpus luteum diameter and/or echogenicity in Nelore heifers. *Anim Reprod Sci.* 2019;209:106144.

- [42] Wiltbank M, Lopez H, Sartori R, Sangsritavong S, Gumen A. Changes in reproductive physiology of lactating dairy cows due to elevated steroid metabolism. *Theriogenology*. 2006;65:17-29.
- [43] Wiltbank MC, Cunha AP, Souza AH, Lopez H, Sartori R, Gumen A, et al. Mechanisms underlying the effect of milk production on duration of estrus and other reproductive traits in lactating dairy cows. *Reprod Domest Anim*. 2008;43:20-20.
- [44] Wiltbank MC, Sartori R, Herlihy MM, Vasconcelos JLM, Nascimento AB, Souza AH, et al. Managing the dominant follicle in lactating dairy cows. *Theriogenology*. 2011;76:1568-82.
- [45] Wessel T, Schuchter U, Walt H. Ciliary motility in bovine oviducts for sensing rapid non-genomic reactions upon exposure to progesterone. *Horm Metab Res*. 2004;36:136-41.
- [46] Crisman RO, McDonald LE, Thompson FN. Effects of progesterone or estradiol on uterine tubal transport of ova in the cow. *Theriogenology*. 1980;13:141-54.
- [47] Binelli M, Gonella-Diaza AM, Mesquita FS, Membrive CMB. Sex Steroid-Mediated Control of Oviductal Function in Cattle. *Biology*. 2018;7. 15.
- [48] Youngquist RS, Bierschwal CJ, Elmore RG, Jenkins AL, Schultz RH, Widmer JH. Induction of abortion in feedlot Heifers with Cloprostenol (ICI 80,996). *Theriogenology*. 1977;7:305-12.
- [49] Domingues RR, Ginther O, Gomez-Leon V, Castro T, Wiltbank MC. Endometrial and luteal responses to a prostaglandin F2alpha pulse: A comparison between heifers and mares. *Biol Reprod*. 2022; 106:979-991.

[50] Mann GE, Lamming GE. Timing of prostaglandin F(2alpha) release episodes and oxytocin receptor development during luteolysis in the cow. *Anim Reprod Sci.* 2006;93:328-36.

[51] Domingues RR, Ginther OJ, Gomez-Leon VE, Wiltbank MC. Up-regulation of endometrial oxytocin receptor is associated with the timing of luteolysis in heifers with two and three follicular waves. *Biol Reprod.* 2020;102:316-26.

[52] Robinson RS, Mann GE, Lamming GE, Wathes DC. Expression of oxytocin, oestrogen and progesterone receptors in uterine biopsy samples throughout the oestrous cycle and early pregnancy in cows. *Reproduction.* 2001;122:965-79.

[53] Kieborz-Loos KR, Garverick HA, Keisler DH, Hamilton SA, Salfen BE, Youngquist RS, et al. Oxytocin-induced secretion of prostaglandin F-2 alpha in postpartum beef cows: Effects of progesterone and estradiol-17 beta treatment. *J Anim Sci.* 2003;81:1830-36.

[54] Mann GE, Lamming GE. Effect of the Level of Estradiol on Oxytocin-Induced Prostaglandin-F2-Alpha Release in the Cow. *J Endocrinol.* 1995;145:175-80.

[55] Middleton E, Minela T, Ahearne M, Arnold H, Santos A, Pursley J. Dairy heifers have an earlier increase in serum pregnancy-specific protein B compared with lactating dairy cows. Is this an indicator of earlier conceptus attachment? *JDS communications.* 2022;3:291-95.

[56] Vasconcelos JLM, Sangsritavong S, Tsai SJ, Wiltbank MC. Acute reduction in serum progesterone concentrations after feed intake in dairy cows. *Theriogenology.* 2003;60:795-807.

[57] Wiltbank MC, Sartori R, Vasconcelos JLM, Nascimento AB, Souza AH, Cunha AP, et al. Managing the dominant follicle in high-producing dairy cows. *Reproduction in Domestic Ruminants Vii.* 2010;67:231-45.

[58] Wiltbank MC, Carvalho PD, Kaskin A, Hackbart KS, Meschiatti MA, Bastos MR, et al. Effect of Progesterone Concentration During Follicle Development on Subsequent Ovulation, Fertilization, and Early Embryo Development in Lactating Dairy Cows. *Biol Reprod.* 2011;85.

[59] Monteiro PLJ, Sartori R, Canavessi AMO, Melo LF, Motta JCL, Consentini CEC, et al. Accessory corpus luteum regression during pregnancy I: timing, physiology, and P4 profiles. *Reproduction.* 2021;162:473-82.

[60] Monteiro PLJ, Gamarra CA, Genari RS, Prata AB, Barletta RV, Duran PG, et al. Accessory corpus luteum regression during pregnancy II: reproductive outcomes. *Reproduction.* 2021;162:483-95.

[61] Giordano JO, Fricke M, Wiltbank MC, Cabrera VE. An economic decision-making model for comparing reproductive management programs in dairy herds. *J Dairy Sci.* 2010;93:2301-01.

[62] Rial C, Laplacette A, Giordano J. Effect of a targeted reproductive management program designed to prioritize insemination at detected estrus and optimize time to insemination on the reproductive performance of lactating dairy cows. *J Dairy Sci.* 2022;105:8411-25.

[63] Pugliesi G, Miagawa BT, Paiva YN, Franca MR, Silva LA, Binelli M. Conceptus-induced changes in the gene expression of blood immune cells and the ultrasound-accessed luteal function in beef cattle: how early can we detect pregnancy? *Biol Reprod.* 2014;91:95.

[64] Pugliesi G, Bisinotto DZ, Mello BP, Lahr FC, Ferreira CA, Melo GD, et al. A novel strategy for resynchronization of ovulation in Nelore cows using injectable progesterone (P4) and P4 releasing devices to perform two timed inseminations within 22 days. *Reprod Domest Anim.* 2019;54:1149-54.

[65] Andrade JPN, Domingues RR, Monteiro PJ, Dias JR, Pimenta C, Guimarães AS, et al. Identification of nonpregnant beef cows based on CL size vs. luteal blood perfusion at 21 days after timed artificial insemination. *Theriogenology*. 2024;230:15-20.

[66] Ginther OJ, Silva LA, Araujo RR, Beg MA. Temporal associations among pulses of 13,14-dihydro-15-keto-PGF(2alpha), luteal blood flow, and luteolysis in cattle. *Biol Reprod*. 2007;76:506-13.

[67] Domingues RR, Andrade JPN, Cunha TO, Madureira G, Hoppman AS, Teixeira NN, et al. Profiles of interferon-stimulated genes in multiple tissues and circulating pregnancy-associated glycoproteins and their association with pregnancy loss in dairy cows. *Biol Reprod*. 2023;110:558-68.

**Chapter 6: ReBreed21-ET: Evaluation of a rapid resynchronization program that allows
timed embryo transfer every 21 days**

João Paulo N. Andrade ^a, Victor E. Gomez-León ^{a,b}, Guilherme Madureira ^a, Luma C. Sartori^a;
Gustavo F. Grillo ^{a,c}, Rafael R. Domingues ^{a,d}, Meliton Fosado ^e, Rodrigo V. Sala^e, Milo C.
Wiltbank ^{a*}

^a Department of Animal and Dairy Sciences, University of Wisconsin-Madison, Madison, WI
53706, USA

^b Department of Animal Sciences and Industry, Kansas State University, Manhattan, KS 66506,
USA

^c Departamento de Reprodução e Avaliação Animal, Universidade Federal Rural do Rio de
Janeiro, Seropédica, Rio de Janeiro 23897-000, Brasil

^d Department of Animal Sciences, The Ohio State University, Columbus, OH, 43210, USA

^e ST Genetics-Volm Farm ST Heifer Center, Kewaskum, WI, 53040, USA

*Corresponding Author: wiltbank@wisc.edu

Under review in Theriogenology (THERIO-S-24-011150).

Abstract

This study evaluated the efficiency of a rapid resynchronization of ovulation program to allow timed embryo transfer (TET) every 21d in heifer embryo recipients. Holstein heifers (n=550) had synchronized ovulation using a modified 5d CoSynch program for a TET (D7) after induced ovulation (D0). After TET, heifers were blocked by number of previous TETs and randomized into one of two resynchronization of ovulation programs: Resynch28 (n=279), a traditional resynch program for TET 35d after previous TET; or ReBreed21-ET, a program designed to allow TET 21d after previous TET. Once assigned into one of the two programs, heifers were kept in the same program during a 105-d TET period. In Resynch28, heifers received an intravaginal progesterone (P4) insert on D28, on D33 the P4 was removed, and pregnancy diagnosis was performed using rectal ultrasound to determine embryonic heartbeat. Nonpregnant heifers received a PGF2 α treatment with a later GnRH treatment on D35 for a potential TET on D42 (35d after previous TET). Heifers in ReBreed21-TET received an intravaginal P4 insert on D14, on D19 the P4 was removed, and on D21 a GnRH treatment was given to synchronize a new ovulation. On D28, pregnancy diagnosis was performed using transrectal ultrasound to detect an embryonic heartbeat and nonpregnant heifers that had a corpus luteum (CL) \geq 18mm in diameter received a TET (21d after the previous TET). Pregnancy per ET (P/ET) from the first TET was greater for heifers in ReBreed21-ET (52%) than Resynch28 (39.4%). In contrast, the subsequent TET (second and later) had similar P/ET for ReBreed21-ET (40.4%) and Resynch28 (40.8%). The overall pregnancy loss from D28 to D63 did not differ between programs (ReBreed21-ET [18.5%] and Resynch28 (16.3%]). Nevertheless, there were fewer pregnancy losses from D28 to D33 for Resynch28 (3.5%) than ReBreed21-ET (10.1%), while from D33 to D47, there was greater pregnancy loss for Resynch28 (10.1%) than ReBreed21-ET (4.9%). Time

to pregnancy was approximately 7d earlier for ReBreed21-ET (45 ± 3 d) than Resynch28 (53 ± 3 d). Overall accumulative pregnancies at the end of a 105-d TET season were greater for ReBreed21-ET (75.2%) than Resynch28 (64%). Thus, the ReBreed21-ET program can improve the efficiency of TET programs.

Keywords: embryo transfer, resynchronization, ReBreed21, progesterone, pregnancy loss.

Introduction

There were over 2 million embryos produced worldwide in 2022, an ~80% increase from 2012, primarily due to increased embryo production in North America. The embryo production in the USA grew ~155% between 2012 and 2022, with current production of over half of all embryos worldwide [1]. Regardless of the source of the embryos (*in vivo* or *in vitro* produced), the main advantage of embryo transfer (ET) is the multiplication of high genetic merit female offspring, allowing a dam to produce more than one calf/year, in some cases more than a hundred [2]. Furthermore, ET maximizes the use of sex-sorted semen from high genetic merit sires as a single semen straw can produce multiple embryos [3]. In addition, with the advent of the beef-on-dairy strategy, use of ET became a unique opportunity for farmers to produce pure beef calves with premium value compared with beef/dairy crossbred calves. Combining these strategies, the adoption of ET leads to rapid genetic improvement in cattle herds, boosting animal reproductive and productive performance [4, 5]. Thus, ET has become a reproductive biotechnology that has been utilized throughout the world and is commercially available in many different types of cattle operations [4].

A fundamental aspect of ET programs is the selection and use of embryo recipients. Embryo recipients must be on the desired day of the estrous cycle (~7d after estrus) to match the embryo's developmental stage [6]. To achieve this purpose, a variety of strategies have been utilized, including daily detection of estrus, synchronization of estrus, or synchronization of ovulation. Synchronization of ovulation is a strategy that allows about 85% of females to be eligible to receive an ET on a scheduled day producing a Timed-ET (TET) [7-9]. Due to the improved efficiency of animal use, TET has become a standard reproductive management strategy to maximize the use of recipients and increase their reproductive efficiency [4, 9, 10].

For instance, a direct comparison of heifers randomized to receive ET after detected estrus or after a TET program found that pregnancy per ET (P/ET) was not different (TET=45.2% vs 41.1% after estrus; $P = 0.29$), but the utilization rate of recipients was much greater ($P < 0.001$) for TET (89.8%) than estrus (69.2%) [9] consistent with other similar studies [2].

In an ET program, it is critical to establish strategies to manage non-synchronized recipients, recipients that remained nonpregnant after a previous ET, and recipients that undergo pregnancy loss. Ideally, any nonpregnant recipient should be rapidly re-enrolled in a resynchronization protocol for the next TET. Often, resynchronization programs begin before pregnancy diagnosis with females that are found pregnant not continuing in the program, whereas nonpregnant ones continue to another service, usually 35 to 42d after the previous service [11]. Of particular importance, pregnant recipients that lose the pregnancy after pregnancy diagnosis will produce economic losses due to both the death of an offspring with high genetic merit and the prolonged feeding of a nonpregnant recipient [12, 13]. Hence, to develop an efficient reproductive program for embryo recipients, one needs to consider the integration of synchronization, pregnancy diagnosis, and resynchronization programs targeting rapid TET combined with efforts to mitigate pregnancy loss in ET recipients.

ReBreed21 is a reproductive program for cattle that allows timed AI (TAI) every 21d through efficient resynchronization of females combined with determination of non-pregnancy using Color Doppler ultrasound of the CL on D21 after previous AI [14-16]. Adapting this program to embryo recipients could allow ET every 21d and potentially optimize the efficiency of a TET program. In a TET program, the selection of nonpregnant animals could be performed on D28 of pregnancy through standard ultrasonography to detect embryonic heartbeat rather than the Doppler ultrasound normally used with ReBreed21 TAI programs. Thus, a nonpregnant

recipient could be identified as nonpregnant and be ready to receive a subsequent TET seven days after a synchronized ovulation or 21d since the previous ET. This strategy could reduce time between ETs, improving reproductive efficiency, while facilitating the labor schedule by keeping animal management and TET on the same day of the week. Furthermore, in the ReBreed21 resynchronization program, the intravaginal P4 insert is strategically used during the embryo elongation period, and this may benefit embryonic development and placentation, potentially decreasing pregnancy losses [17-20].

Thus, the objective of this study was to evaluate the efficiency of a ReBreed21 modification for dairy heifers used as embryo recipients (ReBreed21-ET) to produce a potential TET every 21d in comparison to another efficient reproductive program for TET every 36d (Resynch28) during a 105-d TET breeding season. Our central hypothesis is that the ReBreed21-ET program will increase the reproductive efficiency of Holstein embryo recipients during a 105-d TET program. Specifically, we hypothesized that: (1) The ReBreed21-ET protocol would produce a hormonal/ovarian milieu that would be favorable for synchronization of ovulation on d21 in nonpregnant heifers, (2) ReBreed21-ET would have similar P/ET as Resynch28 for first and later TETs, and (3) Recipients enrolled in ReBreed21-ET would have earlier time to pregnancy compared to Controls (Resynch28).

Materials and Methods

Animals, location, and management

The experiment was performed in a commercial operation of STgenetics in Kewaskum-WI. The overall experiment used 550 dairy heifers with BCS of 3.05 ± 0.01 (1 to 5 scale). Many of these recipients had received previous TET (0 to 6 times) before enrollment in the study.

Animals were managed under the Guide for the Care and Use of Agricultural Animals and approved by the Animal Care and Use Committee of the College of Agriculture and Life Sciences, University of Wisconsin. The heifers were housed in a free-stall barn, had ad libitum access to water, and were fed ad libitum once daily with a total mixed ration (TMR) formulated to meet or exceed the nutritional requirements of Holstein heifers weighing 360 Kg and gaining 0.8 kg/d (NRC, 2001).

Reproductive programs for TET

During eight weeks, dairy embryo recipients received a TET and were randomized into Resynch28 (n=279) or ReBreed21-ET (n=271) blocked by the previous number of TET (0 or ≥ 1). Once enrolled in one of the two programs, each recipient was maintained in the program for 105-d. For the first TET in the experiment, synchronization of ovulation was carried out with a modified 5d CoSynch program [9]. Five days after the induced ovulation with GnRH (D0), all heifers had their ovaries evaluated using ultrasound, and those with at least one CL > 15 mm in diameter were assigned to receive an ET 2d later (D7) in the uterine horn ipsilateral to the CL. The embryo evaluation was performed under the IETS guidelines [21]. Embryos used in the current trial were classified by: 1) type of embryo: fresh IVF embryo (n=445) and frozen IVF embryos (n=65); 2) quality: 1 (n=454) and 2 (n=56); and 3) development stage: d6 (n=75), d7 (n=406), and d8 (n=29). Heifers that received a cloned embryo at one of the ET (n=27 [Resynch28 n=9; ReBreed21-ET n=18]) or died during the trial (n=8 [Resynch28 n=3; ReBreed21-ET n=5]) or had missing information (n=5 [Resynch28 n=3; ReBreed21-ET n=2]) were excluded from the analysis. The final number of females was 510 dairy embryo recipient heifers randomly assigned to Resynch28 (n=264) and ReBreed21-ET (n=246).

Experimental design

Resynchronization program for TET: Resynch28

The control program for this experiment used the standard resynchronization protocol for the operation (Resynch28). Resynchronization was started on D28 (D0 = GnRH) with the insertion of an intravaginal P4 insert (CIDR) in all heifers without knowledge of the pregnancy status of the heifer. On D33, the CIDR was removed, and a pregnancy diagnosis was performed by ultrasound (detection of embryonic heartbeat). In nonpregnant heifers, cloprostenol sodium (500mg, PGF2 α analog) was applied, followed by treatment with gonadorelin (100mg, GnRH analog) 3d later. Thus, the modified 5d CoSynch program used for the second TET was essentially the same as that used for the first TET [9]. As in the first TET, all heifers had their ovaries evaluated by ultrasound on D5 after final GnRH, and heifers with a CL > 15 mm diameter received a TET. Thus, all procedures were similar for the first and second TET, allowing 36d between TET.

Resynchronization program for TET: ReBreed21-ET

For ReBreed21-ET, resynchronization started on D14 (D0 = GnRH) with CIDR inserted and removed on D19, followed by GnRH treatment in the afternoon of D21. On the morning of D28, pregnancy diagnosis was performed by ultrasound (detection of embryonic heartbeat). Nonpregnant heifers with a CL >18 mm in diameter were assigned to a new TET on the same day in the afternoon, allowing a 21d interval between TET.

Pregnancy diagnosis and further management information

Pregnancy diagnosis was performed by ultrasound (detection of embryonic heartbeat) on D 28, 33, 47, and 63. Heifers in either program with pregnancy loss were resynchronized with a modified 5d CoSynch [9] and kept in the same reproductive resynchronization program as previously enrolled (Resynch28 or ReBreed21-ET). During the resynchronization program,

heifers that did not meet the CL requirements for ET (described in sections 2.2 and 2.4) were also resynchronized with a 5-d CoSynch for another potential TET. Therefore, the number of TET for Resynch28 were: first TET (n = 264), second or later ETs (n=240). The number of TET for ReBreed21-ET were: first TET (n=246), second or later TET (n = 253) (includes TET on D28 after nonpregnancy diagnosis [n = 141] and resynchronized with 5-d CoSynch after pregnancy loss or ovulation failure [n = 112]).

Blood sample collection and P4 measurement

Blood samples on D19 and D21 were collected into evacuated tubes (Vacutainer, Dickinson, Franklin Lakes, NJ) by the coccygeal tail vein puncture. Immediately after collection, tubes were placed on ice and refrigerated until centrifuged at 2500 x g for 20 min. The serum was recovered and stored frozen at -20°C in 2 mL Eppendorf tubes until assayed for P4. Circulating P4 was determined using a solid-phase RIA kit containing antibody-coated tubes and I¹²⁵-labeled P4 (ImmuChem Coated Tube P4 125I RIA Kit, MP Biomedicals, CA, USA) following the commercial recommendation for a short incubation time of 120 min in a water bath (set at 37 °C) followed by aspiration of tubes. The intra- and inter-assay CVs were 1.1 and 1.8 % and sensitivity was 0.07 ng/mL.

Statistical analysis

Binary data such as P/ET, pregnancy loss, cumulative pregnancies, utilization rate, proportion of ovulation from D19 to 21, and proportion of animals with P4<1ng/mL on D21 were analyzed with the generalized linear model using the GLIMMIX procedure of SAS (Version 9.4; SAS Institute). The reproductive program was included in the model as fixed effects. Embryo quality, type of embryo, embryo developmental stage, number of TET during the experiment, and the technician that performed the TET were used as explanatory variables in

the model and kept in the model if $P < 0.20$. Interaction between the reproductive program and the other explanatory variables were evaluated in the model and kept if significant. The mean time to pregnancy was analyzed using the PROCLIFETEST procedure, using the Wilcoxon test. Continuous variables (circulating P4 and largest follicle diameter) were analyzed using the PROC MIXED procedure for linear models. Assumptions (linearity, independence, and normality) were assessed by plotting the residuals and by the Shapiro-Wilk test. If deviations from assumptions were found, data were transformed guided by BoxCox transformation to select the best transformation: natural logarithms, square root, or ranks. Significant differences between treatment groups were deemed for $P \leq 0.05$, whereas differences between $P > 0.05$ and $P \leq 0.10$ were considered a tendency.

Results

The results from the first TET for the two reproductive programs at the different pregnancy diagnoses are shown in Figure 2A. For the first TET, P/ET was greater on D28 ($P < 0.01$) and D47 ($P = 0.03$) for ReBreed21-ET (52% and 43.9%) than Resynch28 (39.4% and 35.2%), while P/ET tended to be greater on D33 ($P = 0.08$) and D63 ($P = 0.09$) for ReBreed21-ET (46% and 41.5%) compared to Resynch28 (38.6% and 34.5%). For second and later TET, P/ET was similar for ReBreed21-ET and Resynch28 at all pregnancy diagnoses (D28 to D63, Figure 3A). The P/ET for heifers in ReBreed21-ET that were resynchronized with 5-d CoSynch (after a pregnancy loss or previous ovulation failure) was 37.5% (42/112), 35.7% (40/112), 33.9% (38/112), and 33.9% (38/112) on D28, D33, D47, and D63, respectively (not shown on Figure 3A) and similar to second and later TET for ReBreed21-ET and Resynch28.

Pregnancy loss for the first TET (Figure 2B) from D28 to D33 was lower ($P < 0.05$) in Resynch28 (1.9%) than in ReBreed21-ET (11.7%) but did not differ for other periods and was similar for all periods after the second or later TET (Figure 3B). To further explore the occurrence of pregnancy loss in TET programs, we combined all TET (first, second, and later TET) during the experimental period (Table 1). Pregnancy loss between D28 to D33 was 2.9-fold greater ($P < 0.01$) for ReBreed21-ET (10.1%) than Resynch28 (3.5%), whereas between D33 to D47 it was 2.1-fold greater ($P = 0.04$) in Resynch28 (10.1%) than ReBreed21-ET (4.9%). There was no difference in pregnancy loss between ReBreed21-ET vs. Resynch28 for D47 to D63 (4.6% vs. 3.4%; $P = 0.45$) or D28 to D63 (18.5% vs. 16.3%; $P = 0.69$).

The synchronization dynamics are shown in Table 2. Heifers in Resynch28 had a greater diameter of the largest follicle on D19 but smaller diameter on D21 compared with ReBreed21-ET, mainly because of the greater percentage of heifers with ovulation between D19 to D21, 39.7% vs. 24.3%, respectively. Heifers in ReBreed21 had greater circulating P4 on D19 but lower on D21 compared with Resynch28. On D19, the proportion of nonpregnant heifers with P4 < 1 ng/mL was greater ($P < 0.001$) for the Resynch28 (35.4%, 103/291) than ReBreed21-ET (1.9%, 5/263). Whereas on D21, it was lower ($P < 0.001$) for Resynch28 (52.2%, 152/291) compared to ReBreed21-ET (61.6%, 162/263).

The utilization rate of recipients synchronized with modified 5-d CoSynch (both first and later TET) was 87.9% (911/1036). Only considering the nonpregnant heifers that had P4 < 1 ng/mL on D21, the potential utilization rate was ReBreed21-ET 78.4% (127/162). However, including all the nonpregnant on D28 the utilization rate of nonpregnant embryo recipients on D28 for was 58.8% (160/272). On the other hand, For nonpregnant heifers that received TET on D28, 19.4% (31/160) had P4 > 0.8 ng/mL on D21, which tended ($P = 0.06$) to negatively affect

the P/ET (25.8% [8/31]) compared with heifers that received TET on D28 and had circulating P4 on D21 < 0.8 ng/mL (44.7% [46/103]). Considering the nonpregnant heifers on D28 in ReBreed21-ET that had CL, 19 were after the 105th day of the ET season, thus, they did not receive an ET in the trial.

The cumulative pregnancy percentage during the 105-d TET season was greater ($P < 0.01$) for ReBreed21-ET (75.2%) compared with Resynch28 (64%, Figure 5A). Thus, there was a relative increase of 17.5% in pregnant embryo recipients in ReBreed21-ET compared to Resynch28. Using survival curves, the percentage of nonpregnant heifers during the 105-d TET season was 8.4d earlier ($P < 0.01$) to pregnancy for ReBreed21-ET (44.9 ± 3 d) compared with Resynch28 (day 53.3 ± 3 d).

Discussion

Efficient reproductive biotechnologies and strategies can allow cattle operations to achieve high reproductive and productive performance [22]. Herein, we report a novel resynchronization program designed specifically for embryo recipients targeting potential TET every 21 d, using a modified protocol of ReBreed21 [14-16]. The present study supported our main hypothesis that ReBreed21-ET increases the performance of Holstein embryo recipients during a 105-d TET program. Performing an earlier TET at 21d after the previous TET with ReBreed21 increased the percentage of recipients that were pregnant at the conclusion of the TET period and produced an earlier time to pregnancy compared to a very efficient TET program (Resynch28) that allowed TET every 35d. In addition, there was an observed P4 supplementation effect on pregnancy outcomes due to the P4 insert, increasing P/ET in ReBreed21-ET (D14-19 P4 supplementation increased P/ET on D28) while decreasing

pregnancy loss during a short period in Resynch28 (D28-33 P4 supplementation decreased pregnancy loss from D28-33). Therefore, the present study provides important insights regarding whether ReBreed21-ET can be integrated into embryo-recipient operations and provides biological insights into the effect of P4 supplementation on pregnancy loss.

Our first hypothesis was supported that the ReBreed21-ET protocol would produce a favorable hormonal/ovarian milieu for synchronization of ovulation on D21 in nonpregnant heifers. An efficient synchronization of ovulation protocol designed for TET programs must induce high ovulation to increase embryo recipients' utilization rate (typically ~85%) [9]. Indeed, using the modified 5-d CoSynch in the present experiment reached a utilization rate of 87.9%. In other resynchronization protocols, including the modified 5-d CoSynch protocol, exogenous treatment with PGF2 α is critical in the synchronization of ovulation programs to decrease circulating P4 and increase the recipient utilization rate. Furthermore, residual P4 by D21 (~7d before TET in nonpregnant heifers in ReBreed21-ET) decreased P/ET due to not properly achieving the optimal hormonal milieu (low P4 plus high estradiol near estrus) for high fertility after AI or ET [23, 24]. On the other hand, in early resynchronization protocols such as ReBreed21-ET, all hormonal treatments are performed prior to the pregnancy diagnosis, and therefore PGF2 α analogs must be avoided to prevent iatrogenic abortion [25]. Nevertheless, compared to Control, ReBreed21-ET decreased ovulation before D21 due to retention of the P4 insert until D19, resulting in a larger follicle on D21 and a greater percentage of heifers with P4 < 1ng/mL on D21. Conversely, even with a reasonably favorable hormonal/ovarian milieu in the ReBreed21-ET, the percentage of nonpregnant heifers eligible for TET on D28 was only 59%. Thus, one aspect associated with the poor utilization rate of nonpregnant recipients on D21 was

likely the fact that exogenous PGF2 α analogs could not be used during the resynchronization of ovulation protocol.

Pregnancy loss is another factor associated with low efficiency in ET programs that appears to be reduced during the ReBreed21-ET program, at least on D28. Thus, the second hypothesis was partially supported as the P/ET was 32% greater for the first TET in the ReBreed21-ET (52% [128/246]) compared to Resynch28 (39.4% [104/264]). Previous research indicates that P4 supplementation can increase embryo elongation, potentially increasing fertility. For example, transferring an embryo into a recipient uterus primed with high P4 results in increased embryonic length during elongation [17, 26] and fetal development on D42 [27]. In the present study, we speculate that the increase in P/ET at the first TET in ReBreed21 may be due to the P4 supplementation from D14 to D19 of gestation, potentially favoring embryo development during the critical elongation period. Paradoxically, P/ET was similar between reproductive programs for the second and subsequent TET, suggesting a lack of effect of P4 supplementation on fertility in these later TETs. These results continue the uncertainty/confusion about the impact of P4 supplementation on fertility [28, 29]. Still, ReBreed21-ET achieved acceptable P/ET compared to the control program in this study or to other programs using dairy heifer embryo recipients (~ 40 to 50%, pregnancy diagnosis on D32 of gestation) [9]. Nevertheless, a major aspect of ET programs that were also observed in this study was the pregnancy loss from D28 to D60, which decreases the number of pregnancies by 15 to 25% [9, 20].

Of particular interest in the present study was that pregnancy loss occurred at different times in the two resynchronization programs, perhaps due to the timing of P4 supplementation with the intravaginal P4. Pregnancy loss from D28 to D33 was lower ($P < 0.01$) for Resynch28

(3.5%) compared to ReBreed21-ET (10.1%), while there was an increase ($P = 0.04$) in losses from D33 to D47 for Resynch28 (10.1%) compared to ReBreed21-ET (4.9%). Pregnancy losses in lactating dairy cows and beef cows range from 5% to 20% between D28 to D33 of gestation, representing about half of the pregnancy losses that occur from D28 to D60 [20, 30-32]. This is consistent with unpublished data from our laboratory from more than 2,500 TET in Holstein heifer embryo recipients in which 11.1% (128/1156) of losses were observed in five days (D28 to D33) using pregnancy diagnosis by ultrasound with the presence of an embryonic heartbeat. Exploring pregnancy loss following ET provides a unique model for evaluating the effects of P4 supplementation since all ET recipients, in theory, receive a viable embryo on D7. In the present experiment, although there were fewer pregnancy losses from D7 to D28 in ReBreed21 than Resynch28, perhaps due to P4 from D14 to D19, pregnancy loss from D33 to D47 was less for Resynch28 than ReBreed21-ET (10.1 vs 3.5%), perhaps due to intravaginal P4 during this time of pregnancy. The intravaginal P4 supplementation from D28 to D33 in Resynch28 may have rescued some pregnancies that would have been lost due to CL regression. We recently reported that about 50% of pregnancy losses from D20 to D33 of gestation in lactating dairy cows are due to spontaneous CL regression [33]. Consistent with this concept, the removal of P4 supplementation on D33 was potentially associated with the increased pregnancy loss between D33 and D47 in Resynch28. Nonetheless, overall pregnancy losses from D28 to D63 were similar for ReBreed21-ET (18.5%) and Resynch28 (16.3%). Future studies, specifically designed to explore the effect of P4 supplementation during key stages of pregnancy establishment, should be performed to confirm the findings of the present manuscript and help understand the pregnancy loss mechanisms in cattle.

Our third hypothesis was also supported, that ReBreed21-ET would result in earlier pregnancy than Resynch28. The ReBreed21-ET program resulted in 17% more pregnancies by 105-d of the TET season compared to Resynch28 (75.2% [185/246] vs. 64% [169/264]; $P < 0.01$) and led to an 8d earlier average time to pregnancy (45 ± 3 vs. 53 ± 3 d; $P < 0.01$). The rapid establishment of pregnancy is key to an efficient ET program since the main goal is to produce high genetic merit calves with the least total costs. Similar to the economics in TAI of heifers [34], the fixed costs of housing and feeding of nonpregnant heifers are important expenses in heifer embryo recipient operations. To optimize efficiency, TET programs allow TET of most embryo recipients on the first day of the TET season and, combined with resynchronization strategies, can result in a quicker time to pregnancy, similar to dairy cow TAI programs [35, 36]. An effective formula to succeed in the development of a reproductive program is to link optimal fertility in the first service, rapid and accurate nonpregnancy diagnosis, and optimal fertility in the second and subsequent services. The ReBreed21-ET resynchronization program integrates acceptable P/ET in first and subsequent TET with shorter intervals between ETs, thereby merging the main features of optimized reproductive programs. Thus, the ReBreed21-ET program can lead to highly efficient reproductive performance in embryo recipient operations.

Finally, practical implementation of complete TET programs can be facilitated by simple integration into a weekly calendar [4, 37]. Figure 6 models an example of a practical schedule for implementing ReBreed21-ET in a TET season. The animal handling is concentrated to 3d of the week, with all TET on Thursday and no requirement for weekend labor. Additionally, the potential second TET in ReBreed21-ET is on the same day that heifers have the first pregnancy diagnosis, optimizing the skilled technician workday and minimizing animal stress due to excessive handling [38]. Thus, using the ReBreed21-ET program can optimize workflow and

animal welfare while improving reproductive performance by increasing the number of pregnancies and reducing the time to pregnancy.

Conclusion

In summary, the ReBreed21-ET program, increased fertility in the first TET, increasing the pregnancies during a 105-d TET season, with an earlier time to pregnancy compared to Resynch28. Interestingly, there were different timings of pregnancy losses in the different programs, potentially due to different periods of P4 supplementation in ReBreed21-ET vs. Resynch28. Of practical importance, the ReBreed21-ET is a new reproductive program that can be incorporated into commercial embryo recipient operations, and this study provides evidence that it will enhance reproductive performance in ET operations. Future research should focus on reducing pregnancy losses after TET using P4 supplementation and on improving the synchronization of ovulation on D21 in ReBreed21-ET to optimize the utilization rate of recipients and improve efficiency of this TET program.

Acknowledgments

The authors thank the Volm's Farms for assistance with management and animal handling during field work (ST Genetics, Kewaskum, WI). Partial funding was provided by ST genetics, Hatch Project WIS01240 to MCW, and USDA-NIFA project 2019-67015-29704 to MCW.

Table 1. Pregnancy loss of Holsteins embryo recipients resynchronized with two different resynchronization programs.

Pregnancy loss period (days)	ReBreed21-ET	Resynch28	P-value
28 to 33	10.1% (23/227)	3.5% (7/202)	< 0.01
33 to 47	4.9% (10/204)	10.1% (20/198)	0.04
47 to 63	4.6% (9/194)	3.4% (6/178)	0.45
28 to 63	18.5% (42/227)	16.3% (33/202)	0.69

Table 2. Dynamics of ReBreed21 synchronization from D19 to D21 after the previous TET in nonpregnant embryo recipients on D28

Item	Resynch28	ReBreed21-ET	P-value
Ovulation from D19 to 21 (n/n)	39.7% (120/302)	24.3% (66/272)	<0.0001
D19 LF diameter mm (\pm SEM)	12.2 (0.2)	12.0 (0.2)	0.5
D21 LF diameter mm (\pm SEM)	11.4 (0.2)	12.5 (0.2)	<0.0001
D19 circulating P4 ng/mL (\pm SEM)	3.9 (0.2)	4.3 (0.2)	<0.01
D21 circulating P4 ng/mL (\pm SEM)	3.2 (0.2)	2.4 (0.2)	<0.0001
D19 Heifers with P4<1ng/mL (n/n)	35.4% (103/291)	1.9% (5/263)	<0.0001
D21 Heifers with P4<1ng/mL (n/n)	52.2% (152/291)	61.6% (162/263)	0.03

Figure Legends

Figure 1. Schematic timeline of the experimental groups with two resynchronization of ovulation programs for TET ReBreed21-ET and Resynch28. ET = embryo transfer, NP = nonpregnant, PGF = PGF2 α analog, G = GnRH analog, CL = nonpregnant heifer on D28 with CL \geq 18 mm diameter, w/o CL = nonpregnant heifer on D28 without CL or CL <18mm diameter. Other examinations: BS = Blood sampling for P4; US indicates ovarian ultrasound; PD = Pregnancy diagnosis by ultrasound

Figure 2. Pregnancy/ET for the first TET in the trial (Panel A) and pregnancy loss (Panel B) between pregnancy diagnosis by ultrasound from day 28 to 63.

Figure 3. Pregnancy/ET for the second and later TET in the trial (Panel A) and pregnancy loss (Panel B) between pregnancy diagnosis by ultrasound from D28 to D63 for second and later TET.

Figure 4. Reproductive performance at different times in a 105-d TET season using Resynch28 or ReBreed21-ET. **Panel (A)** cumulative pregnancies by each 21-d period. **Panel (B)** Survival percentage of nonpregnant heifers during the 105-d TET season and time to pregnancy for Resynch28 (day 53.3 [\pm 3]) and ReBreed21-ET (day 44.9 [\pm 3]) P<0.01.

Figure 5. Practical schedule for implementing a TET season using ReBreed21-ET and Resynch28, showing days relative to first Timed-ET (TET), pregnancy diagnosis (PD) by ultrasound to evaluate embryonic heartbeat and hormonal treatments (GnRH analogs [G],

intravaginal progesterone insert [P4 insert], PGF2 α analogs [PGF]). In ReBreed21-ET, after PD on D28, nonpregnant (NP) heifers with corpus luteum (CL) received a new TET on D28, and NP heifers without CL or CL<18mm, received a modified 5-d CoSynch initiated on D28 for a potential second TET 42d after first TET.

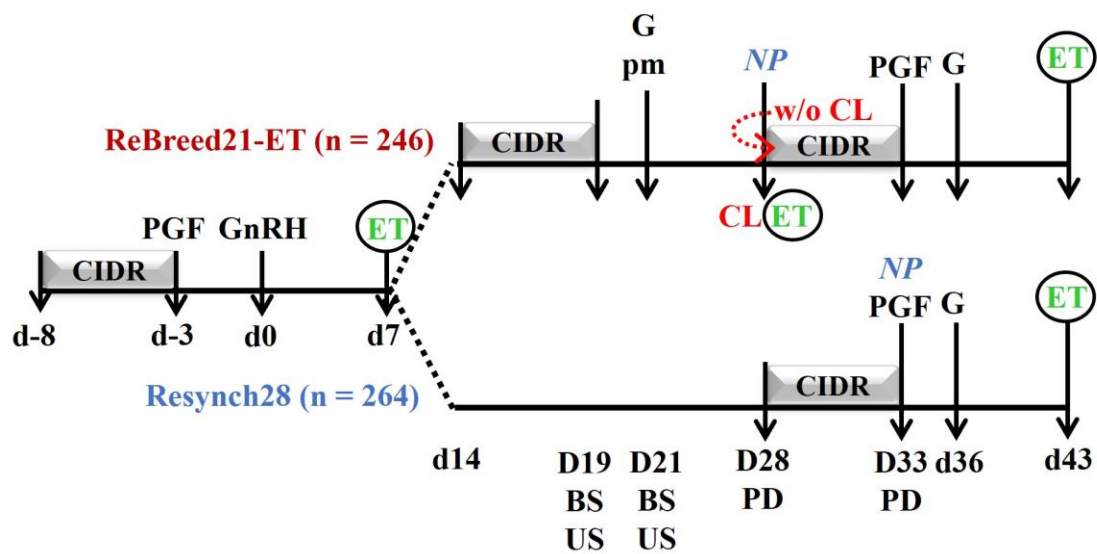


Figure 1

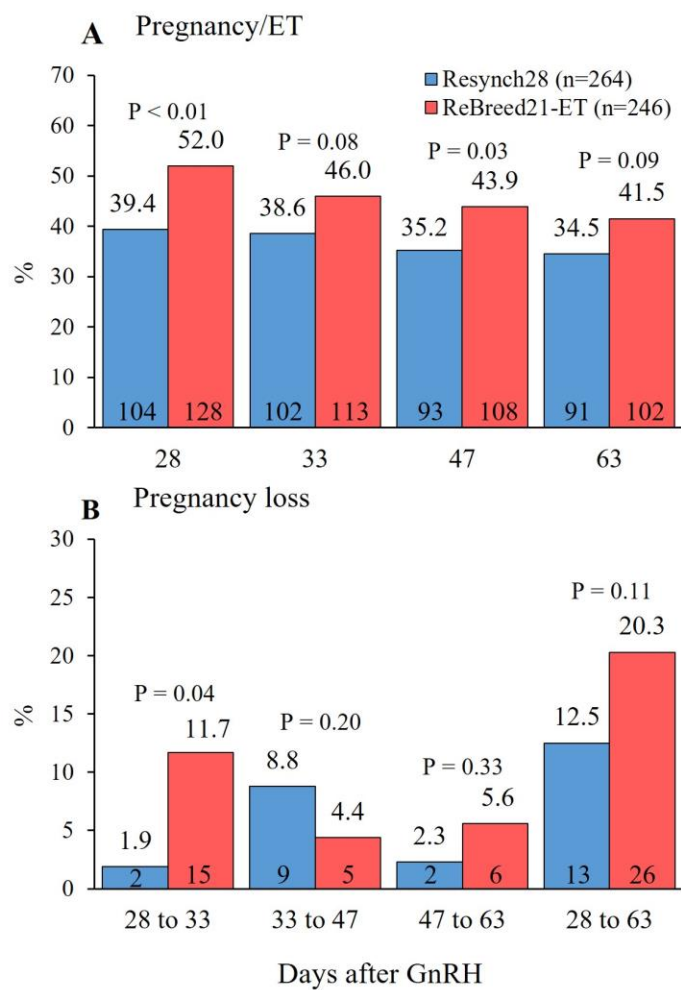


Figure 2

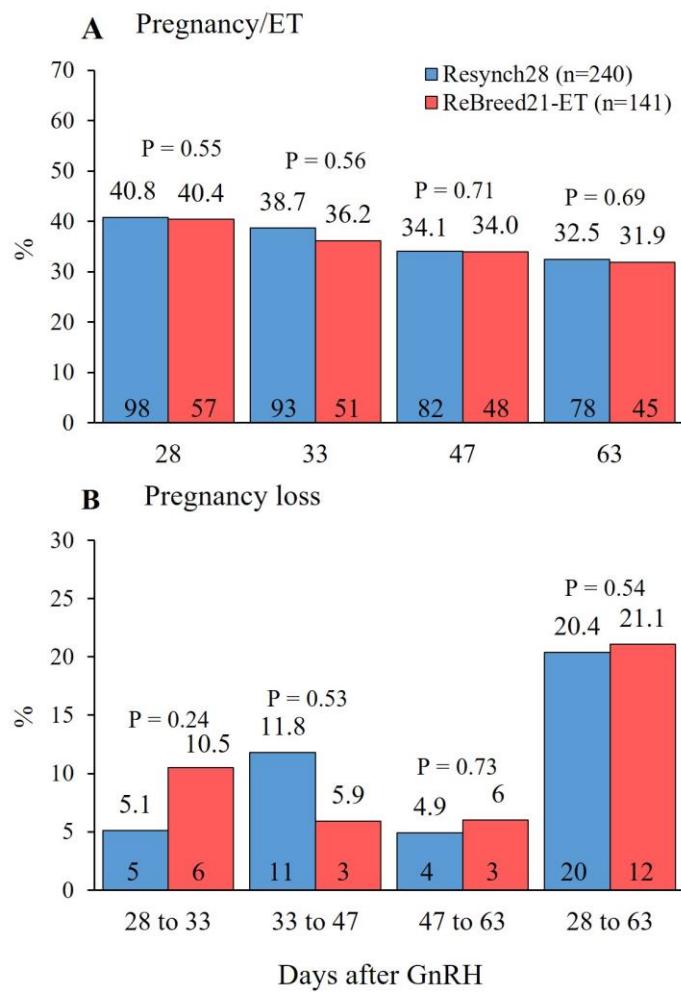


Figure 3

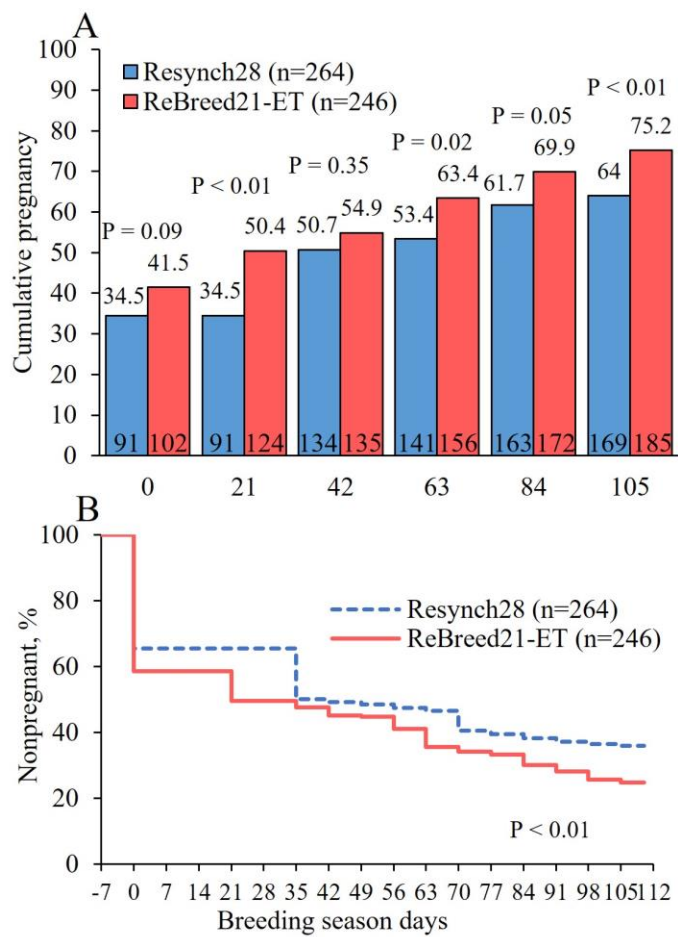


Figure 4

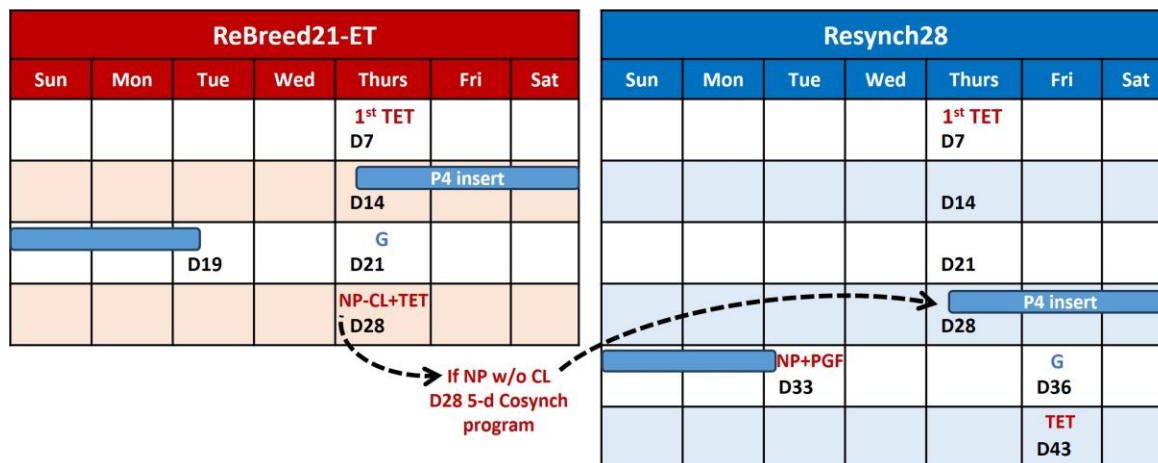


Figure 5

References

- [1] Viana J. 2022 Statistics of embryo production and transfer in domestic farm animals. *IETS Newsletter*. 2023;41:25.
- [2] Bo GA, Baruselli PS, Moreno D, Cutaia L, Caccia M, Tribulo R, et al. The control of follicular wave development for self-appointed embryo transfer programs in cattle. *Theriogenology*. 2002;57:53-72.
- [3] Lu KH, Cran DG, Seidel GE. In vitro fertilization with flow-cytometrically-sorted bovine sperm. *Theriogenology*. 1999;52:1393-405.
- [4] Baruselli PS, de Souza AH, de Sa MF, Marques MO, Sales JND. Genetic market in cattle (Bull, AI, FTAI, MOET and IVP): financial payback based on reproductive efficiency in beef and dairy herds in Brazil. *Anim Reprod*. 2018;15:247-55.
- [5] Nicholas FW, Smith C. Increased Rates of Genetic Change in Dairy-Cattle by Embryo Transfer and Splitting. *Anim Prod*. 1983;36:341-53.
- [6] Baruselli PS, Ferreira RM, Sales JN, Gimenes LU, Sa Filho MF, Martins CM, et al. Timed embryo transfer programs for management of donor and recipient cattle. *Theriogenology*. 2011;76:1583-93.
- [7] Nasser LF, Reis EL, Oliveira MA, Bo GA, Baruselli PS. Comparison of four synchronization protocols for fixed-time bovine embryo transfer in *Bos indicus* x *Bos taurus* recipients. *Theriogenology*. 2004;62:1577-84.
- [8] Baruselli PS, Ferreira RM, Sa Filho MF, Nasser LF, Rodrigues CA, Bo GA. Bovine embryo transfer recipient synchronisation and management in tropical environments. *Reprod Fertil Dev*. 2010;22:67-74.

[9] Sala RV, Melo LF, Motta JCL, Leffers-Neto L, Carrenho-Sala LC, Fosado M, et al. Optimization of a 5-day fixed-time embryo transfer (FTET) protocol in heifers I. Manipulation of circulating progesterone through reutilization of intravaginal progesterone devices during FTET. *Theriogenology*. 2020;156:171-80.

[10] Ferraz PA, Burnley C, Karanja J, Viera-Neto A, Santos JEP, Chebel RC, et al. Factors affecting the success of a large embryo transfer program in Holstein cattle in a commercial herd in the southeast region of the United States. *Theriogenology*. 2016;86:1834-41.

[11] Sa Filho M, Marques M, Giroto R, Santos F, Sala R, Barbuio J, et al. Resynchronization with unknown pregnancy status using progestin-based timed artificial insemination protocol in beef cattle. *Theriogenology*. 2014;81:284-90.

[12] Lee JI, Kim IH. Pregnancy loss in dairy cows: the contributing factors, the effects on reproductive performance and the economic impact. *J Vet Sci*. 2007;8:283-88.

[13] De Vries A. Economic value of pregnancy in dairy cattle. *J Dairy Sci*. 2006;89:3876-85.

[14] Andrade JPN, Gomez-Leon VE, Andrade FS, Carvalho BP, Lacouth KL, Garcia FZ, et al. Development of a novel 21-day reinsemination program, ReBreed21, in *Bos indicus* heifers. *Theriogenology*. 2020;155:125-31.

[15] Andrade JPN, Monteiro PJJ, Prata AB, Robl AJ, Neto J, Lippe B, et al. Optimizing ReBreed21 II: Fertility and reproductive efficiency in different parities during a shortened breeding season in beef cattle. *Theriogenology*. 2024;224:41-49.

[16] Andrade JPN, Domingues RR, Carvalho BP, Gomez-Leon V, Prata AB, Sartori R, et al. Optimizing ReBreed21 I: Evaluation of endocrine and ovarian dynamics in non-bred *Bos indicus* heifers. *Theriogenology*. 2024;220:77-83.

[17] Clemente M, de la Fuente J, Fair T, Al Naib A, Gutierrez-Adan A, Roche JF, et al. Progesterone and conceptus elongation in cattle: a direct effect on the embryo or an indirect effect via the endometrium? *Reproduction*. 2009;138:507-17.

[18] Nascimento AB, Bender RW, Souza AH, Ayres H, Araujo RR, Guenther JN, et al. Effect of treatment with human chorionic gonadotropin on day 5 after timed artificial insemination on fertility of lactating dairy cows. *J Dairy Sci* 2013;96:2873-82.

[19] Niles AM, Fricke HP, Carvalho PD, Wiltbank MC, Hernandez LL, Fricke PM. Effect of treatment with human chorionic gonadotropin 7 days after artificial insemination or at the time of embryo transfer on reproductive outcomes in nulliparous Holstein heifers. *J Dairy Sci*. 2019;102:2593-606.

[20] Wiltbank MC, Baez GM, Garcia-Guerra A, Toledo MZ, Monteiro PL, Melo LF, et al. Pivotal periods for pregnancy loss during the first trimester of gestation in lactating dairy cows. *Theriogenology*. 2016;86:239-53.

[21] Stringfellow DA, Seidel SM. *Manual of the International Embryo Transfer Society (IETS)*. 4th ed. Champaign, IL1998.

[22] Bo GA, Peres LC, Cutaia LE, Pincinato D, Baruselli PS, Mapletoft RJ. Treatments for the synchronisation of bovine recipients for fixed-time embryo transfer and improvement of pregnancy rates. *Reprod Fertil Dev*. 2012;24:272-77.

[23] Pereira MH, Sanches CP, Guida TG, Rodrigues AD, Aragon FL, Veras MB, et al. Timing of prostaglandin F₂alpha treatment in an estrogen-based protocol for timed artificial insemination or timed embryo transfer in lactating dairy cows. *J Dairy Sci*. 2013;96:2837-46.

[24] Giordano JO, Wiltbank MC, Guenther JN, Pawlisch R, Bas S, Cunha AP, et al. Increased fertility in lactating dairy cows resynchronized with Double-Ovsynch compared with Ovsynch initiated 32 d after timed artificial insemination. *J Dairy Sci* 2012;95:639-53.

[25] Youngquist RS, Bierschwal CJ, Elmore RG, Jenkins AL, Schultz RH, Widmer JH. Induction of abortion in feedlot Heifers with Cloprostenol (ICI 80,996). *Theriogenology*. 1977;7:305-12.

[26] Lonergan P, Sánchez JM. Progesterone effects on early embryo development in cattle. *J Dairy Sci* 2020;103:8698-707.

[27] Rabaglino MB, Sanchez JM, Mc Donald M, Crowe MA, O'Callaghan E, Lonergan P. Transfer of bovine embryos into a uterus primed with high progesterone concentrations positively impacts fetal development at 42 days of gestation. *Theriogenology*. 2023;200:25-32.

[28] Alkan H, Tekindal MA, Demirel MA, Soyuturk BI, Golbasi M, Deniz YE, et al. Effect of strategies to increase progesterone levels on fertility of bovine embryo transfer recipients - A meta-analysis. *Theriogenology*. 2024;215:177-86.

[29] Monteiro PL, Jr., Nascimento AB, Pontes GC, Fernandes GO, Melo LF, Wiltbank MC, et al. Progesterone supplementation after ovulation: Effects on corpus luteum function and on fertility of dairy cows subjected to AI or ET. *Theriogenology*. 2015;84:1215-24.

[30] Reese ST, Franco GA, Poole RK, Hood R, Montero LF, Oliveira RV, et al. Pregnancy loss in beef cattle: A meta-analysis. *Anim Reprod Sci*. 2020;212.

[31] Stangaferro ML, Toledo MZ, Gennari RS, Perez MM, Gamarra CA, Sitko EM, et al. Effects of feeding rumen-protected methionine pre- and postpartum on reproductive outcomes of multiparous Holstein cows. *J Dairy Sci*. 2021;104:11210-25.

[32] Domingues RR, Andrade JPN, Cunha TO, Madureira G, Hoppman AS, Teixeira NN, et al. Profiles of interferon-stimulated genes in multiple tissues and circulating pregnancy-associated glycoproteins and their association with pregnancy loss in dairy cows. *Biol Reprod.* 2023;211:558-68.

[33] Domingues RR, Andrade JPN, Cunha TO, Madureira G, Moallem U, Gomez-Leon V, et al. Is pregnancy loss initiated by embryonic death or luteal regression? Profiles of pregnancy-associated glycoproteins during elevated progesterone and pregnancy loss. *JDS Communications.* 2022;4:149-54.

[34] Lauber MR, Cabrera EM, Santos VG, Carvalho PD, Maia C, Carneiro B, et al. Comparison of reproductive management programs for submission of Holstein heifers for first insemination with conventional or sexed semen based on expression of estrus, pregnancy outcomes, and cost per pregnancy. *J Dairy Sci* 2021;104:12953-67.

[35] Giordano JO, Fricke PM, Cabrera VE. Economics of resynchronization strategies including chemical tests to identify nonpregnant cows. *J Dairy Sci* 2013;96:949-61.

[36] Carvalho PD, Santos VG, Giordano JO, Wiltbank MC, Fricke PM. Development of fertility programs to achieve high 21-day pregnancy rates in high-producing dairy cows. *Theriogenology.* 2018;114:165-72.

[37] Bo GA, de la Mata JJ, Baruselli PS, Menchaca A. Alternative programs for synchronizing and resynchronizing ovulation in beef cattle. *Theriogenology.* 2016;86:388-96.

[38] Gebresenbet G, Wikner I, Bobobee EYH, Maria G, Villarroel M. Effect of transport time and handling on physiological responses of cattle. *J Agr Sci Tech.* 2012;2:800.

Chapter 7: Delayed embryonic attachment and profile of pregnancy-associated glycoproteins are associated with pregnancy loss at different stages of gestation in Holstein embryo recipients

João Paulo N. Andrade ^a, Rafael R. Domingues ^{a,b}, Victor E. Gomez-León ^{a,c}, Guilherme Madureira ^a, Luma C. Sartori^a, Gustavo F. Grillo ^{a,d}, Meliton Fosado ^e, Rodrigo V. Sala ^e, Milo C. Wiltbank ^a

^a Department of Animal and Dairy Sciences, University of Wisconsin-Madison, 1675 Observatory Drive, Madison, WI 53706, USA

^b Department of Animal Sciences, The Ohio State University, 2027 Fyffe Road, Columbus, OH 43210, USA

^c Department of Animal Sciences and Industry, Kansas State University, 1530 Mid-Campus Drive North, Manhattan, KS 66506, USA

^d Departamento de Reprodução e Avaliação Animal, Universidade Federal Rural do Rio de Janeiro, Seropédica, Rio de Janeiro 23897-000, Brasil

^e ST Genetics-Volm Farm ST Heifer Center, Kewaskum, WI, USA, 53040

Corresponding Author: Milo C. Wiltbank

E-mail: wiltbank@wisc.edu

To be submitted to Theriogenology

Abstract

The aim was to investigate the occurrence and mechanisms of pregnancy loss (PL) in Holstein embryo recipient cows based on the profile of pregnancy-associated glycoproteins (PAGs) and progesterone (P4) and the impact of P4 supplementation on the timing of PL. In experiment 1, 1003 fresh IVF embryos were transferred into heifers and the overall P/ET based on transrectal ultrasonography was 42.3% on 28d (424/1003), 39.1% on 33d (392/1003), 37.0% on 47d (371/1003), and 35.5% on 63d (356/1003). Thus, PL was 7.5% (n=32) between 28-33d, 5.4% (n=21) from 33-47d, and 4.0% (n=15) from 47-63d. Using an observational retrospective cohort experimental design, each PL heifer were matched to one non-pregnant (NP) and one pregnant (Preg) heifer. Heifers were designated as having embryonic attachment (Eatt) on 21d based on circulating PAGs (above 0.06 ng/mL). The percentage of Eatt on 21d were similar for Preg (80%), PL 33-47d (76.2%), and PL 47-63d (80%). The PL 28-33d had fewer Eatt on 21d (50%) compared to Preg and by contrast with PL 33-47d with 47-63d (77.8%). The PL 28-33d and 33-47d had intermediary circulating PAGs compared to NP and Preg on 21d. Nevertheless, on 28d, only PL 28-33d had lower circulating PAGs compared with Preg (10.1 vs. 15.3), while a tendency between PL and Preg in the second period (12.4 vs. 15.9). In experiment 2 Holstein heifers received an ET (n = 540) and were randomized to a P4 supplementation (PlusP4 n = 246) from 14-19d or remain untreated (Control n = 264). For each heifer, a 50% increase in circulating PAGs between 19-21d was indicative for Eatt. The Eatt on 21d and P/ET on 28d were greater for PlusP4 (64% and 52%) compared to Control (55% and 39%). When considering only heifers that have P4 \geq 1ng/mL on 21d, the PlusP4 group had greater P/ET (67% vs. 49%), greater Eatt (72.3% vs. 55.3%) and decreased PL (16% vs. 24%) compared to the Control group. For heifers with P4 < 1ng/mL, Eatt was greater in PlusP4 (28%) compared to Control (26%).

Pregnancy outcomes evaluated according to tercile of PAGs on 21d and within each groups indicated decreased PL from 21 to 28d in the PlusP4 group (65%) compared to Control (100%) and increased P/ET on 28d for PlusP4 (12%) compared to the Control (2%) for heifers in Low PAGs tercile. In the medium tercile, Eatt and P/ET was greater in the PlusP4 group (78% and 59%) compared to the Control (57% and 39%). In conclusion, heifers with PL on d28-33 and d33-47 had signs of underdevelopment placentation several days before the PL (d21 and d28). Alternatively, P4 supplementation reduced PL in heifers with suboptimal placentation.

Keywords: embryo transfer, bovine, embryonic loss, progesterone.

Introduction

Bovine embryo transfer has been a consistently growing market worldwide, and pregnancy loss after ET is still the primary barrier to achieve the full potential for this technique [1-4]. Many improvements in laboratory techniques and embryo recipient management have been made that can now be matched to our current knowledge of bovine pregnancy establishment in order to reduce pregnancy losses [2, 5]. The main events for pregnancy establishment include the maintenance of the corpus luteum, extensive morphological and physiological changes in the conceptus, and the development of a functional placenta [6]. Specifically, an embryo reaches the uterus in the morula stage, undergoes multiple cell divisions to develop into a blastocyst, sheds off the zona pellucida, and then finally elongates, producing large amounts of interferon tau, the first signal for maternal recognition of pregnancy and prevention of luteolysis. Subsequently, the elongated embryo attaches to the uterine endometrium usually around day 20-23 and this marks the initiation of placental development [7]. This process is histologically characterized by the formation of multinuclear cells that secrete pregnancy-associated glycoproteins (PAG) [8, 9]. After embryonic attachment, the placenta continues to develop, forming a fully developed chorioallantoic placenta, reaching high circulating PAG concentrations by 30d of gestation, when the embryo is fully dependent on the placenta for nutrition, oxygen, and waste exchange [10-12]. Interestingly, by 17-21d of gestation, 70-80% of cows have a live embryo secreting interferon tau; however, by 35d, around 40% of cows have undergone pregnancy loss [13-15]. Thus, the period of embryo elongation and interferon-tau secretion, transitioning to embryo attachment and PAG secretion, and finally establishment of embryonic circulatory system and chorioallantoic placenta are key periods to target in research to reduce pregnancy losses. This project focuses on exploring the profile of

PAG to gain insight into the biology of pregnancy establishment, placental development, corpus luteum maintenance, and to develop strategies to mitigate pregnancy loss in embryo recipients.

Maternal circulating concentrations of PAG have been used as a marker of pregnancy and as a method of pregnancy diagnosis [16-19]. Early studies of PAG assays showed intriguing differences in profiles among different stages of pregnancy, fetal sex, and embryo recipient breeds and parities [20]. In addition, the circulating concentrations of PAG on specific days of gestation have been associated with pregnancy losses either in beef and dairy cattle likely due to delayed or suboptimal placental development and/or embryonic death [21-23]. As commercial implementation and sensitivity of assays for PAG have increased during the last few decades, a creative approach targeting the timing of the first PAG increase within an animal from days 17 to 24 after AI were able to attain 100% sensitivity in early pregnancy diagnosis [24]. The timing of the first significant PAG increase in an animal has also been used to evaluate timing of presumed embryo attachment and this has been strongly associated with pregnancy losses in lactating dairy cows before 30d after AI. Interestingly, using this method it was reported that lactating dairy cows have a later time to presumed embryo attachment compared with heifers, and the authors speculated this was due lower circulating progesterone (P4) due to high steroid metabolism in lactating cows [25]. The same research group also found a greater risk of pregnancy loss in cows that had an increase in PAG after day 21 of gestation [26, 27]. Daily PAG evaluation has been particularly useful and allowed our research group to find that about half of pregnancy losses are initiated by inappropriate CL regression without embryonic death and about half are initiated by death of the embryo without associated CL regression in lactating dairy cows [28]. Thus, progress has been made in understanding the mechanisms of pregnancy losses in lactating dairy cows using the early increase in PAG as an indicator of presumed embryo attachment, however

no studies have reported this concept in embryo recipients. Exploring the early PAG increase in embryo recipients will help distinguish losses due to improper or delayed placentation and to validly develop strategies to mitigate the substantial losses that have been reported in embryo recipients from ET to 30d of gestation [3]. To evaluate this idea in a large number of recipients key timepoints have been chosen to evaluate PAG concentrations, based on data obtained from daily sampling done in lactating cows and heifers.

Therefore, to gain further insight into pregnancy establishment and pregnancy loss in bovine embryo recipients, the present research focused on the relationships among maternal circulating concentrations of PAG, pregnancy status, placental function, and the occurrence of pregnancy loss at key gestational times (from embryo attachment to development of the chorioallantoic placenta). In addition, we explored the effect of P4 supplementation during embryo elongation on the concentrations of PAG on days 21 and 28 of gestation. We used data from two experiments in Holstein embryo recipient heifers following 1,003 timed embryo transfers (TET) to explore pregnancy loss in an observational retrospective cohort experimental design and in 510 TET to evaluate the effect of targeted P4 supplementation on pregnancy outcomes. We hypothesized that (1) pregnancy loss at different gestational periods (from day 28 to 33, 33 to 47, and 47 to 63) is associated with different circulating PAG concentrations before pregnancy loss, and (2) that P4 supplementation during embryo elongation will increase the percentage of heifers with embryonic attachment by 21d and subsequent pregnancy per ET (P/ET).

Materials and Methods

The two experiments were performed in a commercial operation for bovine ET recipients from ST Genetics in Fond du Lac WI. The overall trial was composed of 550 dairy heifers with an average BCS of 3.05 (\pm 0.01) on a scale of 1 to 5. Heifers received 0 to 6 TET before enrollment in the study. The heifers were housed in a free-stall barn and fed a TMR once daily formulated to meet or exceed the nutritional requirements of Holstein heifers weighing 360 Kg and gaining 0.8 kg/d [29], with ad libitum access to feed and water. Heifers were managed under the Guide for the Care and Use of Agricultural Animals under an approved protocol by the Animal Care and Use Committee of the College of Agriculture and Life Sciences, University of Wisconsin.

In experiment 1, data included 1,003 embryo transfers using a synchronization of ovulation programs developed for TET using a modified 5-d CoSynch protocol that has been previously validated [30]. Embryos used in this experiment were IVF fresh (n=944) and frozen (n=59); quality: 1 (n=902) and 2 (n=101); and developmental stage: 6 (n=159), 7 (n=785), and 8 (n=59).

Blood samples were collected on days 19, 21, 28, 33, 47, and 63 into evacuated tubes (Vacutainer, Dickinson, Franklin Lakes, NJ) by puncture of the coccygeal vein. Immediately after collection, tubes were placed on ice and kept refrigerated until centrifuged at 2500 x g for 20 min. Serum was recovered and stored frozen at -20°C . Circulating P4 was determined using a solid-phase RIA kit containing antibody-coated tubes and I^{125} -labeled P4 (ImmuChem Coated Tube P4 125I RIA Kit, MP Biomedicals, CA, USA) following the commercial recommendation for a short incubation time of 120 min in a water bath (set at 37°C) followed by aspiration of tubes. Intra- and inter-assay CVs were 2.4 and 2.1 % and sensitivity was 0.02 ng/mL. Circulating

PAG was determined by ELISA (BioPRYN; BioTracking LLC, Moscow, ID). The sensitivity was 0.02 ng/mL, and intraassay CV and interassay CV were ng/mL, 3.0%, and 4.2%, respectively.

Pregnancy diagnosis was performed on days 28, 33, 47, and 63 by transrectal ultrasonography based on visualization of embryonic heartbeat. We used an observational retrospective cohort experimental design to compare the profiles of PAG among nonpregnant, pregnant, and heifers with pregnancy loss for each period of pregnancy loss (first, second, or third). For each heifer with pregnancy loss, we selected a nonpregnant and a pregnant matched-control heifer as described. For the nonpregnant group, a heifer must have been diagnosed nonpregnant on 28d and had circulating P4 ≤ 1 ng/mL at 19d. For the pregnant group, a heifer must have been diagnosed as pregnant at all pregnancy diagnoses (days 28, 33, 47, and 63). In addition, for both pregnant and nonpregnant groups, each heifer must match with a heifer in the pregnancy loss group for TET date, embryo quality, embryo development, embryo source, and number of previous TET. Therefore, each heifer with a pregnancy loss event had a positive (pregnant) and negative (nonpregnant) counterpart.

The identification of presumed embryonic attachment by 21d for pregnant heifers and heifers with pregnancy loss was based on concentrations of PAG. Circulating concentrations of PAG on 21d in nonpregnant heifers (based on transrectal ultrasonography performed on 28d) were used as a baseline. In the nonpregnant group, the highest concentration of PAG on 21d was 0.06 ng/mL. Therefore, any cow in the pregnancy loss groups with a PAG concentration above 0.06 ng/mL on 21d was considered to have embryonic attachment and, therefore, considered pregnant.

In experiment 2, data included 510 embryo transfers using synchronization of ovulation programs for TET–modified 5-d CoSynch, as described [30]. Embryos used in this experiment were IVF fresh (n=445) and frozen (n=65); quality: 1 (n=454) and 2 (n=56); and developmental stage: 6 (n=75), 7 (n=406), and 8 (n=29). This experiment was a completely randomized design to either receive P4 supplementation (CIDR, Eazi-Breed cattle insert, 1.38 g of P4, Zoetis, Florham Park, NJ) from 14d to 19d of gestation (n = 246) or remain untreated (control; n = 264) until 28d. We explored the impact of P4 supplementation between 14d and 19d on concentrations of PAG and pregnancy outcomes on 28d.

Blood samples were collected on days 19, 21, and 28 and processed as described in Experiment 1. For each heifer, a 50% increase in circulating PAG between 19d and 21d indicated embryonic attachment. To evaluate the relationship among pregnancy outcomes, circulating PAG, and P4 supplementation, each group was divided into terciles according to circulating PAG (Low, Medium, and High [PlusP4-Low tercile n =82, Control-Low tercile n =88, PlusP4-Medium tercile n =82, Control-Medium tercile n =88, PlusP4-High tercile n =82, and Control-High tercile n =88]). Pregnancy diagnosis was performed on 28d of gestation by transrectal ultrasonography based on the identification of embryonic heartbeat. Pregnancy loss was considered when the heifer had embryonic attachment at 21d but was not pregnant on 28d.

Statistical analysis

In Experiment 1, the pregnancy status (Pregnant, Pregnancy Loss, and Nonpregnant) was included as fixed effects as well as the parameters used to select counterpart heifers (TET date, embryo quality, embryo development, embryo source, and number of previous TET). In Experiment 2, the effect of P4 supplementation, embryo quality, embryo development, embryo source was included as a fixed effect. Binary data (P/ET, percentage of embryo attachment on

21d, and pregnancy loss) was analyzed using the GLIMMIX procedure of SAS (Version 9.4; SAS Institute). Differences in circulating PAG and P4 among nonpregnant, pregnancy loss, and pregnant groups were analyzed using the PROC MIXED procedure for linear models.

Assumptions (linearity, independence, and normality) were assessed by plotting the residuals, quantile-quantile plot, and the Shapiro-Wilk test. If case deviations from assumptions were found, data was transformed guided by BoxCox transformation to select the best transformation: natural logarithms, square root, or ranks. The interaction between treatment and embryo features (quality, source, and stage) was offered as an explanatory variable and kept in the model if significant. Significant differences between treatment groups were considered for $P \leq 0.05$, whereas differences between $P > 0.05$ and $P \leq 0.10$ were considered as a tendency.

Results

In experiment 1, the P/ET was 42.3% (424/1003) on 28d, 39.1% (392/1003) on 33d, 36.9% (369/1003) on 47d, and 35.3% (354/1003) on 63d. Pregnancy losses between pregnancy diagnosis were 7.5% (32/424) from 28d to 33d (first period), 5.9% (23/392) from 33d to 47d (second period), and 4.1% (15/369) from 47d to 63d (third period). Circulating concentrations of PAG on 21d and 28d, including individual PAG profiles, are shown at different stages of gestation (Figure 2, Table 1). Concentrations of PAG on 21d and 28d were lower in heifers with pregnancy loss between 21d and 28d compared to pregnant heifers, and both were greater than nonpregnant heifers. For the period of pregnancy loss between 33d and 47d, heifers with pregnancy loss had higher circulating PAG than non-pregnant but lower compared with pregnant on 21d. However, on 28d, there was only a tendency ($P = 0.08$) for lower concentrations than pregnant heifers. Lastly, concentrations of PAG on 21d and 28d were not different between

heifers with pregnancy loss on days 47-63 and pregnant heifers. Both groups had greater concentrations of PAG compared with nonpregnant heifers.

The percentage of heifers with embryonic attachment on 21d, according to the period of pregnancy loss, is shown in Figure 3. The incidence of embryonic attachment on 21d was 37.5% less for heifers with pregnancy loss in the first period (28d to 33d; 50%) than for pregnant heifers (80%). Compared to heifers with pregnancy loss in the second and third periods (77.8%, 28/36), the frequency of embryonic attachment for heifers with pregnancy loss in the first period was 35.7% lower ($P = 0.02$). Heifers with pregnancy loss in the second and third periods had a similar percentage of embryonic attachment on 21d compared with pregnant heifers.

In experiment 2, pregnancy outcomes for the control and PlusP4 groups are shown (Figure 4). The percentage of embryonic attachment on 21 and P/ET on 28d was greater ($P < 0.05$) in the PlusP4 (64.2% and 52.0%) compared to the control (54.9% and 39.4%). The incidence of pregnancy loss between 21d and 28d was not different between groups. On 21d, the percentage of embryonic attachment differed ($P < 0.01$) according to PAG terciles (Table 2). Embryonic attachment was greatest for the high tercile (86.5%, 147/170), intermediate for the medium tercile (67.1%, 114/170), and lowest for the low tercile (24.7%, 42/170). Furthermore, P/ET also differed ($P < 0.01$) according to PAG terciles: greater for the high tercile (81.2%, 138/170), intermediate for the medium tercile (48.2%, 82/170), and lowest for the low tercile (7.1%, 12/170). Pregnancy loss between 21d and 28d also differed ($P < 0.01$) according to PAG tercile: it was lowest in the high tercile (10.2%, 15/147), intermediate in the medium tercile (36%, 41/114), and greatest in the low tercile (78.6%, 33/42). The P/ET on d28 was greater in the PlusP4 for the low (+430%) and medium (+52%) terciles compared with the Control, while

the pregnancy loss between 21d and 28d was reduced with P4 supplementation only in the low tercile.

There were 26.9 % (39/145) of heifers that had an embryonic attachment on d21 despite concentrations of P4 below 1 ng/mL, indicating luteolysis (Table 3). All heifers with P4 below 1 ng/mL underwent pregnancy loss by 28d. Progesterone supplementation increased pregnancy per ET on 21d and 28d in heifers with P4 above 1 ng/mL on 21d and tended to decrease pregnancy loss between 21d and 28d compared with Control.

Discussion

As embryonic attachment and placental development occur, the number of giant multinucleated cells increases along with the overall placental PAG secretory capacity, which has been associated with pregnancy outcomes [31, 32]. Thus, exploring the profile of PAG can provide insights into pregnancy establishment, placental development, and embryonic-maternal interactions [33]. The main novelties in our study are that (1) pregnancy loss is associated with the abnormal profile of PAG prior to the occurrence of pregnancy loss only in pregnancy losses before 43d and (2) P4 supplementation increases the percentage of animals with embryonic attachment by 21d and mitigates pregnancy loss. Our hypotheses that pregnancy loss at different periods is associated with different profiles of PAG and that P4 supplementation increases the percentage of heifers with embryonic attachment by 21d were accepted. Moreover, P4 supplementation from days 14 to 19 increased pregnancy rates on 21d and 28d with some impact on pregnancy loss in heifers that maintained corpus luteum function beyond 21d. Overall, our study reveals novel aspects related to the timing of embryonic attachment to the endometrium

and the occurrence of pregnancy loss, and these findings have potential to be useful for the future development of strategies to increase P/ET and mitigate pregnancy loss in embryo recipients.

Embryonic attachment is a vital process for pregnancy establishment; however, its time is decisive for future pregnancy maintenance [34]. In a recent report, the day of embryonic attachment was a day later in cows with pregnancy loss compared to cows that maintained their pregnancy [28]. In the present study, not only was the incidence of attachment by 21d lower, but also low concentrations of circulating PAG on 21d were associated with pregnancy loss between 28d and 33d, indicating improper embryonic attachment. Taken together, both findings corroborate the concept that later embryonic attachment is associated with pregnancy loss in cattle. Nevertheless, only pregnancy losses before 47d (days 28 to 33 and days 33 to 47) were associated with lower concentrations of PAGs on 21d and 28d, suggesting suboptimal placental function. Interestingly, it has been the period with the greatest incidence of pregnancy loss in multiple studies in heifers, lactating dairy cows, and beef cows [13, 14, 35].

As the secretion of PAG rapidly increases from the moment of embryonic attachment and is dynamically secreted through gestation, the circulating concentrations of PAG have been used as a marker for placental function [8, 21, 32, 36, 37]. Indeed, low concentrations of PAGs have been associated with pregnancy loss in the present study and others [22], likely related to suboptimal placental development and function. Our study presents a novel concept for the association among concentrations of PAG, placental function, and the period of pregnancy loss. As the pregnancy advances beyond the establishment of the allantochorion placenta, pregnancy loss appears not to be associated with circulating PAG prior to the pregnancy loss event. Heifers with pregnancy loss between 33d and 47d had characteristics that partially resemble pregnancy loss before 33d as well as some aspects similar to pregnancy loss after 47d. Similar to heifers

with pregnancy loss between 28d and 33d, heifers with pregnancy loss between 33d and 47d had lower concentrations of PAG on 21d and 28d, indicating suboptimal placentation prior to pregnancy loss. However, similarly to heifers with pregnancy loss between 47d to 63d, the frequency of embryonic attachment was similar to the pregnant heifers, and neither circulating PAG were different comparing heifers that had or did not have pregnancy loss. Overall, these results suggest that embryonic attachment or early placental development is not the only cause of pregnancy loss in this period. Interestingly, circulating PAG in lactating dairy cows on day 35 revealed that the risk for fetal loss from days 35 to 63 was 10 and 6.8 times greater in cows with low and high PAG compared with medium PAG, respectively [38]. A negative association between high circulating PAG and pregnancy is not expected since several studies point towards a consistent positive relationship with fertility [22, 23, 31, 39]. However, cloned embryos have high circulating PAG and a high incidence of pregnancy loss [40, 41]. It appears that pregnancy loss after day 47 is unrelated or less related to issues with embryonic attachment and early placentation, at least based on circulating PAG.

An important yet poorly understood aspect of pregnancy establishment and maintenance in cattle is the mechanism for CL maintenance beyond the classical period of maternal recognition of pregnancy (16d-25d) [42, 43]. Between 16d and 25d of gestation, the elongating embryo secretes large amounts of interferon tau that prevents $\text{PGF2}\alpha$ release from the endometrium [44]. However, after attachment to the endometrium, embryonic secretion of interferon tau decreases dramatically [45] to low/undetectable concentrations by day 25. The mechanisms that maintain the CL after cessation of interferon tau secretion remain to be elucidated. It has been proposed that increased uterine vein blood flow or decreased prostaglandin transporter on the uterovarian plexus decrease the transport of $\text{PGF2}\alpha$ to the CL

[43]. In addition, the action of a luteotropic/protector such as prostaglandin E2 or PAG has been proposed [46, 47]. Interestingly, as explored in the present study, the increase in maternal circulating concentrations of PAG coincides with the transition in the mechanism of luteal maintenance. We recently reported that about 50% of pregnancy loss in lactating dairy cows between days 22 and 33 of gestation is due to inappropriate luteal regression. In contrast, the other 50% is lost due to conceptus failure without luteal regression [48]. In experiment 2, 42.9% of heifers with pregnancy loss between day 21 and 28 had embryonic attachment on d21 despite concentrations of P4 below 1 ng/mL on day 21, suggesting that luteal regression may be the main cause of pregnancy loss from day 21 to 28. A limitation of our study is that only two samples (days 19 and 21) were available to assess embryonic attachment and luteal regression; therefore, we likely underestimated the potential overall pregnancy loss up to day 28, considering that few heifers are expected to have embryo attachment after 21d [25]. Similarly, we were not able to identify inappropriate luteal regression between 21d and 28d as the cause of pregnancy loss. Nonetheless, it appears that delayed embryonic attachment to the uterine endometrium and lower concentrations of PAG are associated with diminished conceptus survival [48]. However, we have been unable to identify a direct relationship between delayed embryonic attachment to the endometrium, lower concentrations of PAG, and luteal maintenance/regression. Future studies with more frequent samples should be performed to more comprehensively assess the role of embryonic vs maternal causes for pregnancy loss associated with P4.

It has been suggested that the later embryonic attachment in lactating dairy cows compared to heifers is likely due to decreased circulating P4 in lactating cows, given their greater liver catabolism of P4, likely delaying embryonic development and attachment [34]. Only heifers

were used in the present study. Yet, P4 supplementation increased pregnancy per ET and decreased pregnancy loss but only in the medium tercile for concentrations of PAG. In addition, the present finding indicates that while pregnancy outcomes from heifers in different PAG terciles were markedly different, P4 supplementation specifically improved P/ET in heifers in the low and medium tercile groups for circulating PAG, that is, in heifers with suboptimal embryonic attachment/placentation. The P4 support during embryo elongation mitigates pregnancy loss in heifers with lower PAG while increasing the percentage of heifers with embryonic attachment by day 21. More specifically, it was suggested that P4 supplementation through the formation of an accessory CL mitigates pregnancy loss only in expanded blastocyst [49]. Nevertheless, it has been shown that P4 can increase embryo size during the elongation stage, and more recently, it has been demonstrated that heifers with uterine primed with P4 positively impacts fetal development at 42 days of gestation [50-52]. Taken together, the present study provides new insight into the mechanisms of pregnancy loss, suggesting that inadequate early embryonic attachment/placentation is a significant contributor to pregnancy loss that occurs from 28d to 47d. Additionally, we reported improvement in pregnancy outcomes for heifers with suboptimal placentation supplemented with P4. Future research endeavors should focus on understanding embryonic and maternal determinants for appropriate embryonic attachment and placentation in embryo recipients.

Conclusion

In conclusion, heifers with pregnancy loss between 28d to 33d had a lower incidence of attachment on 21d and lower circulating PAG on 21d and 28d, whereas heifers with pregnancy loss between 33d to 47d had lower PAG on days 21d and 28d despite the incidence of

attachment on 21d similar to pregnant heifers. On the contrary, pregnancy loss between days 47 to 63 was associated with a similar incidence of attachment on day 21 and had similar circulating PAG on days 21, 28, and 33 compared to pregnant heifers. Lastly, P4 supplementation increased pregnancy outcomes in ET recipients with suboptimal placentation. Taken together, lower circulating PAG in early gestation is associated with greater pregnancy loss in embryo recipients from TET (7d) to 33d, and P4 supplementation is suggested to mitigate those losses. Future studies should be performed to replicate these findings.

Acknowledgments

The authors thank the Volm's Farms for assistance with management and animal handling during field work (ST Genetics, Kewaskum, WI). Partial funding was provided by ST genetics, Hatch Project WIS01240 to MCW, and USDA-NIFA project 2019-67015-29704 to MCW.

Table 1. Circulating PAG in Holstein embryo recipient heifers with pregnancy losses in different periods during the first two months of gestation.

Day	Non-Pregnant	Pregnancy loss	Pregnant	P
<i>First period (days 28 to 33; n = 32)</i>				
21	0.02 (0.01) c	0.26 (0.07) b	0.52 (0.12) a	P<0.01
28	0.02 (0.01) c	10.1 (1.5) b	15.3 (1.1) a	P<0.01
<i>Second period (days 33 to 47; n = 21)</i>				
21	0.02 (0.01) c	0.30 (0.07) b	0.41 (0.07) a	P<0.01
28	0.03 (0.01) c	12.4 (1.3) b	15.9 (1.3) a	P<0.01
33	n/a	21.0 (2.03)	22.8 (1.7)	P=0.11
<i>Third period (days 47 to 63; n = 15)</i>				
21	0.02 (0.01) b	0.28 (0.07) a	0.38 (0.09) a	P<0.01
28	0.02 (0.01) b	15.8 (2.0) a	16.4 (1.9) a	P<0.01
33	n/a	30.6 (3.6)	25.1 (2.04)	P=0.21
47	n/a	14.0 (2.8)	19.4 (3.3)	P=0.19

Different lowercase letter means $P \leq 0.05$ and different uppercase letter means $P > 0.05$ and \leq

0.10 across rows.

Table 2. Pregnancy outcomes in Holstein embryo recipient heifers according to circulating PAG on day 21.

Item	Plus P4	Control	P-Value
<i>Low tercile</i>			
d21 Attachment	24.4% (20/82)	25% (22/88)	0.92
d28 Embryo heartbeat	12.2% (10/82)	2.3% (2/88)	0.02
Pregnancy Loss	65% (13/20)	100% (22/22)	0.03
<i>Medium tercile</i>			
d21 Attachment	78.1% (64/82)	56.8% (50/88)	<0.01
d28 Embryo heartbeat	58.5% (48/82)	38.6% (34/88)	0.01
Pregnancy Loss	32.8% (21/64)	40% (20/50)	0.42
<i>High tercile</i>			
d21 Attachment	90.2% (74/82)	83% (73/88)	0.17
d28 Embryo heartbeat	85.4% (70/82)	77.3% (68/88)	0.18
Pregnancy Loss	9.5% (7/74)	11% (8/73)	0.76

Table 3. Pregnancy outcomes in Holstein embryo recipient heifers according to circulating progesterone on day 21.

Item	Plus P4	Control	P-Value
<i>Progesterone < 1ng/mL</i>			
d21 Attachment	27.5% (19/69)	26.3% (20/76)	0.86
d28 Embryo heartbeat	0% (0/69)	0% (0/76)	0.99
Pregnancy Loss	100% (19/19)	100% (20/20)	0.99
<i>Progesterone ≥ 1ng/mL</i>			
d21 Attachment	78.5% (139/177)	66.5% (125/188)	0.01
d28 Embryo heartbeat	72.3% (128/177)	55.3% (104/188)	<0.01
Pregnancy Loss	15.8% (22/139)	24% (30/125)	0.09

Figure Legends

Figure 1. Experiment 1. an observational retrospective cohort experimental design to explore circulating pregnancy-associate glycoproteins with pregnancy loss (PL) from 28 d to 33 d (n = 32), 33 d to 47 d (n = 21), and 47 d to 63 d (n = 15) after 1003 timed embryo transfer (TET). Each heifer that had a pregnancy loss event was matched with a heifer in the same cohort that was nonpregnant (NP on 28 d, and the P4 was <1ng/mL [Low P4] on 19 d) and Pregnant in all pregnancy diagnoses (PD). In addition, for both pregnant and nonpregnant groups, each heifer must match with a heifer in the pregnancy loss group for TET date, embryo quality, embryo development, embryo source, and number of previous TET. BS = blood samples.

Experiment 2. Experimental design for the progesterone supplementation (Plus P4) using an intravaginal P4 insert (CIDR) from 14 d to 19 d after induced ovulation using a modified 5-d CoSynch for TET.

Figure 2. Individuals circulating PAGs on day 21 and 28 of the gestation for Holstein embryo recipients with pregnancy loss in three periods compared with pregnant and nonpregnant. For the period from **day 28 to 33**: (Nonpregnant [n = 32], Pregnancy loss [n = 33], and Pregnant [n = 32]; period from **day 33 to 47**: Nonpregnant [n = 27], Pregnancy loss [n = 23], and Pregnant [n = 27]; period from **day 47 to 63**: Nonpregnant [n = 16], Pregnancy loss [n = 15], and Pregnant [n = 16]. Different lowercase letters mean $P \leq 0.05$.

Figure 3. Percentage of embryonic attachment on day 21 of the gestation for Holstein embryo recipients with pregnancy loss in three periods. Different lowercase letters mean $P \leq 0.05$ and different uppercase letters means $P > 0.05$ and < 0.1 .

Figure 4. Percentage of embryonic attachment and pregnancy per embryo transfer for Holstein embryo recipients that receive a P4 insert (Plus P4) from days 14 to 19 compared with no P4 supplementation (Control). Different lowercase letters mean $P \leq 0.05$ and different uppercase letters means $P > 0.05$ and < 0.1 .

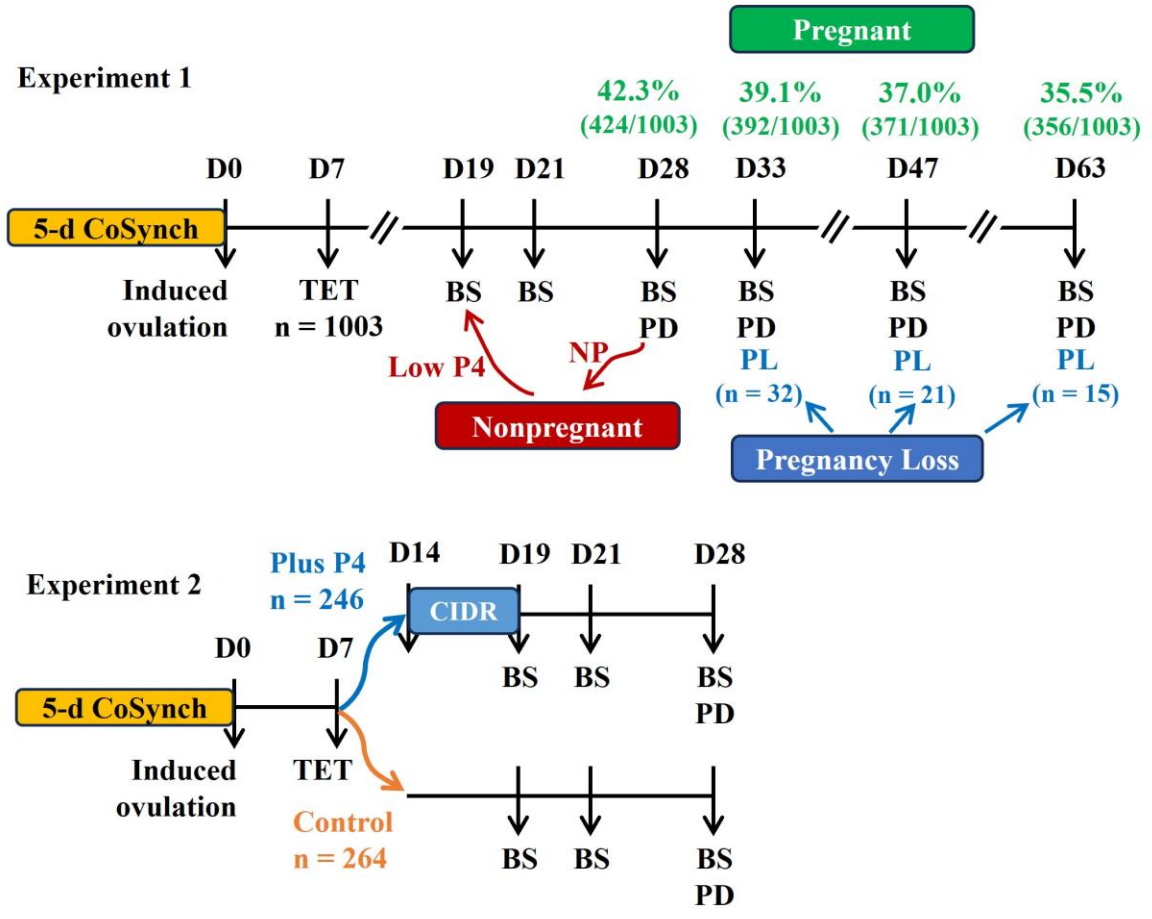


Figure 1

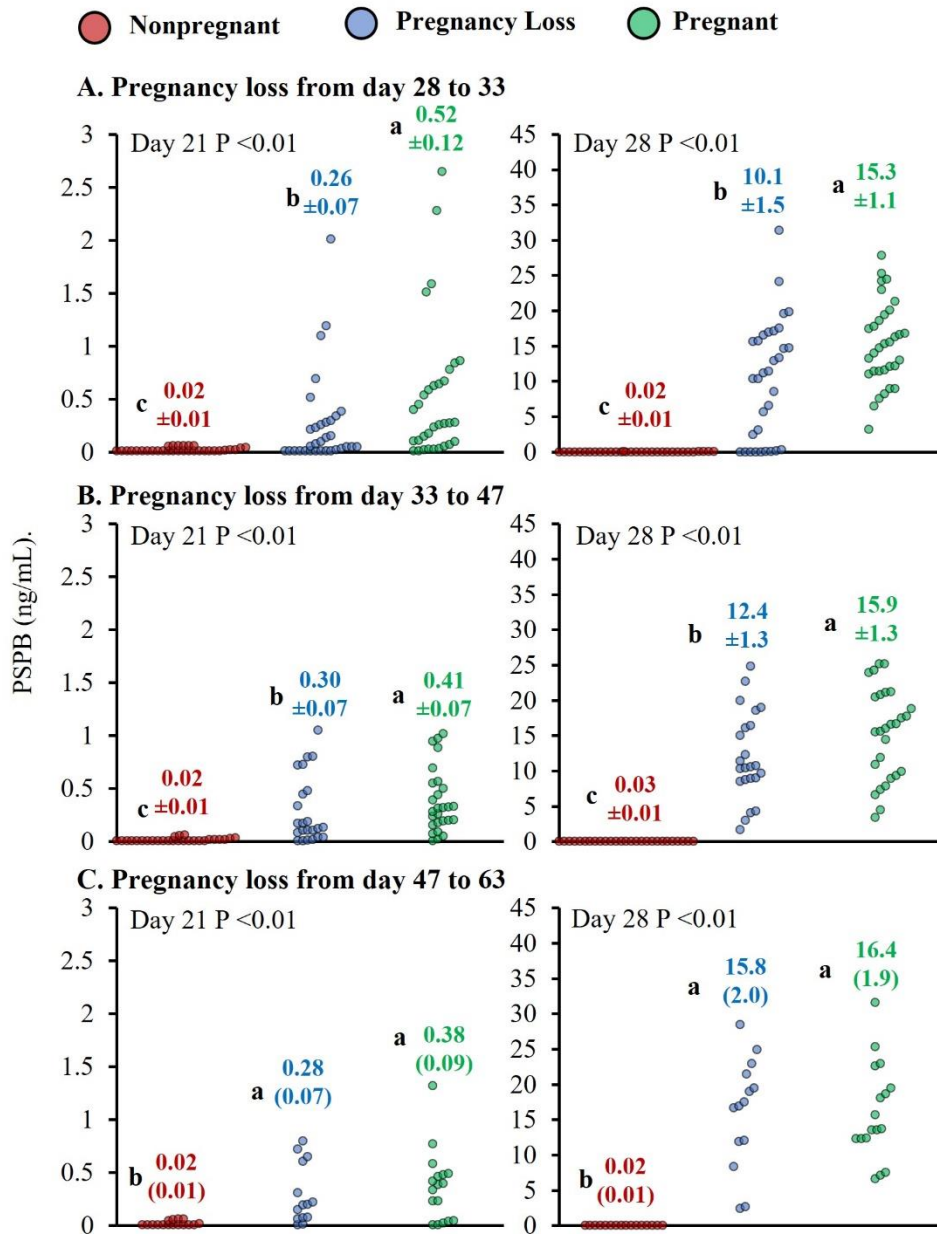


Figure 2

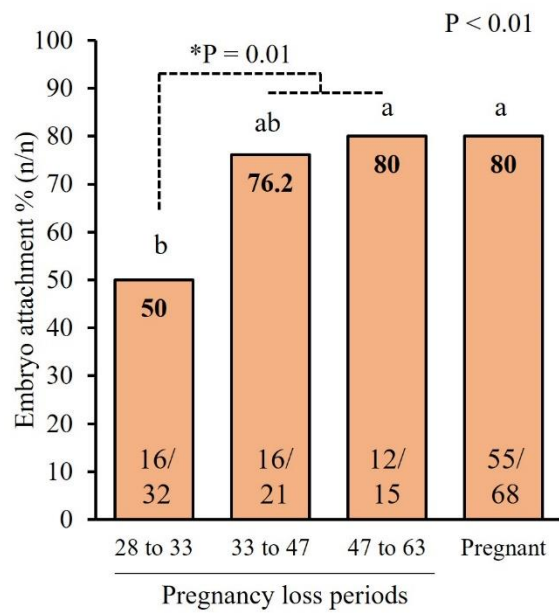


Figure 3

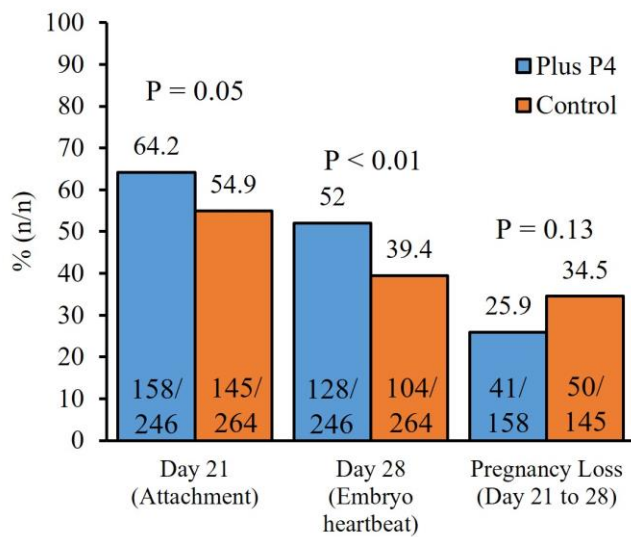


Figure 4

References

- [1] Viana J. 2022 Statistics of embryo production and transfer in domestic farm animals. IETS Newsletter. 2023;41:25.
- [2] Hansen PJ. The incompletely fulfilled promise of embryo transfer in cattle-why aren't pregnancy rates greater and what can we do about it? J Anim Sci. 2020;98.
- [3] Munhoz SK, Cooke RF, Munhoz AK, Prado CP, Pereira MHC, Vasconcelos JLM. Pregnancy losses in Bos indicus-influenced beef and dairy recipients assigned to a fixed-time embryo transfer protocol. Anim Reprod Sci. 2024;264.
- [4] King K, Seidel Jr G, Elsdon R. Bovine embryo transfer pregnancies. I. Abortion rates and characteristics of calves. J Anim Sci. 1985;61:747-57.
- [5] Seneda MM, Costa CB, Zangirolamo AF, Anjos MMd, Paula GRd, Morotti F. From the laboratory to the field: how to mitigate pregnancy losses in embryo transfer programs? Anim Reprod. 2024;21:e20240032.
- [6] Bazer FW, Kim J, Song G, Ka H, Tekwe CD, Wu G. Select nutrients, progesterone, and interferon tau affect conceptus metabolism and development. Annals of the New York Academy of Sciences. 2012;1271:88-96.
- [7] Placentation in Mammals: Tribute to E.C. Amoroso's Lifetime Contributions to Viviparity. Placentation in Mammals: Tribute to EC Amoroso's Lifetime Contributions to Viviparity. 2021;234:1-254.
- [8] Wallace RM, Pohler KG, Smith MF, Green JA. Placental PAGs: gene origins, expression patterns, and use as markers of pregnancy. Reproduction. 2015;149:R115-26.

[9] Seo H, Melo GD, Oliveira R, Franco-Johannsen GA, Bazer FW, Pohler KG, et al. Immunohistochemical examination of the uteroplacental interface of cows on days 21, 31, 40, and 67 of gestation. *Reproduction*. 2024;167.

[10] Arosh JA, Banu SK, McCracken JA. Novel concepts on the role of prostaglandins on luteal maintenance and maternal recognition and establishment of pregnancy in ruminants. *J Dairy Sci*. 2016;99:5926-40.

[11] Absalon-Medina VA, Butler WR, Gilbert RO. Preimplantation embryo metabolism and culture systems: experience from domestic animals and clinical implications. *J Assist Reprod Genet*. 2014;31:393-409.

[12] Bazer FW, Burghardt RC, Johnson GA, Spencer TE, Wu GY. Interferons and progesterone for establishment and maintenance of pregnancy: interactions among novel cell signaling pathways. *Reprod Biol*. 2008;8:179-211.

[13] Wiltbank MC, Baez GM, Garcia-Guerra A, Toledo MZ, Monteiro PL, Melo LF, et al. Pivotal periods for pregnancy loss during the first trimester of gestation in lactating dairy cows. *Theriogenology*. 2016;86:239-53.

[14] Reese ST, Franco GA, Poole RK, Hood R, Montero LF, Oliveira RV, et al. Pregnancy loss in beef cattle: A meta-analysis. *Anim Reprod Sci*. 2020;212.

[15] Domingues RR, Andrade JPN, Cunha TO, Madureira G, Hoppman AS, Teixeira NN, et al. Profiles of interferon-stimulated genes in multiple tissues and circulating pregnancy-associated glycoproteins and their association with pregnancy loss in dairy cows. *Biol Reprod*. 2023;110:558-68.

[16] Green JA, Parks TE, Avalle MP, Telugu BP, McLain AL, Peterson AJ, et al. The establishment of an ELISA for the detection of pregnancy-associated glycoproteins (PAGs) in the serum of pregnant cows and heifers. *Theriogenology*. 2005;63:1481-503.

[17] Reese ST, Pereira MC, Vasconcelos JLM, Smith MF, Green JA, Geary TW, et al. Markers of pregnancy: how early can we detect pregnancies in cattle using pregnancy-associated glycoproteins (PAGs) and microRNAs? *Anim Reprod*. 2016;13:200-8.

[18] Durocher J, Moore RK, Castonguay MH, Albaaj A, LeBlanc SJ, Dufour S. Bayesian estimation of sensitivity and specificity of a milk pregnancy-associated glycoprotein ELISA test for pregnancy diagnosis between 23 and 27 days after insemination in Holstein dairy cows. *J Dairy Sci*. 2022;105:6985-96.

[19] Ricci A, Carvalho PD, Amundson MC, Fourdraine RH, Vincenti L, Fricke PM. Factors associated with pregnancy-associated glycoprotein (PAG) levels in plasma and milk of Holstein cows during early pregnancy and their effect on the accuracy of pregnancy diagnosis. *J Dairy Sci*. 2015;98:2502-14.

[20] Zoli AP, Guilbault LA, Delahaut P, Ortiz WB, Beckers JF. Radioimmunoassay of a bovine pregnancy-associated glycoprotein in serum - its application for pregnancy diagnosis. *Biol Reprod*. 1992;46:83-92.

[21] Pohler KG, Geary TW, Johnson CL, Atkins JA, Jinks EM, Busch DC, et al. Circulating bovine pregnancy associated glycoproteins (bPAGs) are associated with late embryonic/fetal survival but not ovulatory follicle size in suckled beef cows. *J Anim Sci*. 2013; 91:4158-67.

[22] Pohler KG, Pereira MHC, Lopes FR, Lawrence JC, Keisler DH, Smith MF, et al. Circulating concentrations of bovine pregnancy-associated glycoproteins and late embryonic mortality in lactating dairy herds. *J Dairy Sci.* 2016;99:1584-94.

[23] Pohler KG, Peres RFG, Green JA, Graff H, Martins T, Vasconcelos JLM, et al. Use of bovine pregnancy-associated glycoproteins to predict late embryonic mortality in postpartum Nelore beef cows. *Theriogenology.* 2016;85:1652-9.

[24] Middleton EL, Pursley JR. Short communication: Blood samples before and after embryonic attachment accurately determine non-pregnant lactating dairy cows at 24 d post-artificial insemination using a commercially available assay for pregnancy-specific protein B. *J Dairy Sci.* 2019;102:7570-5.

[25] Middleton E, Minela T, Ahearne M, Arnold H, Santos A, Pursley J. Dairy heifers have an earlier increase in serum pregnancy-specific protein B compared with lactating dairy cows. Is this an indicator of earlier conceptus attachment? *JDS communications.* 2022;3:291-5.

[26] Santos A, Minela T, Branen J, Pursley J. Time to increase in pregnancy-specific protein B following artificial insemination is a direct determinant of subsequent pregnancy loss in lactating dairy cows. *J Dairy Sci.* 2023;106:3734-47.

[27] Pursley JR, Santos A, Minela T. Review: Initial increase in pregnancy-specific protein B in maternal circulation after artificial insemination is a key indicator of embryonic survival in dairy cows. *Animal.* 2023;17.

[28] Domingues RR, Andrade JPN, Cunha TO, Madureira G, Moallem U, Gomez-Leon V, et al. Is pregnancy loss initiated by embryonic death or luteal regression? Profiles of pregnancy-associated glycoproteins during elevated progesterone and pregnancy loss. *JDS Communications.* 2022;4:149-54

[29] NRC. Nutrient requirements of dairy cattle. Washington, DC, USA: Natl Acad Sci; 2001.

[30] Sala RV, Melo LF, Motta JCL, Leffers-Neto L, Carrenho-Sala LC, Fosado M, et al. Optimization of a 5-day fixed-time embryo transfer (FTET) protocol in heifers I. Manipulation of circulating progesterone through reutilization of intravaginal progesterone devices during FTET. *Theriogenology*. 2020;156:171-80.

[31] Reese S, Geary T, Franco G, Moraes J, Spencer T, Pohler K. Pregnancy associated glycoproteins (PAGs) and pregnancy loss in high vs sub fertility heifers. *Theriogenology*. 2019;135:7-12.

[32] Griffin CK, Lemley CO, Pohler KG, Sun X, Lear AS. Characterization of placentome vascular perfusion in relation to pregnancy associated glycoproteins throughout gestation in pregnant beef heifers. *Theriogenology*. 2024;219:94-102.

[33] Bazer FW, Spencer TE. Hormones and Pregnancy in Eutherian Mammals. *Hormones and Reproduction of Vertebrates, Vol 5: Mammals*. 2011:73-94.

[34] Middleton E, Minela T, Ahearne M, Arnold H, Santos A, Pursley J. Dairy heifers have an earlier increase in serum pregnancy-specific protein B compared with lactating dairy cows. Is this an indicator of earlier conceptus attachment? *JDS Communications*. 2022;4:291-95.

[35] Santos JE, Thatcher WW, Chebel RC, Cerri RL, Galvao KN. The effect of embryonic death rates in cattle on the efficacy of estrus synchronization programs. *Anim Reprod Sci*. 2004;82-83:513-35.

[36] Niles AM, Fricke HP, Carvalho PD, Wiltbank MC, Hernandez LL, Fricke PM. Effect of treatment with human chorionic gonadotropin 7 days after artificial insemination or at the time

of embryo transfer on reproductive outcomes in nulliparous Holstein heifers. *J Dairy Sci.* 2019;102:2593-606.

[37] Bai HAK, Kawahara M, Takahashi M, Imakawa K. Recent progress of interferon-tau research and potential direction beyond pregnancy recognition. *J Reprod Dev.* 2022;68:299-306.

[38] Lopez-Gatius F, Hunter R, Garbayo J, Santolaria P, Yaniz J, Serrano B, et al. Plasma concentrations of pregnancy-associated glycoprotein-1 (PAG-1) in high producing dairy cows suffering early fetal loss during the warm season. *Theriogenology.* 2007;67:1324-30.

[39] Oliveira Filho R, Franco G, Reese S, Dantas F, Fontes P, Cooke R, et al. Using pregnancy associated glycoproteins (PAG) for pregnancy detection at day 24 of gestation in beef cattle. *Theriogenology.* 2020;141:128-33.

[40] Chavatte-Palmer P, De Sousa N, Laigre P, Camous S, Ponter A, Beckers J-F, et al. Ultrasound fetal measurements and pregnancy associated glycoprotein secretion in early pregnancy in cattle recipients carrying somatic clones. *Theriogenology.* 2006;66:829-40.

[41] Constant F, Camous S, Chavatte-Palmer P, Heyman Y, De Sousa N, Richard C, et al. Altered secretion of pregnancy-associated glycoproteins during gestation in bovine somatic clones. *Theriogenology.* 2011;76:1006-21.

[42] Thatcher WW, Guzeloglu A, Mattos R, Binelli M, Hansen TR, Pru JK. Uterine-conceptus interactions and reproductive failure in cattle. *Theriogenology.* 2001;56:1435-50.

[43] Wiltbank MC, Meidan R, Ochoa J, Baez GM, Giordano JO, Ferreira JCP, et al. Maintenance or regression of the corpus luteum during multiple decisive periods of bovine pregnancy. *Anim Reprod.* 2016;13:217-33.

[44] Bazer FW, Wu GY, Spencer TE, Johnson GA, Burghardt RC, Bayless K. Novel pathways for implantation and establishment and maintenance of pregnancy in mammals. *Molecular Human Reproduction*. 2010;16:135-52.

[45] Imakawa K, Bai R, Nakamura K, Kusama K. Thirty years of interferon-tau research; Past, present and future perspective. *Anim Sci J*. 2017;88:927-36.

[46] Wiltbank MC, Monteiro PL, Domingues RR, Andrade JPN, Mezera MA. Maintenance of the ruminant corpus luteum during pregnancy: interferon-tau and beyond. *animal*. 2023;17:100827.

[47] Ginther OJ. Uteroovarian pathway for embryo-empowered maintenance of the corpus luteum in farm animals. *Theriogenology*. 2024;216:103-10.

[48] Domingues RR, Andrade JPN, Cunha TO, Madureira G, Moallem U, Gomez-Leon V, et al. Is pregnancy loss initiated by embryonic death or luteal regression? Profiles of pregnancy-associated glycoproteins during elevated progesterone and pregnancy loss. *JDS Commun*. 2023;4:149-54.

[49] Garcia-Guerra A, Sala RV, Carrenho-Sala L, Baez GM, Motta JCL, Fosado M, et al. Postovulatory treatment with GnRH on day 5 reduces pregnancy loss in recipients receiving an in vitro produced expanded blastocyst. *Theriogenology*. 2020;141:202-10.

[50] Spencer TE, Forde N, Lonergan P. The role of progesterone and conceptus-derived factors in uterine biology during early pregnancy in ruminants. *J Dairy Sci*. 2016;99:5941-50.

[51] Lonergan P, Sánchez JM. Progesterone effects on early embryo development in cattle. *J Dairy Sci* 2020;103:8698-707.

[52] Rabaglino MB, Sanchez JM, Mc Donald M, Crowe MA, O'Callaghan E, Lonergan P. Transfer of bovine embryos into a uterus primed with high progesterone concentrations positively impacts fetal development at 42 days of gestation. *Theriogenology*. 2023;200:25-32.

Chapter 8: General Conclusions and Future Directions

Challenges and opportunities in developing ReBreed21: where does this concept come from?

My experience, particularly as a practicing veterinarian, has taught me to appreciate the importance of reproduction for a livestock operation. Due to my passion for reproduction, during my Vet school I focused my effort in mastering bovine reproductive biology and reproductive biotechnologies under the guidance of Dr. Marco Mello and Dr. Roberto Azevedo. In addition, after hearing a seminar from Dr. Roberto Sartori and Dr. Manuel Sa Filho I was inspired to challenge myself to “think out of the box”, questioning the current concepts of bovine reproduction. This mindset combined with the skills that I gained during my Veterinary school training, helped me to successfully work as a practicing veterinarian on commercial herds during for two years. In this commercial situation, I felt challenged to improve reproductive efficiency in different types of dairy farms. I began to wonder if it was possible to develop an ideal program to rapidly rebreed nonpregnant cows to optimize reproductive performance and simultaneously produce a program that would be feasible to execute in a commercial farm. Driving back from a dairy farm I realized that a timed rebreed every 21 d (ReBreed21) would, in theory, maximize the service rate in dairy herds and with reasonable fertility could result in a shortened 42-d breeding period length. I realized this type of program could also be potentially attractive for beef cattle operations. From there on my whole life was changed and after three failed attempts waiting Dr. Milo in a Zoom call, Milo hired my wife as a research assistant. My wife is also a veterinarian, and she became the J1 Visa holder since she could speak English. I became the J2 Visa holder until I learned to speak English. I worked as a volunteer in the Wiltbank and

Hernandez laboratory to gain English skills including a notable scientific discussion about hypocalcemia with Dr. Hernandez at the DCC guided by the google translator app. In the Fall of 2021, I started my Ph.D. program with the support of Dr. Milo Wiltbank. We have been able to dive into the ReBreed21 idea exploring and optimizing this program for cows of different breeds, *Bos indicus* and *Bos taurus*, and under various physiological and production situations.

The pioneering aspect of this thesis was the integration of basic reproductive biology with robust on-farm strategies to envision the development and optimization of ReBreed21. Exploring the three most important sectors in bovine production (dairy, beef, and embryo transfer recipients), ReBreed21 was successfully developed for beef heifers and embryo recipients. The series of studies in dairy brought to light the challenges and opportunities of developing such an ambitious program for high-producing lactating dairy cows. Finally, after exploring the biology and fertility in randomized research experiments with over 8,236 cows/heifers, we also found new insights into P4 supplementation to mitigate pregnancy loss and corpus luteum maintenance in lactating dairy cows after oxytocin challenges.

Challenges and opportunities in developing ReBreed21: why target an intense reproductive program with a shorter breeding period?

Reproductive management is a pillar to achieving high animal performance and profitability in bovine operations. The time cows become pregnant is critical to their productive life, and from a herd management perspective, it can be targeted by narrowing the breeding period length. In the first chapter of this thesis, the nuances among bovine productive purposes were dissected from the big picture to specific practical and biological details on improving bovine performance by optimizing reproductive programs. The time of pregnancy for optimal

profitability should be considered based on the bovine productive purpose (dairy, beef, or embryo recipient) and the particularities of each herd, such as average milk production, climate, and production profile.

In beef cow operations, heifers and cows that become pregnant in the first 21 d of the breeding season will be those weaning the heavier calves. The weight of the calf at weaning is associated with high performance for females (high pregnancy rate in their first breeding season) or males (more kg after feedlot). For embryo recipients, a rapid time for pregnancy is critical due to the central goal of producing a high-merit genetic calf for the least cost, particularly the major cost of a recipient program, the cost of housing nonpregnant recipients. Therefore, increasing the number of pregnancies at the beginning of the breeding season is also desirable for embryo recipients. On the other hand, for lactating dairy cows the optimal time for pregnancy is variable mainly according to the level of milk production, thus each dairy farm and dairy cows may have an optimal time to pregnancy. Nevertheless, the current reproductive programs for lactating dairy cows produce, at minimum, a 70 d to 100 d breeding period. Taken together, no matter the bovine purpose, a reproductive program that allows a narrow breeding period can be a livestock precision tool to optimize animal performance and boost farm profitability.

Challenges and opportunities in developing ReBreed21: a program built at the intersection of basic biology and applied biotechnology

This thesis suggests a model for developing ReBreed21 for high bovine reproductive performance by attaining high fertility in the first service (AI or ET), earlier selection of nonpregnant cows, and assuring reasonable fertility in the second and later services (Figure 1). For the first step in the model, a solid and broad understanding of bovine physiology was required to ensure that the optimal hormonal milieu for high fertility in dairy and beef was

achieved with current modifications in TAI programs, as illustrated in Figure 2. In addition, in all the ReBreed21 approaches (beef, ET, and dairy) sought to take advantage of resynchronization treatments to provide a P4 supplementation to the first service and potentially increase fertility by decreasing the earlier pregnancy loss. The selection of nonpregnant animals was evaluated using on-farm milk P4 or CL morphological/blood perfusion changes in cows undergoing luteolysis. Finally, to achieve high fertility in the second and later service, the approach was to adjust the ReBreed21 to closely mimic what has been reported to be the optimal hormonal milieu to achieve high pregnancies per service according to the bovine biotypes (Figure 2).

To pursue our goal in beef cattle, the first challenge was to find them in America's Dairyland. We made the strategic decision to use our contact in Brazil and found a large beef cattle operation in Central Brazil to develop the ReBreed21 program for beef cattle. Our studies using beef cattle, specifically in Chapter Two, show how the physiology matches the early potential timed rebreeding 21 d after the previous AI. We also tested a modification using a GnRH treatment at 12 d designed to improve the heifer synchrony, but it achieved overall follicular/hormonal synchronization that was similar to the original ReBreed21. The fertility evaluations for ReBreed21 were performed in a large cow/calf commercial operation, with an impressive increase in pregnancies in a shortened 42-d breeding season using ReBreed21 compared with the conventional Resynch program for heifers. In addition, the estradiol supplementation was tested as a new modification of ReBreed21 and was found to not be necessary in heifers but was essential to optimize P/AI in primiparous cows. Conversely, there was poor fertility in multiparous cows bred with ReBreed21, and estradiol supplementation did not improve the results. Noteworthy, the biological model of ReBreed21 was first drawn based on heifer reproductive physiology; further modifications must be considered to enhance fertility

in multiparous cows. Another critical step is the early identification of nonpregnant cows. In Chapter Four, we compared two methods to determine nonpregnant cows at 21 d after the previous AI, based on CL size or blood perfusion using ultrasound. Both methods had few false negatives, which is extremely important to avoid iatrogenic abortus. Finally, the accuracy was >90% for both methods, but there were more false positives when the selection of nonpregnant was made by CL size rather than CL blood flow by Doppler ultrasound.

Aware of the importance of ET programs in bovine reproduction and of a skyrocketing number of bovine ET in the US, we tested our creativity in developing the ReBreed21 for ET recipients (ReBreed21-ET). Adapting ReBreed21 to a timed embryo transfer setting resulted in a ten-percentage point increase in pregnancies after a 105-d timed embryo transfer season compared to a high-performance reproductive program for embryo recipients. The utilization rate of nonpregnant heifers on D28 with ReBreed21-ET was only about 60%, but rapidly returning nonpregnant heifers to another timed ET with fertility as good as the standard protocol was one of the reasons that allowed ReBreed21-ET to reach more pregnancies at the end of the season. Another reason for the ReBreed21-ET success was decreased pregnancy loss in the first Timed-ET. Interestingly, the strategic use of P4 inserts from 14 d to 19 d of the estrous cycle in ReBreed21 was precisely during the embryo elongation period, which resulted in a greater P/ET on 28 d. To further explore the relationship between the P4 supplementation from ReBreed21 resynchronization in embryo recipients, Chapter Seven was dedicated to diving into pregnancy loss by assessing early circulating PAGs, embryo attachment, and P4 supplementation. The circulating PAGs showed a high association with earlier pregnancy loss. Furthermore, P4 supplementation decreased the embryo losses from ET to embryo attachment on 21 d in underdeveloped placentation heifers. Those results dissected potential conditions in which

interventions such as P4 supplementation can improve P/ET, which can help develop new strategies to mitigate pregnancy loss.

The ReBreed-21 idea has a great potential to optimize reproductive performance in lactating dairy cows. To pursue this goal, I was blessed to be in Wisconsin-US to work in THE pioneer synchronization of ovulation lab worldwide. Chapter Five of this thesis condensed the series of experiments with the challenges and opportunities of ReBreed21 in high-producing lactating dairy cows. The later and wider luteolysis dispersion in dairy cows was the first barrier to be defeated, allowing the development of ReBreed21. Exogenous treatments with PGF2 α analogs are often used to synchronize luteolysis, but in ReBreed21, most of the treatments are performed before the nonpregnancy diagnosis, and then PGF2 α analogs must be avoided to prevent iatrogenic abortus. Therefore, our lab explored exogenous oxytocin treatment, taking advantage of the oxytocin-induced PGF2 α restricted in nonpregnant cows. Experiments 1 and 2 of Chapter Five validate the concept of luteolysis synchronization using oxytocin, requiring a minimum of 2 treatments on 18 d and 19 d since the previous TAI, without affecting fertility. In experiment 3, ReBreed21 was compared with Resynch25, the state-of-art timed rebreeding program available. Conflicting with what was found in experiment 2, there was a decrease in P/AI in experiment 3 for cows treated with oxytocin, particularly in multiparous cows in their first TAI using the Double-Ovsynch program. One modification of ReBreed21-Dairy was also tested, including accessories CLs using GnRH treatments on 5 d and 11 d after TAI. The rationale of this modification was, on the one hand, to synchronize the follicular wave for nonpregnant cows bred on 21 and to provide a P4 supplementation from the previous TAI. However, neither the P/AI for nonpregnant cows bred was improved nor was pregnancy loss in the last TAI mitigated. With about a ten percentage points decrease in pregnancies in the first AI

associated with poor P/AI for cow timed rebred on 21 d, ReBreed21 did not improve reproductive performance in lactating dairy cows. Interestingly, even failing to check 2 of the 3 points in our model to develop a high reproductive program, the proportion of pregnancies by 180 days in milk and average time to pregnancy for ReBreed21+G and Resynch25 were not significantly different. Therefore, significant improvements are still needed to optimize ReBreed21 in lactating dairy cows.

Future directions

To develop ReBreed21, many questions must be stated that challenge current concepts in bovine reproductive management. We accepted the challenges, taking risks to go beyond what was never attempted, putting in a single PhD thesis a broad approach to improve bovine reproductive performance, explore the challenges and opportunities of dairy cows, ET recipients, and beef cattle, and finally consider the unique reproductive biology in each of them with the ultimate goal of optimizing bovine performance and farm profitability. Nevertheless, bringing to light a novel concept, many questions were answered, while many more have arisen, thereby opening new avenues to explore from now on.

The lack of fertility in beef multiparous cows using ReBreed21 affirms that a new model, especially one that considers parity, is needed. The ReBreed21 model was based on the expected follicular wave pattern in beef heifers, and according to our studies, only a P4 insert was required to promote a proper hormonal milieu to achieve repeatable fertility. For TAI in beef cattle, the most common approach to starting a synchronization of ovulation protocols is combining an intravaginal P4 insertion with an intramuscular injection of GnRH or estradiol analogs. Therefore, one possibility to improve the ReBreed21 in multiparous beef cows is to bring back the modification of the GnRH treatment on 12 d after induced ovulation with heifers to assure

high follicle synchrony. Another ongoing challenge in the beef cattle reproductive program is reducing the number of days to handle animals in the chute. Thus, some strategies such as an oral progestin treatment could be an opportunity to explore resynchronization protocol to mitigate animal handling, improving welfare.

In ET programs, an important aspect of optimizing reproductive performance is the mitigation of pregnancy loss. Undoubtedly, one of the most used interventions to decrease pregnancy loss is P4 supplementation. It is well-known that circulating P4, particularly CL-produced P4, is crucial to bovine pregnancy maintenance, fulfilling the word's etymology (a ketone that supports gestation). On the other hand, there is a tremendous scientific contradiction between P4 supplementation and pregnancy loss mitigation. In agreement with the overall scientific results, we also reported a contradiction in pregnancy loss after P4 supplementation. The P4 inserts from 14 d to 19 d in the ReBreed21-ET decrease the loss from ET to 28 d but only in the first TET. Assuming that the benefits of P4 supplementation are in animals with suboptimal placentation, an opportunity is to design strategies that early identify those individuals associated with other strategies of P4 supplementation. Another aspect is to focus on the differentiation and identification of pregnancy loss causes to help understand the literature contradictions and support the development of more effective and replicable strategies to mitigate pregnancy loss.

In relation to ReBreed21-dairy, the excitement in developing a novel method to synchronize luteolysis with oxytocin was mixed brought with it the frustration regarding the decrease in fertility. As was discussed in this thesis, the decrease in fertility in multiparous cows raises questions about what has been assumed to be the physiology model in CL maintenance. Does the high liver metabolism of steroids alter the time oxytocin receptor regulation? Or do

multiple doses of exogenous oxytocin signals to upregulation of oxytocin receptor? Nonetheless, there are still opportunities to explore and integrate oxytocin in early-timed rebreeding programs since there is no indication of negative effects on fertility in first-lactation cows. Thus, oxytocin can certainly be an important strategy to optimize the performance of ReBreed21-beef and ReBreed21-ET. Lastly, a more straightforward opportunity to develop a rapid timed rebreeding program is by delaying the time to rebreed for 23 d to 25 d, waiting for more cows to naturally undergo luteolysis instead of an indirect induction of luteolysis with oxytocin for a ReBreed23 or 25.

Conclusion

In summary, this thesis presents the successful development/optimization of a novel program for AI and ET every 21 d for beef cows and embryo recipients (Figure 3). We also reported the challenges and opportunities in developing the ReBreed21 for multiparous beef cows and high-producing lactating dairy cows. Additionally, we report new findings into pregnancy establishment/loss associated with P4 supplementation and challenges with oxytocin-induced PGFM. Therefore, the work in this thesis expands the frontiers in timed rebreeding programs and greatly advances bovine reproductive performance while bringing new insights into basic bovine reproductive physiology. We hope the work of this thesis benefits farmers worldwide and inspires scientists to advance the development of new technologies to optimize bovine performance.

Figures

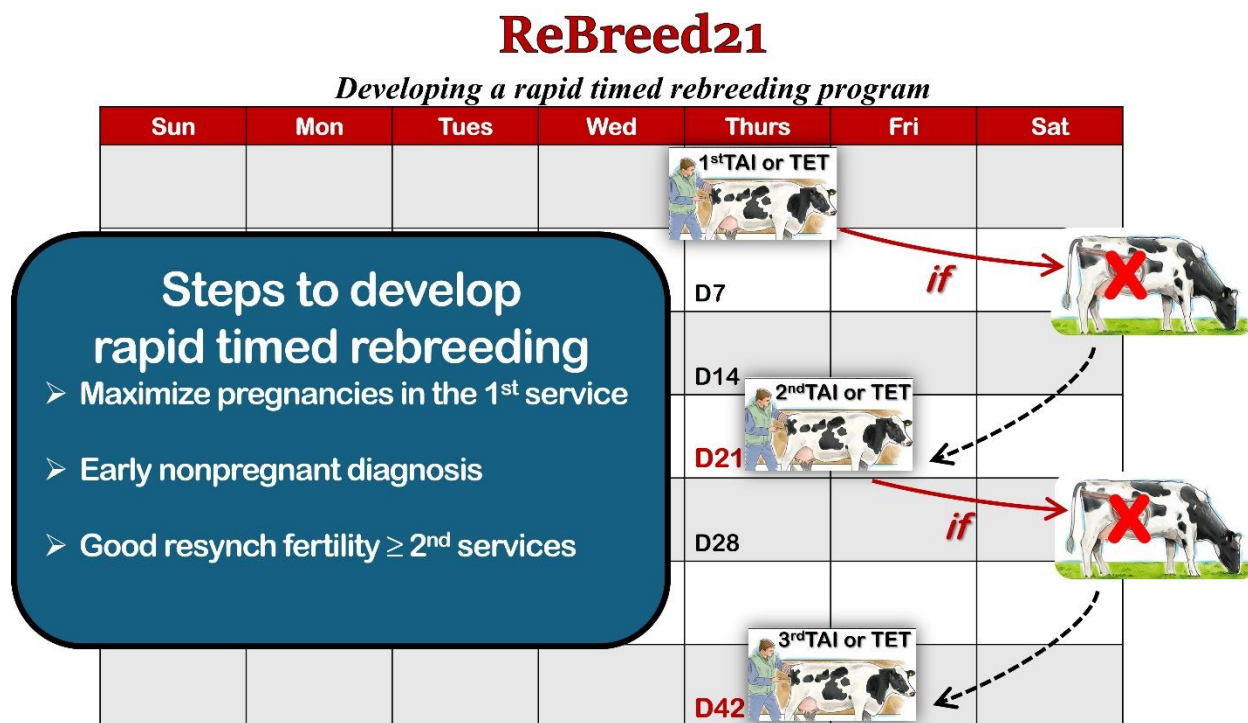


Figure 1. Model for the ReBreed21 strategy. Three fundamental precepts for development of the ReBreed21 approach: (1) Maximize or No decrease in fertility to previous 1st service, (2) Accurate early pregnancy diagnosis, (3) Adequate fertility to rebreeding services.

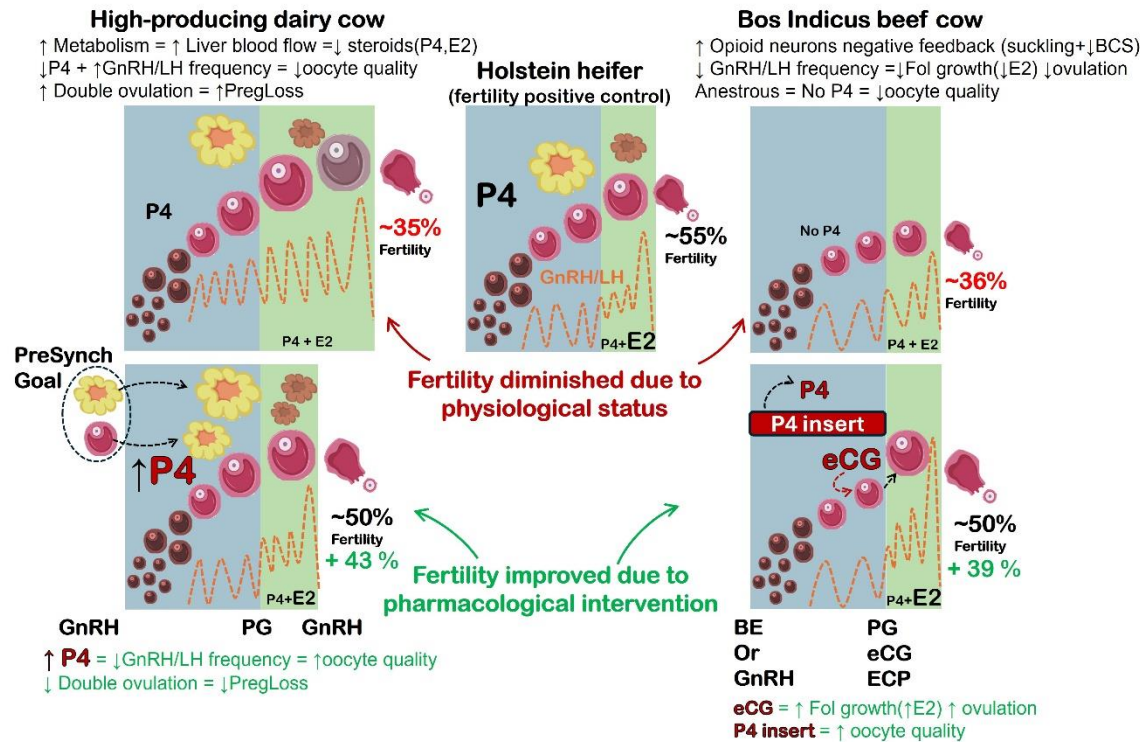
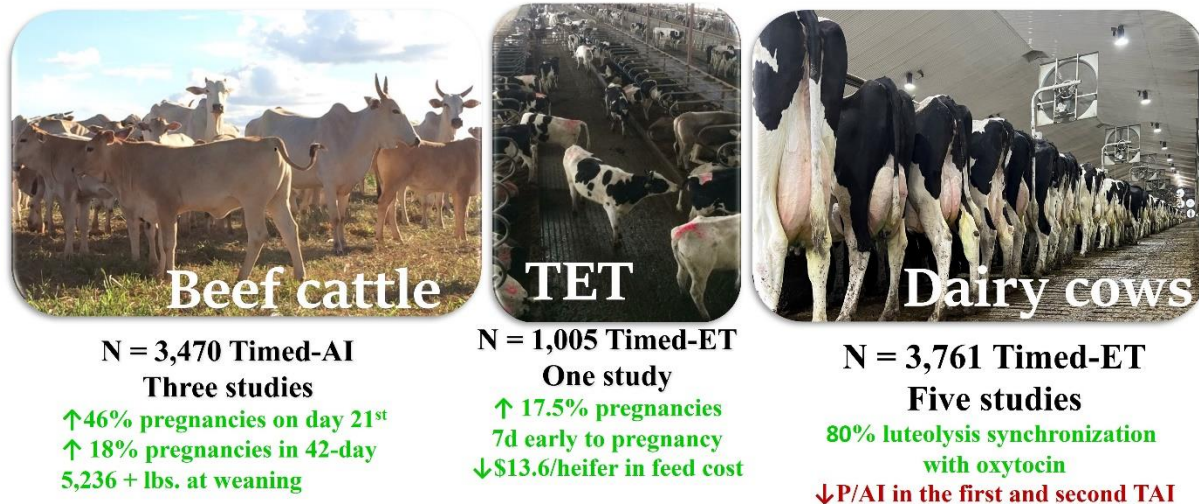


Figure 2. Model of optimal hormonal milieu to achieve high pregnancy per service in beef or dairy cows. **High-producing dairy cow. Hormonal disruption:** high clearance of circulating steroid hormones (progesterone and estradiol) due to high metabolism increases GnRH/LH frequency, resulting in prolonged follicular development, delayed and double ovulation, low-quality oocytes, and lower fertility per AI and high pregnancy loss. **Exogenous hormonal intervention:** Resynchronization (PreSynch) aims to concentrate the number of cows at day seven of the estrous cycle at the initiation of the synchronization protocol. Thus, most cows will have a mature corpus luteum (CL), assuring high progesterone to decrease the GnRH/LH frequency by negative feedback in the hypothalamus, decreasing follicular growth and double ovulation. **Bos indicus beef cattle. Hormonal disruption:** negative feedback hypothalamus on GnRH/LH release (calf presence, low body condition score, and/or low energy intake) triggers a sequence of negative effects (reduced follicular growth, lower circulating estradiol during the follicular dominance phase, lack of estradiol-positive GnRH feedback, lower amplitude/surge of LH, anovulatory condition, and/or smaller ovulatory follicle which results in smaller corpus luteum [lower circulating progesterone in diestrus]) that culminates in lower fertility per AI. **Exogenous hormonal intervention:** Synchronization of a new follicular wave emergence with eCG treatment that binds to LH/FSH receptors, increases follicular growth, and indirectly mitigates the negative feedback effect of GnRH release.

ReBreed21

Developing a rapid timed rebreeding program



8,236 cows (Timed-AI or TE)

Figure 3. Graphical abstract of the main results of ReBreed21 in beef cattle, ET recipients, and dairy cows.