University of São Paulo
“Luiz de Queiroz” College of Agriculture

Holistic vision of dairy herds: reproductive management, timed-artificial insemination programs, nutrition and heat stress

Carlos Eduardo Cardoso Consentini

Thesis presented to obtain the degree of Doctor in Science Area: Animal Science and Pastures

Piracicaba
2022

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Dados Internacionais de Catalogação na Publicação
DIVISÃO DE BIBLIOTECA – DIBD/ESALQ/USP

Consentini, Carlos Eduardo Cardoso

Holistic vision of dairy herds: reproductive management, timed-artificial insemination programs, nutrition and heat stress / Carlos Eduardo Cardoso Consentini. - - Piracicaba, 2022.

133 p.

Tese (Doutorado) - - USP / Escola Superior de Agricultura "Luiz de Queiroz".

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DEDICATION

To my grandfather Luiz Morais Cardoso. In some way, he was responsible to my decision to become a veterinarian.

To my uncle Roberto Rennó Raphaelli who was so proud of my academic career and of my choice to do my Master's and PhD at ESALQ/USP. He always thought that I was unbeatable. We really miss you, buddy.

ACKNOWLODGEMENTS

I want to express my gratitude to my parents Carlinhos and Sonia, my sister Luciana and my brother Dedé for all the support during my academic life and for always encourage me to make tough decisions about what would make me happy. There isn't one single day that I don't think about you guys and want to make you proud. Thanks for everything. Love you all.

Thanks to Dr. Roberto Sartori Filho. The guy I asked to be a speaker in a college symposium on a Sunday morning of 2015, in which he accepted saying that he was there because he loved doing that, regardless if it was a Sunday morning. Life was generous to me giving the opportunity to be a researcher intern at the Department of Animal and Dairy Sciences of the University of Wisconsin-Madison, and who was there? Yes, Dr. Sartori. I am really thankful for all the work, teachings and friendship back at that time, and I appreciate the opportunity of pursuing a Master's and PhD degree at "Luiz de Queiroz" College of Agriculture - University of São Paulo, under your orientation. I admire you as a teacher and educator, and your dedication and way of doing science. Thanks for orientation, friendship and all the opportunities you gave me during almost 8 years now. Oh, I saw your badge of that 2015 symposium in your office.

I would like to thank the University of São Paulo (USP) that has been my house during the past 11 years of my life. I am grateful for the School of Veterinary Medicine and Animal Sciences for the education during my years as a vet student. A special thanks to Dr. Pietro Sampaio Baruselli and Dr. Francisco Palma Rennó for all the help and knowledge. It was (still is) a pleasure to be your student, and I appreciate everything you guys have done to me.

A special thanks to the ESALQ. I am proud of become a PhD in this outstanding school. I appreciate the knowledge provided for the professors from the Animal Sciences Department, and for their orientation and friendship. A really special thanks to Dra. Carla Bittar for always having your office's door open and for your willingness to solve problems and support the students. More than teaching us many topics (calves, biochemistry, nutrition, etc.) as a professor, you are an important person in the student's life, as you were in mine. You were present, helped me and always supported me during really important parts of my life. I will always be grateful.

I am thankful for the great friends I made here, who made life in Piracicaba very pleasant. Thanks all the guys from CPZ (Brasa, K-uzo, Tarja, Larguei, Leiteira, Lupa, Ricardo and Laureano). Thanks, Velho Tita, for the friendship during these years, buddy. Thanks to Gustavo Salvati and Willian Pereira for the friendship that we built up.

I would like to express my tremendous gratitude to Dr. Milo Wiltbank. Thanks for the opportunity to work and learn so much with you, and be your student at UW-Madison. I am very grateful for all your orientation during my Master’s and PhD, and for keep forcing me to understand how to better develop experimental designs, which questions we should ask and how we can answer them. You made a lot for me and so many other people. I am very grateful for all the opportunities and experiences you provided me. Thanks for the friendship, buddy. I am always here for you.

Special thanks to my friend Pedrinho Monteiro. I guy I always admired and had the opportunity to work with and learn so much during the year at UW-Madison. Thanks for the friendship and for always being willing to help with anything we need. Despite the loss against the 49ers, it was a very unique day we had together at that Packes vs. 49ers at the Lambeau Field (Go Pack Go, and to register here, Flamengo is the Champion of 1987 Brasileirão hahahahahahah). To another brother of Madison, João Paulo Andrade (JP), thanks for the friendship. I admire your hard work and way of living with your family. It was a pleasure to help you in your trials. Love you guys and your family. JP, big kiss on Fabi, Maria e Teteu. Pedrinhossauro, big kiss on Fer, Mama e Lilo.

Still in Madison, I cannot forget to thank dear friends who were with during a very special period of my like. Thanks, my dear friends Marcelão and Michele for always being present, showing love and supporting me. It was a pleasure be with you guys almost daily and share so many memories with you guys. Love you and I am very proud of you and I wish a lot of success in your plans. My buddies Tiagão e Tadeuzão. What a hell of a ride we had together palls. We have been through a lot together, from very tough episodes to many great moments. Thank you very much for the friendship. Love you guys.

My friend Alexandre Prata. I admire your way to face challenges and for always wanting to make the difference. Thanks for taking me out on the road and teaching me so much during these years. Thanks for fighting with me about important thing I must improve. I am always grateful for the friendship, the support and the opportunities you gave me. You can count on me, Xandi.

Leonardo de França e Melo (“The Old of the River”). I am so thankful for all you have done for me since we met in 2015 in Madison. Thanks for always being ready to answer the phone, discuss about anything that is happening in life or with the cows. I appreciate your friendship and all the Campari and Jack and Coke we had together. Lasty, thank you very much for your help and hard work on projects that we have done in the past years.

Thanks Alexandre Henrily de Souza for always being able to help (or better, to solve) every issue we faced throughout the years. I admire you for your outstanding knowledge, the professional you are and how you see our profession, the academy, the industry and life. I have learned so much with you, and I am really grateful for you always trusting me and for many opportunities. I appreciate your friendship, support and advices. You were always there during really important moments and decisions of my life. Thanks, and count on me, Sr. Alex.

To my dear friend Tiago Carneiro, thanks for the tremendous training you provided to me on reproductive techniques during vet School, all the learning and for the friendship and partnership in so many projects during the last years. Really admire you, Ti. You were so important for the formation of so many people. “Gotta go now, baby, if I hurry, I can still make Cheyenne”.

Really special thanks to Céu Azul Farm’s crew for the hard work during the fertility program’s trial. Thanks, Danilo, Ernane, Tatti, Fernando, Marcelinho, Tiago, Luis, Tuanne, Taynara, Paulinho and family, and Indio. I appreciate your friendship and the year we spend together, working, fishing and having fun. I want to express my gratitude to the students and interns that helped during this year-long experiment. A special thanks to Yasmin Pazini, Pedro Ferro and Anna Muller. A special thanks to my friend Juan Galindez, that even being a beef cattle guy, accepted the challenge and worked very hard during the project.

To GlobalGen vet science, thanks for supporting many studies with products and years of partnership. I am very thankfull for the trust you guys put on me and the incredible opportunities during these years. A special thanks to Prata, Mauro, Gabriel, Moacir, Sergio and Faleiros. It is a pleasure to have your friendship and work with you guys.

I would like to thank São Paulo Research Foundation (FAPESP - Fundação de Amparo à Pesquisa do Estado de São Paulo; process: # 2018/03798-7), Coordination for the Improvement of Higher Education (CAPES, Brasília, Brazil) and Brazilian National Council for Scientific and Technological Development (CNPq, Brasília, Brazil) for scholarships and/or other financial support during this trial and my Master’s.

Now, to Guilherme Madureira (Careca), Rodrigo Lemos (Digão), Lucas Silva (Giri), Jessica Motta, one BIG SPECIAL thanks for these friends for all their hard work and friendship. I appreciate Jessica and Giri for the tremendous effort during the dairy projects. A lot of the information we produced is because of you guys. Thanks, Digão and Carequinha (the beef cattle guys) for so many memories and friendship. I wish you guys all the best in your lives. You can count on me.

Last, but not least. As in my Master’s, I want to express my special gratitude to the dairy cows, especially those that participated in the studies. You deserve all respect, and I will always work trying to provide you a healthy, comfortable and happy life, in order to you girls keep providing such a noble product to us that is the milk.

SUMMARY

RESUMO

Visão holística de fazendas leiteiras: manejo reprodutivo, programas de inseminação artificial em tempo fixo, nutrição e estresse térmico

O objetivo dessa tese de doutorado foi abordar diferentes aspectos que afetam a performance reprodutiva de fazendas leiteiras. O primeiro capítulo é uma revisão que discute como o manejo reprodutivo afeta a reprodução, apresentando estratégias para trabalhar o primeiro serviço pós-parto e as re-inseminações das vacas. Além disso, a revisão discute pontos importantes que afetam a fertilidade dos programas de inseminação artificial em tempo fixo (IATF) como promover sincronização de onda folicular no início dos protocolos, alta concentração de progesterona (P4) durante o desenvolvimento do folículo pré-ovulatório, e a importância do ambiente hormonal no período próximo à inseminação e pós-ovulação. Além disso, a revisão discute como a nutrição, genética, estresse térmico, escore de condição corporal, saúde e o período de transição afetam a performance reprodutiva. O segundo capítulo envolve um experimento no qual foi comparado duas estratégias de pré-sincronização e dois protocolos de IATF, estabelecendo 4 programas reprodutivos para a 1ª IATF pós-parto. Apesar das diferentes bases farmacológicas, os programas reprodutivos promoveram resultados similares e alta fertilidade, e os resultados mostraram uma mesma eficiência de um programa estabelecido, o Duplo-Ovsynch, comparado com o programa inovador elaborado pelo nosso grupo de pesquisa, o Duplo E-Synch. No estudo do capítulo 3, otimizações durante o protocolo de IATF á base de estradiol (E2) e P4 foram avaliadas e observamos um aumento na fertilidade quando um tratamento com hormônio liberador de gonadotrofina (GnRH) foi adicionado ao início do protocolo convencional iniciado apenas com benzoato de E2. O capítulo 4 engloba um artigo em que informações relacionadas a nutrição e reprodução de fazendas leiteiras foram avaliadas retrospectivamente. São dados de campo que sugeriram que dietas com alta concentração de carboidratos não fibrosos no início da lactação podem ter efeito negativo na fertilidade e performance reprodutiva de fazendas comerciais de alta produção de leite. O quinto e último capítulo discute outro importante fator que afeta a fertilidade, o estresse térmico. Dados foram coletados durante um ano em uma fazenda comercial e os resultados mostram efeitos negativos na fertilidade de variáveis associadas ao estresse térmico, como temperatura retal no momento da IA, estação do ano e índice de temperatura e umidade (THI). Por fim, concluímos que é importante que as fazendas e profissionais tenham uma visão holística do sistema de produção, buscando entender e controlar fatores que afetam a fertilidade, além de implementar estratégias de manejo reprodutivo e programas de IATF otimizados, objetivando atingir alta eficiência reprodutiva em conjunto com alta produção de leite.

Palavras-chave: Vaca leiteira, Fertilidade, Inseminação artificial em tempo fixo, Nutrição, Estresse térmico

ABSTRACT

Holistic vision of dairy herds: reproductive management, timed-artificial insemination programs, nutrition and heat stress

The objective of this PhD dissertation was to address different aspects that affect reproductive performance of dairy herds. The first chapter is a review that discusses how reproductive management affects reproduction, presenting strategies for the first postpartum service and re-inseminations. In addition, the review discusses key points that affect fertility of timed-artificial insemination (TAI) programs, such as promoting follicular wave emergence at the beginning of the protocols, high concentration of progesterone (P4) during preovulatory follicle development, and the importance of the hormonal environment in the periovulatory period and post-ovulation. Furthermore, the review discusses how nutrition, genetics, heat stress, body condition score, health and the transition period affect reproductive performance. The second chapter involves an experiment in which two pre-synchronization strategies and two TAI protocols were compared, establishing four reproductive programs for the first TAI postpartum. Despite the difference on pharmacological bases, the reproductive programs promoted similar results and high fertility, and the results showed a same efficiency of an established program, Double-Ovsynch, compared with the novel program developed by our research group, Double E-Synch . In the study of chapter 3, optimizations during the estradiol (E2) plues P4-based TAI protocol were evaluated, and we observed an increase in fertility when a treatment with gonadotropin-releasing hormone (GnRH) was added at the beginning of the conventional protocol initiated with only E2 benzoate. Chapter 4 includes an article in which information related to nutrition and reproduction from commercial dairy herds was retrospectively evaluated. These are field data that suggested that diets with a high concentration of non-fiber carbohydrates in early lactation may have a negative effect on fertility and reproductive performance in high production commercial dairy farms. The fifth chapter discusses another important factor that affects fertility, heat stress. Data were collected during one year on a commercial farm and the results confirmed negative effects on fertility of variables associated with heat stress, such as rectal temperature at the time of AI, season of the year and temperature and humidity index (THI). Finally, we conclude that it is important for farms and professionals to have a holistic vision within the herd, looking for to understand and control factors that affect fertility, in addition to implementing optimized TAI programs and effective reproductive management strategies to guarantee high service rates and fertility, aiming to achieve high reproductive efficiency with high milk production.

Keywords: Dairy cow, Fertility, Timed-artificial insemination, Nutrition, Heat stress

CHAPTER 1: REVIEW - FACTORS THAT OPTIMIZE REPRODUCTIVE EFFICIENCY IN DAIRY HERDS WITH AN EMPHASIS ON TIMED ARTIFICIAL INSEMINATION PROGRAMS

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animals MDPI

Review

Factors That Optimize Reproductive Efficiency in Dairy Herds with an Emphasis on Timed Artificial Insemination Programs

Carlos Eduardo Cardoso Consentini , Milo Charles Wiltbank and Roberto Sartori

https://doi.org/10.3390/ani11020301

Abstract

Reproductive efficiency is closely tied to the profitability of dairy herds, and therefore successful dairy operations seek to achieve high 21-day pregnancy rates in order to reduce the calving interval and days in milk of the herd. There are various factors that impact reproductive performance, including the specific reproductive management program, body condition score loss and nutritional management, genetics of the cows, and the cow comfort provided by the facilities and management programs. To achieve high 21-day pregnancy rates, the service rate and pregnancy per artificial insemination (P/AI) should be increased. Currently, there are adjustments in timed artificial insemination (TAI) protocols and use of presynchronization programs that can increase P/AI, even to the point that fertility is higher with some TAI programs as compared with AI after standing estrus. Implementation of a systematic reproductive management program that utilizes efficient TAI programs with optimized management strategies can produce high reproductive indexes combined with healthy cows having high milk production termed "the high fertility cycle". The scientific results that underlie these concepts are presented in this manuscript along with how these ideas can be practically implemented to improve reproductive efficiency on commercial dairy operations.

Keywords: cattle; fertility; timed-AI; dairy cows; management; reproductive tools

1. Introduction

For decades, genetic selection in dairy cattle was primarily focused on milk production. This genetic selection for production, combined with advances in nutrition, management, facilities, and veterinary programs have generated the modern dairy herds with high milk production (9000 to >12,000 kg of milk in a 305-day period). It has been recognized that primary selection for production lead to cows with poorer reproductive efficiency and health traits [1]. During the last two decades, increased selection for traits linked to reproduction combined with the reliability gains that genomics provided for less heritable traits, such as reproduction, has led to tremendous progress among dairy herds regarding genetic potential for reproduction in the modern dairy cow [2–4]. Nevertheless, there are so many factors that affect reproductive efficiency in dairy cattle that a multifaceted approach is required to optimize reproductive performance on high production dairy herds. One approach that has been used with great success on many dairy farms across the globe is to have a systematic reproductive management program that includes timed artificial insemination (TAI) [5,6]. In this review, first, we consider the key physiology that underlies the development of high fertility TAI programs (Section 2) and how this physiology can be practically implemented in TAI programs (Section 3). Specific high-fertility TAI programs are presented in Section 4. Subsequently, key management/cow factors that can alter the efficiency of these reproductive programs are considered (Section 5), and then we conclude with thoughts on practically combining these concepts in programs that optimize reproductive efficiency on dairy herds (Section 6). The goal of this review is to provide scientists, veterinarians, dairy consultants, and dairy producers with up-to-date scientific information on reproductive efficiency in dairy herds that use TAI.

Quantification of reproductive efficiency on dairy farms can be accomplished through a variety of measures. In this review, we primarily use the 21-day pregnancy rate (21 d-PR) because of the utility of this measure for making immediate management decisions on a dairy farm. The 21 d-PR is defined as the percentage of eligible cows that become pregnant every 21 d. The 21 d-PR is most efficiently calculated on a computer. First, the number of eligible cows during each 21-day period must be calculated (i.e., cows past the voluntary waiting period (VWP), that are not pregnant, and not designated as “do not breed”) including whether a cow should be included that is eligible for only a portion of the 21-day period (usually, if eligible for >11 d in a 21-day period they are included). Thus, it would be better to consider “eligible cows” to be “eligible 21-day periods” because a cow can be eligible during multiple 21-d periods before she becomes pregnant. Next, the number of cows that became pregnant during that 21-d period, either due to AI after estrus or TAI, are determined and divided by the number of eligible cows in that 21-day period. Thus, the 21 d-PR can only be determined after definitive pregnancy diagnosis. Two other key measures determine the 21 d-PR on a dairy and should be calculated when evaluating reproductive efficiency on a dairy farm. The 21-day service rate (21 d-SR) is a calculation of the percentage of eligible cows that are serviced (receive AI) during a 21-day period. The 21 d-SR can be calculated immediately after finishing the 21-day period because it does not include determination of whether a cow became pregnant. The final key measure is the pregnancies per artificial insemination (P/AI), inaccurately called conception rate in some circles. The P/AI should be calculated separately for the first AI (first AI P/AI) and second and later AIs (2nd + AIs). This is because programs that yield differences in fertility are generally used for the first vs. later services.

A brief consideration of the link between reproductive efficiency and profitability is appropriate, although this is considered in much greater depth in other manuscripts [7–14]. One key consideration is that cows in the first third of lactation provide greater income over feed cost as compared with cows in the middle or at the end of lactation [15]. In addition, multiparous cows generally have much greater milk production during the first third of their lactation than primiparous cows. Hence, greater milk production per cow per day and efficiency of milk production can be achieved by increasing the percentage of cows in early lactation and the percentage of cows in later lactations (older cows). Thus, one goal of reproductive management programs is to maximize the number of cows that become pregnant early in lactation in order to increase production efficiency and production per cow per day. For instance, [15] reported that a reduction in calving interval of 60 d increased milk production per day (1.51 and 1.11 kg/d) and during entire lactation (~498 and ~366 kg/lactation) in both high-production (12,500 kg in 305 d of lactation) and moderate-production herds (9000 kg in 305 d of lactation), respectively.

An additional key profit generator from efficient reproduction is a reduction in the need for culling high merit cows due to poor reproduction, resulting in either reduced culling or a shift in culling to cows with lower milk production, disease problems such as mastitis, and udder, genetic, or foot and leg issues. Thus, there is an improvement in the overall quality of the herd when reproductive efficiency is improved. Economic benefits may also arise in herds with greater reproductive efficiency due to reduction in reproductive costs, such as costs for semen, reproductive hormones, and veterinary costs such as pregnancy diagnoses, although this will vary with the method used to improve reproductive efficiency [7,11,13]. Finally, as discussed in detail later in this review, greater reproductive efficiency will cause a greater percentage of cows to enter “the high fertility cycle” leading to many benefits in terms of improved health, production, and reproduction [16].

2. Five Key Physiologic Factors That Influence Fertility in Timed Artificial Insemination (TAI) Protocols

Protocols for TAI can be broadly divided into two the following pharmacological bases: (1) Ovsynch-type protocols using gonadotropin releasing-hormone (GnRH) and (2) protocols that use estradiol (E2) compounds plus treatment with progesterone (E2/P4 protocols). Regardless of the hormonal combinations, the overall physiological objectives are similar, as summarized in Figure 1. First, the protocol attempts to synchronize emergence of a new follicular wave either by ovulating a dominant follicle after GnRH treatment or by inhibiting gonadotropins after treatment with E2 compounds plus P4 to induce turnover of follicles in the current follicular wave. Second, circulating P4 is maintained at elevated concentrations during development of the new preovulatory follicular wave. Third, efficient regression of the corpus luteum (CL) using prostaglandin F2α (PGF) minimizes P4 and enhances circulating E2 near TAI. Fourth, a follicle with adequate size and age is synchronously ovulated using either GnRH or E2 to correspond with proper scheduling of TAI. Finally, elevated and consistent circulating P4 is maintained from properly functioning CL generated after the final ovulation.

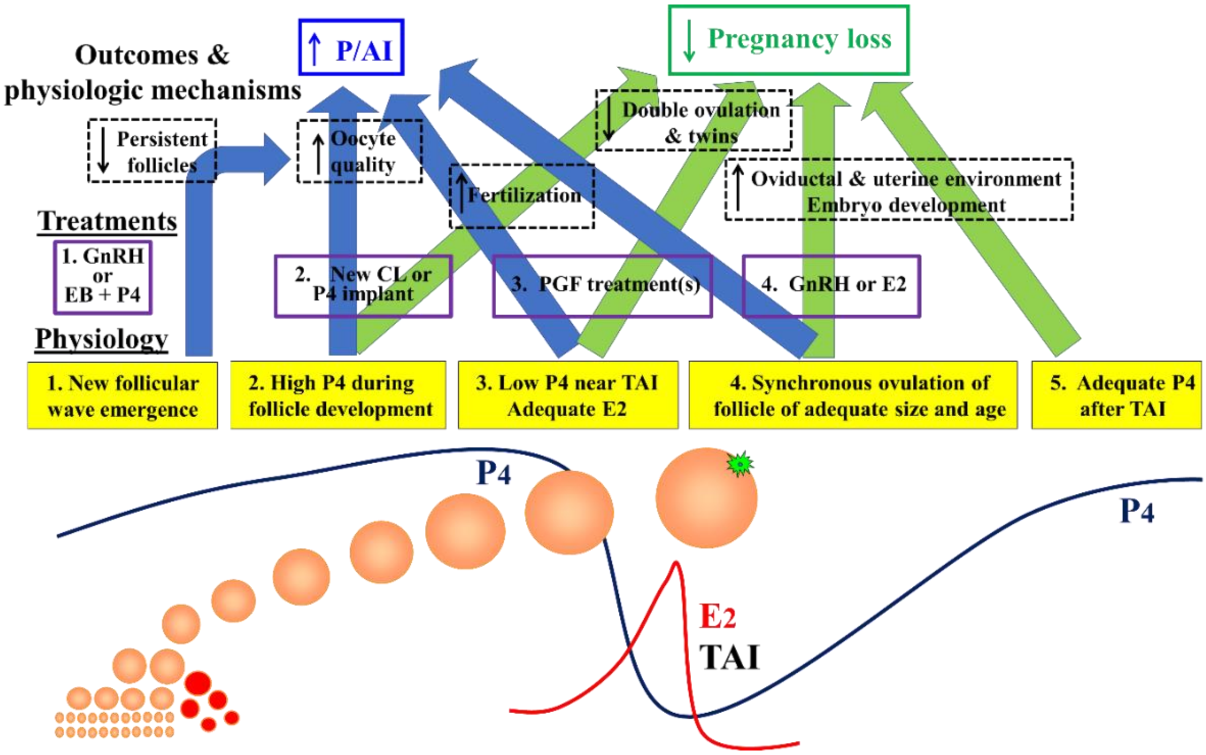

Figure 1. Key physiology (yellow rectangles) that should occur during timed artificial insemination (TAI) protocols in lactating dairy cows. Rectangles with purple lines show common treatments that are used to achieve these results and rectangles with dashed black lines show the mechanisms that produce increased pregnancy per artificial insemination (P/AI) or reduced pregnancy loss in TAI protocols. Corpus luteum (CL), estradiol (E2) benzoate (EB), gonadotropin-releasing hormone (GnRH), progesterone (P4), prostaglandin F2α (PGF).

Synchronized emergence of a new follicular wave minimizes development of persistent follicles during the protocol (Figure 1). Previous studies have shown that ovulation of follicles that have prolonged periods of follicular dominance can dramatically reduce fertility of lactating dairy cows [17]. Prolonged dominance may reduce fertility by decreasing oocyte quality, possibly by allowing premature meiotic resumption due to high luteinizing hormone (LH) pulse frequency [18]. Although oocytes from these persistent follicles appear to be efficiently fertilized, the embryo stops developing prior to the blastocyst stage [19]. In a study that evaluated ovarian dynamics during an E2/P4 TAI protocol, cows without follicle wave emergence at the beginning of the protocol that subsequently ovulated persistent follicles at the end of the protocol had lower P/AI compared to cows that had emergence of a new follicular wave (21 vs. 43%) [17]. Similarly, in GnRH-based protocols, P/AI was greater in cows that ovulated follicles of intermediary size (15–19 mm, 47%) as compared with those ovulating smaller (<14 mm, 36%) or larger (>20 mm, 38%) follicles [20]. Thus, optimizing the follicle size and oocyte quality near TAI depends on the efficiency of the strategy used to initiate emergence of a new follicular wave at the beginning of the protocol.

Secondly, circulating P4 concentrations during preovulatory follicle development have dramatic effects on the subsequent fertility of high-producing lactating dairy cows. Lower circulating P4 during follicular growth, either due to an anovulatory condition [21] or due to the higher catabolism of this hormone in high-producing cows [22,23], is associated with greater pulsatility of LH, which can result in premature resumption of oocyte meiosis and germinal vesicle breakdown, decreasing oocyte quality, and consequently fertility [18,24]. A study by [25] reported that cows yielding over 40 kg/d of milk that were superstimulated to produce multiple ovulations during the first follicular wave (low P4 during follicle growth) had a greater percentage of degenerate embryos (23.5%) as compared with cows superovulated during the first follicular wave but with P4 supplementation (7.1%) or those superovulated during the second follicular wave (3.9%). Moreover, the percentage of transferable embryos was much greater after superovulation during the second follicular wave (88.5%) and the first wave with supplementary P4 (78.6%) as compared with superovulation during the first follicular wave (55.9%). An elegant study evaluated the effect of circulating P4 concentration on embryo quality of cows synchronized and with single ovulation [26], in which the ovulatory follicle developed under a higher or lower circulating P4 milieu. Although fertilization was similar (78% on average), the percentage of grade 1 and 2 embryos (high quality embryos) was greater for cows ovulating follicles that developed under higher P4 (86.5%) than follicles that developed under lower P4 (61.5%). Moreover, cows with higher circulating P4 had fewer degenerate embryos (8.1%) than cows with lower circulating P4 (34.6%).

Many studies have reported greater P/AI when cows were submitted to TAI programs in which a CL was present or the P4 milieu during follicle development was high [27–29]. In a compilation of data from studies of our laboratory [30] using P4-based protocols, that started with estradiol benzoate (EB), GnRH, or both, the presence of CL at the beginning of TAI protocols or at the time of PGF increased P/AI by 15–24% (Figure 2), and the best fertility was achieved when CL was present at both times of the protocol (Figure 2).

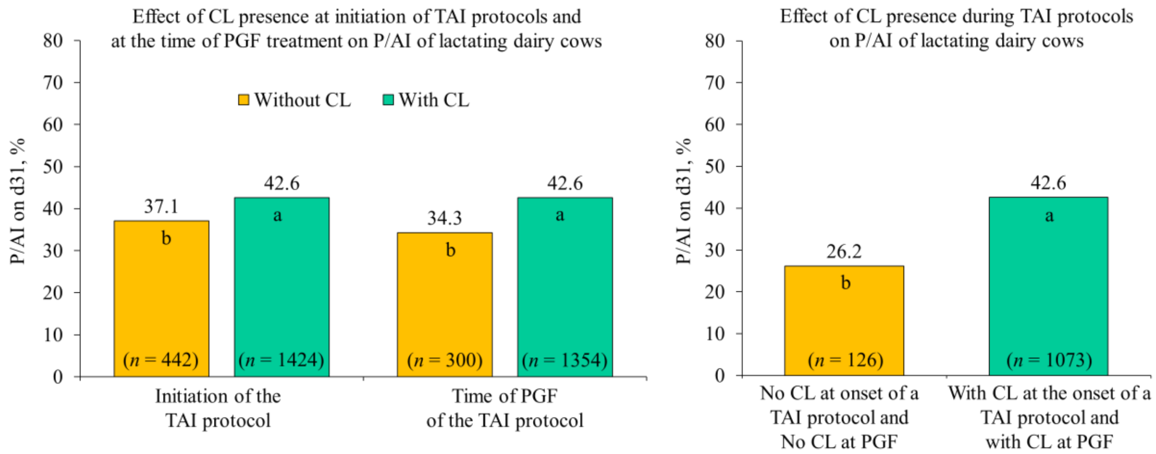

Figure 2. Effect of the presence of corpus luteum (CL) during timed artificial inseminations (TAI) protocols on pregnancy per AI (P/AI) of lactating dairy cows. [a,b] Different letters represent differences ($p < 0.05$). Prostaglandin F2α (PGF). Data from 3 experiments from our lab in which cows were submitted to TAI protocols initiated with progesterone implant and estradiol benzoate, GnRH, or both (from [30])

Another important aspect of circulating P4 concentration during TAI programs is related to double/multiple ovulation and twinning. Double ovulation is more frequent when there is low circulating P4 during the protocol [31, 32] and in cows with higher milk yield [33, 34]. Another factor that influences double ovulation is parity, in which multiple ovulations have been described to be more frequent in multiparous compared to primiparous cows [35], and this can be explained by the greater milk production in multiparous cows. Double ovulation in dairy cattle is undesirable because it increases the incidence of twin pregnancies [36], which are associated with calving problems, calf mortality, freemartins, and problems with calf development. Moreover, twinning is associated with greater pregnancy loss after 30 d of pregnancy [37, 38]. Thus, during preovulatory follicle development, increasing circulating P4 optimizes follicle size and oocyte quality and also can decreases development of co-dominant follicles, multiple ovulation, and twins; this effect may decrease pregnancy loss.

The third key physiologic outcome to achieve during TAI programs is to efficiently regress the CL, having minimal circulating P4 near TAI. Many studies have reported a relationship between circulating P4 concentrations near TAI and ovulation or fertility [17, 20, 39–42] with even small concentrations of P4 near TAI producing dramatic decreases in fertility. For example, in a large data set compiled by [6], there was a 66% relative decrease in P/AI for cows with P4 ≥ 0.4 ng/mL (14%, 161/435) as compared with cows with P4 < 0.4

ng/mL (41%, 1125/2713) at the time of the second GnRH treatment (G2) during the Ovsynch protocol (Figure 3). This outcome is likely to be due to the negative effects of residual P4 on ovulation at the end of a TAI protocol, and on gamete transport [43], hampering fertilization efficiency (Figure 1).

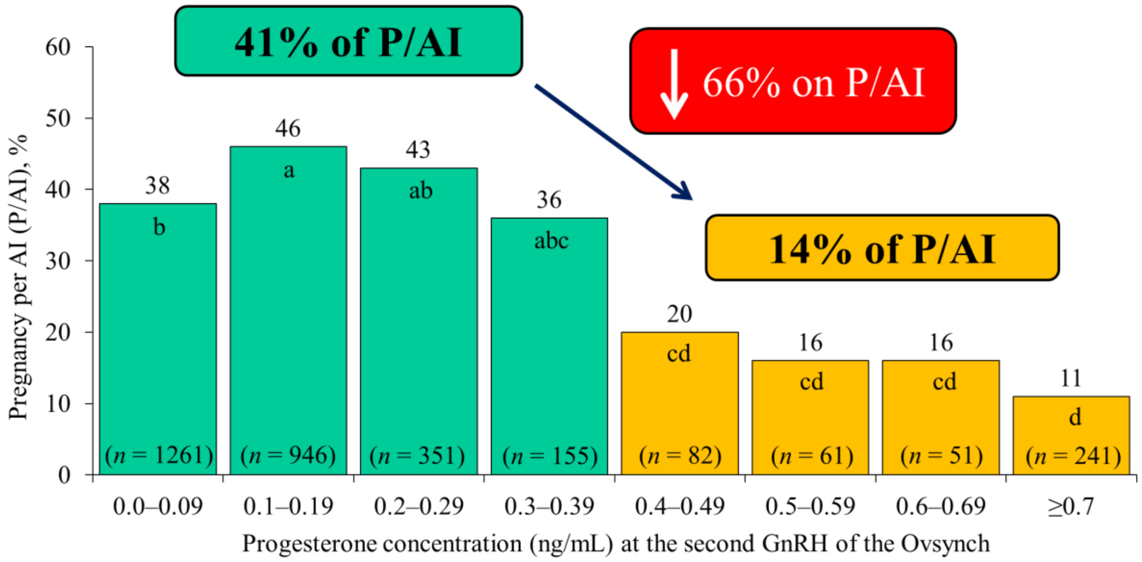

Figure 3. Pregnancy per AI (P/AI) in lactating dairy cows in relation to progesterone concentration at the time of the second gonadotropin releasing-hormone (GnRH) of the Ovsynch protocol. [a,b,c,d] Different letters represent differences ($p < 0.05$). From [6].

This residual P4 near AI is due to a lack of complete luteolysis after the PGF treatment during the protocols, which may occur in 13 to 44% of cows [44,45], and is more problematic when young CL are present at the time of PGF, due to their lower responsiveness to a single treatment with PGF [46]. Therefore, new strategies have been used in TAI programs to overcome the issue of incomplete CL regression at the end of the protocol, and those strategies are discussed later in this manuscript.

The fourth key point is related to optimal size and synchronized ovulation of the follicle in relation to TAI. A more optimal size will result in greater E2 concentrations prior to TAI resulting in greater expression of estrus at the end of TAI protocols. In general, cows that express estrus before TAI achieve greater P/AI, in both Ovsynch-type [47] and E2/P4-based protocols [48]. Another positive effect of expression of estrus is a decrease in pregnancy loss, as reported in a study with 5430 cows, in which cows expressing estrus had ~28% lower pregnancy loss than cows not expressing estrus [48]. Cows expressing estrus may also have greater fertility due to greater likelihood of ovulation [49], although an analysis of only cows that ovulated to an E2/P4 protocol still showed an increase in fertility in cows expressing estrus [48]. Similarly, in cows synchronized with GnRH-based protocols, estrus is related to circulating E2, which is greater for cows ovulating larger follicles at the end of the protocol [20], and higher circulating E2 before AI is also associated with greater fertility [50]. Adequate circulating E2 prior to AI is associated with a differential expression of genes in the endometrium and conceptus, likely producing conditions that are favorable to pregnancy [51], and gamete transport [52]. Thus, ovulation of a more optimal size of follicle will result in greater circulating E2 during proestrus and greater expression of estrus. Use of different strategies to induce ovulation can also result in more synchronized ovulation in relation to TAI and this may help fertility.

Finally, the absolute requirement for P4 (or the CL hormone) in pregnancy maintenance was demonstrated over 100 years ago [53, 54]. The numerous studies investigating whether P4 supplementation increased fertility in lactating dairy cows starting in the 1950s [55, 56], until today, have been less definitive [17, 57–59]. Among 30 trials that we evaluated for a review manuscript [43], the vast majority (25/30) showed a numeric improvement in fertility with P4 supplementation, but only six of these trials showed significance ($p < 0.05$). Only two [58, 60] of these trials that found significance had groups of more than 100 animals per treatment.

The most extensive trials to increase P4 have been done by inducing formation of an accessory CL with hCG or GnRH treatment. When hCG or GnRH is administered on Day 5 after AI, there is generally formation of an accessory CL and increased P4 during the mid-luteal phase [61, 62]. For example, we observed 93% ovulation after treatment with 3300 IU of hCG on Day 5 with an increase in circulating P4 by 3 d after hCG treatment from Day 8 until 16 of the cycle [63]. We performed a meta-analysis of 10 previous trials that analyzed the effect of hCG on fertility in a total of 4397 lactating cows [58]. There was a modest (p = 0.04) increase of 3% comparing hCG (37.0%, 808/2184) to control cows (34.0%, 752/2213). On the basis of these results, we designed a manipulative study that included data from 2979 lactating dairy cows on six commercial dairies [58]. Treatment with hCG 5 d after AI increased (p = 0.01) fertility by 3.5% from 37.3% in controls (566/1519) to 40.8% in hCG-treated cows (596/1460). A surprising observation was that all of the effect of hCG on fertility was due to a dramatic increase in primiparous cows (39.5%, 215/544 control primiparous and 49.7%, 266/535 hCG-treated primiparous) with no change due to hCG treatment in multiparous cows (36.0%, 351/975 control multiparous and 35.7%, 330/925 hCG-treated multiparous). A more recent meta-analysis [59], evaluating about 18,000 cows per treatment group, reported that buserelin (>10 µg) or hCG (>2500 IU) increased P/AI in primiparous cows, particularly, if they had lower fertility (<45% P/AI). Although it is clear that P4 can alter many aspects of endometrial gene expression, uterine histotroph, and increased embryo elongation [64], none of these experiments provide definitive evidence for the mechanisms causing the differences between parities in the fertility effects of hCG. Further research is clearly needed to clarify whether the timing of the P4 increase or other factors can explain the relatively low effect of hCG on fertility and the unexpected parity influence on the hCG effect, or the inconclusive effects of other strategies for P4 supplementation post AI on P/AI in dairy cows.

3. Hormonal Strategies to Improve TAI Protocols

3.1. Hormonal Strategies to Initiate TAI Protocols

There are two main strategies used to initiate TAI protocols and to promote a new follicular wave emergence. The first one aims to synchronize emergence of a new follicular wave by causing atresia of the follicles present in the ovaries due to a negative feedback in follicle stimulating-hormone (FSH) and LH, promoted by a combination of an increase in circulating E2 (from an E2 ester) and P4 (from intravaginal P4 implants, IVP). This is the physiologic basis for initiation of E2/P4-based protocols. The second strategy, which is the basis for Ovsynch-type protocols, stimulates emergence of a new follicular wave by inducing ovulation of a dominant follicle by exogenous GnRH treatment.

The most used E2 ester along with P4 implants on Day 0 (d0) of TAI protocols is EB using a dose of 2 mg. However, this strategy did not properly synchronize emergence of a new follicular wave in more than 25% of lactating dairy cows [17]. Another study reported 24.2% of cows ovulating a persistent follicle at the end of a protocol starting with EB, GnRH, and a P4 implant [65]. Therefore, this issue can impair fertility considering that older/persistent follicles may ovulate overstimulated oocytes, and therefore result in poorer embryo development in lactating dairy cows.

Studies from our laboratory have focused on strategies to initiate TAI protocols that improve synchronization rates and fertility. In one of those studies [17], increasing the dose of EB to 3 mg did not improve synchronization of emergence of a new follicle wave as compared with treatment with 2 mg (71.4 vs. 81.6%, respectively). Moreover, initiating the protocol with EB plus P4 implants in the presence of young (3 d after a GnRH treatment) or dominant follicles (7 d after GnRH) produced similar wave emergence efficiency (78.7 vs. 82.3%). The overall synchronization rate (follicular wave emergence at the beginning and ovulation at the end) for these traditional E2/P4-based protocols was 32 to 60% in studies from our lab, and P/AI was much greater for synchronized cows than cows that were not properly synchronized (61.3 vs. 15.7%) [17].

Another potential negative factor in P4-based TAI protocols that start with E2 protocols is that treatment with EB at the beginning is associated with a greater incidence of luteolysis between d0 and the time of PGF treatment, decreasing the percentage of cows with CL and the number of CL at PGF, which is related to lower circulating P4 during development of the preovulatory follicle [29, 66], compromising fertility. About ~40% of the cows that had a CL present on d0 underwent CL regression between d0 and PGF when EB treatment was at the beginning of a TAI protocol [17, 29, 66]. Table 1 shows a compilation of data from four studies that compared treatment with EB vs. GnRH or EB vs. EB plus GnRH at the beginning of TAI protocols. Treatment with GnRH increased (22.2%) the percentage of cows with CL at the time of PGF, indicating an increase in circulating P4 during the protocol (Table 1).

Table 1. Percentage of lactating dairy cows with a corpus luteum at the time of prostaglandin F2α (PGF) treatment comparing timed artificial insemination (TAI) protocols that utilized only estradiol benzoate (EB) vs. protocols with gonadotropin releasing-hormone (GnRH) either alone or combined with EB.

Study	*n*	EB on d0 of the TAI Protocol	GnRH on d0 of the TAI Protocol	Difference [1]	*p*-Value
[67] Pereira et al. (2013) [2]	1190	43.3%	72.5%	29.1%	<0.01
[68] Pereira et al. (2015) [3]	1474	55.4%	69.5%	14.1%	<0.01
[29] Melo et al. (2016) [3]	417	57.3%	76.4%	19.0%	<0.01
[66] Consentini et al. (2020) [3]	369	56.6%	89.8%	33.2%	<0.01
Total	3450	52.0%	74.3%	22.2%	<0.001

[1] The difference is in absolute percentage points. [2] Comparison between EB vs. EB plus GnRH on d0 of FTAI protocols. [3] Comparison between EB vs. GnRH on d0 of FTAI protocols.

The objective of TAI protocols that begin with GnRH is to induce ovulation, resulting in emergence of a new follicular wave and increasing circulating P4 during preovulatory follicle development. Ovulation to GnRH increased circulating P4 at PGF in multiple studies [41, 65, 69] and increased P/AI [69,70]. Ovulation to GnRH primarily increases P/AI in cows initiating the protocol without CL or with low circulating P4 [66, 69, 70].

Since ovulation after d0 is associated with greater circulating P4 during follicle development and greater P/AI, optimized TAI programs seek to maximize this response. One strategy is to use presynchronization strategies. Another approach to increase ovulation after d0 of a FTAI protocol is related to the dose and analogue of GnRH. When increased the dose of gonadorelin acetate from 100 to 200 µg [71], there was a greater LH peak, and this was particularly important in cows with greater circulating P4, due to an inhibitory effect of P4 on the GnRH-induced LH peak. In fact, in a study using nonlactating Holstein cows, the dose of 100 µg of gonadorelin induced ovulation in only 58.1% of cows with a 7-day-old CL present compared to 95.5% ovulation in cows without a CL [72].

When comparing two analogues of GnRH, studies from our laboratory [73] have shown that 100 µg gonadorelin acetate produced a lower LH peak compared to 10 µg buserelin acetate in Nelore (*Bos indicus*) heifers (5.4 vs. 11.7 ng/mL) and cows (3.4 vs. 6.9 ng/mL) on Day 7 of the estrous cycle. When the dose of these two analogues was doubled, buserelin increased the LH peak in heifers (11.7 vs. 23.2 ng/mL) and cows (6.9 vs. 13.2 ng/mL), whereas the double dose of gonadorelin only increased the LH peak in cows (3.4 vs. 6.3 ng/mL) but not in heifers (5.4 vs. 5.2 ng/mL). Considering the main effects of the study, buserelin induced a greater LH peak and ovulation than gonadorelin [73]. Other studies have reported greater efficiency of buserelin and lecirelin than gonadorelin [74, 75].

Table 2 presents fertility data of studies that compared protocols initiated only with EB vs. GnRH alone or EB plus GnRH, all associated with insertion of a P4 implant. There was greater P/AI (6.1 absolute percentage increase, on average, ranging from 4.5 to 9.5) and 18.5% (relative P/AI) in protocols initiated with GnRH or GnRH plus EB compared to those initiated only with EB. Therefore, it is recommended that TAI protocols in lactating dairy cows should be initiated with GnRH instead of EB, or at least a GnRH treatment should be included at the beginning of the protocol. In addition, doubling the dose of GnRH at the beginning of a TAI protocol may be advantageous to increase the ovulatory response, especially in cows expected to have a CL on d0.

Table 2. Pregnancy per AI (P/AI) of lactating dairy cows submitted to timed artificial insemination (TAI) protocols initiating with estradiol benzoate (EB), gonadotropin releasing-hormone (GnRH), or including a GnRH at the beginning. In all treatments, a progesterone (P4) implant was inserted on d0.

Study	n	Only EB on d0	GnRH on d0	Difference [1]	*p*-Value
[68] Pereira et al. (2015)	1808	30.7%	36.8%	5.9% (19.2%)	<0.05 [2]
[29] Melo et al. (2016)	1035	33.7%	38.2%	4.5% (13.4%)	0.07 [3]
[76] Carneiro et al. (2017)	871	28.7%	38.2%	9.5% (33.1%)	<0.05 [4]
		28.7%	34.5%	5.8% (20.2%)	0.10 [2]
[66] Consentini et al. (2020)	943	37.5%	42.8%	5.3% (14.1%)	NS [3]
		37.5%	42.0%	4.5% (12.0%)	NS [5]
		37.5%	44.3%	6.8% (18.1%)	<0.05 [4]
Total	4657	33.5%	39.5%	6.1% (18.2%)	<0.05

[1] The difference is in absolute percentage points (relative % increase and difference/only EB). [2] Comparison between EB vs. EB plus GnRH on d0 of TAI protocols. [3] Comparison between EB vs. GnRH on d0 of TAI protocols. [4] Comparison between EB vs. EB on d0 plus GnRH on d2 of TAI protocols. [5] Comparison between EB vs. GnRH on d0 and d2 of TAI protocols.

3.2. Intravaginal P4 Implants during TAI Protocols

Although intravaginal P4 implants may be used to improve fertility during TAI protocols, it should be noted that P4 implants do not increase circulating P4 in lactating dairy cows compared to the concentrations that are achieved in cows with an active CL. For example, in the study by [77], circulating P4 on d7 and d14 of an estrous cycle in lactating dairy cows was 2.1 and 4.2 ng/mL, respectively. In contrast, in a study from our laboratory, when comparing two commercial P4 devices (1.9 and 2.0 g of P4) in postpartum cows without CL and producing 40.0 kg of milk per day, the peak of circulating P4 was similar between devices (1.6 ng/mL) and the mean P4 during Day 9 of insertion was 0.85 ng/mL (unpublished data). Studies by [27, 28] reported greater circulating P4 in cows with a CL during the protocol compared to those without CL that were supplemented with two P4 devices (1.38 g), even though P4 supplementation increased circulating P4 to 1.9 ng/mL. Therefore, TAI protocols can be improved by increasing the proportion of cows that initiate the protocol with a CL, either by decreasing anovulatory conditions or by using presynchronization programs.

A study [28], with more than 600 cows per group, compared cows initiating the Ovsynch protocol with a CL present on d0 to cows without CL on d0 supplemented or not with two P4 implants with 1.38 g of P4, each. Cows without CL at the beginning of the protocol had the lowest fertility (31.3%), but P/AI on d32 did not differ between cows with CL and those without CL, but supplemented with P4 (38.4 and 42.2%, respectively). In a study with ~160 cows per group, using E2/P4-based TAI protocols and analyzing only cows that ovulated at the end of the TAI protocol, cows treated with two P4 implants tended to have greater P/AI on d60 compared to cows receiving only one implant (48.1 vs. 37.7%) [78].

In a meta-analysis done in 2015 [79], with 25 studies and more than 16,000 cows supplemented or not with one P4 implant, there was a greater risk of pregnancy on d32 and d60 in P4-supplemented cows, but mainly in cows without CL at the beginning of the TAI protocol. Moreover, P4 supplementation tended to reduce pregnancy loss. It is important to mention that in the meta-analysis, cows inseminated in estrus during the TAI program had no benefit from P4 supplementation [79].

Therefore, besides the need for P4 implants in E2/P4-based protocols, Ovsynch-type protocols may also benefit by the addition of P4 implants due to better synchronization of wave emergence, improved oocyte quality, improved luteolysis after single PGF treatments, and reduced double ovulation and twins.

3.3. Additional PGF Treatment during TAI Protocols

Complete luteolysis is essential for optimal fertility during TAI protocols (Figure 3). Therefore, the following two strategies have been used to achieve this outcome: (1) increasing the dose of PGF [45,69], and (2) adding a second treatment with PGF, in general, 24 h after the first one [39, 41, 44, 45, 80, 81].

Increasing the dose of cloprostenol sodium from 500 to 750 µg during a double-Ovsynch program increased the percentage of multiparous cows with complete luteolysis (87.7 vs. 79.2%) but not primiparous cows (92.8 vs. 89.7%) [69]. Interestingly, doubling the dose of dinoprost tromethamine from 25 to 50 mg during the Ovsynch protocol [45] did not increase the percentage of cows with complete luteolysis (88 vs. 88%) and did not increase P/AI (30.2 vs. 32.4%). However, two treatments with PGF 24 h apart increased the proportion of cows with complete luteolysis (88 vs. 94%) and increased P/AI (30.2 vs. 35.4%).

A meta-analysis with seven studies, 5356 cows analyzed for P/AI and 1856 cows analyzed for luteolysis, evaluated the effect of an additional treatment with PGF during the Ovsynch protocol [82]. This analysis reported that 11.6% (6 to 14%) more cows had complete luteolysis when two PGF treatments were employed, and there was a 4.6% increase in P/AI (13.5% relative P/AI, Table 3).

Table 3. Effect of an additional treatment with prostaglandin F2α (PGF) during the Ovsynch protocol on complete luteolysis at the end of the protocol and on pregnancy per artificial insemination (P/AI).

Item	1 PGF during Ovsynch	2 PGF during Ovsynch	Range	*p*-Value
Complete luteolysis at the end of Ovsynch, % (*n*/*n*)	83.5 (788/944)	95.1 (867/912)	6–14	<0.001
P/AI, % (*n*/*n*)	34.0 (915/2689)	38.6 (1029/2667)	3–9	<0.001

Adapted from [82]. Meta-analysis with seven studies with randomized controlled designs.

3.4. Strategies to Induce Final Ovulation in TAI Programs

Synchronized ovulation of the dominant follicle at the end of TAI protocols can be induced by E2 esters, such as EB or E2 cypionate (EC) [29], or with GnRH, as in Ovsynch [83], potentially altering fertility. The use of EC is convenient because it can be administered concomitant with the final PGF of the protocol or P4 implant withdrawal [29]. However, the timing of ovulation induced by EC is more variable than when GnRH is used [84,85]. On the other hand, when GnRH is used at the end of TAI protocols, optimal fertility is only achieved if cattle are handled one additional time. Moreover, expression of estrus is reduced.

Our group designed a large experiment to compare fertility in response to different inducers of ovulation that were administered at times that were considered to be ideal for fertility in TAI protocols [86]. A protocol for synchronization of ovulation was initiated (d0) after a novel presynchronization with 16.8 µg of buserelin acetate and insertion of a 2.0 g P4 implant, followed by a PGF treatment on d6, and a second PGF on d7, concomitant with the removal of the P4 implant. In Group EC, cows received 1.0 mg EC on d7 as an inducer of ovulation. In Group G, cows received 8.4 µg GnRH at 56 h after the first PGF (16 h before TAI). In Group EC/G, cows received both EC and GnRH. The TAI was performed on d9 (48 h after P4 withdrawal) in all experimental treatments, and pregnancy diagnosis was performed 31 and 60 d after TAI.

Our hypothesis was that the EC/G group would have the greatest P/AI, due to a more synchronized ovulation in response to GnRH plus greater estrus expression because of E2 supplementation, but this idea was not supported. Pregnancy per AI on d31 was not different among the strategies to induce final ovulation (~43%). Other studies that compared EC to GnRH as ovulation inducers also reported similar fertility between treatments [84,85]. Although pregnancy loss tended ($p = 0.09$) to be greater in cows receiving only GnRH as ovulation inducer (Table 4), there was no detectable difference ($p = 0.54$) in P/AI on d60 among treatments. Additionally, when the two groups that received EC (EC and EC/G) were combined, there was lower pregnancy loss compared to cows receiving only GnRH [11.2 (21/188) vs. 19.8 (17/86), $p = 0.05$). The potential for greater pregnancy loss in group G is rationally explained by a lower circulating E2 concentration during proestrus. This could alter the oviductal or uterine environment, potentially increasing pregnancy loss, similar to the increased pregnancy loss observed in cows that did not express estrus during E2/P4 protocols [48]. Therefore, the traditional strategies to induce final ovulation in TAI programs such as GnRH 16 h and EC 48 h before TAI, in general, provide good overall fertility and can be chosen by dairy operations according to management and costs.

Table 4. Pregnancy per artificial insemination (P/AI) 31 and 60 d after timed AI (TAI) and pregnancy loss according to the strategy to induce final ovulation. EC (estradiol cypionate 48 h before TAI), EC/G (estradiol cypionate 48 and GnRH 16 h before TAI), and G (GnRH 16 h before TAI).

Item	Strategy to Induce Final Ovulation in the TAI Protocol			*p-Value*
	EC	EC/G	G	
P/AI on d31, % (*n*/*n*)	42.5 (99/233)	43.0 (95/221)	42.8 (89/208)	0.45
P/AI on d60, % (*n*/*n*)	37.1 (86/232)	37.5 (81/216)	33.7 (69/205)	0.42
Pregnancy loss, % (*n*/*n*)	12.2 (12/98) ^{A}	10.0 (9/90) ^{A}	19.8 (17/86) ^{B}	0.09

^{A,B} Different letters represent differences ($p < 0.05$). When the two groups that received EC (EC and EC/G) were combined, there was lower pregnancy loss compared to cows receiving only GnRH [11.2 (21/188) vs. 19.8 (17/86), $p = 0.05$). Adapted from [86].

4. Fertility Programs: First AI and Resynch Protocols with Improved Fertility

4.1. Protocols for First TAI That Produce Better Fertility than AI to Estrus

It is well known that one of the greatest benefits of reproductive programs using TAI is the increase in service rate. In addition, optimized TAI protocols can provide extra benefits associated with greater P/AI when compared to programs for AI after estrus synchronization/detection. Thus, those optimized TAI protocols have been termed "fertility programs" [5, 6] and four of these programs that are discussed below are presented in Figure 4.

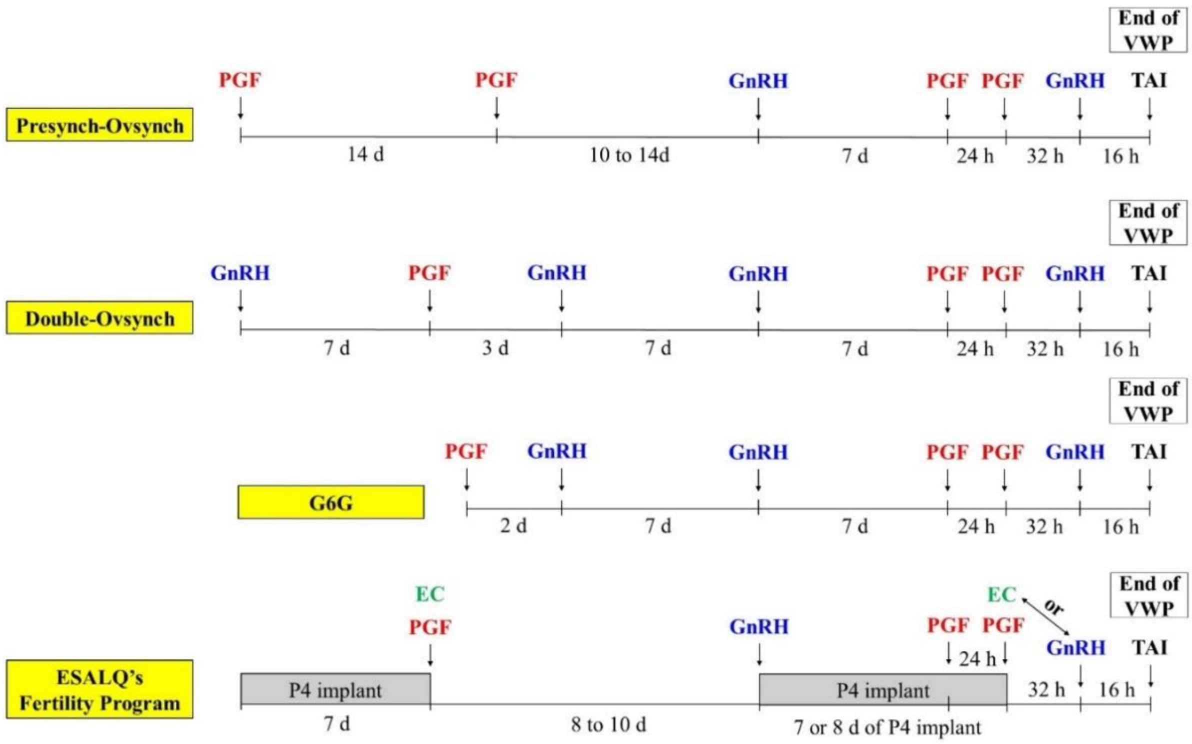

Figure 4. Hormonal treatments used in four fertility programs that are discussed in the text. These programs can be used to achieve 100% service rate and improved pregnancy per artificial insemination compared to the first postpartum timed artificial insemination (TAI) in lactating dairy cows. Voluntary waiting period (VWP), gonadotropin-releasing hormone (GnRH), prostaglandin F2α (PGF), progesterone (P4), and estradiol cypionate (EC).

Fertility programs use many of the principles and strategies discussed above to optimize synchronization, ovarian dynamics, and hormonal environment during TAI protocols. A critical aspect of these programs is the use of a presynchronization strategy in order to ensure that most of the cows initiate the breeding protocol (initiated with GnRH) at an ideal stage of the estrous cycle (6–8 d of the cycle), in which cows have an approximately seven-day-old CL and the first wave dominant follicle that will be responsive to the first GnRH. As previously discussed, increasing ovulation response to the first GnRH will increase the percentage of cows with synchronized emergence of a follicular wave. Causing a new ovulation in the presence of a seven-day-old CL results in cows with two CL throughout the protocol, thus, increasing circulating P4 during the development of the preovulatory follicle. Due to the longer duration of these fertility programs, in general, they are used exclusively for the first postpartum AI, especially because presynchronization strategies can be applied before the end of the VWP, resulting in no delay in receiving the first AI.

One of the earlier presynchronization programs developed was based on PGF administrations, known as the Presynch-Ovsynch protocol (PO) [87]. The PO is based on a PGF treatment, followed by a second PGF 14 days later, and initiation of an Ovsynch-type protocol 10 to 14 days after the second PGF [87–90]. In general, PO increased fertility compared to Ovsynch [88, 89]. In the study by [87] P/AI was 42.8 (113/264) vs. 29.4% (80/272) for PO and Ovsynch, respectively, but it should be mentioned that PO only increased fertility in cyclic cows. Therefore, one disadvantage of the PO is that it is only effective in cyclic cows. Thus, if the percentage of cows that are anovular is high in the herd during early lactation, other strategies that induce ovulation during the presynchronization are likely to be more efficient. In addition, PO does not precisely synchronize cows to be in the ideal day of the cycle on d0 of Ovsynch, because it is based on inducing cows to be in estrus with variable timing after PGF treatments [90, 91]. This may produce a less than ideal timing for starting the Ovsynch protocol (6–8 d). A final aspect to be considered is whether cows that are observed in estrus during the Presynch-Oyvsynch protocol should receive AI. The percentage of cows detected in estrus after the second PGF can be over 50% [90–92], and it is common for dairy operations to inseminate those cows. Although the cows are being inseminated earlier postpartum, fertility is generally lower compared to not breeding cows that show estrus and inseminating all cows at the TAI after PO [93]. Thus, if the herd submits cows to TAI at the end of the PO, this can be considered to be a fertility program, even with the considerations regarding anovulatory condition and accuracy of the presynchronization with PGF.

The second fertility program presented in this manuscript is the G6G or G7G. Commonly, cows receive a PGF treatment and 2 days later a GnRH treatment, 6 or 7 dayds before initiating the Ovsynch protocol. Therefore, the G6G/G7G should increase the percentage of cows at the ideal stage of the estrous cycle to initiate Ovsynch. In addition, the inclusion of GnRH during the presynchronization may benefit anovular cows. The G6G/G7G is commonly used in commercial dairy herds and several studies have tested this strategy [44, 94, 95]. One of them [50] reported greater ovulation to the first GnRH (85 vs. 54%), greater response to PGF (96 vs. 69%), better synchronization rate (92 vs. 69%), and greater P/AI (50 vs. 27%) in cows submitted to G6G compared to Ovsynch initiated at random days of the estrous cycle.

The third fertility program is the double-Ovsynch program (DO) [96]. The DO was developed to optimize the response to hormonal treatments during the breeding Ovsynch protocol, increasing synchronization and the hormonal milieu during follicle development. In the study by [96], when compared to PO, DO decreased the percentage of cows with P4 < 1.0 ng/mL (9.4 vs. 33.3%) at the time of the first GnRH of the breeding Ovsynch, increased circulating P4 at PGF (4.2 vs. 3.2 ng/mL), and increased P/AI (49.7 vs. 41.7%). In a study, which compared the DO and the PO, in relation to circulating P4 concentrations and ovulation to GnRH treatments [97], 94% of the cows in the DO had CL at the time of the first GnRH compared to 68% of the cows in the PO. Moreover, ovulation to the first GnRH was greater in the DO (80%) compared to the PO (69.9%), and the percentage of cows with P4 ≥ 1.0 ng/mL at PGF was greater in the DO than the PO (88 vs. 76%). Another study, with ~1700 cows, which compared the DO and the PO, reported a greater uniformity of intermediary P4 concentrations at first GnRH treatment of the breeding Ovsynch protocol in cows submitted to the DO, and only ~6% of the cows had P4 < 0.5 ng/mL at the beginning of the breeding protocol compared to ~25% in the PO program [98]. There was a clear benefit of the DO to anovular cows and greater incidence of ovulation in response to the presynchronization treatments. In this study, P/AI was greater with the DO (46.3 vs. 36.8%), with a greater effect in primiparous (52.5 vs. 42.3%, $p = 0.02$) than multiparous (40.3 vs. 34.3%, $p = 0.07$) cows. This increased fertility in cows synchronized with DO has also been described in an elegant study that submitted cows to TAI after the DO compared to a protocol designed to increase expression of estrus, with all cows being inseminated at similar days in milk (DIM) (~77 DIM) [99]. Cows in the DO group for first AI had 100% service rate compared to 77.5% in cows bred to estrus. There was also an increase in P/AI from 38.6 to 49.0%, and a 27% relative increase in P/AI when the DO was used (Table 5). Due to the increase in both service rate and P/AI with DO, there was more than a 50% increase in the 21-day PR (Table 5).

Table 5. Effect of Double-Ovsynch or management aimed to inseminate cows in estrus on submission rate, pregnancies per artificial insemination (P/AI) 33 and 63 d after insemination, and percentage of pregnant cows at 33 and 63 d after first insemination in lactating Holstein cows with similar days in milk.

Item	Strategy for first AI		Difference, % (p-Value)
	Double-Ovsynch	Estrus	
No. of cows	294	284	
Submission rate, % (*n*/*n*)	100.0 (294/294)	77.5 (220/284)	29 (<0.01)
P/AI at 33 d, % (*n*/*n*)	49.0 (144/294)	38.6 (85/220)	27 (002)
Pregnant cows at 33 d, % (*n*/*n*)	49.0 (144/294)	29.9 (85/284)	64 (<0.01)
P/AI at 63 d, % (*n*/*n*)	44.6 (131/294)	36.4 (80/220)	23 (0.05)
Pregnant cows at 63 d, % (*n*/*n*)	44.6 (131/294)	28.2 (80/284)	58 (<0.01)

Adapted from [99].

Recent experiments from our laboratory used a novel presynchronization strategy prior to breeding protocols that are initiated with GnRH [86]. The presynchronization was based on E2 and P4, using an intravaginal P4 implant that was removed after 7 d. At the time of P4 implant withdrawal, cows were treated with PGF and EC to induce estrus and ovulation. Eight to 10 d later, the cows were treated with GnRH to initiate the first postpartum TAI protocol and an intravaginal P4 implant was inserted and kept for 7 or 8 d. One d before and at the time of P4 implant removal, PGF treatments were given. Ovulation at the end of the protocol was synchronized with EC (given at the time of P4 implant withdrawal), GnRH (given 16 h prior to FTAI), or both. The P/AI varied from 32 to 58% among six farms, with an overall P/AI of 43%. Compared to regular TAI protocols that were initiated at random stages of the estrous cycle, the fertility program increased P/AI (59.9 vs. 43.9% [*n* = 663] and 46.4 vs. 30.1% [*n* = 416], for data set 1 and 2, respectively).

Therefore, use of TAI protocols can increase SR by allowing AI of all cows without the need for detection of estrus. Use of more optimized TAI protocols have the advantage of increasing P/AI compared to AI to detected estrus and thereby can dramatically increase the percentage of cows that become pregnant during the first week after the end of the VWP.

4.2. Reinsemination Strategies: Reducing the Interval between AI and Optimizing Fertility

After submitting cows to the first postpartum AI, it is imperative to identify nonpregnant cows as soon as possible and to reinseminate them as early as possible. The most common strategies to reinseminate nonpregnant cows in dairy herds are either detecting estrus or inseminating them using TAI programs after nonpregnant diagnosis (NPD). Several strategies were developed to increase reinsemination rates of cows by detection of estrus [100–102]. In general, it was concluded that, in herds with relatively high estrus detection rates and good fertility at AI by estrus, the reproductive performance can be similar to those using or including TAI. However, the herd will always have less control of the interval between inseminations, which can be longer than when submitting cows to TAI Resynch programs.

Regarding resynchronization of ovulation to reinseminate cows using TAI, many studies have been carried out to either understand the physiology or to improve the efficiency of resynchronization (Resynch) programs. It is common to initiate the resynchronization protocol at the time of the NPD. However, there are strategies that initiate the TAI protocol before NPD, which include presynchronization protocols or use P4 supplementation [40, 41, 102–109].

Initiating the resynchronization protocol at NPD diagnosis either at d32 or d39 after a previous AI did not differ in terms of P/AI [40, 110]. However, presynchronization with a GnRH treatment 7 days before the onset of the protocol improved fertility [40,110], and P4 supplementation increased P/AI, especially in cows without CL or with P4 < 1.0 ng/mL at the beginning of the resynchronization protocol [109, 110].

In one study [111], cows received a GnRH treatment 32 days after a previous AI, and 7 days later (time of NPD, 39 d after a previous AI), and were divided into three groups as follows: (1) no CL, CL < 15 mm or cystic (cows initiated an Ovsynch + P4 protocol and were inseminated on d49); (2) cows with CL > 15 mm (cows received the final treatments of the Ovsynch, with PGF at NPD, GnRH 16 h before TAI, and were inseminated on d42); (3) no CL, CL < 15 mm or cystic (cows received a GnRH on d39 and had the Ovsynch initiated on d46, being reinseminated 56 days after the previous AI). Cows in suboptimal conditions for fertility, when received an Ovsycnh + P4 protocol or a presynchronization with a GnRH treatment before the Ovsynch, had their fertility restored [111]. However, the authors did not observe improved performance for Ovsynch + P4 (cows reinseminated earlier) as compared with cows reinseminated after the presynch + Ovsynch program. It should be mentioned that all protocols in that study included only one PGF treatment at the end.

Another study [108] evaluated whether the interbreeding interval could be shortened by using a shortened resynchronization strategy that used treatments based on ovarian structures found at the D32 NPD. Thus, two strategies were compared as follows: (1) Resynch-32, a conventional Resynch-32 (with only one PGF) and TAI at d42 after previous AI and (2) shortened Resynch. Cows were evaluated for ovarian structures at the D32 NPD, cows with a CL ≥ 15 mm and a follicle ≥ 10 mm were treated with two PGF, 24 h apart, and GnRH 16 h before TAI, and received TAI at d35 after previous AI or cows that did not have a CL ≥ 15 mm at NPD were treated with an Ovsynch + P4 protocol that included two PGF and were inseminated on d42 after previous AI. The shortened Resynch strategy reduced time to pregnancy by 16 days (79 vs. 95 days), improved the likelihood of achieving pregnancy (1.18 hazard ratio), but did not affect overall P/AI (33.9 vs. 31.0%) compared to the conventional Resynch-32 protocol [108]. This could be an efficacious Resynch program for herds that use ultrasound for NPD at d32 after AI.

A more recent study [109] compared Resynch-32 (with two PGF treatments) to a management strategy in which nonpregnant cows with CL at NPD received two PGF 24 h apart, and GnRH 16 before TAI (on Day 35), while cows without CL at NPD were enrolled in a Ovsynch + P4 protocol, with two PGF at the end, and were reinseminated 42 days after a previous AI. The authors reported that the management strategy that evaluated ovarian structures to enroll cows in the selected Resynch strategies was a viable alternative to reduce inter-insemination intervals [109].

Most of the studies discussed above included detection of estrus as part of their management strategy. Therefore, the efficacy of the TAI Resynch programs can be confounded by differences in detection of estrus before NPD and the potential that fertility would be different in cows that were detected in estrus compared to if all the cows had entered the Resynch TAI program.

Thus, dairy operations have several options for managing their reinsemination program including using strategies that utilize or do not utilize detection of estrus and TAI programs that reduce the interval between inseminations. In addition, strategies can be used that optimize fertility by identifying cows that were not properly synchronized by the Resynch strategy. Figure 5 shows two common Resynch strategies. The upper strategy is the classical Resynch-32 strategy with Ovsynch initiated at the NPD at d32 after previous AI and TAI done at d42 after previous AI. The lower panel shows a more aggressive Resynch strategy using GnRH treatment at d25 after previous AI and 1 week later (D32) using ultrasound for NPD and to perform a “CL check”. Cows with a CL continue the Ovsynch protocol with TAI at d35 after previous AI (80–85% of non-pregnant cows). Cows without a CL at the NPD would have very low fertility if they continued in the Ovsynch protocol (<10%), and therefore are resynchronized with an Ovsynch + P4 protocol to receive TAI at d42 after previous AI (15–20% of non-pregnant cows). By eliminating non-synchronized cows (no CL at NPD) and by using two doses of PGF in the protocol, the P/AI can be increased by 10–15%, thereby producing a higher fertility Resynch program.

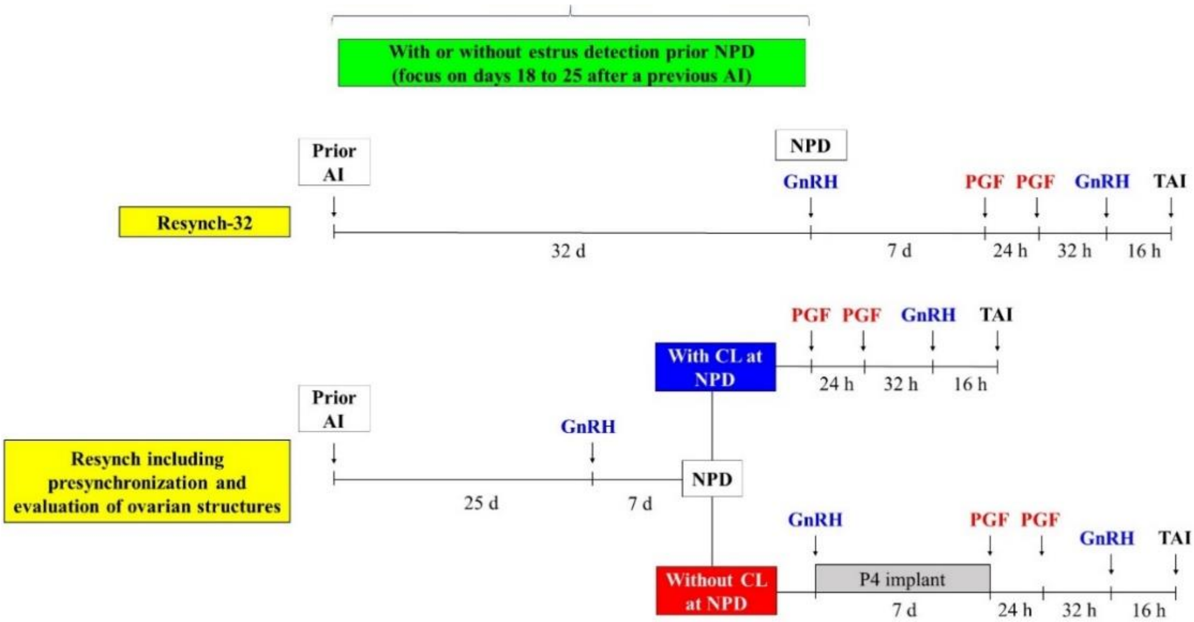

Figure 5. Schematic representation of two commonly used reinsemination programs, designed to reduce the interval between timed artificial insemination (TAI) and optimize fertility. The timing of nonpregnancy diagnosis (NPD) is the same in both protocols (32 days after AI) and is a commonly used timing in herds using transrectal ultrasound for pregnancy diagnosis. The upper strategy is Resynch-32, while the lower strategy is designed to produce earlier TAI in most non-pregnant cows and to improve fertility to the TAI by using a corpus luteum (CL) evaluation at the NPD ultrasound. Gonadotropin-releasing hormone (GnRH), prostaglandin F2α (PGF), progesterone (P4).

5. Key Factors That Alter Reproductive Efficiency in Dairy Herds

As shown in Figure 6, there are multiple factors that determine the success of a reproductive management program. In herds using exclusively TAI programs (right side in purple), anovulation is less of a problem than in herds using exclusively AI to estrus (left side in blue). This is because TAI programs can induce cyclicity leading to AI in all cows, including anovular cows. In herds using TAI, the DIM at first AI can be chosen by the design of the program. In addition, herds using fertility programs such as double-Ovsynch can have high fertility at first TAI. The efficiency of the rebreeding program will depend on the timing of NPD and the design of the Resynch program. In contrast, herds that use AI to estrus are dependent upon cows returning to cyclicity (making anovulation a critical problem) and detection of estrus in these cows with proper timing of AI during all days of the week. Similarly, the rebreeding program is dependent upon detection of estrus. The fertility after AI to estrus may be more controlled by certain factors such as level of milk production than observed in TAI programs that more fully control the follicle size, length of dominance, and hormonal environment [5, 6, 99].

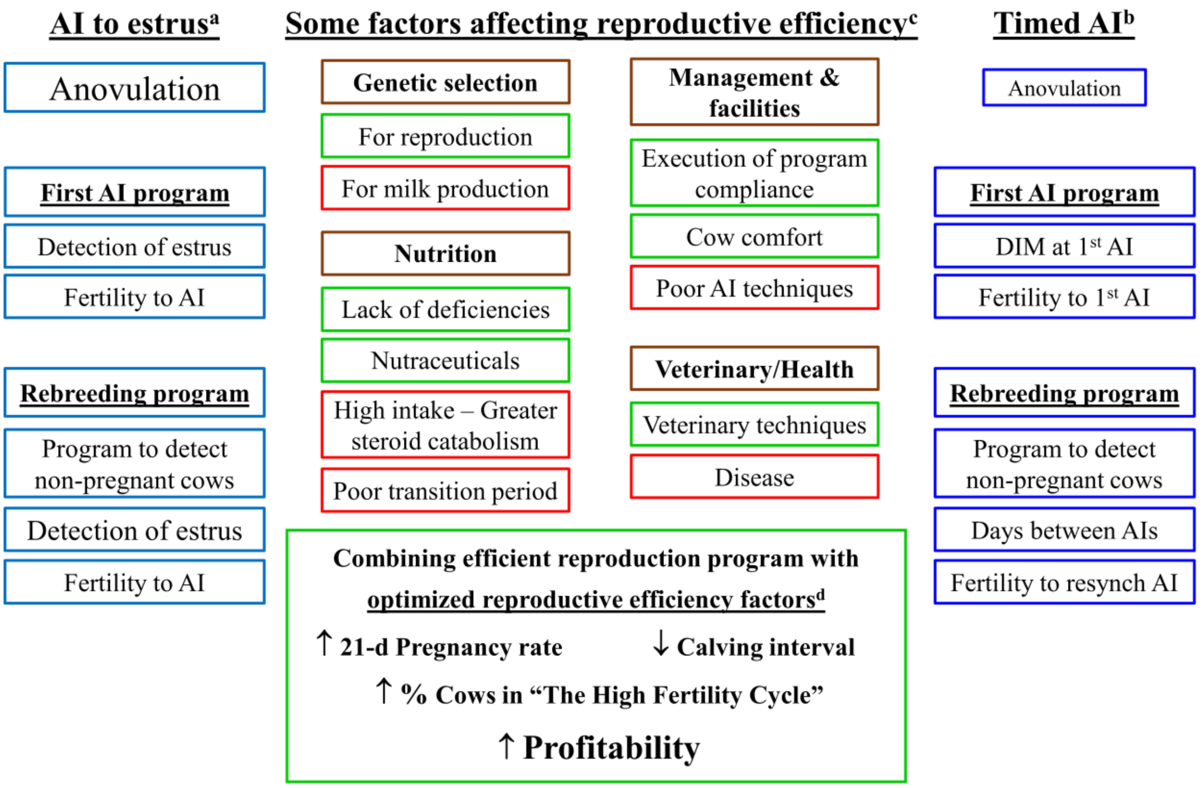

Figure 6. Representation of the key reproductive factors that directly affect a reproductive management program using artificial insemination (AI) to estrus (**left** [a]) or timed AI (**right** [b]). Some of the key factors that affect reproductive efficiency in either or both types of programs are shown (**center** [c]). Factors shown in red squares tend to decrease fertility, whereas factors shown in green rectangles tend to increase fertility. (**Bottom** [d]) Shows that implementation of effective reproductive management programs, combined with optimization of factors that alter reproductive efficiency allows farms to reach the goal of improved reproductive efficiency and profitability on a high-producing dairy farm. Days in milk (DIM).

In the center section of Figure 6 is shown four categories (brown rectangles) of factors that can affect reproductive efficiency in herds that use TAI, AI to estrus, or a combination of the two methods in their reproductive management program. Some of these factors are expected to increase reproductive efficiency (shown in green rectangles), while other factors tend to decrease reproductive efficiency (shown in red rectangles). These factors are discussed in more depth in this section of the manuscript. At the bottom is shown the overall goals for optimizing all of these factors into an efficient reproductive management system so that there is an increase in 21 d-PR, decreased calving interval, increased percentage of cows in the high fertility cycle, and ultimately increased profitability for the dairy operation.

5.1. Genetic Selection for Health and Reproductive Traits

One key change in genetic selection during the last 20 years has been the shift to selection for reproductive and health traits rather than milk production alone [1–4]. This has been a key factor related to the increase in daughter pregnancy rate (DPR) that has been observed since 2000. The more dramatic increase in phenotypic DPR since 2000 compared to genotypic DPR indicates that management factors, along with selection for high fertility genetics, have played a key role in the improvement in phenotypic DPR, including development of systematic breeding programs using TAI [5, 112]. A recent study reinforced the importance of genetics in reproductive performance by evaluating primiparous and multiparous cows based on their genomic DPR, using quartiles [113]. The herds used the same reproductive management program on all cows but found greatly improved reproductive performance using multiple measures (P/AI at first AI, number of services/pregnancies, percentage of cows pregnant at the end of lactation, and interval between calving and pregnancy) for cows in the top 25% for DPR as compared with the lowest 25%. For example, primiparous cows in the top quartile for DPR became pregnant 30 days earlier (165 vs. 195 d) and multiparous cows became pregnant 36 days earlier (140 vs. 176 d) as compared with cows in the lowest quartile [113]. Another recent study [114] randomized ~2400 primiparous cows by genetic merit for fertility (high, medium, and low) and to reproductive management strategy (TAI vs. primarily AI to estrus). Although fertility was greater for TAI (double-Ovsynch TAI) than for AI to estrus, the cows with the highest genetic merit for fertility had greater P/AI than the cows with lower genetic potential for fertility in either type of reproductive management strategy. Thus, selection of cows with high genetic potential for fertility is a strategy that can and should be utilized by all dairy herds regardless of whether they manage reproduction primarily with TAI or using estrus.

5.2. Optimizing Cow Comfort and Reducing Heat Stress

Many physiological, production and reproduction responses can be influenced by management and facilities, and in this section, we focus on heat stress, which has a tremendous negative impact on dairy operations, especially in tropical and subtropical regions. The negative impact of heat stress on reproduction is extensively reported in the literature, with the P/AI of hot seasons being 20 to 50% lower than cooler months of the year [115–117]. However, in addition to reproduction, the negative impacts of heat stress can also impact many other aspects of production, health, and profitability in dairy operations. For example, heat stress of dairy cows can influence dry matter intake [118], animal welfare [119], immune system and health [120], and have carryover effects into the next generation [121]. Effects of heat stress on reproduction can impact reproduction in the short term but also may have effects on oocyte quality for 40 to >100 days after the end of heat stress [122, 123]. Lactating dairy cows under heat stress had reduced oocyte competence and quality, reduced fertilization rates, and poorer embryo quality [23, 124].

Thus, heat abatement strategies are needed on all types of dairies, particularly, in areas with greater heat index and humidity. Some of the most common strategies for cooling cows are fans, shade, natural ventilation, and water-cooling-systems, such as misters and sprinklers. For instance, a study that compared shade with water-cooling systems, over 18 weeks, reported a greater efficiency of water-cooling systems in reducing rectal temperature and respiration rate [125]. In a study using cows on a pasture-based system, cooling for a short period of time (90 min before afternoon milking) or providing shade reduced body temperature and respiration rate compared to control cows; however, combining shade with sprinklers was the most efficient system during days with more intense heat stress (temperature-humidity index ≥69) [126].

The duration and location for cooling cows can vary, with some herds cooling cows in the holding pen, the feed line, or both. Using data from Israel dairy herds [127], cows not cooled compared to those cooled for 7.5 h/d (holding pen + feed line), or 4.5 h/d (only holding pen) had a greater decrease in summer milk production (3.6, 1.6, and 0.6 kg/d, respectively), lower summer/winter production ratio (90.7, 96.1, and 98.5%, respectively), and lower P/AI at first AI of the summer (15, 34, and 34%, respectively).

In herds that are implementing strategies to cool the cows, there are different issues to consider including volume of water used, water size droplet, sprinkler flow rate, length of time spent dampening compared to drying during the cooling cycle, among others, which have been extensively discussed in the scientific literature [128–131].

One exciting aspect, which has been introduced and discussed in the past few years, is the benefit of minimizing heat stress of dry-pregnant cows during the dry period. [121] reported interesting results on the impact of cooling multiparous dams with shade, fans, and water soakers during the dry period on the performance of the daughters and granddaughters. In the study, there was greater culling before first calving and productive life was reduced in daughters from dams that were not cooled compared to dams that were cooled. In addition, more granddaughters were culled before first breeding from dams that were not cooled. In terms of milk production, daughters from heat-stressed dams produced less milk in the first, second, and third lactations (2.2, 2.3, and 6.5 kg/d, respectively), and the granddaughters produced 1.3 kg less milk/d in their first lactation. Thus, the results highlight the importance of cooling cows to optimize their own production and reproduction, but also to improve performance of their offspring.

Lastly, thinking about reproductive management strategies to improve performance during heat stress periods, an interesting manuscript reported that embryo transfer (ET) could be an alternative tool for improving fertility [117]. The fertility was reduced in hot seasons of the years using either TAI or ET; however, P/ET was notably greater than P/AI in hot seasons and had less variation throughout the year compared to P/AI [117]. Thus, use of ET can reduce the negative impact of heat stress on fertility, and this may be especially important in tropical regions.

5.3. Importance of the Transition Period on Subsequent Fertility

Another critical factor for optimizing fertility in dairy herds is related to the transition period, defined as the period from 21 days before calving to 21 days after calving. Issues during the transition period can impact health during the subsequent lactation [132, 133] body condition score (BCS) change, and fertility [134, 135]. These factors impact the likelihood for a cow becoming pregnant early in lactation and entering “the high fertility cycle” [16], as discussed in Section 6.

A healthier transition period is key to the profitability of a dairy herd, due to the usually high incidence of diseases and associated costs [133, 136], high incidence of culling and mortality [137, 138], and the impact on production and reproduction [133, 135]. For example, cows that did not have diseases within the first 21 DIM reached the peak of milk production earlier, produced more milk at peak, and produced ~360 and ~703 more kg of milk in a 305-day period compared to cows with one or more than one disease, respectively (10,453 vs. 10,096 vs. 9750 kg) [133].

Return to cyclicity during the postpartum period is critical for the success of reproductive programs, and the nutrition and management during the transition period is a critical determinant of time to first ovulation. Anovulation at ~60 DIM can range from 5 to 45% in different dairy herds [139] and is greater in cows that have a greater incidence of health problems and cows losing excessive BCS during the postpartum period [133,135,140]. For example, one recent study by [140] evaluated 943 Holstein cows during the postpartum period and reported that, at 50 DIM, a 17.9% prevalence of anovulation in healthy cows (38.4% of the cows) as compared with 29.8% prevalence in cows with one disease (33.7% of the cows) and 39.5% prevalence in those that had more than one disease (27.9% of the cows). As in many dairy herds, most (~60%) cows had at least one health problem in this study.

The timing of return to cyclicity can influence the timing of first AI and also the fertility to the first AI. For example, if a farm uses AI after detection of estrus as the main reproductive strategy for first service, anovulatory cows will receive their first AI later in lactation and may also have lower fertility to first AI as compared with cows that had undergone more cycles prior to first AI. In herds that use TAI, the synchronization strategy may allow early first AI, but the fertility is lower in cows that initiate TAI protocols in the absence of a CL or in low P4 concentrations as illustrated in Figure 1.

Diseases also negatively impact fertility. [132] evaluated 5719 cows from seven herds and reported greater P/AI at first service in healthy cows compared to those having one or more than one disease in early lactation (51.4 vs. 43.3 vs. 34.7%, respectively). Pregnancy loss (PL) was also affected by diseases, with 8% of PL in healthy cows compared to 13.9% and 15.9% in cows with one and more than one disease [132].

A large study that evaluated ~5000 cows reported a long-term negative effect of diseases on reproductive performance [133]. Cows, up to 150 DIM, that had diseases during the first 21 DIM had lower P/AI in early lactation and when inseminated. Moreover, the PL of those cows that had diseases was elevated. The lower P/AI combined with the greater PL among cows with diseases resulted in fewer cows calving/AI from those inseminated, up to 200 DIM. In addition, days open was lower in healthy cows (133.5 d) compared to cows with one disease (145.5 d) or multiple diseases (157.2 d). The percentage of cows calving again in the farm was greater for healthy cows than those with one or multiples diseases (72.8 vs. 59.6 vs. 47.3%, respectively). Therefore, these results show a clear and important impact of diseases during the transition period on early lactation and first AI, and a long-term negative impact on P/AI and PL, affecting the reproductive efficiency of cows during the entire lactation.

Continuing the discussion on the importance of the transition period, several studies have evaluated the physiology of energy and BCS changes of high-producing dairy cows, during the transition period and beginning of lactation. Two of those studies [134, 135], discussed below, had a similar experimental design that focused on evaluations of cows that lost, maintained, or gained BCS during the transition period.

One initial interesting result is that only 50% or fewer cows lost BCS during the transition period. The percentage of cows that gained, maintained, or lost BCS during the transition period was 22.4, 35.8, and 41.8% in one of the studies [134] and 28, 22 and 50% of cows in the other study [135]. In both studies, the BCS in the prepartum or at calving was lower for cows gaining BCS compared to cows that lost BCS in the transition period (2.57 vs. 2.97 [135] and 2.85 vs. 2.93 [134]). These results show that not all high-producing dairy cows lose BCS after calving and that cows with BCS of 3.0 or greater were the most likely to lose BCS.

Barletta et al. [135] reported that postpartum cows with increasing BCSs resumed cyclicity earlier than cows with decreasing BCSs (33.9 vs. 47.1 d), and that 100% of the cows with increasing BCSs as compared with 81.1% of the cows with decreasing BCSs were cyclic around 50 DIM. Moreover, the percentage of cows with more than one disease in early lactation was lower in cows gaining BCS compared to cows losing BCS (39.4 vs. 62.9%). The P/AI at first service was approximately three times greater for cows that gained BCS than cows that lost BCS during the transition period (53.9 vs. 18.3%). In the study by [134], with 1887 Holstein cows, the P/AI at first FTAI postpartum was 25%, 38%, and 84% for cows that lost, maintained, or gained BCS during the postpartum period. Therefore, these studies indicate that cows gaining BCS during the transition period have better health and cyclicity status in early lactation, and excellent fertility in the first service compared to cows losing BCS.

In summary, to optimize the transition period and reduce postpartum BCS loss, it is critical that cows calve with a relatively low BCS. On the one hand, overconditioned cows at calving ($\geq$3.25) will have greater BCS loss, more health problems, later resumption of cyclicity, and lower fertility. Nevertheless, it is not advantageous for overconditioned cows to lose BCS during the dry period in an attempt to reduce BCS at calving. Loss of BCS during the dry period was associated with greater incidence of disease and lower fertility after calving [141]. On the other hand, underconditioned cows at calving ($\leq$2.5) will have much later return to cyclicity, greater health problems, and lower fertility. In addition, other nutritional and management strategies can be implemented to prevent or decrease BCS loss and health problems in early lactation and this will improve performance and health of dairy cows and increase the likelihood of early pregnancy.

5.4. Nutritional Strategies to Optimize Reproductive Performance

Diets for lactating cows should be balanced to provide the required nutrients for milk production and for the reproductive process. Any deficiencies, whether in required vitamins, minerals, or nutrients, could produce nutritional conditions that might compromise reproduction. Nevertheless, provision of nutritional components in excess of requirements are not likely to lead to increased reproductive efficiency. This was clearly demonstrated by overfeeding of phosphorus (P) in dairy cattle diets during the 1980s and 1990s. On the basis of early studies that suggested cattle maintained on P deficient pastures had decreased calf crop, prolonged periods of anestrus, and poor reproductive performance, many nutritionists recommended feeding P in excess of requirements to improve reproductive performance. However, when we evaluated cows fed two levels of P (0.37%, recommended and 0.57%, excess), we found no differences in any measure of reproduction including return to cyclicity, expression of estrus, length of estrus, P/AI, or time to pregnancy [142–144]. Thus, although deficiencies of many nutrients may reduce reproductive performance, supplementing nutrients in excess of requirements may be expensive and is unlikely to improve fertility.

Nutrition during the dry period can have important implications for subsequent reproductive performance. A retrospective analysis of seven studies that compared higher energy diets and controlled energy diets (higher fiber) found that cows fed higher energy diets during the prepartum period had lower DIM during the postpartum period, greater BCS loss, and increased days open [145]. Thus, higher energy diets during the dry period should not be recommended. In some herds, vitamin E may be limiting during the dry period. In a study from our lab, dry dairy cows were receiving supplementation with less than the dietary recommendation for vitamin E and were randomized to either receive no treatment or to be treated weekly with three injections of 1000 IU each of DL-α-tocopherol administered during the last 3 prepartum weeks [146]. Vitamin E supplementation reduced the incidence of retained placenta (13.5 vs. 20.1%) and stillbirth (6.8 vs. 14.9%). Additionally, after first postpartum AI, for cows receiving vitamin E, pregnancy loss was reduced (12.5 vs. 20.5%) and considering all inseminations up to 200 DIM, Ps/AIs on Day 30 (38.4 vs. 34.5%) and Day 60 (32.8 vs. 26.9%) were greater ($p < 0.05$) and the pregnancy loss was lower (14.5 vs. 21.5%) as compared with cows who did not receive vitamin E. In addition, cows treated with the prepartum vitamin E injections had reduced days open (126 vs. 137 d). Thus, delivery of nutrients during the dry period, including vitamin E, need to meet requirements or there can be fairly severe consequences near calving with increased stillbirth and retained placenta, and subsequent reduction in reproductive performance. In this study, cows supplemented with vitamin E did not have increased milk production suggesting that the requirement for vitamin E, and possibly other nutrients, may differ for reproductive traits compared to production traits.

Hypocalcemia is another condition that is tied to the nutritional program during the transition period and can have important consequences for subsequent reproductive performance. In U.S. dairy herds, clinical hypocalcemia (<1.4 mM total calcium concentration) occurs in 5–10% of dairy cows, with subclinical hypocalcemia (1.4–2.0 mM total calcium concentration) occurring in an additional 50% of dairy cows [147]. Hypocalcemia in dairy cows is considered to be a gateway to disorders of the immune system and metabolism, leading to health and metabolic issues in periparturient dairy cows. The focus of recent research has been on the association of hypocalcemia with neutrophil function and development of metritis [148]. Recent studies have reported that cows developing clinical or subclinical hypocalcemia had decreased dry matter intake, greater negative energy balance, impaired immune function, increased risk of health problems, increased risk of metritis, decreased neutrophil number and activity, and decreased fertility and reproductive performance [147–150].

One of the most common and efficient strategy to reduce incidence of hypocalcemia is the use of acidogenic diets during the prepartum period. A recent meta-analysis [151] showed that a reduction in the dietary cation-anion difference (DCAD) in the prepartum period increased Ca concentration in the peripartum period in both primiparous and multiparous cows and increased milk production. The same meta-analysis reported that decreased DCAD in the diets reduced retained placenta and metritis and reduced health problems per cow in both multiparous and primiparous [151]. The DCAD does not need to be lower than −150 mEq/Kg of dry matter [151]; however, it is important to monitor the efficiency of the strategy by measuring urine pH in prepartum cows, to determine if the diet is inducing metabolic acidosis. Thus, using negative DCAD diets during the prepartum period is the most scientifically justified strategy for reducing hypocalcemia in dairy cattle.

Another nutritional strategy that has been evaluated for improving health, production, and reproduction is the inclusion of choline and methionine in the diets of lactating dairy cows. It is reported that increasing rumen-protected choline (RPC) in the prepartum diet increased pre- and postpartum DIM and milk production [152]. Moreover, RPC in the prepartum diet has been reported to decrease inflammation pre and postpartum and improved immune function, as evidenced by a greater proportion of neutrophils undergoing phagocytosis and oxidative burst in the postpartum period [153]. Feeding RPC to cows during pre and postpartum periods reduced the incidence of clinical ketosis, mastitis, and morbidity; however, in primiparous, RPC in the prepartum increased cases of fever and metritis [154]. Another nutrient that has been evaluated for effects on reproduction is feeding rumen-protected methionine (RPM). There is a consistent increase in milk protein percentage and protein yield by feeding RPM. In addition, methionine concentrations appear to be associated with optimal early embryonic development [144, 155–157], and modulation of gene expression in early bovine embryos [156]. In addition, [158] fed RPM from 30 DIM until d60 of pregnancy and found that embryo size was increased in multiparous cows supplemented with RPM, as evidenced by greater increased embryonic abdominal diameter and volume and amniotic vesicle volume. There was no significant effect of RPM on P/AI, however, pregnancy loss was decreased by RPM feeding in multiparous cows [158]. In addition, feeding polyunsaturated fatty acids (PUFA) has been found to increase fertility in some experiments. For example, a study [159] with more than 700 dairy cows fed marine algae that was rich in PUFA, i.e., docosahexaenoic acid, daily from 27 to 147 DIM, reported increased P/AI in both primiparous and multiparous cows (41.6 vs. 30.7%) and a reduction in days to pregnancy by 22 d (102 vs. 124 d). Thus, RPC, RPM, and PUFAs may be considered to be “nutraceuticals” that can improve reproductive performance when included in diets of lactating dairy cows.

6. Implementation of Efficient Reproductive Management Programs: Achieving the High Fertility Cycle

On the basis of the data discussed above related to BCS loss and fertility [134,135], the research group of Richard Pursley at Michigan State University introduced the concept of “the high fertility cycle” [16]. They experimentally explored this idea in a study of 851 lactating Holstein cows (primiparous and multiparous), with an average milk production of 42 kg per d. Cows that had a calving interval (CI) of 13 months (~395 d) had lower BCS at parturition (≤2.7) as compared with cows with greater CI. Moreover, all cows with a 13-month CI maintained or gained BCS after calving [16]. Conversely, all cows with CI > 14 months had BCS ≥ 3.1 at parturition and all of them lost 0.5 or more points of BCS after calving [16].

Following a similar pattern as shown previously [134,135], the authors observed fewer health issues (7%) in cows that maintained or gained BCS as compared with those losing ≥0.5 points of BCS (30%). In addition, P/AI was greater (50.0 vs. 39.9%) and PL was lower (0.0 vs. 8.3%) for cows maintaining or gaining BCS as compared with those that lost BCS during postpartum [16].

Thus, these results are consistent with the concept that cows that become pregnant earlier postpartum (prior to 130–150 DIM), in other words, achieving a CI close to 13 months, it is likely that they will not be overconditioned at the time of calving, and therefore will have reduced BCS loss after calving and have fewer health issues. Of special importance to this review, those cows will have improved fertility and reduced pregnancy loss, allowing them to become pregnant earlier in lactation and thereby entering the high fertility cycle.

Figure 7 attempts to synthesize all of the ideas discussed in this manuscript into a concluding figure that illustrates how to optimize reproductive efficiency in dairy herds that use TAI. A key concept is that a systematic program is designed by the management team ,and then carried out on a consistent basis each week. The overall goal is to achieve a 21 d-PR above 25% with over 80% of cows pregnant by 150 DIM, thus, thrusting most cows in the herd into the high fertility cycle.

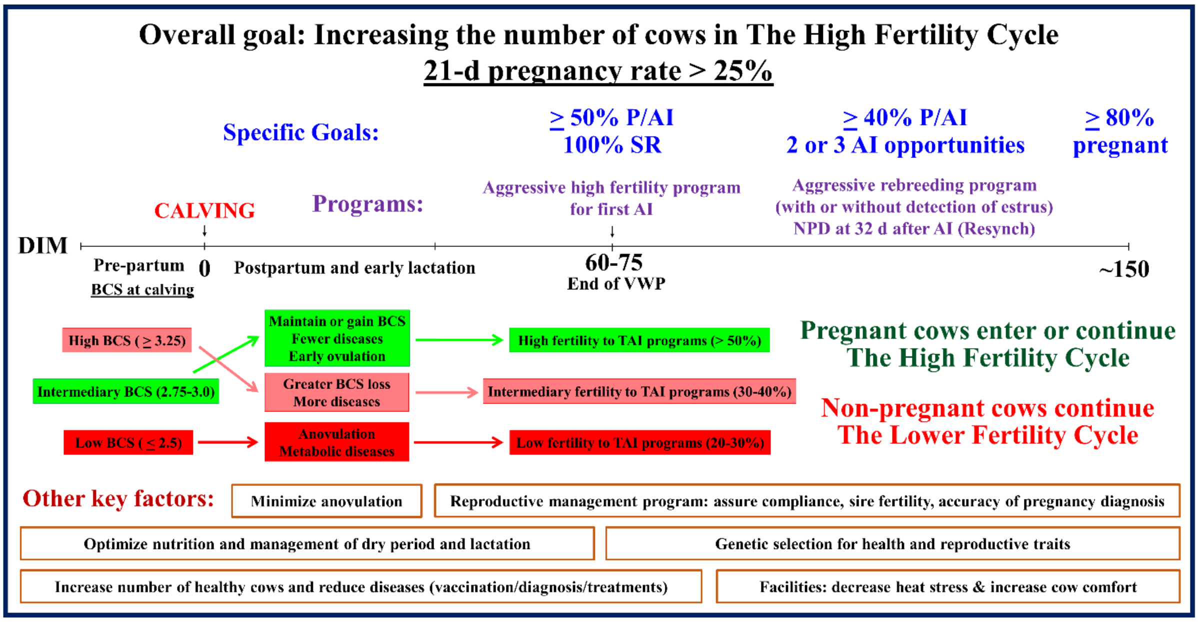

Figure 7. Concluding figure on how to increase the number of cows entering "the high fertility cycle", including physiological aspects, reproductive management strategies, and factors that impact reproductive performance. Body condition score (BCS), voluntary waiting period (VWP), timed AI (TAI), nonpregnancy diagnosis (NPD), days in milk (DIM), and pregnancy per AI (P/AI).

The first thing to consider when designing this program is how to achieve a cow with the optimal physiology to respond to a TAI or estrus detection program with high fertility. One of the critical factors to consider is achieving an intermediate BCS of 2.7–3.0 in cows as they approach calving. The nutrition and management programs need to be optimized to have excellent nutrition, health, and cow comfort, thereby reducing anovulation and increasing likelihood of cows achieving high fertility. As shown in Figure 7, the herd health program should be efficient in preventing, diagnosing, and treating diseases in order to maximize number of healthy cows that enter the first AI program.

The reproductive program used at first AI is critical for assuring the proper CI for the majority of cows. In most dairy farms, it would be advantageous to use a high fertility TAI program at first AI. This type of program can produce more than 50% pregnancies at first AI, with all cows receiving first AI at the end of the VWP. These fertility programs have been developed based on optimization of the physiology that produces high fertility. This includes a presynchronization strategy to induce cyclicity and assure that cows are at the proper reproductive state during the breeding protocols (Figure 4), promoting superior fertility as compared with strategies that inseminate cow based on standing estrus [6,99]. Nevertheless, some of the key cow factors prior to first AI, such as BCS loss, can produce subfertility results even to optimized TAI programs. In addition, other key reproductive factors must be considered, such as fertility of sires and use of proper AI technique, including thawing of semen, when suboptimal results are obtained in herds using fertility protocols at first AI, such as double-Ovsynch.

When cows do not become pregnant at first service postpartum, they need to be rapidly identified as nonpregnant and reinseminated. Therefore, to shorten the interbreeding interval, herds can detect estrus for second and greater services, although the success of this strategy will rely on the efficiency of the herd in detecting estrus and on the fertility of the cows inseminated in estrus. Alternatively, insemination of nonpregnant cows can be performed after resynchronization of ovulation using TAI protocols (commonly termed Resynch), usually initiated at the time of, or before, the NPD (Figures 5 and 6) [40, 107, 111]. The 75 to 90 d from the first TAI to 150 DIM are critical; rebreeding strategies should be aggressive and optimized to achieve at least a two- or three-times higher fertility rate of AIs prior to 150 DIM. Rebreeding strategies that achieve 40% or more P/AI should ensure over 80% of cows are pregnant by 150 DIM. Programs should be designed in which fewer cows become pregnant after 150 DIM because these cows are likely to have excessive BCS at the next calving and are likely to not achieve the high fertility cycle.

Tools are available to dairy producers and veterinarians to achieve the high fertility cycle in high producing dairy herds. Some of these tools, such as optimized TAI protocols, have been extensively discussed in this manuscript and should be readily implemented in a dairy herd. Other tools for detection of estrus, such as activity monitors, tail-paint, and estrus detection patches are also available for herds that want to increase efficiency of estrus detection. These tools are likely to increase service rate but are unlikely to change P/AI directly, since they only allow more accurate detection of cows in estrus but do not change the underlying reproductive physiology. A more holistic or complete view of the reproductive management program also needs to be achieved by the management team in charge of optimizing reproduction. Many important nutritional, health, and facility details may be overlooked for their critical role in driving reproductive efficiency. The successful team should utilize the tools that match their reproductive management strategy and attempt to optimize all aspects of the program. The final detail that is important is: consistency, consistency, consistency.

Author Contributions: Conceptualization, C.E.C.C., M.C.W., and R.S.; data curation, C.E.C.C.; writing—original draft preparation, C.E.C.C.; writing—review and editing, M.C.W. and R.S.; supervision, R.S. All authors have read and agreed to the published version of the manuscript.

Funding: Results from Consentini et al. [66,86] were from experiments supported by a grant from São Paulo Research Foundation (FAPESP, São Paulo, Brazil) number 2018/03798-7, and also by scholarship from FAPESP (2017/15904-3). The National Council for Scientific and Technological Development (CNPq, Brasília, Brazil) and the Coordination for the Improvement of Higher Education Personnel (CAPES, Brasília, Brazil) supported Carlos Consentini and other students with scholarships.

Institutional Review Board Statement: The Animal Care and Use Committee of "Luiz de Queiroz" College of Agriculture of University of São Paulo (ESALQ/USP) approved all procedures involving cows in the study from Consentini et al. protocol #2017.5.11620.11.3).

Acknowledgments: The authors were supported by scholarships from the National Council for Scientific and Technological Development (CNPq, Brasília, Brazil), the Coordination for the Improvement of Higher Education Personnel (CAPES, Brasília, Brazil), and from the São Paulo Research Foundation (FAPESP, São Paulo, Brazil). Some of the data presented in this manuscript were from studies supported by a grant from FAPESP (2018/03798-7).

Conflicts of Interest: The authors declare no conflict of interest.

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CHAPTER 2 (TO BE SUBMITTED FOR JOURNAL OF DAIRY SCIENCE): FERTILITY PROGRAMS FOR LACTATING DAIRY COWS: A NOVEL PRESYNCH + TIMED AI PROGRAM (DOUBLE E-SYNCH) PRODUCES SIMILAR OVARIAN DYNAMICS, SYNCHRONIZATION, AND FERTILITY AS DOUBLE-OVSYNCH

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Abstract

Fertility programs were implemented for the first postpartum timed-AI (TAI) in 800 (primiparous and multiparous) lactating dairy cows, evaluating 2 presynchronization (presynch) strategies and 2 TAI protocols, in a 2×2 factorial design. Weekly, cows were enrolled into 1 of 4 groups (Ovs+Ovs, Ovs+OvsP4/E2, PreP4/E2+Ovs and PreP4/E2+OvsP4/E2). On d-17 (34 ± 3 days in milk), the Ovs presynch initiated with 10 µg buserelin acetate (GnRH), and cows received 0.5 mg cloprostenol (PGF) on d-10, and 10 µg GnRH on d-7. The PreP4/E2 presynch initiated on d-17 with a used 2 g P4 implant. On d-10, implant was removed and 0.5 mg PGF and 1 mg E2 cypionate (EC) were given. For TAI protocols, Ovs was Ovsynch: d0: 20 µg GnRH (double dose), d7: PGF, d8: PGF, d9.5: 10 µg GnRH, and d10: TAI (16 h after GnRH). Cows submitted to OvsP4/E2 received on d0: 20 µg GnRH (double dose) and a 2 g P4 implant, d7: PGF, d8: P4 implant removal, PGF and EC, and d10: TAI. For all cows, ultrasound was performed on d-17, d0, d7 and d17, and expression of estrus until TAI was evaluated. The presence of CL on d-17 (average = 68.8%) was similar among treatments and parity. Presence of CL on d0 of TAI protocols was high, and Ovs as a presynch slightly increased the proportion of cows with CL (95.5 vs. 90.8%). However, at the first PGF of breeding protocols, there was no effect of presynch, and 99.0% (396/400) of the cows had at least 1 CL. Ovulation after d0 was greater in cows submitted to PreP4/E2 than Ovs (72.0 vs. 64.3%), and those ovulating had greater P/AI (51.0 vs. 41.6%). Overall, multiple ovulation after TAI was low and similar between TAI protocols and presynch strategies (7.2% [54/753]). Expression of estrus in OvsP4/E2 protocols was greater than Ovs (69.4 vs. 41.5%), and an interaction was detected, in which cows not expressing estrus ovulated more after TAI in Ovs compared to OvsP4/E2 protocol (93.2 vs. 77.7%). Cows expressing estrus had greater P/AI in both Ovs (58.3 vs. 45.3%) and OvsP4/E2 (58.2 vs. 30.9%), but there was an interaction, and cows not showing estrus had greater P/AI on Ovs compared to OvsP4/E2 (45.3 vs. 30.9%). There was no interaction between presynch and TAI protocol on P/AI on d32 (48.4, 49.7, 53.3, and 52.5% for Ovs+Ovs [Double-Ovsynch], Ovs+OvsP4/E2, PreP4/E2+Ovs and PreP4/E2+OvsP4/E2 [Double E-Synch], respectively), and no differences on pregnancy loss between days 32 and 90. In summary, the two presynchronization strategies and both TAI protocols, despite differences in pharmacological bases, induced similar and well-controlled ovarian dynamics, high synchronization, and excellent fertility outcomes, providing 4 outstanding options of high fertility TAI programs.

Keywords: dairy cow, timed-AI, fertility

Introduction

Timed-artificial insemination (**TAI**) programs are divided within two main pharmacological bases: Ovsynch-type programs, and estradiol (**E2**) plus progesterone (**P4**)-based. Moreover, combinations of both and hormonal manipulations have been implemented to increase fertility. Currently, it is established that TAI programs including a presynchronization, the so-called fertility programs, can increase pregnancy per AI (**P/AI**) compared to cows inseminated by estrus (Santos et al., 2017), and they are an attractive strategy to increase reproductive efficiency, particularly used for the first postpartum service. These fertility programs can increase fertility by improving the hormonal milieu during ovulatory follicle development and overall synchronization (Bello et al., 2006; Wiltbank and Pursley, 2014). The Double-Ovsynch (**DO**) is an example of fertility programs that benefit anovulatory cows, and it is reported to promote better reproductive outcomes than other programs such as Presynch-Ovsynch or cows inseminated by estrus (Herlihy et al., 2012; Ayres et al., 2013; Santos et al., 2017).

Although presynchronization strategies improve reproductive outcomes, there are key factors that impact fertility of lactating dairy cows submitted to TAI programs (Consentini et al., 2021), such as ovulatory follicle age (Monteiro et al., 2015); circulating P4 during follicle development and near AI (Carvalho et al., 2018); ovulatory follicle size, circulating E2 and expression of estrus at the end of TAI protocols (Bello et al., 2006; Souza et al., 2009; Bisinotto et al., 2015). Final ovulation for TAI is induced by two main hormonal treatments: gonadotropin releasing-hormone (**GnRH**) and E2 esters (e.g., E2 cypionate; **EC**), both differing on the timing of ovulation in relation to AI and the proportion of cows expressing estrus (Pancarci et al., 2002; Souza et al., 2009). Nevertheless, expression of estrus is associated with greater fertility in TAI programs inducing final ovulation with EC and GnRH (Bisinotto et al., 2015a; Pereira et al., 2016). On the other hand, estrus is associated with a decrease in milk production during that short period of time (Lopez et al., 2005), and it can be associated with injuries and management issues due to estrus behavior.

In the present study, our general proposal was to increase overall fertility by implementing TAI programs including presynchronization strategies and other adjustments such as a double dose of GnRH at the beginning of breeding protocols and two prostaglandin F2α (**PGF**) treatments. A novel E2/P4-based presynchronization approach, which promoted exciting ovarian dynamics and fertility results in a prior study, was compared to the Ovsynch as presynchronization. In addition, two frequently used TAI protocols were also compared, which was the traditional Ovsynch and a protocol including a P4 implant and final ovulation induced with EC. Thus, four TAI programs were compared, including the DO and the novel program proposed (Double E-Synch; **DES**). The name of the program was based on the pharmacological base of the presynch strategy and the TAI protocol, in which both induce final ovulation with EC, so the Double E refers to the two E2-induced ovulations.

Three main hypotheses were proposed for the study: 1) both presynchronization would promote similar proportion of cows with corpus luteum (**CL**) at the beginning and at the time of first PGF of the breeding protocols, as well as ovulation rate after the first GnRH given on d0 would be similar; 2) the TAI protocol with EC at the end would promote better fertility due to a higher proportion of cows expressing estrus prior to AI; and 3) Expression of estrus at the end of the TAI protocols would be associated with greater fertility in both TAI protocols.

Materials and Methods

The experiment was conducted in a commercial dairy farm located in the Midwest of Brazil, from January to December of 2021. The Animal Care and Use Committee of Luiz de Queiroz College of Agriculture of the University of São Paulo (ESALQ/USP) approved all procedures involving cows in this study (protocol # 5112290720).

Animals and Herd Management

Cows were housed in free stall barns with sand bedding and had free access to water, mineral salt, and were fed *ad libitum* with a total mixed ration diet balanced to meet or exceed the nutritional requirements of lactating dairy cows producing 40 kg/d of milk (National Research Council – NRC, 2001). Throughout the experiment, cows were milked thrice a day and the 305-d average milk production of the herd was 9,500 kg of milk.

A total of 800 lactating Holstein cows (389 multiparous and 411 primiparous) were used for their first TAI postpartum, and the presynchronization protocols (d-17; Figure 1) were initiated at 34 ± 3 days in milk (**DIM**) for cows to be inseminated at 61 ± 3 DIM. The average milk yield for primiparous on d-17, d0 and at TAI were 27.9 ± 0.3, 31.8 ± 0.3 and 33.7 ± 0.3 kg/d, respectively, whereas multiparous yielded 39.4 ± 0.3, 41.1 ± 0.3 and 41.6 ± 0.3 kg/d. The average body condition score (**BCS**) on d-17 was 3.32 ± 0.02 for primiparous and 3.15 ± 0.02 for multiparous.

Treatments and Experimental Design

Following a 2x2 factorial design, weekly cohort of cows were randomly allocated into 1 of 4 TAI programs (Ovs+Ovs, Ovs+OvsP4/E2, PreP4/E2+Ovs and PreP4/E2+OvsP4/E2) according to parity and milk production. Thus, the two presynchronization protocols were Ovs and PreP4/E2, and TAI breeding protocols were Ovs and OvsP4/E2. Cows in the Ovs presynchronization received 10 µg buserelin acetate (GnRH, Maxrelin, GlobalGen, Jaboticabal, Brazil) on d-17, 0.5 mg cloprostenol sodium (PGF, Induscio, GlobalGen) on d-10, and 10 µg GnRH im on d-7. The PreP4/E2 presynchronization initiated on d-17 with a previously used (for 8 or 16 days) 2 g P4 implant (Reprosync, GlobalGen), which was removed on d-10, with treatments of 0.5 mg PGF and 1 mg EC (Cipion, GlobalGen). Regarding breeding protocols, the Ovs initiated with 20 µg GnRH (double-dose) on d0, the first PGF was given on d7 followed by a second dose on d8, and 10 µg GnRH was given on d9.5 (16 h before TAI on d10). Cows submitted to OvsP4/E2 protocol received 20 µg GnRH (double-dose) and an intravaginal 2 g P4 implant on d0, the first PGF on d7 and a second dose on d8, concomitant with P4 implant removal and 1 mg EC, and the TAI was performed on d10. All injectables were given intramuscular. During the entire year of the study, AIs were performed by the same technician and only conventional frozen/thawed Holstein semen was used.

Body Condition Score, Ovarian Structures, Expression of Estrus and Pregnancy Diagnosis

Information related to BCS, ovarian dynamics and expression of estrus were recorded for all cows during the study. The BCS was evaluated at the beginning of presynchronization (d-17) and at TAI (d10). Ultrasound (**US**) to evaluate CL presence and measurements were performed on d-17 (initiation of presynch programs), d0 (beginning of breeding protocols), d7 (time of first PGF) and d17 (7 days after TAI). All cows received a tail-head patch for detection of estrus at the time of first PGF, and were evaluated on d8, d9.5 and at TAI. Cows were considered expressing estrus if the device was activated and/or based on other signs of estrus such as standing mounting.

The first pregnancy diagnosis was performed 32 d after TAI by transrectal ultrasonography of the reproductive tract by confirming an embryo heartbeat. The confirmation of pregnancy was performed by US on d60 and 90 after TAI.

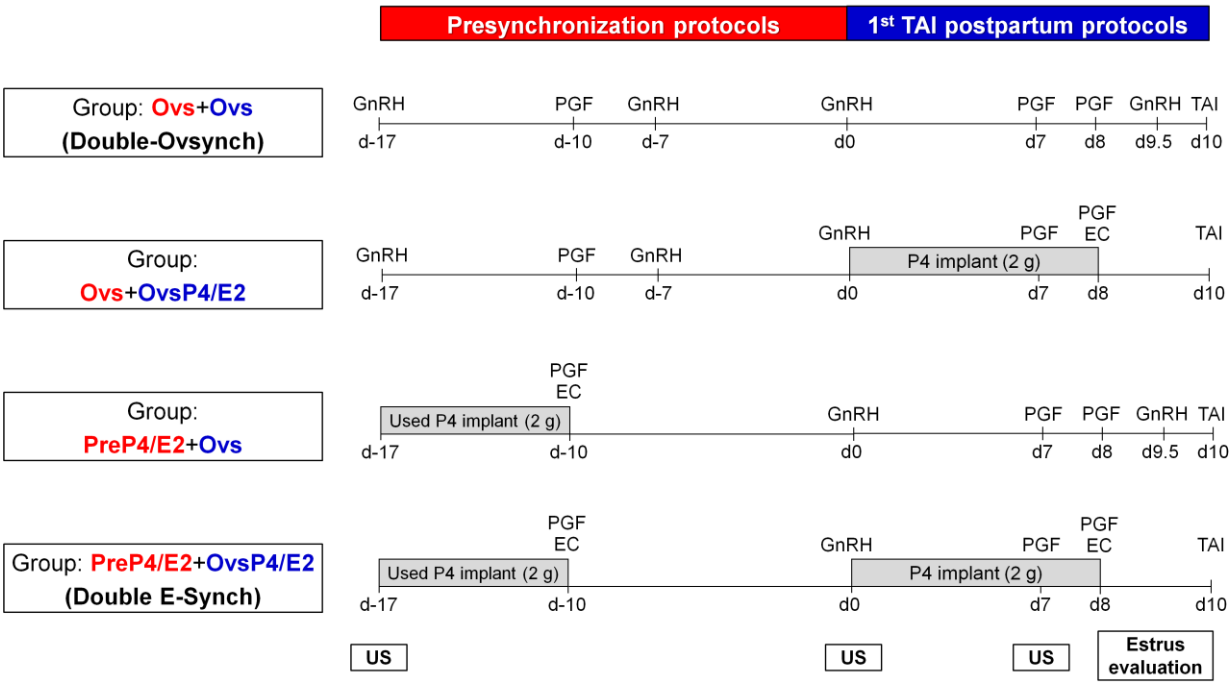

Figure 1. Experimental design with hormonal treatments and procedures performed during the presynchronization and timed-artificial insemination (TAI) protocols. Cows in the Ovs presynchronization received on d-17: GnRH, d-10: PGF and d-7: GnRH. The PreP4/E2 presynchronization received on d-17: previously used P4 implant and d-10: P4 removal, PGF and EC. Regarding breeding protocols, cows in the Ovs received on d0: 20 µg GnRH, d7: PGF, d8: PGF, d9.5: 10 µg GnRH, and d10: TAI (16 h after GnRH). Cows submitted to OvsP4/E2 received on d0: 20 µg GnRH and a 2 g P4 implant, d7: PGF, d8: P4 implant removal, PGF and EC, and d10: TAI. In all cows, ultrasound (US) evaluations were performed on d-17, d0, d7 and d17 (7 days after TAI), and expression of estrus between d8 and TAI was evaluated. Abbreviations: GnRH (buserelin acetate), PGF (prostaglandin F2α, cloprostenol sodium), P4 (progesterone) and EC (estradiol cypionate).

Statistical Analysis

Statistical analyses were performed using the Statistical Analysis System (SAS, Version 9.4 for Windows SAS Institute Inc., Cary, NC). The analyses of binary variables were performed using the GLIMMIX procedure, fitting a binomial distribution with Link Logit function. Additionally, the option ddfm = kenwardroger was included in the model statement to adjust the degrees of freedom for variances.

For presence of CL on d-17, d0 and d7, the model included the effects of presynchronization, parity and BCS. The model for ovulation after d0 included effects of presynchronization, parity, BCS and presence of CL on d0. For expression of estrus, the variables studied were presynchronization, TAI protocol, parity, and ovulation after d0. Regarding ovulation rate and multiple ovulation after TAI, the model included effects of presynchronization, protocol, parity, and expression of estrus. The models for P/AI and pregnancy loss included effects of presynchronization, TAI protocol, parity, ovulation after TAI and expression of estrus. Moreover, for pregnancy loss, effect of multiple ovulation was studied. The interaction between presynchronization strategies and TAI protocols was maintained in the models, and other biologically valuable interactions between treatments and the variables were evaluated, and they are presented and discussed throughout the manuscript. When an interaction was detected, the SLICE command within the LSMEANS was used to interpret the results. Tukey honest significant difference post hoc test was performed to determine differences. Values are presented as percentage and significant differences were declared when $P \leq 0.05$, whereas tendencies were considered when $0.10 > P > 0.05$.

The GLIMMIX procedure was used for regression to model the probability of pregnancy and pregnancy loss according to milk production. Logistic regression curves were created using the coefficients provided by the interactive data analysis from SAS and the formula $Y = \exp(\alpha \text{ x } X + \beta)/[1 + \exp(\alpha \text{ x } X + \beta)]$, where Y = probability of occurrence; exp = exponential; α = slope of the logistic equation; β = intercept of the logistic equation; and X = analyzed outcome.

Results and Discussion

The two presynchronizations strategies and both TAI protocols evaluated in the study, although differing in their pharmacological bases, promoted excellent and similar reproductive outcomes in terms of ovarian dynamics and synchronization, and a relatively high fertility was achieved. This first manuscript presents and discusses the ovarian dynamics, synchronization, and overall fertility outcomes. The second companion paper presents the relationship between milk production, expression of estrus, type of protocol and fertility. Lastly, a third paper discusses the impact of BCS changes and health problems on cows submitted to the fertility programs.

Presence of CL During the Reproductive Programs and Ovulation After First GnRH

The overall presence of CL at the beginning of presynchronization programs (34 DIM) was 68.8% (550/800, Table 1), which is similar to studies reporting ~30% of anovulatory condition at the beginning of lactation, up to 60 DIM (Chebel et al., 2010; Manríquez et al., 2022). Parity and BCS influenced the presence of CL on d-17, with more multiparous with CL compared to primiparous cows (71.7 [279/389] vs. 65.9% [271/411]; $P = 0.03$), and less thinner cows (≤ 2.75) with CL than cows with BCS > 2.75 (62.2 [84/135] vs. 70.1% [466/665]; $P = 0.03$).

All cows were submitted to presynchronization approaches including strategies to induce ovulation, and 93.1% (745/800) of the cows initiated the breeding protocols with CL, and Ovs presynchronization slightly increased the proportion of cows with CL on d0 (Table 1). More multiparous had CL on d0 compared to primiparous cows (95.4 [371/389] vs. 91.0% [374/411]; $P = 0.005$), and at that moment, there was no effect of BCS ≤ or >2.75 on CL incidence (91.9 [124/135] vs. 93.4% [621/665]; $P = 0.34$). Several studies using presynchronization programs described that 80% or less cows had CL on d0 of the breeding protocol (Ayres et al., 2013; Herlihy et al., 2013; Dirandeh et al., 2015). However, the results from our study indicate that both presynchronization strategies were very effective to induce ovulation in cows, consequently, initiating the breeding protocol with CL, similar to previous studies reporting ≥ 90% of cows with CL at first GnRH of breeding protocols in programs such as DO (Ayres et al., 2013; Luchterhand et al., 2019). Although smaller differences are more likely to be detected in the range of 90-95%, which is the case of presence of CL on d0 in our study, we consider that Ovs may have increased the proportion of cows ovulating at the end of the presynchronization due to the following reasons. The Ovs as a presynchronization approach consists in a synchronization of ovulation protocol with known synchronization and ovulatory rates. On the other side, the PreP4/E2 presynchronization only used a P4 implant to maintain a follicle developing to have its ovulation induction at the end. Thus, a follicle turnover during the latter presynchronization protocol could have happened in some cows. Moreover, GnRH as ovulation inducer in Ovs can have promoted greater ovulation than EC in particular classes of cows.

Table 1. Ovarian dynamics during reproductive programs according to the presynchronization strategy: presence of corpus luteum (CL) at the beginning of presynchronization and at first GnRH of breeding timed-artificial insemination (TAI) protocols, ovulation after first GnRH, and presence of CL at first PGF treatment.

Item, % (n/n)	Presynchronization strategy[1]		*P*-value
	Ovsynch	PreP4/E2	
CL at the beginning of presynch	68.5 (274/400)	69.0 (276/400)	0.85
CL at first GnRH of TAI protocols	95.5 (382/400)	90.8 (363/400)	0.04
Ovulation after first GnRH	64.3 (257/400)	72.0 (288/400)	0.04
CL at first PGF of TAI protocols	99.0 (396/400)	98.0 (392/400)	0.54

[1]Cows in the Ovsynch presynchronization received on d-17: GnRH, d-10: PGF and d-7: GnRH. The PreP4/E2 presynchronization received on d-17: used P4 implant and d-10: P4 removal, PGF and EC. All cows initiated the breeding TAI protocols with 20 µg of GnRH on d0 and the first PGF was given on d7.

The ovulation incidence after the GnRH administered on d0 was higher in cows submitted to PreP4/E2 (Table 1), which can be partially explained by the numerically lower CL presence on d0. Indeed, cows without CL on d0 had much greater ovulation than cows with CL present (85.5 vs. 66.8%; P = 0.002). Although we expected high ovulation after d0, due to the use of presynchronizations and doubled the recommended dose of buserelin, ovulatory responses of 57.5% (187/325) and 60.3% (70/116) were reported in previous studies using DO (Giordano et al., 2013; Carvalho et al., 2015). The high proportion of CL at the time of first GnRH (93%) may have contributed to the lack of ovulation in a proportion of cows, since those with CL and high P4 have reduced LH peak and ovulatory responses (Giordano et al., 2012; Carvalho et al, 2015).

One of the most important aspects associated with fertility of TAI programs in lactating dairy cows is the presence of CL and circulating P4 at the time of PGF treatment. Consistently, presence of CL and higher P4 during follicle development has been associated with greater P/AI (Bisinotto et al., 2015a; Consentini et al., 2021), and the reproductive programs implemented in the study were extremely efficient in having 98.5% of cows with at least one CL at the time of PGF treatment of the breeding protocols (Table 1).

Therefore, despite differences on their physiological bases, it can be considered that both presynchronization strategies of this study were very efficient to induce well-controlled ovarian dynamics, assuring adequate P4 (presence of CL) and high synchronization rates during the breeding protocols.

Expression of Estrus at the End of the Breeding Protocols and Ovulation After TAI

There was no difference between presynchronization strategies regarding estrus expression or ovulation at the end of the TAI breeding protocols (Table 2). Moreover, a notable relatively low multiple ovulation after TAI (Table 2) reinforces the fact that the programs implemented in the study were able to optimize CL presence and circulating P4 during development of the preovulatory follicle. It is important to provent multiple ovulations since twinning is undesirable in dairy operations due to an increase in pregnancy loss (Martins et al., 2018), higher proportion of postpartum health problems, and impaired calf development and mortality.

Table 2. Effect of the presynchronization strategy on expression of estrus at the end of timed-artificial insemination (TAI) protocols and ovulation after TAI.

Item, % (n/n)	Presynchronization strategy[1]		*P*-value
	Ovsynch	PreP4/E2	
Estrus by d9.5	17.3 (69/400)	18.0 (72/400)	0.61
Estrus by TAI	54.3 (217/400)	56.3 (225/400)	0.58
Ovulation after TAI	93.3 (373/400)	95.0 (380/400)	0.28
Multiple ovulation after TAI	5.9 (22/373)	8.4 (32/380)	0.15

[1]Cows in the Ovsynch presynchronization received on d-17: GnRH, d-10: PGF and d-7: GnRH. The PreP4/E2 presynchronization received on d-17: used P4 implant and d-10: P4 removal, PGF and EC. All cows initiated the breeding TAI protocols with 20 µg of GnRH on d0 and the first PGF was given on d7.

Regarding breeding protocols, a greater expression of estrus near TAI in cows receiving EC compared to GnRH was expected. In fact, more cows in OvsP4/E2 group expressed estrus by d9.5 and at the time of AI compared to the Ovs breeding protocol (Table 3). A slightly greater proportion of cows submitted to the Ovs protocol ovulated after TAI compared to OvsP4/E2. However, incidence of multiple ovulation did not differ between the TAI protocols (Table 3). The greater ovulation incidence in cows submitted to the Ovs protocol may have happened because when GnRH is used to synchronize the final ovulation, an exogenous LH peak is induced and can stimulate ovulation even in cows with smaller follicles (Sartori et al., 2002) or those that would not have an endogenous LH peak. On the other hand, when EC is the ovulation inducer, the E2 coming from both EC and the dominant follicle should promote the GnRH peak, which will lead to the LH peak. So, a lack of ovulation might happen in cows with smaller follicles and with low circulating E2, not enough to induce a GnRH peak, such as from cows not expressing estrus.

Table 3. Effect of breeding timed-artificial insemination (TAI) protocol on expression of estrus and ovulation after TAI.

Item, % (n/n)	Breeding TAI protocol[1]		*P*-value
	Ovsynch	OvsynchP4/E2	
Estrus by d9.5	10.9 (44/405)	24.6 (97/395)	<0.001
Estrus by TAI	41.5 (168/405)	69.4 (273/395)	<0.001
Ovulation after TAI	96.1 (389/405)	92.2 (364/395)	0.02
Multiple ovulation after TAI	8.0 (31/389)	6.3 (23/364)	0.36

[1]The Ovsynch protocol consisted of: d0: 20 µg GnRH, d7: PGF, d8: PGF, d9.5: 10 µg GnRH, and d10: TAI (16 h after GnRH). Cows submitted to OvsynchP4/E2 received on d0: 20 µg GnRH and a 2 g P4 implant, d7: PGF, d8: P4 implant removal, PGF and EC, and d10: TAI.

In fact, there was an overall effect of estrus on the proportion of cows ovulating, however, when the effect of the TAI protocol was separately studied, the Ovs protocol increased ovulation after TAI in cows not expressing estrus, while ovulation was extremely high in cows expressing estrus, regardless of the TAI protocol (Table 4). Multiple ovulation was not influenced by expression of estrus or interaction between estrus and TAI protocol (Table 4). The present study provided valuable novel information about ovulation after TAI according to type of protocol and expression of estrus in a large number of cows submitted to fertility programs. Previous studies did not report differences on ovulation after TAI between EC and GnRH (Souza et al., 2009; Ferreira et al., 2015). Moreover, the number of cows in those studies was much smaller and the TAI protocols were not optimized.

Table 4. Effect of the breeding timed-artificial insemination (TAI) protocol and expression of estrus on ovulation after TAI.

Item, % (n/n)	Breeding TAI protocol[1]		*P*-value[2]		
	Ovsynch	OvsynchP4/E2	P	E	I
Ovulation after TAI					
No estrus	93.2 (220/236)	77.7 (94/121)	<0.001	<0.001	<0.001
Estrus	100 (168/168)	98.5 (259/273)	0.97		
Multiple ovulation after TAI					
No estrus	9.6 (21/220)	8.5 (8/94)	0.88	0.18	0.83
Estrus	6.0 (10/168)	5.6 (15/269)	0.65		

[1]The Ovsynch protocol consisted of: d0: 20 µg GnRH, d7: PGF, d8: PGF, d9.5: 10 µg GnRH, and d10: TAI (16 h after GnRH). Cows submitted to OvsynchP4/E2 received on d0: 20 µg GnRH and a 2 g P4 implant, d7: PGF, d8: P4 implant removal, PGF and EC, and d10: TAI.

[2]P = effect of TAI protocol within class of cows; E = main effect of expression of estrus; and I = interaction between TAI protocol and estrus expression.

Related to the effect of parity on ovulation after TAI, there was no interaction between parity and TAI protocol, and multiparous had greater ovulation than primiparous (96.7 [376/389] vs. 91.7% [377/411]; *P* = 0.001). Moreover, multiple ovulation was almost 3 times greater in multiparous than primiparous cows (11.4 [43/376] vs. 2.9% [11/377]; *P* < 0.0001), which is expected since milk production is highly correlated with multiple ovulation (Lopez et al., 2005; Martins et al., 2018) and multiparous were producing more milk during the TAI protocol in the present study (41.6 ± 0.31 vs. 33.7 ± 0.25 kg/d).

Interestingly, there was an interaction between parity and TAI protocol on expression of estrus. In the Ovs protocol, primiparous expressed more estrus than multiparous cows. However, there was no effect of parity in the OvsP4/E2 protocol (Figure 2). It is stablished the relationship between milk production and estrus, and as milk production increases the expression of estrus reduces (Lopez et al., 2004). Hence, when the TAI protocol did not include EC as ovulation inducer (Ovsynch group), multiparous cows with greater milk production had a lower expression of estrus. On the other hand, the OvsP4/E2 protocol increased the overall proportion of cows expressing estrus, and there was no effect of parity.

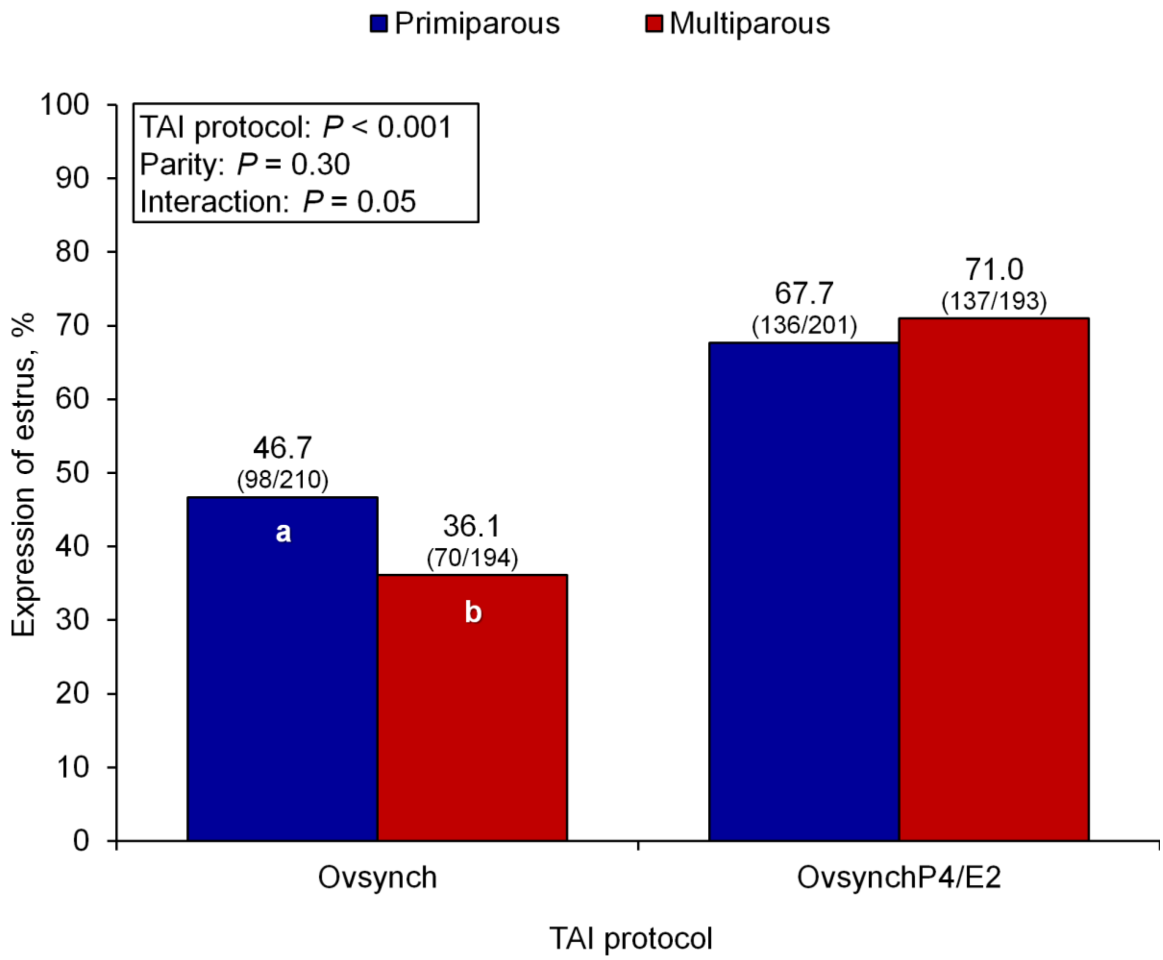

Figure 2. Expression of estrus by the time of timed-artificial insemination (TAI) according to parity and TAI protocol. The Ovsynch protocol consisted of: d0: 20 µg GnRH, d7: PGF, d8: PGF, d9.5: 10 µg GnRH, and d10: TAI. Cows submitted to OvsynchP4/E2 received on d0: 20 µg GnRH and a 2 g P4 implant, d7: PGF, d8: P4 implant removal, PGF and EC, and d10: TAI.

[a,b]Primiparous presented greater expression of estrus than multiparous when submitted to Ovsynch protocol (P = 0.03). When submitted to OvsynchP4/E2, there was no effect of parity (P = 0.54).

To evaluate the effect of milk production on expression of estrus regardless of parity, probability curves were generated for estrus according to milk production within each TAI protocol (Figure 3). Interestingly, the probability of estrus occurrence decreased as milk yield increased only in cows submitted to the Ovs protocol. This result indicates that when EC is used to induce final ovulation (e.g., OvsP4/E2 protocol), the expression of estrus is high, and it is not influenced by milk yield.

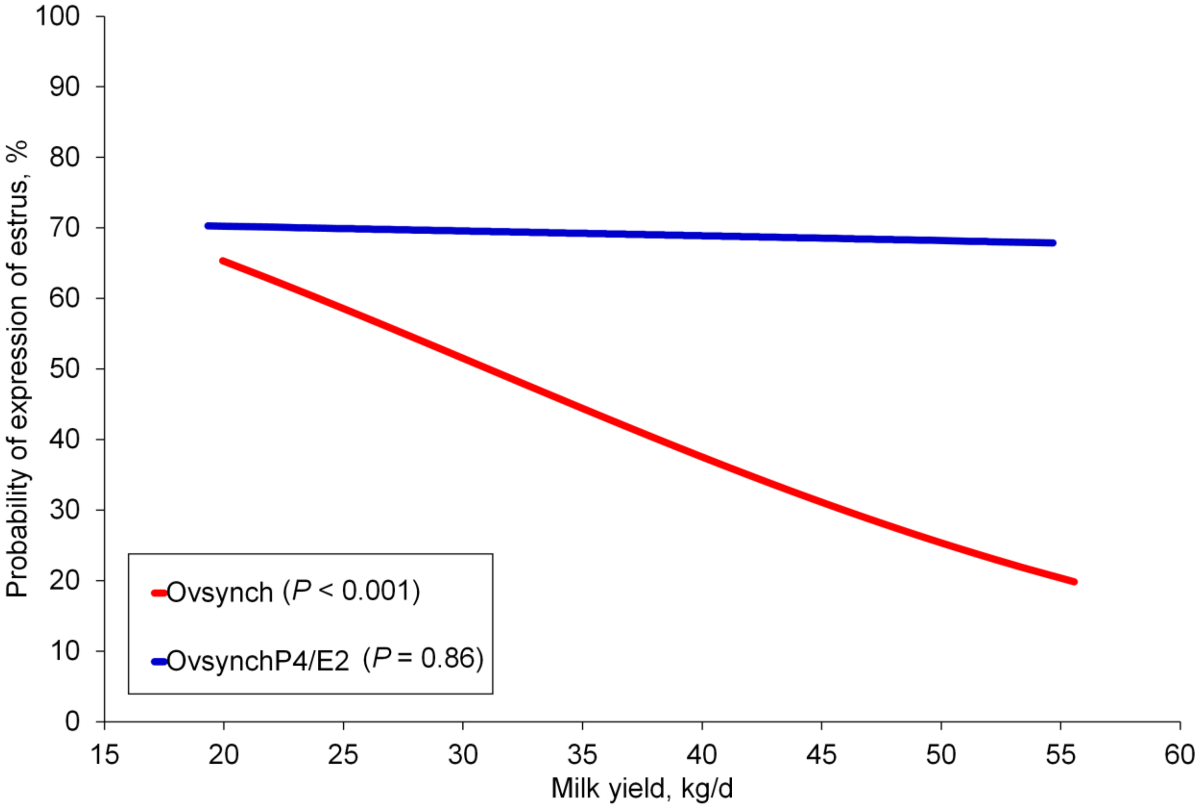

Figure 3. Probability of estrus at the end of the timed-artificial insemination (TAI) according to milk yield during the protocol. The Ovsynch protocol (n = 373) consisted of: d0: 20 µg GnRH, d7: PGF, d8: PGF, d9.5: 10 µg GnRH, and d10: TAI. Cows submitted to OvsynchP4/E2 (n = 362) received on d0: 20 µg GnRH and a 2 g P4 implant, d7: PGF, d8: P4 implant removal, PGF and EC, and d10: TAI.

Fertility According to Reproductive Programs and Other Variables

Interestingly, despite differences in the physiology, the outcomes for fertility and pregnancy loss were similar among the TAI programs (Table 5). Our hypotheses were that both presynchronization strategies would reflect in similar proportion of CL and ovulation on d0 and CL at PGF, and despite the small differences on CL on d0 (~5%) and ovulation (~8%), fertility was similar between presynchronizations. Regarding TAI protocols, it was expected that the greater expression of estrus in OvsP4/E2 would promote better fertility or lower pregnancy loss, and despite differences in estrus and the slightly higher ovulatory response at the end of the Ovs protocol (4%), fertility was also similar between the TAI protocols, and no differences on pregnancy loss was detected. When only ovulated cows were considered for fertility analyses, also no differences were detected (Table 5). These results are exciting, demonstrating that the 4 TAI programs are excellent options to promote high overall fertility. Moreover, the study revealed similar fertility between the already established Double-

Ovsynch and the novel program (Double E-Synch) which has been frequently used by our research group and commercial dairy herds.

Table 5. Pregnancy per AI (P/AI) and pregnancy loss of lactating Holstein cows submitted to 4 fertility programs (Presynch + TAI protocol) for the first pospartum service.

Item, % (n/n)	Reproductive program (Presynch + TAI protocol)[1]				*P*-value[3]		
	Ovs + Ovs	Ovs + OvsP4/E2	PreP4/E2 + Ovs	PreP4/E2 + OvsP4/E2	Pres	Prot	Int
All cows							
P/AI on d32	46.0 (93/202)	45.5 (90/198)	51.7 (105/203)	48.7 (96/197)	0.22	0.60	0.66
P/AI on d60	39.1 (79/202)	36.9 (73/198)	41.4 (84/203)	40.6 (80/197)	0.41	0.64	0.91
P/AI on d90	36.1 (73/202)	34.3 (68/198)	37.0 (75/203)	38.6 (76/197)	0.48	0.95	0.70
Preg. loss d32 to 90	21.5 (20/93)	24.4 (22/90)	28.6 (30/105)	20.8 (20/96)	0.70	0.61	0.26
Ovulated cows[2]							
P/AI on d32	48.4 (93/192)	49.7 (90/181)	53.3 (105/197)	52.5 (96/183)	0.33	0.96	0.71
P/AI on d60	41.2 (79/192)	40.3 (73/181)	42.6 (84/197)	43.7 (80/183)	0.54	0.99	0.86
P/AI on d90	38.0 (73/192)	37.6 (68/181)	38.1 (75/197)	41.5 (76/183)	0.63	0.71	0.66
Preg. loss d32 to 90	21.5 (20/93)	24.4 (22/90)	28.6 (30/105)	20.8 (20/96)	0.70	0.61	0.26

[1]On d-17, cows in the Ovs presynchronization received 10 µg buserelin acetate (GnRH), 0.5 mg cloprostenol (PGF) on d-10, and 10 µg GnRH on d-7. The PreP4/E2 presynchronizatio initiated on d-17 with a 2 g progesterone (P4) implant, that was removed on d-10, with 0.5 mg PGF and 1 mg estradiol cypionate (EC). Cows in the Ovs TAI protocol received on d0: 20 µg GnRH, d7: PGF, d8: PGF, d9.5: 10 µg GnRH, and d10: TAI (16 h after GnRH). Cows submitted to OvsP4/E2 received on d0: 20 µg GnRH and a 2 g P4 implant, d7: PGF, d8: P4 implant removal, PGF and EC, and d10: TAI.

[2]Only cows with corpus luteum 7 days after TAI, which were considered as ovulating after TAI.

[3]Pres: main effect of presynchronization protocol. Prot: main effect of TAI breeding protocol. Int: Interaction between presynchronization and TAI protocol

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Primiparous commonly have greater fertility compared to multiparous cows, and it was not different in the present study (51.6 [212/411] vs. 44.2% [172/389]). Moreover, there was no interaction between parity and presynchronization or TAI protocols, and no effect of parity on pregnancy loss was detected. In several times, milk yield has been associated with decreased fertility or lower reproductive efficiency. However, genetic selection towards fertility traits, associated with optimized comfort, nutrition and reproductive management allow current modern dairy herds to achieve high reproductive performance despite individual milk yield. The effect of milk yield on fertility in TAI-related studies is variable in the literature, with studies describing a negative relationship (Pereira et al., 2020) or no effect (Santos et al., 2009). Since all TAI programs were considered optimized and promoted similar and high fertility, we were instigated to evaluate the possible effect of milk yield on fertility. Interestingly, there was no effect or interaction between milk yield and resynchronization strategy or TAI protocol. For instance, cows submitted to Ovs and OvsP4/E2 had similar fertility in both classes (below or above the median) of milk yield ≤ 38 kg/d (50.3 [93/185] vs. 49.0% [96/196]; $P = 0.89$) and > 38 kg/d (47.1 [89/189] vs. 45.0% [76/169]; $P = 0.69$). When a logistic regression was performed to evaluate the effect of milk yield on the probability of pregnancy and pregnancy loss, no effect was detected (Figure 4). These results are exciting since they indicate that milk yield is not necessarily associated with lower reproductive efficiency, especially when cows are submitted to TAI programs that optimize fertility.

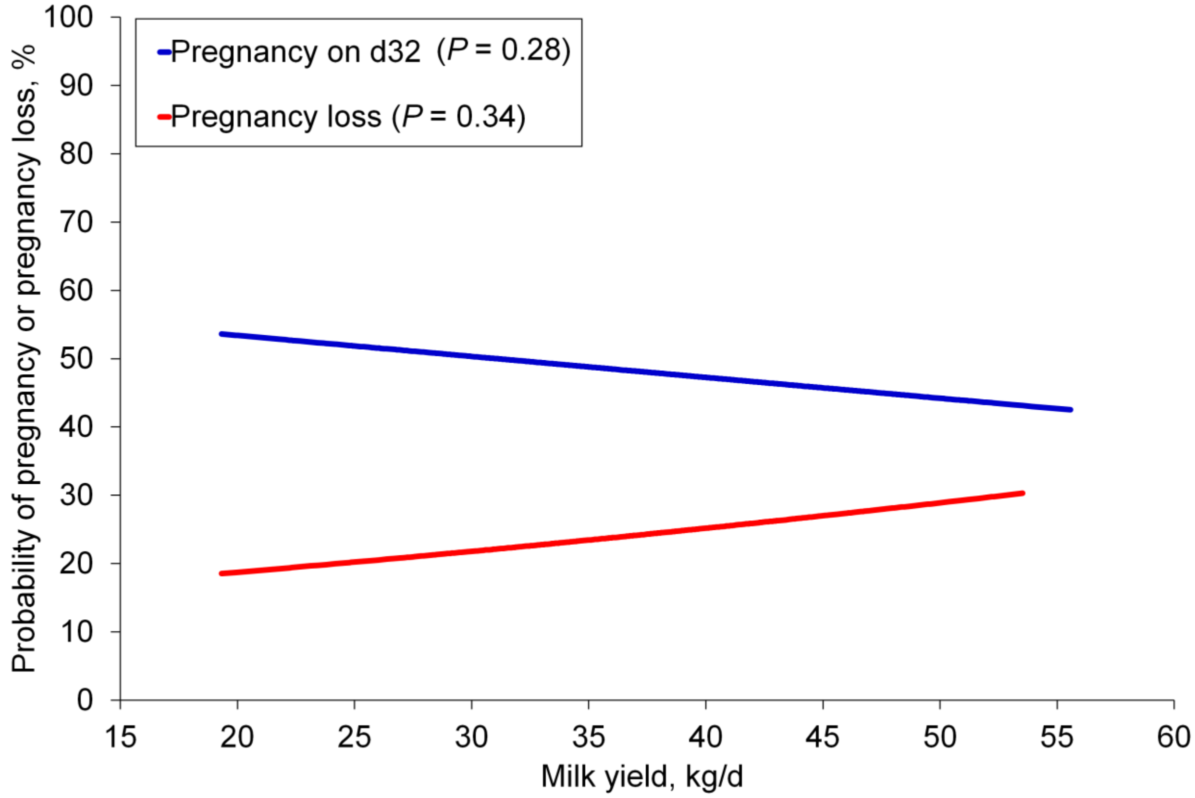

Figure 4. Effect of milk yield on probability of pregnancy on d32 (n = 735) and pregnancy loss (n = 352) between d32 and d90 after timed-artificial insemination in lactating Holstein cows submitted to fertility programs (Presynch + TAI protocol).

Presence of CL during the protocol and ovulation after the first GnRH can impact fertility. The low number of cows without CL on d0 (n = 55) and at PGF (n = 12) did not allow us to effectively evaluate the effect of CL at time points. However, when presence of CL at the beginning of the presynchronization strategies was evaluated, cows without CL had lower fertility compared to cows with CL by 34 DIM (49.9 [303/607] vs. 42.0% [81/193]; P = 0.05). This result suggests that even when cows undergo optimized TAI programs, early resumption of cyclicity is important to optimize fertility. In fact, anovulatory condition during early lactation is associated with greater BCS loss and health problems, which have short- and long-term negative impact on fertility (Carvalho et al., 2014; Barletta et al., 2017; Carvalho et al., 2019).

Cows ovulating at the beginning of the breeding protocols had greater fertility (51.0 [278/545] vs. 41.6% [106/255]; P = 0.01), and no interaction with TAI protocol was detected. Several studies reported greater fertility in cows ovulating after the first GnRH, especially in cows without CL or with low circulating P4 (Giordano et al., 2013; Borchardt et al., 2020). Ovulation is crucial to promote a well synchronized new follicular wave emergence and the presence of a developing CL during the protocol, both aspects associated with greater fertility (Monteiro et al., 2015; Melo et al., 2018). One difference between the TAI protocols implemented in the present study, in addition to the strategy to induce final ovulation, is the inclusion of a P4 implant in the OvsP4/E2 group. Progesterone supplementation during TAI protocols has been associated with greater fertility, particularly in cows without CL at the beginning of the protocol (Bisinotto et al., 2015a,b). However, we understand that the P4 implant in the OvsP4/E2 protocol in the present study may have benefited a low proportion of cows, i.e. those 10% initiating the protocol without CL. Even though, the majority of these cows ovulated after the first GnRH, which also increases fertility in cows without CL. Another aspect on how the P4 implant can have a positive impact on fertility during TAI protocols is preventing early expression of estrus or premature ovulation. However, that should be a minor issue in the present study since 98.5% of the cows had at least one CL at the time of the first PGF of the breeding protocols. Thus, it is not possible to conclude there was any benefit of the presence of a P4 implant in the OvsP4/E2 protocol on P/AI.

There was no difference between cows ovulating or not at the beginning of the TAI protocols on pregnancy loss (22.7 [63/278] vs. 27.4% [29/106]; P = 0.34). The number of cows with multiple ovulation was reduced (n = 54) but, considering only cows that conceived after AI, multiple ovulation was associated with higher pregnancy loss (39.3 [11/28] vs. 22.8% [81/356]; P = 0.04), which was expected based on data from previous studies (Martins et al., 2018).

Fertility According to Reproductive Programs and Expression of Estrus

As discussed, TAI protocols influenced expression of estrus, which influenced ovulation after TAI. Thus, to evaluate the effect of expression of estrus on fertility, only ovulated cows were included in the analyses, and there was a clear effect of expression of estrus on fertility in both protocols (Table 6). Cows submitted to Ovs and OvsP4/E2 protocols that did not express estrus had decreases of 23% and 47% on P/AI, respectively. When all cows were considered (ovulated and non-ovulated), the difference in fertility between TAI protocols in cows without estrus was even greater (42.2 [100/237] vs. 24.0% [29/121] for Ovs and OvsP4/E2, respectively; P < 0.001), which was expected due to the differences in ovulation. For instance, 78% of cows without estrus in OvsP4/E2 ovulated after TAI compared to 93% in the Ovs protocol. Since it is impossible for cows that do not ovulate after TAI to become pregnant, the greater fertility of cows without estrus in the Ovs protocol is understandable. However, the reasons for greater P/AI in the Ovs protocol considering only ovulated cows not expressing estrus cannot be totally explained.

Table 6. Effect of the breeding timed-artificial insemination (TAI) protocol and expression of estrus on pregnancy per AI (P/AI) on d32 of lactating dairy cows ovulating after TAI.

Item, % (n/n)	P/AI of the breeding TAI protocol[1]		*P*-value
	Ovsynch	OvsynchP4/E2	
No estrus	45.3 (100/221)	30.9 (29/94)	0.02
Estrus	58.3 (98/168)	58.2 (157/270)	0.88
P-value	0.03	< 0.001	

[1]The Ovsynch protocol consisted of: d0: 20 µg GnRH, d7: PGF, d8: PGF, d9.5: 10 µg GnRH, and d10: TAI (16 h after GnRH). Cows submitted to OvsynchP4/E2 received on d0: 20 µg GnRH and a 2 g P4 implant, d7: PGF, d8: P4 implant removal, PGF and EC, and d10: TAI.
There was a main effect of expression of estrus ($P < 0.001$), but no main effect of TAI protocol ($P = 0.96$). Moreover, there was an interaction between estrus and TAI protocol ($P = 0.04$).

We speculated that cows not showing estrus in OvsP4/E2 may have follicles too small at the end of the protocol, since even receiving an E2 supplementation (i.e., EC), they were not detected in estrus. Thus, ovulation may have occurred too late in relation to TAI in these cows. In contrast, during the Ovs protocol, the GnRH as ovulation inducer practically guarantee that ovulation occurs between 24 and 32 h after GnRH treatment, even in cows with smaller follicles. Therefore, fertility may have been more compromised in OvsP4/E2 cows not expressing estrus due to a non-optimized timing of the ovulation (Pursley et al., 1998; Saacke, 2008).

Pregnancy loss did not differ among the reproductive programs as presented in Table 5. It is important to mention that the overall pregnancy loss of the farm was high, at least compared to other studies with large number of animals that reported 12-14% of pregnancy loss (Wiltbank et al., 2016; Fernandez-Novo et al., 2020; Sigdel et al., 2021). Although the protocol with EC resulted in higher expression of estrus, our hypothesis of lower pregnancy loss in cows submitted to the OvsP4/E2 protocol was not supported, since there was no significant difference in pregnancy loss between OvsP4/E2 and Ovs (22.6 [42/186] vs. 25.3% [50/198]; $P = 0.61$). On the other hand, expression of estrus did not interact with TAI protocol or parity, and it was associated with decreased pregnancy loss (Table 7). Cows not expressing estrus up to the time of TAI had 39.2% greater pregnancy loss between d32 and 90 after TAI (Table 7).

Table 7. Effect expression of estrus on pregnancy loss (PL) of lactating dairy cows submitted to fertility programs (Presynch + TAI protocol).

Pregnancy loss, % (n/n)	Expression of estrus near TAI		*P*-value
	No estrus	Estrus	
PL between d32 and 60	20.2 (26/129)	16.6 (42/255)	0.37
PL between d60 and 90	11.7 (12/103)	5.6 (12/213)	0.04
PL between d32 and 90	29.5 (38/129)	21.2 (54/255)	0.05

The expression of estrus near TAI has been associated with pregnancy loss. In protocols with EC as ovulation inducer, cows expressing estrus had 28.5% lower pregnancy loss (14.4 [255/1,785] vs. 20.1% [43/222]; Pereira et al., 2016). Similarly, cows submitted to Ovsynch-type protocols expressing estrus at the end of the protocol also had reduced pregnancy loss compared to cows without estrus (6.5 [68/1,041] vs. 10.9% [161/1,482]; Consentini et al., unpublished data). Expression of estrus is associated with several aspects favorable to the establishment and maintenance of pregnancy. For instance, cows expressing estrus had greater ovulatory follicle and circulating P4 after TAI (Rodrigues et al., 2018; Cooke et al., 2019), and higher circulating pregnancy associated glycoproteins on d28 (Pohler et al., 2016), which are both associated with increased fertility. Moreover, expression of estrus is associated with changes in the reproductive tract and expression of genes favorable to embryo implantation, placentation, and pregnancy (Davoodi et al., 2016; Cooke et al., 2019).

Conclusions

In conclusion, the study validated 2 presynchronizations strategies and 2 TAI protocols, establishing 4 possible fertility programs, all of them inducing well-controlled ovarian dynamics, excellent synchronization, and high fertility. Moreover, one of our goals was to compare the Double-Ovsynch with the Double E-Synch, and despite differences in pharmacological bases, both promoted similar results. Finally, the data generated from this study offers to dairy operations effective options of TAI programs to be used according to their management and preferences.

Notes

The authors thank the owners and staff of Céu Azul Farm for the use of their cows and facilities, and their collaboration throughout the study. We appreciate the Coordination for the Improvement of Higher Education (CAPES, Brasília, Brazil) and Brazilian National Council for Scientific and Technological Development (CNPq, Brasília, Brazil) for the financial support during the experiment. We also thank the São Paulo Research Foundation (FAPESP, São Paulo, Brazil) for the support by the fund grant # 2018/03798-7. Our appreciation is extended to GlobalGen vet science (Jaboticabal, São Paulo, Brazil) for providing all hormones used in the presynchronization and TAI protocols.

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CHAPTER 3: IMPROVED FERTILITY FOLLOWING A GONADOTROPIN-RELEASING HORMONE TREATMENT ON DAY 2 OF AN ESTRADIOL AND PROGESTERONE-BASED TIMED-ARTIFICIAL INSEMINATION PROTOCOL IN LACTATING DAIRY COWS

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JDS Communications®
2022; 3:212–216
https://doi.org/10.3168/jdsc.2022-0212
Short Communication
Physiology

Improved fertility following a gonadotropin-releasing hormone treatment on day 2 of an estradiol and progesterone-based timed-artificial insemination protocol in lactating dairy cows

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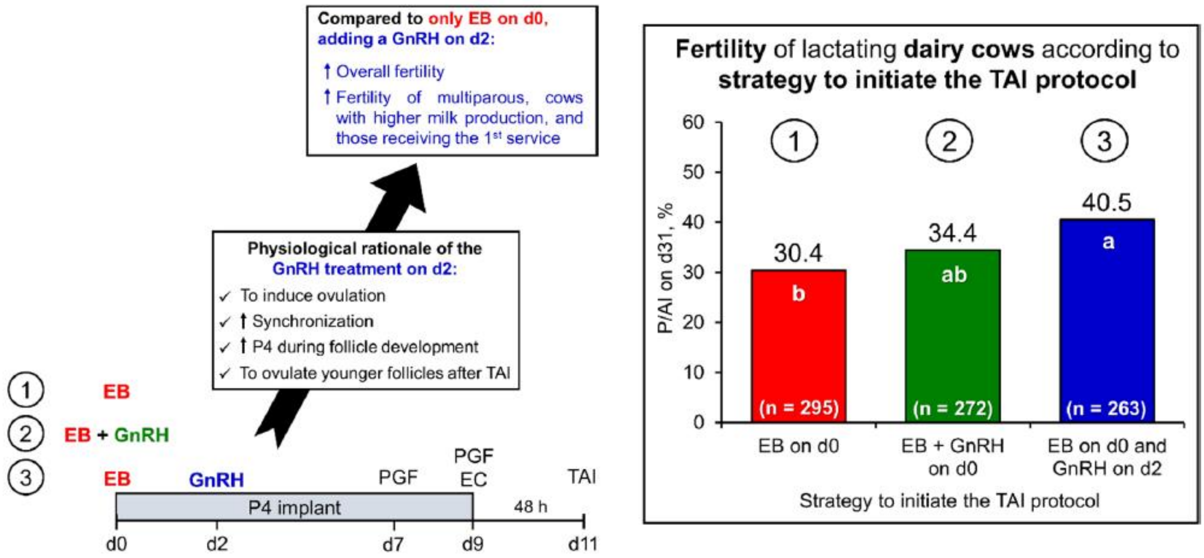
Graphical Abstract



Summary

This study evaluated 3 strategies to initiate an estradiol/progesterone-based timed-artificial insemination protocol: (1) estradiol benzoate (EB) only on d 0, (2) EB plus GnRH on d 0, and (3) EB on d 0 and GnRH on d 2. Compared with the negative control group (EB only on d 0), adding GnRH on d 2 increased overall fertility, and particularly benefited the following groups of cows: multiparous cows, cows with higher milk production, and cows receiving the first postpartum service.

Highlights

- GnRH on d 2 of timed AI (TAI) protocols initiated with estradiol benzoate increases fertility.
- Only estradiol benzoate on d 0 of TAI protocols decreases fertility of dairy cows.
- GnRH on d 2 of TAI protocol increased pregnancy per AI of multiparous and higher-producing cows.

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Abstract

The present study evaluated the addition of a gonadotropin-releasing hormone (**GnRH**) concomitant or 2 days after the beginning of protocols initiated with estradiol benzoate (**EB**). A total of 459 multiparous and 371 primiparous lactating Holstein cows were enrolled in the study. Weekly cohort of cows were randomly assigned to 1 of 3 experimental groups that differed in the strategy to initiate the TAI protocol. On d0, all cows received a 1.55 g progesterone (**P4)** implant and, in **EBd0** group, cows received 2 mg of EB i.m.. Cows assigned in **EBd0-GnRHd0** group were treated simultaneously on d0 with 2 mg ofEB plus 100 µg of gonadorelin diacetate tetrahydrate (GnRH) i.m. and, in **EBd0-GnRHd2** group, cows received 2 mg of EB on d0 and 100 µg of GnRH 48 hours later, on d2. The remaining treatments in the protocol were similar among groups, and included 0.53 of mg i.m. cloprostenol sodium (**PGF**) on d7, followed by a second PGF treatment on d9, at the time of P4 implant withdrawal and 1 mg of estradiol cypionate i.m.. The TAI was performed on d11 (48 hours after P4 removal) in all experimental groups. Regarding pregnancy per AI (**P/AI**) on d30, a treatment effect was detected, in which cows from EBd0-GnRHd2 group presented greater fertility than EBd0 cows, while EBd0-GnRHd0 group did not differ among EBd0 and

EBd0-GnRHd0 (40.5 vs. 30.4 vs. 34.4%; respectively). Interestingly, GnRH treatment on d2 increased fertility in multiparous, cows with greater milk production and cows receiving the first postpartum service. In summary, GnRH treatment at the beginning of an estradiol/P4-based TAI protocol increased fertility, only when the GnRH was given on d2. Moreover, there was a more pronounced positive effect of this strategy in particular classes of cows such as multiparous, cows with greater milk production, and those receiving the first service.

Introduction

There are critical points during timed-artificial insemination (**TAI**) programs that can optimize fertility of lactating dairy cows (Consentini et al., 2021). Initially, it is important to properly synchronize the emergence of a new follicular wave; this is an essential event to control the age of the ovulatory follicle (Monteiro et al., 2015). Moreover, the presence of corpus luteum (**CL**) and high circulating progesterone (**P4**) concentrations during development of the preovulatory follicle are positively associated with P/AI (Bisinotto et al., 2015; Melo et al., 2016).

Regarding synchronization of a new follicular wave emergence, gonadotropin-releasing hormone (**GnRH**) can be administered to induce ovulation, followed by emergence of a new follicular wave within 24 hours, as commonly used in Ovsynch-type protocols (Pursley et al., 1995). In a recent compilation of studies by Borchardt et al. (2020), it was demonstrated an overall ovulation incidence after a GnRH treatment of 51.4% (2,204/4,291). However, there is a variation in the ovulatory response among studies, influenced by a number of physiological aspects such as CL presence (Borchardt et al., 2020), steroid hormone concentrations (Stevenson and Pulley, 2016), stage of the estrous cycle (Vasconcelos et al., 1999), use of presynchronization protocols (Belo et al., 2006), and dose of GnRH (Giordano et al., 2013). Another often used strategy to synchronize follicular emergence is by causing atresia of the follicles in response to the combination of estradiol (**E2**) and P4, such as in E2/P4-based protocols (Bó et al., 1995; Barros et al., 2000). The circulating P4 profiles during the TAI protocol may differ according to the strategy used at the beginning. For instance, when GnRH causes ovulation, a new follicular wave initiates simultaneously to the development of a new CL throughout the protocol, and both factors are associated with greater pregnancy per AI (**P/AI**; Giordano et al., 2013; Melo et al., 2016; Borchardt et al., 2020). In contrast, in E2/P4-based protocols, previous studies reported that approximately 25% of cows failed to have a new follicular wave emergence, and about 40% of cows underwent CL regression before the scheduled treatment with prostaglandin F2α (**PGF**). These events were associated to lower fertility in lactating dairy cows (Monteiro et al., 2015; Melo et al., 2016; Melo et al., 2018).

In a previous study, initiating the TAI protocol with GnRH instead of estradiol benzoate (**EB**) improved ovarian dynamics (CL presence at PGF), P4 milieu (higher P4 at PGF) and fertility in lactating dairy cows (Melo et al., 2016). A frequently implemented TAI protocol in commercial dairy herds, initiates with EB and has an extended length and proestrus, with the first PGF on d7, the second treatment on d9 (at P4 implant removal), and cows being inseminated on d11 (Pereira et al., 2015). Adding a GnRH at the beginning of this protocol increased fertility of lactating dairy cows (Pereira et al., 2015). However, the GnRH given on d2 could promote better fertility, because in cows ovulating after a GnRH given on d0, the ovulatory follicle may be too old or overexposed to luteinizing hormone (**LH**) due to protocol length and a longer proestrus.

Thus, the objective of the present study was to evaluate 3 strategies to initiate TAI protocols in lactating dairy cows, that included: treatment with estradiol benzoate (**EB**) plus P4 implant only; or an additional treatments with GnRH, either simultaneously to the EB treatment (d0) or 2 days later (d2). The main hypothesis was that the inclusion of a GnRH treatment on d0 or d2 would increase P/AI of lactating dairy cows, and the GnRH on d2 would promote greater fertility than GnRH given on d0 with the EB.

Material and Methods

Expecting an increase on P/AI ranging from 5 to 10 percentage points (e.g. 30 vs. 35 to 40%), the minimum sample size of 300 cows was determined after a power calculation using the PROC POWER of SAS 9.4 (power = 0.80 and $\alpha = 0.05$). The experiment was conducted in 2 commercial dairy farms located in Southeast of Brazil, both with 305-d average milk production of 9,000 kg. The Animal Research Ethics Committee of Luiz de Queiroz College of Agriculture of the University of São Paulo (ESALQ/USP) approved all procedures involving cows in this study (CEUA - 5112290720). Farms had approximately 700 lactating Holstein cows milked thrice daily and fed twice with a total mixed ration based on corn silage and a corn and soybean meal-based concentrate with minerals and vitamins balanced to meet or exceed the nutritional requirements of lactating dairy cows producing 40 kg/d of milk (National Research Council – NRC, 2001). All cows had *ad libitum* access to water and were housed in free-stall barns bedded with sand and equipped with fans.

A total of 459 multiparous and 371 primiparous lactating Holstein cows were enrolled in the study from November of 2015 to August of 2016. Weekly cohort of cows were randomly assigned, according to parity and number of service (first postpartum TAI and resynchronization of ovulation protocols initiated at nonprenant diagnosis 31 d after a prior

AI), to 1 of 3 experimental groups that differed in the strategy to initiate the TAI protocol (Figure 1). On d0, all cows received a 1.55 g P4 implant (PRID Delta, Ceva, France) and, in **EBd0** group, cows received 2 mg of EB (Estrogin, Biofarm, Brazil). Cows assigned to **EBd0-GnRHd0** group were treated simultaneously on d0 with 2 mg of EB plus 100 µg of gonadorelin diacetate tetrahydrate (GnRH, Cystorelin, Merial, Brazil) and, in **EBd0-GnRHd2** group, cows received 2 mg of EB on d0 and 100 µg of GnRH 48 hours later, on d2. The remaining treatments in the protocol were similar among all groups, and included 0.53 mg of cloprostenol sodium (PGF, Veteglan, Hertape Calier, Brazil) on d7, followed by a second PGF treatment on d9, at the time of implant removal and 1 mg of estradiol cypionate (**EC**, Cipionato-HC, Hertape Calier, Brazil). The TAI was performed on d11 (48 hours after P4 removal) with conventional Holstein semen in all experimental groups, and pregnancy diagnosis was performed by ultrasound examination 31 d after TAI.

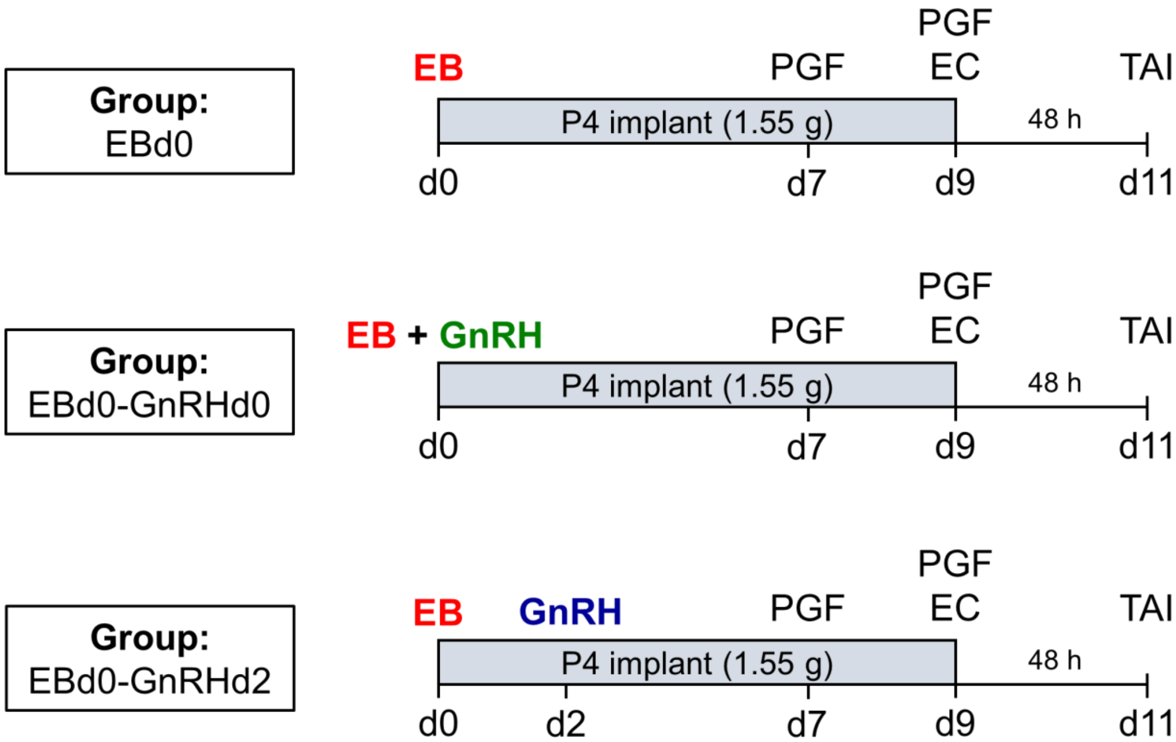

Figure 1. Experimental design with the hormonal treatments during timed-artificial insemination (TAI) protocols. On d0, all cows received a 1.55 g progesterone (P4) implant and, in EBd0 group, cows received 2 mg of estradiol benzoate (EB). In EBd0-GnRHd0 group, cows received 2 mg of EB plus 100 µg of gonadorelin diacetate tetrahydrate (GnRH) simultaneously on d0, and, in EBd0-GnRHd2 group, cows received 2 of mg EB on d0 and 100 µg of GnRH 48 h later, on d2. The remaining treatments in the protocol were similar among groups, including 0.53 mg of cloprostenol sodium (PGF) on d7, followed by a second PGF on d9, concomitant with P4 implant withdrawal and 1 mg of estradiol cypionate (EC). The TAI was performed on d11 (48 h after P4 removal) in all experimental groups.

Statistical analyses were performed using the Statistical Analysis System (SAS, Version 9.4 for Windows SAS Institute Inc., Cary, NC). Analyses for continuous variables, such as days in milk (**DIM**) and milk production near TAI (7-d average production before TAI), were performed using the GLIMMIX procedure fitting a Gaussian distribution. Analyses of binary response variable (P/AI on d31) were performed using the GLIMMIX procedure, fitting a binomial distribution with Link Logit function. Additionally, the option ddfm = kenwardroger was included in the model statement to adjust the degrees of freedom for variances.

The initial model for P/AI on d31 included the effect of treatment, farm, parity (primiparous and multiparous), milk production class (< or ≥ 33.1 kg/d; Lopez et al., 2004), number of AI (first or later services), and the interaction between treatment and these variables. For the final model, only the interaction between farm and treatment was removed. To independently evaluate the effect of treatment in each class of cows within parity, milk production and service number, the SLICE command was used in the GLIMMIX procedure.

Tukey honest significant difference post hoc test was performed to determine differences. Values are presented as least square means (**LSM**) ± standard error of the mean (**SEM**). Significant differences were declared when $P < 0.05$, whereas tendencies were considered when $0.10 > P \geq 0.05$.

Results and Discussion

The average DIM was 168.1 ± 4.1 and did not differ among treatments ($P = 0.74$) or between farms ($P = 0.92$). Similarly, milk production was not different among treatments ($P = 0.64$) or farms ($P = 0.17$), and multiparous had slightly greater milk production than primiparous cows (30.9 ± 0.4 vs. 29.1 ± 0.4 kg/d; $P = 0.003$).

Regarding P/AI on d31, a treatment effect was detected ($P = 0.04$), in which cows from EBd0-GnRHd2 group had greater fertility than EBd0 cows, whereas EBd0-GnRHd0 group did not differ from the other groups (Figure 2).

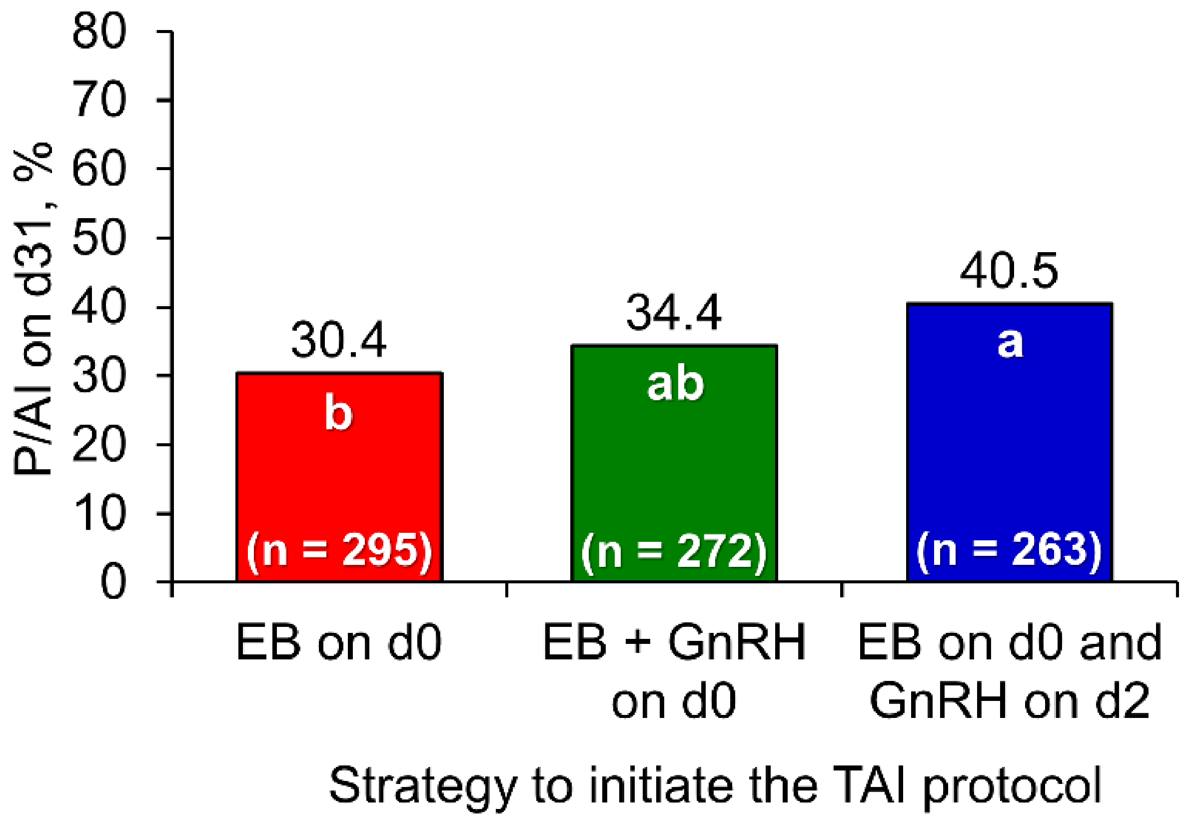

Figure 2. Pregnancy per artificial insemination (P/AI) 31 days after timed-AI (TAI) according to the strategy to initiate the TAI protocol (*P* = 0.04). Treatments were estradiol benzoate on d0 (EBd0), estradiol benzoate plus GnRH on d0 (EBd0-GnRHd0), or estradiol benzoate on d0 and GnRH on d2 (EBd0-GnRHd2) of the TAI protocol. [a,b]Means with different letters are different ($P < 0.05$).

In a recent compilation of studies comprising 4,657 lactating dairy cows, Consentini et al. (2021) reported that the administration of only GnRH at the beginning of TAI protocols or its inclusion on d0 or d2 of an E2/P4-based protocol increased fertility by 17.9% when compared to E2/P4-based protocols initiated only with EB (39.5 vs. 33.5%). Treatment with GnRH at the beginning of E2/P4-based protocols seems to increase fertility because of the induction of ovulation, which increases the proportion of cows with a functional CL at the time of treatment with PGF and improves circulating P4 concentrations during the protocol (Pereira et al., 2015; Melo et al., 2016; Consentini et al., 2021). The study by Cerri et al. (2011) demonstrated that higher circulating P4 concentrations reduced LH pulse frenquency during follicular development in a synchronization protocol, which is fundamental to ensure an adequate growth of the dominant follicle in lactating dairy cows (Wiltbank et al., 2011). Moreover, studies reported that higher circulating P4 during the protocol were associated to better embryo quality and greater fertility in dairy cows (Rivera et al., 2011; Wiltbank et al., 2012).

In the present study, treatment with GnRH concomitant with EB on d0 did not increase P/AI, in contrast to Pereira et al. (2015), that reported a greater P/AI when GnRH was added on d0 (30.7 vs. 26.8%). One reasonable explanation for the lack of effect of GnRH treatment on d0 on fertility may be associated to the age of the ovulatory follicle at the end of the protocol. Because of the length of the protocol (11 days), cows from EBd0-GnRHd0 group that ovulated to the GnRH given on d0, although synchronized, possibly had an older ovulatory follicle at the time of AI. Moreover, because of the 4-day period of proestrus (due to the first PGF treatment on d7) this follicle may have experienced an overexposure to LH pulse-frquency at the end of the protocol, compromising oocyte quality, which impairs fertility (Revah and Butler, 1996; Cerri et al., 2009; Monteiro et al., 2015). Conversely, results from the present study suggest that when cows ovulate to the GnRH treatment on d2, the end of the protocol works similarly as the traditional 5-d synchronization protocol, which results in a younger ovulatory follicle at the time of AI, producing greater P/AI. Indeed, according to Santos et al. (2010), the 5-day Cosynch72 with 2 PGF treatments promoted greater P/AI than the 7-day Cosynch72 with 1 PGF treatment (37.9 vs. 30.9%). In addition, an interesting study comparing the 5-day Ovsynch protocol and the traditional Ovsynch, both with 2 PGF treatments, reported similar fertility between these TAI programs (43.8 and 41.4%; Santos et al., 2016).

Additionally, an aspect that can explain the lower fertility of the EBd0 group is the expected lack of emergence of a new follicular wave after EB plus P4 implant treatment in a percentage of cows (25-35%, Monteiro et al, 2015; Melo et al., 2018), resulting in a low overall synchronization to the protocol in lactating dairy cows (32 to 60%; Monteiro et al., 2015). In this sense, the ideia of adding a GnRH treatment on d2 in the present study aimed to induce ovulation in cows that did not respond to the treatment with EB plus P4 implant, increasing the proportion of cows synchronized to the protocol. In addition, studies reported that about 40% of cows with a CL on d0 underwent CL regression during the synchronization protocol when treated with EB at the beginning of the synchronization protocol (Monteiro et al., 2015; Melo et al., 2016; Consentini et al., 2021), reducing circulating P4 concentrations during follicular development. These two situations can be partially overcome when a GnRH treatment is added at the beginning of the protocol.

Furthermore, there was no effect of farm (P = 0.55) nor interaction between farm and treatment (P = 0.92; Table 1). Likewise, there was no effect of number of AI on fertility (P =

0.25). Previous studies reported a marked decrease in P/AI as the number of services or DIM increased (Lopes et al., 2013). Although it is hard to draw final conclusions on why the number of AI did not affect fertility in the current study, it is possible that greater incidence of metabolic problems and more acute heat stress may have played an important role and could partially explain these contrasting results. Unsurprisingly, primiparous had greater P/AI than multiparous cows (P = 0.005; Table 1), as previously reported (Carvalho et al., 2014, 2015). This can be mainly explained by the lesser challenges related to liver steroid metabolism due to the lower milk production, and fewer health issues in the postpartum period in primiparous cows (Reinhardt et al., 2011; Pascottini et al., 2017).

When additional analyses were performed to better understand the effect of treatment within specific classes of cows (Table 1), the EBd0-GnRHd2 group presented greater fertility in cows with greater milk production (≥ 33.5 kg/d). This effect was also observed in multiparous cows and cows receiving the first service (Table 1). Normally, these classes of cows have higher milk production (multiparous > primiparous, and 1st service > later services), which is closely related to a greater steroid hormone menabolic rate (Sangsritavong et al., 2002). This condition could be compromising the emergence of a new follicular wave in response to EB plus P4 implant, besides reducing circulating P4 concentrations during follicular development, resulting in an older (and overexposed to LH) ovulatory follicle. Another possible explanation for the greater P/IA observed in EBd0-GnRHd2 group into these classes, althouth not properly evaluated, is the expected greater incidence of cows in anovulatory condition, mainly in the first service (Monteiro et al., 2021), which would result in a greater number of cows without CL at the beginning of the protocol. In both situations, addition of a GnRH treatment at the beginning of the TAI protocol could optimize the synchronization and potentially improve fertility of lactating dairy cows. In the present study, this could parcially explain the greater P/IA observed specially in EBd0-GnRHd2 group compared to EBd0 group.

Table 1. Pregnancy per artificial insemination (P/AI) 31 days after timed-artificial insemination (TAI) according to the strategy to initiate the TAI protocol, farm, parity, milk production, and number of AI.

Item	Overall	Strategy to initiate the TAI protocol[1]			*P*-value[2]		
		EB on d0	EB+GnRH on d0	EB on d0 and GnRH on d2	T	V	I
Farm							
1	33.4 (137/398)	29.5 (55/161)	31.2 (40/125)	39.8 (42/112)	0.24	0.35	0.78
2	36.6 (156/432)	31.3 (43/134)	37.8 (54/147)	41.1 (59/151)	0.26		
Parity							
Primiparous	40.0 (149/371)[x]	35.3 (54/140)	43.2 (52/119)	41.7 (43/112)	0.46	0.005	0.27
Multiparous	30.3 (144/459)[y]	25.9 (44/155)[b]	26.6 (42/153)[b]	39.2 (58/151)[a]	0.03		
Milk production, kg/d							
< 33.5	33.2 (187/540)	30.6 (65/190)	35.4 (64/178)	33.7 (58/172)	0.65	0.32	0.16
≥ 33.5	36.8 (106/290)	30.1 (33/105)[b]	33.5 (30/94)[b]	47.6 (43/91)[a]	0.04		
Number of AI							
First service	32.7 (98/294)	24.6 (21/86)[b]	34.1 (35/103)[ab]	40.6 (42/105)[a]	0.04	0.20	0.25
Later services	37.3 (195/536)	36.9 (77/209)	34.8 (59/169)	40.4 (59/158)	0.64		

[1]Treatments were estradiol benzoate on d0 (EBd0), estradiol benzoate plus GnRH on d0 (EBd0-GnRHd0), or estradiol benzoate on d0 and GnRH on d2 (EBd0-GnRHd2) of the TAI protocol.

[2]T: effect of treatment within class of cows; V: main effect of the variable (farm, parity, milk production, and number of AI); and I: interaction between treatment and the variable.

[a,b] Least square means with different superscripts within a row are different ($P < 0.05$).

[x,y]Least square means with different superscripts within a column are different ($P < 0.05$) considering the main effect of the specific variable (farm, parity, milk production, and number of AI)

.

Compliance and consistency of hormonal treatments is an important aspect when implementing synchronization protocols in dairy herds. The hormonal schedule must fit into the herd's weekly routine to make it as simple as possible. Thus, besides improvement in fertility, GnRH given on d2 is ideal for a weekly routine of hormonal treatments, in which it falls right into the same day for P4 device removal in cows starting the synchronization protocol the week before. It has an important practical aspect because the additional GnRH on d2 can be handled simultaneously to the device removal of cows synchronized the previous week, making it easy to be implemented and assuring good compliance while avoiding extra labor for managing cows during breeding routines.

Conclusions

In conclusion, addition of a GnRH treatment at the beginning of the E2/P4-based TAI protocol increased fertility, only when GnRH was given on d2. Moreover, the positive effect of this strategy was more pronounced in multiparous, cows with greater milk production, and cows in the first service, which could be more benefited from a better synchronization, higher circulating P4 concentrations during the protocol, and a younger (and not overexposed to LH) ovulatory follicle at the end of the protocol.

Notes

The authors thank the owners and staff of farms J-IDA and Tucaninha for the use of their cows and facilities. We appreciate the Coordination for the Improvement of Higher Education (CAPES, Brasília, Brazil) and Brazilian National Council for Scientific and Technological Development (CNPq, Brasília, Brazil) for the financial support during the experiment. We also thank the São Paulo Research Foundation (FAPESP, São Paulo, Brazil) for the support in the study by the scholarship grant # 2017/15904-3 and fund grant # 2018/03798-7.

The authors have not stated any conflicts of interest.

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CHAPTER 4 (ACCEPTED FOR PUBLICATION IN THE JOURNAL OF DAIRY SCIENCE COMMUNICATIONS, OCTOBER OF 2022): RELATIONSHIPS BETWEEN TOTAL MIXED RATION NUTRITIONAL COMPONENTS AND REPRODUCTIVE PERFORMANCE IN HIGH-PRODUCING DAIRY HERDS

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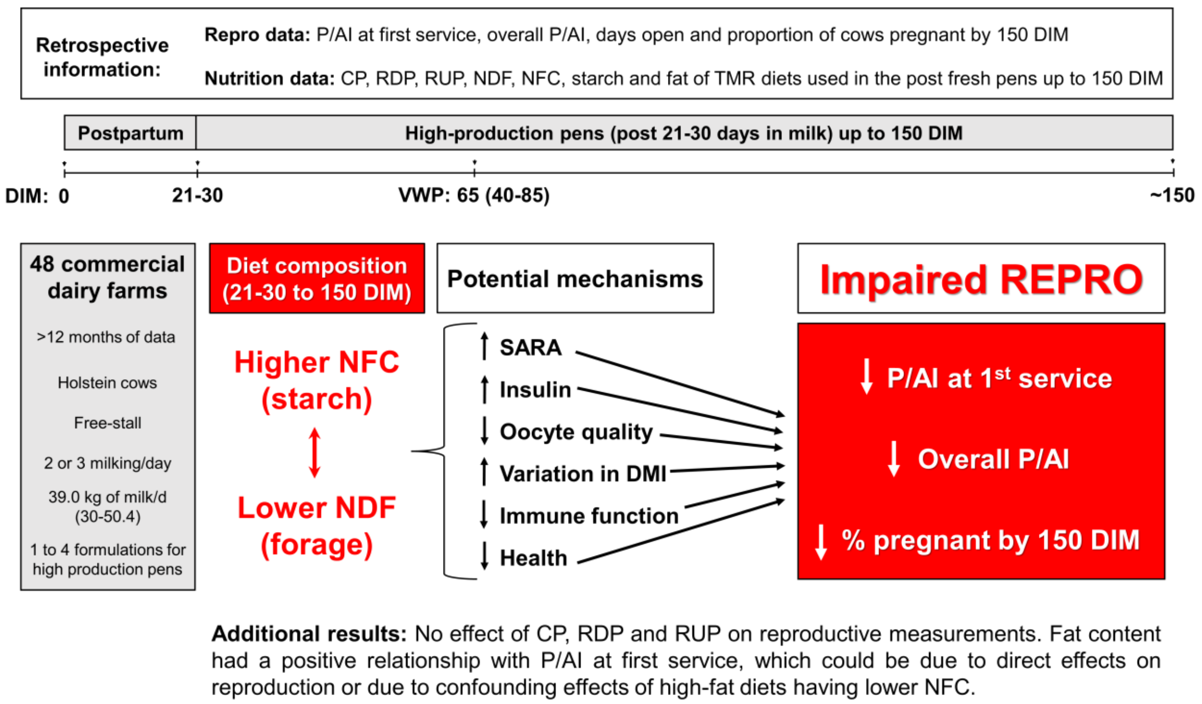
GRAPHICAL ABSTRACT



Abstract

The main objective of the present study was to determine whether composition of total mixed ration (**TMR**) influences reproductive performance in high-producing commercial dairy farms. Dairy producers and nutritional consultants from 48 dairy farms located in Wisconsin-US agreed to provide reproductive data and dietary information on high-milk production pens during main breeding period for previous 12 months. Dietary components (percentage in dry matter) were: crude protein (**CP**), rumen degradable (**RDP**) and undegradable (**RUP**) protein, neutral detergent fiber (**NDF**), non-fiber carbohydrates (**NFC**), starch, and fat. Reproductive data were: service rate (**SR**), overall pregnancy per artificial insemination (**P/AI**) and P/AI at the first service, 21-d pregnancy rate (**PR**), days open (**DOPN**), and percentage of cows pregnant by 150 DIM (**PREG150**). Participating herds had lactating Holstein cows (range = 143 to 2,717) housed in free-stall facilities. Statistical analyses were performed with CORR and GLIMMIX of SAS. Daily average milk production of herds was 38.9 ± 0.60 kg/d (30.0 to 50.4 kg/d). Overall SR was 58.5% (39-73) and P/AI was 36.1% (22-49). Overall 21-d PR was 20.3% (10-42%). Correlation between SR and PR was 0.59 (*P*<0.0001), while correlation of overall P/AI and P/AI at first service with PR were both 0.72 (*P*<0.0001). Similarly, for PREG150, correlation with overall P/AI (0.63; *P*<0.0001) and P/AI at first service (0.66; *P*<0.0001) were greater than with SR (0.48; *P*=0.001). There was large variation in diet composition, with CP varying from 16.0-18.7%, NDF from 24.9-35.1%, NFC from 31.7-46.6%, starch from 20.1-30.8%, and fat from 3.1-6.7%. Overall, there were no detectable associations of CP, RDP, and RUP with reproductive measures. The strongest relationship was a decrease in reproductive performance with increasing dietary NFC including: overall P/AI (-0.48; *P*=0.001), P/AI at first service (-0.51; *P*=0.0005), and PREG150 (-0.33; *P*=0.03). Starch also had a negative relationship with P/AI at first service (-0.35; *P*=0.05). Conversely, greater NDF was positively associated with P/AI at first service (0.34; *P*=0.01). Fat content was also positively associated with P/AI at first service (0.34; *P*=0.02). When NFC was divided in tertiles (<40, 40 to 42.2 and >42.2 % NFC), the highest tertile had lower overall P/AI (39 vs. 36 vs. 31%), P/AI at first service (43 vs. 40 vs. 33%) and PREG150 (54 vs. 53 vs. 47%). In conclusion, farms with greater dietary NFC may have compromised reproductive performance. Correspondingly, herds with greater NDF content can achieve high milk production and potentially have positive effects on reproduction. Other effects of dietary components on reproduction were not as obvious in this herd-level analysis.

Key words: dairy cows, diet composition, nutrition, fertility

Body of the paper

Reproductive performance is an important determinant of dairy herd efficiency with an optimized calving interval increasing milk production, subsequent reproductive performance, and farm profitability (Middleton et al., 2019). Efficiency of reproduction in high-producing dairy cows is impacted by numerous factors including: heat stress (Baruselli et al., 2020), body condition score (**BCS**) and BCS changes (Carvalho et al., 2014), health problems (Carvalho et al., 2019), timed-artificial insemination (**TAI**) programs (Consentini et al., 2021), and nutrition (Rodney et al., 2018). This study focused on the impact of specific nutritional components in the total mixed ration (**TMR**) on various measures of reproductive performance in well-managed Midwestern dairy farms.

Previous studies have focused on the impact on reproduction of specific nutritional manipulations such as: acidogenic diets (Santos et al., 2019), supplementation of specific fatty acids (Rodney et al., 2015), amino acids such as methionine and methyl-group donors like choline (Zhou et al., 2016), and manipulation of dietary energy and starch sources (Cardoso et al., 2020; Albornoz and Allen 2018). Despite the potential impact of nutrition on dairy cow reproduction, it is challenging to perform valid nutrition-reproduction experiments, due to the necessity for continuous manipulation of the diet in a large number of animals to validly quantify changes in binomial fertility values such as pregnancy per AI (**P/AI**). Thus, the relationships among key components of the TMR, that have known effects on milk production, have not been systematically connected to reproductive performance. The main objective of the present study was to determine whether composition of TMR influences reproductive measures on high-producing commercial dairy farms. The approach was to use dietary data from nutrition consultants, such as concentrations of protein, fiber, carbohydrate and fat in the TMR, and to correlate this information with reproductive data collected during the same time period. This experimental approach did not allow testing of specific dietary components but was designed to identify key components of the TMR that may impact reproduction to help direct future manipulative studies on nutrition-reproduction interactions in high-producing dairy cows.

Data from 48 commercial dairy farms located in Wisconsin-US were retrieved directly from nutrition consultants on each dairy to create the dietary component database. All participating herds had more than 100 (range = 143 to 2,717) lactating Holstein cows housed in free-stall facilities. The herds consented to provide their complete diets and accurate production and reproductive records with archive files for the previous 12 months that matched the period of the TMR. Nutritional information included all dietary ingredients and nutrient compositions of the diets for the high-production cow pens post 21 to 30 days in milk (**DIM**). Thus, the diet information retrieved from all herds coincided with the main breeding period after calving, which started after the end of the voluntary waiting period (**VWP**) and up to ~150 DIM.

A total of 64 diet ingredients were identified including: forage and concentrate sources, fat and amino acid (**AA**) supplements, byproduct feeds, minerals, and vitamins. Complete dietary composition was analyzed by each nutronal consultant at multiple times during the experimental period with mean values obtained for each farm on the content (percentage in dry matter; **DM)** of crude protein (**CP**), rumen degradable (**RDP**) and undegradable (**RUP**) protein, neutral detergent fiber (**NDF**), non-fiber carbohydrates (**NFC**), starch, and fat.

The reproductive data were retrieved by the same technician from the Dairy Comp 305 and PCDart herd management software, and excluded “do not breed” cows. The main data retrieved were the percentage of TAI used for first service and for all AIs, service rate (**SR**), overall P/AI and P/AI at the first service, 21-d pregnancy rate (**PR**), days open (**DOPN**), and percentage of cows pregnant by 150 DIM (**PREG150**).

Statistical analyses were performed using the Statistical Analysis System (SAS, Version 9.4 for Windows SAS Institute Inc., Cary, NC). Data were tested for normality of residuals with the Shapiro-Wilk test, using the UNIVARIATE procedure of SAS. Correlation tests between dietary components and reproductive measures were performed with the CORR procedure, and logistic regressions were performed using the GLIMMIX procedure fitting a Gaussian distribution. For some variables with significant correlations, the intercept and slope of equations were obtained using the option solution in the GLIMMIX procedure. Additionally, the option ddfm = kenwardroger was included in the model statement to adjust the degrees of freedom for variances. In addition to the logistic regressions performed considering the diet components as continuous variables, tertiles were created according to the level of the component, for example NDF and NFC, in order to study the effect of those components as class independent variables.

Tukey honest significant difference post hoc test was performed for mean separation. Values are presented as mean ± standard error of the mean (**SEM**). Significant differences were declared when $P \leq 0.05$, whereas tendencies were considered when $0.05 < P \leq 0.10$.

Daily average milk production of the herds was 38.9 ± 0.60 kg/d, varying from 30.0 to 50.4 kg/d. The average milk fat and protein percentage and somatic cell count were 3.67 ± 0.03, 3.05 ± 0.01 and 246,500 ± 13,999, respectively, and there was no effect ($P > 0.10$) of herd size on any of these milk parameters.

The VWP was 65 DIM, on average, ranging from 40 to 85 DIM. For reproductive management, most of the herds used exclusively TAI for first service, with an average across herds of 80% (25-100) for the first service, and the average for all inseminations of 65% (15-99). As expected, the SR (58.5% overall, ranging from 39 to 73) increased as the percentage of TAI use increased. However, interestingly, the percentage of TAI use for first service had a stronger relationship (R = 0.53; $P = 0.0003$) with SR than overall TAI use (R = 0.33; $P = 0.03$).

The overall P/AI was 36.1% (22-49), with primiparous cows having 19% greater fertility than multiparous cows (40.4 vs. 34.0%). The overall fertility at first service was 39.7% (20-51), with a P/AI of 45.9% in primiparous and 36.2% in multiparous cows. Overall 21-d PR from all farms was 20.3%, ranging from 10 to 42%. Percentage of cows pregnant by 150 DIM and overall DOPN was 52% (30-75) and 129 days (96-189), respectively. The 21-d PR and PREG150 are important measures of reproductive efficiency, and both are influenced by SR and P/AI. Interestingly, in our database, overall P/AI and P/AI at first service had greater relationships with 21-d PR and PREG150 compared to SR. The correlation coefficient between SR and PR was 0.59 ($P < 0.0001$), while the correlation between overall P/AI and P/AI at first service with PR were both 0.72 ($P < 0.0001$). Similarly, for PREG150, the correlation with overall P/AI (0.63; $P < 0.0001$) and P/AI at first service (0.66; $P < 0.0001$) were greater than with SR (0.48; $P = 0.001$). As discussed previously, reproductive efficiency is associated with the efficiency, timing, and fertility to the first and later AI programs (Giordano et al., 2012). The stronger association of P/AI with reproductive performance compared to SR highlights the importance of using programs to increase SR (such as use of TAI), but also using programs and management to maximize fertility (Consentini et al., 2021), for example, implementing fertility programs at first service (Fricke and Wiltbank, 2022) since fertility at first AI is a major driver of reproductive performance.

Regarding general nutritional information, the percentage of forage in the diets varied from 48 to 60% (average = 56.1%), and the variation in the main components of the diet is depicted in Figure 1. As shown, there is considerable variation in TMR between herds, particularly in forage, starch, NDF, and NFC content of the diets. The variation in vitamin content in the TMR diets was surprisingly large, with vitamin A ranging from 93,000 to 401,000 IU, vitamin D from 28,700 to 72,800 IU, and vitamin E ranging from 460 to 2,868 IU per cow per day. Several factors could influence ingredients used within a farm and, thus, TMR composition, such as quality and type of forage, price and availability of ingredients, and the necessity or desire to include a particular ingredient by a nutritionist or dairy producer.

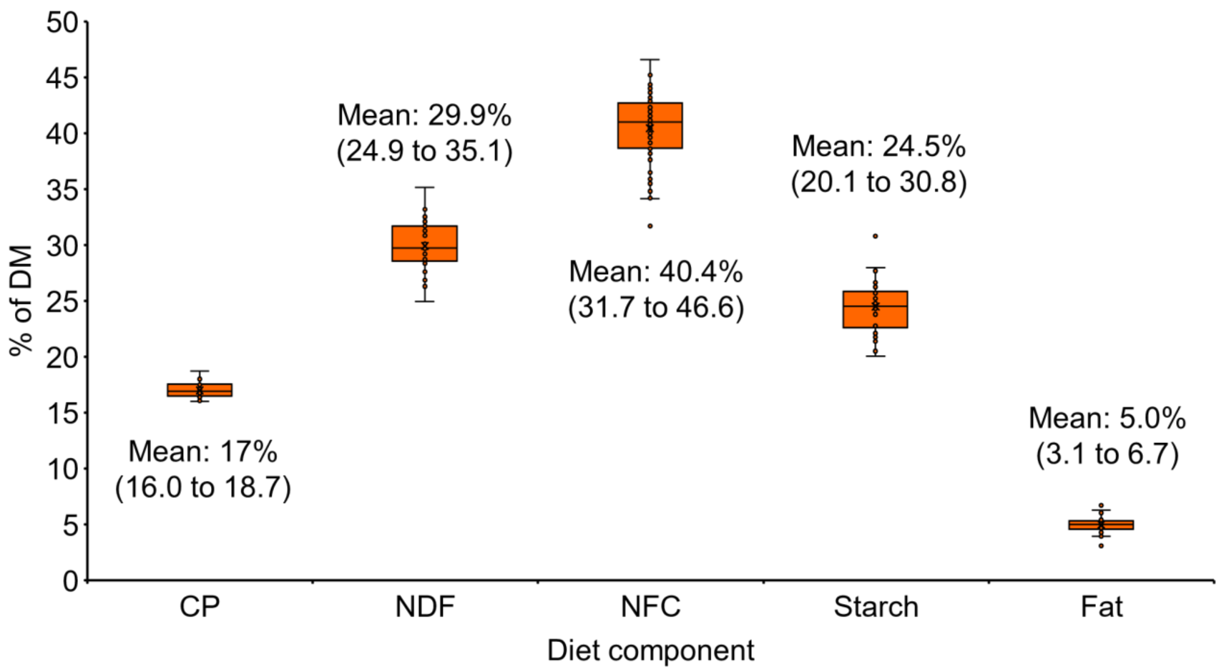


Figure 1. Variation in dietary components (% of dry matter, DM) among high producing dairy herds. CP: crude protein, NDF: neutral detergent fiber, NFC: non-fiber carbohydrate.

There was no correlation between NFC (R = -0.14; *P* = 0.32), NDF (R = 0.08; *P* = 0.57), CP (R = 0.06; *P* = 0.70), RDP (R = 0.04; *P* = 0.79), RUP (R = 0.02; *P* = 0.98), starch (R = -0.22; *P* = 0.22), or fat (R = 0.23; *P* = 0.12) content of the diets in high production pens with herd average milk production. Moreover, the variation in NFC (32 to 47) and starch (20 to 31) among the farms in this study is within the range of NFC and starch values reported by for high producing cows (NRC, 2001, National Academies of Sciences and Medicine, 2021). Thus, it may be possible for dairy herds to feed well-formulated diets with controlled starch and NFC levels, with adequate forage and non-forage ingredients, and still achieve high milk production. There are various factors that influence milk production, many of which were not controlled or evaluated in the present study. However, these results are encouraging in terms of attempting to better understand variation in the main components of the diets among farms and their influence on milk production. For instance, it would be interesting to experimentaly evaluate if controlled levels of NFC and higher NDF would allow high milk production while improving reproduction.

The relationships between various aspects of the TMR and three measures of reproductive efficiency are in Table 1. The three measures of reproductive performance were chosen because there was no correlation between any of the dietary components and SR and correlations with PREG150 were very similar to correlations with PR. Overall, there were no detectable associations of dietary protein, expressed as CP, RDP, or RUP, on these reproductive measures across the dairy herds. The strongest relationship was found for NFC with decreasing reproductive performance with increasing NFC. This negative relationship of NFC was significant for P/AI at first service, overall P/AI, or percentage pregnant at 150 DIM. Conversely, greater NDF was associated with greater P/AI at first service. The NFC content of the TMR was also associated with a decrease in reproductive performance, expressed as P/AI at first service or overall P/AI and tended to be negatively associated with PREG150 (Table 1).

Table 1. Correlation between dietary components and reproductive measures in high producing commercial dairy herds.

Item (% of DM)	Reproductive measurement		
	P/AI at 1st service	Overall P/AI	Pregnant by 150 DIM
CP	0.05 (0.73)	0.16 (0.31)	-0.12 (0.45)
RDP	-0.11 (0.48)	-0.03 (0.85)	-0.16 (0.32)
RUP	0.23 (0.14)	0.26 (0.10)	0.06 (0.70)
NDF	0.34 (0.03)	0.25 (0.11)	0.11 (0.48)
NFC	-0.51 (0.0005)	-0.48 (0.001)	-0.33 (0.03)
Starch	-0.35 (0.05)	-0.20 (0.28)	-0.16 (0.38)
Fat	0.34 (0.02)	0.24 (0.12)	0.24 (0.17)

The table shows correlation coefficient (R) and *P* value (between parenthesis).

CP: crude protein, RDP: rumen degradable protein, RUP: rumen undegradable protein, NDF: neutral detergent fiber, NFC: non-fiber carbohydrate.

Figure 2 illustrates the relationships between NDF and NFC with reproductive measures. As shown, NDF is positively associated with P/AI at first service either when comparisons were made with all individual herd data or if herds were divided by tertiles for NDF and compared to reproductive measures. Conversely, there was a strong negative association between NFC, on an individual herd basis or when herds were divided by tertiles, and all three measures of reproductive performance (Figure 2).

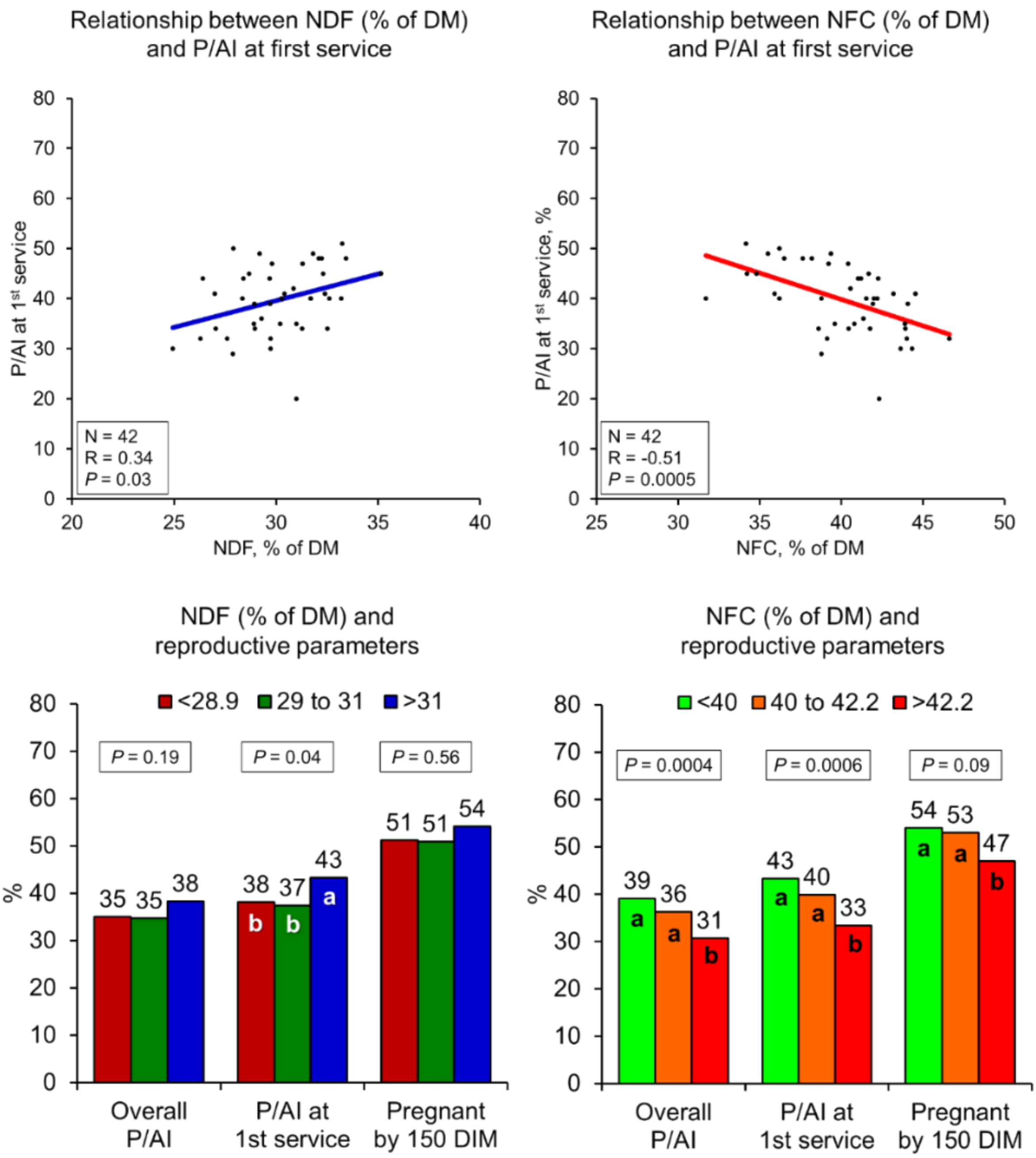


Figure 2. Relationship between dietary levels of neutral detergent fiber (NDF) and non-fiber carbohydrate (NFC) and reproductive outcomes in high producing commercial dairy herds.

The methodology used in the present study does not allow us to determine the reasons that specific dairy herds had greater or less NFC in their diets or the mechanisms that produced the negative correlations with overall P/AI (-0.48), P/AI at first service (-0.51) and PREG150 (-0.33). When the effect of starch level of diets was evaluated, similar to NFC, starch had a negative relationship with P/AI at first service (-0.35; *P* = 0.05), but had no detectable effect on overall P/AI or PREG150 (Table 1). Since the starch, particularly coming from corn (e.g. dry ground corn or high moisture corn), is the main source of NFC in the diets, we expected a negative effect of high levels of starch on reproduction. High starch leads to increased insulin that can reduce fertilization of oocytes, increase degeneration of embryos, and cause an overall reduction in fertility (Bender et al., 2014, Wiltbank et al., 2014). There are previous studies reporting a decrease in DMI for cows receiving high-starch diets or starch sources with greater fermentability (Albornoz and Allen 2018). Moreover, high starch diets can induce upregulation of genes associated with inflammation (Albornoz et al., 2020). In addition, the occurence of subacute ruminal acidosis (**SARA**) is related to the starch and NFC content of diets (Khorrami et al., 2021). In a study (Khafipour et al., 2009) with lactating dairy cows, the authors exposed cows that were consuming a 50:50 forage to concentrate diet (starch: 26.1% and NFC: 32.7%) to a high starch/NFC diet (starch: 33.4% and NFC: 40.4%) or maintained the cows in the control group. Cows fed the high starch diet had decreased DMI (16.5 vs. 19.0 kg/d) and milk production (28.3 vs. 31.6 kg/d). In addition, the high starch diet induced a lower average ruminal pH and more than doubled the minutes per day of pH below 5.6 (279 vs. 118 min/d). In the same study, rumen lipopolysaccharide (**LPS**) was increased, and also plasma LPS (0.52 vs. < 0.05 EU/mL). The authors suggested that a high NFC/starch diet may trigger an inflammatory response, which was further evidenced by an increase in haptoglobin and LPS biding protein in cows consuming the high starch/NFC diets (Khafipour et al., 2009). Cows with inflammatory response have greater energy (e.g. glucose) utilization, potentially producing a more negative energy state (Kvidera et al., 2017). In addition, a negative relationship between inflammation markers and fertility has been reported (Zebeli et al., 2015). Thus, these negative aspects of high NFC diets could impair reproductive performance, in spite of potential benefits of increased dietary energy coming from NFC. Figure 2 shows the negative associations of NFC and reproductive performance, particularly in herds with higher NFC, as P/AI decreased from 43 to 33% (> 20% reduction in relative P/AI) with corresponding decreases in overall P/AI and PREG150.

The effect of fat on reproduction has been extensively studied in past research and our study also found a positive correlation of percentage fat in the TMR with P/AI at first service. This could be due to multiple reasons. First, when dietary starch and NFC are reduced, fat may be added to the diet to increase the energy content of the diet. Indeed, level of fat had a negative correlation with NFC in our database (-0.49; $P = 0.0004$) providing evidence for this potential indirect effect of dietary fat on reproduction. Second, several studies have evaluated effects of dietary fat on milk production, health, and reproduction with studies generally supplementing cows during the transition period and/or early lactation (Rodney et al., 2018). Generally, there is a positive effect of fat supplementation, particularly unsaturated fatty acids, on health, follicle and corpus luteum (**CL**) development, and on pregnancy outcomes (Santos et al., 2009). For instance, Sinedino et al. (2017) supplemented cows after the transition period (from 27 to 147 DIM) with docosahexaenoic acid and reported better cyclicity and greater P/AI at first service, particularly in primiparous, and greater overall P/AI. In another study, cows supplemented with fish oil during the breeding period (30 to 160 DIM) had greater overall P/AI on d 60 and lower pregnancy loss (Silvestre et al., 2011). Altogether, the current findings argue for a positive effect of fat supplementation, although it is hard to draw final conclusions whether this is a direct effect or due to the fact that a greater use of fat in the diet allows for less NFC to meet the energy demands of postpartum cows.

The lack of an effect of dietary protein on any of our reproductive measures is interesting, however it should be noted that the variation in dietary CP, RDP, and RUP levels was not as large as variation in other components of the diets. Some previous studies have noted a negative effect of blood or milk urea nitrogen (**MUN**) on reproduction (**MUN**; Webb and Bruyn, 2021). The observed MUN can be influenced by CP, RDP, and RUP levels, as well as the quality of protein, and energy in the diet. We expected no effect of protein on reproduction, since modern well-formulated diets generally do not have issues with elevated MUN. Consistent with our results, a previous meta-analysis also reported no effect of CP, RDP, or RUP on P/AI or interval from calving to pregnancy (Rodney et al., 2018).

Finally, the limitations of this type of study need to be emphasized. There are numerous dietary and management factors that can greatly impact reproductive performance such as cow comfort, reproductive program, pen size, stocking density, type of housing to name just a few factors that could cause variation in reproductive performance between dairies (Chebel et al., 2016; Wang et al., 2016; Jensen and Proudfoot, 2017). Some other management factors with potentially important effects on fertility such as homogeneity of TMR provided within pens or across days, consistency in feeding times, or even feeding deviations due to external factors (rainfalls, etc) were not accounted in this study. In addition, other characteristics of the ingredients and diets that were not evaluated in this study could influence DMI, energy balance, milk production, behaviour, and reproduction. For example, fat supplementation in our database was not detailed in depth. It is known that fat supplementation can impact NDF digestibility, DMI, milk production, and NDF content based on type of fatty acids (saturated, n-3, n-6) level of inclusion and period of lactation (Weld and Armentano, 2017; Piantoni et al., 2015; Souza et al., 2019; Souza et al., 2021) and these factors could change reproductive performance. Similarly, the quality and physical characteristics of the forage sources could differ substantially among farms, and it is reported that type of forage, fiber content and digestibility, and particle size influence DMI, and behaviour patterns such as eating, rumination, chewing, and resting (Jiang et al., 2017; Grant and Ferraretto, 2018;). Finally, negative energy balance and BCS changes during the transition period and early lactation are likely to differ substantially among farms and it is well-established that BCS changes during early lactation dramatically impact health, fertility at first service, and reproductive performance (Carvalho et al., 2014; Barletta et al., 2017). Thus, since our analysis was based on differences in reproductive performance between different dairy herds that were not controlled for numerous confounding factors, the results should not be used as definitive proof for any specific theory. Instead, these results can be used as the rationale for further studies on the critical topic of the effects of nutrition on reproduction in lactating dairy cows.

In conclusion, the results of this study suggest that farms with greater dietary NFC, particularly during early lactation, may have compromised reproductive performance, such as decreased P/AI at first service, lower overall fertility, and fewer cows pregnant by 150 DIM. On the other hand, herds with greater NDF content potentially have positive effects on reproduction. Other effects of dietary components on reproduction were not as obvious in this herd-level analysis.

Notes

The authors thank the owners and staff of all farms that kindly agreed to participate in the study, allowing complete access to their nutritional and reproduction data. The authors have not stated any conflicts of interest.

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CHAPTER 5 (TO BE SUBMITTED FOR JOURNAL OF DAIRY SCIENCE COMMUNICATIONS): RELATIONSHIP BETWEEN HEAT STRESS VARIABLES AND FERTILITY OF LACTATING DAIRY COWS DURING A REPRODUCTIVE YEAR IN A COMMERCIAL DAIRY HERD

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Body of the paper

Numerous aspects impact milk production and reproductive performance of high producing dairy herds, such as body condition score (**BCS**) changes and health problems during the transition period (Carvalho et al., 2014; Manríquez et al., 2021), anovulatory condition (Vieira-Neto et al., 2014), genetic traits (Lima et al., 2020), nutrition (Rodney et al., 2018), timed-articial insemination (**TAI**) programs and reproductive management (Consentini et al., 2021). Nonetheless, one key aspect that significantly impacts animals and industry overall performance, is the heat stress (**HS**).

Lactating high producing dairy cows, mainly due to milk production and greater dry matter intake, have lower termoregulatory capacity than heifers (Sartori et al., 2002), and it is reported that greater milk production is associated with lowered threshold for HS (Yan et al., 2021). The HS in lactating dairy cows is associated with changes in behaviour and physiological alterations that eventually can impact milk production, health and fertility (Becker et al., 2020). In addition, several impacts on reproduction are reported, such as impaired fertilization and embryo development (Sartori et al., 2002; Kasimanickan et al., 2021), compromised follicular and corpus luteum (**CL**) development and fuction, as well as hormonal patterns and reduced pregnancy per AI (**P/AI**; Schuller et al., 2014, 2017; Wolfenson and Roth, 2018).

Retrospective cohort studies, evaluated the effect of HS on fertility (Schuller et al., 2014, 2017; Baruselli et al., 2020), Likewise, our objective was to evaluate the association of HS variables on P/AI of lactating dairy cows during one reproductive year in a commercial dairy herd located in a tropical environment/climate. Additionally, we studied the relationships between HS and type of service (AI by estrus vs. TAI), service number, BCS and milk yield. Database included rectal temperature (**RT**) at the time of AI, temperature-humidity index (**THI**), and seasonal weather (hot vs. cool). We anticipate that cows inseminated in HS would have lower fertility, but the negative effect of HS would be greater in particular classes of cows, such as those with higher milk production for instance.

Data were retrieved for entries from January 1st, 2014, until December 31, 2014. The commercial dairy herd was located in Minas Gerais state, Brazil (Latitude: 20° 58' 17" S, Longitude: 46° 7' 57" W). The farm had approximately 1,500 lactating Holstein cows milked thrice daily and fed twice with a total mixed ration based on corn silage and a corn and soybean meal-based concentrate with minerals and vitamins. The database included only primiparous cows. A total of 1,463 AI events was recorded, with the majority being TAI (n = 1,078) compared to AI by estrus (n = 385). The TAI protocol used in the herd during the period of the study was a common estradiol plus progesterone-based protocol. Before each AI procedure, RT was measured and recorded. The climate variables were the daily dry bulb temperature (T; °C) and relative humidity (RH; %) recorded by the Instituto Nacional de Meteorologia (INMET, Estação: Machado/MG (A567), Brazil). Temperature and RH of the day were used to calculate the daily THI based on the equation from Ravagnolo and Misztal (2000).

Statistical analyses were performed using the Statistical Analysis System (SAS, Version 9.4 for Windows SAS Institute Inc., Cary, NC). Correlation between climate variables (temperature, RH and THI) were performed using the CORR procedure. Analyses of P/AI on d30 were performed using the GLIMMIX procedure, fitting a binary distribution with Link logit function. Additionally, the option ddfm = kenwardroger was included in the model statement to adjust the degrees of freedom for variances. All models to study the effect of HS variables included the effects of type of AI (estrus or TAI), service number (1^{st} or ≥2), BCS (≤2.75 or >2.75), milk production class (≤30 or >30 kg/d, with 30 being the calculated median), and the interaction between the HS and the other class variables. The HS variables considered were RT (<39.1 and ≥39.1 ºC), THI (≤68 and >68), and season of the year (Hot:

Spring [September/22 to December/21] and Summer [December/22 to March/19], and Cool: Autumn [March/20 to June/20] and Winter [June/21 to September/21]).

Tukey honest significant difference *post hoc* test was performed to determine differences. Values are presented as mean ± standard error of the mean (SEM). Significant differences were declared when $P \leq 0.05$, whereas tendencies were considered when $0.10 > P > 0.05$.

The LOGISTIC procedure was used for logistic regression to model the probability of pregnancy on d30 according to RT and THI. Logistic regression curves were created using the coefficients provided by the interactive data analysis from SAS and the formula $Y = exp\ (\alpha \times X + \beta) / [1 + exp\ (\alpha \times X + \beta)]$, where Y = probability of occurrence; *exp* = exponential; α = slope of the logistic equation; β = intercept of the logistic equation; and X = analyzed variable.

The average milk production of the herd was 30.3 ± 0.20 kg/d, and number of AI was 3.1 ± 0.06 (1^{st} service: n = 469; 2^{nd} or greater services: n = 994). Mean THI during the period evaluated was 67.2 ± 0.12, and the correlationship between THI and temperature of day (R = 0.99; $P < 0.0001$) was much stronger than THI and RH (R = -0.19; $P < 0.0001$). The monthly THI and P/AI are presented on Figure 1, and as expected, it is possible to identify the pattern in which THI increases at the end of the winter and remain high (average above 68) during the entire hot season, while during the most of the fall and winter, the mean THI was below 68. It is interesting to mention that, in this particular year and location, the low THI from May to August is explained mainly by the the 5 ºC drop in average temperature (data not shown), because the RH remained elevated (73-79) during these months, only decreasing during the months of August, September and October (60-62).

Despite few fluctuations during the year, it is possible to identify that the highest P/AI did not coincide with the months with lowest THI (Figure 1), and this pattern is also observed in previous studies (Baruselli et al., 2020). Generally, the months with greater fertility are those at the end of the cool season, and not at the beginning or in the middle of season. This result reveals the importance of HS not only causing short-term, but also long-term negative effects. According Roth, 2017, cows inseminated in September had most of their follicle development (early primordial, antral and preovulatory stages) under moderate or lower levels of HS, which can be important in terms of oocyte and embryo quality, and fertility. Conversely, during the hot months, the fertility rapidly decreases, following the increase in HS, evidenced by elevations on THI.

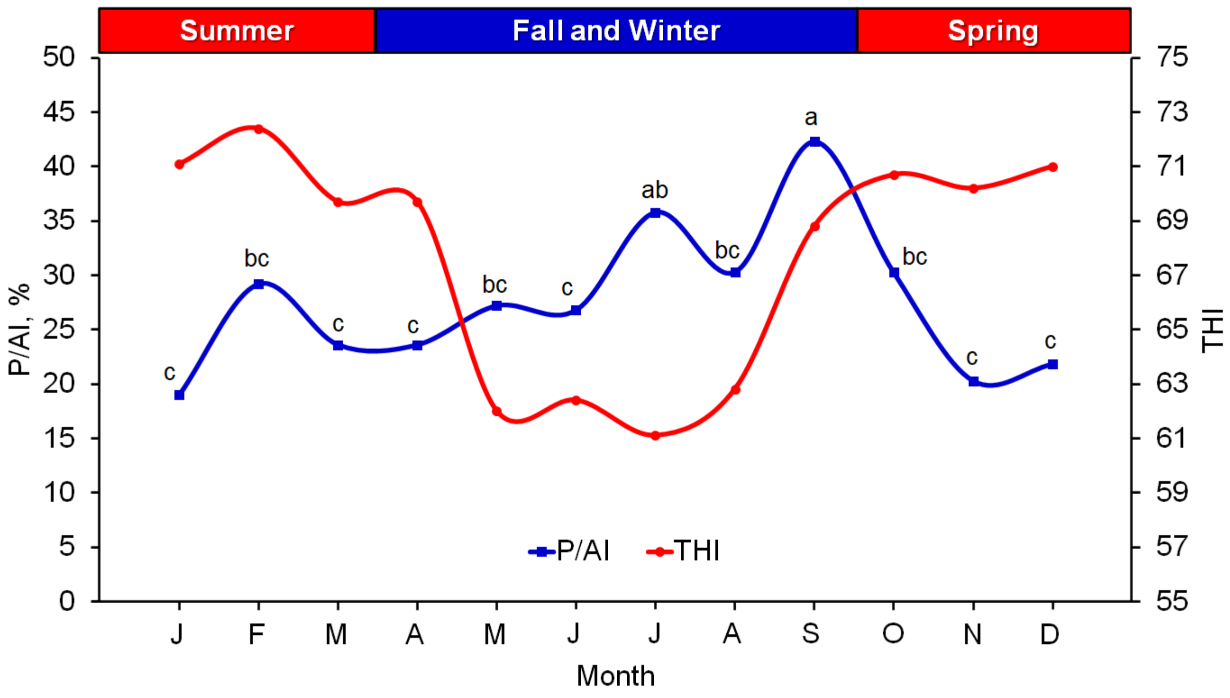

Figure 1. Monthly temperature-humidity index (THI) and pregnancy per artificial insemination (P/AI) throughout one reproductive year in a commercial dairy farm (n = 1,463 AI). [a,b,c]Different letters denote differences on P/AI among the months (P = 0.01).

There is a relationship among HS variables, so, at a certain level, it is expected that HS variables similarly impact fertility, and that happened in the study, with RT ≥39.1 (22.5 [101/448] vs. 29.1% [295/1,014]; P = 0.01), hot season (24.2 [165/683] vs. 29.7% [232/780]; P = 0.05) and THI greater than 68 (24.1 [182/754] vs. 30.3% [215/709]; P = 0.04) decreasing fertility in about 20%. To highlight the impact of RT and THI, probability curves for pregnancy according to these variables are presented in Figure 2. There are numerous studies reporting the negative effect of elevated RT or THI on P/AI in cows receiving AI after estrus or TAI (Schuller et al., 2014; Schuller et al., 2017; Pereira et al., 2017). Higher THI was associated with reduced fertility in a previous study evaluating 7,252 AI, particularly THI above 70 (Schuller et al., 2014), and RC above 39.1 ºC decreased fertility in 33% (22.8 [162/709] vs. 34.1% [279/817]) in lactating Holstein cows with similar milk production and location of the present study (Pereira et al., 2017).

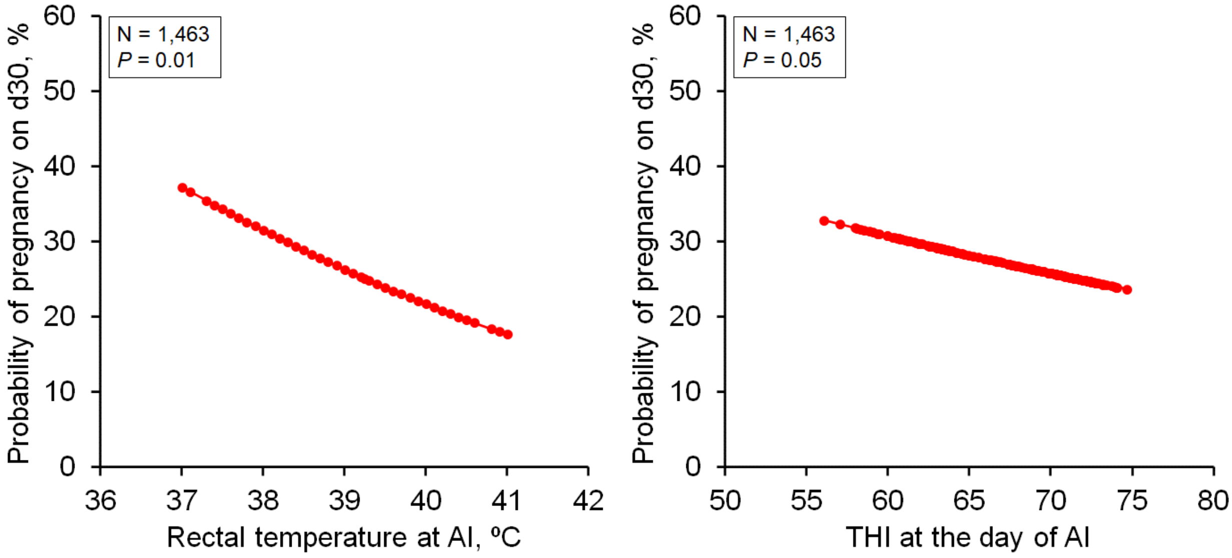

Figure 2. Probability curves for pregnancy on d30 according to rectal temperature at the time of artificial insemination (AI) and the temperature-humidity index (THI) at the day of AI during one entire reproductive year of a commercial dairy farm.

There was no interaction between any of the HS variables and the other class variables, and effect of season, RT and THI was similar within the classes of cows stablished to perform the analysis. Thus, we decided to present a table comprising the effect of type and number of AI, BCS and milk production, and their interactions with only one of the HS variables, electingTHI (Table 1).

Table 1. Pregnancy per artificial insemination on d30 according to type and number of service, body condition score (BCS) and milk production, as well as their interaction with temperature-humidity index (THI) at the day of AI.

Item	Overall	THI at day of AI		*P*-value[1]		
		>68	≤68	THI	Var	Int
Type of AI						
Estrus	26.5 (102/385)	25.3 (50/198)	27.8 (52/187)	0.04	0.73	0.31
TAI	27.4 (295/1,078)	23.7 (132/556)	31.2 (163/522)			
Service						
1st	35.6 (166/469)	31.8 (83/261)	39.9 (83/208)	0.005	<0.0001	0.96
≥2	23.3 (231/994)	20.1 (99/493)	26.4 (132/501)			
BCS						
≤2.75	24.7 (163/660)	21.5 (75/349)	28.3 (88/311)	0.004	0.05	0.62
>2.75	29.1 (230/790)	26.4 (106/401)	31.9 (124/389)			
Production						
≤30 kg/d	22.4 (119/532)	19.2 (40/208)	24.4 (79/324)	0.03	0.0002	0.94
>30 kg/d	33.1 (183/553)	29.5 (72/244)	35.9 (111/309)			

[1]THI: main effect of THI; Var: main effect of each variable; Int: interaction between the variable and THI.

There was no effect of type of AI on fertility, although the type of cows receiving AI by estrus or TAI may vary throughout the year due to several reasons, such as number of service, anovulatory condition and farm insemination decisions and criteria. There was no interaction between THI (or season and RT) and type of AI, which is reasonable since the impact of HS on the oocyte, for instance, would compromise fertility regardless of type of AI. The lack of difference on fertility according to type of AI can be attributed to the fact that the TAI protocol implemented during the year is considered non optimized, and there are several adjustments and manipulations which are known to increase fertility of lactating dairy cows submitted to TAI programs compared to estrus (Consentini et al, 2021; Fricke and Wiltbank, 2022).

There was no interaction between BCS and THI, in which HS conditions had similar negative effects on fertility of both BCS classes of cows. As expected, cows with lower BCS had reduced fertility, which is completely established in the literature (Carvalho et al., 2014).

Not only the BCS near AI is important to fertility, but the BCS changes, particularly in the postpartum period, significantly influence health and reproductive performance (Carvalho et al., 2014; Manríquez et al., 2021). In this sense, we studied the relationship between BCS and number of AI, and an interaction was detected, in which the BCS was more important for fertility when cows were receiving the first postpartum service (28.7 [58/202] vs. 40.4% [107/265], for BCS ≤2.75 and >2.75, respectively; P = 0.009) compared to later AI (22.9 [105/458] vs. 23.4% [123/525], for BCS ≤2.75 and >2.75, respectively; P = 0.85).

Cows receiving the first service had greater fertility than cows receiving later AIand there was no interaction between number of service and type of AI (P = 0.84). Despite no differences between estrus and TAI for first service were observed in this database, currently, it is known that fertility programs can promote greater service rate and fertility compared to managements based on estrus detection (Santos et al., 2017). We hypothesized that negative HS impact on fertility would be more important for cows receiving the first service since they would be more challenged due to postpartum BCS loss in most of them and elevated milk production near the lactation peak. However, there was no interaction between HS variables, such as THI, and number of service. In fact, in our dataset, milk production was positively related with fertility and also did not interact with HS variables (Table 1).

Aiming to evaluate the association between milk yield and fertility, the database was partitioned according to the median (≤30: average = 24.9 ± 0.17 kg/d or >30: average = 35.4 ± 0.15 kg/d) indicating higher P/AI on cows with higher milk production (Table 1). These results are intriguing since several times milk production is considered negatively associated with fertility, however, in well managed dairy herds, with optimized cows' comfort, nutrition and high fertility TAI programs, it is possible to achieve high fertility along with high milk production (Fricke and Wiltbank, 2022). We failed to confirm a hypothesis that HS have greater impact on cows with greater milk production, even with milk production being probably associated with greater HS or lowered threshold for HS (Yan et al., 2021). Interestingly, in a previous study that partitioned herds in low and high production to evaluate effect of intensive or moderate colling programs during the summer, showed results in wich moderate colling was related with lower fertility, but the negative effect of not implementing intensive heat abatement strategies was more pronounced in herds with low milk production (Flamembaum and Galon, 2010). However, it is worthy to mention that this type of study had limitation in terms of controlling factos that impact reproduction. Several non recorded or controlled aspects could be influencing why cows with higher milk production achieved greater fertility, such as allocation of them in better and well managed facilities in the farm and an improved nutrition of cows producing more milk. Moreover, social stressors (Chebel et al., 2016), stocking density (Wang et al., 2016; Creutzinger et al., 2021a,b), nutrition (Rodney et al., 2018), BCS and BCS changes (Carvalho et al., 2014) are some of other factors that may vary throughout the year and can be influencing fertility and the effects of HS variables.

In conclusion, this is one additional study that supported the importance of HS variables on herd-level fertility, reinforcing the importance of implementing heat abatement strategies in order to alleviate extreme reduction on fertility, particularly during periods of heat stress. Moreover, in this dataset, it was not stablished an interaction of heat stress variables and other aspects such as type of service, number of AI, BCS and level of milk yield.

The authors thank the owners and staff of Fazendas Reunidas that kindly agreed to participate on the study, allowing access to their record. Moreover, we extend our appreciation to Heloíse Duarte from IDEAGRI company for helping with retrieving cows information from the Ideagri software. The authors have not stated any conflicts of interest.

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