Theriogenology 224 (2024) 41–49

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Original Research Article

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

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ARTICLE INFO

Keywords:
Bos indicus
Breeding season
Doppler ultrasound
Timed AI

ABSTRACT

This study evaluated the efficiency of a rapid reinsemination program allowing timed AI (TAI) every 21d (ReBreed21) in a commercial beef cow-calf operation. Nelore females from different parities (n = 2085) were synchronized for first TAI (D0 = TAI) using an estradiol/progesterone (E2/P4) protocol and assigned to one of three reinsemination programs: Resynch33 (n = 753), traditional resynch program with second TAI at D42 after first TAI; ReBreed21 (n = 687); or ReBreed21+EC (n = 670). The ReBreed females (n = 1357) received intravaginal P4 insert on D12, on D19 P4 was removed, and a dose of equine chorionic gonadotropin (eCG) was administered, then, ReBreed21 females received 0.6 mg of EC (ReBreed21+EC) or nothing (ReBreed21) and on D21, nonpregnancy (NP) was determined using Doppler ultrasound to detect corpus luteum (CL) blood flow (BF) (NP: <25 % BF pixels of total CL area) and NP cows received immediate TAI and GnRH to induce ovulation. Pregnancy diagnosis was performed at D33 after TAI following all TAIs. Cows considered pregnant at D21, based on CL BF, but NP on D33 were designated False-Positives (FP) and false negatives (FN) were number of nonpregnant cows/heifers on d21 based on the CL BF found to subsequently be pregnant on D33 divided by the total number pregnant. Pregnancy/AI (P/AI) did not differ for the first TAI (55.1 %) among the treatments. Heifers had similar P/AI at the second AI in all groups and similar to the first AI. Primiparous had greater P/AI in ReBreed21+EC and Resynch33 at s TAI compared to ReBreed21, 51.7 %, 55.8 %, 34.2 %, respectively. Multiparous had greater P/AI at second TAI in Resynch33 (60.9 %) than ReBreed21 programs (34.7 %). The percentage FP and FN among ReBreed21 programs did not differ, 13.8 and 0.2 %, respectively. Overall accumulative pregnancies on D21 of the breeding season were greater for ReBreed21 and ReBreed21+EC than Resynch33 (69.7 %, 71.6 %, and 55.5 %, respectively). However, on D42 of the breeding season, only heifers had greater pregnancies in ReBreed21 programs than Resynch33 (73.3 %, 74.3.6 %, and 63.2 %, respectively). Average days to pregnancy were less (P = 0.01) for ReBreed21 and ReBreed21+EC than Resynch33. Thus, the ReBreed21 strategy can improve the efficiency of TAI programs in beef cattle. Of interest, ReBreed21 was particularly effective in nulliparous, somewhat effective in primiparous when EC was added to the program, but relatively ineffective in multiparous beef cattle.

1. Introduction

Timed artificial insemination (TAI) is a biotechnology that allows cows to receive AI without detection of estrus [1]. It has been particularly useful in beef cattle because it allows the entire herd to potentially receive AI near the start of the breeding season with a minimal number of animal handlings [2]. Protocols that allow TAI also induce cyclicity in non-cycling cattle [3]. In addition, TAI allows the use of high genetic sires from anywhere in the world to produce higher-quality F1 offspring or improve the herd's genetics. Using TAI, a beef cattle operation can

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https://doi.org/10.1016/j.theriogenology.2024.04.019
Received 27 November 2023; Received in revised form 26 April 2024; Accepted 29 April 2024
Available online 30 April 2024
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have 50 % of cows pregnant on the first day of the breeding season [4–6]. Thus, the use of TAI reduces the length of the breeding season, improving the productivity and profitability of beef cattle operations [7]. In all beef cattle systems, the most productive and profitable cows are those that become pregnant during the first 21d of the breeding season, followed by those that become pregnant during the second 21d cycle, followed by a decrease in economic value and efficiency (lighter steers and lower fertility heifers) in cows pregnant later in the breeding season [8–10]. Nevertheless, modeling of the breeding season shows that, even in high efficiency operations, more than 40 % of heifers and cows become pregnant after the first 21d from the start of the breeding season, reducing profitability and increasing the risk of involuntary culling of females [5].

To increase the number of cows pregnant in the early breeding season and during a shortened breeding season, strategies have been developed, termed Resynch strategies, that seek to reduce the interval between TAIs [11]. The method of pregnancy diagnosis is a key determinant of the type of Resynch strategy that can be utilized in a herd. The gold standard for pregnancy diagnosis is the use of transrectal ultrasound, utilizing the B mode, to visualize the embryonic heartbeat around 30d after TAI. Utilizing this method of pregnancy diagnosis, a Resynch strategy can be initiated in nonpregnant cows allowing TAI at about 40d after the first TAI. Alternatively, a Resynch strategy can begin at D22 after TAI, without knowing the pregnancy status of the cow, with the pregnancy diagnosis at about 30d and TAI two days later [12–16]. In addition, research has utilized Doppler ultrasound to indirectly determine the pregnancy status based on CL blood flow around 21d post-TAI [17,18]. Based on this technology, an early Resynch can be initiated that will allow TAI two days after the Doppler pregnancy diagnosis, i.e. ~23d after previous TAI [19,20]. Despite potential improvements with these different Resynch programs, all these strategies produce a second TAI that is after the first 21d of the breeding season.

One additional complication is that early Resynch strategies have been difficult to practically implement on commercial beef cattle operations due to: 1) Treatments occurring on different days of the week than the typical days used for the first TAI and may be required to be performed on weekends, 2) Early pregnancy evaluations can produce false positive diagnoses that need to be accounted for in the breeding program, 3) Doppler ultrasound can be expensive and technically challenging to perform. Our research group has recently been working with a program termed ReBreed21 that allows TAI every 21d and that matches a similar daily schedule as used for the first TAI [21]. The pregnancy diagnosis and TAI are both performed on D21 after the previous TAI to optimize the labor schedule. Finally, unlike other early Resynch strategies, there is no treatment with prostaglandin $F_{2\alpha}$ (PGF) during the Resynch protocol, thus reducing the risk of iatrogenic abortions in false-negative cows [14,22,23]. In the ReBreed21 protocol, the intravaginal P4 implant is removed two days before the early pregnancy diagnosis and treatment with equine chorionic gonadotropin (eCG) is used to stimulate preovulatory follicle growth from d19 to 21. Another potential improvement to the protocol may be the addition of estradiol cypionate (EC) to increase circulating estradiol-17B (E2) during the proestrus period and potentially increase synchrony of luteolysis in nonpregnant cows. Previous research has shown that E2 treatment during early pregnancy (d18 to 21) will only induce luteolysis in nonpregnant cows, due to inhibition of endometrial ESR1 expression by embryonic interferon-tau in early pregnant cows [24,25]. Hence, fertility may be optimized and potentially false positives reduced by increasing circulating E2 during the period of proestrus [26–30].

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

2. Materials and methods

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

2.1. Animals, location, and reproductive management

Nelore *Bos-indicus* beef cattle (n = 2163) at Roncador Farm, located in Querencia, MT, Brazil were used in the present study. Animals were kept on pasture condition (*Brachiaria brizantha*) supplemented with mineral salt and had ad libitum access to water. The experiment was done with a total of 844 heifers, maintained in two different pastures, a total of 638 primiparous cows maintained in three different pastures, and a total of 603 multiparous cows maintained in three different pastures. Animals within a pasture were randomized to treatments so that all treatments were represented in each group. The treatments began when multiparous and primiparous cows were approximately 35d after calving, and heifers were at 14–20 months of age with ≥280 kg of body weight.

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

For management reasons (missing in the pasture or escape into another pasture) 78 cows/heifers were not found on the day of pregnancy diagnosis and these cows were excluded from further analyses, making 2085 as the final number of cows and heifers in the study.

2.2. Reinsemination protocols and pregnancy diagnoses (PD)

As summarized in Fig. 1, twelve days after the first TAI cows/heifers were randomized into one to three treatments: ReBreed21 (n = 687), heifers/cows received an intravaginal P4 implant (either new or used) on D12. Seven days later (D19), the implant was removed, and eCG (cows - 300 IU; heifers - 200 IU) was administered; ReBreed21+EC (n = 670), cows/heifers received an intravaginal P4 implant on D12. Seven d later (D19), the implant was removed, eCG (cows - 300 IU; heifers - 200 IU) and 0.6 mg of EC were administered. In both ReBreed21 groups, heifers/cows had ovaries evaluated by an experienced technician to determine pregnancy status two days later (D21) using subjective CL blood flow scores [33]. Heifers/cows considered to be nonpregnant, based on the Doppler ultrasound, received immediate TAI together with i.m. treatment with 25 μg of lecirelin acetate, a GnRH agonist (TecRelin®, Uniao Quimica, São Paulo, SP, Brazil). This procedure was replicated one more time using the same treatments in each heifer/cow to replicate the ReBreed21 program, allowing a 42-day breeding season with three potential TAIs. Pregnancy diagnoses were evaluated at D33

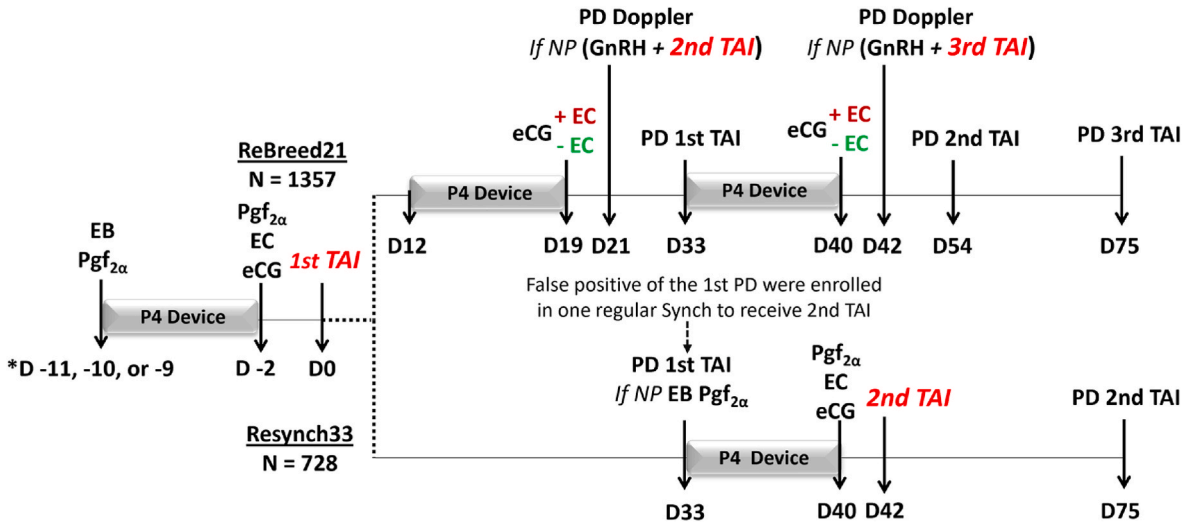

Fig. 1. Illustration of the treatments for ReBreed21 and Resynch33 including timing of intravaginal progesterone (P4) device (*kept for 7, 8, or 9 days [previously shown to produce similar outcomes [32]]), estradiol benzoate (EB), estradiol cypionate (EC), equine chorionic gonadotropin (eCG), cloprostenol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

after the first TAI in all cows/heifers (ReBreed21 and Resynch33) by B mode ultrasound. The presence of an amniotic vesicle containing an embryo with a heartbeat was the determinant of pregnancy. In the Resynch33 group (n = 728), nonpregnant cows/heifers were enrolled in a traditional Resynch breeding program: Insertion of an intravaginal P4 implant together with 2 mg EB (D33 after previous AI), 7d later, removal of P4 and treatment with PGF, EC, and eCG (200 IU – heifers; 300 IU – cows) and 2d later TAI (D42 after previous AI). Heifers/cows enrolled in ReBreed21 or ReBreed21+EC received the D33 pregnancy diagnosis to confirm the diagnosis on D21. Heifers/cows that were diagnosed pregnant on D21 by CL blood flow but were not pregnant on D33 were considered false positives. These heifers/cows were enrolled in the synchronization program using the same protocol as the Resynch33 group. Thus, they received a second TAI on D42 of the breeding season.

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

2.3. Statistical analyses

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

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

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

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

3. Results

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

The overall pregnancies at D21 of the breeding season was 24 % greater ($P < 0.01$) for cows enrolled in the ReBreed21 programs (70.7 %; 959/1357) compared to Resynch33 (55.5 %; 404/728) (Fig. 2). The most dramatic effect of the ReBreed21 programs compared with Resynch33 was on pregnancies at D21: 45 % more pregnancies for heifers (62.9 % [353/561] vs. 43.6 % [123/283]; $P < 0.01$), followed by a 26 % increase in primiparous (76.1 % [303/398] vs. 60.4 % [145/240]; $P < 0.01$), and 15 % for multiparous (76.1 % [303/398] vs. 66.3 % [136/205]; $P = 0.01$). Thus, the magnitude of the increase in pregnancy at D21 for ReBreed21 groups vs Resynch33 was greatest for heifers (45 %), less for primiparous (26 %), and least for multiparous (15 %), consistent with the differences in fertility at first TAI (highest in multiparous, least in heifers) and the reduction in fertility at second TAI

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

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

Lowercase letters indicate differences ($p \leq 0.05$) among the pregnancy/AI in each endpoint and Uppercase letters indicate tendency ($p > 0.05 \leq 0.1$).
[a] Pregnancies at the end of the breeding season.
[b] FP breeding is defined as a cow that had a functional CL on D21 but was nonpregnant on D33 and had second TAI at D42 using the Resynch33 program.
[c] Contrast Resynch33 vs. ReBreed21 + ReBreed21+ECP.
[d] Contrast ReBreed21 vs. ReBreed21+ECP.

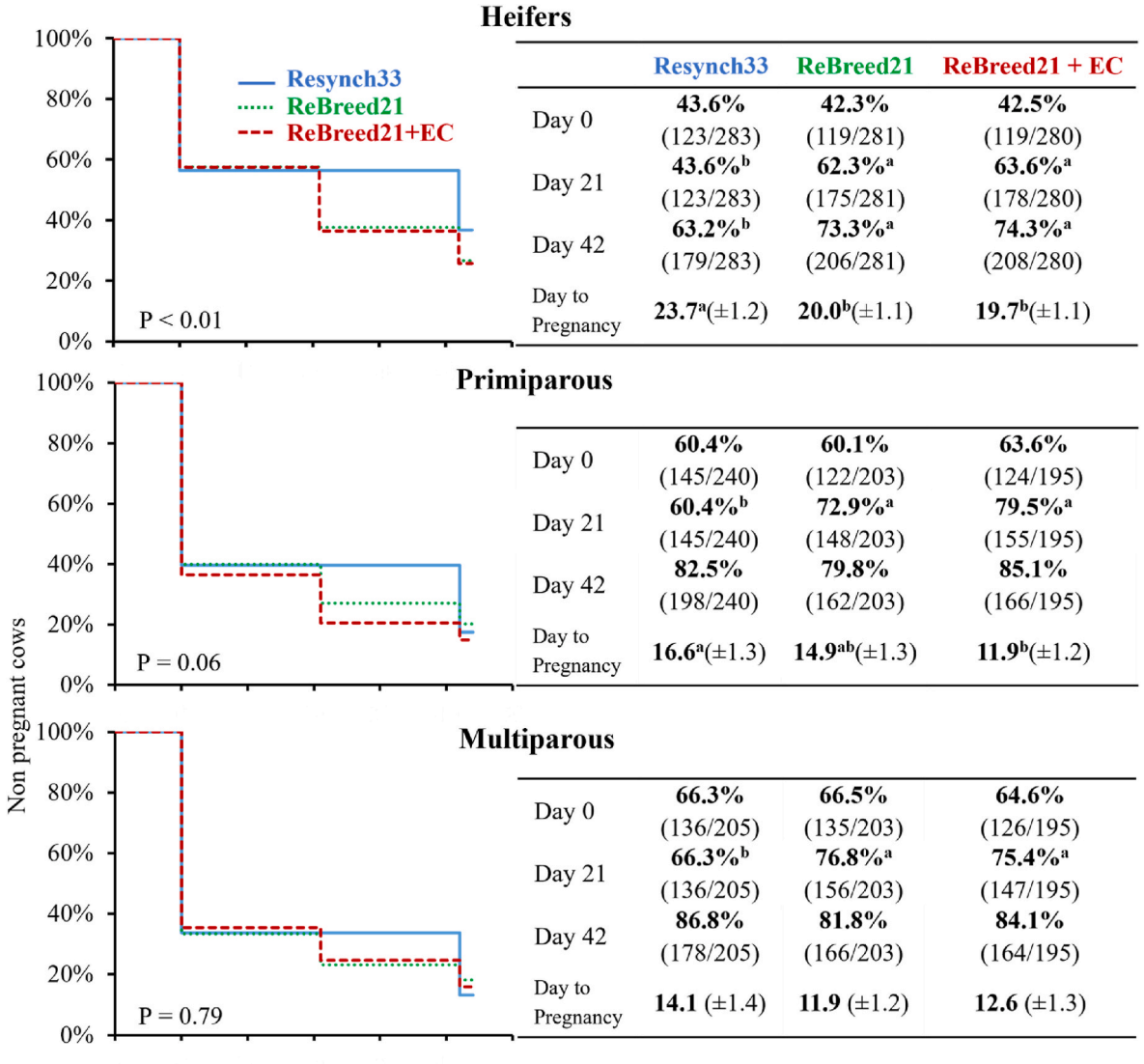

Fig. 2. Survival analysis of the nonpregnant heifers and cows in 42d breeding season with reproductive programs that allow TAIs at every 21 or 42 d. The right side of survival curve the cumulative pregnancy at d 0, 21, and 42 of the breeding season with the average day to pregnancy (±SEM) according to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

in multiparous but not in heifers (Fig. 3).

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

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

Animals were kept in eight different pastures in this experiment, within each pasture all three treatment groups represented, bred, and pregnancy diagnosed on the same day. Fig. 4 shows the results for each individual pasture at 21d and 42d of the breeding season. At 21d of the breeding season, all parities had increased pregnancy with ReBreed21 programs. Nevertheless, at 42d of the breeding season, only the heifers pastures were increased with no difference in cumulative pregnancy in primiparous and multiparous cows (Fig. 4).

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

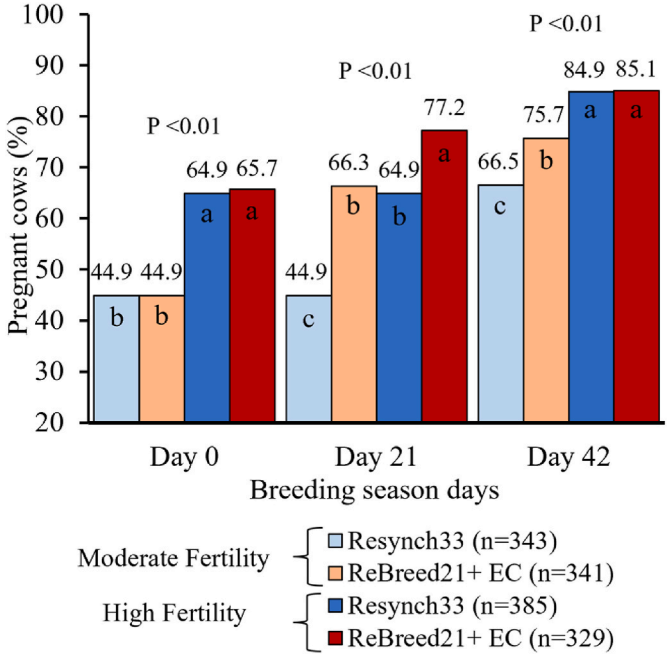

Fig. 3. Cumulative pregnancy during 42d breeding season according to the fertility of the first TAI (moderate fertility P/AI < median - high fertility P/AI > median) for cows enrolled in 2 different reproductive programs Resynch33 and ReBreed21+EC.

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

4. Discussion

Our first study with ReBreed21 introduced the basic concept for the rapid reinsemination program [21] and a companion paper [35] evaluates the physiology associated with this program, however these studies were done in only in a limited number of heifers. In the present study, the ReBreed21 program was evaluated in more than 2000 animals of different parities in a shortened breeding season. Overall, doing a second TAI at 21d after first TAI: 1) increased the percentage of heifers/cows pregnant at 21d of the breeding season, 2) increased the percentage of heifers pregnant at the end of the breeding season, and 3) shortened the day to pregnancy, based on survival analysis. Thus, this large, randomized, controlled trial on a commercial cow-calf operation provides critical information on parity and fertility at first TAI to consider in determining whether to utilize ReBreed21 in a reproductive management program.

The important physiological and fertility differences between parities make it critical to analyze all results by individual parity and, importantly, the number of animals used in this study allowed this analysis. Heifers had the lowest P/AI at first TAI and relative P/AI increased by 43.3 % for primiparous (42.8 vs 61.3 %, respectively) and increased 53.9 % comparing heifers to multiparous cows (42.8 % vs 65.8 %). For heifers, pregnancy early in the breeding season is critical for productive life, subsequent reproductive performance of the heifer, and for overall economic outcomes in a commercial cow-calf operation. Our results in this study (43.3 %) were somewhat lower than the average results in the scientific literature during the last 27 years with P/TAI in *Bos indicus* heifers of 47.0 % (7443/15,841) and 55.1 % (30,333/55,012) for *Bos taurus* heifers [36]. Most of the reported *Bos indicus* results are from older heifers (~20 months of age), whereas our study had many younger Nelore heifers (most less than 18 months of age) with many heifers likely to be prepubertal, based on other reports [37–39]. The ReBreed21 strategy was developed and optimized in Nelore beef heifers and, comparable to our previous results [21], the P/TAI was similar for heifers at second ReBreed21 TAI as at first TAI (41.8 vs 42.4 %, respectively). The P/AI was also similar to heifers bred to the Resynch33 strategy (35.0 %) but with the obvious advantage that ReBreed21 heifers were bred at 21d after previous TAI compared to 42d after AI for Resynch33 heifers. Accordingly, on the D21st of the breeding season there were 45 % more heifers pregnant in ReBreed21 than Resynch33 (62.9 % vs. 43.6 %). Most non-pregnant heifers in the ReBreed21 programs also had the chance for 2 TAIs by the end of the breeding season (42d) and, accordingly, there were 17 % more heifers pregnant by the end of the breeding season in ReBreed21 programs compared to Resynch33 (73.8 vs 63.2 %). Of particular importance, the synchronization produced with Rebreed21 allows a synchronized ovulation soon after the previously induced cycle, thus reducing the likelihood that heifers will return to an anovular state after TAI. In herds

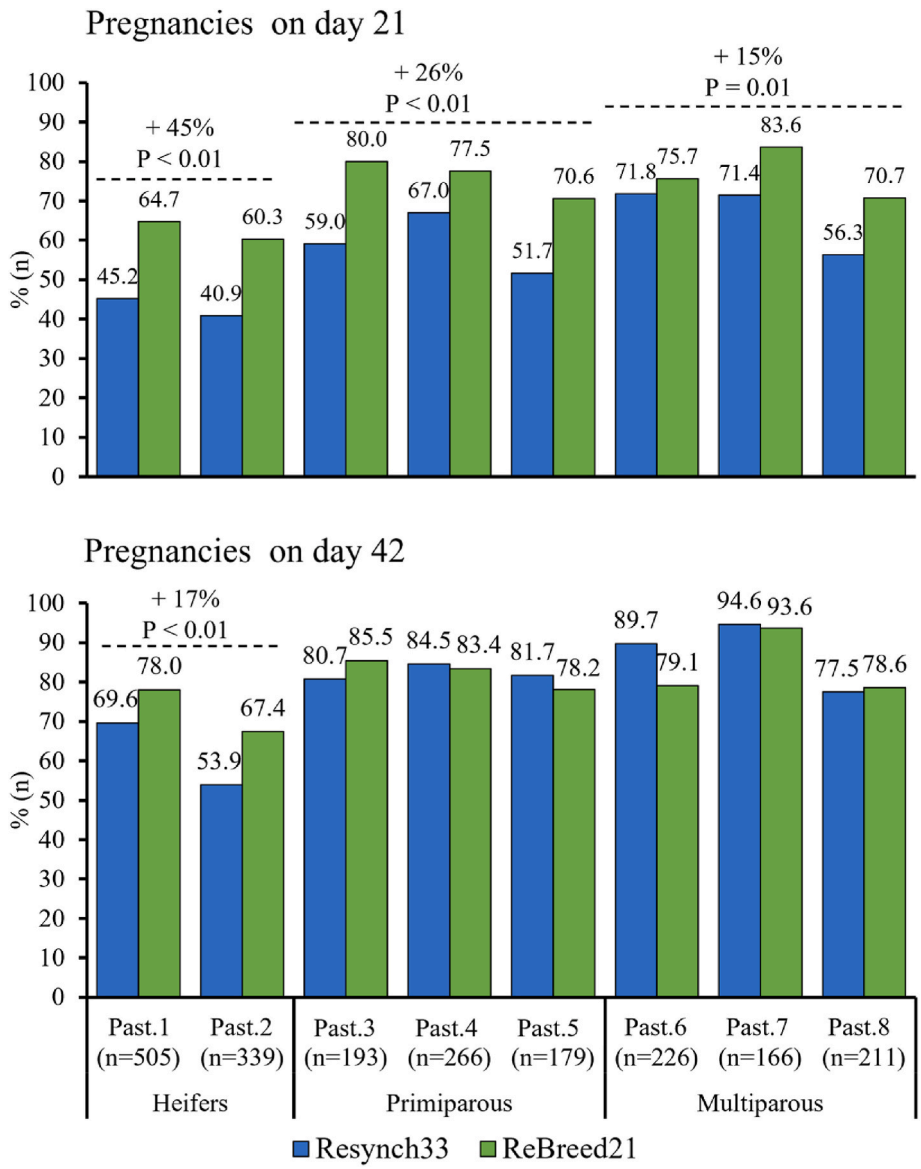

Fig. 4. Cumulative pregnancy at 21d of the breeding season for each of 8 pastures (Past. in Figure) of cow/heifers (2 heifers, 3 primiparous, and 3 multiparous) enrolled in Resynch33 and ReBreed21with and without EC.

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

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

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

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

that use natural service after first TAI, heifers that do not become pregnant to first TAI and return to an anovular state would not be rebred, thus reducing the efficiency of reproductive programs in young non-cycling heifers. The efficiency produced by ReBreed21 in heifers is well-illustrated in the survival curves with, not only more heifers becoming pregnant during the breeding season, but heifers also having earlier pregnancy compared to Resynch33 heifers. Thus, the results with ReBreed21 in heifers are exceptional and warrant continued testing of this protocol in this parity group.

Primiparous beef cattle also face substantial challenges with energy demands of first lactation competing with the metabolic demands of continuing development of the structural frame of the animal [39,40]. Our review of over 275,000 TAI in beef cattle indicated that primiparous *Bos indicus* had lower P/TAI (39.2 %) in published scientific manuscripts than either heifers or multiparous cows [36]. In contrast, the primiparous cows in our study had much greater P/TAI than heifers (42.6 % higher P/TAI; 61.3 vs 43.0 %) and only slightly lower fertility than multiparous (7.7 % higher). Thus, this particular *Bos indicus* herd did not appear to exhibit the typical low fertility problems in primiparous cows. Arguably the most interesting result with primiparous cows was the

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

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

improvement in the ReBreed21 program by adding EC to the protocol, resulting in 51.2 % more pregnancies compared to the ReBreed21 without exogenous estradiol (51.7 vs 34.2 %). This was consistent with our third hypothesis, that EC would increase fertility in the ReBreed21 program, but this hypothesis was only supported in primiparous and not in heifers or multiparous cows. This indicates that primiparous cows may have insufficient endogenous estradiol production during the ReBreed21 protocol used in this study. Other studies are consistent with increasing estradiol during the proestrus period being associated with an increase in fertility [15,41,42]. The ReBreed21 program with EC increased (32 %) the percentage of primiparous cows pregnant by the 21st day of the breeding season to 79.5 % (155/195), compared with Resynch33 (60.4 % [145/240]), leading to pregnancy 4.7d earlier during the breeding season. Nevertheless, there was no difference in percentage of primiparous cows pregnant at D42 of the breeding season comparing ReBreed21 programs to Resynch33, likely due to the high fertility in primiparous cows in this herd allowing excellent results with Resynch33 using only two TAI during the 42d breeding season.

In multiparous cows, the results with the present ReBreed21 program, even with the addition of EC, did not indicate an advantage in using this intensive reinsemination program. This herd was clearly well-managed in a way that optimized reproductive performance with about two-thirds (65.8 %) of multiparous cows pregnant after the first TAI. This was much greater than what we summarized from previous TAI studies with multiparous *Bos indicus* beef cattle showing only 50.9 % (22,649/44,463) pregnant to first TAI [36]. After only two TAI, a total of 86.8 % of multiparous cows were pregnant in our study, a value that would be difficult to surmount, even using a reinsemination program that was optimized for multiparous cows. The current ReBreed21 program was unmistakably not optimal for multiparous cows as P/AI was only about half the fertility observed at first TAI or after the Resynch33 program.

The dramatically improved reproductive performance of heifers in this study highlights the value of using ReBreed21 during the first breeding season in Bos indicus females. The first 21d of the breeding season are critical for obtaining high performance in beef operations [10]. Heifers/cows that become pregnant in this period will wean heavier calves (21 kg heavier than dams calving in the second 21d [9]). In addition, heifers that were born in the first 21d of the calving season had greater fertility during their first breeding season [9]. Models on the length of breeding seasons, showed an increase in profitability when heifers and cows become pregnant during the first 21d of the breeding season, due to greater longevity in the herd and more kg of calf weaned during the whole productive life [8,43]. In this study, 77.4 % of cows were pregnant by 21d of the breeding season, indicating a high percentage of cows (77.4 % represents 91 % of the total pregnant cows) will be eligible for the first TAI and all subsequent TAIs during the next breeding season. This is particularly important for heifers that are entering their primiparous breeding season, resulting in an outstanding beginning to a productive herd life for these animals. Early pregnancy in replacement heifers should result in: production of sufficient replacement cows, increased voluntary culling, earlier parturition and shorter calving season, and calving in more optimal seasonal conditions with greater likelihood of pregnancy as primiparous cow [8,44,45]. Potential return on investment (ROI) of 11 % increase in pregnancies in heifers for ReBreed21 compared with Resynch33 in a hypothetical breeding season for 200 head is ~21 more calves, thus an increase of ~4620 kg of calf at weaning (R$36,960.00 in gross profit at R$8/kg; Brazilian Real). Assuming R$942.00 in extra synchronization costs and R$2824 extra veterinary fees (2d), this results in a net profit of R$33,194.00 and an ROI of 881.4 %. This value does not include the increase in kg of calf due to earlier pregnancy.

In addition to the major parity effects that were observed with ReBreed21, other observations provided noteworthy physiologic information on the ReBreed21 protocol that may be useful for modification of this protocol or development of more optimized protocols in the future. For example, our second hypothesis, that EC treatment would not reduce fertility to the first TAI, was supported by our results in all three parities with no reduction in P/AI to first TAI by EC treatment in ReBreed21+EC. On D19 after breeding, cows/heifers should be in the interferon-tau period of pregnancy, which should inhibit any upregulation by estradiol of endometrial oxytocin receptors [24,28,46]. In another study, treatment with estradiol benzoate on D22 after TAI was also reported to not decrease P/AI to previous TAI [15].

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

consistent with previous studies [19,48,51]. False positives may be due to later CL regression in some heifers/cows or pregnancy loss. Our recent study with non-bred heifers indicates that 22.2 % (12/54) of unquestionably non-pregnant heifers did not have CL regression (circulating progesterone <1 ng/mL) by D21, suggesting that later CL regression may account for at least half of false positives [35]. Importantly, the ReBreed21 strategy includes an ultrasound pregnancy diagnosis at D33 that will allow detection of both false negatives (minimal numbers) and false positives, allowing appropriate management decisions to deal with these animals. Evaluation of pregnancy on D33 allows any non-pregnant animals to be resynchronized and receive their second TAI at d42 after previous AI. Thus, the use of the complete ReBreed21 program will allow a minimum of 2 TAIs in non-pregnant females during a 42d breeding season with most non-pregnant animals having the opportunity for 3 TAIs in 42d.

Finally, the practical implementation of the ReBreed21 strategy on commercial beef cattle operations is illustrated by the calendar that can be used to implement a 42d breeding season with 2 ReBreed21 protocols in 4 pastures of cows (Fig. 5). First, the priority on this farm is to have no labor on Sundays. The first TAIs are done on Friday and Saturday and the next week on Wednesday and Thursday. The ReBreed21strategy is implemented and completed during the next 2 weeks with the Doppler pregnancy diagnosis and second TAI done on the same d of the week but 21d later, thus ReBreed21. A critical practical aspect of earlier reinsemination programs is the intense work schedule that can overlap with other treatment days or weekends, according to the chosen strategy [6,11]. Using the ReBreed21 schedule allows execution of an entire breeding season (3 TAIs) in only 2 months in four groups of cows without work on Sundays, without overlapping workdays, and optimizing the work in the middle of the week. This allows cows that become pregnant to the first and second TAI (ReBreed21) to receive the first and all potentials breeding in the next breeding season. This can be particularly important for heifers to optimize the number and timing of primiparous calving and the productive life of the cows.

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

CRediT authorship contribution statement

João Paulo N. Andrade: Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Pedro J.L. Monteiro:** Writing – review & editing, Formal analysis. **Alexandre B. Prata:** Writing – review & editing, Resources, Project administration, Methodology. **Adelino J. Robl:** Methodology, Investigation. **José Neto:** Methodology, Investigation. **Beatriz Lippe:** Methodology, Investigation, Data curation. **Heuller S. Ribeiro:** Methodology, Investigation. **Diego Hartmman:** Resources, Methodology, Investigation. **Roberto Sartori:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Formal analysis. **Milo C. Wiltbank:** Writing – review & editing, Writing – original draft, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Acknowledgments

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

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