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Development of a novel 21-day reinsemination program, ReBreed21, in *Bos indicus* heifers

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ABSTRACT

The aim was to develop a program for resynchronization of ovulation (ReBreed21) that allowed reinsemination of non-pregnant *Bos indicus* heifers every 21 d using timed AI (TAI) without the need for detection of estrus. The Rebreed21 program begins 12 d after previous TAI (Day 0) by inserting an intravaginal P4 implant (Day 12) that is removed 7 d later (Day 19) combined with treatment with 300 IU of eCG. On Day 21, early pregnancy diagnosis (Doppler PD) is performed based on CL vascularity. Non-pregnant (NP) heifers immediately received AI combined with 100 μg of GnRH. The program is replicated 12 d after second TAI to produce a breeding season (BS) of 42 d with 3 potential TAIs. Two experiments were conducted as a proof of concept for this rapid rebreeding program. In Experiment 1, 76 heifers were enrolled in ReBreed21, as explained above. In Experiment 2, 300 Nellore heifers were synchronized for 1st TAI and randomly assigned to one of two groups: ReBreed21 (n = 147) or another early resynchronization procedure, Resynch14 (n = 153) with P4 implant inserted 14 d after previous TAI plus 50 mg of long-acting injectable P4; 8 d later P4 implant removed (Day 22) and early Doppler PD performed; NP heifers received 150 μg of cloprostenol, 0.5 mg of ECP, and 300 IU of eCG with TAI on Day 24. In both experiments, the largest follicle (LF) was measured at each Resynch TAI. Ultrasound was later used to confirm the early Doppler PD and to determine ovulation (OV) to Resynch at 12 d after TAI in ReBreed21 (Day 33 of pregnancy) and 14 d after TAI in Resynch14 (Day 38 of pregnancy). Final PD was performed 40 d after 3rd TAI. Results for Experiment 1 were: diameter of LF 11.8 ± 0.23 mm; 88.9% OV; 20.5% false positives; 38.1% P/AI at 1st TAI; 44.4% overall P/AI for ReBreed21 TAIs; 72.3% total pregnant at end of BS. In experiment 2, Rebreed21 vs. Resynch14 were different for: diameter of LF (10.9 ± 0.17 vs. 10.0 ± 0.17 mm, P = 0.0003), heifers with LF < 8.5 mm (10.2 vs. 26.4%, P = 0.04), or LF ≥ 11 mm (50.0 vs. 37.2%, P = 0.001), and P/AI at first TAI (29.3% [43/147] vs. 20.3% [31/153], P = 0.074) but similar for OV (overall 86.8% [239/275], P = 0.82), false positives (P = 0.52) overall P/AI for Resynch TAIs (33.6 vs. 28.8%, P = 0.4), and total pregnant at end of BS (58.5% [86/147] vs. 55.6% [85/153], P = 0.64). In addition, median time to pregnancy was 9 d earlier (P = 0.0007) for ReBreed21 than Resynch14. Thus, ReBreed21 is a novel protocol that allows earlier reinseminations than Resynch14 but with similar fertility.

1. Introduction

Development of efficient timed AI programs has provided important tools for reproductive management of both dairy and beef cattle herds [1,2]. Fixed time AI (TAI) programs permit all eligible cows to receive AI at the beginning of the breeding season.

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In addition, they can also reduce the negative effects of several pathophysiologic conditions such as anovulation due to undernutrition or calf suckling [3], which represent the two most important challenges for beef cattle production in grazing tropical conditions. Programs for TAI are currently being successfully utilized worldwide on a large scale with satisfactory results in pregnancy per AI (P/AI) [4].

Following the initial development of TAI programs, methods were modified to be utilized at second and later breedings, termed resynchronization programs, allowing complete reproductive management programs utilizing only TAI [5–8]. Use of TAI programs in beef cattle has been reported to improve genetic merit and weaning weight of calves, reduce time to pregnancy, and increase the percentage of cows pregnant at the end of the breeding season compared to breeding with natural service sires [9,10]. Thus, improving efficiency of TAI programs, including resynchronization TAI programs, could be practically utilized to increase efficiency of reproductive management and to enhance utilization of superior genetics in either beef or dairy cattle operations [4].

In beef cattle, a resynchronization strategy has been proposed allowing TAI every 32 days, i.e. three TAIs during a 64 day breeding season [9]. However, hormonal treatments begin prior to determination of pregnancy status and only cows that became pregnant to the first TAI are likely to calve early enough to be eligible for the first TAI during the next breeding season. Recent studies have reported a new resynchronization strategy that allows TAI every 24 days by using Doppler ultrasonography to determine the functional status of the CL [11,12]. However, the timing of treatments occurs on different days of the week as utilized for the first TAI making it challenging to practically implement this strategy on commercial operations. Experiment 1 was performed to provide a proof of concept related to a novel approach for breeding Nellore heifers every 21 days, termed ReBreed21. The study allowed us to observe preliminary measures from heifers submitted to the Rebreed21 program such as P/AI and false positives. Experiment 2 was a comparison of the ReBreed21 program to a previously published early resynchronization strategy that permitted TAI every 24 days (Resynch14). The primary hypothesis for this study was that ReBreed21 and Resynch14 would produce similar P/AI, ovulation rate to the protocol, and frequencies of false positives.

2. Materials and methods

Two experiments were performed in the current study. In each experiment, animals on random days of the estrous cycle were enrolled in a first TAI protocol and received their AI on Day 0. Subsequently, in Experiment 1, all heifers were assigned to only one resynchronization program with TAI every 21 days (ReBreed21); whereas in Experiment 2, heifers were assigned to ReBreed21 or Resynch14 (TAI every 24 days). Details of each experiment are provided below and in Fig. 1. All the animal handlings and experimental procedures were approved (protocol #: 1730040918) by the Animal Use and Ethics Committee from the Veterinarian Institute of the Universidade Federal Rural of Rio de Janeiro.

2.1. Experiment 1, ReBreed21: Heifers, location, and reproductive management

Experiment 1 was conducted during the 2017/2018 breeding season in a commercial beef cattle operation in the southeast of Brazil. This experiment used Nellore heifers (n = 76) that were considered eligible for insemination based on their weight (333.4 ± 3.3 kg) and no apparent abnormalities in the reproductive tract, as evaluated by ultrasound. The heifers were kept on *Brachiaria brizantha* pastures with ad libitum access to water and mineralized salt.

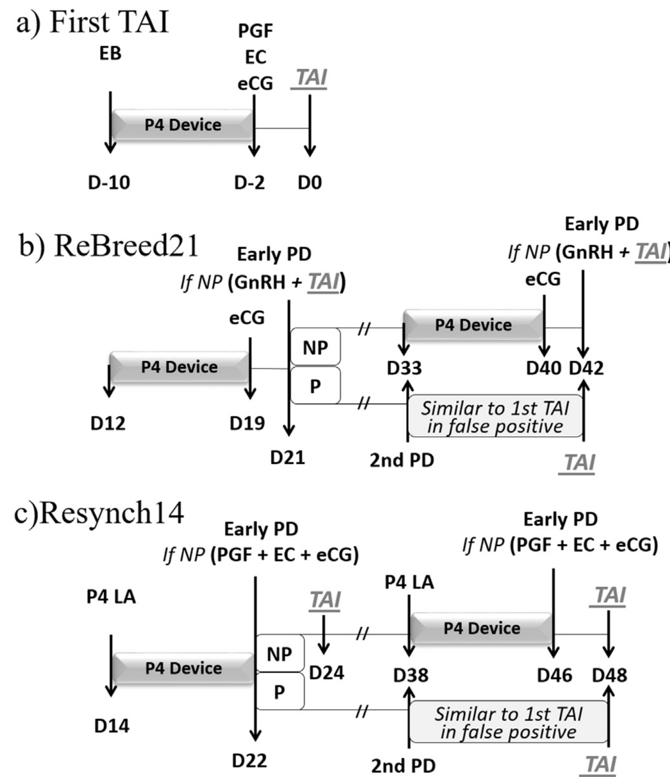

Fig. 1. Schematic design for the treatments used in the a) first TAI, and the two resynchronization strategies: b) ReBreed21 and c) Resynch14. The schemes included: timing of intravaginal P4 device, estradiol benzoate (EB), estradiol cypionate (EC), equine chorionic gonadotropin (eCG), cloprostenol (PGF), long-acting injectable P4 (50 mg P4), gonadorelin (GnRH), pregnancy diagnosis (PD) by Doppler (D21 or D22) or B-mode (D33 or D38) ultrasonography with non-pregnant (NP) heifers receiving rebreeding. Experiment 1 used protocols 1a and 1b, whereas Experiment 2 used protocol 1a, 1b, and 1c.

Fig. 1 shows the protocol that was used for first TAI (Fig. 1a) and for rebreeding TAIs (Fig. 1b) during Experiment 1. At the beginning of the breeding season, heifers of unknown cyclicity status were submitted to a first TAI (Fig. 1a). The synchronization was initiated on Day −10 (Day 0 = TAI) with the insertion of a third use (previously used for 16 days) intravaginal progesterone (P4) insert (CIDR® initially containing 2 g of P4, Zoetis, São Paulo, SP, Brazil) and i.m. administration of 2 mg of estradiol benzoate (Sincrodiol®, Ourofino, Cravinhos, SP, Brazil). On Day −2, the P4 insert was removed, and heifers were treated i.m. with 150 μg of d-cloprostenol (Veteglan®, Hertape Calier, Juatuba, MG, Brazil), 1 mg of estradiol cypionate (ECP®, Zoetis, São Paulo, SP, Brazil) and 300 IU of equine chorionic gonadotropin (eCG®; Folligon, MSD, São Paulo, SP, Brazil). On Day 0, the heifers received the first fixed TAI. The reutilization of P4 devices is common practice performed during P4-based protocols in beef cattle [13]. After each use, the CIDRs were dipped for 15 min in chlorhexidine-water solution (0.08%), scrubbed, rinsed, air-dried, and stored until the next use [14,15]. The P4 concentration resulting from a third use CIDR has been reported to be ~3 ng/mL in post pubertal Nellore heifers [16] and ~1.5 ng/mL in ovariectomized beef cows [17].

Twelve days after first TAI, all heifers were enrolled in the ReBreed21 (Fig. 1b) resynchronization program by receiving the insertion of a third use (previously used for 16 days) intravaginal P4 device (CIDR®, Zoetis, São Paulo, SP, Brazil). Seven days later (Day 19), the P4 device was removed, and the heifers received 300 IU of eCG (eCG®; Folligon, MSD, São Paulo, SP, Brazil). Two days later

(Day 21), the heifers had their ovaries evaluated by an experienced technician to diagnose pregnancy status based on the CL vascular perfusion (described in section 2.3.). Heifers diagnosed as non-pregnant (n = 37) received the subsequent TAI in addition to 100 μg of GnRH i.m. (Fertagyl®, MSD, São Paulo, SP, Brazil) immediately after the ultrasound examination. These heifers were also used to replicate the ReBreed21 program allowing a short breeding season (42 days) with three potential TAI.

2.2. Experiment 2, ReBreed21 vs Resynch14: Heifers, location, and management

Experiment 2 was conducted during the 2018 breeding season (June–September) of a commercial beef cattle operation in the state of Acre in northern Brazil. This study used a total of 300 Nulliparous Nellore heifers with an average weight of 291.5 ± 1.9 kg and no apparent abnormalities in the reproductive tract as evaluated by ultrasound and transrectal palpation. Heifers were housed on two pastures of *Panicum Maximum* grass with ad libitum access to water and mineralized salt with both treatment groups represented on each pasture. Weight gain per animal was similar on the two pastures (24.98 ± 4.03 kg) during the experiment with no differences (P = 0.46) between the ReBreed21 and the Resynch14 heifers.

As summarized in Fig. 1a for experiment 2, ovarian function was synchronized for first TAI in heifers of unknown cyclicity status (Day 0) with a 10-day protocol. At the beginning of the protocol (Day −10), the heifers were treated i.m. with 2.0 mg of estradiol benzoate (Bioestrogen® Biogénesis Bagó, Curitiba, PR, Brazil) and received an intravaginal implant containing 1 g of P4 (Cronipres® Monodose M-24 Biogénesis Bagó, Curitiba, PR, Brazil). On Day −2, the P4 implant was removed and heifers were treated i.m. with 150 μg of sodium cloprostenol (Croniben® Biogénesis Bagó, Curitiba, PR, Brazil), 0.5 mg of estradiol cypionate (Croni-Cip® Biogénesis Bagó, Curitiba, PR, Brazil) and 300 IU of eCG (Ecegon® Biogénesis Bagó, Curitiba, PR, Brazil). The TAI was performed 48 h after removal of intravaginal implant by a single inseminator using semen from one of two bulls. During the subsequent TAIs the heifers were inseminated using semen primarily (90%) from Bull 2.

All heifers enrolled in the ReBreed21 (n = 147) program began the protocol on Day 12 after first TAI, as described previously (section 2.1.) but using the commercial products used in the rest of Experiment 2: intravaginal P4 implant (Cronipres® Monodose M-24) and eCG (Ecegon®).

The Resynch14 (Fig. 1c) program was done as previously reported [5]. The program began on Day 14 after the previous TAI. All enrolled heifers (n = 153) received an intravaginal P4 insert (Cronipres® Monodose M-24) and i.m. treatment with 50 mg of long-acting P4 (Sincrogest LA®). Eight days later (Day 22) the P4 device was removed and an ultrasound exam was performed to determine pregnancy status based on CL vascularity, as described below (section 2.3). Heifers diagnosed as non-pregnant received, immediately after the ultrasound examination, 150 μg of cloprostenol (Croniben®), 0.5 mg of estradiol cypionate (Croni-Cip®) and 300 IU of eCG (Ecegon®). Two days later (Day 24), non-pregnant heifers received the subsequent TAI.

The treatments within each program (ReBreed21 or Resynch14) were replicated to allow a potential third TAI in non-pregnant heifers and a breeding season of 42 and 48 days, respectively.

2.3. Ultrasound evaluations and pregnancy diagnosis

In the current experiments, a color Doppler ultrasound machine (Z5, Mindray®, North America, Mahwah, NJ, USA) was used for all evaluations. The ultrasound machine was set to a 5.0 MHz frequency, 54 color gain, 222 color wall filter, and 1.0 kHz pulse repetition frequency. All evaluations were conducted by the same experienced technician. Early pregnancy diagnosis was performed based only on CL vascularity without examination of uterine contents. Heifers that had a CL with <25% vascular perfusion were considered to have a non-functional CL and were designated as non-pregnant [18]. The early pregnancy diagnosis was performed on Day 21 after previous TAI for the ReBreed21 and on Day 22 after previous TAI for the Resynch14 program. In Experiment 1, early pregnancy diagnosis was confirmed by the presence of an embryo 33 days after TAI. In Experiment 2, the presence of an embryo was confirmed 33 days after each TAI for the ReBreed21 or 38 days after each TAI for the Resynch14 program. At the time of the ReBreed21 and Resynch14 TAIs, all heifers were evaluated by ultrasound to measure the diameter of the largest follicle. Subsequently, ovulation was confirmed 12 days after TAI for ReBreed21 and 14 days after TAI for Resynch14.

Heifers that were diagnosed as pregnant by the CL vascular perfusion but subsequently were not pregnant at the second pregnancy diagnosis were considered as false positive. The resulting false positive heifers from the 1st TAI, were resynchronized again using a similar protocol as used at the first TAI [ReBreed21 (EB + P4 insert on Day −9; P4 insert removed and treatment with PGF + ECP + eCG on Day −2; TAI Day 0) and Resynch14 (EB + P4 insert on Day −10; P4 insert removed and treatment with PGF + ECP + eCG Day −2; TAI Day 0)]. These breedings were performed along with the last (Third)TAI on Day 42 and Day 48 of the breeding season for ReBreed21 and Resynch14, respectively. These pregnancies from these breedings are included in the total pregnant heifers during the breeding season of each experiment (Tables 1 and 2).

2.4. Statistical analyses

In Experiment 1, pregnancies per AI (P/AI) at first TAI was compared with P/AI to the Resynch TAIs by the GLIMMIX procedure of SAS (Version 9.4; SAS Institute).

In Experiment 2 binomial variables, including percentage of false positive results, final percentage pregnant, pregnancies per AI (P/AI), and percentage of heifers per category of follicle size were evaluated by the GLIMMIX procedure of SAS. The effect of bull was considered in the model evaluating P/AI. Continuous variables, such

Table 1
Results of Experiment I, including first TAI and Rebreed21 (second and third TAIs) in Nellore heifers during a 42 days breeding season.

Endpoint	% (n/n)
P/AI	
1st TAI	38.1 (29/76)
2nd TAI (1st ReBreed21)[a]	46.1 (18/39)
3rd TAI (2nd ReBreed21)[b]	40.0 (6/15)
Rebred False positives[c d]	25.0 (2/8)
Pregnant during Breeding Season [e]	72.3 (55/76)
FALSE POSITIVES [c]	
Based on non-pregnant heifers	20.5 (14/68)
Based on pregnant heifers	22.9 (14/61)

[a] The number of heifers enrolled in the 2nd TAI correspond to those diagnosed as non-pregnant (n = 47) after the 1st TAI minus the false positive heifers (n = 8).
[b] The number of heifers enrolled in the 3rd TAI correspond to those diagnosed as non-pregnant (n = 21) after the 2nd TAI minus the false positive heifers (n = 6).
[c] False positives were determined by CL vascular perfusion (≥25%) on Day 21 and embryo absent on Day 33 (Day 0 = TAI).
[d] False positive heifers of 1st TAI were rebred before the end of the breeding season.
[e] The number of pregnant heifers during the breeding season corresponds to pregnancies from the 1st TAI, 2nd TAI, 3rd TAI, and rebred false positive heifers from the first TAI.

Table 2
Results for Experiment 2, comparison of 1st TAI and resynchronization (2nd and 3rd TAIs) for ReBreed21 and Resynch14 protocols on Nellore heifers.

Endpoint	ReBreed21	Resynch14	P-value
	% (n/n)	% (n/n)	
P/AI			
1st TAI	29.2 (43/147)	20.2 (31/153)	0.07
2nd TAI (1st Resynchronization)[a]	41.2 (33/80)	33.7 (32/95)	0.3
3rd TAI (2nd Resynchronization)[b]	19.0 (8/42)	22.0 (13/59)	0.7
Rebred False positives[c d]	8.3 (2/24)	33.3 (9/27)	0.03
Pregnant during Breeding Season [e]	58.5 (86/147)	55.6 (85/153)	0.6
FALSE POSITIVES [c]			
Based on non-pregnant heifers	19.2 (29/151)	16.5 (31/187)	0.5
Based on pregnant heifers	27.6 (29/105)	32.9 (31/94)	0.3

[a] The number of heifers enrolled in the 2nd TAI correspond to those diagnosed as non-pregnant after the 1st TAI (ReBreed21[n = 104] and Resynch14[n = 122]) minus the false positive heifers (ReBreed21[n = 24] and Resynch14[n = 27]).
[b] The number of heifers enrolled in the 3rd TAI correspond to those diagnosed as non-pregnant after the 2nd TAI (ReBreed21[n = 47] and Resynch14[n = 63]) minus the false positive heifers (ReBreed21[n = 5] and Resynch14[n = 4]).
[c] False positives were determined by CL vascular perfusion (≥25%) on Day 21 for ReBreed21 or Day 22 for Resynch14 and embryo absent on Day 33 for ReBreed21 or Day 38 for Resynch14 (Day 0 = TAI).
[d] False positive heifers of 1st TAI were rebred before the end of the breeding season.
[e] The number of pregnant heifers during the breeding season corresponds to pregnancies from the 1st TAI, 2nd TAI, 3rd TAI, and rebred false positive heifers from the 1st TAI.

as diameter of the ovulatory follicle and weight gain during the experiment, were analyzed by the ttest procedure of SAS. The median time to pregnancy was determined by survival curve analysis using the PROC LIFETEST procedure of SAS. Significant differences between treatment groups were considered for $P \leq 0.05$, whereas differences between $P > 0.05$ and $P \leq 0.10$ were considered as a tendency. Data are presented as means ± SEM and as percentages for continuous and binary outcomes, respectively.

3. Results

In experiment 1 (Table 1), a total of 88.9% (48/54) of heifers ovulated during the 2nd and 3rd TAI with an average follicle diameter of 11.8 ± 0.2 mm. There were no differences (P = 0.71) among the P/AI of 1st, 2nd, and 3rd TAI (40.8% [53/130]) resulting in 72.3% of heifers pregnant at the end of the 42 d breeding season. A total of 8 and 6 heifers (1st and 2nd TAI respectively) were found to be false positives based on CL perfusion ≥25% on Day 21 and embryo absent on Day 33. Calculation of false positive heifers based on heifers diagnosed pregnant at Day 21 was 22.9% (14/61), whereas based on non-pregnant heifers was 20.5% (14/68).

In experiment 2 (Tables 2 and 3), during resynchronizations (2nd and 3rd TAI) follicle diameter at TAI was larger for ReBreed21 (10.9 ± 0.17 mm) than Resynch14 (10.0 ± 0.17 mm) although ovulation rate was similar (88.6%). Further analysis (Fig. 2) showed that there were more heifers with larger follicles (≥11 mm) in ReBreed21 than Resynch14 and fewer heifers with small follicles (<8.5 mm). Ovulation rate and P/AI (Table 3) were greater for the large follicle category than for the small one. In addition, if only the heifers that ovulated are utilized in the calculation, the large follicle category had 173% greater relative P/AI than the small follicle category.

The P/AI tended to be greater (46% increase) for ReBreed21 than Resynch14, P/AI was similar (30.1%) for resynchronization TAIs (2nd and 3rd TAI). Percentage of heifers pregnant at the end of the breeding season was similar for ReBreed21 and Resynch14 but median time to pregnancy was 9 d earlier (P = 0.0007) for ReBreed21 (12 d) than for Resynch14 (21 d), based on survival curve analysis.

A total of 199/477 (41.7%) heifers were diagnosed as pregnant, based on the CL vascularity after first and second TAI, with a tendency (P = 0.056) for Rebreed21 (46.2% [105/227]) to have greater P/AI than Resynch14 (37.6 [94/250]). From these, 60 heifers were considered to have a false positive diagnosis based on the absence of an embryo at the second pregnancy diagnosis. The false positive rate was similar for ReBreed21 and Resynch14 calculated either for heifers diagnosed pregnant at the early Doppler pregnancy diagnosis (30.1%) or calculated based on non-pregnant heifers at second pregnancy diagnosis (17.8%). The false positive frequency after first TAI was similar (P = 0.70) for Rebreed21 and Resynch14.

There was an effect (P < 0.01) of sire on fertility regardless of the resynchronization protocol in Experiment 2. That is, bull 1 had greater P/AI (59.2% [29/49]) compared to bull 2 (24.8% [130/525]). Calculation of the incidence of false positives based on heifers that were pregnant at first diagnosis by CL vascular perfusion produced greater (P < 0.01) frequency of false positives for bull 2 (34.1% [57/167]) than bull 1 (6.5% [2/31]), whereas, calculation based on non-pregnant heifers produced a similar (P = 0.75) frequency for bull 2 (14.4% [57/395]) and bull 1 (10.0% [2/20]).

4. Discussion

This manuscript introduces the development (Experiment 1) of a new resynchronization strategy that we have termed ReBreed21 based on the possibility of rebreeding non-pregnant heifers every 21 days, and compares the results (Experiment 2) to another early rebreeding program that was termed Resynch14 [11], based on the timing of initiation of the resynchronization strategy. Consistent with our primary hypothesis, Experiment 2 resulted in no difference between ReBreed21 and Resynch14 for ovulation rate, frequency of false positive diagnoses, P/AI at each resynchronization breeding, and overall P/AI at the end of the breeding season. Ovulation to both resynchronization protocols was around 86%, although heifers in ReBreed21 had a larger follicle at TAI, a shorter breeding season (42 vs 48 d), and earlier time to pregnancy. The similar results for P/AI between the two synchronization strategies must be interpreted with caution because the low number of heifers used in these initial "proof of concept" studies have insufficient statistical power to adequately test fertility. In addition, we did not attempt to directly compare results from the two experiments, since many factors differed between the studies including animals, farm, and vaginal implant. Valid comparisons of reproductive results can only be done by randomization of treatments within a group of animals since reproductive outcomes depend on multiple factors [4,11]. Overall, the P/AI results were lower than expected for both resynchronization programs due to a significant effect of sire on fertility. Nonetheless, a shorter resynchronization program has been developed in this study that appears to produce satisfactory results. Future fertility trials will be needed before this strategy can be routinely recommended on commercial beef cattle operations.

Various types of resynchronization programs have been previously developed and validated for shortening the time from previous AI to rebreeding [4,11]. Although some resynchronization strategies in dairy cattle began the Resynch protocol on day 20 after AI [19], most early resynchronization programs in beef cattle began the Resynch protocol on day of pregnancy diagnosis (~Day 30 after AI) and therefore breedings happened at about 40 d intervals [1,2,5–8,11]. More recent programs for beef cattle began Resynch protocols prior to pregnancy diagnosis and cows that were subsequently found to be non-pregnant would complete the protocol and receive a TAI at 30–35 d after previous AI [9]. Recently, new approaches [5] for resynchronization protocols have been

Table 3
Results from all Resynch TAIs (2nd and 3rd TAI) in Experiment 2, comparing ReBreed21 and Resynch14 for largest follicle at TAI, ovulation rate, pregnancy/AI, pregnancy/AI only in heifers that ovulated, and similar comparisons among three different categories for largest follicle at TAI.

	Rebreed21	Resynch14	P-value	By category			P-value
Follicle at TAI (mm)	10.9 ± 0.17	10.0 ± 0.17	0.0003	**< 8.5**	**8.5–10.9**	**≥ 11**	
Ovulation, % (n/n)	86.9 (107/123)	86.8 (132/152)	0.8	58.8 (30/51) a	89.1 (90/101) b	96.5 (110/114) b	0.0001
Pregnancy/AI, % (n/n)	33.6% (41/122)	28.8% (45/156)	0.33	13.7 (7/51) a	28.7 (29/101) b	43.9 (50/114) c	0.0009
P/AI for ovulators, % (n/n)	38.5% (40/104)	34.9% (44/126)	0.57	16.6 (5/30) a	32.2 (29/90) ab	45.4 (50/110) b	0.01

Lowercase letters indicate differences ($p < 0.05$) among the follicle categories within each endpoint.

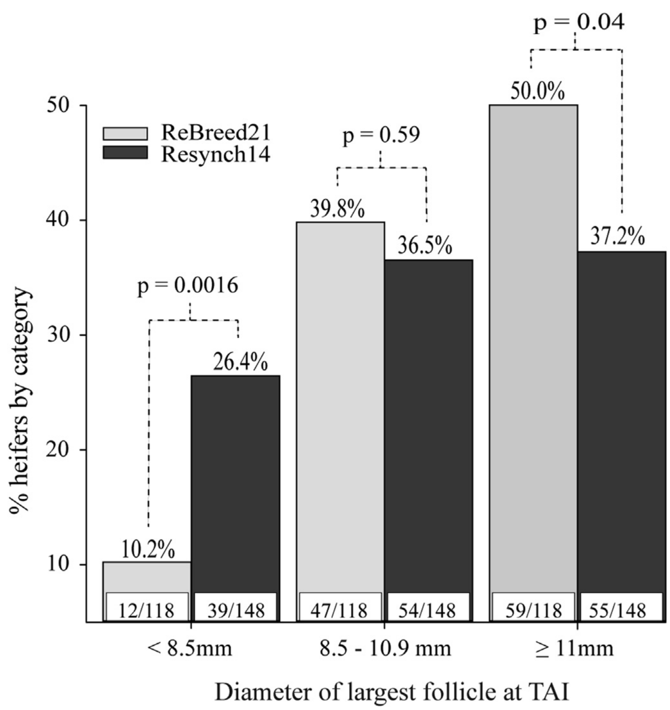

Fig. 2. Experiment 2 distribution of heifers into small (<8.5 mm), intermediate (8.5–10.9 mm), or large (≥11 mm) categories based on size of the follicle at pregnancy diagnosis on Day 21 or Day 22 (Day 0 = TAI) for ReBred21 and Resynch14, respectively. P-value is shown for differences between treatment groups within each follicle category.

developed based on the use of earlier pregnancy diagnosis using Doppler ultrasound of CL vascular perfusion [20]. One of these programs was tested in Experiment 2 by beginning the protocol at 14 d after previous TAI and using Doppler ultrasound for early pregnancy diagnosis on Day 22. Thus, breeding interval was 24 d with a three TAI breeding season of 48 d. The ReBreed21 program that was developed in this study can better facilitate the handling of cows because tasks are performed on the same day of the week as a 7 d TAI protocol. In addition, the early Doppler pregnancy diagnosis was performed at the same time as the rebreeding TAI. The breeding interval is therefore 21 d with a three AI breeding season of only 42 d. Thus, in theory, ReBreed21 represents an interesting and potentially practical new strategy for reproductive management of beef cattle.

Despite differences in the type and timing of treatments in the two strategies in Experiment 2, there were no differences in percentage of heifers pregnant at the end of the breeding season or in P/AI at the resynchronization TAIs. There was a tendency for a difference in P/AI at 1st TAI with ReBreed21 having slightly greater P/AI than Resynch14. Previous studies have also observed a possible effect of P4 treatment near the end of the cycle on fertility in both beef [21] and dairy [22] cattle. Differences in timing and magnitude of the P4 supplementation could explain any differences between the two protocols in fertility [23,24]; however, clearly future large experiments that address this important question are needed. The reduction in average days to pregnancy by about 9 d in the ReBreed21 compared to the Resynch14 protocol probably reflect a combination of numerical differences in P/AI at 1st and 2nd TAI that favor ReBreed21 and the shorter interval between breedings. There is the potential that shorter time to pregnancy could reduce the calving interval and increase the reproductive and productive performance of livestock systems [25]. Future experiments are needed to validly evaluate the economic value/costs of using ReBreed21 or other rapid resynchronization programs for management of rebreeding.

The variation in bull fertility that was observed in Experiment 2 was a major limitation in this study. Unfortunately, the low fertility results were associated with the principal sire that was used in the research project. A previous study with more than 60,000 TAI found a range of 7.2–77.3% in P/AI between beef bulls used in TAI [26]. Thus, future studies need to be conducted with a larger number of sires and breedings to definitely evaluate the novel rebreeding concept introduced in this manuscript.

One key concept related to resynchronization strategies is that the earlier the pregnancy diagnosis, the greater the number of false positives. Early pregnancy diagnosis in rapid resynchronization programs, are based on the evaluation of the CL vascular perfusion [12] near the time of normal CL regression (Days 19–22 after TAI). False positives from an early pregnancy diagnosis done by luteal blood flow could be due to early pregnancy losses [27], however it could also be caused by later CL regression in a non-pregnant animal. Indeed, luteolysis occurs later than Day 22, in many heifers with three follicular waves [28,29]. Consistent with the idea that later luteolysis is causing false positives, a previous experiment observed 10.8% (4/37) of non-bred Nellore heifers that had CL regression after Day 21 of estrous cycles (Roberto Sartori, personal communication). Thus, in all management strategies using an early pregnancy diagnosis, a second pregnancy diagnosis is necessary to confirm the presence of the embryo and validate the pregnancy diagnosis [30,31]. The frequency of false positives in these experiments were in agreement with earlier studies [30,32,33] but seemed to be greater than some recent reports [34,35]. Rebreeding the heifers that were false positive to the 1st TAI resulted in a surprisingly low number of pregnancies (2 extra pregnancies in either Expt 1 or 2) and this may not be economically advantageous.

Previous studies have generally calculated the frequency of false positives using number of pregnant animals in the denominator, however, it may be more correct to calculate the false positive frequency using number of non-pregnant animals in the denominator. For example, the poor fertility sire gave a particularly high false positive value (34.5%) compared to the high fertility sire (6.5%) based on pregnant heifers but had similar false positive rates if the calculations were based on number of non-pregnant heifers (10–14.4%). If it is assumed that the low fertility sire had a low fertilization rate due to low sperm quality then the false positive rate (10–14.4%) for the two groups could be entirely explained by heifers that did not have an embryo but had not yet undergone luteolysis by Day 21 or 22. This idea is important because methods that stimulate earlier CL regression in non-pregnant heifers (in a manner that does not cause CL regression in pregnant heifers due to protective actions of interferon-tau during early pregnancy) may help to reduce the false positive frequency and improve the efficiency of the resynchronization program.

The decision to administer PGF on day 22 (Resynch14) or to breed the heifers on day 21 (ReBred21), based on the early pregnancy diagnosis by using the CL vascular perfusion, did not allow us to evaluate the rate of false negative pregnancy diagnoses in this study. A previous study (Guimaraes et al., 2015) reported no false negative pregnancy diagnoses based on CL vascular perfusion on day 21 compared to a definitive diagnosis based on the presence of the embryonic vesicle on day 35 after expected ovulation. We have also observed no false negatives in two larger studies using Holstein heifers. Future experiments are warranted, that are adequately designed to evaluate the false negative rate with larger number of Nellore cattle.

Physiologically, one of the most interesting results was the larger follicle diameter observed during the ReBreed21 protocol, as compared to the Resynch14 protocol. The average follicle diameter was about 1.0 mm larger at the TAI on Day 21 in ReBreed21 than at TAI on Day 24 in Resynch14. It is not possible, in the absence of direct comparisons of follicular dynamics in the two protocols, to definitely state the physiological reason(s) for the difference in follicular diameter between Resynch14 and ReBreed21 heifers. In our opinion, it seems most likely that there was a later time of emergence of the preovulatory follicular wave in Resynch14 than ReBreed21. Alternatively, treatment with the long-acting injectable P4 in Resynch14 may have reduced follicle growth rate [36], although a recent study observed no effect of treatment with a long-acting P4 on follicle size at TAI during a resynchronization program [27].

Along with the average follicle diameter being larger for ReBreed21 than Resynch14, there was a difference in distribution of the largest follicle diameter with Resynch14 having more heifers with small follicles (<8.5 mm) and ReBreed21 having more heifers with larger follicles (≥11 mm). Heifers with follicles smaller than 8.5 mm had a lower frequency of ovulation (58.8%) and much lower P/AI (13.7%) than heifers with larger follicles. In *Bos taurus* cattle, follicles smaller than 8.5 mm are rarely observed to ovulate, even when treated with an ovulatory stimulus [37,38]. However, follicles of *Bos indicus* cattle, such as Nellore, undergo selection of a dominant follicle and acquire ovulatory capacity at a smaller diameter than *Bos taurus* cattle [39–41]. For example, follicles in Nellore heifers underwent deviation at 6.2 mm and ovulation occurred in 33.3% (3/9) of heifers with follicles between 7.0 and 8.4 mm in diameter treated with 25 mg of porcine LH [42]. Nevertheless, the differences in frequency of ovulation with smaller follicles only partially explained the differences in fertility with P/AI being strongly related to follicle diameter even after correcting for differences in ovulation between follicle size categories. Previous studies have shown an effect of preovulatory follicle diameter on fertility in beef cattle synchronization programs, perhaps due to larger preovulatory follicles producing greater expression of estrus, greater likelihood of ovulation or earlier ovulation, better synchronization, or greater circulating estradiol near AI [43,44]. In addition, ovulation of a larger follicle could lead to development of a larger CL and potentially greater circulating P4 after AI that could improve fertility and decrease pregnancy loss. Future studies with a larger number of animals are needed to unravel the effect of these physiologic differences on the reproductive efficiency of each resynchronization program.

In conclusion, this research has introduced a new resynchronization program, ReBreed21, that produces synchronized ovarian function, based on the distribution of follicle diameters at TAI and high efficiency of ovulation to the protocol. This novel protocol produced similar fertility but with an earlier time to pregnancy compared with another early resynchronization protocol, Resynch14. Rebreed21 may be practical to implement on commercial beef cattle operations based on the convenient timing of the treatments, however, further studies are needed to compare the fertility, efficiency, and practicality of this protocol. It seems possible that this new program could improve reproductive efficiency in Nellore heifers and potentially in other types of cattle by reducing the time between breedings and length of the breeding season.

CRediT authorship contribution statement

João P.N. Andrade: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration. **Victor E. Gomez-León:** Formal analysis, Writing - original draft, Writing - review & editing, Visualization. **Fabiana S. Andrade:** Conceptualization, Methodology, Data curation, Writing - original draft. **Bruno P. Carvalho:** Methodology, Data curation, Investigation. **Karen L. Lacouth:** Data curation. **Felipe Z. Garcia:** Methodology, Data curation. **Júlio C.F. Jacob:** Resources. **José N.S. Sales:** Methodology, Writing - review & editing. **Milo C. Wiltbank:** Formal analysis, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration. **Marco R.B. Mello:** Methodology, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration.

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