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Progesterone-based timed AI protocols for *Bos indicus* cattle III: Comparison of protocol lengths

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ABSTRACT

This study aimed to validate a 7 d progesterone (P4)-based fixed-time AI (FTAI) protocol for *Bos indicus* cattle by comparing to 8 and 9 d-type protocols. The first study compared 7 vs. 8 d protocols in Nelore heifers (Exp. 1.1; n = 742) and cows (Exp. 1.2; n = 2488), and the second study compared 7 vs. 9 d protocols in cows (Exp. 2; n = 1343). On experimental Day −10 and Day −11 the 8 and 9 d groups received an intravaginal P4 implant, 2.0 mg estradiol benzoate (EB) and 0.5 mg cloprostenol sodium (PGF). On Day −9 the 7 d group received the same treatments (P4, EB, and PGF). Then, on Day −2 all groups had the P4 implants removed, and PGF, 0.6 mg estradiol cypionate, and 300 IU equine chorionic gonadotropin (eCG) was administered. Fixed-time AI was performed 48 h later (Day 0) and 8.4 mg buserelin acetate (GnRH) was administered to 7d-G, 8d-G and 9d-G groups, whereas 7d-0, 8d-0 and 9d-0 groups did not receive GnRH at AI. Estrus was detected using tail-chalk between Day −2 and Day 0. Pregnancy per AI (P/AI) was evaluated by ultrasound 30 d after AI. Effects were considered significant when $P \leq 0.05$, whereas a tendency was designated when $P \leq 0.10$ and $P > 0.05$. In heifers (Exp. 1.1), incidence of estrus was similar regardless of protocol length (7 or 8 d). There was no independent treatment effect on P/AI or interaction between protocol length and GnRH at AI for P/AI (7d-0: 46.9, 7d-G: 51.4, 8d-0: 47.7, and 8d-G: 43.6%). Heifers in estrus had greater P/AI, and GnRH had no additional effect. More cows (Exp. 1.2) from the 8 d protocol were in estrus than cows submitted to the 7 d protocol. Additionally, despite no interaction between protocol length and GnRH on P/AI (7d-0: 55.9, 7d-G: 60.9, 8d-0: 56.2, and 8d-G: 60.8%), GnRH at AI increased P/AI. There was no interaction between estrus and GnRH, but cows displaying estrus had greater P/AI. Cows not expressing estrus tended ($P = 0.06$) to have greater P/AI when receiving GnRH. In Exp. 2, more 9 d cows were in estrus than 7 d cows. Protocol length did not affect P/AI but tended ($P = 0.08$) to interact with GnRH (7d-G had greater P/AI [57.9%] than 7d-0 [47.6%], but 9d-0 [54.6%] and 9d-G [55.4%] were not different from other groups). Moreover, GnRH increased P/AI only for the 7 d protocol. No interaction between estrus and GnRH was detected but estrus improved P/AI, and GnRH tended ($P = 0.09$) to improve P/AI of cows in estrus. In conclusion, despite longer protocols being more conducive to expression of estrus, there were no detectable effects of protocol length on P/AI. In addition, GnRH at FTAI may improve fertility in cows, particularly when cows are treated with shorter protocols.

1. Introduction

Artificial insemination (AI), including fixed-time AI (FTAI) programs, have become critical management tools [1] to optimize genetic improvement and reproductive efficiency, and

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consequently, increase the profitability of commercial beef [2–5] and dairy farms [6–8]. In particular, the use of FTAI has enabled the service of a large number of animals in a reduced period of time and facilitated cross-breeding and genetic improvement in extensive beef cattle operations [9]. Therefore, different strategies have been studied to properly control ovarian function through the use of exogenous hormones during the synchronization protocols that allow FTAI.

There are three key features of successful synchronization of ovulation programs. First, it is necessary to induce the emergence of a new follicular wave and control follicle development to ensure that, at the end of the protocol, an adequately sized growing follicle is available for ovulation [10–13]. Second, the length of the protocol must be defined (i.e. period of progesterone [P4] exposure, generally varying from 5 to 9 d) [14–18]. Finally, at the end of the protocol, circulating P4 concentrations need to be minimal [19–21] with a synchronous induction of ovulation that matches the schedule for FTAI to optimize fertility [22].

Focusing on the protocol length, the most common options for *Bos indicus* cattle have been the 8 [17] or 9 d protocols [18,23]. In addition, the 7 d P4-based protocols have been studied as an alternative protocol length for synchronization of ovulation and satisfactory fertility has been observed [16,24,25]. Some of the positive aspects of the 7d protocol compared to 8 or 9 d protocol are the shorter duration and the day of the week to start the protocol is the same week day as P4 implant removal, making this protocol potentially very applicable in many commercial situations. However, no controlled research study has been published that validly compares the different lengths of protocol in *Bos indicus* cattle during an E2/P4-based protocol.

Thus, this study directly compared reproductive outcomes during a 7, 8, or 9 d E2/P4-based FTAI protocol. Our hypotheses were: 1. The 7 d protocol would induce lower expression of estrus compared to 8 or 9 d protocols; 2. Regardless of the length of the protocol, administration of gonadotropin-releasing hormone (GnRH) at the time of AI would increase pregnancy per AI (P/AI) of females not expressing estrus by the time of AI; 3. Despite differences in expression of estrus, P/AI would be similar among treatments due to the additional effect of GnRH treatment at AI on fertility of females not displaying estrus.

2. Material and methods

The experiments were performed at Roncador Farm, located in Querência, MT, Brazil. Animals were kept on pasture (*Brachiaria brizantha*) supplemented with mineral salt and had *ad libitum* access to water. The Animal Research Ethics Committee of Luiz de Queiroz College of Agriculture of the University of São Paulo (ESALQ/USP) approved all procedures involving heifers and cows (Protocol # 2017.5.1618.11.9).

2.1. Experiment 1.1

A total of 742 Nelore (*Bos indicus*) nulliparous heifers were enrolled (averaging 18–24 mo of age and body condition score [BCS] 2.8 ± 0.02 on a scale from 1 to 5 [26]). All heifers were previously submitted to a protocol for induction of cyclicity (Day −34: insertion of an intravaginal implant with 1.0 g of P4 [Repro neo, GlobalGen Vet Science, Jaboticabal, Brazil] previously used for 14 d; Day −22: P4 implant withdrawal and administration of 0.6 mg estradiol cypionate im [EC; Cipion, GlobalGen Vet Science]). After 12 d (Day −10) all heifers, regardless of corpus luteum (CL) presence, were randomly assigned to the treatments (Fig. 1A): 7d-0 (7 d of P4 implant + no GnRH at AI; n = 192), 7d-G (7 d of P4 implant + GnRH at AI; n = 179), 8d-0 (8 d of P4 implant + no GnRH at AI; n = 176), or 8d-G (8 d of P4 implant + GnRH at AI; n = 195). On Day −10, 8 d groups received an intravaginal P4 implant with 0.5 g (Repro one, GlobalGen Vet Science) and 2.0 mg estradiol benzoate (EB; Syncrogen, GlobalGen Vet Science) and 0.5 mg cloprostenol sodium (PGF; Induscio, GlobalGen Vet Science) were administered im. In order to breed all animals on the same day, on Day −9 the 7 d group received the same treatments described above. All P4 implants were removed on Day −2 and 0.5 mg PGF, 0.6 mg EC, and 300 IU equine chorionic gonadotropin (eCG; ECGen, GlobalGen Vet Science) were administered im. Additionally, on Day −2, heifers had tail-chalk spread on the base of their tailhead. After 48 h, on Day 0, heifers were checked for expression of estrus, inseminated, and only G groups (7d-G and 8d-G) received 8.4 μg buserelin acetate im (GnRH; Maxrelin, GlobalGen Vet Science).

2.2. Experiment 1.2

A total of 2488 Nelore (*Bos indicus*) cows with mean BCS of 3.0 ± 0.08 and distinct parity (non-lactating [n = 753, and BCS = 3.2 ± 0.05]; multiparous [n = 1,284, and BCS = 2.8 ± 0.05]; and primiparous [n = 301, and BCS = 2.9 ± 0.07]) were submitted to the same experimental treatments as described for heifers (Fig. 1A). Randomly, and equally distributed among treatments, cows received either a new or a previously 7 d used P4 implant with 1.0 g (Repro neo, GlobalGen Vet Science). The P4 implants were reused because previously studies have shown similar P/AI between new and reused P4 implants in zebu cows [18]. Thus, treatments were 7d-0 (7 d of P4 implant + no GnRH at AI; n = 592), 7d-G (7 d of P4 implant + GnRH at AI; n = 576), 8d-0 (8 d of P4 implant + no GnRH at AI; n = 589), or 8d-G (8 d of P4 implant + GnRH at AI; n = 581).

2.3. Experiment 2

A total of 1343 Nelore cows with a mean ± SEM BCS of 2.9 ± 0.05, and classified as non-lactating (n = 611, and BCS = 3.0 ± 0.04) or multiparous (n = 732, and 131 BCS = 2.8 ± 0.05), were treated similarly to what was described for cows during Exp. 1.2. The same doses of hormones were used but the duration of treatment with an intravaginal P4 releasing insert was either 7 or 9 d (Fig. 1B). Thus, in order for all cows to be bred on the same day, 7 d protocol cows were initiated on Day −9 during this experiment, and the 9 d protocol started on Day −11. Treatments were: 7d-0 (7 d of P4 implant + no GnRH at AI; n = 357), 7d-G (7 d of P4 implant + GnRH at AI; n = 342), 9d-0 (9 d of P4 implant + no GnRH at AI; n = 317), or 9d-G (9 d of P4 implant + GnRH at AI; n = 327). Similar to Exp. 1, cows had tail-chalk spread on the base of their tailhead at the time of P4 implant removal for estrus detection, and AI was performed on Day 0.

The FTAI was performed by two experienced technicians in each experiment, using 20.0 × 10^6 frozen/thawed proven semen of five Rubia Gallega sires.

2.4. Ultrasound examinations

A subset of cows from Exp. 1.2 (n = 173) and Exp. 2 (n = 313) were evaluated by ultrasound 14 d after FTAI to determine the presence or absence of a CL and to estimate the percentage of cows that ovulated more than one follicle to the protocols. Pregnancy diagnosis was performed by ultrasound at 28–35 d after FTAI.

2.5. Statistical analysis

Statistical analyses were performed using the Statistical Analysis System (SAS, Version 9.4 for Windows SAS Institute Inc., Cary, NC), and all experiments were performed as a completely randomized 2 by 2 factorial design.

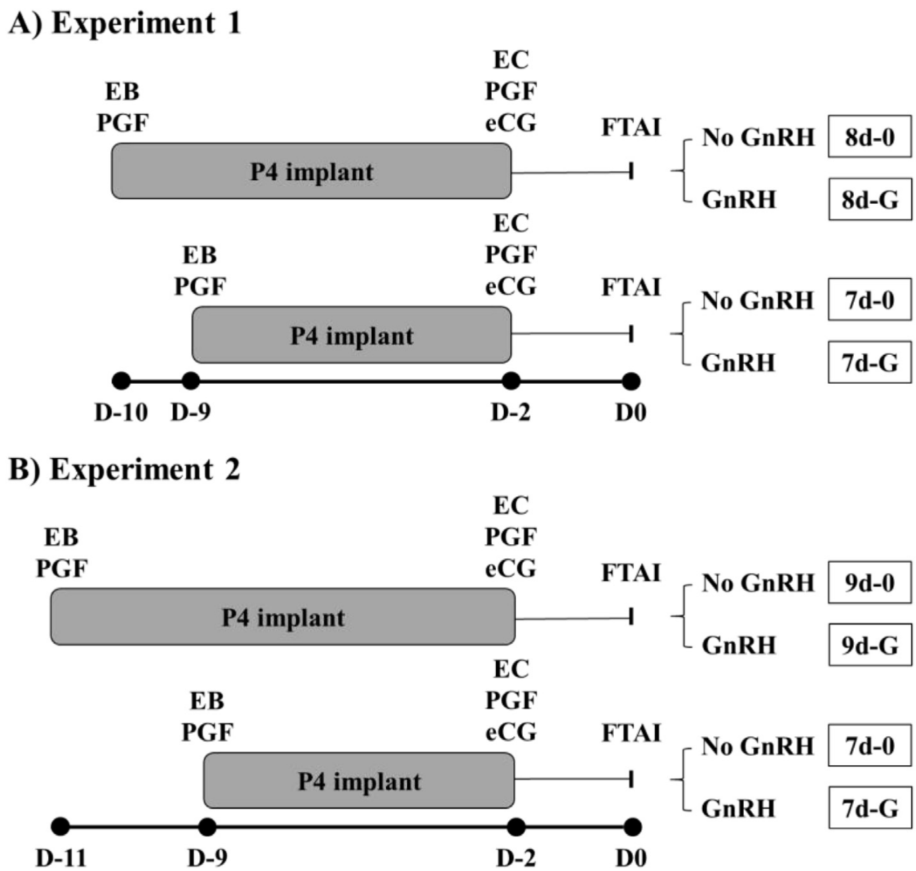

Fig. 1. Experimental design of experiments 1 (*Bos indicus* heifers and cows) and 2 (*Bos indicus* cows). During Exp. 1, the 7 and 8 d progesterone (P4)-based fixed-time AI (FTAI) protocols were compared. Exp. 2 compared 7 and 9 d P4-based FTAI protocols. On Day −10 and Day −11, respectively, the 8 and 9 d groups received an intravaginal P4 implant, estradiol benzoate (EB) and cloprostenol sodium (PGF) im. After 1 (Exp. 1) or 2 d (Exp. 2), which will be Day −9 for both experiments, 7 d groups received the same treatments described above (P4 implant, EB and PGF). At the same time as P4 implant removal (Day −2), estradiol cypionate (EC), PGF, and equine chorionic gonadotropin (eCG) were administered. Then, after 48 h (Day 0), females were inseminated and G-groups (7d-G, 8d-G and 9d-G) received buserelin acetate (GnRH) whereas others (7d-0, 8d-0 and 9d-0) did not receive GnRH. Doses: EB (1.5 mg for heifers and 2.0 mg for cows); PGF (0.5 mg); EC (0.5 mg); eCG (300 IU); and GnRH (8.4 μg). For heifers, only new implants with 0.5 g of P4 were used. For cows, new and previously used implants with 1.0 g of P4 were used.

Discrete responses of measured variables were analyzed using the generalized linear mixed model (GLIMMIX) procedure and fit to a binary distribution (expression of estrus, P/AI, and incidence of double ovulation after AI).

Selection of the model that best fit each response variable of interest was determined by finding the model with the lowest value for the *Akaike Information Criterion Corrected (AICC)* using the backward elimination procedure that removed independent variables with $P > 0.10$ from the model. Treatment was considered a fixed effect and the tested covariates were parity and BCS on Day 0 of the protocol for all analyses. Additionally, the effects of bull, inseminator and the type of P4 implant (new or reused) were tested during fertility analyses.

Differences were considered significant for $P \leq 0.05$, whereas a tendency was designated when $P \leq 0.10$ and $P > 0.05$. Mean comparisons were performed by the adjusted Tukey test. The results are expressed as least squares means ± standard error of the mean (LSM ± SEM), unless otherwise indicated.

3. Results

3.1. Experiment 1.1

Expression of estrus was very high for heifers submitted to both the 7 or 8 d protocols (97.3%; 722/742) and was not different between treatments (Table 1). Pregnancies per AI were not affected by protocol length (49.1 [182/371] vs. 45.6% [169/371]) for the 7 and 8 d protocols, respectively, or by GnRH treatment at AI (no GnRH: 47.3 [174/368]; GnRH: 47.3% [177/374]) and the interaction of length and GnRH was also not significant. Heifers showing estrus by the time of AI tended ($P = 0.10$) to have greater P/AI compared to those without expression of estrus (47.8 [346/724] vs. 27.8% [5/18]), with no interaction ($P = 0.30$) between estrus and GnRH (Fig. 2A). Moreover, no effect on P/AI ($P > 0.40$) was detected when analyzing protocol length, expression of estrus, and GnRH treatment at AI (Fig. 3A).

3.2. Experiment 1.2

Parity and protocol length both had effects on expression of estrus but there was no interaction ($P = 0.80$) between parity and protocol length on expression of estrus. The 8 d protocol increased the percentage of cows detected in estrus (72.0% [842/1170]) compared to the 7 d protocol (65.8% [769/1168]; Table 1) and a greater percentage of non-lactating cows were detected in estrus (80.5% [606/753]) compared to primiparous cows (43.2% [130/301]), while multiparous cows had an intermediary result (68.2% [875/1284]). During the P/AI analysis, the effects of bull, inseminator and the type of P4 implant were not significant. Additionally, the protocol length had no effect on P/AI ($P = 0.90$) and did not

Table 1
Relationship between protocol length (7, 8 or 9 d of exposure to a progesterone [P4] implant) and administration of GnRH at the time of AI on expression of estrus and pregnancy per AI (P/AI).

	7d		8 or 9d		*P* value		
	No GnRH	GnRH	No GnRH	GnRH	L[1]	G[2]	L*G
Heifers (7 vs. 8d)							
Estrus, % (n/n)	97.0 (360/371)		98.1 (364/371)		0.34	.	.
P/AI, % (n/n)	46.9 (90/192)	51.4 (92/179)	47.7 (84/176)	43.6 (85/195)	0.34	0.96	0.24
Cows (7 vs. 8d)							
Estrus, % (n/n)	65.8 (769/1168)		72.0 (842/1170)		<0.01	.	.
P/AI, % (n/n)	55.9 (331/592)	60.9 (351/576)	56.2 (331/589)	60.8 (353/581)	0.98	0.02	0.91
Cows (7 vs. 9d)							
Estrus, % (n/n)	54.7 (382/699)		71.3 (459/644)		<0.01	.	.
P/AI, % (n/n)	47.6^{A} (170/357)	57.9^{B} (198/342)	54.6^{AB} (173/317)	55.4^{AB} (181/327)	0.43	0.04	0.08

A,B Values in the same row with different superscripts differ ($P > 0.05$ and ≤ 0.1).
1 L, Length.
2 G, GnRH.

interact with GnRH treatment at AI ($P = 0.90$; Table 1). Thus, the 7 d protocol had similar P/AI compared to the 8 d protocol (7 d: 58.4 [682/1168]; 8 d: 58.5% [684/1170]; $P = 0.98$). Moreover, GnRH at AI had a positive effect on P/AI (no GnRH: 56.1 [662/1181]; GnRH: 60.9% [704/1157]; $P = 0.02$) and there was a tendency for a greater impact of GnRH for the 7 d (no GnRH: 55.9 [331/592]; GnRH: 60.9% [351/576]; $P = 0.08$) than the 8 d protocol (no GnRH: 56.2 [331/589]; GnRH: 60.8% [353/581]; $P = 0.11$), inducing 5.0 and 4.6% absolute increases (8.9 and 8.2% relative increases) on P/AI, respectively. Expression of estrus positively affected P/AI (no estrus: 50.2% [365/727]; estrus: 62.1% [1001/1611]; $P < 0.01$). However, there was no interaction between estrus incidence and GnRH treatment ($P = 0.40$; Fig. 2B). Furthermore, cows without expression of estrus that received GnRH at AI had a tendency ($P = 0.06$) for greater P/AI compared to those that did not receive GnRH (54.0 [182/337] vs. 46.9% [183/390]). Finally, in cows that did not express estrus, there was no effect of GnRH at AI in cows submitted to the 7 d protocol ($P = 0.40$) although, GnRH treatment increased P/AI in cows submitted to the 8 d protocol (Fig. 3B). The incidence of double ovulation after AI was similar ($P = 0.50$) between 7 and 8 d protocol (2.6 [5/190] vs. 1.6% [3/193], respectively).

3.3. *Experiment 2*

No interaction between protocol length and parity was detected for expression of estrus but both had significant effects when analyzed separately. A greater ($P < 0.01$) percentage of cows were detected in estrus after the 9 d protocol compared to the 7 d protocol (71.3 [459/644] vs. 54.7% [382/699]; Table 1), and a lesser percentage of non-lactating cows were detected in estrus ($P = 0.03$) compared to multiparous cows (59.4 [363/611] vs. 65.3% [478/732]). During the P/AI analysis (Table 1) the effect of bull, inseminator and the type of the P4 implant was non-significant. A tendency for interaction between protocol length and GnRH treatment at AI was observed for P/AI ($P = 0.08$) in which 7d-G cows had greater P/AI compared to 7d-0. In addition, 9d-0 and 9d-G cows had intermediary results which were not different from the other treatments (Table 1). Nevertheless, analysis of independent effects indicated that both protocol lengths had similar P/AI (7 d: 52.7 [368/699]; 9 d: 55.0% [354/644]; $P = 0.40$) and GnRH treatment improved P/AI (no GnRH: 50.9 [343/674]; GnRH: 56.7% [379/669]; $P = 0.03$). There was a positive effect of GnRH ($P = 0.007$) during the 7 d protocol, with a 10.3% absolute increase (21.6% relative increase) on P/AI (no GnRH: 47.6 [170/357]; GnRH: 57.9% [198/342]). In addition, no interaction was observed between expression of estrus and GnRH although, cows in estrus tended ($P = 0.09$) to have greater P/AI when receiving GnRH (no GnRH: 55.5 [227/409]; GnRH: 61.3% [265/432]; Fig. 2C). As expected, expression of estrus had a positive effect on P/AI (no estrus: 45.8 [230/502]; Estrus: 58.5% [492/841]; $P < 0.01$). Finally, GnRH at AI increased ($P = 0.02$) P/AI of cows submitted to the 7 d protocol that had expressed estrus and tended ($P = 0.10$) to increase P/AI of those that did not express estrus (Fig. 3C). Both protocols had similar ($P = 0.90$) incidence of double ovulation after AI (7 d: 0 [0/70]; 9 d: 1.4% [1/74]).

4. Discussion

The aims of this study were to evaluate FTAI protocols by comparing the 7 d protocol to longer protocols (8 and 9 d-long) used in *Bos indicus* cattle and by identifying variables that were associated with improved fertility within each protocol, such as expression of estrus prior to AI or GnRH treatment at the time of AI. It has been shown that the time of exposure to P4 can change the development pattern of the DF and consequently, affect the size of the preovulatory follicle, expression of estrus and fertility outcomes [27]. There were concerns that a shorter P4-based protocol, although very producer-friendly, might result in lower fertility due to reduced expression of estrus and reduced preovulatory follicle size at FTAI. On the other hand, a longer protocol (9 d of P4 implant), could have reduced fertility due to turnover [13] of the synchronized follicle wave prior to the controlled decrease in circulating P4.

The first hypothesis of this study was that longer protocols would result in a greater percentage of cattle expressing estrus prior to FTAI. All protocols that were evaluated in this study had treatment with EB at the beginning of the protocol along with administration of the P4 implant. This would likely result in initial inhibition of circulating gonadotropins by EB followed by consistent emergence of a new follicle wave between 2 and 4 d after initiation of the protocol [10,13]. For example, our recent evaluation of ovarian dynamics in *Bos indicus* cattle found that 92.4% (62/66) had emergence of a new follicular wave 2.4 ± 0.3 d after EB administration [13]. Consequently, at the time of P4 implant removal, each protocol length would produce a DF differing in age and size. Thus, 8 and 9 d protocols should result in a larger DF, on average, and higher circulating E2 concentrations resulting in greater incidence of estrus expression [28]. In heifers, essentially all animals showed estrus whether they were treated for 7 or 8 d (97–98%), whereas in cows, the 7 d protocol resulted in lower expression of estrus compared to 8 and 9 d protocols with a 6.2% difference between 7 and 8 d protocols and a 16.6% difference between 7 and 9 d protocols (absolute increase). The lack of effect of protocol length on expression of estrus in heifers might have been related to the physiological condition of the heifers, since they were

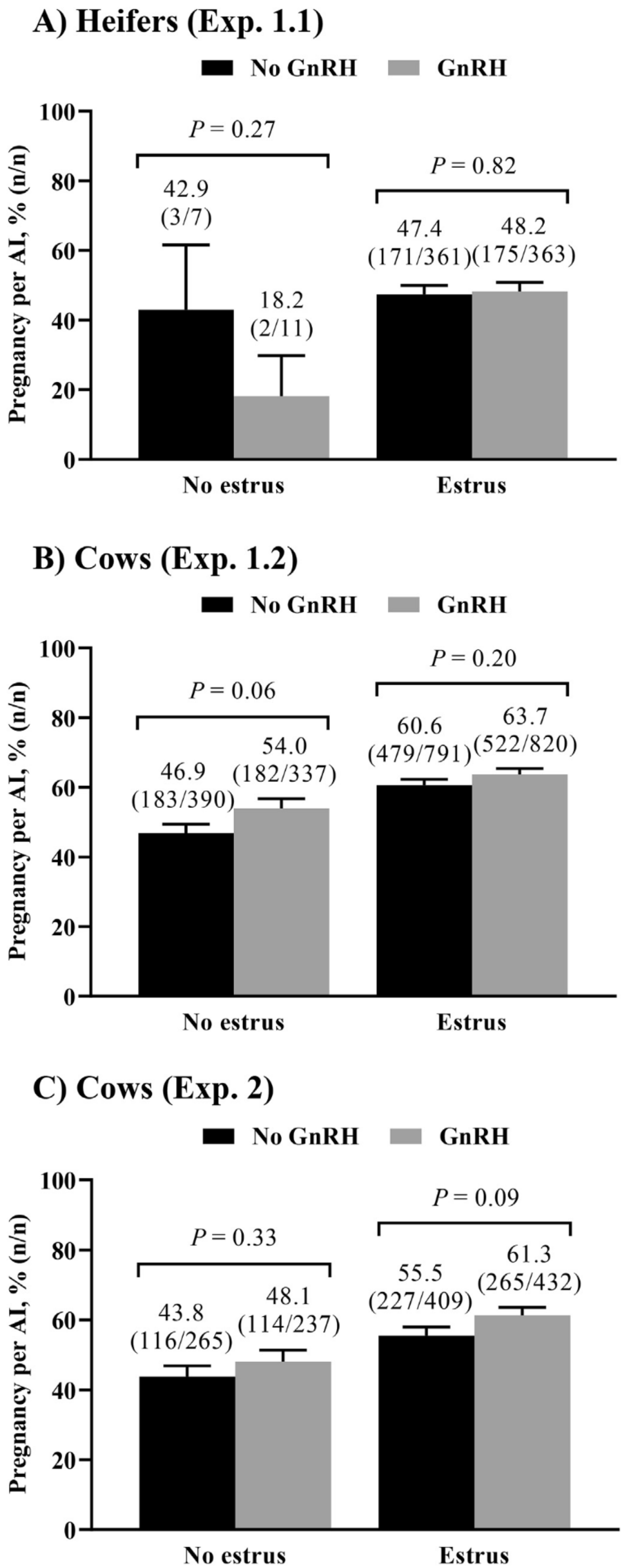

Fig. 2. Pregnancy per AI (mean ± SEM) by estrus expression and GnRH treatment at the time of AI for *Bos indicus* cattle submitted to 7 vs. 8 d progesterone (P4)-based fixed-time AI (FTAI) protocols (Exp. 1.1: heifers [A]; Exp. 1.2: cows [B]) or submitted to 7 vs. 9 d FTAI protocols (Exp. 2: cows [C]).

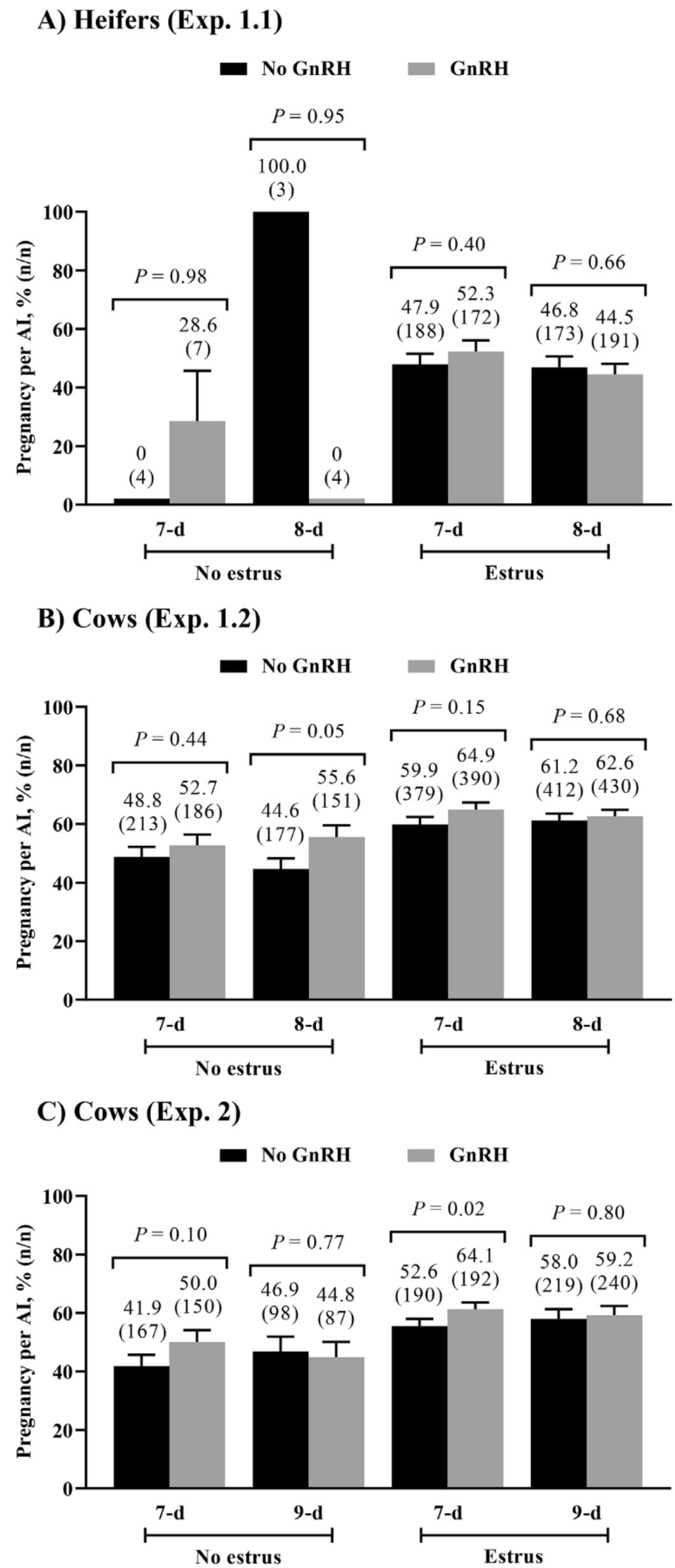

Fig. 3. Relationships among the length (7, 8 or 9 d) of the progesterone (P4)-based fixed-time AI (FTAI) protocols, expression of estrus, and GnRH treatment at the time of AI in *Bos indicus* cattle through the comparison between 7 vs. 8 d protocol (Exp. 1.1: heifers [A]; Exp. 1.2: cows [B]) and comparison between 7 vs. 9 d protocols (Exp. 2: cows [C]). Pregnancy per AI data are shown as mean ± SEM.

submitted to a hormonal protocol to induce ovulation prior to the FTAI protocol and, therefore, ~80% of heifers should have had a CL at the beginning of the FTAI protocol [29,30]. Additionally, administration of PGF on Day 0 of the protocol is likely to have induced luteolysis at the beginning of the protocol, resulting in low circulating P4 concentrations during the protocol, and consequently increased follicle growth and expression of estrus [27] due to a greater LH pulse frequency [31]. The high incidence of estrus in heifers could also be explained by the EC administration at the time of P4 implant removal, which increases expression of estrus and has been known to induce a pharmacological estrus [32,33].

It is well known that some females do not express estrus at the end of the synchronization protocol and that lack of estrus can compromise P/AI outcomes [17,18,23]. In contrast, administration of GnRH at the time of AI has been used as a tool to induce an LH surge and, consequently, cause a synchronized ovulation in those females with no estrus expression prior to AI. Previous studies reported both a positive [25,34] or no effect of GnRH treatment at the time of a FTAI on P/AI [35]. Possible explanations for these controversial results may be related to the semen quality/longevity used in the studies, or lack of synchronization to the protocols in some of the animals. It is known that, by treating with GnRH, it is assured that ovulation will occur within a 28–30 h period after GnRH if a dominant follicle is present at the time of AI [22]. In other words, GnRH treatment at the time of AI is intended to prevent delayed ovulation related to smaller follicles at the time of AI [36] or lack of ovulation due to lack of an LH surge. In the present study, it has been hypothesized that regardless of the length of the protocol, administration of GnRH at the time of AI would increase P/AI of females not expressing estrus. This hypothesis was not supported because there was no interaction between estrus incidence and GnRH treatment (Fig. 2), despite administration of GnRH had increased P/AI, independent of estrus. In the experiments in cows, there was a 5.0%, and 10.3% absolute increase in P/AI by using GnRH with the 7 d protocol. Moreover, treatment with GnRH had a positive effect in the cows treated with the 8 d protocol (4.6%) but little effect in cows treated with the 9 d protocol (0.8%), perhaps because of a larger preovulatory follicle and greater expression of estrus in the 9 d protocol.

An unexpected result was the improvement in fertility caused by GnRH treatment in cows that expressed estrus, based on removal of tail-chalk. For example, in Exp. 2 cows treated with the 7 d protocol had a significant improvement in fertility when estrus was expressed (from 52.6% to 64.1%). It is unclear if this positive effect is because of inaccuracies with detection of estrus using tail-chalk or due to a positive effect of GnRH, even in cows that expressed estrus. It is possible that a pharmacological estrus induced by EC may not be accompanied by ovulation with the GnRH treatment [32,33]. Future experiments on the effect of GnRH on P/AI, particularly in cows treated with shortened protocols, should continue to randomize all cows into treatments to see if the GnRH effects are only observed in cows that do not show estrus, in line with physiologic expectations, or if there is a positive effect of GnRH even in cows that express estrus.

The third hypothesis of this study was that P/AI would be similar among treatments due to the additional effect of GnRH treatment at AI on fertility, compensating for an expected lower expression of estrus in cows treated with shorter protocols. This hypothesis was partially confirmed because, although there was similar P/AI among treatments, this was not necessarily due to the additive effect of GnRH exclusively in cows not expressing estrus, because, in some instances, cows detected in estrus were also benefited by the GnRH treatment. Therefore, the combination of EC and GnRH as ovulation inducers may have overcome the potential negative effect of smaller ovulatory follicles on fertility of cows submitted to shorter protocols. Moreover, this study demonstrated that the rate of double ovulation after AI was similar among the different protocol lengths, likely because follicular deviation occurred before the administration of eCG at P4 implant removal [13].

In conclusion, despite longer protocols being more conducive to expression of estrus, there were no detectable effects of protocol length on P/AI. In addition, GnRH seems to be an important tool to improve fertility in cows, particularly when cows are treated with shorter protocols, such as protocols with 7 or 8 d of P4 treatment. These results, using more than 4000 animals (n = 4423), clearly show similar fertility among these protocols of different lengths, allowing for flexibility in the schedules for FTAI protocols for managing reproduction on an operation of *Bos indicus* cattle.

CRediT authorship contribution statement

Alexandre B. Prata: Conceptualization, Methodology, Investigation, Writing - original draft. **Guilherme Madureira:** Formal analysis, Data curation, Writing - original draft, Writing - review & editing. **Adelino J. Robl:** Investigation. **Heuller S. Ribeiro:** Investigation. **Milton Sagae:** Investigation. **Manoel C.V. Elias:** Investigation. **César Pimenta:** Investigation. **Jhonny Barrios:** Investigation. **Diego Hartmman:** Investigation. **Althuir A. Schneider:** Investigation. **Gabriel A.F. Sandoval:** Resources. **Milo C. Wiltbank:** Conceptualization, Methodology, Resources, Supervision, Formal analysis, Writing - original draft. **Roberto Sartori:** Conceptualization, Methodology, Funding acquisition, Visualization, Investigation, Formal analysis, Supervision, Project administration, Writing - original draft, Writing - review & editing.

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