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Effect of increasing GnRH and $PGF_{2\alpha}$ dose during Double-Ovsynch on ovulatory response, luteal regression, and fertility of lactating dairy cows

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ABSTRACT

Ovsynch-type synchronization of ovulation protocols have suboptimal synchronization rates due to reduced ovulation to the first GnRH treatment and inadequate luteolysis to the prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) treatment before timed artificial insemination (TAI). Our objective was to determine whether increasing the dose of the first GnRH or the $PGF_{2\alpha}$ treatment during the Breeding-Ovsynch portion of Double-Ovsynch could improve the rates of ovulation and luteolysis and therefore increase pregnancies per artificial insemination (P/AI). In experiment 1, cows were randomly assigned to a two-by-two factorial design to receive either a low (L) or high (H) doses of GnRH (Gonadorelin; 100 vs. 200 μg) and a $PGF_{2\alpha}$ analogue (cloprostenol; 500 vs. 750 μg) resulting in the following treatments: LL ($n = 263$), HL ($n = 277$), LH ($n = 270$), and HH ($n = 274$). Transrectal ultrasonography and serum progesterone (P4) were used to assess ovulation to GnRH1, GnRH2, and luteal regression after $PGF_{2\alpha}$ during Breeding-Ovsynch in a subgroup of cows ($n = 651$ at each evaluation). Pregnancy status was assessed 29, 39, and 74 days after TAI. In experiment 2, cows were randomly assigned to LL ($n = 220$) or HH (n = 226) treatment as described for experiment 1. For experiment 1, ovulation to GnRH1 was greater ($P = 0.01$) for cows receiving H versus L GnRH (66.6% [217/326] vs. 57.5% [187/325]) treatment, but only for cows with elevated P4 at GnRH1. Cows that ovulated to GnRH1 had increased ($P < 0.001$) fertility compared with cows that did not ovulate (52.2% vs. 38.5%); however, no effect of higher dose of GnRH on fertility was detected. The greater $PGF_{2\alpha}$ dose increased luteal regression primarily in multiparous cows ($P = 0.03$) and tended to increase fertility ($P = 0.05$) only at the pregnancy diagnosis 39 days after TAI. Overall, P/AI was 47.0% at 29 days and 39.7% at 74 days after TAI; P/AI did not differ ($P = 0.10$) among treatments at 74 days (LL, 34.6%; HL, 40.8%; LH, 42.2%; HH, 40.9%) and was greater ($P < 0.001$) for primiparous cows than for multiparous cows (46.1% vs. 33.8%). For experiment 2, P/AI did not differ ($P = 0.21$) between H versus L treatments (44.2% [100/226] vs. 40.5% [89/220]). Thus, despite an increase in ovulatory response to GnRH1 and luteal regression to $PGF_{2\alpha}$, there were only marginal effects of increasing dose of GnRH or $PGF_{2\alpha}$ on fertility to TAI after Double-Ovsynch.

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1. Introduction

A strategy to maximize insemination risk after the end of the voluntary waiting period and fertility at first artificial insemination (AI) in lactating dairy cows is to combine a

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program for presynchronization of the estrous cycle, such as Presynch, with a synchronization of ovulation program, such as Ovsynch [1–3]. Depending on the management approach, presynchronization programs are initiated at specific days in milk (DIM) postpartum to allow completion of the program and first AI service either by the end of voluntary waiting period or shortly thereafter. The purpose of presynchronization is to initiate the Ovsynch protocol at the ideal time in the estrous cycle to optimize fertility [2–6].

Recently, a presynchronization program, termed Double-Ovsynch (DO), has been developed and tested [6]. The DO protocol consists of a modified Ovsynch protocol 7 days before a regular Ovsynch-56 hour-timed artificial insemination (TAI) program (Pre-Ovsynch; GnRH-7 days-prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-3 days-GnRH and Breeding-Ovsynch; GnRH-7 days-$PGF_{2\alpha}$-56 hour-GnRH-16 to 20 hour-TAI). A number of studies have reported results with the DO protocol at first service [6–10] or later services [11]. Comparison of the DO protocol with the Presynch-Ovsynch protocol with a 12-day interval from the second $PGF_{2\alpha}$ of Presynch to initiation of Ovsynch found greater fertility with DO [6,9]. Nevertheless, improved responses to each of the hormonal treatments in the DO protocol would be expected to further improve the fertility of lactating dairy cows treated with this protocol before TAI.

Cows that ovulate to the first GnRH treatment of the Ovsynch protocol have greater fertility than cows that do not ovulate to this GnRH treatment [5,12,13]. Ovulation to the first GnRH treatment of Ovsynch (GnRH1) results in the formation of a new CL, which, in turn, increases circulating progesterone (P4) during the Ovsynch protocol. Furthermore, ovulation to GnRH1 results in the initiation of a new follicular wave within ~2 days of treatment [14], thereby increasing the overall response of cows to the protocol [11,15] and reducing the variability in follicle size [4,5,16]. The DO protocol is designed to optimize ovulation to the first GnRH treatment of Breeding-Ovsynch by initiating the protocol on Day 7 of the estrous cycle, a time when the dominant follicle of the first follicular wave should be present and likely at an optimal stage of development for ovulation in response to a GnRH treatment. Nevertheless, ovulation was not detected in all cows treated with GnRH on Day 7 after a GnRH treatment [6,11,17]. This may be, at least in part, because of a suboptimal LH surge when GnRH treatment is applied in the presence of high circulating P4 as would be expected on Day 7 of the estrous cycle [18]. In lactating dairy cows, the use of a higher dose of GnRH can dramatically increase the magnitude of the LH surge, either in the presence or absence of circulating P4 [17,18]; hence, this may be a viable alternative to increase ovulatory response to the first GnRH treatment of Breeding-Ovsynch to optimize synchronization and fertility.

Optimal response to the $PGF_{2\alpha}$ treatment before TAI of Ovsynch is desirable because lack of complete luteolysis after this treatment reduces fertility during Ovsynch-type programs in lactating dairy cows [7,11,19]. This may be particularly problematic in a program such as DO in which increased ovulation to the first GnRH treatment of Breeding-Ovsynch is expected to increase the proportion of cows with a CL that is less mature and potentially less responsive to $PGF_{2\alpha}$. For example, Brusveen et al. [7] reported that after the use of DO for first service, ~15% of cows did not undergo complete luteolysis (P4 < 0.4 ng/mL 56 hours after GnRH), a problem which was almost completely eliminated after a second treatment with $PGF_{2\alpha}$ 24 hours after the first treatment. Similarly, Giordano et al. [11], using the same criteria to determine CL regression, reported that when using the DO protocol for resynchronization of ovulation, ~10% of cows failed to regress their CL. Giordano et al. [11] also reported a lower rate of luteal regression for multiparous (83.9%) than for primiparous cows (89.7%) and for cows that ovulated (79.4%) versus those that did not ovulate (93.0%) in response to the first GnRH treatment of Breeding-Ovsynch. In agreement, other investigators have reported lower rates of luteal regression in multiparous than in primiparous lactating dairy cows [19]. Thus, optimizing CL regression in specific groups of cows may be particularly challenging; hence, new alternatives should be explored to increase CL regression during the DO protocol. As opposed to administering two doses of $PGF_{2\alpha}$ 24 hours apart as performed by Brusveen et al. [7], it is possible that increasing the dose of $PGF_{2\alpha}$ may be a simple alternative to maximize the percentage of cows that undergo complete CL regression before TAI.

This research focused on increasing the doses of the first GnRH and the $PGF_{2\alpha}$ treatments of the Breeding-Ovsynch portion of the DO protocol in an attempt to increase ovulatory response to GnRH1 and the rate of luteolysis after $PGF_{2\alpha}$ and before TAI. Thus, the specific objectives of the two studies presented herein were the following: (1) to assess ovulatory response after treatment with 100 μg versus 200 μg of GnRH at the time of the first GnRH of the Breeding-Ovsynch portion of the DO protocol and (2) to determine the rates of CL regression in response to 500 μg versus 750 μg of a synthetic $PGF_{2\alpha}$ analogue during the Breeding-Ovsynch portion of the DO protocol in lactating dairy cows. In addition, the fertility of cows treated with the different GnRH and $PGF_{2\alpha}$ doses was evaluated. We hypothesized that cows receiving the greater dose of GnRH would have a greater ovulatory response and that more cows receiving the greater dose of $PGF_{2\alpha}$ would undergo complete luteal regression after the $PGF_{2\alpha}$ treatment. We also hypothesized that because of the greater ovulatory response and percentage of cows with complete luteal regression, cows treated with the greater doses of GnRH and $PGF_{2\alpha}$ would have superior pregnancies per artificial insemination (P/AI) than cows receiving the lower doses.

2. Materials and methods

2.1. Animals and management

Lactating dairy cows from a commercial dairy milking ~1800 cows in south-central Wisconsin (Brodhead, WI, USA) were used in two experiments performed from December 2007 to November 2008 (experiment 1) and November 2008 to January 2009 (experiment 2). Cows were housed in free stall barns, fed a total mixed ration once daily with *ad libitum* access to feed and water. Throughout the experimental period, cows were milked three times daily at ~8-hour intervals, and all cows were

treated with bovine somatotropin (Posilac, 500 mg, Elanco Animal Health, Indianapolis, IN, USA) at 14-day intervals beginning at ~60 days postpartum. The rolling herd average for the herd was 13,463 kg, average daily milk yield of ~40 kg/cow/day with 3.4% fat and 3.1% protein yield. All procedures including hormonal treatments, ovarian ultrasonography, pregnancy diagnosis, blood collection, and TAI were performed while cows were restrained in self-locking head gates at the feedline.

2.2. Experiment 1: Treatments

Weekly cohorts of 30 to 50 cows that were 56 ± 3 DIM were enrolled in a DO protocol (Pre-Ovsynch, GnRH-7 days-$PGF_{2\alpha}$-3 days-GnRH; 7 days later Breeding-Ovsynch, GnRH-7 days-$PGF_{2\alpha}$-56 hour-GnRH-16 to 20 hour-TAI; Fig. 1) for synchronization of ovulation for first TAI at 83 ± 3 DIM. A total of 1084 cows (519 primiparous and 565 multiparous) completed the experimental treatments to receive their first TAI. Cows enrolled that completed the Pre-Ovsynch portion of the DO protocol were treated with GnRH (100 μg of Gonadorelin diacetate tetrahydrate, im, Fertagyl, Intervet/Schering-Plough Animal Health, Millsboro, DE, USA), 7 days later were treated with $PGF_{2\alpha}$ (500 μg of cloprostenol sodium, im, Estrumate, Schering-Plough Animal Health, Summit, NJ, USA), and 3 days later received another treatment with GnRH (100 μg of Gonadorelin, im). At the time of the second GnRH treatment of the Pre-Ovsynch portion of the DO protocol, the cows were stratified by parity and randomly assigned to receive one of four treatment combinations in a two-by-two factorial arrangement of treatments. Seven days after completion of Pre-Ovsynch, the cows initiated the Breeding-Ovsynch portion of DO by receiving either a 100- or 200-μg dose of GnRH (GnRH1), and then within each of the GnRH dose groups, the cows received either a 500- or 750-μg dose of $PGF_{2\alpha}$ at the time of the $PGF_{2\alpha}$ of Breeding-Ovsynch (Fig. 1). This treatment arrangement resulted in the following treatment combinations: 100 μg GnRH–500 μg $PGF_{2\alpha}$ (n = 263), 100 μg GnRH–750 μg $PGF_{2\alpha}$ (n = 277), 200 μg GnRH–500 μg $PGF_{2\alpha}$ (n = 270), and 200 μg GnRH–750 μg $PGF_{2\alpha}$ (n = 274). Fifty-six hours after $PGF_{2\alpha}$, all cows received GnRH (GnRH2; 100 μg of Gonadorelin, im) to complete the Breeding-Ovsynch portion of the DO protocol and received TAI 16–20 hours later (Fig. 1).

2.3. Experiment 1: Blood sampling and P4 analysis

Blood samples for the analysis of serum P4 concentrations were collected for a subset of cows (n = 651) via puncture of the median caudal vein or artery into evacuated tubes (Vacutainer, BD, Franklin Lakes, NJ, USA). The blood samples were centrifuged (2000 × g, 20 minutes), and serum was harvested and stored at −20 °C until assayed. The blood samples were collected at the time of GnRH1, $PGF_{2\alpha}$, and GnRH2 of the Breeding-Ovsynch portion of the DO protocol (Fig. 1). P4 concentrations were estimated using a solid-phase, no-extraction radioimmunoassay (Coat-a-Count, Diagnostic Products Corporation, Los Angeles, CA, USA). The average sensitivity for the assays was 0.03 ng/mL of P4. Intra- and interassay coefficients of variation were 7.0% and 9.6%, respectively.

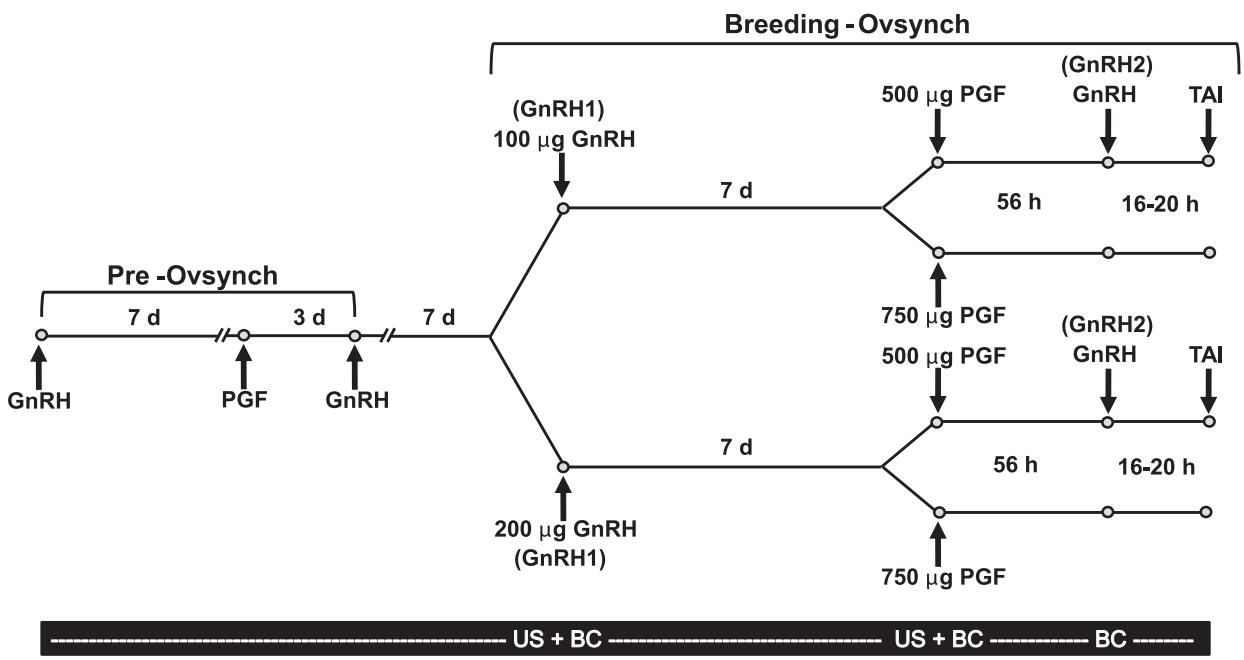

Fig. 1. Schematic representation of experimental procedures during experiment 1. Enrolled cows completed the Pre-Ovsynch portion of a DO protocol (GnRH-7 d-$PGF_{2\alpha}$-3 d-GnRH). At the time of the second GnRH injection of the Pre-Ovsynch portion of the DO protocol, cows were stratified by parity and were randomly assigned to receive either 100 or 200 μg of GnRH (GnRH1) and then, within each of the GnRH dose groups, cows received either 500 or 750 μg of a $PGF_{2\alpha}$ analogue at the time of the $PGF_{2\alpha}$ of the Breeding-Ovsynch portion of a DO protocol. Fifty-six hours after $PGF_{2\alpha}$, all cows received GnRH (GnRH2) to complete the Breeding-Ovsynch portion of the DO protocol and received TAI 16 to 20 hours later. US, ovarian ultrasonography; BC, blood collection.

At GnRH1 and the $PGF_{2\alpha}$ of Breeding-Ovsynch, P4 concentrations were used to determine the percentage of cows with a functional CL (≥1 ng/mL of P4), whereas P4 concentrations at $PGF_{2\alpha}$ and GnRH2 of the Breeding-Ovsynch were used to determine luteal regression after the $PGF_{2\alpha}$ treatment. For the evaluation of luteal regression in response to $PGF_{2\alpha}$, the cutoff value used to determine complete luteal regression was selected on the basis of the effect of P4 concentrations at the time of GnRH2 (56 hours after the $PGF_{2\alpha}$ treatment) of Breeding-Ovsynch on P/AI [7,11]. The cutoff value selected was 0.3 ng/mL of P4 on the basis of a significant decrease in P/AI at 29 days after TAI for groups of cows created on the basis of their circulating concentrations of P4 at the time of GnRH2 (P4 groups: <0.05 ng/mL, P/AI = 47.4% [n = 95]; 0.05–0.099 ng/mL, 55.7% [n = 140]; 0.1–0.149 ng/mL, P/AI = 47.1% [n = 138]; 0.15–0.199 ng/mL, P/AI = 50.0% [n = 110]; 0.2–0.299 ng/mL, P/AI = 62.5% [n = 64]; ≥0.3 ng/mL, P/AI = 30.5% [n = 59]).

2.4. Experiment 1: Ovarian ultrasonography and pregnancy diagnosis

Transrectal ultrasound (US) was performed in the same subgroup of cows (n = 651) from which the blood samples were collected using a portable scanner (Easi-Scan, BCF Technology Ltd., Livingston, UK) fitted with a 7.5-MHz linear array transducer to determine the presence and size of the ovarian structures (CL and follicles) at GnRH1 and 7 days later at the $PGF_{2\alpha}$ treatment of the Breeding-Ovsynch portion of the protocol (Fig. 1). The diameter of all follicles ≥4 mm and CL were estimated and recorded using on-screen background gridlines comprising squares with 10-mm sides. Ovulation to GnRH was defined as the appearance of a new CL on either ovary where a follicle of ovulatory size (≥10 mm) was present at the time of the previous US examination 7 days earlier.

Pregnancy diagnosis was performed by US 29 days after TAI in all cows using the same US equipment as for ovarian US. Reconfirmation of pregnancy status was performed by transrectal palpation of the uterine contents by the herd veterinarian at 39 and 74 days after TAI.

2.5. Experiment 2: Treatments

For experiment 2, 446 cows (193 primiparous and 253 multiparous) were enrolled in the DO protocol as described in experiment 1; however, the cows were randomly assigned to receive one of two treatments—(1) control, 100 μg GnRH–500 μg $PGF_{2\alpha}$ (n = 220) or (2) treatment, 200 μg GnRH–750 μg $PGF_{2\alpha}$ (n = 226). Pregnancy diagnosis and reconfirmation of pregnancy were performed similar to experiment 1 with all cows evaluated for pregnancy 29 days after TAI with reconfirmation of pregnancy 74 days after TAI (n = 72 pregnant cows) or 53 days after TAI (n = 117 pregnant cows).

2.6. Statistical analysis

The experimental design for experiments 1 and 2 was a complete randomized block design with parity as the blocking factor. In experiment 1, for outcomes (P/AI, CL regression, cows with their estrous cycle synchronized)measured after cows received the $PGF_{2\alpha}$ of the Breeding-Ovsynch portion of DO, the arrangement of treatments was a two-by-two factorial consisting of the following factors: dose of GnRH (100 vs. 200 μg) and dose of $PGF_{2\alpha}$ (500 vs. 750 μg).

Before statistical analyses of quantitative variables (P4 concentration and follicle size), normality of the data distribution was tested using the Shapiro-Wilk statistic and graphical methods (Q-Q plot, probability plot, and histograms) obtained with the UNIVARIATE procedure of SAS (version 9.2, SAS Institute Inc., Cary, NC, USA). Due to a lack of normality of the data distribution, P4 concentrations at GnRH2 were log-transformed.

Circulating concentrations of P4 at GnRH1 and the $PGF_{2\alpha}$ of the breeding portion of the DO protocol and size of the dominant follicle at GnRH1 were evaluated by ANOVA using PROC MIXED of SAS. The models used to evaluate the concentrations of P4 and size of the dominant follicle at GnRH1 included the following: dose of GnRH (100 vs. 200 μg of GnRH) and parity (primiparous vs. multiparous) as explanatory variables; whereas, the model for P4 concentration at $PGF_{2\alpha}$ contained the following as explanatory variables: dose of GnRH, dose of $PGF_{2\alpha}$ (500 vs. 750 μg of $PGF_{2\alpha}$), the dose of GnRH by dose of $PGF_{2\alpha}$ interaction, and parity.

Binary response data such as the percentage of cows with a functional CL (P4 ≥ 1.0 ng/mL) at the time of GnRH1 and the $PGF_{2\alpha}$ of the breeding portion of the DO protocol, percentage of cows with follicles ≥10 mm, ovulatory response to GnRH1, and luteal regression after the $PGF_{2\alpha}$ treatment of the Breeding-Ovsynch portion of the DO protocol were evaluated by logistic regression using the GLIMMIX procedure of SAS. The final models used for percentage of cows with a functional CL, percentage of cows with follicles ≥10 mm, and ovulatory response to GnRH1 included dose of GnRH and parity as explanatory variables. The model for ovulatory response to GnRH also included the presence of a functional CL as explanatory variable and size of the dominant follicle as a covariate. The model for percentage of cows with a CL at the time of $PGF_{2\alpha}$ of the breeding portion of the DO protocol contained the following as explanatory variables: dose of GnRH, dose of $PGF_{2\alpha}$, the dose of GnRH by dose of $PGF_{2\alpha}$ interaction, and parity and ovulation to GnRH. The effect of concentration of P4 on ovulatory response to GnRH1 was further analyzed by creating groups of cows on the basis of their circulating P4 and the dose of GnRH received. Three groups of cows were created on the basis of the following P4 concentrations at GnRH1: 0.00 to 0.99, 1.00 to 2.69, and ≥2.70 ng/mL. The first group was created to represent cows without a functional CL at GnRH1, whereas the other two groups represented cows with P4 below and above the mean (2.7 ng/mL) at GnRH1 in experiment 1. Ovulatory response to GnRH1 was compared by the dose of GnRH within each of these P4 concentration groups.

Cows were categorized by their circulating P4 concentrations at the time of GnRH2, and the P/AI of each category were compared by logistical regression using the GLIMMIX procedure of SAS with a model that contained P4 concentration as explanatory variable. The model used to

determine the differences between treatments on luteal regression was similar to the model that was used for evaluation of cows with a functional CL at the time of $PGF_{2\alpha}$. Finally, the percentage of cows that responded to the treatments was calculated by determining the percentage of cows with a functional CL (P4 $\geq$ 1 ng/mL) at $PGF_{2\alpha}$ and that had complete luteal regression by 56 hours after $PGF_{2\alpha}$. Once the percentage of cows that were synchronized was determined, P/AI at 29 days after TAI for each treatment combination was evaluated with logistical regression using the GLIMMIX procedure of SAS with a model that contained the effect of dose of GnRH, dose of $PGF_{2\alpha}$, their interaction, and parity of cows.

At the time of GnRH1, the relationship between the predicted probability of ovulation to GnRH and size of the largest follicle present on the ovaries as well as dose of GnRH was determined with logistic regression using PROC LOGISTIC of SAS. To eliminate inaccurate skewing of the model because of the low number of observations with follicle sizes unlikely to ovulate, cows with follicles <10 mm (n = 9) and >22 mm (n = 8) in diameter were eliminated from the dataset. The effect of dose of GnRH and follicle size and the interaction between dose of GnRH and follicle size were evaluated. Linear, quadratic, and cubic effects of follicle size were tested. The final model contained the effect of dose of GnRH and the linear and quadratic effects of follicle size at GnRH1. In addition, the relationship between the concentrations of P4 at GnRH1 and at the $PGF_{2\alpha}$ of Breeding-Ovsynch on the predicted probability of pregnancy was determined. To eliminate inaccurate skewing of the models due to the low number of observations with extremely high values for P4 concentration (n = 7 at GnRH1 and n = 28 at $PGF_{2\alpha}$), these observations were considered to have a maximum of 8 ng/mL, which was selected as the highest possible P4 concentration in this dataset. In all cases, the linear, quadratic, and cubic effects of P4 on the predicted probability of pregnancy were tested. For concentrations of P4 at GnRH1, the linear effect of P4 remained in the model, whereas for P4 at $PGF_{2\alpha}$, both the linear and quadratic effects remained in the model.

Analyses of P/AI and pregnancy loss were performed by logistic regression using the GLIMMIX procedure of SAS. For experiment 1, P/AI at 29, 39, and 74 days after TAI, the initial model contained the following categorical explanatory variables: dose of GnRH, dose of $PGF_{2\alpha}$, and their respective interaction. The model also contained the effect of parity, AI technician (one vs. two), and the interaction between treatment and parity. Model selection was performed by finding the model with the lowest value for the Akaike Information Criterion using a backward elimination procedure that removed all variables with P > 0.10 from the model. Both treatment and parity were forced to remain in each model. Parity was kept in the final models because it was used as a blocking factor for randomization of cows to treatments. Therefore, for P/AI at 29, 39, and 74 days after AI, the final model contained the fixed effects of treatment and parity. For analysis of pregnancy loss, the same categorical variables (except AI technician) and interactions used for P/AI were used to obtain the models for pregnancy loss from 29 to 39 and 29 to 74 days after AI. Procedures and criteria used for model selection were similar to those used for P/AI. The final model included the effect of treatment and parity.

For experiment 2, analyses of P/AI at 29 days after TAI and pregnancy loss were performed in a similar manner as for experiment 1. For P/AI 29 days after TAI, the initial model contained the following categorical explanatory variables as fixed effects: treatment (control vs. treatment), parity (primiparous vs. multiparous), AI technician (one vs. two), and the interaction between treatment and parity. For pregnancy loss, the variables included for analysis were similar to those of P/AI, except that AI technician was not included. For pregnancy loss, two analyses were run to evaluate pregnancy loss separately for cows rechecked at 53 or 74 days after TAI. Both models contained the same explanatory variables (treatment and parity).

A significant difference was considered as P < 0.05, whereas differences between P $\geq$ 0.05 and P $\leq$ 0.10 were considered a statistical tendency. A one-tailed test was used to test the hypotheses that the higher doses of GnRH and $PGF_{2\alpha}$ would increase ovulatory response, luteal regression, and fertility, whereas a two-tailed tested was used for all other analyses. Furthermore, the least significant difference post-hoc mean separation test was used to determine differences between least square means. Data included in the text are presented as arithmetic means (±SEM) obtained using PROC MEANS or PROC FREQ of SAS.

3. Results

3.1. Experiment 1: Ovarian status and responses for cows receiving 100 versus 200 μg of GnRH

At the time of GnRH1 treatment of the Breeding-Ovsynch portion of the DO protocol, the overall percentage of cows with a functional CL (P4 $\geq$ 1.0 ng/mL) was 92.8% (604/651), circulating P4 concentrations averaged 2.7 ± 0.1 ng/mL, and 96.0% (625/651) of the cows had a follicle $\geq$10 mm, with the average diameter of the largest follicle being 15.4 ± 0.1 mm. At GnRH1, no difference between 100 and 200 μg of GnRH was observed for the percentage of cows with a functional CL (P = 0.46), percentage of cows with a follicle $\geq$10 mm (P = 0.24), or follicle size (P = 0.50).

As expected, treatment with the 200-μg dose of GnRH increased (P = 0.01) ovulation to GnRH1 compared with 100 μg (Table 1) and was similar (P = 0.33) for primiparous (59.9%) and multiparous cows (64.0%). Nevertheless, the greater dose of GnRH only increased ovulation to GnRH1 in primiparous (66.9% vs. 53.5%; P = 0.01) but not in multiparous cows (66.3% vs. 61.5%; P = 0.18). The percentage of cows with a functional CL (P4 $\geq$ 1.0 ng/mL) at the $PGF_{2\alpha}$ of the Breeding-Ovsynch was similar (P = 0.79) for cows treated with either 100 or 200 μg of GnRH. P4 concentrations at the time of $PGF_{2\alpha}$ were greater (P = 0.03) for cows receiving 200 μg of GnRH than receiving 100 μg, with differences in P4 concentrations between doses in the primiparous (P = 0.01) but not the multiparous cows (P = 0.54). Complete CL regression after $PGF_{2\alpha}$ tended (P = 0.09) to be greater for cows receiving the 200 compared with the 100-μg dose of GnRH (Table 1). Finally, the percentage of cows considered to have their estrous cycle synchronized

Table 1
Ovarian status and responses in cows treated with 100 versus 200 μg of GnRH at the time of the first GnRH injection of the Breeding-Ovsynch portion of a DO protocol.

Item	Dose of GnRH		
	100 μg	200 μg	P
Ovulation to GnRH1[a] (%) (*n*/*n*)	57.5 (187/325)	66.6 (217/326)	0.01
Cows with P4 ≥ 1.0 ng/mL at $PGF_{2\alpha}$ (%) (*n*/*n*)	91.4 (297/325)	90.8 (296/326)	0.79
P4 at $PGF_{2\alpha}$ (ng/mL)	4.1 ± 0.1	4.4 ± 0.1	0.03
Complete CL regression[b] (%) (*n*/*n*)	84.9 (252/297)	89.5 (265/296)	0.09
Synchronized cows[c] (%) (*n*/*n*)	77.5 (252/325)	81.3 (265/326)	0.24

[a] GnRH1, first GnRH injection of the Breeding-Ovsynch portion of DO.
[b] Complete CL regression, P4 concentration ≥1.0 ng/mL at $PGF_{2\alpha}$ and <0.3 ng/mL at the second GnRH injection of the Breeding-Ovsynch portion.
[c] Synchronized cows, a cow was considered synchronized when P4 ≥ 1 ng/mL at the time of the $PGF_{2\alpha}$ injection and had complete luteal regression.

was similar (P = 0.24) for cows treated with either 100 or 200 μg of GnRH (Table 1).

Ovulatory response to the first GnRH1 (combined for both GnRH doses) was similar (P = 0.99) for cows with low (68.1%) or medium (68.0%) P4 at the time of GnRH1 but was lower (P = 0.08 for high vs. low and P < 0.001 for high vs. medium) for cows with high P4 at of GnRH1 (54.1%). Cows with low concentrations of P4 had a similar (P = 0.66) ovulatory response regardless of the dose of GnRH. However, treatment with 200 μg of GnRH tended (P = 0.06) to increase the ovulatory response in cows with medium circulating P4. In cows with high circulating P4, a clear increase in ovulatory response (P = 0.03) was observed for cows treated with 200 μg of GnRH compared with 100 μg (Fig. 2A).

In addition, an effect of dose of GnRH (P = 0.02) and a quadratic (P = 0.04) relationship between follicle size and the predicted probability of ovulation at GnRH1 was observed (Fig. 2B). The predicted probability of ovulation increased until follicle size was 16 to 18 mm followed by a downward trend of the predicted probability for follicle size up to 22 mm. At all follicle sizes, the model calculated a greater predicted probability of ovulation for cows receiving 200 μg of GnRH compared with the 100 μg.

3.2. Experiment 1: Ovarian status and responses for cows ovulating or not ovulating to the first GnRH of Breeding-Ovsynch (Table 2)

The impact of ovulation to GnRH1 was evaluated for various physiological measures (Table 2). At GnRH1, almost all cows (92.8%; 604/651) had a functional CL (P4 ≥ 1.0 ng/mL) and a follicle ≥10 mm (96.0%; 625/651) with no differences between cows on the basis of ovulatory response to GnRH1 (P = 0.38 for CL; P = 0.444 for follicle). However, concentrations of P4 at GnRH1 were greater (P < 0.001) for cows that did not subsequently ovulate to GnRH1 than in cows that ovulated to GnRH1 (Table 2). In addition, the overall size of the largest follicle at the time of GnRH1 was greater (P = 0.03) for cows that ovulated than for cows that failed to ovulate (Table 2).

The percentage of cows with a functional CL at the $PGF_{2\alpha}$ of Breeding-Ovsynch was greater for cows that ovulated to GnRH1 (P < 0.001; Table 2). Almost all cows (96.5%) that ovulated to GnRH1 had concentrations of P4 ≥ 1 ng/mL at the time of $PGF_{2\alpha}$, whereas this decreased (P < 0.001) to only 82.2% in cows that did not ovulate to GnRH1 (Table 2). Similarly, cows that ovulated to GnRH1 had greater concentrations of P4 at $PGF_{2\alpha}$, either including all cows in the analysis (P < 0.001; Table 2) or only primiparous (P < 0.001) or multiparous (P < 0.001) cows (data not shown). Among all cows that had a functional CL at the time of

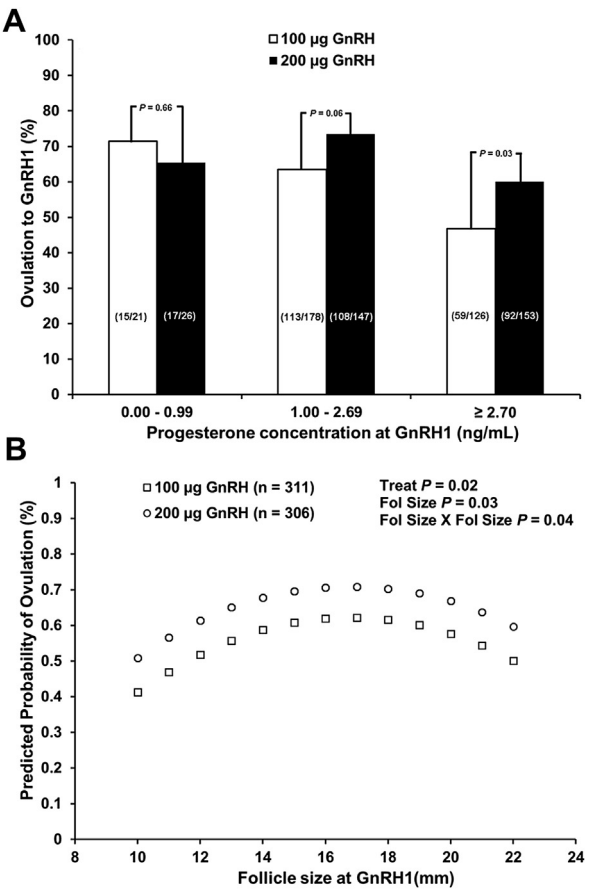

Fig. 2. Effect of circulating concentrations of P4 on ovulatory response to 100 or 200 μg of GnRH at the time of the first GnRH injection (GnRH1) of the Breeding-Ovsynch portion of a DO protocol (A). Three groups of cows were created on the basis of the following P4 concentrations at GnRH1: 0.00 to 0.99, 1.00 to 2.69, and ≥2.70. These groups represent cows without a functional CL at GnRH1 and cows with P4 below and above the mean (2.7 ng/mL) at GnRH1 in experiment 1. Predicted probability of ovulation in response to a 100- or 200-μg dose of GnRH treatment (Treat) at the time of the first GnRH injection of the Breeding-Ovsynch portion of the DO protocol according to size of the largest follicle (Fol Size) present on the ovaries (B).

Table 2
Concentrations of P4, follicle size, and fertility on the basis of ovulatory response of cows to the first GnRH treatment of the Breeding-Ovsynch portion of a DO protocol.

Item	Ovulation to GnRH1[a]		
	No	Yes	P
Cows with P4 ≥ 1.0 ng/mL at GnRH1 (%) (*n*/*n*)	93.9 (232/247)	92.1 (372/404)	0.38
P4 at GnRH1 (ng/mL)	3.0 ± 0.1	2.5 ± 0.1	<0.001
Follicle size at GnRH1 (mm)	15.0 ± 0.2	15.6 ± 0.2	0.03
Cows with P4 ≥ 1.0 ng/mL at $PGF_{2\alpha}$ (%) (*n*/*n*)	82.2 (203/247)	96.5 (390/404)	<0.001
P4 at $PGF_{2\alpha}$ (ng/mL)	3.6 ± 0.1	4.7 ± 0.1	<0.001
Synchronized cows[b] (%) (*n*/*n*)	73.3 (181/247)	83.2 (336/404)	0.01
P/AI 29 days all cows (%) (*n*/*n*)	38.5 (95/247)	52.2 (211/404)	<0.001
P/AI synchronized cows (%) (*n*/*n*)	43.7 (79/181)	55.7 (187/336)	0.010

Abbreviation: AI, artificial insemination.
[a] GnRH1, first GnRH injection of the Breeding-Ovsynch portion of DO.
[b] Synchronized cows, a cow was considered synchronized when P4 ≥ 1 ng/mL at the time of the $PGF_{2\alpha}$ injection and had complete luteal regression.

$PGF_{2\alpha}$, there were no differences (P = 0.30) observed in the percentage of cows with complete luteal regression after $PGF_{2\alpha}$ between cows that ovulated (86.2%; 336/390) or did not ovulate (89.2%; 181/203) to GnRH1.

When the two criteria used to classify whether cows were synchronized or not (presence of a CL at $PGF_{2\alpha}$ and luteal regression) were considered, a greater percentage of cows that ovulated to GnRH1 were synchronized compared with cows that did not ovulate to GnRH1 (P = 0.01). The overall P/AI of cows regardless of their synchronization status was greater (P < 0.001) for cows that ovulated than for cows that failed to ovulate to GnRH1. Similarly, the P/AI of synchronized cows was greater (P = 0.01) for cows that ovulated than for cows that did not ovulate to the GnRH treatment (Table 2).

When the overall impact of ovulation to GnRH1 on P/AI was analyzed, P/AI were 13.7 percentage points greater (P < 0.001) for cows that ovulated in response to GnRH1 (52.2%; 211/404) than for cows failing to ovulate after GnRH1 (38.5%; 211/404), which calculates to 35.6% (13.7/38.5) more pregnancies (Table 2). A detailed analysis of the effect of ovulation on P/AI within each of the P4 concentration groups created to evaluate ovulatory response to GnRH1 indicated that for cows with low P4 at GnRH1, ovulation tended (P = 0.09) to increase P/AI. However, for cows with medium P4 concentration at GnRH1, no effect of ovulation to GnRH1 on P/AI was observed (P = 0.15). For cows with high P4 concentrations at GnRH1, ovulation to GnRH1 resulted in greater (P = 0.001) P/AI (Fig. 3).

3.3. Experiment 1: Ovarian status and responses at the time of $PGF_{2\alpha}$ and final GnRH treatment

The overall percentage of cows with a functional CL at the time of the $PGF_{2\alpha}$ treatment was 91.1% (593/651) and was similar (P = 0.61) among the four treatments (Table 3). Concentrations of P4 at $PGF_{2\alpha}$ were greater (P = 0.03) for cows receiving 100 μg of GnRH compared with 200 μg (4.1 ± 0.1 vs. 4.4 ± 0.1 ng/mL, respectively), for primiparous than for multiparous cows (4.7 ± 0.1 vs. 3.9 ± 0.1 ng/mL, respectively; P < 0.001), for cows that ovulated than for cows that did not ovulate to GnRH1 (4.7 ± 0.1 vs. 3.6 ± 0.1 ng/mL, respectively; P < 0.001), and for cows that had a CL versus those that did not have a CL at GnRH1 (4.4 ± 0.1 vs. 2.0 ± 0.3 ng/mL, respectively; P < 0.001). Conversely, no difference (P = 0.87) in concentrations of P4 at $PGF_{2\alpha}$ was observed for cows receiving either 500 or 750 μg of $PGF_{2\alpha}$ (4.2 ± 0.1 vs. 4.3 ± 0.1 ng/mL).

Luteal regression in response to the $PGF_{2\alpha}$ treatment of the Breeding-Ovsynch for cows with a functional CL was greater (P = 0.03) for cows receiving the 750-μg dose of $PGF_{2\alpha}$ than the 500-μg dose (Table 3) and was greater (P = 0.01) for primiparous (91.2%; 260/285) than for multiparous cows (83.4%; 257/308). Conversely, no differences were observed between cows treated with either 100 or 200 μg of GnRH (P = 0.12; Table 3). Further evaluation of the effect of dose of $PGF_{2\alpha}$ on luteal regression for primiparous and multiparous cows indicated that the greater dose of $PGF_{2\alpha}$ did not increase (P = 0.18) the percentage of primiparous cows with luteal regression (Fig. 4A). In contrast, more (P = 0.03) multiparous cows treated with the 750-μg dose of $PGF_{2\alpha}$ than the 500-μg dose had complete luteal regression (Fig. 4A). P/AI were similar for primiparous (P = 0.22) and multiparous (P = 0.21) cows receiving the 500- or the 750-μg dose of $PGF_{2\alpha}$ (Fig. 4B).

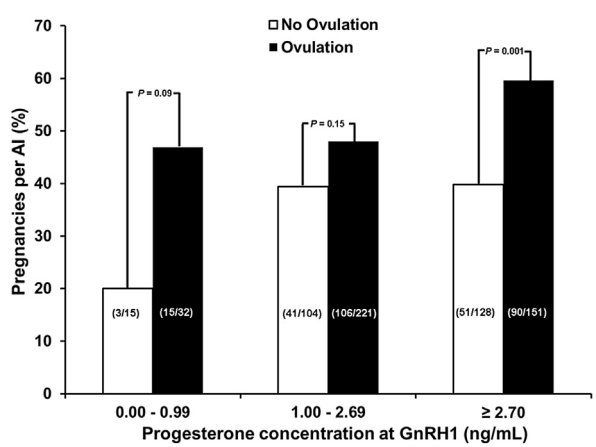

Fig. 3. P/AI according to circulating concentrations of P4 at the time of the first GnRH injection (GnRH1) of the Breeding-Ovsynch portion of a DO protocol in cows on the basis of ovulatory response to the GnRH injection. Three groups of cows were created on the basis of the following P4 concentrations at GnRH1: 0.00 to 0.99, 1.00 to 2.69, and ≥2.70. These groups represent cows without a functional CL at GnRH1 and cows with P4 below and above the mean (2.7 ng/mL) at GnRH1 in experiment 1.

Table 3
Ovarian status and responses at the time of the $PGF_{2\alpha}$ and final GnRH treatments in cows treated with 500 versus 750 μg or a $PGF_{2\alpha}$ analogue during the Breeding-Ovsynch portion of a DO protocol.

Item	Treatment				P value		
	100 μg GnRH		200 μg GnRH		GnRH dose	$PGF_{2\alpha}$ dose	GnRH dose × $PGF_{2\alpha}$ dose
	500 μg $PGF_{2\alpha}$	750 μg $PGF_{2\alpha}$	500 μg $PGF_{2\alpha}$	750 μg $PGF_{2\alpha}$			
Cows with P4 ≥ 1.0 ng/mL at $PGF_{2\alpha}$ (%) (*n*/*n*)	92.6 (150/162)	90.2 (147/163)	90.9 (150/165)	90.7 (146/161)	0.77	0.55	0.61
Complete CL regression (%) (*n*/*n*)	80.7 (121/150)	89.1 (131/147)	88.0 (132/150)	91.1 (133/146)	0.12	0.03	0.50
Synchronized cows[a] (%) (*n*/*n*)	74.7 (121/162)	80.4 (131/163)	80.0 (132/165)	82.6 (133/161)	0.25	0.20	0.69
P/AI synchronized cows (%) (*n*/*n*)	46.3 (56/121)	53.4 (70/131)	56.1 (74/132)	49.6 (66/133)	0.50	0.94	0.12

Abbreviation: P/AI, pregnancies per AI.
[a] Synchronized cows = a cow was considered synchronized when P4 ≥ 1 ng/mL at the time of the $PGF_{2\alpha}$ injection and had complete luteal regression.

The percentage of synchronized cows calculated as previously described was 79.4% (517/651) and was not affected by the dose of GnRH (P = 0.25), the dose of $PGF_{2\alpha}$ (P = 0.20), or their interaction (P = 0.69; Table 3). P/AI for nonsynchronized cows were 30.0% (40/134) across all treatment groups, whereas overall P/AI for synchronized cows in the four treatment combinations was 51.4% (266/517).

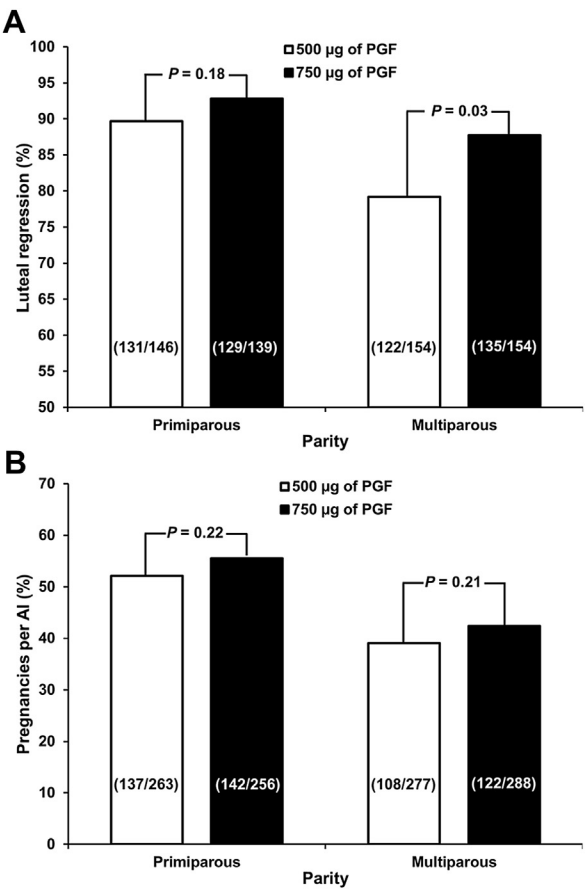

Fig. 4. Luteal regression for primiparous and multiparous cows in response to 500 or 750 μg of a $PGF_{2\alpha}$ analogue during the Breeding-Ovsynch portion of a DO protocol (A). Complete luteal regression was defined as the presence of a functional CL (P4 ≥ 1.0 ng/mL) at the time of $PGF_{2\alpha}$ and circulating concentrations of P4 of <0.3 ng/mL at the time of the last GnRH treatment of the Breeding-Ovsynch portion of a DO protocol. P/AI for primiparous and multiparous cows receiving 500 or 750 μg of a $PGF_{2\alpha}$ analogue during the Breeding-Ovsynch portion of a DO protocol (B).

3.4. Experiment 1: Impact of P4 concentrations at the first GnRH and $PGF_{2\alpha}$ treatments of Breeding-Ovsynch on the predicted probability of pregnancy

A tendency for a positive linear association (P = 0.09) was observed between the predicted probabilities of pregnancy 29 days after TAI and concentrations of P4 at GnRH1 (Fig. 5A). In addition, a quadratic relationship (P = 0.02) between concentrations of P4 at $PGF_{2\alpha}$ and the predicted probability of pregnancy 29 days after TAI was detected (Fig. 5B). The predicted probability of pregnancy increased from about 20% for cows with 0 ng/mL up to above 55% for cows with P4 concentration of 5 ng/mL or more at the time of $PGF_{2\alpha}$.

3.5. Experiment 1: Effect on P/AI of increasing the dose of GnRH and $PGF_{2\alpha}$

Overall, P/AI at 29, 39, and 74 days after TAI for all cows included in experiment 1 were 47.0% (509/1084), 43.2% (468/1084), and 39.7% (430/1084), respectively. At 29 days after TAI, dose of GnRH (P = 0.28), dose of $PGF_{2\alpha}$ (P = 0.13), or their interaction (P = 0.16) did not affect P/AI (Table 4). At 39 days after TAI, however, cows receiving the 750-μg dose of $PGF_{2\alpha}$ tended to have greater (P = 0.05) P/AI (45.4%) than cows receiving the 500-μg dose of $PGF_{2\alpha}$ (40.9%) regardless of GnRH dose treatment (P = 0.28). At 74 days after TAI, there was no effect (P = 0.19) of dose of GnRH, whereas cows receiving the 750-μg dose of $PGF_{2\alpha}$ tended (P = 0.08) to have greater P/AI (41.5%) than cows receiving 500-μg of $PGF_{2\alpha}$ (37.8%; Table 4).

Conversely, primiparous cows had greater P/AI than multiparous cows at 29 (53.8% vs. 40.7%; P < 0.001), 39 (50.1% vs. 36.8%; P < 0.001), and 74 days (46.1% vs. 33.8%; P < 0.001) after TAI. No effect of AI technician was detected at any of the time points evaluated. Overall, pregnancy loss from 29 to 39 days after TAI and total pregnancy loss from 29 to 74 days after TAI were 8.1% (41/509) and 15.5% (79/509), respectively. The incidence of pregnancy loss from 29 to 39 and 29 to 74 days after TAI was not affected by the dose of GnRH, dose of $PGF_{2\alpha}$, or their interaction (Table 4).

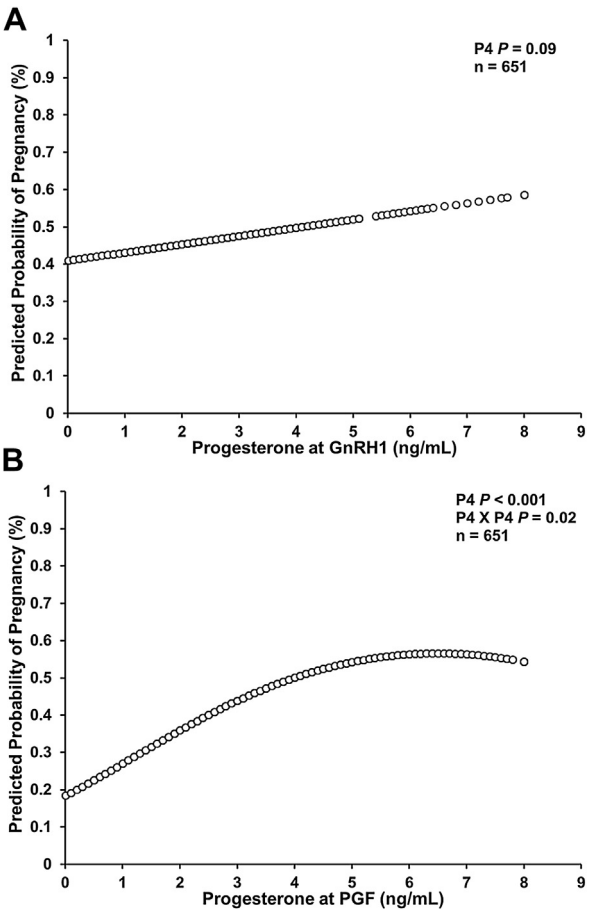

Fig. 5. Predicted probability of pregnancy 29 days after TAI on the basis of circulating concentrations of P4 at the time of the first GnRH treatment (GnRH1; A) and the $PGF_{2\alpha}$ treatment (B) of the Breeding-Ovsynch portion of a DO protocol.

3.6. Experiment 2: Effect on P/AI of increasing the dose of GnRH and $PGF_{2\alpha}$

At 29 days after TAI, P/AI were similar (P = 0.21) for cows receiving the 100-μg GnRH–500 μg $PGF_{2\alpha}$ (40.5%; 89/220) or 200-μg GnRH–750 μg $PGF_{2\alpha}$ (44.2%; 100/226) dose treatment combinations. In addition, P/AI were similar (P = 0.31) for primiparous and multiparous cows (45.1%; 87/193 vs. 40.3%; 102/253, respectively). Overall pregnancy loss for the subgroup of cows tested at 29 and 74 days after TAI was 16.7% (12/72) and did not differ between treatment groups (P = 0.15) or parities (P = 0.21). Similarly, pregnancy loss for the subgroup of cows tested at 29 and 53 days after TAI was 12.8% (15/117) and did not differ between treatment groups (P = 0.15) or parities (P = 0.84).

4. Discussion

The purpose of these experiments was to explore new strategies to optimize the response to key hormonal treatments of the DO protocol. Experiment 1 was designed to test the hypothesis that increasing the dose of GnRH from 100 to 200 μg at the time of the first GnRH of the Breeding-Ovsynch portion of the DO protocol would increase ovulatory response to GnRH and that increasing the dose of $PGF_{2\alpha}$ from 500 to 750 μg would increase the percentage of cows with luteal regression after the $PGF_{2\alpha}$ treatment. A greater ovulatory response to GnRH was expected to improve the fertility of first postpartum TAI service as reported in other studies [5,12,13]. Furthermore, more cows were expected to undergo luteal regression after the $PGF_{2\alpha}$ treatment and have minimal concentrations of P4 at GnRH2, a situation that has been associated with greater fertility in synchronized lactating dairy cows [7,11,20].

The higher dose of GnRH increased the overall ovulatory response by 9 percentage points in support of our first hypothesis. We speculate that the reason for the difference in ovulatory response between the dose groups was the greater GnRH-induced LH release elicited by the 200-μg dose of GnRH in the high circulating P4 environment (mean P4 = 2.7 ng/mL) present at the time of the first GnRH of the Breeding-Ovsynch. This idea is supported by the observation that cows without a functional CL at GnRH1 had a similar ovulatory response to either 200 or 100 μg of GnRH. In contrast, cows with a functional CL and elevated P4 at GnRH1 had increased ovulatory response to 200 μg of GnRH compared with 100 μg. Further evidence that the magnitude of the LH surge may be limiting in high P4 is the observation that almost all cows (96%) had a follicle of sufficient size to have ovulatory capacity at the time of GnRH1. The practical implications of these data are that increasing the dose of GnRH may be more beneficial for cows with high circulating P4 at GnRH1 than for cows without a functional CL. In agreement, Galvao and Santos

Table 4
Results from experiment 1 for P/AI at 29, 39, and 74 days after TAI in cows synchronized with a DO protocol that received 100 versus 200 μg of GnRH at the first GnRH treatment and 500 versus 750 μg $PGF_{2\alpha}$ during the Breeding-Ovsynch portion of a DO protocol.

Item	Treatment				P value		
	100 μg GnRH		200 μg GnRH		GnRH dose	$PGF_{2\alpha}$ dose	GnRH dose × $PGF_{2\alpha}$ dose
	500 μg $PGF_{2\alpha}$	750 μg $PGF_{2\alpha}$	500 μg $PGF_{2\alpha}$	750 μg $PGF_{2\alpha}$			
P/AI 29 days (%) (*n*/*n*)	43.0 (113/263)	49.3 (133/270)	47.7 (132/277)	47.8 (131/274)	0.28	0.13	0.16
P/AI 39 day (%) (*n*/*n*)	38.0 (100/263)	46.3 (125/270)	43.7 (121/277)	44.5 (122/274)	0.24	0.05	0.11
P/AI 74 days (%) (*n*/*n*)	34.6 (91/263)	42.2 (114/270)	40.8 (113/277)	40.9 (112/274)	0.19	0.08	0.10
Pregnancy loss 29–39 days after TAI (%) (*n*/*n*)	11.5 (13/113)	6.0 (8/133)	8.3 (11/132)	6.9 (9/131)	0.74	0.16	0.48
Pregnancy loss 29–74 days after TAI (%) (*n*/*n*)	19.5 (22/113)	14.3 (19/133)	14.4 (19/132)	14.5 (19/131)	0.48	0.45	0.46

[16] reported that the presence of a follicle of ovulatory size at the time of a GnRH treatment in lactating dairy cows does not guarantee the occurrence of ovulation. Thus, it seems likely that the major limiting factor for reduced ovulation to GnRH1 during the DO protocol in lactating dairy cows is the suppression by P4 of the GnRH-induced LH surge as previously reported [18]. Although increasing the dose of GnRH from 100 to 200 μg in lactating dairy cows results in an LH surge of greater magnitude [18], ovulatory response in this experiment did not maximize synchronization to the protocol and fertility. For unknown reasons, the higher dose of GnRH seemed to be more effective in increasing ovulation in primiparous cows (13.4%) than multiparous cows (4.8%). This may be due to the greater P4 concentrations in primiparous than multiparous cows or differing responses to different GnRH doses in cows of different parities.

It may seem counterintuitive that a higher GnRH dose increased ovulatory response at GnRH1 but did not improve fertility; however, the increase in ovulatory response was only about 10 percentage points. The improvement in fertility for cows that ovulated to GnRH1 compared with cows that did not ovulate to GnRH1 was 13.7 percentage points. Thus, only a 1.37 percentage point improvement in P/AI would be expected due to this increase in ovulatory response, such a small difference as to be undetectable except in a much larger experiment with more statistical power. It seems clear that increasing the dose of GnRH at GnRH1 is likely to provide a diminishingly small benefit and is unlikely to be of practical economic benefit to dairy producers. Nevertheless, it seems clear that any benefit is only likely to be observed in cows with elevated P4 at GnRH1.

The second working hypothesis was that increasing the dose of $PGF_{2\alpha}$ would increase the percentage of cows with complete luteal regression by 56 hours after $PGF_{2\alpha}$ treatment. The results of this experiment support this hypothesis with more cows undergoing complete luteal regression when treated with 750 μg of $PGF_{2\alpha}$ compared with 500 μg. Similar to previous reports, cows that did not have complete luteal regression after the $PGF_{2\alpha}$ treatment of Ovsynch had lower fertility using a calculated threshold value for P4 (0.3 ng/mL) that was found to be similar to the values selected in other studies [7,11,21]. Interestingly, the higher dose of $PGF_{2\alpha}$ was only effective in increasing the luteal regression in multiparous cows but not in primiparous cows. The lower percentage of multiparous cows that respond to $PGF_{2\alpha}$ and the increase caused by the higher dose of $PGF_{2\alpha}$ in multiparous cows could be due to greater metabolism of cloprostenol in multiparous than primiparous cows. The practical implication of this observation is that older cows are more likely to benefit than younger cows from an increased dose of $PGF_{2\alpha}$. This agrees with the observations by others of reduced luteal regression after a $PGF_{2\alpha}$ treatment in multiparous compared with primiparous cows [11,19].

The P/AI data from experiment 1 did not support our first hypothesis of increased fertility with a greater dose of GnRH and only partially supported our second hypothesis that a greater dose of $PGF_{2\alpha}$ would increase fertility of lactating dairy cows. However, P/AI were greater for cows treated with 750 μg of $PGF_{2\alpha}$ only at 39 (4.5 percentage points) and 74 (4.5 percentage points) days after TAI. Similar to the observations for the greater dose of GnRH, the improvement in luteal regression with the greater dose of $PGF_{2\alpha}$ was insufficient to generate a detectable difference in P/AI for all cows or when primiparous and multiparous cows were analyzed separately at 29 days after TAI (data not shown). Despite the clear improvement in luteal regression (8.5%) in multiparous cows, P/AI at 39 days after TAI only improved by 4.5 percentage points. Assuming that cows with luteal regression had 50% P/AI and cows without complete luteal regression had 0% P/AI, it is logical that an improvement of just over 4 percentage points in P/AI (8.5 percentage points increased luteal regression *50% P/AI) is all that could be expected with improved luteal regression. Using a different approach to increase luteal regression, Brusveen et al. [7] reported that adding an extra dose of $PGF_{2\alpha}$ 24 hours after the $PGF_{2\alpha}$ treatment of Breeding-Ovsynch for first TAI service increased luteal regression by 11.4 percentage points in cows of all parities. Despite a numerical trend in favor of the treated group (5.7%), P/AI were similar for cows treated (52.7%, $n = 221$) or not treated (47.0%, $n = 232$) with the additional $PGF_{2\alpha}$ treatment. Thus, the magnitude of improvement in fertility by increasing the dose of $PGF_{2\alpha}$ seems logical from both of these studies; however, even larger future studies are needed to confirm this result and to provide a valid economic assessment of this strategy.

As expected, there were no significant treatment effects on pregnancy loss. Nevertheless, the numerically greater pregnancy loss for the low doses of $PGF_{2\alpha}$ could account for the differential effect of $PGF_{2\alpha}$ dose on P/AI when it was evaluated at later pregnancy diagnoses.

The aim of experiment 2 was to collect additional P/AI data for the treatment combination that was expected to have the greatest effect on fertility. In agreement with experiment 1, no significant differences in P/AI were observed in cows treated with the higher doses of GnRH and $PGF_{2\alpha}$ despite ~4 percentage points higher P/AI for the higher dose combination compared with the lower dose combination in both experiments. Although this difference could be of economic value to dairy producers, it is still uncertain whether this improvement would be consistently observed in larger, more extensive trials on multiple commercial dairies.

Even though these experiments were not designed to compare the DO protocol with other presynchronization strategies for first TAI, it is worth noting the high percentage of cows (92.8%; 604/651) with a functional CL at initiation of the Breeding-Ovsynch portion of DO. These results contrast with those observed in cows presynchronized with two $PGF_{2\alpha}$ treatments 14 days apart (presynch), in which the percentage of cows without a CL has been reported in the 15% to 30% range [6,22–24]. Direct comparisons of Presynch-Ovsynch versus DO protocols have consistently reported a reduction in the percentage of anovular cows at GnRH1 of the Breeding-Ovsynch protocol (33.3%–9.4% [6], 32.2%–6.0% [10], 24.7%–6.3% [9]). Thus, the percentage of anovular lactating dairy cows is clearly reduced by the DO protocol. Nevertheless, this may not translate into fertility improvements in anovular cows because a recent study using a 5-day

Cosynch protocol in grazing dairy cows did not observe an improvement in P/AI in anovular cows by presynchronizing with DO compared with presynch, although a decrease in pregnancy loss was noted with DO compared with presynch [25]. In our study, similar to what has been reported in other studies [11,15,24], cows with a CL at GnRH1 had improved fertility, although our experimental design did not allow us to determine which cows were anovular before the DO protocol. Furthermore, higher circulating P4 at GnRH1 was associated with increased fertility to the timed AI. Even more dramatic was the association between circulating P4 at the time of $PGF_{2\alpha}$ and subsequent fertility. High P4 at the time of $PGF_{2\alpha}$ would indicate better synchronization and a better hormonal environment for growth of the ovulatory follicle [5,26,27].

Another interesting finding of this study was the significant effect of parity on fertility with 13.1 percentage points greater P/AI for primiparous than for multiparous cows in experiment 1. These observations are in agreement with Souza et al. (+28%) [6] and Herlihy et al. (+12.2%) [9], who reported greater P/AI for primiparous than multiparous cows treated with DO for first postpartum TAI service. However, other studies have reported no difference between primiparous and multiparous cows treated with DO at first postpartum TAI (experiment 2 and Brusveen et al. [7]) or second or later TAI [11]. The greater rate of CL regression in primiparous compared with multiparous cows could explain part of the parity differences in fertility. It is intriguing, however, that no differences in fertility were observed between parities in experiment 2, even though the experiment was done with the same pharmaceutical products and on the same commercial dairy.

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