Comparison of 2 protocols to increase circulating progesterone concentration before timed artificial insemination in lactating dairy cows with or without elevated body temperature

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ABSTRACT

Two treatments designed to increase circulating progesterone concentration (P4) during preovulatory follicle development were compared. One treatment used 2 intravaginal P4 implants (controlled internal drug-releasing inserts; CIDR) and the other used a GnRH treatment at beginning of the protocol. Lactating Holstein cows that had been diagnosed as nonpregnant were randomly assigned to receive timed artificial insemination (TAI) following 1 of 2 treatments ($n = 1,638$ breedings): (1) GnRH: CIDR + 2 mg of estradiol (E2) benzoate + 100 μ g of GnRH on d -11, PGF_{2 α} on d -4, CIDR withdrawal + 1.0 mg of E2-cypionate + PGF_{2 α} on d -2, and TAI on d 0; or (2) 2CIDR: 2 CIDR + 2 mg of E2-benzoate on d -11, 1 CIDR withdrawn + PGF_{2 α} on d -4, second CIDR withdrawn + 1.0 mg of E2-cypionate + PGF_{2 α} on d -2, and TAI on d 0. Milk yield was measured daily between d 0 and d 7. Rectal temperature was measured using a digital thermometer at d 0 and 7, and elevated body temperature was defined as an average rectal temperature $\geq 39.1^{\circ}\text{C}$. Pregnancy diagnoses were performed on d 32 and 60 after TAI. We detected no effect of treatments on pregnancy per AI or pregnancy loss regardless of elevated body temperature, body condition score, parity, milk yield, or presence or absence of a corpus luteum (CL) on d -11 or d -4. Pregnancy per AI at 60 d was reduced [elevated body temperature = 22.8% (162/709), no elevated body temperature 34.1% (279/817)] and pregnancy loss tended to increase [elevated body temperature = 20.2% (41/203), no elevated body temperature 14.4% (47/326)] in cows with elevated body temperature. Various physiological measurements associated with greater fertility were also reduced in cows with elevated body temperature, such as percentage of cows with a CL at PGF_{2 α} (de-

creased 7.9%), ovulatory follicle diameter (decreased 0.51 mm), expression of estrus (decreased 5.1%), and ovulation near TAI (decreased 2.8%) compared with cows without elevated body temperature. A greater proportion of cows (30.2%) had a CL at PGF_{2 α} in the GnRH treatment [74.1% (570/763)] than in the 2CIDR treatment [56.9% (434/763)]; however, circulating P4 concentration was greater at the time of PGF_{2 α} treatment (d -4) for cows 2CIDR (4.26 ± 0.13 ng/mL) than in cows in GnRH (3.99 ± 0.14 ng/mL). Thus, these 2 protocols yield similar fertility results that might be due to somewhat different physiological alterations. Treatment with GnRH increased the proportion of cows with a CL at PGF_{2 α} ; however, the 2CIDR protocol increased circulating P4 under all circumstances.

Key words: synchronization protocols, progesterone, elevated body temperature

INTRODUCTION

Elevated progesterone (P4) during preovulatory follicle development is associated with greater fertility in cows submitted to AI after natural estrus (Folman et al., 1973; Erb et al., 1976; Fonseca et al., 1983; Meisterling and Dailey, 1987), after PGF_{2 α} -induced luteolysis (Diskin et al., 2006) or during timed AI (TAI) protocols (Giordano et al., 2010; Martins et al., 2011). Some of the reasons for reduced circulating P4 concentrations during growth of the preovulatory follicle in TAI protocols can be lack of a corpus luteum (CL) at the beginning of a synchronization protocol for first AI (Stevenson et al., 2008; Bisinotto et al., 2010a, 2013) or subsequent AI services (Fricke et al., 2003; Sterry et al., 2006; Silva et al., 2009), elevated steroid metabolism in lactating dairy cows (Sangsritavong et al., 2002; Vasconcelos et al., 2003; Wiltbank et al., 2006), or regression of the CL during the protocol (Thatcher et al., 1986; Araujo et al., 2009; Monteiro et al., 2015). Thus, lactating dairy cows can have reduced circulating P4 concentrations during preovulatory follicular devel-

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opment during natural or synchronized cycles, resulting in reduced pregnancy per AI (**P/AI**; Souza et al., 2008; Bisinotto et al., 2010a,b, 2013; Denicol et al., 2012; Dirandeh et al., 2015a,b; Wiltbank et al., 2015).

Two different approaches have been used to increase circulating P4 during preovulatory follicle development. First, treatment with GnRH at the beginning of a protocol can ovulate a follicle, even in the presence of a primary CL, thereby increasing circulating P4 by creation of an accessory CL. As expected, in GnRH-based protocols, increased circulating P4 concentration at the beginning of the protocol (Bisinotto et al., 2010b, 2013; Denicol et al., 2012) and the presence of a functional CL at the time of PGF_{2α} treatment have been associated with improved fertility (Giordano et al., 2010; Martins et al., 2011). Protocols that use GnRH on d 6 or 7 of the estrous cycle have a particularly high percentage of cows that ovulate to the GnRH treatment compared with cows treated with GnRH on other days of the cycle. This results in a new accessory CL, increased circulating P4, and better synchronization of the preovulatory follicular wave, resulting in increased P/AI with these protocols (Vasconcelos et al., 1999; Souza et al., 2008; Herlihy et al., 2012; Dirandeh, 2014; Wiltbank and Pursley, 2014). Similarly, addition of GnRH at the beginning of estradiol (**E2**)- and P4-based protocols results in greater circulating P4 at PGF_{2α} and greater fertility with these protocols (Pereira et al., 2015). Increased P4 during E2- or P4-based protocols is particularly important because these protocols result in lower circulating P4 concentrations at the time of PGF_{2α} treatment (2.29 ± 0.15 ng/mL) compared with GnRH protocols (2.89 ± 0.15 ng/mL; Vasconcelos et al., 2011a). In addition, ovulation to GnRH at the beginning of a synchronization protocol initiates a new follicular wave and may therefore improve synchronization of the follicular wave and potentially improve fertility during a TAI protocol. A second method to increase circulating P4 during preovulatory follicle growth is by using a P4 implant such as a controlled internal drug-releasing insert (**CIDR**). However, lactating cows have greatly elevated metabolism of circulating P4 due to elevated liver blood flow and this results in a sub-optimal increase in circulating P4 (<1 ng/mL) during treatment with a single P4 implant (Stevenson et al., 2006). Alternatively, Bisinotto et al. (2013) evaluated supplementation of cows with low circulating P4 at the beginning of a protocol with 2 CIDR in a GnRH-based 5-d TAI program (d -8 GnRH, d -3 and -2 PGF_{2α}, d 0 GnRH and AI). Treatment with 2 CIDR increased circulating P4 to 2.65 ng/mL and restored fertility in lactating dairy cows lacking a CL at the initiation of the TAI program to a value that was similar to cows that began the protocol with a CL, compared with

cows with low circulating P4 without CIDR. In a recent study from our group (Pereira et al., 2017), we evaluated supplementation with 2 instead of 1 CIDR in an E2-/P4-based protocol in cows with reduced circulating P4 concentration at the beginning of the protocol (<1.0 ng/mL). Treatment with 2 CIDR tended to increase P/AI compared with 1 CIDR in TAI (1 CIDR: 42.8% vs. 2 CIDR: 52.6%; $n = 326$) but not in timed embryo transfer ($n = 445$) programs. Thus, increased circulating P4 and improved P/AI have been shown for protocols that produce a new CL using GnRH treatment or directly increase circulating P4 by treating with 2 P4 implants instead of 1 P4 implant during the protocol.

The objective of this study was to compare these 2 strategies for increasing circulating P4 concentration during preovulatory follicular development in lactating dairy cows. We hypothesized that treatment with GnRH or treatment with 2 CIDR would produce similar circulating P4 concentrations during the protocol, similar percentage of cows ovulating near TAI, and similar P/AI and pregnancy losses in lactating dairy cows.

MATERIALS AND METHODS

This experiment was conducted at 4 commercial dairy farms in Minas Gerais State, Brazil, from May 2013 to January 2014. All animal procedures followed the recommendations of the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (FASS, 1999). During the experimental period, cows were housed in freestall barns and had access to an adjoining sod-based paddock. Throughout the experiment, cows were milked 3 times daily. All procedures, including injections, ovarian ultrasonography, pregnancy diagnosis, blood collection, and TAI, were performed while cows were restrained in self-locking head gates at the feedline. Cows were fed ad libitum a TMR based on corn silage and Tifton hay as forages, with concentrates composed of corn and soybean meal, and added minerals and vitamins, which was balanced to meet or exceed the nutritional requirements of lactating dairy cows (NRC, 2001).

Animals and Treatments

This study used 1,638 breedings in lactating Holstein cows (throughout the text, “n” refers to the number of breeding events that received a particular protocol). At the beginning of the experiment (d -11), cows averaged 187.8 ± 13.5 DIM, yielding 31.4 ± 2.23 kg of milk/d, BCS of 2.94 ± 0.03 [on a 1 (emaciated) to 5 (obese) scale; Wildman et al., 1982], lactation number of 2.43 ± 0.36 [primiparous (=1) $n = 457$; multiparous (≥ 2) $n = 1,181$], and had been bred 3.38 ± 0.08 times.

All cows were diagnosed nonpregnant before being enrolled in this trial. Within each farm, cows were divided in batches by parity (primiparous and multiparous), DIM, and milk production. All cows found to be not pregnant after a previous AI were randomized into the study, regardless of whether they had been previously utilized in the study. Cows received the following TAI protocol: an intravaginal P4 insert containing 1.9 g of P4 (CIDR, Zoetis, São Paulo, Brazil) and 2.0 mg of estradiol benzoate (EB, i.m.; 2.0 mL of Estrogin, Farmavet, São Paulo, Brazil) on d -11; 25 mg (i.m.) of dinoprost tromethamine (PGF_{2α}; 5.0 mL of Lutalyse, Zoetis) on d -4; CIDR withdrawal and 1.0 mg (i.m.) of estradiol cypionate (ECP; 0.5 mL of E.C.P., Zoetis) and PGF_{2α} on d -2; and TAI on d 0. Cows were randomly assigned to receive 1 of 2 treatments (Figure 1): (1) **GnRH treatment**: 100 µg of GnRH injection on d -11 (i.m.; 2.0 mL of Cystorelin, Merial, SP, Brazil) or (2) **2CIDR treatment**: an additional CIDR placed on d -11, which was removed at d -4.

The TAI was performed (d 0) using commercial, frozen-thawed semen from 36 bulls. Devices for detection of estrus (Estrotest, Rockway Inc., Spring Valley, WI) were placed on the tail head of cows at the time of CIDR removal. The Estrotest patches were not used for detection of estrus or timing of AI because all cows

were bred to TAI by study personnel during the experimental period. The Estrotest was monitored at TAI by the research personnel, and cows with an activated Estrotest (complete color change) were designated as expressing estrus during the protocol.

Ultrasonography

In both groups, ovaries were evaluated by transrectal ultrasonography (US, Aloka SSD-500 with a 7.5-MHz linear-array transducer, Aloka, Tokyo, Japan) on d -11, -4 and 7 to determine presence of a CL (independent of its diameter), and at d 0 to measure the diameter of the largest follicle present. Ovulation at the beginning of the protocol was defined by the presence of a follicle >8 mm on d -11 and with a corresponding new CL at the time of first PGF_{2α} treatment (d -4). In addition, the percentage of cows with a new CL on d -4, independent of the presence of a follicle >8 mm on d -11, was also determined and used in some analyses. The size of the ovulatory follicle was determined by the largest follicle present on the ovary on d 0 that corresponded to an observed CL on d 7. Cows with follicles <8 mm on d 0 but with a CL on d 7 were defined as “early ovulators” and were not used in analyses of ovulatory follicle diameters.

Figure 1. Diagram of activities. Ovaries were evaluated by ultrasound (US) in all cows on d -11, -4, 0 and 7. Cows received the following timed AI (TAI) protocol: an intravaginal progesterone (P4) insert containing 1.9 g of P4 (CIDR, Zoetis, São Paulo, Brazil) and 2.0 mg of estradiol benzoate (EB, i.m.; 2.0 mL of Estrogin, Farmavet, São Paulo, Brazil) on d -11; 25 mg (i.m.) of dinoprost tromethamine (PGF_{2α}; 5.0 mL of Lutalyse, Zoetis) on d -4; CIDR withdrawal, 1.0 mg (i.m.) of estradiol cypionate (ECP; 0.5 mL of ECP, Zoetis) and PGF_{2α} on d -2; and TAI on d 0. Cows were randomly assigned to receive 1 of 2 treatments: (1) GnRH treatment: 100-µg GnRH injection at d -11 (i.m.; 2.0 mL of Cystorelin, Merial, São Paulo, Brazil) or (2) 2CIDR treatment: an additional CIDR at d -11, which was removed at d -4. P4 = blood sampling and analysis of P4 concentration (sample on d -11, -4, and 7); US = ultrasonography of the ovaries (d -11, -4, 0, and 7).

Ovulation to ECP, Pregnancy Success, and P/AI

Cows with CL present on d 7 were defined as having ovulated to the ECP. Pregnancy per AI was calculated by dividing the number of pregnant cows at the pregnancy diagnosis 32 or 60 d after TAI by the total number of cows that received TAI. Pregnancy loss was calculated by dividing the number of cows that lost their pregnancy by the number of pregnant cows at the 32-d pregnancy diagnosis.

Sample Collection

Milk yield was measured daily between d 0 and 7, and average daily production through this interval was used in the analysis. Rectal temperature was measured using a digital thermometer (Jumbo Display Lab Thermometer; DeltaTrak, Pleasanton, CA) at d 0 and 7. Elevated rectal temperature, as a surrogate for elevated body temperature, was defined as the average rectal temperature $\geq 39.1^{\circ}\text{C}$ ($<39.1^{\circ}\text{C}$ = no elevated body temperature), given that 39.1°C is considered a threshold for elevated body temperature in dairy cattle (Berman et al., 1985; West, 2003). Blood samples were collected on d -11 ($n = 1,574$), d -4 ($n = 1,548$), and d 7 ($n = 1,516$), by coccygeal venipuncture into 10-mL blood collection tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ). After blood collections, tubes were placed on ice immediately, maintained at 4°C for 12 h, and centrifuged at $1,500 \times g$ for 15 min at room temperature for serum collection. Serum was stored at -20°C for subsequent P4 analysis. Serum concentrations of P4 were analyzed using the Coat-A-Count solid phase ^{125}I RIA kit (Diagnostic Products Inc., Los Angeles, CA) that had been previously validated in our laboratory (Santos and Vasconcelos, 2006). The intra-assay coefficient of variation was 5.6%, the inter-assay coefficient of variation was 10.2%, and the assay sensitivity was 0.01 ng/mL.

Receiver operating characteristic (ROC) curves were generated to determine the optimum concentration of P4 at $\text{PGF}_{2\alpha}$ that resulted in the highest sensitivity and specificity for prediction of P/AI at 60 d in dairy cows. Thus, there were 2 classes of P4 concentration, based on the ROC curves, as above or below the calculated threshold of 3.66 ng/mL.

Statistical Analysis

The experiment was analyzed as a completely randomized design. The binomial variables (elevated body temperature, class of circulating P4 concentration, ovulation to GnRH, estrus detection, ovulation to ECP,

double ovulation, P/AI on d 32 and 60, and pregnancy losses from 32 to 60 d) were analyzed using the GLIMMIX procedures of SAS (ver. 9.4, SAS Institute Inc., Cary, NC) with Satterthwaite approximation to determine the denominator degrees of freedom for tests of fixed effects, with farms as a random effect. Backward elimination was used for final model selection based on Wald statistics criterion when $P \leq 0.15$ for the following covariables (used as covariables if not the subject of the analysis): elevated body temperature, treatment, lactation number, DIM, BCS, and milk yield. Thus, independent statistical models were constructed to analyze each variable and the interaction between treatment and each variable (e.g., 8 different statistical models were used to construct Table 1).

The continuous discrete variables (i.e., lactation number, DIM, BCS, milk production, previous AI number, P4 concentrations at d -11, -4 and 7, and follicular diameter) were analyzed using the MIXED procedures of SAS. Backward elimination was also used for final model selection using the same potential covariables as used for binomial analyses.

The GLM procedure of SAS was used to determine whether each individual measurement influenced P/AI linearly, quadratically, or cubically. When significant, the logistical regression models were fit by the method of maximum likelihood using PROC LOGISTIC of SAS to determine the intercept and slope(s) values according to maximum likelihood estimates from each significant continuous order effect, and the P/AI was determined. The logistical regression models were designed using the intercept and slope values on the following equation: Probability = $(e^{\text{logistic equation}})/(1 + e^{\text{logistic equation}})$. Logistic curves were constructed using the minimum and maximum values detected for each individual measurement.

Covariables that were found to be not significant ($P > 0.15$) were removed from the statistical model. The results are expressed as least squares means $\pm$ standard errors of the mean (SEM) for continuous variables. In all analyses, differences were considered significant when $P \leq 0.05$, and differences between $P > 0.05$ and $P \leq 0.10$ were considered tendencies.

RESULTS

No differences were detected between GnRH and 2CIDR treatments for lactation number (2.45 ± 0.36 vs. 2.41 ± 0.36 ; $P = 0.46$), DIM (187.8 ± 13.5 vs. 187.8 ± 13.5 ; $P = 0.99$), BCS (2.92 ± 0.03 vs. 2.95 ± 0.03 ; $P = 0.26$), milk yield (31.5 ± 2.23 vs. 31.3 ± 2.23 kg/d; $P = 0.63$), presence of CL at start of protocol d -11 [67.3% (551/817) vs. 68.1% (560/821); $P = 0.74$], or

Table 1. Effects of different cow variables on pregnancy per AI (P/AI) using the d-60 pregnancy diagnosis (LSM; no./no. in parentheses)

Variable	Protocol ¹		P-value		
	GnRH	2CIDR	Treatment	Variable	Interaction
Cow temperature (°C)					
<39.1	34.0 (138/402)	33.6 (141/415)	0.91	<0.01	0.79
≥39.1	22.0 (81/361)	22.9 (81/348)			
BCS					
<2.75	25.2 (50/202)	22.6 (40/178)	0.44	0.09	0.91
≥2.75	28.6 (176/615)	31.0 (199/643)			
Parity order					
Primiparous	34.1 (74/224)	35.1 (79/233)	0.62	<0.01	0.89
Multiparous	24.9 (152/593)	26.5 (160/588)			
Milk yield (kg/d)					
<30	26.2 (107/406)	29.4 (120/407)	0.52	0.63	0.44
≥30	29.1 (119/411)	28.7 (119/414)			
Corpus luteum (CL) presence on d -11					
No CL	25.7 (68/266)	24.7 (64/261)	0.75	0.05	0.46
CL	28.7 (158/551)	31.2 (175/560)			
CL presence on d -4					
No CL	18.2 (37/206)	20.6 (71/345)	0.18	<0.01	0.74
CL	31.8 (188/590)	35.9 (163/455)			
Progesterone on d -11 (ng/mL)					
<1.0	24.3 (72/297)	28.2 (83/294)	0.52	0.16	0.30
≥1.0	30.0 (148/492)	29.1 (143/491)			
Estrus expression					
No	9.2 (8/90)	5.1 (4/82)	0.69	<0.01	0.46
Yes	30.9 (216/701)	32.1 (228/712)			

¹Protocols: GnRH = controlled internal drug-release insert (CIDR) + 2 mg of estradiol benzoate + 100 µg of GnRH on d -11, PGF_{2α} on d -4, CIDR withdrawal + 1.0 mg of estradiol cypionate (ECP) + PGF_{2α} on d -2, and timed AI on d 0; 2CIDR = 2 CIDR inserts + 2 mg of estradiol benzoate on d -11, PGF_{2α} on d -4, CIDR withdrawal + 1.0 mg of ECP + PGF_{2α} on d -2, and timed AI on d 0.

circulating P4 concentration at d -11 (2.92 ± 0.13 vs. 2.97 ± 0.13 ng/mL; $P = 0.68$).

Overall Effects on P/AI

The effects of treatment on P/AI were stratified by elevated body temperature, BCS, parity, milk yield, CL at d -11 and -4, circulating P4 concentration on d -11, and expression of estrus (Table 1). Treatment did not affect P/AI, using either d-32 or d-60 pregnancy diagnosis, and there were no interactions between treatment and these various measures on P/AI. For example, the 2 treatment groups were not different for P/AI ($P > 0.10$) regardless of elevated body temperature, BCS, parity, milk yield, expression of estrus, presence or absence of a CL on d -11 or -4, or circulating P4 concentrations. There were effects or tendencies for effects of some of these variables to lower P/AI, such as cows with elevated body temperature ($P < 0.01$) or lower BCS (<2.75 ; $P = 0.09$) in multiparous compared with primiparous cows ($P < 0.01$), in cows without versus with a CL on d -11 ($P = 0.05$) and d -4 ($P < 0.01$), and in cows with no expression of estrus ($P < 0.01$). In contrast, circulating P4 concentration at d -11 ($P = 0.16$) and milk yield ($P = 0.63$) did not affect P/AI.

Effects of Treatment and Elevated Body Temperature on Reproductive Measures

Elevated body temperature was an important factor for altered reproductive physiology and decreased reproductive success in both treatments (Table 1). Table 2 shows the effect of elevated body temperature on various measures of reproductive physiology in cows treated with the 2 synchronization strategies. A greater proportion of ($P < 0.01$) cows in the GnRH treatment ovulated a follicle and had a new CL at PGF_{2α} compared with the 2CIDR treatment; however, elevated body temperature did not affect ovulation of a follicle on d -11 ($P = 0.91$). A greater ($P < 0.01$) proportion of cows in the GnRH treatment than in the 2CIDR treatment had CL at PGF_{2α}. There also was a greater ($P = 0.02$) proportion (5.4 percentage points or 7.9%) of cows with CL in cows without elevated body temperature (68.3%; 558/817) than in cows with elevated body temperature (62.9%; 446/709). Circulating P4 concentration was greater ($P = 0.05$) at the time of PGF_{2α} treatment (d -4) for cows in the 2CIDR group than in the GnRH group. Treatment did not alter expression of estrus at the end of the protocol ($P = 0.55$), although cows with elevated body temperature had reduced ($P < 0.01$; 4.7 percentage points or

Table 2. Effects of average body temperature of cows and treatment protocol¹ on various measures of reproductive physiology (LSM; no./no. in parentheses)

Variable and protocol	Cow temperature (°C)		P-value		
	<39.1	≥39.1	Temperature	Protocol	Interaction
P4 d -11 (ng/mL) ²					
GnRH	2.93 ± 0.15	2.81 ± 0.16	0.34	0.57	0.91
2CIDR	3.02 ± 0.15	2.87 ± 0.16			
Ovulation of d -11 follicle ^{2,3}					
GnRH	42.6 (175/402)	40.7 (153/361)	0.91	<0.01	0.42
2CIDR	5.3 (26/415)	6.8 (29/348)			
Corpus luteum at PGF _{2α} ²					
GnRH	78.1 (314/402)	70.9 (256/361)	0.02	<0.01	0.53
2CIDR	58.8 (244/415)	54.6 (190/348)			
Progesterone (P4) at d -4 (ng/mL) ²					
GnRH	4.12 ± 0.14	3.84 ± 0.14	0.09	0.05	0.67
2CIDR	4.34 ± 0.13	4.17 ± 0.14			
Estrus ²					
GnRH	89.9 (365/402)	85.6 (312/361)	<0.01	0.55	0.84
2CIDR	91.1 (382/415)	86.2 (303/348)			
Follicle diameter at d 0 (mm) ⁴					
GnRH	16.1 ± 0.20	15.1 ± 0.22	<0.01	<0.01	0.02
2CIDR	15.3 ± 0.21	15.0 ± 0.22			
P4 at d 7 (ng/mL) ⁴					
GnRH	3.65 ± 0.15	3.64 ± 0.16	0.69	0.03	0.61
2CIDR	3.43 ± 0.15	3.51 ± 0.16			
Double ovulation ⁴					
GnRH	21.7 (70/367)	25.8 (77/318)	0.08	0.40	0.98
2CIDR	19.8 (65/367)	23.9 (67/306)			

¹Protocols: GnRH = controlled internal drug-release insert (CIDR) + 2 mg of estradiol benzoate + 100 µg of GnRH on d -11, PGF_{2α} on d -4, CIDR withdrawal + 1.0 mg of estradiol cypionate (ECP) + PGF_{2α} on d -2, and timed AI on d 0; 2CIDR = 2 CIDR inserts + 2 mg of estradiol benzoate on d -11, PGF_{2α} on d -4, CIDR withdrawal + 1.0 mg of ECP + PGF_{2α} on d -2, and timed AI on d 0.

²All inseminated cows.

³Percentage of cows with a new corpus luteum on d -4 that had a follicle >8 mm on d -11.

⁴Includes only cows that ovulated to ECP (visible CL on d 7).

5.1%) expression of estrus compared with cows without elevated body temperature [86.7% (615/709) vs. 91.4% (747/817)]. The GnRH protocol resulted in larger follicles near TAI ($P < 0.01$) in cows without elevated body temperature. However, in cows with elevated body temperature, ovulatory follicle size did not differ with treatment protocol. Treatment with GnRH produced greater ($P = 0.03$) circulating P4 concentration (3.54 ± 0.15 ng/mL) on d 7 after TAI compared with 2CIDR protocol (3.37 ± 0.15 ng/mL). Cows without elevated body temperature tended ($P = 0.08$) to have a lower incidence of double ovulation [18.7% (135/734) vs. 23.8% (144/624)].

Ovulation near TAI tended to be greater ($P = 0.09$) in cows without elevated body temperature, but there was no effect of treatment protocol on ovulation near TAI (Table 3). Treatment did not affect P/AI at the 32-d or 60-d pregnancy diagnoses or pregnancy loss between 32 and 60 d of pregnancy. Cows without elevated body temperature during the protocol had greater ($P < 0.01$; increased 11.3 percentage points or 49.6%) P/AI at 60 d [elevated body temperature = 22.8% (162/709), no elevated body temperature 34.1% (279/817); $P < 0.01$]

and tended to have lower pregnancy loss between d 32 and 60 [elevated body temperature = 20.2% (41/203), no elevated body temperature 14.4% (47/326); $P = 0.09$] than cows with elevated body temperature.

Effects of Ovulation at Beginning of Protocol on P/AI

Ovulation to GnRH at the initiation of the protocol was 43.0% (328/763). Therefore, we performed another analysis evaluating cows that did or did not ovulate to GnRH (d -11) and further subdividing cows by elevated body temperature (Table 4). In cows without elevated body temperature, cows that ovulated to GnRH (**GnRH-OV**) tended to have greater ovulation near TAI ($P = 0.09$) compared with cows that did not ovulate to GnRH (**GnRH-NOV**) and cows treated with 2CIDR ($P = 0.10$). Further, in cows without elevated body temperature, the P/AI at 60 d was greater for GnRH-OV ($P = 0.01$) than for GnRH-NOV and tended to be greater ($P = 0.09$) than the 2CIDR treatment. There was no effect on pregnancy loss between the treatments ($P = 0.40$). In cows with elevated body temperature, treatment did not affect ovulation to

Table 3. Effects of treatments¹ and cow temperature on ovulation to estradiol cypionate, pregnancy per AI (P/AI) at the 32-d or 60-d pregnancy diagnosis, and pregnancy loss from 32 to 60 d of gestation (LSM; no./no. in parentheses)

Variable and protocol	Cow temperature (°C)		P-value		
	<39.1	≥39.1	Temperature	Protocol	Interaction
Ovulation near ECP ²					
GnRH	88.1 (355/402)	83.3 (304/361)	0.09	0.93	0.34
2CIDR	86.2 (359/415)	84.8 (298/348)			
P/AI at 32 d for all cows ³					
GnRH	39.7 (162/402)	26.2 (98/361)	<0.01	0.64	0.42
2CIDR	38.9 (164/415)	29.3 (105/348)			
P/AI at 60 d for all cows ³					
GnRH	34.0 (138/402)	22.0 (81/361)	<0.01	0.91	0.79
2CIDR	33.6 (141/415)	22.9 (81/348)			
P/AI at 60 d for synchronized cows ⁴					
GnRH	38.9 (138/355)	26.6 (81/304)	<0.01	0.86	0.98
2CIDR	39.3 (141/359)	27.2 (81/298)			
Pregnancy loss (32–60 d) ⁴					
GnRH	14.8 (24/162)	17.4 (17/98)	0.09	0.48	0.34
2CIDR	14.0 (23/164)	22.9 (24/105)			

¹Protocols: GnRH = controlled internal drug-release insert (CIDR) + 2 mg of estradiol benzoate + 100 µg of GnRH on d –11, PGF_{2α} on d –4, CIDR withdrawal + 1.0 mg of estradiol cypionate (ECP) + PGF_{2α} on d –2, and timed AI on d 0; 2CIDR = 2 CIDR inserts + 2 mg of estradiol benzoate on d –11, PGF_{2α} on d –4, CIDR withdrawal + 1.0 mg of ECP + PGF_{2α} on d –2, and timed AI on d 0.

²Based on presence of CL on d 7.

³Includes all inseminated cows.

⁴Includes only cows that ovulated to ECP (visible corpus luteum on d 7).

ECP ($P = 0.65$) or P/AI at 60 d ($P = 0.96$); however, pregnancy loss was greater in 2CIDR ($P = 0.05$) and in cows that ovulated to GnRH ($P = 0.04$) compared with cows that did not ovulate to GnRH.

Effects of P4 at PGF_{2α} on P/AI

To determine the circulating P4 concentration on d –4 with the greatest accuracy for predicting pregnancy

Table 4. Ovulation near timed AI and pregnancy per AI (P/AI) at 60 d by body temperature of cows comparing treatment protocols¹ with the GnRH protocol divided by whether cows ovulated to GnRH at the start of the protocol and cow temperature (LSM; no./no. in parentheses)

Variable and temperature	Protocol			P-value
	No ovulation to GnRH	Ovulation to GnRH	2CIDR	
Ovulation to ECP				
<39.1°C	85.9 (195/227) ^y	91.4 (160/175) ^x	86.5 (359/415) ^y	0.19
≥39.1°C	80.5 (173/208)	82.8 (131/153)	83.3 (298/348)	0.65
Combined	84.4 (368/435) ^y	88.5 (291/328) ^x	85.8 (657/763) ^{xy}	0.26
P/AI at 60 d ²				
<39.1°C	28.9 (66/227) ^b	41.0 (72/175) ^{a,x}	33.8 (141/415) ^y	0.04
≥39.1°C	22.2 (47/208)	21.8 (34/153)	22.9 (81/348)	0.96
Combined	25.7 (117/456) ^y	31.7 (108/340) ^x	29.1 (239/821) ^{xy}	0.17
P/AI at 60 d ³				
<39.1°C	33.6 (66/195) ^b	45.0 (72/160) ^a	39.2 (141/359) ^{ab}	0.09
≥39.1°C	27.2 (47/173)	26.0 (34/131)	27.2 (81/298)	0.96
Combined	30.8 (113/368)	36.5 (106/291)	33.9 (222/657)	0.30
Pregnancy loss (32–60 d) ³				
<39.1°C	18.5 (15/81)	11.1 (9/81)	14.0 (23/164)	0.40
≥39.1°C	9.6 (5/52) ^a	26.1 (12/46) ^b	22.9 (24/105) ^b	0.08
Combined	14.6	17.5	17.0	0.78

^{a,b}Means in the same row without similar superscript letters differ at $P < 0.05$.

^{x,y}Means in the same row without similar superscript letters differ at $P < 0.10$.

¹Protocols: GnRH = controlled internal drug-release insert (CIDR) + 2 mg of estradiol benzoate + 100 µg of GnRH on d –11, PGF_{2α} on d –4, CIDR withdrawal + 1.0 mg of estradiol cypionate (ECP) + PGF_{2α} on d –2, and timed AI on d 0; 2CIDR = 2 CIDR inserts + 2 mg of estradiol benzoate on d –11, PGF_{2α} on d –4, CIDR withdrawal + 1.0 mg of ECP + PGF_{2α} on d –2, and timed AI on d 0.

²Includes all inseminated cows.

³Includes only cows that ovulated to ECP (visible corpus luteum on d 7).

Table 5. Effect of treatment protocol¹ by progesterone (P4) concentration at PGF_{2α} on pregnancy per AI (P/AI) at 60 d (LSM; no./no. in parentheses)

Variable and protocol	P4 at PGF _{2α}		P4	P-value	
	<3.66 ng/mL	≥3.66 ng/mL		Variable	Interaction
Cow temperature <39.1°C					
GnRH	29.8 (56/186)	38.7 (77/200)	<0.01	0.93	0.16
2CIDR	24.9 (51/202)	43.0 (89/207)			
Cow temperature ≥39.1°C					
GnRH	17.8 (36/196)	26.5 (43/159)	<0.01	0.98	0.97
2CIDR	17.9 (32/174)	26.3 (44/164)			
Combined					
GnRH	23.6 (94/400)	33.5 (123/368)	<0.01	0.79	0.48
2CIDR	22.6 (89/396)	35.7 (137/384)			
Estrus					
No	5.6 (5/92)	9.2 (7/77)	<0.01	0.03	0.25
Yes	25.4 (176/694)	37.4 (252/674)			
Parity order					
Primiparous	28.8 (53/195)	41.7 (91/227)	<0.01	<0.01	0.66
Multiparous	20.8 (130/601)	31.4 (169/525)			

¹Protocols: GnRH = controlled internal drug-release insert (CIDR) + 2 mg of estradiol benzoate + 100 µg of GnRH on d -11, PGF_{2α} on d -4, CIDR withdrawal + 1.0 mg of estradiol cypionate (ECP) + PGF_{2α} on d -2, and timed AI on d 0; 2CIDR = 2 CIDR inserts + 2 mg of estradiol benzoate on d -11, PGF_{2α} on d -4, CIDR withdrawal + 1.0 mg of ECP + PGF_{2α} on d -2, and timed AI on d 0.

versus nonpregnancy on d 60, ROC curves were generated. The circulating P4 concentration that resulted in the greatest combined sensitivity (58.5%) and specificity (55.8%) was ≥3.66 ng/mL ($P < 0.01$). This indicates that 58.5% of the pregnant cows from the 60-d pregnancy diagnosis had P4 ≥3.66 ng/mL, whereas 55.8% of the nonpregnant cows had circulating P4 concentration <3.66 ng/mL. Cows with circulating P4 concentration ≥3.66 ng/mL had greater P/AI at 60 d ($P < 0.01$), independent of elevated body temperature (Table 5). Cows that did not show estrus had reduced P/AI compared with cows that showed estrus ($P < 0.03$), independent of circulating P4 concentration at PGF_{2α} ($P = 0.25$). We detected no interaction ($P = 0.66$) between parity and circulating P4 concentration at PGF_{2α} on P/AI but both P4 ($P < 0.01$) and parity ($P < 0.01$) had independent effects on P/AI.

The 2 treatment groups were combined to analyze the effects of elevated body temperature with circulating P4 at time of PGF_{2α} on ovulation to the protocol and P/AI (Table 6). Ovulation to ECP near the time of AI was only different when comparing cows with greater P4 concentrations without and with elevated body temperature (88.3 vs. 83.1%; $P = 0.04$). For P/AI at the 32-d pregnancy diagnosis, there were effects of elevated body temperature in cows with either greater or lower concentrations of P4 at PGF_{2α} as well as effects of P4 in cows with or without elevated body temperature. Similarly, for P/AI at the 60 d pregnancy diagnosis, there were effects of elevated body temperature and circulating P4 in all combinations ($P < 0.01$). For pregnancy loss, the only effect was an increase in pregnancy loss due to elevated body temperature in

cows with greater circulating P4 at PGF_{2α} (14.4 vs 23.7%; $P = 0.04$).

Logistic Regression of P4 on P/AI and Pregnancy Loss

Combining all data from both treatment groups using logistic regression demonstrated an overall effect ($P < 0.01$) of circulating P4 concentration at PGF_{2α} on P/AI at the 32- and 60-d pregnancy diagnoses, with increases in circulating P4 associated with increases in P/AI. Surprisingly, pregnancy loss was also increased with increasing P4 at the time of PGF_{2α} ($P = 0.05$) when all data were analyzed. Analysis of the 2 groups separately (Figure 2) demonstrated increased P/AI with increasing circulating P4 in both groups at the 32-d pregnancy diagnosis ($P < 0.01$ for 2CIDR and GnRH) and at the 60-d diagnosis (2CIDR, $P < 0.01$; GnRH, $P = 0.06$). The effect of increasing P4 on pregnancy loss (Figure 2C) was only found in the GnRH-treated cows ($P = 0.03$), not in the 2CIDR cows ($P = 0.58$). To further understand these effects, the cows were divided into 3 groups (Figure 3): 2CIDR, GnRH-NOV, and GnRH-OV. At the 32-d pregnancy diagnosis (Figure 3A), increasing circulating P4 was associated with increasing P/AI in 2CIDR ($P < 0.01$) and GnRH-NOV ($P = 0.01$), and tended to be associated ($P = 0.09$) for GnRH-OV cows. At 60 d (Figure 3B), the effect of increasing P4 on P/AI was clear in 2CIDR cows ($P < 0.01$) and less apparent in GnRH-NOV cows ($P = 0.11$), and there was no longer an association ($P = 0.62$) in GnRH-OV cows. For pregnancy loss (Figure 3C), there was no effect of increasing P4 on pregnancy

loss in 2CIDR ($P = 0.58$) or GnRH-NOV ($P = 0.22$) but increasing pregnancy loss was observed with increasing P4 in GnRH-OV cows ($P = 0.05$).

Classification of Cows by Presence of CL at Beginning or End of Protocol

Cows were classified by the presence or absence of CL on d -11 and -4 and by treatment protocol (Table 7). Cows without CL on both days had reduced ovulation to ECP ($P < 0.01$) in both protocols. For both protocols, the circulating P4 concentration at PGF_{2α} (d -4) was greater in cows with CL at both evaluations. Cows without CL at d -11 and with CL at d -4 had greater circulating P4 concentration at PGF_{2α} compared with cows without CL at both evaluations and cows with CL on d -11 and without CL on d -4. In all classes, the 2CIDR treatment had greater circulating P4 concentration at PGF_{2α} compared with the GnRH protocol. There was an effect of presence or absence of a CL at the 2 evaluations on P/AI at the 32-d pregnancy diagnosis for both treatments. Thus, cows without a CL on d -11 but with a CL on d -4 and cows with CL at both evaluations had greater P/AI than the other 2 classes. The 2CIDR treatment tended to have greater P/AI than GnRH in cows without CL at d -11 and with CL at d -4 and cows with CL at d -11 and without CL at d -4. Differences were less pronounced at the 60-d pregnancy diagnosis. Treatment with GnRH increased ($P < 0.01$) the percentage of cows in the 2 classes that had CL at d -4 compared with the 2CIDR treatment.

Effects of Ovulatory Follicle Diameter and P4 at d 7 on P/AI

We detected an effect of ovulatory follicle diameter on P/AI, although it was only observed in the GnRH-OV cows (Figure 4). At the 32-d pregnancy diagnosis (Figure 4A), there was no effect of follicle diameter on P/AI in 2CIDR ($P = 0.73$) or GnRH-NOV ($P = 0.76$) cows but a clear increase in P/AI with increasing ovulatory follicle diameter in GnRH-OV cows ($P < 0.01$). Similarly, at the 60-d diagnosis (Figure 3B), there was no effect of follicle diameter on P/AI in 2CIDR ($P = 0.70$) or GnRH-NOV ($P = 0.65$) cows, but a clear effect of follicle diameter on P/AI in GnRH-OV cows ($P < 0.01$). Pregnancy loss tended to decrease with increasing follicle diameter in GnRH-OV cows ($P = 0.10$) but not in 2CIDR ($P = 0.86$) or GnRH-NOV ($P = 0.68$) cows. There was no effect ($P = 0.78$) of P4 concentrations on d 7 after AI on P/AI when only cows that ovulated near TAI were included in the analyses.

DISCUSSION

This study compared 2 strategies to increase circulating P4 concentrations during growth of the preovulatory follicular wave to improve fertility of TAI protocols that used E2 and P4 to synchronize the estrous cycle of lactating dairy cows. The first strategy combined GnRH treatment with E2 at the beginning of the protocol (Pereira et al., 2015). The second strategy used 2 CIDR to increase P4 concentrations. Direct comparison of these 2 strategies in the present study did not

Table 6. Effect of body temperature of cow by progesterone (P4) concentration at PGF_{2α} on pregnancy per AI (P/AI) at 60 d (LSM; no./no. in parentheses)

Trait and temperature	P4 at PGF _{2α}		P-value
	<3.66 ng/mL	≥3.66 ng/mL	
Ovulation to estradiol cypionate			
<39.1°C	85.6 (313/369)	88.3 (278/324)	0.15
≥39.1°C	84.9 (332/388)	83.1 (362/407)	0.70
P-value	0.79	0.04	
P/AI at 32 d ¹			
<39.1°C	36.3 (81/313)	53.5 (114/278)	<0.01
≥39.1°C	25.8 (122/332)	41.1 (194/362)	<0.01
P-value	<0.01	<0.01	
P/AI at 60 d ¹			
<39.1°C	31.8 (68/313)	46.0 (87/278)	<0.01
≥39.1°C	21.7 (107/332)	31.4 (166/362)	<0.01
P-value	<0.01	<0.01	
Pregnancy loss ¹			
<39.1°C	12.3 (13/81)	14.4 (27/114)	0.59
≥39.1°C	16.1 (15/122)	23.7 (28/194)	0.20
P-value	0.45	0.04	

¹Includes only cows that ovulated to estradiol cypionate (visible corpus luteum on d 7).

demonstrate any differences in P/AI at the 32- or 60-d pregnancy diagnoses or in pregnancy loss. Nevertheless, collection of specific physiological measurements in a large number of lactating cows during different seasons of the year allowed substantial insight into the physiological pathways that underlie fertility outcomes with these protocols. In a previous study (Pereira et al., 2015), the first strategy (GnRH addition) was found to increase P/AI compared with the control protocol without GnRH (GnRH + EB = 50.9% vs. EB = 41.0%). The second strategy (2 intravaginal P4 implants) has also shown an improvement in fertility in conjunction with a 5-d Cosynch protocol (Bisinotto et

Figure 2. Logistic regression analysis of relationship between circulating progesterone (P4) concentration at PGF_{2α} with pregnancies per AI (P/AI) at (A) 32 d or (B) 60 d, or (C) for pregnancy loss for cows in the 2 treatment groups (2CIDR and GnRH; Figure 1). The lines represent the calculated logistic regression with symbols representing each cow that was part of the analysis.

Figure 3. Logistic regression analysis of relationship between circulating progesterone (P4) concentration at PGF_{2α} with pregnancies per AI (P/AI) at (A) 32 d or (B) 60 d, or (C) for pregnancy loss for cows in 2CIDR (Figure 1) or cows in GnRH group that ovulated (GnRH-OV) or did not ovulate (GnRH-NOV) to GnRH treatment. The lines represent the calculated logistic regression with symbols representing each cow that was part of the analysis.

al., 2013) and during an E2/P4 protocol (Pereira et al., 2017). Thus, both of these strategies enhanced fertility in previous studies but resulted in similar fertility in the present study.

Although there was no overall difference between protocols in P/AI, both protocols caused >85% of cows to ovulate at the end of the protocol and >35% P/AI. Both protocols had similar P/AI even when cows were divided by elevated body temperature, BCS, parity, milk yield, presence of a CL on d –11 and –4, circulating P4 concentration on d –11 and –4, or expression

of estrus. Consistent with our expectations, both protocols resulted in high average circulating P4 concentration at PGF_{2α} d -4 (>3.95 ng/mL), with the GnRH protocol having 48% (369/768) and 2CIDR having 49% (384/780) of cows with circulating P4 ≥3.66 ng/mL. In contrast, the GnRH protocol produced a greater proportion of cows with CL at PGF_{2α}, whereas the 2CIDR treatment produced a greater elevation in circulating P4 concentration at the time of PGF_{2α} treatment.

Elevated body temperature was likely to have been caused by heat stress throughout this research, although disease or other cow circumstances can elevate body temperature. Basing the determination of elevated body temperature (heat stress) on measurements on multiple occasions in each cow should reduce errors due to technical imprecision in measurements of individual temperature but leave the possibility that in some cows other physiological or pathological conditions, rather than purely environmental conditions, could have led to an elevation in body temperature. This study found that certain physiological measurements were associated with greater fertility and most of these measures were reduced in cows with elevated body temperature: percentage of cows with CL at PGF_{2α} decreased 7.9%, ovulatory follicle diameter decreased 0.51 mm, ex-

pression of estrus decreased 5.1%, and ovulation near TAI decreased 2.8%. These results are similar to our previous studies (Pereira et al., 2013, 2014, 2015). For example, elevated body temperature reduced follicular growth (Wilson et al., 1998), altered production of steroids by growing follicles (Wolfenson et al., 1995, 1997; Wilson et al., 1998), reduced expression of estrus (Younas et al., 1993), and reduced ovulation at the end of the protocol (Pereira et al., 2013). Each of these physiological changes could account for some of the decrease in fertility, although many studies have also demonstrated effects of elevated body temperature on oocyte health and fertilization (Sartori et al., 2002; Payton et al., 2004; Hackbart et al., 2010; Ferreira et al., 2011; Hooper et al., 2015), early embryonic development (Edwards and Hansen, 1997; Sakatani et al., 2012; Hansen, 2013), and maintenance of pregnancy (Vasconcelos et al., 2011b). This study directly used the temperature of each individual cow near the time of AI, based on the average of 2 independent observations, rather than only air temperature or temperature-humidity index, because cow temperature has been the most sensitive measure of negative effect of elevated body temperature on P/AI in previous studies (Vasconcelos et al., 2011b; Pereira et al., 2015). Along with decreased P/

Table 7. Ovulation to estradiol cypionate (ECP), progesterone (P4) concentration, and pregnancy per AI (P/AI) at 32 and 60 d by presence or absence of corpus luteum (CL) at d -11 and d -4 by treatment¹ (LSM; no./no. in parentheses)

Variable and protocol	CL at d -11/d -4				P-value
	No/No	No/Yes	Yes/Yes	Yes/No	
Ovulation to ECP ²					
GnRH	73.8 (54/73) ^a	83.3 (147/176) ^b	89.4 (353/394) ^c	87.2 (105/120) ^{bc}	<0.01
2CIDR	76.1 (116/152) ^a	85.3 (79/89) ^{bc}	85.3 (296/346) ^b	93.6 (166/177) ^c	<0.01
P-value	0.64	0.23	0.52	0.12	
P4 at d -4 ³					
GnRH	1.40 ± 0.24 ^a	4.20 ± 0.15 ^b	5.20 ± 0.10 ^c	1.24 ± 0.18 ^a	<0.01
2CIDR	2.15 ± 0.16 ^a	5.10 ± 0.22 ^b	5.99 ± 0.11 ^c	2.22 ± 0.15 ^a	<0.01
P-value	<0.01	<0.01	<0.01	<0.01	
P4 ≥3.66 ng/mL at d -4 ^{3,4} (%)					
GnRH	2.7 (2/74)	57.0 (102/179)	68.1 (265/389)	0.0 (0/126)	
2CIDR	7.0 (11/157)	76.9 (70/91)	80.7 (284/352)	10.6 (19/180)	
P-value	0.19	<0.01	<0.01	<0.01	
P/AI at 32 d ³					
GnRH	23.1 (18/77) ^{a*}	34.6 (64/183) ^{b*}	38.9 (160/407) ^b	20.2 (26/129) ^a	<0.01
2CIDR	21.6 (35/160) ^a	44.6 (42/94) ^b	41.9 (152/361) ^b	28.9 (54/185) ^a	<0.01
P-value	0.81	0.09	0.38	0.10	
P/AI at 60 d ³					
GnRH	18.1 (14/77) ^{a*}	29.4 (54/183) ^{b*}	32.7 (134/407) ^b	18.1 (23/129) ^a	<0.01
2CIDR	16.8 (27/160) ^a	38.4 (36/94) ^b	35.2 (127/361) ^b	23.6 (44/185) ^a	<0.01
P-value	0.83	0.11	0.28	0.28	

^{a-c}Means in the same row with different superscript letters differ ($P < 0.05$) except if $*P = 0.07$ (No/No vs. No/Yes).

¹Protocols: GnRH = controlled internal drug-release insert (CIDR) + 2 mg of estradiol benzoate + 100 µg of GnRH on d -11, PGF_{2α} on d -4, CIDR withdrawal + 1.0 mg of estradiol cypionate (ECP) + PGF_{2α} on d -2, and timed AI on d 0; 2CIDR = 2 CIDR inserts + 2 mg of estradiol benzoate on d -11, PGF_{2α} on d -4, CIDR withdrawal + 1.0 mg of ECP + PGF_{2α} on d -2, and timed AI on d 0.

²Based on presence of CL on d 7.

³All inseminated cows.

⁴Distribution of cows with P4 ≥3.66 ng/mL at d -4.

AI in cows with elevated body temperature, pregnancy loss between 32 and 60 d tended to be greater in cows with elevated body temperature in this study. Increased pregnancy loss has been previously reported (Pereira et al., 2013), in which cows with elevated body temperature had greater pregnancy losses [23.8% (17/82)] than cows without elevated body temperature [9.4% (20/241)]. Demetrio et al. (2007) also observed negative effects of elevated body temperature on pregnancy maintenance.

The use of ROC curves indicated that P4 of 3.66 ng/mL at the time of PGF_{2α} was a threshold value

that may be important in regulating fertility in lactating dairy cows, at least for cows in this study. Direct analysis of fertility results was consistent with this value as cows with circulating P4 concentration ≥ 3.66 ng/mL had a 13.2-percentage-point greater P/AI (40.8 vs. 27.6) in cows without elevated body temperature and a 10.6-percentage-point greater P/AI in cows with elevated body temperature (33.6 vs. 23.0). Thus, there were about 50% more pregnancies per AI in cows with high P4 ($13.2/27.6 = 47.8\%$ or $11.6/23.0 = 50.4\%$) compared with cows with P4 < 3.66 ng/mL at PGF_{2α}, independent of elevated body temperature or treatment protocol. This is the first study that has attempted to define the critical circulating level of P4 associated with the TAI during an E2-/P4-based synchronization program in dairy cows. A previous study using an Ovsynch-type of TAI protocol calculated an optimal value of 3.78 ng/mL, based on ROC curves (Ribeiro et al., 2012), which is very similar to the value that we obtained. Inclusion of a GnRH treatment at the start of the E2/P4 protocol in the GnRH treatment group increased the percentage of cows with P4 that exceeded the critical fertility value due to provision of an accessory CL during the protocol. Previous studies reported that E2/P4 protocols have reduced percentage of cows with CL at time of PGF_{2α} compared with GnRH-based protocols [5-d Cosynch = 73.6% (597) vs. E2/P4 = 44.3% (593); Pereira et al., 2013] and reduced circulating P4 concentration at PGF_{2α} (E2/P4: 2.29 ± 0.15 vs. GnRH: 2.89 ± 0.15 ng/mL; Vasconcelos et al., 2011a). Conversely, in the 2CIDR protocol there were more cows above this critical value for circulating P4, due to provision of exogenous P4, in spite of fewer cows with a CL at PGF_{2α}. A previous study reported that treatment of cows without a CL at the initiation of a GnRH-based TAI protocol with 2 CIDR increased plasma P4 to 2.65 ng/mL and restored fertility to a value that was similar to that of cows with a CL (no-CL control cows: 28.6%, no-CL cows with 2CIDR: 43.7%, cows with CL: 47.3%; Bisinotto et al., 2013). All of these results support the idea that elevations in P4 during preovulatory follicle development increase fertility, with a value around 4 ng/mL as a threshold value, based on ROC curves.

The effects of increased circulating P4 before AI have been linked to a reduced size of the preovulatory follicle, improved condition of the ovulated oocyte, and subsequent improvements in embryo quality during the first week of embryo development (Wiltbank et al., 2014). For example, Cerri et al. (2011b) collected embryos 6 d after AI from cows submitted to protocols with low or high P4, and reported no effect of P4 on fertilization rate but a tendency for greater embryo quality in cows receiving protocols with elevated P4 during preovulatory follicle development. Similarly,

Figure 4. Logistic regression analysis of relationship between ovulatory follicle diameter with pregnancies per AI (P/AI) at (A) 32 d or (B) 60 d, or (C) for pregnancy loss for cows in 2CIDR (Figure 1) or cows in GnRH group that ovulated (GnRH-OV) or did not ovulate (GnRH-NOV) to GnRH treatment. The lines represent the calculated logistic regression with symbols representing each cow that was part of the analysis.

Rivera et al. (2011) reported an increased proportion of transferable embryos (78.6 vs. 55.9%) when donors that were superovulated during the first follicular wave (low P4) received 2 P4 implants during the protocol. However, in our results, the only group that demonstrated an effect of follicle diameter on fertility was the group that received GnRH and ovulated to the GnRH treatment and, paradoxically, P/AI increased as follicle diameter increased in these cows. The increase in P/AI with increasing follicle diameter was clear at both the 32-d ($P < 0.01$) and 60-d ($P < 0.01$) pregnancy diagnoses, and pregnancy loss tended ($P = 0.10$) to decrease with increasing follicle diameter in cows that ovulated to GnRH but this relationship was not observed in cows that received 2CIDR or that received GnRH but did not ovulate ($P > 0.65$ for all logistic regressions). In a previous study, we found that initiation of a new follicular wave during the middle of a Ovsynch protocol by aspiration of the dominant follicle on d 3 or 4 after GnRH treatment resulted in ovulation of a much smaller follicle and greatly reduced fertility, perhaps due to reduced estradiol concentrations near AI or decreased circulating P4 after AI caused by the development of a smaller CL (Vasconcelos et al., 2001). Cows that ovulate to the GnRH will initiate a new follicular wave during the next day (Pursley et al., 1995), and the ovulatory follicle would be expected to have a large diameter by the end of the protocol, particularly with the 11-d protocol that was used in the present study. Thus, ovulation of a smaller follicle in cows that ovulated to GnRH is likely to be related to turnover of the dominant follicle before the end of the protocol, perhaps resulting in reduced fertility for the same reasons that were present in the previous manipulative study (Vasconcelos et al., 2001). It seems that extremely elevated circulating P4 would make it more likely for turnover of the follicular wave during the protocol, which may help explain the puzzling observation that increasing circulating P4 was associated with increased pregnancy loss but only in cows that ovulated to the GnRH treatment. Thus, initiation of a new follicular wave near the end of the protocol, as likely demonstrated by cows that ovulate small follicles in spite of ovulation to GnRH at the start of the protocol, may result in reduced expression of estrus that could reduce fertility (Pereira et al., 2016) or production of a smaller CL, resulting in reduced circulating P4 that may reduce fertility (Wiltbank et al., 2014). A shorter protocol may be more optimal when GnRH is used at the beginning of the protocol because loss of follicular dominance can occur in some animals before induction of ovulation when using a longer protocol.

In addition, previous studies have indicated that cows with low circulating P4 concentration during develop-

ment of the ovulatory follicle are more likely to prematurely develop pathways leading to $\text{PGF}_{2\alpha}$ secretion by the endometrium, causing short luteal lifespan (Cerri et al., 2011a,b; Bisinotto et al., 2013). For example, Bisinotto et al., (2013) observed that the proportion of cows that were re-inseminated from d 5 to 17 after AI was greater ($P = 0.02$) for cows without CL at the beginning of the protocol than for cows with a CL or cows without a CL that were treated with 2CIDR during the TAI protocol. Nevertheless, in the study of Pereira et al. (2017), the authors did not observe an increase in short luteal phases and did not observe an effect of 2CIDR treatment on pregnancy in cows submitted to embryo transfer, suggesting that short luteal phases are unlikely to be an explanation for the greater fertility with increased P4 supplementation in cows that are already receiving a protocol that contains an CIDR. Sá Filho et al. (2009) reported that treatments with P4 (3 or 6 d) before induction of ovulation in anestrous Nelore (*Bos indicus*) cows increased the percentage of animals with a normal luteal lifespan. Thus, in protocols that use P4 implants during the protocol and end the protocol with ECP treatment, it seems unlikely, although not tested in this study, that short luteal phases are the primary mechanism underlying improvements in fertility with increased preovulatory P4. It seems more likely that changes in the ovulated oocyte, reproductive tract environment, or a combination of both processes underlie the improved fertility.

The evaluation of physiologic measures in over 1,500 cows allowed the interesting observations that the 2 treatments seem to have differing advantages and disadvantages. The 2CIDR treatment consistently produced greater circulating P4 concentrations. For example, in cows with no CL at the time of $\text{PGF}_{2\alpha}$ (d -4), circulating P4 was 0.89 ng/mL greater in 2CIDR than GnRH cows (2.19 vs. 1.30 ng/mL), which translates to a >60% increase in circulating P4 ($0.89/1.30 = 68.5\%$). Cows with a CL on d -4 had a similar absolute increase in P4 of 0.92 ng/mL (5.80 vs. 4.88 ng/mL) but the relative increase in P4 was <20% ($18.8\% = 0.92/4.88$) for the 2CIDR cows compared with GnRH cows. Thus, 2CIDR should have an advantage over GnRH based on circulating P4. However, the GnRH treatment had the advantage of causing a much greater percentage of cows to ovulate at the beginning of the protocol (43.0 vs. 7.2%); therefore, a much smaller percentage of cows lacked a CL at the time of $\text{PGF}_{2\alpha}$ (25.1 vs. 43.1%). It should be noted that the proportion of cows with a CL at the time of $\text{PGF}_{2\alpha}$ was greater than the calculated proportion of cows that ovulated to the initial treatment of a protocol because some cows did not have an ovulatory-size follicle at the start of the protocol (>8 mm) but had a CL at the time of $\text{PGF}_{2\alpha}$. We assumed

that these cows had already ovulated at the beginning of the protocol, independent of the treatment. Thus, the overall percentage of cows in each group that were greater than the threshold value for P4 was similar [GnRH: 48.0% (369/768) vs. 2CIDR: 49.2% (384/780)]. However, these 2 strategies increased the percentage of cows above the threshold P4 by different routes: GnRH by increasing the percentage of cows with a CL on d -4 and the 2CIDR protocol by adding an extra ~1 ng/mL of P4, which was necessary for some cows with a CL to exceed the P4 threshold. That the 2CIDR protocol had consistently greater P4 but not increased fertility is consistent with the concept that elevating P4 above the threshold value for fertility may be more important than the absolute magnitude of P4 during the protocol.

Despite the finding that treatment with 2 CIDR increased the circulating P4 concentration, in relation to the protocol with GnRH, there was no effect of treatment on P/AI. We speculate that the results in Table 7 may help to explain this dilemma. In cows with a newly formed CL (no/yes), the 2CIDR group had an increased percentage of cows above the threshold P4 (≥ 3.66 ng/mL at PGF_{2 α}) and this effect may underlie the tendency for increased P/AI (28.9% increase; $P = 0.09$) compared with the GnRH treatment. Nevertheless, in cows without a CL (no/no), treatment with 2CIDR increased P4 but did not increase the percentage of cows above the threshold P4 and had no effects on fertility.

The results from the logistic regression analyses were intriguing and highlight some areas for future research. In particular, the increase in pregnancy loss with increasing P4 at the time of PGF_{2 α} was surprising but may be useful in understanding pregnancy loss. This effect was only apparent in cows that ovulated at the beginning of the protocol; that is, those cows with a newer CL at the time of PGF_{2 α} . Cows with no CL at the beginning of the protocol were more likely to ovulate to the GnRH treatment and therefore only a single, younger CL was present at the end of the protocol. This suggests that an inadequate decrease in circulating P4 near the time of AI, due to incomplete regression of the younger CL, may explain this effect. In addition, this effect was primarily found in cows with elevated body temperature as shown in Table 6 in which pregnancy loss was greater in cows with elevated body temperature but only in cows with greater circulating P4. In a previous study done during summer with elevated body temperature, Pereira et al. (2013) reported that cows that ovulate to GnRH treatment at CIDR insertion in a 5-d Cosynch protocol tended to have greater pregnancy loss (25.7%) than cows that did not ovulate to the GnRH injection at CIDR insertion (12.7%). One additional insight from our logistic regression analyses

was that cows that ovulated larger follicles at the end of the protocol had reduced pregnancy loss but, again, only cows that ovulated at the beginning of the protocol (Figure 4). These 2 phenomena may be related to lower P4 and greater circulating E2 near the time of AI producing a more favorable environment that may reduce subsequent pregnancy loss between 32 and 60 d of pregnancy. We discussed this intriguing concept in our previous studies in which we observed that cows that displayed estrus had reduced pregnancy loss either for cows bred to AI or by embryo transfer (Pereira et al., 2013, 2014, 2016). Thus, pregnancy loss probably involves uterine alterations related to the hormonal environment near AI.

CONCLUSIONS

We compared 2 protocols that were designed to increase circulating P4 during preovulatory follicle development. The 2 protocols provided similar results, with elevated body temperature having a major effect on fertility in both protocols. These results continue to provide support for the concept that cows with increased P4 during the preovulatory follicle wave have improved fertility, with the threshold P4 value calculated in this experiment being 3.66 ng/mL. This study also provided information on elevated body temperature in dairy cows, with temperatures $>39.0^{\circ}\text{C}$ being associated with lower P/AI and higher pregnancy loss.

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