Effect of adding a gonadotropin-releasing-hormone treatment at the beginning and a second prostaglandin F_{2α} treatment at the end of an estradiol-based protocol for timed artificial insemination in lactating dairy cows during cool or hot seasons of the year

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ABSTRACT

Our hypothesis was that fertility could be increased in a timed artificial insemination (TAI) protocol based on estradiol (E2) and progesterone (P4) by combining GnRH with E2-benzoate at the start of the protocol to increase circulating P4 during preovulatory follicle development and by using 2 prostaglandin F_{2α} (PGF) treatments at the end to decrease P4 near TAI. Lactating Holstein cows ($n = 1,808$) were randomly assigned during the cool or hot season of the year to receive TAI (d 0) following 1 of 3 treatments: (1) control: controlled internal drug-release insert + 2 mg of E2-benzoate on d -11, PGF on d -4, controlled internal drug-release insert withdrawal + 1.0 mg of E2-cypionate on d -2, and TAI on d 0; (2) 2PGF: identical to control protocol with addition of a second PGF treatment on d -2; (3) GnRH: identical to 2PGF protocol with addition of a 100- μ g GnRH treatment on d -11. Pregnancy diagnoses were performed on d 32 and 60 after TAI. Season had major effects on many reproductive measures, with cool season greater than hot season in percentage of cows with corpus luteum (CL) at PGF (62.9 vs. 56.2%), ovulatory follicle diameter (15.7 vs. 14.8 mm), expression of estrus (86.7 vs. 79.9%), ovulation following the protocol (89.7 vs. 84.3%), and pregnancies per artificial insemination (P/AI; 45.4 vs. 21.4%). The GnRH protocol increased percentage of cows with CL (control = 56.9%; 2PGF = 55.8%; GnRH = 70.5%) and P4 at PGF (control = 3.28 ± 0.22 ; 2PGF = 3.35 ± 0.22 ; GnRH = 3.70 ± 0.21 ng/mL), compared with control and 2PGF protocols. The GnRH protocol increased P/AI at the pregnancy diagnosis at 32 d [37.3% (219/595)] and 60 d [31% (179/595)] after TAI, compared with control

[30.0% (177/604); 25.1% (145/604)], with intermediate results with 2PGF protocol [33.2% (196/609); 28.0% (164/609)]. The positive effects of GnRH treatment on P/AI were only detected during the cool season (GnRH = 50.9%; 2PGF = 44.2%; control = 41.0%) and not during the hot season. In addition, the effect of GnRH was only observed in cows with low P4 (<3 ng/mL) at the start of the protocol and not in cows that began the protocol with high P4. Furthermore, presence of CL at PGF interacted with follicle diameter such that cows with a CL at PGF had greater P/AI if they ovulated larger rather than smaller follicles near TAI. Thus, fertility to TAI can be improved by inducing ovulation at the beginning of an E2/P4-based protocol using GnRH treatment, particularly during the cool season of the year and in cows with low P4 at the start of the protocol.

Key words: gonadotropin-releasing hormone, follicle diameter, progesterone

INTRODUCTION

Protocols that synchronize the time of ovulation and allow timed AI (TAI) can improve reproductive efficiency and profitability of dairy herds (Pursley et al., 1997; Giordano et al., 2010; Ribeiro et al., 2012b). Currently, 2 major types of pharmaceutical approaches exist to synchronize ovulation in dairy cattle (Wiltbank et al., 2011a; Wiltbank and Pursley, 2014). The first approach uses GnRH at the beginning and end of the protocol based on the classic Ovsynch protocol (Pursley et al., 1995). The second approach uses a progesterone (P4) implant combined with estradiol (E2) treatments at the start and end of the protocol to synchronize the follicular wave and synchronize ovulation (Bo et al., 1995; Souza et al., 2009; Baruselli et al., 2012). The underlying goals for these 2 pharmaceutical approaches are similar: synchronization of the follicular wave and

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corpus luteum (CL) function to produce an optimal endocrine environment with final synchronous ovulation of an ideal diameter of follicle (Wiltbank et al., 2011a). However, the advantages and disadvantages of these 2 approaches are only beginning to be appreciated (Vasconcelos et al., 2011b; Pereira et al., 2013a; Wiltbank and Pursley, 2014).

The GnRH-based protocols are based on ovulation of the dominant follicle at the beginning of the protocol leading to initiation of a new follicular wave and presence of an accessory CL during the preovulatory follicular wave (Pursley et al., 1995; Wiltbank and Pursley, 2014). However, only 50 to 65% of cows ovulate to a GnRH treatment administered at a random stage of the estrous cycle (Vasconcelos et al., 1999; Galvão and Santos, 2010; Giordano et al., 2012b), and cows that do not ovulate to the first GnRH treatment generally have reduced fertility (Thatcher et al., 2002; Giordano et al., 2013). In contrast, E2/P4-based programs rely on the E2-benzoate treatment at the beginning of the protocol to suppress circulating gonadotropins resulting in follicular atresia and initiation of a new follicular wave 3 to 5 d later (Bo et al., 1995; Cavalieri et al., 2003; Souza et al., 2009). The E2/P4-based protocols also do not have 100% efficiency in synchronizing follicular-wave emergence (Souza et al., 2009), although detailed ovarian studies with large groups of cows are still needed. Of particular interest for this study, these 2 protocols may be acting on follicles of different diameters (Wiltbank et al., 2011a). For example, large dominant follicles ovulate after GnRH, whereas small follicles regress after the FSH suppression induced by E2. Thus, combining these 2 treatments, GnRH and E2, at the beginning of the protocol may provide greater efficiency of follicular-wave synchronization.

The circulating hormonal concentrations, particularly circulating P4 concentrations, are critical for optimal fertility during either protocol. Increased P4 concentrations at the start (Pursley and Martins, 2011; Wiltbank et al., 2011b) or presence of a CL at the end (Pereira et al., 2013a) of the protocol have been associated with improved fertility. On the other hand, the presence of a new CL may reduce the percentage of cows that have complete regression of the CL at the end of the protocol. Indeed during Ovsynch protocols, circulating P4 has slight elevations near the time of AI (≥ 0.4 ng/mL) in 10 to 25% of cows, and these P4 elevations are associated with a dramatic reduction in fertility to the timed AI (Souza et al., 2007; Brusveen et al., 2009; Martins et al., 2011). Thus, complete luteolysis in response to the PGF treatment, as determined by circulating P4 near the time of AI, is likely to be critical for fertility in both types of protocols. To increase the likelihood of luteolysis in Ovsynch-type protocols, researchers have

increased the dose of PGF (Giordano et al., 2013) or the number of PGF treatments (Brusveen et al., 2009; Santos et al., 2010; Ribeiro et al., 2012a) at the end of the protocol. Thus, addition of GnRH at the beginning of an E2/P4-based TAI program could allow improvements in follicular-wave synchronization and increased P4 concentrations at time of PGF but could bring the disadvantage of incomplete luteolysis at the end of the protocol in some cows. Previous studies have not yet tested the effect of an additional PGF treatment in E2/P4-based TAI protocols.

Heat stress has been found to reduce fertility to AI (Vasconcelos et al., 2011a; Pereira et al., 2013a; Pereira et al., 2014). Potential reasons for this may be that heat stress can affect the hormonal environment (Wolfenson et al., 1995; Wolfenson et al., 1997; Wilson et al., 1998), reduce follicular growth (Wilson et al., 1998) and oocyte competence (de S Torres-Júnior et al., 2008; Rispoli et al., 2013), and have detrimental effects on embryo development and survival (Hansen and Aréchiga, 1999; Wolfenson et al., 2000; Hansen et al., 2001). In a direct comparison of a GnRH-based protocol (5-d Cosynch) and an E2/P4-based protocol during summer, the E2/P4-based protocol was superior to the GnRH-based protocol in cows with heat stress, as measured by elevated body temperature, but the fertility was similar in cows that were not heat stressed (Pereira et al., 2013a). Thus, fertility effects of certain treatments may be enhanced or suppressed during different environmental conditions, particularly heat stress.

This study was designed to evaluate the physiology and fertility when a GnRH treatment is added at the beginning of an E2/P4-based protocol in lactating dairy cows. To ensure regression of the CL at the end of the protocol, we also tested whether addition of a second PGF treatment at the end of the protocol would enhance fertility. In addition, to determine whether season affected the response to these protocols, this experiment was performed in both the cool time of year and during increasing heat stress in lactating dairy cows. The specific hypothesis for the study was that addition of a GnRH treatment at the beginning of an E2/P4-based TAI protocol would increase P4 concentrations and the percentage of cows with a CL at the time of PGF, and that use of 2 PGF treatments at the end of the protocol would increase fertility of lactating dairy cows.

MATERIALS AND METHODS

This experiment was conducted at 4 commercial dairy farms in Minas Gerais State, Brazil, from July 2012 to January 2013. 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 free-stall barns, with 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 feed line. Cows were fed *ad libitum* a TMR based on corn silage and Tifton hay as forages, with a concentrate based on corn and soybean meal, and 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 a total of 1,808 lactating Holstein cows. At the beginning of the experiment (d -11), cows averaged 145 ± 2.1 DIM, yielding 27.4 ± 0.2 kg of milk/d, with BCS of 2.78 ± 0.01 [in a 1 (emaciated) to 5 (obese) scale; Wildman et al., 1982], lactation number of 2.3 ± 0.04 [primiparous (= 1), n = 841; multiparous (≥ 2), n = 967], and had been bred 1.95 ± 0.1 times (first AI, n = 557; second AI, n = 446; third AI or more, n = 805). Within each farm, cows were blocked by parity (primiparous and multiparous); all cows that were past the voluntary waiting period and not pregnant were used and randomized into the study, without regard to whether they had been previously used in the study. Within each block, cows were randomly assigned to receive 1 of 3 treatments (Figure 1): (1) control treatment: an intravaginal P4 insert containing 1.9 g of P4 (CIDR, Zoetis, São Paulo, Brazil), and 2.0 mg (i.m.) of estradiol benzoate (2.0 mL of Estrogin, Farmavet, São Paulo, SP, Brazil) on d -11, 25 mg (i.m.) of dinoprost tromethamine (PGF; 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) on d -2, and TAI on d 0; (2) 2PGF treatment: the same protocol used in the control group, using 2 PGF injections, the first at d -4 and the second at d -2; (3) GnRH treatment: the same protocol used in the 2PGF group, adding a GnRH injection at d -11 (2.0 mL of Cystorelin, Merial, SP, Brazil). The TAI was performed (d 0) using commercial frozen-thawed semen from 10 different bulls. Devices for detection of estrus (Estroject, Rockway Inc., Spring Valley, WI) were placed on the tail head of cows at the time of CIDR removal. The estroject 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 estroject was monitored at TAI by the research per-

sonnel, and cows with an activated estroject (complete color change) were designated as expressing estrus during the protocol. The season was classified as cool (July to September) and hot (October to January) seasons based on previous historical temperatures and meteorological data in the region. The temperature averaged 19.4°C during the cool season and 24.2°C during the hot season, with much greater precipitation during the hot season (741 mm of rain per month) compared with the cool season (20.9 mm/mo).

Ultrasonography

In both groups ovaries were evaluated by transrectal ultrasonography (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 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 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. 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.

Ovulation to ECP, Pregnancy Success, and Pregnancy Loss

Cows with CL present on d 7 were defined as having ovulated to the ECP. Pregnancy success (P/AI) was calculated by dividing the number of pregnant cows at the pregnancy diagnosis at 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 production was measured daily between d 0 to 7, and average daily production through this interval was used in the analysis. Blood samples were collected on d -11 (n = 1,453), d -4 (n = 1,443), and d 7 (n = 1,630), by coccygeal venipuncture into commercial, 10-mL blood-collection tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ). After bleedings, 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

Figure 1. Diagram of activities. The ovaries were evaluated by ultrasound (US) in all cows on d -11, -4, 0, and 7. On d -11 cows were randomly assigned to begin the protocols: control = controlled internal drug-release insert (CIDR) and 2.0 mg of estradiol benzoate (EB), 7 d later PGF, 48 h later the CIDR was removed and cows received 1.0 mg of estradiol cypionate (ECP), 48 h after CIDR removal timed AI (TAI) was performed; 2PGF = was the same as the control protocol but cows received another PGF treatment on d -2; GnRH+2PGF = the same as the 2PGF protocol, but cows received a GnRH and EB on d -11. P4 = blood sampling and analysis of progesterone concentration (sample on d -11, -4, and 7).

temperature (20–25°C) 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 radioimmunoassay kit (DPC Diagnostic Products Inc., Los Angeles, CA) that had been previously validated in our laboratory (Santos and Vasconcelos, 2006). The intraassay coefficient of variation was 5.6% and the interassay coefficient of variation was 10.2%, and the assay sensitivity was 0.01 ng/mL.

Statistical Analysis

The experiment was analyzed as a completely randomized design. The binomial variables (season, ovulation at d -11, new CL at PGF, percentage of CL at PGF, 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 (SAS Institute Inc., Cary, NC) with farm as a random effect and other variables included in the models, if appropriate ($P \leq 0.15$), including effects of treatment, parity, and their interactions, as well as DIM, BCS, CIDR use, and milk yield as covariates. The continuous dependent 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, and other variables that were included in the

models were the effects of treatment, parity, and their interactions, as well as DIM, BCS, CIDR use, and milk yield as covariables. The GLM procedure of SAS was used to determine whether each individual measurement influenced pregnancy rates linearly, quadratically, or cubically. The LOGISTIC procedure was used to determine the intercept and slope values according to maximum likelihood estimates from each significant continuous order effect, and the probability of pregnancy was determined. Logistic curves were constructed using the minimum and maximum values detected for each individual measurement.

Satterthwaite approximation was used to determine the denominator degrees of freedom for tests of fixed effects with the random statement containing effect of group. 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 error of the mean for continuous variables. In all analyses, differences were considered significant when $P \leq 0.05$, whereas differences between $P > 0.05$ and $P \leq 0.15$ were considered tendencies.

RESULTS

Season had dramatic effects on many of the physiological and fertility measures (Table 1). The number of cows with a CL at PGF increased ($P = 0.01$), and

there tended to be more cows with a new CL ($P = 0.09$) in the cool season. The follicle diameter near TAI was reduced ($P < 0.01$) during the hot season, although circulating P4 on d 7 after TAI was not altered ($P = 0.35$) by season. A greater ($P < 0.01$) percentage of cows during the cool than hot season showed estrus and ovulated to the ECP treatment ($P < 0.01$), although more cows had double ovulation in the hot than cool season ($P < 0.01$). The fertility (P/AI) was more than 2-fold greater ($P < 0.01$) in the cool than hot season at either the 32- or 60-d pregnancy diagnoses, although pregnancy loss between 32 and 60 d was not different ($P = 0.41$) between seasons. Differences in fertility between seasons were still observed ($P < 0.01$) when only cows that ovulated to the ECP were analyzed (Table 1). Seasons also differed for DIM (cool = 137 ± 3.0 vs. hot = 151 ± 2.9 ; $P < 0.01$), milk production (cool = 28.5 ± 0.27 vs. hot = 26.3 ± 0.28 ; $P < 0.01$), and BCS (cool = 2.76 ± 0.01 vs. hot = 2.80 ± 0.01 ; $P < 0.01$). Parity of cows that were enrolled in the study did not differ between season ($P = 0.73$). Season did not interact with DIM ($P = 0.92$), BCS ($P = 0.80$), milk production ($P = 0.52$), or parity ($P = 0.48$) on P/AI at 60 d.

Control, 2PGF, and GnRH protocols did not differ for lactation number (2.11 ± 0.42 vs. 2.09 ± 0.42 vs. 2.01 ± 0.42 ; $P = 0.41$), DIM (140 ± 7.3 vs. 150 ± 7.3 vs. 144 ± 7.3 d; $P = 0.15$), BCS (2.76 ± 0.04 vs. 2.77 ± 0.04 vs. 2.78 ± 0.04 ; $P = 0.55$), and milk production (27.9 ± 1.37 vs. 28.4 ± 1.37 vs. 28.1 ± 1.37 kg/d; $P = 0.47$). Table 2 shows the effect of treatment protocol on

reproductive measures, divided by season if the initial analyses (Table 1) showed an effect of season on the measured variable. More ($P < 0.01$) cows ovulated and had a new CL at PGF in the GnRH protocol than in control or 2PGF protocols. More ($P < 0.01$) cows had a CL at PGF in the GnRH protocol than in the other 2 protocols in either the cool or hot seasons. Circulating P4 was greater at the time of PGF treatment (d -4) for cows treated with GnRH than the other protocols, although follicle diameter ($P = 0.61$) was not different between protocols. Treatment with 2PGF tended ($P = 0.11$) to have greater P4 concentration on d 7 than the GnRH protocol. Treatment protocol did not alter expression of estrus in the cool season, although in the hot season the GnRH protocol tended to increase expression of estrus ($P = 0.10$) compared with the control. Ovulation to ECP was greater ($P = 0.05$) in GnRH than control cows with both seasons combined, and GnRH tended ($P = 0.13$) to have greater ovulation to ECP than control during the cool season. Double ovulation was reduced for cows in the GnRH protocol during the cool season ($P < 0.01$) but was not different ($P = 0.40$) during the hot season. For cows receiving the GnRH protocol, double ovulation had more than a 2-fold increase (26.5 vs. 11.0% ; $P < 0.01$) during the hot compared with cool season.

Table 3 shows the effects of treatment protocol on fertility (P/AI) in cows during the cool or hot season. At the 32-d pregnancy diagnosis, treatment had an effect ($P = 0.02$), with the GnRH protocol having greater fertility ($P < 0.01$) than control and tending to have

Table 1. Effects of season on measures of reproductive physiology and fertility

Item ¹	Cool season	Hot season	P-value
Ovulation of d -11 follicle ²	19.6 (168/857)	17.7 (109/617)	0.35
New CL at PGF ³	27.8 (238/857)	23.8 (147/617)	0.09
CL at PGF ⁴	62.9 (539/857)	56.2 (347/617)	0.01
P4 d -11 ⁴ (ng/mL)	3.05 ± 0.09	3.27 ± 0.11	0.13
P4 d -4 ⁴ (ng/mL)	3.39 ± 0.09	3.51 ± 0.10	0.41
Follicle diameter d 0 ⁵ (mm)	15.7 ± 0.1	14.8 ± 0.1	<0.01
P4 d 7 ⁵ (ng/mL)	3.42 ± 0.06	3.33 ± 0.07	0.35
Estrus ⁴	86.7 (742/856)	79.9 (739/925)	<0.01
Ovulation to ECP	89.7 (736/821)	84.3 (685/813)	<0.01
Double ovulation ⁵	17.5 (129/736)	24.2 (166/685)	<0.01
P/AI for all cows ²			
32 d	45.4 (389/857)	21.4 (203/951)	<0.01
60 d	37.0 (317/857)	18.0 (171/951)	<0.01
Pregnancy loss (d 32–60)	18.5 (72/389)	15.8 (32/203)	0.41
P/AI for synchronized cows ⁵			
32 d	50.5 (372/736)	25.3 (173/685)	<0.01
60 d	41.3 (304/736)	20.9 (143/685)	<0.01
Pregnancy loss (d 32–60)	18.3 (68/372)	17.3 (30/173)	0.79

¹Least squares means, % (no./no.) or mean $\pm$ SEM; P4 = progesterone; P/AI = pregnancies per AI.

²Percentage of cows with a new corpus luteum (CL) on d -4 that had a follicle >8 mm on d -11.

³Percentage of cows with a new CL on d -4 independent of presence of follicle >8 mm on d -11.

⁴All inseminated cows.

⁵Includes only cows that ovulated to estradiol cypionate (ECP; visible CL on d 7).

Table 2. Effects of treatment protocol on measures of reproductive physiology

Item ¹	Protocol ²			P-value
	Control	2PGF	GnRH	
Ovulation of d -11 follicle ³	11.1 (55/494) ^b	9.0 (44/488) ^b	36.2 (178/492) ^a	<0.01
New CL at PGF ⁴	19.2 (95/494) ^b	16.4 (80/488) ^b	42.7 (210/492) ^a	<0.01
CL at PGF ⁵				
Cool season	59.0 (167/283) ^b	56.5 (160/283) ^b	72.9 (212/291) ^a	<0.01
Hot season	51.7 (109/211) ^b	52.7 (108/205) ^b	64.7 (130/201) ^a	0.01
P-value	0.10	0.40	0.05	
Combined	56.9 (276/494) ^b	55.8 (268/488) ^b	70.5 (342/492) ^a	<0.01
P4 d -4 ⁶ (ng/mL)	3.28 ± 0.22 ^b	3.35 ± 0.22 ^b	3.7 ± 0.21 ^a	0.01
P4 d 7 ⁶ (ng/mL)	3.42 ± 0.08 ^{xy}	3.44 ± 0.08 ^x	3.27 ± 0.08 ^y	0.22
Follicle at AI ⁶ (mm)	15.2 ± 0.2	15.4 ± 0.2	15.4 ± 0.2	0.61
Estrus ⁵				
Cool season	87.6 (248/283)	86.9 (245/282)	85.6 (249/291)	0.76
Hot season	76.8 (242/315) ^y	80.8 (253/313) ^{xy}	82.2 (244/297) ^x	0.23
P-value	<0.01	0.05	0.26	
Combined	81.3 (490/598)	83.1 (498/595)	83.0 (493/588)	0.62
Ovulation to ECP ⁶				
Cool season	87.4 (236/270) ^y	90.1 (245/272) ^{xy}	91.4 (255/279) ^x	0.30
Hot season	82.5 (231/280)	84.3 (231/274)	86.1 (223/259)	0.52
P-value	0.10	0.04	0.05	
Combined	84.9 (467/550) ^b	87.2 (476/546) ^{ab}	88.9 (478/538) ^a	0.15
Double ovulation ⁶				
Cool season	23.3 (55/236) ^b	18.8 (46/245) ^b	11.0 (28/255) ^a	<0.01
Hot season	21.2 (49/231)	25.1 (58/231)	26.5 (59/223)	0.40
P-value	0.59	0.10	<0.01	
Combined	22.3 (104/467) ^x	21.9 (104/476) ^{xy}	18.2 (87/478) ^y	0.24

^{a,b,xy} Means in the same row without similar superscripts differ at $P < 0.05$ and $P < 0.1$, respectively.

¹Least squares means, % (no./no.) or mean ± SEM; P4 = progesterone.

²Control = controlled internal drug-release insert + 2 mg of estradiol benzoate on d -11, PGF on d -4, controlled internal drug-release insert withdrawal + 1.0 mg of estradiol cypionate on d -2, and timed AI on d 0; 2PGF = identical to control protocol with addition of a second PGF treatment on d -2; GnRH = identical to 2PGF protocol with addition of a 100-μg GnRH treatment on d -11.

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

⁴Percentage of cows with a new CL on d -4 independent of presence of follicle >8 mm on d -11.

⁵All inseminated cows.

⁶Includes only cows that ovulated to estradiol cypionate (ECP; visible CL on d 7).

($P = 0.09$) greater fertility than 2PGF when data from cows in both seasons were combined. However, the treatment effect was only apparent during the cool ($P = 0.05$) but not in the hot season ($P = 0.40$). At the 60-d pregnancy diagnosis, GnRH treatment had greater P/AI than control using data from either the combined seasons ($P = 0.02$) or the cool season ($P = 0.03$) but not using data from only the hot season ($P = 0.35$). If analyses were done using only cows that ovulated to ECP and using the 32-d pregnancy diagnosis (Table 3), GnRH treatment had greater fertility than control ($P = 0.01$) and tended to be greater than ($P = 0.13$) 2PGF for the combined seasons and in the cool season (GnRH vs. control, $P = 0.04$; GnRH vs. 2PGF, $P = 0.13$) but not during the summer ($P = 0.47$). Differences were less pronounced at the 60-d pregnancy diagnosis using only data from cows that ovulated to ECP.

At the beginning of the protocols (d -11), the P4 concentrations were similar ($P = 0.29$) between the treatments (control = 2.96 ± 0.25 vs. 2PGF = $3.23 \pm$

0.25 vs. GnRH = 3.04 ± 0.25 ng/mL). Cows were classified by the P4 concentration on d -11 as either low P4 [<3.0 ng/mL, 49% of the animals (716/1,453)] or high P4 [≥ 3.0 ng/mL, 51% of the animals (737/1,453)], as shown in Table 4. The majority of cows in the low P4 group had very low P4 concentrations [<0.5 ng/mL = 56% (401/716), 0.5 to 0.99 ng/mL = 17.7% (127/716), 1 to 2.99 ng/mL = 26.3% (188/716)]. The GnRH protocol increased ($P < 0.01$) the percentage of cows with ovulation at the start of the protocol and new CL at the time of PGF, independent of P4 concentration at d -11 ($P < 0.01$). In cows with high P4, the control and 2PGF groups had almost no ovulations or new CL. In all 3 protocols, more ($P < 0.01$) ovulations and new CL were present at PGF in cows that began the protocols with low rather than high P4. In addition, the GnRH protocol increased the percentage of cows with any age of CL at the PGF treatment in cows with low ($P < 0.01$) and high ($P = 0.02$) P4 on d -11. Ovulation to the ECP was greater for cows that had high rather

Table 3. Effects of treatment protocol on pregnancies per AI (P/AI) in the hot and cool seasons

Item ¹	Protocol ²			P-value
	Control	2PGF	GnRH	
P/AI at 32 d for all cows ³				
Cool season	41.0 (116/283) ^b	44.2 (125/283) ^y	50.9 (148/291) ^{a,x}	0.05
Hot season	19.0 (61/321)	21.8 (71/326)	23.4 (71/304)	0.40
P-value	<0.01	<0.01	<0.01	
Combined	30.0 (177/604) ^b	33.2 (196/609) ^{b,y}	37.3 (219/595) ^{a,x}	0.02
P/AI at 60 d for all cows ³				
Cool season	32.9 (93/283) ^b	36.4 (103/283) ^{ab}	41.6 (121/291) ^a	0.09
Hot season	16.2 (52/321)	18.7 (61/326)	19.1 (58/304)	0.59
P-value	<0.01	<0.01	<0.01	
Combined	25.1 (145/604) ^b	28.0 (164/609) ^{ab}	31.0 (179/595) ^a	0.06
P/AI at 32 d for synchronized cows ⁴				
Cool season	46.6 (110/236) ^b	49.0 (120/245) ^{ab}	55.7 (142/255) ^a	0.11
Hot season	22.5 (52/231)	26.0 (60/231)	27.4 (61/223)	0.47
P-value	<0.01	<0.01	<0.01	
Combined	34.7 (162/467) ^b	37.8 (180/476) ^y	42.5 (203/478) ^{a,x}	0.05
P/AI at 60 d for synchronized cows ⁴				
Cool season	37.7 (89/236) ^y	40.4 (99/245) ^{xy}	45.5 (116/255) ^x	0.20
Hot season	19.5 (45/231)	21.7 (50/231)	21.5 (48/223)	0.81
P-value	<0.01	<0.01	<0.01	
Combined	28.7 (134/467) ^y	31.3 (149/476) ^{xy}	34.3 (164/478) ^x	0.18

^{a,b,y}Means in the same row without similar superscripts differ at $P < 0.05$ and $P < 0.1$, respectively.

¹Least squares means, % (no./no.). P4 = progesterone.

²Control = controlled internal drug-release insert + 2 mg of estradiol benzoate on d -11, PGF on d -4, controlled internal drug-release insert withdrawal + 1.0 mg of estradiol cypionate on d -2, and timed AI on d 0; 2PGF = identical to control protocol with addition of a second PGF treatment on d -2; GnRH = identical to 2PGF protocol with addition of a 100- μ g GnRH treatment on d -11.

³All inseminated cows.

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

than low P4 at the start of control ($P = 0.01$) and 2PGF ($P = 0.03$) protocols but not for cows in the GnRH protocol ($P = 0.19$). In cows with low P4 on d -11, ovulation to ECP was greater ($P = 0.05$) for GnRH than control cows, but this was not observed ($P = 0.78$) in cows with high P4 on d -11. Follicle diameter at AI, double ovulation, and circulating P4 on d 7 did not substantially differ by treatment group and circulating P4 at the start of the protocols.

Fertility differences (at 60-d pregnancy diagnosis) based on treatment, season, and P4 concentration at the start of the protocol are shown in Table 5. In cows with low P4 at the start of the protocol, GnRH treatment increased fertility compared with control ($P < 0.01$) and 2PGF protocol ($P = 0.04$); however, this treatment effect was not observed ($P = 0.81$) in cows with high P4 at the start of the protocol. Interestingly, high P4 on d -11 was associated with increased P/AI ($P = 0.05$) in control cows. In contrast in GnRH-treated cows, low P4 on d -11 was associated with greater ($P = 0.04$) fertility than high P4 on d -11. The treatment effects in cows with low P4 were observed in both the cool ($P = 0.02$) and hot ($P = 0.07$) seasons, with GnRH-treated cows always greater than control cows and intermediate values for 2PGF cows. Oddly,

GnRH treatment was associated with reduced fertility in cows with high P4 on d -11 during the hot season ($P = 0.01$) but not during the cool season ($P = 0.60$). If only cows that ovulated to ECP were used in the analysis (bottom of Table 5), similar effects were observed with GnRH treatment having greater P/AI ($P = 0.01$) than control and tending to be greater ($P = 0.08$) than 2PGF in cows with low P4 on d -11 but not in cows with high P4 ($P = 0.77$). Treatment effects were similar in cows that ovulated to ECP during the cool and hot seasons, with reduced fertility ($P < 0.01$) in cows with high versus low P4 on d -11 continuing to be observed in GnRH-treated cows during the hot season.

Cows were classified based on presence or absence of CL on d -11 and -4 (data not shown). Treatment had no effect on P/AI in cows that had a CL on d -11 and -4 ($P = 0.58$; $n = 601$; 29.8%); in cows that had a CL present on d -11 but not on d -4 ($P = 0.58$; $n = 358$; 24.6%); or in cows that had no CL on d -11 but had a CL on d -4 ($P = 0.81$; $n = 285$; 33.7%). Surprisingly, in cows with no detectable CL on d -11 or -4 (anovular cows), the 2 PGF (32.1%; 25/78) increased ($P = 0.05$) P/AI compared with control (19.4%; 19/98). The GnRH treatment (31.5%; 17/54) also tended to increase P/AI compared with control ($P = 0.10$).

Table 4. Effects of treatment protocol divided by progesterone (P4) concentration on d -11 on measures of reproductive physiology

Item ¹	Protocol ²			P-value
	Control	2PGF	GnRH	
Ovulation at d -11 ³				
P4 <3.0	19.8 (50/252) ^b	16.9 (38/225) ^b	46.9 (112/239) ^a	<0.01
P4 ≥3.0	1.7 (4/233) ^b	1.6 (4/257) ^b	26.3 (65/247) ^a	<0.01
P-value	<0.01	<0.01	<0.01	
New CL at PGF ⁴				
P4 <3.0	34.1 (86/252) ^b	32.0 (72/225) ^b	58.6 (140/239) ^a	<0.01
P4 ≥3.0	3.0 (7/233) ^b	2.3 (6/257) ^b	27.5 (68/247) ^a	<0.01
P-value	<0.01	<0.01	<0.01	
CL at PGF				
P4 <3.0	54.4 (137/252) ^b	56.0 (126/225) ^b	73.2 (175/239) ^a	<0.01
P4 ≥3.0	57.5 (134/233) ^b	54.1 (139/257) ^b	66.0 (163/247) ^a	0.02
P-value	0.49	0.67	0.08	
Ovulation to ECP ⁵				
P4 <3.0	82.0 (200/244) ^b	84.3 (183/217) ^{ab}	88.2 (202/229) ^a	0.16
P4 ≥3.0	89.9 (205/228)	91.0 (232/255)	91.8 (225/245)	0.78
P-value	0.01	0.03	0.19	
Follicle at AI ⁶ (mm)				
P4 <3.0	15.2 ± 0.2	15.3 ± 0.2	15.5 ± 0.2	0.61
P4 ≥3.0	14.9 ± 0.2	15.1 ± 0.2	15.1 ± 0.2	0.57
P-value	0.35	0.70	0.30	
Double ovulation ⁶				
P4 <3.0	20.0 (40/200)	23.5 (43/183)	17.5 (36/202)	0.38
P4 ≥3.0	30.7 (63/205) ^b	25.9 (60/232) ^{ab}	22.7 (51/225) ^a	0.16
P-value	0.01	0.58	0.21	
P4 at d 7 ⁶ (ng/mL)				
P4 <3.0	3.62 ± 0.13	3.70 ± 0.13	3.45 ± 0.13	0.36
P4 ≥3.0	3.57 ± 0.12 ^x	3.50 ± 0.11 ^{xy}	3.30 ± 0.11 ^y	0.22
P-value	0.78	0.22	0.40	

^{a,b,x,y}Means in the same row without similar superscripts differ at $P < 0.05$ and $P < 0.1$, respectively.

¹Least squares means, % (no./no.) or mean ± SEM.

²Control = controlled internal drug-release insert + 2 mg of estradiol benzoate on d -11, PGF on d -4, controlled internal drug-release insert withdrawal + 1.0 mg of estradiol cypionate on d -2, and timed AI on d 0; 2PGF = identical to control protocol with addition of a second PGF treatment on d -2; GnRH = identical to 2PGF protocol with addition of a 100-μg GnRH treatment on d -11.

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

⁴Percentage of cows with a new CL on d -4 independent of presence of follicle >8 mm on d -11.

⁵All inseminated cows.

⁶Includes only cows that ovulated to estradiol cypionate (ECP; visible CL on d 7).

The ovulatory follicle diameter differed by season, with an interaction between season and presence of the CL on follicle diameter (Table 6). Cows in the cool season ovulated larger follicles than cows in the hot season either in the presence or absence of a CL on d -4. However, in the cool season, cows that had a CL present on d -4 ovulated smaller follicles than cows without a CL ($P < 0.01$), whereas presence of a CL had no effect on follicle diameter in the hot season ($P = 0.55$). Classification of cows based on presence or ovulation of larger (≥ 14 mm) or smaller (< 14 mm) follicles (Table 6) showed that cows with larger follicles had greater ovulation to the ECP either in the presence or absence of a CL on d -4 and in the hot or cool season. However, for cows with a smaller follicle (< 14 mm) at TAI, cows with a CL present on d -4 had a

greater percentage of cows that ovulated to the ECP than cows without a CL on d -4 during the cool ($P = 0.02$) but not hot ($P = 0.66$) season. The fertility, based on the 60-d pregnancy diagnosis, was also affected by the ovulatory follicle diameter and presence of a CL on d -4 (Table 6). Cows with a CL on d -4 had greater fertility for cows ovulating a large follicle than a small follicle in both the cool ($P = 0.02$) or hot ($P < 0.01$) seasons. This effect of ovulating a larger follicle was not observed in cows that did not have a CL present on d -4 in either the cool ($P = 0.73$) or hot ($P = 0.37$) seasons. Similar effects were observed if the analyses were done with only cows that ovulated to ECP (Table 6).

There was also an interaction ($P = 0.05$) between ovulatory follicle diameter and presence of a CL on d -4 on P/AI. For this analysis we used the 60-d preg-

Table 5. Effects of treatment protocol divided by progesterone (P4) concentration on d -11 on pregnancies per AI (P/AI) at d-60 pregnancy diagnosis

Item ¹	Protocol ²			P-value
	Control	2PGF	GnRH	
P/AI at 60 d for all cows ³				
P4 <3.0	21.8 (55/252) ^b	28.0 (63/225) ^b	36.4 (87/239) ^a	<0.01
P4 ≥3.0	29.6 (69/233)	30.0 (77/257)	27.5 (68/247)	0.81
P-value	0.05	0.64	0.04	
Cool-season P/AI at 60 d ³				
P4 <3.0	28.3 (43/152) ^b	35.3 (48/136) ^y	43.3 (65/150) ^{a,x}	0.02
P4 ≥3.0	38.3 (49/128)	38.2 (55/144)	40.3 (56/139)	0.92
P-value	0.07	0.62	0.60	
Hot-season P/AI at 60 d ³				
P4 <3.0	12.0 (12/100) ^b	16.9 (15/89) ^{ab}	24.7 (22/89) ^a	0.07
P4 ≥3.0	19.1 (20/105) ^x	19.5 (22/113) ^x	11.1 (12/108) ^y	0.18
P-value	0.17	0.64	0.01	
P/AI at 60 d for synchronized cows ⁴				
P4 <3.0	27.0 (54/200) ^b	32.8 (60/183) ^y	41.1 (83/202) ^{a,x}	0.01
P4 ≥3.0	32.2 (66/205)	32.8 (76/232)	29.8 (67/225)	0.77
P-value	0.25	0.99	0.01	
Cool-season P/AI at 60 d ⁴				
P4 <3.0	35.0 (42/120) ^b	41.3 (45/109) ^{ab}	48.4 (61/126) ^a	0.10
P4 ≥3.0	40.7 (46/113)	40.3 (54/134)	43.3 (55/127)	0.87
P-value	0.37	0.88	0.45	
Hot-season P/AI at 60 d ⁴				
P4 <3.0	15.0 (12/80) ^b	20.3 (15/74) ^{ab}	29.0 (22/79) ^a	0.10
P4 ≥3.0	21.7 (20/92) ^x	22.5 (22/98) ^x	12.2 (12/98) ^y	0.13
P-value	0.26	0.73	<0.01	

^{a,b; x,y} Means in the same row without similar superscripts differ at $P < 0.05$ and $P < 0.1$, respectively.

¹Least squares means, % (no./no.).

²Control = controlled internal drug-release insert + 2 mg of estradiol benzoate on d -11, PGF on d -4, controlled internal drug-release insert withdrawal + 1.0 mg of estradiol cypionate on d -2, and timed AI on d 0; 2PGF = identical to control protocol with addition of a second PGF treatment on d -2; GnRH = identical to 2PGF protocol with addition of a 100-μg GnRH treatment on d -11.

³All inseminated cows.

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

nancy diagnosis and only cows that ovulated to ECP (Figure 2). Using data from both seasons, cows with a CL on d -4 had greater ($P < 0.01$) fertility as ovulatory follicle diameter increased; however, no effect of follicle diameter on P/AI was observed in cows without CL on d -4 ($P = 0.80$). When the data were divided by season, the increase in P/AI with increasing follicle diameter was observed in both the cool ($P = 0.05$) and hot ($P = 0.01$) season. In contrast to cows with no CL, in the cool season, ovulatory follicle diameter tended ($P = 0.12$) to have a negative effect on fertility. In the hot season, ovulatory follicle diameter had no effect on P/AI in cows without CL on d -4.

Expression of estrus increased ($P < 0.01$) the percentage of cows that ovulated to ECP [no estrus = 62.6% (171/273), estrus = 92.0% (1,245/1,354)]. Expression of estrus was associated with increased ($P < 0.01$) P/AI in cows that ovulated to ECP at both the 32-d [no estrus = 26.3% (45/171), estrus = 40.1% (499/1,245)] and 60-d [no estrus = 22.2% (38/171), estrus = 32.8% (408/1,245)] pregnancy diagnoses.

DISCUSSION

Protocols that synchronize ovulation using E2 and P4 are currently used in many parts of the world. The results of the present study provide practical knowledge about how these specific TAI protocols can be improved and also provide new physiological information into how fertility can be regulated in lactating dairy cows. One of the major practical insights is that provision of a GnRH treatment at the beginning of an E2/P4-based TAI program can increase fertility; however, this increase was only observed in the cool season of the year. During the hot season, fertility was less than half the fertility observed during the cool time of year, and treatment protocol had no effect on fertility. Also, subtle but potentially important changes in reproductive physiology during heat stress existed. Other important physiological insights were also obtained when fertility data were analyzed in relation to specific measures of reproductive physiology, such as circulating P4 at the beginning of the protocol, presence of CL

Table 6. Effects of season and follicle diameter at timed AI [small (<14 mm) or large (≥14 mm) follicles] on ovulation to estradiol cypionate (ECP) at the end of the protocol [corpus luteum (CL) present on d 7], and pregnancies per AI (P/AI) based on 60-d pregnancy diagnosis

Item ¹	Cool season			Hot season		
	<14 mm	≥14 mm	<i>P</i> -value	<14 mm	≥14 mm	<i>P</i> -value
Distribution ² (%)						
No CL at d -4	9.0 (68/758)	27.0 (206/758)		17.0 (90/532)	24.0 (126/532)	
CL at d -4	23.0 (176/758)	41.0 (308/758)		23.0 (125/532)	36.0 (191/532)	
Ovulation to ECP ³						
No CL at d -4	73.1 (49/67)	92.1 (187/203)	<0.01	80.0 (72/90)	88.9 (112/126)	0.07
CL at d -4	86.2 (144/167)	94.2 (276/293)	<0.01	82.4 (103/125)	93.2 (178/191)	<0.01
<i>P</i> -value	0.02	0.36		0.66	0.18	
P/AI at 60 d ²						
No CL at d -4	38.2 (26/68)	35.9 (74/206)	0.73	12.2 (11/90)	16.7 (21/126)	0.37
CL at d -4	33.0 (58/176)	43.5 (134/308)	0.02	9.6 (12/125)	26.2 (50/191)	<0.01
<i>P</i> -value	0.44	0.08		0.54	0.05	
P/AI at 60 d ³						
No CL at d -4	51.0 (25/49)	39.6 (74/187)	0.15	15.3 (11/72)	18.8 (21/112)	0.55
CL at d -4	37.5 (54/144)	46.4 (128/279)	0.05	11.7 (12/103)	28.1 (50/178)	<0.01
<i>P</i> -value	0.10	0.15		0.48	0.07	
Follicle diameter ³ (mm)						
No CL at d -4		16.1 ± 0.2			14.7 ± 0.2	<0.01
CL at d -4		15.2 ± 0.1			14.6 ± 0.2	<0.01
<i>P</i> -value		<0.01			0.55	

¹Least squares means, % (no./no.) or mean ± SEM; P/AI = pregnancies per AI.

²Includes all inseminated cows.

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

at time of PGF, and ovulatory follicle diameter. Thus, this study provides important insights into the interaction between heat stress, key measures of reproductive physiology, and fertility.

The performance of this study during 2 seasons of the year with sufficient numbers of cows evaluated in both cool ($n = 857$) and hot ($n = 617$) seasons allowed analysis of the effect of heat stress on physiological measures and treatment effects. Physiological measurements associated with greater fertility were generally reduced during the hot season, such as percentage of cows with CL at PGF decreased 6.7%, ovulatory follicle diameter decreased 0.9 mm, expression of estrus decreased 6.8%, and ovulation to the ECP decreased 5.4%. Previous studies have also reported that heat stress reduces follicular growth (Wilson et al., 1998), alters production of steroids by growing follicles (Wolfenson et al., 1995; Wolfenson et al., 1997; Wilson et al., 1998), reduces expression of estrus (Younas et al., 1993), and reduces ovulation (Pereira et al., 2013a). Each of these physiological changes could account for some decrease in fertility. For example, lack of ovulation would decrease fertility from potentially ~50% (overall cool-season fertility) to 0% in 5.4% of cows, causing a reduction of 2.7% in overall P/AI. The heat stress-induced changes in the other physiological measures would be expected to have less effect on fertility than lack of ovulation (expression of estrus is +10.6% in 6.8% of cows = -0.72%; smaller follicle = -1.2%;

no CL on d -4 = -0.90), suggesting that only about 5.5% of the reduction in fertility could be potentially explained by changes in these physiological measures. Thus, most of the dramatic effects of heat stress on P/AI in this study are likely to be explained by well-described effects of heat stress (Hansen, 2009; Hansen, 2013) on oocyte competence (de S Torres-Júnior et al., 2008; Rispoli et al., 2013), fertilization (Hackbart et al., 2009), early embryo development (Hansen and Aréchiga, 1999; Wolfenson et al., 2000; Hansen et al., 2001), and embryo development after d 7 (Biggers et al., 1987; Ryan et al., 1993; Demetrio et al., 2007).

Another intriguing observation was that the improvements with the GnRH protocol on the P/AI were only observed in cows with low P4 concentration (<3.0 ng/mL) at the beginning of the protocol (Table 5). These cows had only 21.8% pregnant to the control protocol but 36.4% pregnant to the GnRH protocol, a 14.6% absolute increase but, more accurately, 67% (14.6/21.8) more pregnancies with the GnRH protocol compared with the control protocol. The increase with the GnRH compared with the control protocol was observed in the cool (53%) or hot (106%) seasons. In sharp contrast, cows with high P4 concentration at the beginning of the protocol had no improvement in P/AI by using the GnRH protocol compared with the control, perhaps because of the decrease in ovulation to the GnRH from ~50% in low P4 to 26.3% in high P4. This depression in ovulation to the first GnRH due to high P4 concentra-

Figure 2. Effect of follicle diameter at time of AI (d 0) in the presence or absence of a corpus luteum (CL) at time of first PGF (d -4) on pregnancies per AI (P/AI) at the 60-d pregnancy diagnosis using only cows that ovulated to the estradiol cypionate (CL detected on d 7). All cows from both seasons are combined in panel A, whereas only cows during the cool season (panel B) or hot season (panel C) are shown below. In cows with a CL on d -4, follicle diameter had a linear effect on P/AI in all cows ($P < 0.01$) and a similar effect using only cows in the cool ($P = 0.05$) or hot ($P = 0.01$) season.

tions has been reported previously and is likely due to a decreased LH surge in response to GnRH in the presence of high P4 (Giordano et al., 2012a, 2013). The GnRH-treated cows had more cows with a CL and with a new CL at the time of PGF treatment than control cows. The presence of a CL or 2 CL would increase cir-

culating P4 concentration during follicle development. In Ovsynch protocols this has been shown to increase P/AI (Bisinotto et al., 2010; Denicol et al., 2012; Bisinotto et al., 2013) and is associated with a greater embryo quality (Cerri et al., 2011; Rivera et al., 2011). The benefits of greater P4 on fertility were also observed in previous studies using E2/P4 protocols (Pereira et al., 2013a). Nevertheless, for cows that started the protocol with elevated P4, the increase in CL and new CL at PGF that was observed in the GnRH group did not translate into improvements in fertility in the cool season and even tended to decrease fertility in the hot season. Thus, cows that begin an E2/P4 protocol with lower circulating P4 show substantial improvements in fertility if GnRH is included in the protocol.

In cows without a CL at PGF, the follicle diameter had no effect on P/AI; in cows with a CL at PGF, cows that ovulated larger follicles had greater P/AI, independent of season. It appears that development of larger follicles is positive for fertility if these follicles are growing in the presence of high circulating P4.

A further unexpected but statistically significant effect was the improvement in fertility with the 2 PGF protocol compared with control in cows with no CL during the protocol (no CL on d -11 and no CL at PGF). These results will need to be repeated, but it is possible that the extra PGF treatment or the timing of the second PGF treatment may have enhanced LH release (Randel et al., 1996; Leonardi et al., 2012) or had some other action, perhaps uterine, to increase the P/AI in anovular cows and cows ovulating small follicles. In cows ovulating larger follicles, the GnRH protocol tended to be superior probably because of the greater proportion of cows with CL and high P4 at time of first PGF.

Finally, as observed in many other studies using E2/P4 protocols, expression of estrus was associated with improved synchronization to the protocol and greater fertility whether calculated for all cows or only for cows that were synchronized (Kasimanickam et al., 2005; Pereira et al., 2013a, 2014). Nevertheless, expression of estrus did not explain the differences in fertility between the protocols because expression of estrus between the protocols did not differ. The results from this study are similar to other reports using E2/P4 protocols (Souza et al., 2009; Pereira et al., 2013b), less than studies using this E2/P4 protocols only in cyclic cows during the cool time of the year (Pereira et al., 2014), but greater than observed in studies using E2/P4 protocols during summer (Pereira et al., 2013a).

Related to the potential economic effect of adding a GnRH treatment and a second PGF treatment, protocol cost increased about ~82% per protocol, from \$5.85 for the control protocol to \$10.67 for the GnRH proto-

col. Using semen prices of \$12.37 per straw, the 60-d pregnancy cost per cow, using only semen and hormone costs, was \$55.38 in the control protocol and \$55.38 in the GnRH protocol. Thus, when semen prices are more than \$12.37 per straw, the actual price per pregnancy will be lower for the GnRH protocol. Obviously, there are numerous other benefits associated with the improved fertility using the GnRH protocol, including reduced labor, reduced culling of high-value cows, reduced days open with all associated improvements in milk production and efficiency, and increased numbers of replacement heifers.

In conclusion, use of GnRH with estradiol-benzoate treatment at the beginning of an E2/P4 protocol for timed AI resulted in improved fertility, particularly in cows during the cool season of the year that had low P4 at the start of protocol. Treatment with GnRH caused ovulation, particularly in cows with low P4, increasing the percentage of cows with a CL at the time of PGF, and these cows demonstrated increasing fertility with increasing follicle diameter. Manipulation of cows to mimic these high-fertility physiological conditions may allow us to optimize fertility with these protocols.

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