UNIVERSIDADE ESTADUAL PAULISTA

FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

CÂMPUS DE BOTUCATU

THE MANIPULATION OF PROGESTERONE PROFILES DURING PROGESTERONE + ESTRADIOL TIMED AI PROTOCOLS IN DAIRY CATTLE: EFFECTS ON FERTILITY

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Thesis presented to the pos graduation program in Animal Science as part of requirements to obtain degree of Doctor

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DEDICATION

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SUMMARY

LIST OF ABBREVIATIONS

AI - Artificial insemination

BCS - Body condition Score

CIDR - Controlled internal drug release

CL - Corpus Luteum

E2 - Estradiol

DIM - Days in milk

EB - Estradiol Benzoate

ECP - Estradiol cypionate

FSH - Follicular stimulant hormone

GnRH - Gonadotropin releasing hormone

LH - Luteinizing hormone

P4 - Progesterone

PGF - Prostaglandin F2α

P/AI - Pregnancy per artificial insemination

ROC - Receiver Operating Characteristic

TAI - Timed artificial insemination

TMR - Total mixed ration

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Chapter 1

INTRODUCTION

The profitability of dairy herds is associated with reproductive efficiency. However, reduced pregnancy per artificial insemination (P/AI) and increased pregnancy losses have been observed in dairy systems (LUCY, 2001). The high milk production is associated with changes in reproductive physiology that may underlie the decline in reproductive efficiency (WILTBANK et al., 2006). Besides the lower P/AI, reduced service rate is another intriguing factor that reduce the reproductive efficiency in dairy herds.

Poor rates of estrous detection combined with poor conception rates make management of reproduction in lactating dairy cows a challenge in most dairy herds (PURSLEY et al., 1995). The timely and accurate detection of estrus generally is below 50 % (SENGER et al, 1994). Hormonal treatments have been developed to synchronize the time of ovulation, allowing successful fixed time artificial insemination (TAI) without the need for detection of estrus (PURSLEY et al., 1997a). These programs are designed to improve artificial insemination (AI) submission rate and fertility is generally similar to AI after detection of estrus (PURSLEY et al., 1997a, PURSLEY et al., 1997b, RABIEE et al., 2005).

A variety of methods have been evaluated to increase fertility during synchronization of ovulation program including: increasing circulating progesterone (P4) concentration during ovulatory follicle development (BISINOTTO et al., 2010c, MARTINS et al., 2011a, WILTBANK et al., 2011b),

This thesis was performed to evaluate strategies to improve fertility in lactating dairy cows. The objectives of the first study was to evaluate the effects of a GnRH treatment at the beginning of an E2/P4-based protocol and whether addition of a second PGF would enhance fertility. The objective of the second was to compare two strategies that improve circulating P4 concentration during follicular development, adding GnRH or 2 CIDR at the beginning of the protocol.

LITERATURE REVIEW

Fertility of dairy cows

Across different levels of milk production, breeds and management systems, 45 to 71% of dairy cows develop metabolic and infectious diseases in the first months of lactation (Ribeiro et al., 2013). In fact, when diseases were classified as clinical (calving problem, metritis, clinical endometritis, mastitis, pneumonia, digestive problems, and lameness), subclinical (subclinical hypocalcemia, subclinical ketosis, and severe negative energy balance), or both, affected cows had increased anovulation and reduced pregnancy per AI (Ribeiro et al., 2013). Infectious diseases, such as infectious bovine rhinotracheitis, bovine viral diarrhea, and leptospirosis have impact on reproductive efficiency too (PEREIRA et al., 2013c).

The negative effects of heat stress on P/AI are associated with negative effects on oocyte competence (DE S TORRES-JÚNIOR et al., 2008, RISPOLI et al., 2013) fertilization (HACKBART et al., 2010), early embryo development (HANSEN and ARÉCHIGA, 1999, WOLFENSON et al., 2000, HANSEN et al., 2001), and embryo development after d7 (BIGGERS et al., 1987, RYAN et al., 1993, DEMETRIO et al., 2007). It is well-established that heat stress can reduce P/AI in lactating cows (VASCONCELOS et al., 2006, VASCONCELOS et al., 2011a, VASCONCELOS et al., 2011c). However, higher milk production appears to increase the susceptibility to heat stress. VASCONCELOS et al., (2011a) detected that increased milk production only impaired pregnancy maintenance in recipient dairy cows when associated with increase in rectal temperatures.

There is a close correlation (r = 0.88) between dry matter intake and milk production in lactating dairy cows (HARRISON et al., 1990). In lactating cows, a continuous high plane of nutrition appears to chronically elevate liver blood flow and the metabolic clearance rate of circulating P4 and E2 concentration (SANGSRITAVONG et al., 2002). This may be related to many of the changes in reproductive measures as the reduced average duration of estrus (less than 8 h) (LOPEZ et al., 2004), reduced embryo quality on d5 post AI (SARTORI et al., 2002), conception rate lower in lactating cows (generally 25–40%) than heifers (60–75%; WILTBANK et al., 2006), pregnancy loss higher in lactating cows than heifers (SANTOS et al., 2004)

and twinning rate in dairy cows is higher than in heifers (RYAN AND BOLAND, 1991).

Timed artificial insemination protocols

Currently there are two major types of pharmaceutical approaches to synchronize ovulation in dairy cattle (WILTBANK et al., 2011a, WILTBANK AND PURSLEY, 2014). The first approach uses gonadotropin releasin hormone (GnRH) at the beginning and end of the protocol based on the classic Ovsynch protocol (PURSLEY et al., 1995). The second approach uses a P4 implant combined with 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., 2009a, BARUSELLI et al., 2012). The underlying goals for these two pharmaceutical approaches are similar: synchronization of the follicular wave and corpus luteum (CL) function in order 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 two approaches are only beginning to be appreciated (VASCONCELOS et al., 2011a, 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 50-65% of cows ovulate after GnRH treatment administered at a random stage of the estrous cycle (VASCONCELOS et al., 1999b, GALVAO AND SANTOS, 2010, GIORDANO et al., 2012a) and cows that do not ovulate after first GnRH treatment generally have reduced fertility (THATCHER et al., 2002, GIORDANO et al., 2013a).

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 3-5 d later (BO et al., 1995, CAVALIERI et al., 2003, SOUZA et al., 2009a). The E2/P4-based protocols also do not have 100% efficiency in synchronizing follicular wave emergence (SOUZA et al., 2009b), although detailed ovarian studies with large groups of cows are still needed. Nevertheless, these

two protocols may be acting on two different sizes of follicles (WILTBANK et al., 2011a); large dominant follicles ovulate after GnRH whereas small follicles regress after the FSH suppression induced by E2.

A variety of methods have been evaluated to increase fertility during synchronization of ovulation programs including: increasing circulating P4 concentration during ovulatory follicle development (BISINOTTO et al., 2010c, MARTINS et al., 2011a, WILTBANK et al., 2011b), increasing length of proestrus (PETERS AND PURSLEY, 2003, PERES et al., 2009, BRIDGES et al., 2010), reducing follicle age (CERRI et al., 2009, SANTOS et al., 2010a), supplementing estrogen (**E2**) during proestrus (CERRI et al., 2004, BRUSVEEN et al., 2009a, SOUZA et al., 2011b) or increasing circulating P4 concentration after AI (LONERGAN, 2011). It has been suggested that these programs improve fertility by improvements in follicle/oocyte and/or oviductal/uterine environmental effects (CERRI et al., 2004, CERRI et al., 2011). In addition to these physiological mechanisms, some of these methods may simply be improving the percentage of cows that are synchronized during the programs (GIORDANO et al., 2012b).

Progesterone concentration during follicular growth before AI

Advantages of TAI are to breed the cows independent of estrous cycle phase and ovulatory condition. However, about 30% of postpartum cows lack a corpus luteum (CL) when synchronization protocol is initiated (STEVENSON et al., 2008, BISINOTTO et al., 2010a, BISINOTTO et al., 2013) and between 15 - 46% of cows lack a CL at pregnancy diagnosis and resynchronization, depending of the moment of pregnancy diagnosis (FRICKE et al., 2003, STERRY et al., 2006, SILVA et al., 2009). When those cows are submitted to TAI protocols, they have reduced circulating P4 concentration during follicular development (SOUZA et al., 2008, BISINOTTO et al., 2010a, DENICOL et al., 2012, BISINOTTO et al., 2013) with result in reduced P/AI (BISINOTTO et al., 2010a, BISINOTTO et al., 2010b).

Circulating P4 concentrations represent a balance between the production of P4, primarily by the corpus luteum (CL), and the metabolism of P4, primarily by the liver (WILTBANK et al., 2014). If P4 production is increased by an increase in luteal tissue

without a change in liver blood flow, then circulating P4 concentration will increase. Conversely, if there is an increase in liver blood flow (e.g. from 1000 to 2000 L/h) then there will be a corresponding decrease in circulating P4 concentration even though P4 production has not been altered (WILTBANK et al., 2014). CUNHA et al. (2008) tested the effects of elevated circulating P4 concentration on fertility on timed AI during the Double Ovsynch programme, cows (n=564) were randomly assigned to have either high or low circulating P4 concentration during the Ovsynch protocol. The cows with low circulating P4 concentration before AI had much lower fertility (37.1% pregnant on Day 29 pregnancy diagnosis) compared with cows with high circulating P4 concentration(51.0%; $P < 0.01$).

Substantial improvements in embryo quality (RIVERA et al., 2011) and P/AI (BISINOTTO et al., 2010b, DENICOL et al., 2012, BISINOTTO et al., 2013) are associated with higher circulating P4 concentrations during follicular development (SOUZA et al., 2008, BISINOTTO et al., 2010a, DENICOL et al., 2012, BISINOTTO et al., 2013). BISINOTTO et al. (2010a) evaluated circulating P4 concentration 7 days before the first GnRH and at the time of the first GnRH of Ovsynch. Cows were classified as anovular at beginning Ovsynch, or with high circulating P4 or low P4 concentration. Cycling cows that began Ovsynch with high circulating P4 concentration had higher numbers of P/AI (43.0%) than cows that had low circulating P4 concentration (31.3%) or that were anovular (29.7%) at the time of initiation of Ovsynch.

The E2/P4 protocols generally use E2-benzoate (EB) and treatment with a intravaginal P4 implant at the start of the protocol without ovulation of the dominant follicle. The circulating P4 concentration at time of PGF are reduced in E2/P4 protocols compared to GnRH protocols (E2 - 2.29 ± 0.15 ng/mL; GnRH - 2.89 ± 0.15 ng/mL; VASCONCELOS et al., 2011b) and have reduced proportion of cows with CL at time of PGF (5dCosynch = 73.6% [597] vs. E2/P4 = 44.3% [593]) compared to GnRH protocols (PEREIRA et al., 2013a). BISINOTTO et al., (2013) evaluated the P4 supplementation with 2 CIDR devices in a GnRH 5-d timed AI program (d −8 GnRH, d −3 and −2 PGF2α, d 0 GnRH and AI). The treatment with 2 CIDR increased P4 concentration in plasma from 0.51 to 2.65 ng/mL and restored fertility in lactating dairy

cows lacking a CL at the initiation of the timed AI program, similar to that of cows in diestrus. These data suggests that strategies to improve circulating P4 concentration during E2/P4 protocols might improve P/AI.

In superstimulated cows RIVERA et al., (2011) showed that higher circulating P4 concentration during superstimulation protocol increased the subsequent quality of embryos collected on Day 7 after estrus. Superstimulation protocols began during the second follicular wave (high P4), during the first follicular wave (low P4) or during the first follicular wave with P4 supplementation using two progesterone-releasing devices to increase circulating P4 concentration. Although the total number of structures that were collected (embryos and oocytes) did not differ between groups, the percentage of transferable embryos was lower for cows superstimulated during the first follicular wave (55.9%), compared to during the second follicular wave (88.5%) or during the first follicular wave with P4 supplementation (78.6%). Similarly, NASSER et al., (2011) reported a dramatic increase in transferable embryos in beef cattle when P4 was supplemented during superstimulation of the first follicular wave.

Follicle dominance period

In Ovsynch programs, cows that ovulate to the first GnRH are more likely to have a synchronized ovulation at the end of the protocol (VASCONCELOS et al., 1999a, RUTIGLIANO et al., 2008), and greater P/AI (RUTIGLIANO et al., 2008). The ovulatory response to GnRH is dependent on phase of the estrus cycle with an average ovulation rate of 64% in lactating dairy cows at arbitrary days of the estrous cycle (VASCONCELOS et al., 1999a). Ovulation to the first GnRH may improve fertility not only by increasing synchronization rate, but also by reducing the period of follicle dominance in dairy cows, which might benefit fertility (CERRI et al., 2009, SANTOS et al., 2010a).

Increasing period of follicular dominance has been associated with reduced fertility and reduced embryo quality on d7 after AI (CERRI et al., 2009; TOWNSON et al., 2002; BLEACH et al., 2004). SANTOS et al., (2010) reported that the 5-d Co-Synch program with TAI at 72 h after PGF resulted in greater P/AI compared to a 7-d Co-Synch protocol, although cows in the 5-d Co-Synch protocol had smaller ovulatory

follicles, reduced peak circulating E2 concentrations, and a smaller proportion of cows in estrus at AI compared with cows treated with the 7-d Co-Synch protocol.

The E2/P4-based programs would also be expected to reduce the period of follicular dominance with emergence of a new follicular wave on average 3.9 days after the initial EB and P4 treatment. These programs have a reported efficiency of synchronizing follicular wave emergence of 84.4% (SOUZA et al., 2009b), suggesting that follicle emergence and subsequent age of the ovulatory follicle may be well controlled with these programs.

Ahmad et al (1995) evaluated fertilization and early embryo development in beef cows with a device that produced subluteal circulating P4 concentration for 7 d after the regression of the CL. Treated cows ovulated an older and larger follicle than non-treated cows and had a lower percentage of embryos developing to 16-cell stage or greater. A possible cause of reduced fertility in persistent follicles is that oocite germinal vesicle breakdown occurred prematurely before ovulation (REVAH AND BUTLER, 1996, MIHM et al., 1999). Subsequent research has indicated that lactating dairy cows that are bred to estrus may also ovulate larger than normal follicles and that extended duration of follicular dominance is associated with reduced fertility (BLEACH et al., 2004).

Proestrus

The proestrus begins at the regression of a functional CL and is characterized by decrease in circulating P4 concentration and increase of E2 from the preovulatory follicles. Estradiol, in the relative absence of progesterone, acts on the hypothalamus to induce estrous behavior (ALLRICH, 1994) which determines the end of proestrus and begging of estrus (PETER et al., 2009).

A number of studies have evaluated the effects of different intervals between initiation of CL regression and AI on fertility during synchronization programs in beef (MENEGHETTI et al., 2009, PERES et al., 2009, BRIDGES et al., 2010) and dairy cattle (PETERS AND PURSLEY, 2003, RIBEIRO et al., 2012b, PEREIRA et al., 2013b). For example, in dairy cows there was a significant ($P < 0.01$) linear trend of increasing P/AI with increased interval between PGF treatment and GnRH treatment (8.8% at 0h; 13.2% at 12 h; 21.4% at 24 h; 28.0% at 36 h).

In *bos taurus* beef cattle, a summary of results from various studies (BRIDGES et al., 2010) suggested that fertility increased as time from PGF until GnRH increased. Similarly, in *bos indicus* beef cattle synchronized with E2 and P4, treatment with PGF 4 d before TAI produced an increase in P/AI compared to PGF treatment 2 d before TAI (time of CIDR withdraw and estradiol cypionate [**ECP**] treatment). Similarly, a study with suckled *bos indicus* beef cattle with CL 4 d before TAI, reported an increase in P/AI (50.3 vs. 36.1%) when the PGF was given 4 d before TAI rather than 2 d before TAI (Meneghetti et al., 2009). In a study with nonlactating *bos indicus* beef cattle (PERES et al., 2009), treatment with PGF 4 d before TAI resulted in lower circulating P4 concentration on the day of CIDR removal, a larger follicle at TAI, increased percentage of cows that ovulated to the protocol (85.4% vs. 77.0%), and increased P/AI (52.0 vs. 36.4% for all cows; 60.9 vs. 47.2% for cows that ovulated), compared to cows treated with PGF 2 d before TAI.

In grazing dairy cows that were presynchronized with 2 PGF treatments (Presynch), increasing the interval between PGF and GnRH + TAI from 58 to 72 h increased P/AI in a 5 d CoSynch TAI protocol (RIBEIRO et al., 2012b). Recent studies from our laboratory evaluated the interval from PGF until TAI in lactating dairy cattle synchronized with an E2/P4-TAI protocol (PEREIRA et al., 2013b). Consistent with the results of previous studies in beef cattle, an increased interval between PGF and TAI (2 d vs. 3 d) increased P/AI in cows that received TAI (19.2 vs. 30.0% at 60 d pregnancy diagnosis) with a more subtle effect in cows that received timed embryo transfer (33.5 vs. 37.9%). The earlier PGF treatment (3 d) resulted in a greater proportion of cows with low circulating P4 concentration (≤ 0.09 ng/mL) at TAI. Reduced circulating P4 concentration at TAI were associated with increased fertility, as has been observed in previous studies using GnRH-based TAI protocols (SOUZA et al., 2007, MARTINS et al., 2011a, GIORDANO et al., 2012b). In addition, reducing interval between PGF and induced ovulation decreased E2 concentrations near TAI, increased short luteal phases, and decreased circulating P4 concentration after AI (VASCONCELOS et al., 2001, BRIDGES et al., 2010). Thus, the improved fertility with a longer interval between luteolysis and induction of ovulation could be related to a greater time for CL regression and thus lower P4 at TAI or, alternatively, greater time for follicle growth, increased ovulatory follicle size, and increased E2 before synchronized ovulation.

In another study, (PEREIRA et al., 2014a), increasing the length of P4 treatment from 8 to 9 days in an E2/P4-based TAI protocol, increased expression of estrus and reduced pregnancy losses between 30 and 60 days. Although there was no overall effect of protocol length on P/AI, there were clear effects of size of the ovulatory follicle and expression of estrus on P/AI. The results indicate that improvements in fertility and reductions in pregnancy loss are likely in E2/P4-based TAI protocols by optimizing the ovulatory follicle diameter, increasing expression of estrus, and optimization of proestrous hormonal environment.

Progesterone concentration near TAI

Inadequate luteolysis can result in increased circulating P4 concentration near AI and a reduced fertility. This is clearly a problem with some cows during timed AI programmes (SOUZA et al., 2007, BRUSVEEN et al., 2009b, PEREIRA et al., 2013b), and may be also a problem in AI programmes based on detection of estrus (DE SILVA et al., 1981, WALDMANN et al., 2001, GHANEM et al., 2006).

In timed AI programmes these problems have been extensively studied during the last few years (WILTBANK et al., 2012). The percentage of cows that do not have complete luteal regression following the PGF treatment of Ovsynch protocol has been reported to range from 5% to 30% (SOUZA et al., 2007, BRUSVEEN et al., 2009b, MARTINS et al., 2011a). A recent study (MARTINS et al., 2011a) evaluated luteolysis at 56, 72 and 96 h after PGF treatment in Ovsynch protocols in first AI, or second and later AI cows. The authors define complete luteolysis when circulating P4 concentration was lower than 0.5 ng/mL. At first AI, 79% of cows underwent complete luteolysis, whereas at second and later AI, 71% underwent complete luteal regression ($P = 0.03$), suggesting that reduced fertility at later inseminations may be partially caused by incomplete luteolyis.

A number of previous studies with Ovsynch-type programs have evaluated the correlation between circulating P4 concentration near the time of AI on fertility. For example SOUZA et al., (2007) evaluated circulating P4 concentration 48 h after the $PGF_{2\alpha}$ treatment of Ovsynch and reported that circulating P4 concentration above 0.5 ng/mL reduce P/AI in 50%. Other researchers have evaluated this value at the time of the second GnRH treatment of Ovsynch (i.e. 56 h after the $PGF_{2\alpha}$ treatment) and

reported values of 0.4 ng/mL (BRUSVEEN et al., 2009a, GIORDANO et al., 2012b) or 0.5 ng/mL (MARTINS et al., 2011a, MARTINS et al., 2011b). Similar to our study, SANTOS et al., (2010a) evaluated the critical circulating P4 concentration using receiving operating characteristic curves (**ROC**) 72 h after PGF treatment and reported a value of 0.24 ng/mL. In E2/P4 protocols, we recently reported that cows with circulating P4 concentration above 0.1 ng/mL near the time of AI had substantial reductions in fertility (≤ 0.09 ng/ml = 34.1% vs. ≥ 0.1 ng/mL = 20.8%; PEREIRA et al., 2013b). Nevertheless, none of these previous studies reported values as low as 0.1 ng/mL as inhibitory to fertility. The use of ECP rather than GnRH to induce ovulation may underlie our observed effect of circulating P4 concentration on fertility at only 0.1 ng/mL. It is possible that cows with P4 > 0.1 ng/mL have a lower ovulation rate or later ovulation in response to the ECP compared to the GnRH treatment.

Thus, complete luteolysis in response to the PGF treatment, as determined by circulating P4 concentration near the time of AI, is likely to be critical for fertility in both types of protocols. In order to increase likelihood of luteolysis in Ovsynch-type protocols, researchers have increased the dose of PGF (GIORDANO et al., 2013b) or the number of PGF treatments (BRUSVEEN et al., 2009b, SANTOS et al., 2010b, RIBEIRO et al., 2012a) at the end of the protocol.

There may be multiple physiological mechanisms that result in the reduced fertility when circulating P4 concentration is elevated near AI. First, P4 may alter spermatozoon or oocyte transport by altering uterine or oviducal contractility, and thus reduce fertilization (HUNTER, 2005). Second, addition of P4 to *in vitro fertilization* reduced the blastocyst rate (SILVA et al., 1999), suggesting that there may be direct effects of P4 on subsequent embryo development. This detrimental effect was reversed with a P4 receptor antagonist (mifepristone – RU486), indicating a specific role for P4 receptors in this action. Elevated P4 *in vitro* also increased total a-inhibin production by the cumulus–oocyte complex, which may reduce embryo development after cleavage (SILVA et al. 1999). Further, the reduced endometrial thickness with slight elevations in circulating P4 concentration (SOUZA et al., 2011a) may indicate other major effects of P4 on the uterus that could result in reduced embryo development. In addition, it seems possible that P4 near the time of AI may alter gamete transport and possibly

reduce fertilization. A study in rats demonstrated that E2 facilitated sperm migration into the oviduct and P4 antagonized this effect (ORIHUELA et al., 1999).

Progesterone concentration post AI

Although there is unequivocal evidence that there is an absolute requirement for circulating P4 concentration in pregnancy maintenance (INSKEEP, 2004), higher circulating P4 concentration after AI is associated with higher fertility in TAI (DEMETRIO et al., 2007, PEREIRA et al., 2014a), but not in TET (DEMETRIO et al., 2007). Lower concentrations of circulating P4 after AI may reduce the embryonic development and survival rate (MANN AND LAMMING, 2001, GREEN et al., 2005). Progesterone controls the uterine environment and influences embryonic development (MANN AND LAMMING, 2001, GREEN et al., 2005, MANN et al., 2006). GREEN et al. (2005) showed that P4 also changes the oviductal environment and acts indirectly on initial embryo development. When MANN et al. (2006) studied P4 supplementation they observed that the insertion of an intravaginal P4 device between d 5 and 9 of the estrus cycle caused an increase in embryo length 16 d after AI. However, the P4 supplementation between d 12 and 16 did not increase the length of the embryo. DEMETRIO et al., (2007) observed that P/ET in recipients was not influenced by concentrations of P4, probably because they received an embryo that was already developed (grade 1 or 2).

More extensive modeling of P4 concentrations with pregnancy, using logistic regression, have demonstrated a relationship between circulating P4 on Days 5, 6 and 7 after AI and P/AI in dairy cows, and a relationship between the rate of P4 increase and P/AI (STRONGE et al. 2005). These authors reported that 60–85% of dairy cows had suboptimal circulating P4 for maintenance of pregnancy, based on absolute P4 concentrations during the early luteal phase or the rate of P4 increase.

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Chapter 2

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EFFECT OF ADDING A GNRH TREATMENT AT THE BEGINNING AND A SECOND PROSTAGLANDIN TREATMENT AT THE END OF AN ESTRADIOL-BASED PROTOCOL FOR TIMED AI IN LACTATING DAIRY COWS DURING COOL OR HOT SEASONS OF THE YEAR

ABSTRACT

Our hypothesis was that fertility could be increased in a timed AI (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 concentration during preovulatory follicle development and by using two prostaglandin (PGF) treatments at the end to decrease circulating P4 concentration near TAI. Lactating Holstein cows (n = 1,808) were randomly assigned during the cool or hot seasons of the year to receive TAI (d0) following one of three treatments: **I. Control:** CIDR + 2 mg of E2-benzoate on d -11, PGF on d -4, CIDR withdrawal + 1.0 mg of E2-cypionate on d -2, and TAI on d 0; **II. 2PGF:** Identical to Control protocol with addition of a second PGF treatment on d -2; **III. GnRH:** Identical to 2PGF protocol with addition of 100 µg GnRH treatment on d -11. Pregnancy diagnoses were performed d32 and d60 after TAI. The GnRH protocol increased percentage of cows with CL (Control = 56.9%; 2PGF = 55.8%; GnRH = 70.5%) and circulating P4 concentration at PGF (Control = 3.28 ± 0.22; 2PGF = 3.35 ± 0.22; GnRH = 3.70 ± 0.21 ng/mL), compared to Control and 2PGF protocols. 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 circulating P4 concentration (<3ng/mL) at the start of the protocol and not in cows that began the protocol with high P4. Further, there was an interaction for presence of CL at PGF 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.Season had major effects on many reproductive measures with cool season greater than hot season in percentage of cows with 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 AI (P/AI; 45.4 vs. 21.4%). 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: GnRH, 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., 2010a, Ribeiro et al., 2012b). Currently there are two major types of pharmaceutical approaches 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., 2009a, Baruselli et al., 2012). The underlying goals for these two pharmaceutical approaches are similar: synchronization of the follicular wave and corpus luteum (**CL**) function in order 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 two 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 50-65% of cows ovulate to a GnRH treatment administered at a random stage of the estrous cycle (Vasconcelos et al., 1999, Galvao 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., 2013a). 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 3-5 d later (Bo et al., 1995, Cavalieri et al., 2003, Souza et al., 2009a). The E2/P4-based protocols also do not have 100%

efficiency in synchronizing follicular wave emergence (Souza et al., 2009b), although detailed ovarian studies with large groups of cows are still needed. Of particular interest for this study, these two 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 two 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 (Wiltbank et al., 2011b, Pursley and Martins, 2012) 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, there are slight elevations in circulating P4 near the time of AI ($\geq$ 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., 2007a, 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. In order to increase likelihood of luteolysis in Ovsynch-type protocols, researchers have increased the dose of PGF (Giordano et al., 2013b) 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), 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 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 assure 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 if there were effects of season on 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 two PGF treatments at the end of the protocol would increase fertility of lactating dairy cows.

MATERIALS AND METHODS

This experiment was conducted at four 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 feedline. Cows were fed ad libitum a TMR based on corn silage and tifton hay as forages, with a

corn-soybean meal-based concentrate, 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 1808 lactating Holstein cows. At the beginning of the experiment (d -11), cows averaged 145 ± 2.1 days in milk (**DIM**), yielding 27.4 ± 0.2 kg of milk/d, with body condition score (**BCS**) of 2.78 ± 0.01 (in a 1 [emaciated] to 5 [obese] scale [(Wildman, 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 utilized and randomized into the study, without regard to whether they had been previously utilized in the study. Within each block, cows were randomly assigned to receive one of three treatments (Figure 1): (**I. Control** treatment) an intravaginal P4 insert containing 1.9 g of P4 (**CIDR**, Zoetis, São Paulo, Brazil), and 2.0 mg (i.m.) estradiol benzoate (**EB**, 2.0 mL of Estrogin, Farmavet, São Paulo, SP, Brazil) on d -11, 25 mg (i.m.) dinoprost tromethamine (PGF; 5.0 mL of Lutalyse, Zoetis, Brazil) on d -4, CIDR withdrawal and 1.0 mg (i.m.) of estradiol cypionate (0.5 mL of **E.C.P**., Zoetis, Brazil) on d -2, and TAI on d0; (**II. 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; (**III. 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 (Estrotect, Rockway, Inc, Spring Valley, WI) were placed on the tail head of cows at the time of CIDR removal. The estrotect 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 estrotect was monitored at TAI by the research personnel and cows with an activated estrotect (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/month) compared to the cool season (20.9 mm/month).

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, d -4 and d7 to determine presence of a CL, and at d0 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 d0 that corresponded to an observed CL on d7. Cows with follicles < 8mm on d 0 but with a CL on d7 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 d7 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 32d or 60d 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 32d pregnancy diagnosis.

Sample collection

Milk production was measured daily between d0 to d7, and average daily production through this interval was used in the analysis. Blood samples were collected on d -11 (n =1453), d -4 (n = 1443) and d7 (n = 1630), by coccygeal venipuncture into commercial, 10 mL blood collection tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA). After bleedings, tubes were placed on ice immediately, maintained at 4°C for 12 h, and centrifuged at 1500 g for 15 min at room temperature (20 – 25ºC) 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, USA) 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.

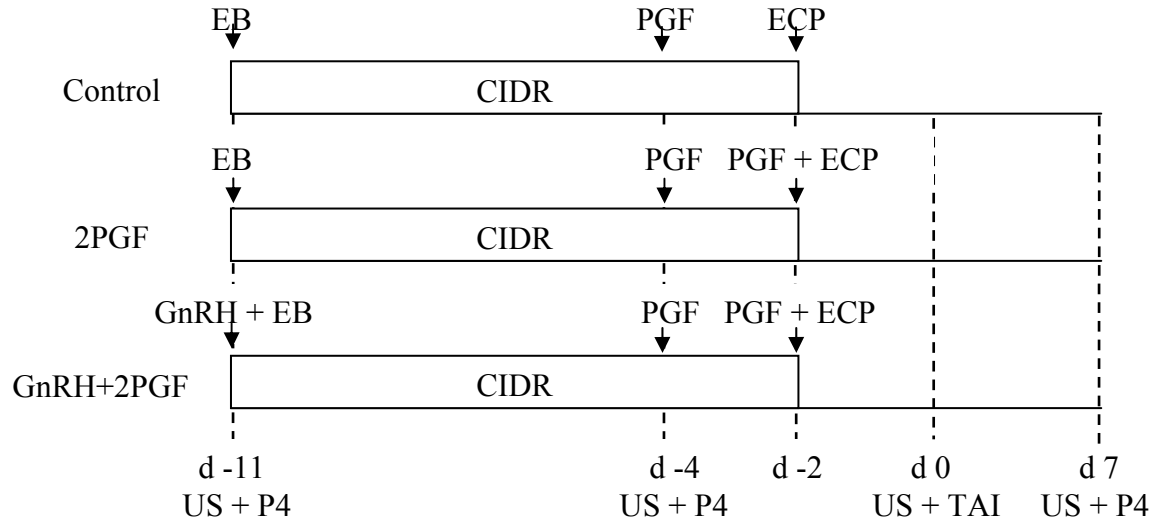

Figure 1. Diagram of activities: The ovaries were evaluated by ultrasound (US) in all cows on d -11, d -4, d0 and d7. On d -11 cows were randomly assigned to begin the protocols: (Control) CIDR and 2.0 mg EB, seven d later PGF, 48 h later the CIDR was removed and cows received 1.0 mg of ECP, 48 h after CIDR removal TAI was performed; (2PGF) was the same as Control protocol but cows received another PGF treatment on d -2; (GnRH+2PGF) was the same as 2PGF protocol, but cows received a GnRH and EB on d -11. 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).

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 d32 and d60, and pregnancy losses from 32 to 60d) were analyzed using the GLIMMIX procedures of SAS (SAS Institute Inc., Cary, NC, USA) 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, d -4 and d7, 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 if each individual measurement influenced pregnancy rates linearly, quadratically, or cubically. The LOGISTIC procedure was used to determine the intercept and slope(s) 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 square means ± SEM 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

There were dramatic effects of season on many of the physiological and fertility measures (Table 1). There were more cows with a CL at PGF ($P = 0.01$) and 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 hot season, although circulating P4 on d7 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 two-fold greater ($P < 0.01$) in the cool than hot season at either the 32 or 60d pregnancy diagnoses, although pregnancy loss between 32 and 60d 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). There were also differences between seasons for days in milk (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 body condition score (cool = 2.76 ± 0.01 vs. hot = 2.80 ± 0.01; P <0.01). There was no differences in parity of cows that were enrolled in the study between season (P = 0.73). There were also no interactions between season and DIM (P = 0.92), BCS (P = 0.80), milk production (P = 0.52), or parity (P = 0.48) on P/AI at 60d.

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

	Season		
*Item	Cool	Hot	*P* Value
Ovulation of d -11 follicle[1]	19.6 (168/857)	17.7 (109/617)	0.35
New CL at PGF[2]	27.8 (238/857)	23.8 (147/617)	0.09
CL at PGF[3]	62.9 (539/857)	56.2 (347/617)	0.01
P4 d -11 (ng/mL)[3]	3.05 ± 0.09	3.27 ± 0.11	0.13
P4 d -4 (ng/mL)[3]	3.39 ± 0.09	3.51 ± 0.10	0.41
Follicle Diameter d0 (mm)[4]	15.7 ± 0.1	14.8 ± 0.1	<0.01
P4 d7 (ng/mL)[4]	3.42 ± 0.06	3.33 ± 0.07	0.35
Estrus[3]	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[4]	17.5 (129/736)	24.2 (166/685)	<0.01
P/AI for all cows[1]			
32d	45.4 (389/857)	21.4 (203/951)	<0.01
60d	37.0 (317/857)	18.0 (171/951)	<0.01
Pregnancy Loss (32-60)	18.5 (72/389)	15.8 (32/203)	0.41
P/AI for synchronized cows[4]			
32d	50.5 (372/736)	25.3 (173/685)	<0.01
60d	41.3 (304/736)	20.9 (143/685)	<0.01
Pregnancy Loss (32-60)	18.3 (68/372)	17.3 (30/173)	0.79

* Least square means (n./n.)
[1] Percentage of cows with a new CL on d -4 that had a follicle >8 mm on d -11; [2] Percentage of cows with a new CL on d -4 independent of presence of follicle >8 mm on d -11; [3] All inseminated cows; [4] Includes only cows that ovulated to ECP (visible CL on d7)

There were no differences between Control, 2PGF, and GnRH protocols for lactation number (2.11 ± 0.42 *vs.* 2.09 ± 0.42 *vs.* 2.01 ± 0.42; P = 0.41), days in milk (140 ± 7.3 *vs.* 150 ± 7.3 *vs.* 144 ± 7.3 d; P = 0.15), body condition score (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.

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

	Protocol			
*item	Control	2PGF	GnRH	*P* Value
Ovulation of d -11 follicle[1]	11.1 (55/494)[b]	9.0 (44/488)[b]	36.2 (178/492)[a]	<0.01
New CL at PGF[2]	19.2 (95/494)[b]	16.4 (80/488)[b]	42.7 (210/492)[a]	<0.01
CL at PGF[3]				
Cool	59.0 (167/283)[b]	56.5 (160/283)[b]	72.9 (212/291)[a]	<0.01
Hot	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 (ng/mL)[4]	3.28 ± 0.22[b]	3.35 ± 0.22[b]	3.7 ± 0.21[a]	0.01
P4 d7 (ng/mL)[4]	3.42 ± 0.08[xy]	3.44 ± 0.08[x]	3.27 ± 0.08[y]	0.22
Follicle at AI (mm)[4]	15.2 ± 0.2	15.4 ± 0.2	15.4 ± 0.2	0.61
Estrus[3]				
Cool	87.6 (248/283)	86.9 (245/282)	85.6 (249/291)	0.76
Hot	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[4]				
Cool	87.4 (236/270)[y]	90.1 (245/272)[xy]	91.4 (255/279)[x]	0.30
Hot	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[4]				
Cool	23.3 (55/236)[b]	18.8 (46/245)[b]	11.0 (28/255)[a]	<0.01
Hot	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

* Least square means (n./n.); [a,b] in the same line = P <0.05; [x, y] In the same line = P <0.1
[1]Percentage of cows with a new CL on d -4 that had a follicle >8 mm on d -11; [2] Percentage of cows with a new CL on d -4 independent of presence of follicle >8 mm on d -11; [3]All inseminated cows; [4]Includes only cows that ovulated to ECP (visible CL on d7)

There were more (P <0.01) cows that ovulated and had a new CL at PGF in the GnRH protocol than in Control or 2PGF protocols. There were more (P <0.01) cows with a CL at PGF in the GnRH protocol than in the other two 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 d7 than 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 to 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 there was more than two-fold increase (26.5 *vs.* 11.0%; $P < 0.01$) in double ovulation during the hot compared to cool season.

Table 3 shows the effects of treatment protocol on fertility (P/AI) in cows during the cool or hot season. At the 32d pregnancy diagnosis, there was an effect ($P = 0.02$) of treatment, with the GnRH protocol having greater fertility ($P < 0.01$) than Control and tending to have ($P = 0.09$) 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 60d 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 32d 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 60d 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 ± 0.25 vs. GnRH = 3.04 ± 0.25 ng/mL). Cows were classified by the P4 concentration on d -11 as either low P4 (<3.0ng/mL 49% of the animals [716/1453]) or high P4 (≥3.0 ng/mL 51% of the animals [737/1453]), 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 there were almost no ovulations or new CL in the Control and 2PGF groups. In all three protocols, there were more (P <0.01) ovulations and new CL 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 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 d7 did not substantially differ by treatment group and circulating P4 at the start of the protocols.

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

	Protocol			
*item	Control	2PGF	GnRH	*P* Value
P/AI 32d for all cows[1]				
Cool	41.0 (116/283)^{b}	44.2 (125/283)^{y}	50.9 (148/291)^{ax}	0.05
Hot	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 60d for all cows[1]				
Cool	32.9 (93/283)^{b}	36.4 (103/283)^{ab}	41.6 (121/291)^{a}	0.09
Hot	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)^{a,b}	31.0 (179/595)^{a}	0.06
P/AI 32d for synchronized cows[2]				
Cool	46.6 (110/236)^{b}	49.0 (120/245)^{ab}	55.7 (142/255)^{a}	0.11
Hot	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)^{ax}	0.05
P/AI 60d for synchronized cows[2]				
Cool	37.7 (89/236)^{y}	40.4 (99/245)^{xy}	45.5 (116/255)^{x}	0.20
Hot	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

* Least square means (n./n.) ^{a,b} in the same line = *P* <0.05; $^{x, y}$ In the same line = *P* <0.1
[1] All inseminated cows, [2] Includes only cows that ovulated to ECP (visible CL on d7)

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

	Protocol			
*item	Control	2PGF	GnRH	*P* Value
Ovulation at d -11[1]				
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[2]				
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[3]				
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 (mm)[4]				
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[4]				
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 d7 (ng/mL)[4]				
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	

* Least square means (n./n.); [a,b] in the same line = $P < 0.05$; [x, y] In the same line = $P < 0.1$

[1] Percentage of cows with a new CL on d -4 that had a follicle >8 mm on d -11; [2] Percentage of cows with a new CL on d -4 independent of presence of follicle >8 mm on d -11; [3] All inseminated cows; [4] Includes only cows that ovulated to ECP (visible CL on d7)

Fertility differences (at 60d 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 to 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 *vs.* low P4 on d -11 continuing to be observed in GnRH-treated cows during the hot season.

Table 5. Effects of treatment protocol divided by P4 concentration on d -11 on pregnancies per AI (P/AI) at d60 pregnancy diagnosis.

	Protocol			
*item	Control	2PGF	GnRH	*P* Value
P/AI 60d for all cows[1]				
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 P/AI 60d[1]				
P4 <3.0	28.3 (43/152)[b]	35.3 (48/136)[y]	43.3 (65/150)[ax]	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 P/AI 60d[1]				
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 60d for synchronized cows[2]				
P4 <3.0	27.0 (54/200)[b]	32.8 (60/183)[y]	41.1 (83/202)[ax]	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 P/AI 60d[2]				
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 P/AI 60d[2]				
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	

* Least square means (n./n.); [a,b] in the same line = P <0.05; [x, y] In the same line = P <0.1

[1] All inseminated cows; [2] Includes only cows that ovulated to ECP (visible CL on d7)

Cows were classified based on presence or absence of CL on d -11 and d -4 (data not shown). There was no effect of treatment on P/AI in cows that had a CL on d -11 and d -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 d -4 (anovular cows) the 2 PGF (32.1%; 25/78) increased ($P = 0.05$) P/AI compared to Control (19.4%; 19/98). GnRH treatment (31.5%; 17/54) also tended to increase P/AI compared to Control ($P = 0.10$).

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 there was no effect of presence of a CL on follicle diameter in the hot season ($P = 0.55$). Classification of cows based on presence/ovulation of larger ($\geq$14 mm) or smaller (<14 mm) follicles (Table 6) showed that cows with larger follicles had greater ovulation to the ECP either in the presence or absence of a CL on d -4 and in the hot or cool season. However for cows with a smaller follicle (<14 mm) at TAI, cows with a CL present on d -4 had a greater percentage of cows that ovulated to the ECP than cows without a CL on d -4 during the cool ($P = 0.02$) but not hot ($P = 0.66$) season. The fertility, based on the 60d pregnancy diagnosis, was also affected by the ovulatory follicle diameter and presence of a CL on d -4 (Table 6). Cows with a CL on d -4 had greater fertility for cows ovulating a large follicle than a small follicle in both the cool ($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).

Table 6. Effects of season and follicle diameter at TAI (small or large follicles) on ovulation to ECP at the end of the protocol (CL present on d7), and pregnancies per AI (P/AI) based on 60d pregnancy diagnosis.

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

* Least square means (n./n.), [a,b] in the same line = $P < 0.05$; [x, y] In the same line = $P < 0.1$

[1] Includes all inseminated cows, [2] Includes only cows that ovulated to ECP (visible CL on d7)

Cows were classified based on presence or absence of CL on d -11 and d -4

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 pregnancy 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 in cows with no CL, there was a tendency ($P = 0.12$) in the cool season for ovulatory follicle diameter to have a negative effect on fertility. In the hot season, there was no effect of ovulatory follicle diameter on P/AI in cows without CL on d -4.

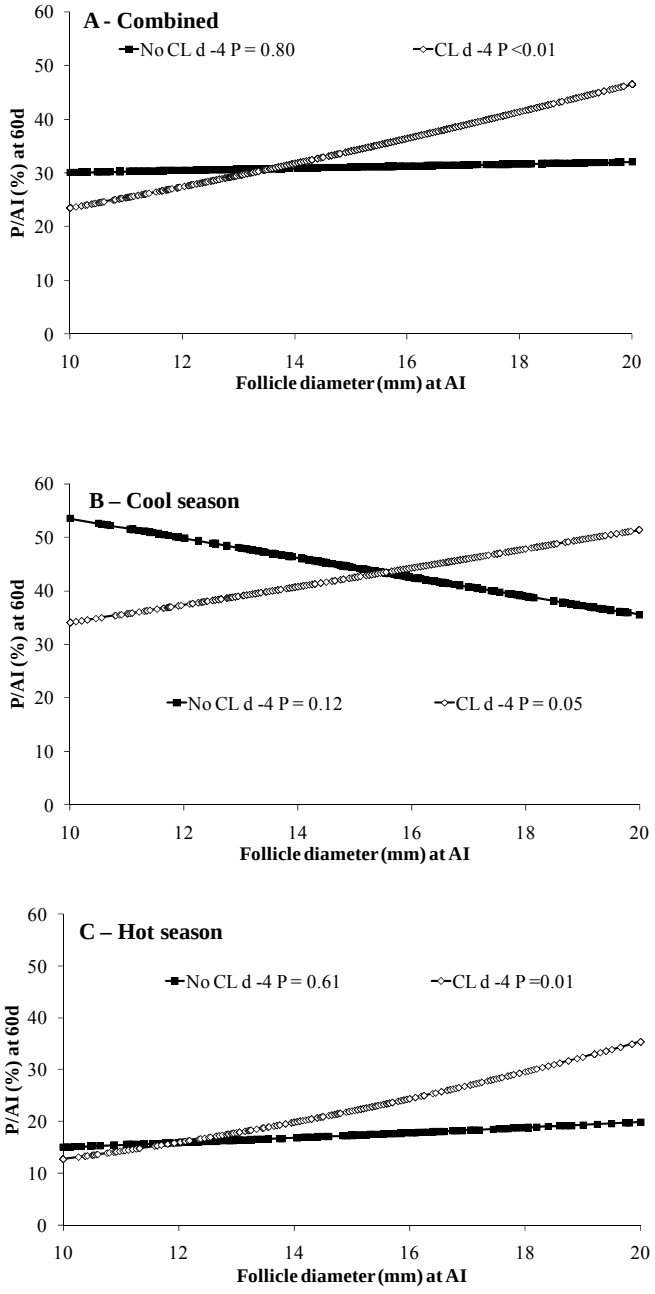

Figure 2. Effect of follicle diameter at time of AI (d0) in the presence or absence of a CL at time of first PGF (d -4) on P/AI at the 60d pregnancy diagnosis using only cows that ovulated to the ECP (CL detected on d7). All cows from both seasons are combined in Panel A, whereas only cows during cool season (Panel B) or during 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 cool ($P = 0.05$) or hot ($P = 0.01$) season.

Expression of estrus increased (P <0.01) the percentage of cows that ovulated to ECP (no estrus = 62.6% [171/273], estrus = 92.0% [1245/1354]). Expression of estrus was associated with increased (P <0.01) P/AI in cows that ovulated to ECP at both the 32d (no estrus = 26.3% [45/171], estrus = 40.1% [499/1245]) and 60d (no estrus = 22.2% [38/171], estrus = 32.8% [408/1245]) pregnancy diagnoses.

DISCUSSION

Protocols that synchronize ovulation using E2 and P4 are currently utilized in many parts of the world. The results of the present study provide practical knowledge into 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 there was no effect of treatment protocol on fertility. There were also subtle but potentially important changes in reproductive physiology during heat stress. 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 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 two 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 impact 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 d7 (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 to 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 to the Control perhaps due to 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 concentrations 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, Giordano et al., 2013b). 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 two CL would increase circulating 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 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 to 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 due to 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, Pereira et al., 2014). Nevertheless, expression of estrus did not explain the differences in fertility between the protocols since there were no differences in expression of estrus between the protocols. The results from this study are similar to other reports using E2/P4 protocols (Souza et al., 2009a, Pereira et al., 2013d), less than studies using this E2/P4 protocols only in cyclic cows during the cool time of the year (Pereira et al., 2014b), but greater than observed in studies using E2/P4 protocols during summer (Pereira et al., 2013c).

Related to the potential economic impact of additing a GnRH treatment and a second PGF treatment, there was an increase in protocol cost of about ~82% per protocol, from $5.85 for the control protocol to $10.67 for the GnRH protocol. Using semen prices of $12.37/straw, the 60d 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/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.

CONCLUSION

In conclusion, use of GnRH with EB 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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Chapter 3

This paper will be submitted to J. Dairy Sci.

COMPARISON OF TWO STRATEGIES TO INCREASE CIRCULATING P4 CONCENTRATION DURING FOLLICULAR DEVELOPMENT IN LACTATING DAIRY COWS INSEMINATED WITH ESTROGEN/PROGESTERONE BASED PROTOCOLS

ABSTRACT

We hypothesized that the 2 CIDR protocol would have similar results on circulating P4 concentration at PGF, ovulation to ECP, P/AI, and pregnancy losses. Lactating Holstein cows (n = 1,638) were randomly assigned to receive TAI (d0) following one of two treatments: (I. GnRH treatment) CIDR+ 2 mg of E2-benzoate + 100 μg GnRH on d -11, PGF on d -4, CIDR withdrawal + 1.0 mg of E2-cypionate + PGF on d -2, and TAI on d 0; (II. 2CIDR treatment) Two CIDR + 2mg of E2-benzoate on d -11, one CIDR withdraw + PGF on d -4, second CIDR withdrawal + 1.0 mg of E2-cypionate + PGF on d -2, and TAI on d 0. Pregnancy diagnoses were performed d32 and d60 after TAI. There was no effect of treatments ($P > 0.10$) on fertility regardless of cow temperature, BCS, parity, milk yield and presence or absence of a CL on d -11 or d -4. Some physiological measurements associated with greater fertility were reduced in cows with elevated temperature (≥ 39.1ºC) as percentage of cows with CL at PGF decreased 5.4%, ovulatory follicle diameter decreased 0.51 mm, expression of estrus decreased 4.7%, and ovulation to the ECP decreased 2.5% compared to cows with reduced temperature (< 39.0ºC), with result in 13.4% decrease ($P < 0.01$) in P/AI and tended to increase ($P = 0.09$) the pregnancy loss between 32 and 60d. There were more ($P < 0.01$) cows with a CL at PGF in the GnRH (< 39.0ºC = 78.1% [314/402]; ≥ 39.1ºC = 70.9% [256/361]) protocol than in 2CIDR protocol (< 39.0ºC = 58.8% [244/415]; ≥ 39.1ºC = 54.6% [190/348], however, circulating P4 concentration was greater ($P = 0.05$) at the time of PGF treatment (d -4) for cows treated 2CIDR (< 39.0ºC = 4.34 ± 0.13; ≥ 39.1ºC = 4.17 ± 0.14 ng/mL) than GnRH (< 39.0ºC = 4.12 ± 0.14; ≥ 39.1ºC = 3.84 ± 0.14 ng/mL), independent of cow temperature. The result of this study shows that the protocols have similar results on circulating P4 concentration at PGF, ovulation to ECP and P/AI.

Key words: protocols, progesterone, heat stress

INTRODUCTION

In postpartum cows, about of 30% of cows lack a corpus luteum (**CL**) at the beginning of the synchronization protocol (Stevenson et al., 2008, Bisinotto et al., 2010a, Bisinotto et al., 2013) and when resynchronization is performed at pregnancy diagnosis between 15 - 46% of cows lack a CL, depending of the moment of pregnancy diagnosis (Fricke et al., 2003, Sterry et al., 2006, Silva et al., 2009). These cows have reduced circulating progesterone (**P4**) concentrations during follicular development (Souza et al., 2008, Bisinotto et al., 2010a, Denicol et al., 2012, Bisinotto et al., 2013) with result in reduced pregnancy per artificial insemination (**P/AI**; Bisinotto et al., 2010b).

In GnRH-based protocols, increased circulating P4 concentration at the beginning of the protocol (Bisinotto et al., 2010b, Denicol et al., 2012, Bisinotto et al., 2013) and the presence of a functional CL at the time of prostaglandin **(PGF)** have been associated with improved fertility (Giordano et al., 2010, Martins et al., 2011). Similarly in estradiol (**E2**) and P4 based protocols, the presence of a CL at the time of PGF treatment is associated with greater fertility (no CL = 16.7% [335] vs. CL = 24.1% [257]; Pereira et al., 2013a). The E2/P4-based protocols result in lower circulating P4 concentrations at the time of PGF treatment (2.29 ± 0.15 ng/mL), than with GnRH protocols (2.89 ± 0.15 ng/mL; Vasconcelos et al., 2011a). Indeed, a direct comparison of cows treated with a GnRH protocol compared to an E2/P4 protocol found an increased proportion of cows with a CL at time of PGF (5dCosynch = 73.6% [n = 597] vs. E2/P4 = 44.3% [n = 593]). Increased circulating P4 concentration with GnRH-based protocol is likely due to ovulation in response to GnRH and formation of an accessory CL in some of the GnRH-treated cows.

In a recent study, Pereira et al., (2015a) evaluated the addition of GnRH at the beginning of an E2/P4 protocol for timed AI, and this protocol increased the circulating P4 concentration at PGF and P/AI (GnRH + EB = 50.9% vs EB = 41.0%). Bisinotto et al., (2013) evaluated the P4 supplementation with 2 CIDR inserts in a GnRH 5-d timed AI program (d −8 GnRH, d −3 and −2 PGF2α, d 0 GnRH and AI) and the treatment with 2 CIDR increased progesterone in plasma to 2.65 ng/mL and restored fertility in lactating dairy cows lacking a CL at the initiation of the timed AI program similar to

that of cows in diestrus. In a recent study from our group (unpublished) we evaluated the supplementation with 2 CIDR in E2/P4 protocols in cows with reduced circulating P4 concentration at the beginning of the protocol (< 1.0 ng/mL), and the 2 CIDR treatment improve the P/AI (1CIDR = 42.8% vs. 2 CIDR = 52.6%).

The objective of this study was to compare two strategies to improve circulating P4 concentration during follicular development in lactating dairy cows, adding GnRH or 2 CIDRs at the beginning of the protocol. We hypothesized that circulating P4 concentration at PGF, ovulation to estradiol cypionate (**ECP**), P/AI, and pregnancy losses, would be similar between these protocols in lactating dairy cows.

MATERIALS AND METHODS

This experiment was conducted at four 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 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 feedline. Cows were fed *ad libitum* a TMR based on corn silage and tifton hay as forages, with a corn-soybean meal-based concentrate, 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 1,638 lactating Holstein cows. At the beginning of the experiment (d -11), cows averaged 187.8 ± 13.5 days in milk (**DIM**), yielding 31.4 ± 2.23 kg of milk/d, with body condition score (**BCS**) of 2.94 ± 0.03 (in a 1 [emaciated] to 5 [obese] scale [(Wildman, 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. Within each farm, cows were divided in batch by parity (primiparous and multiparous), days in milk and milk production, all cows that were past the voluntary waiting period and not

pregnant were utilized and randomized into the study, without regard to whether they had been previously utilized in the study. Within each batch, cows were randomly assigned to receive one of two treatments: (**I. GnRH** treatment) an intravaginal P4 insert containing 1.9 g of P4 (**CIDR**, Zoetis, São Paulo, Brazil), 100 μg GnRH injection (i.m.; 2.0 mL of Cystorelin, Merial, SP, Brazil) and 2.0 mg estradiol benzoate (**EB**, i.m.; 2.0 mL of Estrogin, Farmavet, São Paulo, SP, Brazil) on d -11, 25 mg (i.m.) dinoprost tromethamine (PGF; 5.0 mL of Lutalyse, Zoetis, Brazil) on d -4, CIDR withdrawal, 1.0 mg (i.m.) of estradiol cypionate (0.5 mL of **E.C.P**., Zoetis, Brazil) and PGF on d -2, and TAI on d 0; (**II. 2CIDR** treatment) Two intravaginal P4 insert containing 1.9 g of P4 (**CIDR**, Zoetis, São Paulo, Brazil) and 2.0 mg estradiol benzoate (**EB**, i.m.; 2.0 mL of Estrogin, Farmavet, São Paulo, SP, Brazil) on d -11, 25 mg (i.m.) dinoprost tromethamine (PGF; 5.0 mL of Lutalyse, Zoetis, Brazil) on d -4, CIDR withdrawal, 1.0 mg (i.m.) of estradiol cypionate (0.5 mL of **E.C.P**., Zoetis, Brazil) and PGF on d -2, and TAI on d 0.

The TAI was performed (d 0) using commercial frozen-thawed semen from 36 different bulls. Devices for detection of estrus (Estrotect, Rockway, Inc, Spring Valley, WI) were placed on the tail head of cows at the time of CIDR removal. The Estrotect 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 Estrotect was monitored at TAI by the research personnel and cows with an activated Estrotect (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, d -4 and d 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). 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 < 8mm 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 d 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 d0 to d7, and average daily production through this interval was used in the analysis. Rectal temperature was measured using a digital thermometer (Jumbo Display Lab Thermometer; Delta Track CA, USA) at d 0 and d 7. Heat stress was defined as the average rectal temperature ≥ 39.1°C (< 39.1°C = no heat stress), given that 39.1°C is considered as a threshold for heat stress 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 d7 (n = 1,516), by coccygeal venipuncture into commercial, 10 mL blood collection tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA). After bleedings, tubes were placed on ice immediately, maintained at 4°C for 12 h, and centrifuged at 1500 g for 15 min at room temperature (20 – 25ºC) 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, USA) that had been previously validated in our laboratory. The intra assay coefficient of variation was 5.6% and the inter assay 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 (class of cow temperature, class of P4 concentration, ovulation to GnRH, estrus detection, ovulation to ECP, double ovulation, P/AI on d 32 and d 60, and pregnancy losses from 32 to 60 d) were analyzed using the GLIMMIX procedures of SAS (SAS Institute Inc., Cary, NC, USA) 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, 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, d -4 and d 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, and milk yield as covariables. The GLM procedure of SAS was used to determine if each individual measurement influenced pregnancy rates linearly, quadratically, or cubically. The LOGISTIC procedure was used to determine the intercept and slope(s) 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 ($P > 0.10$) were removed from the statistical model. The results are expressed as least square means ± SEM for continuous variables. In all analyses, differences were considered significant when $P < 0.05$, whereas differences between $P > 0.05$ and $P < 0.10$ were considered tendencies.

Receiver operating characteristic (ROC) curves were generated to determine the optimum concentration of P4 at PGF that resulted in the highest sensitivity and specificity for prediction of P/AI in dairy cows.

RESULTS

There were no differences between GnRH and 2CIDR protocols for lactation number (2.45 ± 0.36 vs. 2.41 ± 0.36; P = 0.46), days in milk (187.8 ± 13.5 vs. 187.8 ± 13.5; P = 0.99), body condition score (2.92 ± 0.03 vs. 2.95 ± 0.03; P = 0.26), milk yield (31.5 ± 2.23 vs. 31.3 ± 2.23; P = 0.63), CL presence at d -11 (67.3% [551/817] vs. 68.1% [560/821]; P = 0.74) and circulating P4 concentration at d -11 (2.92 ± 0.13 vs. 2.97 ± 0.13; P = 0.68).

Effects of cow temperature, BCS, parity, milk yield, CL at d -11 and d -4, P4 circulating P4 concentration at d -11, estrus expression and their interactions with

treatment on pregnancy at 60 d were evaluated following TAI (Table 1). There were no effects of treatment no matter how cows were classified using the various measures. For example, protocols not differ P/AI (P > 0.10) regardless of cow temperature, BCS, parity, milk yield, estrus expression and presence or absence of a CL on d -11 or d -4 and circulating P4 concentrations. There were effects or tendencies of some of these variables with lower P/AI in cows with higher temperature (P < 0.01), BCS < 2.75 (P = 0.09), in multiparous compared to primiparous (P < 0.01), CL absence on days -11 (P = 0.05) and -4 (P <0.01) compared to CL presence, and no estrus expression to the protocol (P < 0.01). In contrast, the circulating P4 concentration at d -11 (P = 0.16) and milk yield (P = 0.63) did not affected P/AI.

Table 1. Effects of variables on P/AI by treatment using d 60 pregnancy diagnosis.

	Protocol[1]		*P* =		
P/AI at 60 d*	GnRH	2CIDR	Protocol	Variable	Interaction
Cow temperature ºC					
< 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)			
≥ 2.75	28.6 (176/615)	31.0 (199/643)	0.44	0.09	0.91
Parity order					
Primiparous	34.1 (74/224)	35.1 (79/233)			
Multiparous	24.9 (152/593)	26.5 (160/588)	0.62	<0.01	0.89
Milk yield					
< 30	26.2 (107/406)	29.4 (120/407)			
≥ 30	29.1 (119/411)	28.7 (119/414)	0.52	0.63	0.44
CL presence on d -11					
No CL	25.7 (68/266)	24.7 (64/261)			
CL	28.7 (158/551)	31.2 (175/560)	0.75	0.05	0.46
CL presence on d -4					
No CL	18.2 (37/206)	20.6 (71/345)			
CL	31.8 (188/590)	35.9 (163/455)	0.18	<0.01	0.74
P4 d -11					
< 1.0	24.3 (72/297)	28.2 (83/294)			
≥ 1.0	30.0 (148/492)	29.1 (143/491)	0.52	0.16	0.30
Estrus expression					
No	9.2 (8/90)	5.1 (4/82)			
Yes	30.9 (216/701)	32.1 (228/712)	0.69	<0.01	0.46

*Least squares means, % (no./no.)

[1]Protocols: **GnRH** = controlled internal drug-release insert + 2 mg of estradiol benzoate + 100 µg GnRH on d −11, PGF on d −4, controlled internal drug-release withdrawal + 1.0 mg of estradiol cypionate + PGF on d −2, and timed AI on d 0; **2CIDR** = Two controlled internal drug-release inserts + 2 mg of estradiol benzoate on d −11, PGF on d −4, controlled internal drug-release withdrawal + 1.0 mg of estradiol cypionate + PGF on d −2, and timed AI on d 0;

The ovulation to the ECP treatment tended to be higher (P = 0.09) in cows with reduced temperature, although, treatment protocol did not alter ovulation to ECP (Table 02). There were no effects of treatment on P/AI at 32, 60 d and pregnancy loss. P/AI was greater (P < 0.01) for cows with reduced temperature at either the 32 or 60d pregnancy diagnoses. Pregnancy loss between 32 and 60 d tended (P = 0.09) to be lower in cows with lower temperature.

Table 2. Effects of treatments and cow temperature on ovulation to ECP, P/AI at 32, 60 d and pregnancy loss.

	Cow temperature ºC				
Item*	<39.1	≥39.1	Temp.	Prot.	Interaction
Ovulation to 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 32d for all cows[1]					
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 for all cows[1]					
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 for synchronized cows[2]					
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)[2]					
GnRH	14.8 (24/162)	17.4 (17/98)	0.09	0.48	0.34
2CIDR	14.0 (23/164)	22.9 (24/105)			

* Least square means (n./n.)
[1] All inseminated cows
[2]Includes only cows that ovulated to ECP (visible CL on d 7)

Table 3 shows the effects of treatment protocol on P/AI in cows with reduced or elevated temperature. There were more (P < 0.01) cows in the GnRH protocol that ovulated a follicle and had a new CL at PGF than in 2CIDR protocol. There were more (P < 0.01) cows with a CL at PGF in the GnRH than in 2CIDR protocol, however, circulating P4 concentration was greater (P = 0.05) at the time of PGF treatment (d -4) for cows treated with 2CIDR. The GnRH protocol resulted in larger follicles (P < 0.01) in cows with reduced temperature, and in cows with elevated temperature there were no differences between the protocols. Treatment with GnRH had greater (P = 0.03) circulating P4 concentration on d 7 than 2CIDR protocol. Treatment protocol did not alter estrus expression and double ovulation. The cow temperature affected some

physiological measures. Cows with reduced temperature (< 39.1ºC) had higher proportion of CL at PGF (P = 0.02) and tended to have higher circulating P4 concentration at PGF (P = 0.09). In cows with elevated temperature (≥ 39.1ºC) the follicle diameter and estrus expression near TAI was reduced (P < 0.01), and these cows tended (P = 0.08) to have higher double ovulation, although circulating P4 concentration on d7 after TAI was not altered (P = 0.69).

Table 3. Effects of treatment protocols on measures of reproductive physiology by cow temperature on P/AI at 60 d

	Cow temperature ºC				
P/AI 60d*	<39.1	≥39.1	Temp.	Protocol	Interaction
Ovulation of d -11 follicle[1,2]					
GnRH	42.6 (197/402)	40.7 (173/361)	0.91	<0.01	0.42
2CIDR	5.3 (67/415)	6.8 (56/348)			
CL at PGF[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)			
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			
P4 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			
Follicle Diameter d0 (mm)[3]					
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 d7 (ng/mL)[3]					
GnRH	3.65 ± 0.15	3.64 ± 0.16	0.69	0.03	0.61
2CIDR	3.43 ± 0.15	3.51 ± 0.16			
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)			
Double ovulation[3]					
GnRH	21.7 (70/367)	25.8 (77/318)	0.08	0.40	0.98
2CIDR	19.8 (65/367)	23.9 (67/306)			

* Least square means (n./n.)

[1] Percentage of cows with a new CL on d -4 that had a follicle >8 mm on d -11

[2] All inseminated cows

[3]Includes only cows that ovulated to ECP (visible CL on d 7)

The ovulation to GnRH was 42.7% (340/796), and another analysis was performed dividing the cows that ovulate to GnRH (d -11) in cows with reduced or elevated temperature (table 4). In cows with reduced temperature, cows that ovulate to

GnRH (**GnRH-OV**) tended to have higher ovulation to ECP (P = 0.09) than cows that not ovulate to GnRH (**GnRH-nOV**) and 2CIDR (P = 0.10). The P/AI at 60 d was higher for GnRH-OV (P = 0.01) than GnRH-nOV, and tended to be higher (P = 0.09) than 2 CIDR treatment. In cows with elevated temperature there was no effect of treatment on ovulation to ECP and P/AI at 60 d.

Table 04 - Ovulation to ECP and P/AI at 60d by ovulation to GnRH and cow temperature

	Protocol			
Item*	No ovulation to GnRH	Ovulation to GnRH	2CIDR	*P*=
Ovulation to ECP [2]				
<39.1 ºC	85.9 (195/227)y	91.4 (160/175)x	86.5 (359/415)y	0.19
≥39.1 ºC	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)	0.26
P/AI 60d[1]				
<39.1 ºC	28.9 (66/227)b	41.0 (72/175)xa	33.8 (141/415)y	0.04
≥39.1 ºC	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)	0.17
P/AI 60d[2]				
<39.1 ºC	33.6 (66/195)b	45.0 (72/160)a	39.2 (141/359)	0.09
≥39.1 ºC	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

* Least square means (n./n.)

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

[1] all inseminated cows

[2]Includes only cows that ovulated to ECP (visible CL on d 7)

ROC curves were generated to determine the circulating P4 concentration on d -4 with the highest accuracy to predict d 60 pregnancy and nonpregnancy. The circulating P4 concentration that resulted in the highest 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 at 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 higher P/AI at 60d (P <0.01), in cows with reduced or elevated temperature (table 5). Cows that not show estrus have reduced P/AI compared to cows that show estrus (P<0.03), independent of circulating P4

concentration at PGF (P = 0.25). There was no interaction (P = 0.66) between parity and circulating P4 concentration at PGF.

Table 5. Effect of treatment protocol by progesterone concentration at PGF on P/AI at 60d

	Progesterone at PGF				
P/AI 60d*	< 3.66 ng/mL	≥3.66 ng/mL	P4	Variable	P=
Cow temperature <39.1					
GnRH	29.8 (56/186)	38.7 (77/200)	<0.01	0.93	0.16
2 CIDR	24.9 (51/202)	43.0 (89/207)			
Cow temperature ≥39.1					
GnRH	17.8 (36/196)	26.5 (43/159)	<0.01	0.98	0.97
2 CIDR	17.9 (32/174)	26.3 (44/164)			
Combined					
GnRH	23.6 (94/400)	33.5 (123/368)	<0.01	0.79	0.48
2 CIDR	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)			

* Least square means (n./n.)

The circulating P4 concentration at PGF affected the pregnancy loss (P < 0.05; figure 01), however, the effect of circulating P4 concentration on pregnancy loss was associated with the GnRH protocol (P = 0.02) and not with the 2CIDR protocol (P = 0.64; Figure 02). This effect was more evident (figure 03), in cows that ovulate to GnRH (P = 0.06) than in cows that not ovulate to GnRH (P = 0.22).

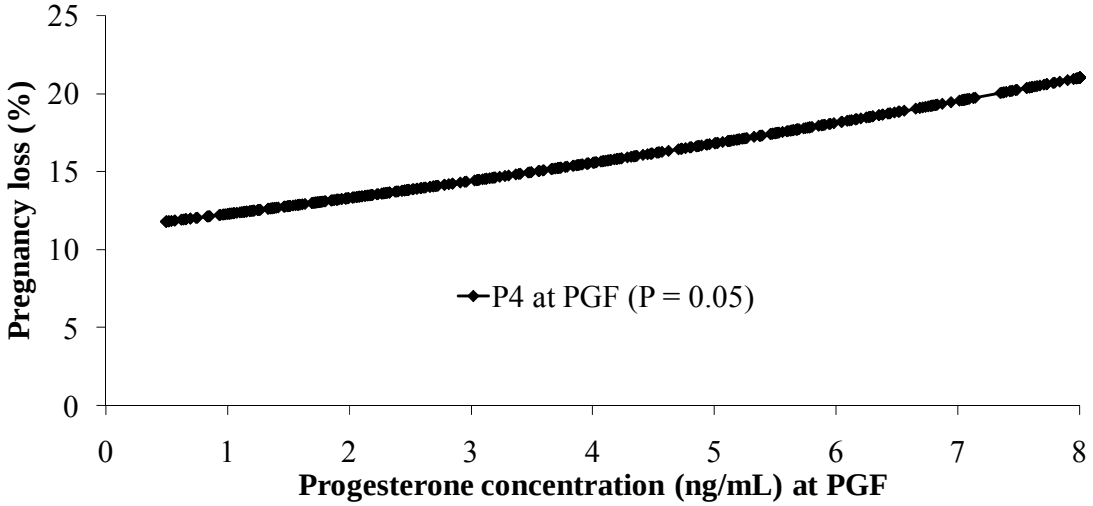

Figure 01. Effect of P4 concentration at PGF on pregnancy loss between 32 and 60 d (P = 0.05)

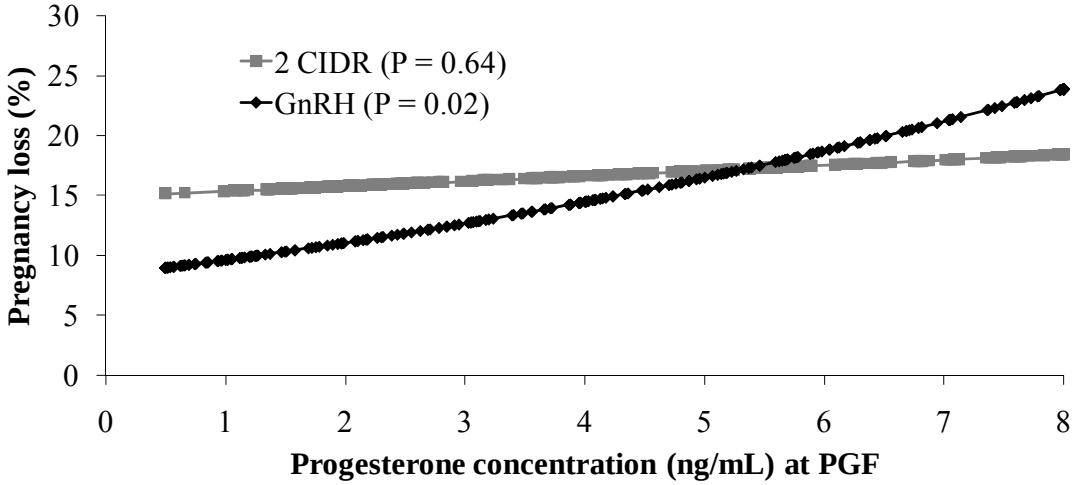

Figure 02. Effect of P4 concentration at PGF on pregnancy loss between 32 and 60 d by protocol (GnRH P = 0.02 and 2CIDR P = 0.64)

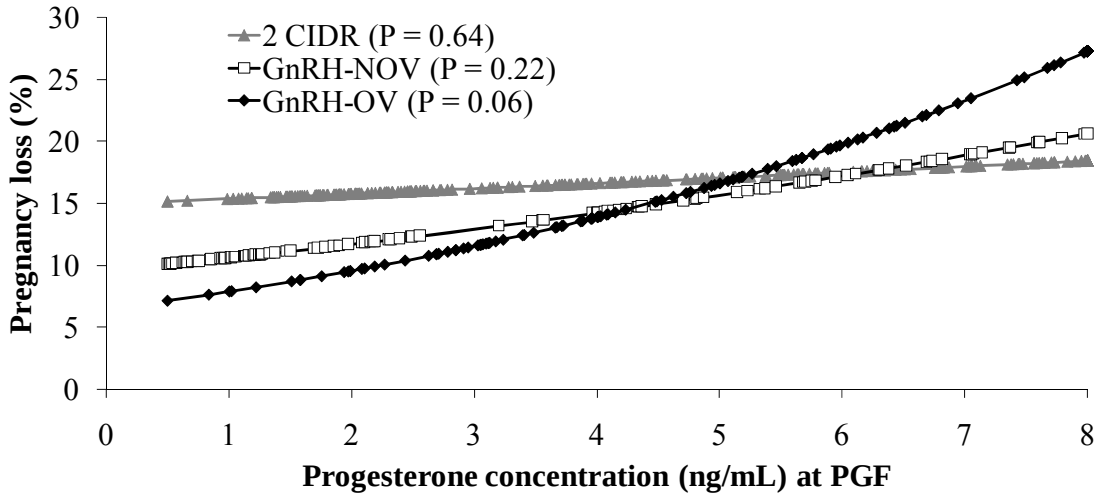

Figure 03. Effect of P4 concentration at PGF on pregnancy loss between 32 and 60 d by ovulation to GnRH (GnRH-NOV P = 0.22; GnRH-OV P = 0.06; 2CIDR P = 0.64)

Cows were classified by the presence or absence of CL on days -11 and -4 (table 6). Cows without CL in both days had reduced ovulation to ECP (P<0.01) in both protocols. For both protocols, the circulating P4 concentration at PGF was higher in cows with CL in both evaluations. Cows without CL at d -11 and with CL at d -4 had higher circulating P4 concentration at PGF than cows without CL in both evaluations and cows with CL at d-11 and without CL at d -4. In all classes the 2 CIDR treatment had higher circulating P4 concentration at PGF. There was effect of class of CL on P/AI at 32 d, for both treatments, cows without CL at d -11 and with CL at d -4 and cows with CL in both evaluations had higher P/AI than the other classes. The 2 CIDR treatment tended to have higher 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.

Table 6. Ovulation to ECP, progesterone concentration, P/AI at 32 and 60d by presence or absence of CL at d -11 and d -4 by treatment

	Corpus Luteum d -11 / d -4				
Item*	No / No	No / Yes	Yes / Yes	Yes / No	*P*=
Ovulation to ECP [2]					
GnRH	73.8 (54/73)b	83.3 (147/176)ad	89.4 (353/394)ac	87.2 (105/120)a	<0.01
2 CIDR	76.1 (116/152)b	85.3 (79/89)a	85.3 (296/346)a	93.6 (166/177)a	<0.01
P=	0.64	0.23	0.52	0.12	
P4 d-4[1]					
GnRH	1.40 ± 0.24b	4.20 ± 0.15ad	5.20 ± 0.10ac	1.24 ± 0.18b	<0.01
2 CIDR	2.15 ± 0.16b	5.10 ± 0.22ad	5.99 ± 0.11ac	2.22 ± 0.15b	<0.01
P=	<0.01	<0.01	<0.01	<0.01	
% P4 ≥3.66 d-4[1]					
GnRH	2.7 (2/74)	57.0 (102/179)	68.1 (265/389)	0.0 (0/126)	
2 CIDR	7.0 (11/157)	76.9 (70/91)	80.7 (284/352)	10.6 (19/180)	
P=	0.19	<0.01	<0.01	<0.01	
P/AI 32d[1]					
GnRH	23.1 (18/77)b	34.6 (64/183)a	38.9 (160/407)a	20.2 (26/129)b	<0.01
2 CIDR	21.6 (35/160)b	44.6 (42/94)a	41.9 (152/361)a	28.9 (54/185)b	<0.01
P=	0.81	0.09	0.38	0.10	
P/AI 60d[1]					
GnRH	18.1 (14/77)b	29.4 (54/183)a	32.7 (134/407) a	18.1 (23/129)b	<0.01
2 CIDR	16.8 (27/160)b	38.4 (36/94)a	35.2 (127/361)a	23.6 (44/185)b	<0.01
P=	0.83	0.11	0.28	0.28	

* Least square means (n./n.)
a,b and c,d in the same row differ (P < 0.01)
[1] all inseminated cows
[2]Includes only cows that ovulated to ECP (visible CL on d 7)

There was no effect (P = 0.78) of P4 concentrations at d 7 after AI on P/AI when only cows that ovulate to ECP were included in the analyses.

DISCUSSION

This study compared two strategies to increase circulating P4 concentration during the follicle development to improve fertility of TAI protocols that utilized E2 and P4 to synchronize the estrous cycle of lactating dairy cows. The first strategy combine GnRH treatment with E2 (Pereira et al., 2015a). GnRH in TAI 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 CL during the preovulatory follicular wave (Pursley et al., 1995, Wiltbank and Pursley, 2014). However, only 50-65% of

cows ovulate to a GnRH treatment administered at a random stage of the estrous cycle (Vasconcelos et al., 1999, Galvao and Santos, 2010, Giordano et al., 2012). The combination of E2 and GnRH at the beginning of the protocol was evaluated in previous study (Pereira et al., 2015a) with resulted in higher P/AI (GnRH + EB = 50.9% vs EB = 41.0%). The second strategy add a second P4 device in the protocol, and this strategy was evaluated in cows with reduced circulating P4 concentration in 5-d cosynch programs (Bisinotto et al., 2013) and E2/P4 protocols (unpublished) with improved fertility. This study, that was performed in a large number of animals and during different seasons of the year allowed analysis of the effect of cow temperature on physiological measures and treatment effects.

Physiological measurements associated with greater fertility were reduced in cows with elevated temperature similarly as in our previous study (Pereira et al., 2015a): percentage of cows with CL at PGF decreased 5.4%, ovulatory follicle diameter decreased 0.51 mm, expression of estrus decreased 4.7%, and ovulation to the ECP decreased 2.5%. 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. The most negative effect on P/AI was cow temperature, as already discussed by Pereira, et al., 2015a and Vasconcelos et al., 2011b. The pregnancy loss between 32 and 60d tended to be higher in cows with elevated temperature in this study. This result was previously observed in other studies (Pereira et al., 2013c) in which cows with heat stress (> 39.1°C) had greater pregnancy losses [23.8% (17/82)] than cows without heat stress [9.4% (20/241)]. Demetrio et al., (2007) also observed negative effect of cow temperature on pregnancy maintenance.

Although, there was no overall change in P/AI both protocols produced a high percentage of cows that responded to the protocols with ovulation (>85%) and great fertility (>39%). Both protocols had similar P/AI regardless of cow temperature, body condition score, parity, milk yield, presence of a CL at d-11 and d -4, circulating P4 concentration at d-11 and d-4 and estrus expression. As our expectations, both protocols resulted in high mean circulating P4 concentration at PGF (> 3.95ng/mL). In

spite of the GnRH protocol had higher proportion of cows with CL at PGF, the 2 CIDR treatment had higher circulating P4 concentration at PGF.

Use of ROC curves allowed selection of important cut-off to evaluate effect of circulating P4 concentration at PGF on fertility. The fertility results gave strong support for these values, cows with circulating P4 concentration ≥ 3.66 ng/mL had greater fertility than cows with circulating P4 concentration < 3.66 ng/mL at PGF, independent of cow temperature or protocol treatment. This is the first study using an E2/P4-based synchronization program in dairy cows that has attempted to define the critical circulating P4 concentration value that improve fertility. Based on ROC curves, the ideal circulating P4 concentration profile on day of PGF in Ovsynch program is 3.78 ng/mL (Ribeiro et al., 2012). Based on these results, the ideal progesterone profile seems to be similar between the Ovsynch and E2/P4 programs. Protocols that use E2/P4 do not induce ovulation of the follicle at the beginning of the protocol, resulting in reduced proportion of cows with CL at time of PGF (5dCosynch = 73.6% [597] vs. E2/P4 = 44.3% [593]) compared to GnRH protocols (Pereira et al., 2013a) and reduced circulating P4 concentration at PGF, compared to GnRH protocols (E2/P4 - 2.29 ± 0.15 vs. GnRH - 2.89 ± 0.15 ng/mL; Vasconcelos et al., 2011a). A recent study evaluated the use of 2 P4 devices (Bisinotto et al., 2013) in 5d Co-synch protocols (d -8 GnRH, d -3 and d -2 PGF, d0 GnRH + IATF), in wich the cows were divided in 3 groups: the cows without CL received 2CIDR or remained untreated if no CL was present (CON), or were considered diestrus if had a CL. Progesterone supplementation with 2 CIDR inserts increased plasma P4 to 2.65 ng/mL and restored fertility similar to that of cows in diestrus (CON 28.6%, 2CIDR 43.7%, Diestrus 47.3%). Previous studies have demonstrated that circulating P4 concentration during follicular development have positive effect on embryo quality. Cerri et al. (2011b) collect embryos 6 d after AI in cows submitted to protocols with low or high P4, find no effects of P4 on fertilization rate and a tendency for higher embryo quality in cows submitted to protocols with high P4. Rivera et al. (2011) demonstrated higher proportion of transferrable embryos (78.6% vs. 55.9%) when superovulated donnors in the first follicular wave (low P4) received 2 P4 devices during the protocol. These results are in agreement with our study, suggesting that higher P4 concentration during follicular development improve P/AI.

Despite treatment with 2 CIDR have increased the circulating P4 concentration in relation to the protocol with GnRH, there was no effect of treatment on P/IA. The results in Table 06, and analysis of the ideal circulating P4 concentration profile helps to understand these results. There was a tendency of the 2CIDR protocol to increase P/AI in cows that had no CL on d-11 and had CL in d-4. These cows had lower circulating P4 concentration at PGF compared to cows in diestrus. In the GnRH protocol, 43% of the cows did not have the ideal circulating P4 concentration, whereas, in the 2CIDR protocol, 76.9% of the cows had circulating P4 concentration above the ideal concentration, resulting in a 10% increase in the P/IA. It was expected that 2 CIDR treatment would increase the P/AI in cows without CL in both evaluations, however no effects of 2 CIDR treatment on P/AI were observed. It seems that the addition of 2 CIDR in those cows was not enough to increase circulating P4 concentration to adequate levels for fertility, give that only 7% of cows had circulating P4 concentration $\geq$ 3.66 ng/mL.

Previous studies suggest that cows with low circulating P4 concentration during development of the ovulatory follicle are more likely to prematurely develop pathways leading to PGF secretion by the endometrium, causing short luteal lifespan (Cerri et al., 2011a,b, Bisinotto et al., 2013). Bisinotto et al., (2013) observed that the proportion of cows that were reinseminated from d 5 to 17 after AI was greater (P = 0.02) for cows without CL at the beginning of the protocol than diestrus and 2CIDR supplemented cows. Sá Filho et al. (2009) detected that treatments with P4 (3 or 6 days) prior to induction of ovulation in anestrous *B. indicus* cows increased the percentage of animals with a normal luteal lifespan. Dos Santos et al., (2009) detected that the concentration of PGFM is lower in animals with lower P4 concentrations 7 d post estrus, and this may have occurred because the lower P4 plasma concentrations stimulated less accumulation of arachidonic acid and COX-2 in endometrial cells, essential elements for the synthesis of PGF2α. In a previous study performed in cows with low circulating P4 concentration at the beginning of an E2/P4 protocol, no effect of treatment was observed on pregnancy per embryo transfer, suggesting that short luteal phases are not associated with P/AI. The differences on short luteal lifespan of GnRH based protocols and E2/P4 based protocols might occur because estrogen based protocol use P4 devices for synchronization in all cows, and in GnRH based protocols without

P4 supplementation (CIDR) some cows do not ovulate to GnRH treatment and have low circulating P4 concentration during follicle development.

Cows detected in estrus have been described to have higher P/AI in E2/P4 protocols (Pereira et al., 2013c, Pereira et al., 2015a, Pereira et al., 2015b) and in Ovsynch protocols (Santos et al., 2010). Bisinoto et al., (2013), using Ovsynch protocol with or without P4 supplementation (CIDR), observed that cows in estrus had greater P/AI, however, the benefits to pregnancy from supplemental progesterone were observed only in cows not detected in estrus at AI. In our study, cows detected in estrus had higher P/AI, and no interaction between circulating P4 concentration at PGF and estrus expression was detected. Interestingly, in Bisinotto et al., (2013) study, in cows without CL at the beginning of the protocol, the estrus detection was greater when they ovulated to the initial GnRH (40.4%) compared to cows that not ovulate to GnRH (21.8%). Anestrus cows treated with P4 devices have increased LH pulses (Smith et al., 1983) which results in higher proportion of cows expressing estrus after P4 device removal (Sá Filho et al., 2010). Some differences between Ovsynch and E2/P4 protocols might explain the results. In Bisinotto et al., (2013) study only 35.1% of all cows were detected in estrus on the day of AI, whereas, in our study 89% of cows were detected in estrus. The addiction of P4 device in all cows in E2/P4 protocols and the use of ECP to induce ovulation might improve the estrus expression and reduce short luteal phases, and in these protocols the benefits of higher circulating P4 concentration should be associated with improved oocyte/embryo quality.

Cows with higher circulating P4 concentration at the time of PGF treatment experienced higher chance to lose the pregnancy up to d 60 and this effect was evident in the GnRH protocol but not on 2CIDR protocol. Pereira et al. (2013b) reported that cows that ovulate to GnRH injection at CIDR insertion in 5-d Cosynch protocol tended to have greater pregnancy losses (25.7%) than cows that did not ovulate to the GnRH injection at CIDR insertion (12.7%). This intriguing result need further investigations, however, it is possible that cows that ovulate to GnRH have lower CL regression at the first PGF, resulting in reduced proestrus length which can result in higher pregnancy loss (Pereira et al., 2013c).

CONCLUSION

Both protocols can be used in lactating dairy cows because no differences on fertility was observed and the results of this study suggests that heat stress have a great impact on fertility.

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Chapter 4

IMPLICATIONS

The results of the present study provide knowledge how TAI protocols can improve fertility 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, and this increase was only observed in the cool season of the year. The second study shows another protocol that can provide fertility similar to the GnRH protocol.

During heat stress, fertility was reduced in both studies.

Related to the potential economic impact of addicting a GnRH treatment and a second PGF treatment, there was an increase in protocol cost of about ~82% per protocol, from $5.85 for the control protocol to $10.67 for the GnRH protocol. Using semen prices of $12.37/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/straw, the actual price per pregnancy will be lower for the GnRH protocol.

The cost of GnRH is the same as with the addition of a second P4 device, being to the responsible technician define what protocol to use.

Obviously there are other benefits associated with the improved fertility using protocols that improve fertility 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.