HORMONAL RESPONSES AND PREGNANCY OUTCOMES AFTER FIVE-DAY OVULATION SYNCHRONIZATION AND PRESYNCHRONIZATION PROGRAMS IN LACTATING DAIRY COWS

by

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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Abstract

Two experiments assessed pregnancy outcomes (pregnancy per AI; P/AI) after 5-d Ovsynch-56 Resynch (RES; GnRH injection 5 d before [GnRH-1; d 0] and 56 h (GnRH-2) after the last $PGF_{2\alpha}$ [PGF] injection on d 6 given 24 h after first PGF injection on d 5, and TAI on d 8) with and without a 5-d progesterone insert. In Exp. 1, only 76% of 1,023 nonpregnant cows enrolled on d 34 post-AI had high (≥ 1 ng/mL) progesterone. The RES-CIDR cows with low progesterone at treatment initiation had greater P/AI than RES-CON (37.7 vs. 29.4%), whereas RES-CIDR cows with high progesterone had lesser P/AI than RES-CON (27.4 vs. 34.3%) suggesting that supplemental progesterone is progesterone-dependent. In Exp. 2, 381 cows were enrolled in similar treatments on d 31 with RES on d 41post-AI plus a third treatment including PG-3-G (Pre-PGF on d 31, Pre-GnRH on d 34, and RES on d 41. The P/AI was similar among treatments but was greater in cows starting RES on d 41 when progesterone was low (44%) than high (33%).Experiment 3 determined LH and ovulatory responses in cows enrolled in two treatments before AI: 1) Pre10 (n = 37): PGF-1 and PGF-2 given 14 d apart (Presynch); or PG3G (n = 33): PGF given concurrent with the PGF-2, 3 d before GnRH-1 followed in 7 d by Ovsynch [injection of GnRH (GnRH-2) 7 d before PGF (PGF-3) and GnRH-3 at either 56 or 72 h after PGF-3] that was initiated 10 d after PGF-2 for Pre10 or 7 d after GnRH-1 of PG3G. The GnRH-1 increased incidences of LH surges and ovulation in PG3G compared with Pre10. The LH in serum of Pre10 was greater than that of cows receiving PG3G after GnRH-2. Following GnRH-3, cows receiving GnRH at 72 h had increased incidence of spontaneous LH surges before GnRH-3. The P/AI for PG3G vs. Pre10 and for 56 vs. 72 h was similar, but the Pre10-72 h treatment combination was less than all other treatment combinations. Release of LH is protocol dependent and flexibility of GnRH timing is an advantage for PG3G before first-service TAI.

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Chapter 1 - Resynch Literature Review

Introduction

Poor expression and inadequate detection of estrus in lactating dairy cows previously submitted to artificial insemination (AI) service continue to challenge dairy herd managers (Bilby et al., 2013). Reducing the inter-insemination interval or increasing the AI service rate increases pregnancy risk. Submission of cows into a resynchronization protocol following non-pregnancy diagnosis (NPD) programs leads to timely rebreeding and increases the AI service rate (Fricke, 2002). Decreased AI service intervals of lactating dairy cows failing to conceive at first and repeat services are essential to improve reproductive efficiency and increase AI service rate in dairy herds (Fricke, 2002). High producing lactating dairy cows have reported AI conception risks of 40% or less (Pursley et al., 1997; Fricke et al., 1998); therefore, more than 60% of lactating cows will have NPD following an AI service. Without timely pregnancy diagnosis and implementation of resynchronization protocols, cows may have extended inter-insemination intervals, extended days in milk (DIM), and reduced milk yield (Dewey et al., 2010). Implementation of an efficient resynchronization protocol and decreased inter-insemination intervals of cows following NPD is critical for judicious reproductive management of dairy cows.

Repeat services following resynchronization protocols have resulted in reduced pregnancies per AI (P/AI) compared with first service timed artificial inseminations (TAI; Bruno et al., 2013). Decreased P/AI may be a result of improperly synchronized estrous cycles following NPD and resynchronization protocols initiated at a random stage of the estrous cycle (Bilby et al., 2013). Studies have indicated large proportions of resynchronized cows are not on the anticipated day of the estrous cycle at the first GnRH injection of the Ovsynch protocol (Bartolome et al., 2009; Bisinotto et al., 2010). The Ovsynch protocol [GnRH injection followed 7 d later by prostaglandin $F_{2\alpha}$ (PGF), 56 h later a second GnRH injection is administered and TAI occurs 16 h later; Pursley et al., 1995] is commonly used to resynchronize ovulation (Resynch) for second and subsequent TAI services (Fricke et al., 2003; Bartolome et al., 2005; Sterry et al., 2006). The day of the estrous cycle at initiation of the Resynch protocol infers the likelihood of ovulation after the first GnRH injection of the Ovsynch protocol, luteolysis, period

of dominance of the ovulatory follicle, and estrous cycle synchrony (Moreira et al., 2001; Fricke et al., 2003). Initiation of the Resynch protocol between d 5 and 9 of the estrous cycle increased the proportion of cows ovulating in response to the first GnRH injection (Vasconcelos et al., 1999) and subsequently increased the percentage of cows with a corpus luteum (CL) present at PGF administration on d 7 of the Resynch protocol. The timing of Ovsynch-Resynch initiation between d 5 and 9 of the estrous cycle results in appropriately timed luteolysis, because the incidence of luteolysis is more than 90% in cows receiving PGF after d 8 of the estrous cycle (Momont and Seguin, 1984). In contrast, a single treatment of 25 mg of PGF on or before d 4 of the estrous cycle fails to induce luteolysis (Rowson et al., 1972; Momont and Seguin, 1984; Beal et al., 1988). Reducing the period of dominance of the preovulatory follicle by decreasing the interval between the initial GnRH and PGF of the Ovsynch protocol from 7 to 5 d increased P/AI in lactating dairy cows (Santos et al., 2010). On d 5 of the estrous cycle, a newly formed CL is less responsive to PGF and previous studies indicated a single standard PGF dose (25 mg) was insufficient to stimulate complete luteal regression (Kasimanickam et al., 2009; Miyamoto et al., 2009; Santos et al., 2010), which is a drawback. Administration of a second standard dose of PGF 24 h after the first improved luteolysis and fertility in beef (Kasimanickam et al., 2009) and dairy (Santos et al., 2010) cows. Most studies report that a bolus dose (200 to 250% of standard dose) of PGF given concurrently or two standard doses given 24 h apart in protocols with 5 d between the first GnRH and PGF treatments induces luteal regression (Santos et al., 2010; Ribero et al., 2012; Stevenson et al., 2013; Valldecabres-Torres et al., 2013).

Another reason for reduced fertility to resynchronization services in cows is that 15 to 26% are without a functional CL, or have decreased circulating progesterone concentrations at the initiation of resynchronization protocols, or both (Fricke et al., 2003; Sterry et al., 2006, Silva et al., 2009). Several studies have indicated the addition of exogenous progesterone via an intravaginally placed controlled internal drug release (CIDR) insert as part of a resynchronization protocol increased P/AI in lactating dairy cows (Bisinotto et al., 2010; Dewey et al., 2010; Bilby et al., 2013). Addition of progesterone via the CIDR seems to increase synchrony of the estrous cycle by decreasing the interval from CIDR withdrawal to ovulation because the CIDR provides adequate circulating progesterone concentrations to prevent estrus and ovulation (Chebel et al., 2010; Bilby et al., 2013). Presynchronization of estrous cycles of cows following NPD improved P/AI after reinsemination (Silva et al., 2007; Dewey et al., 2010). Improved P/AI to

presynchronization protocols is associated with increased ovulation response to the first and second GnRH injections (Vasconcelos et al., 1999; Bello et al., 2006; Rutigliano et al., 2008; Galvão and Santos, 2010; Stevenson et al., 2012), increased circulating concentrations of progesterone (Stevenson et al., 2012), and increased incidence and number of functional CL responsive to the PGF injection (Galvão and Santos, 2010; Stevenson et al., 2012). A presynchronization treatment consisting of administering a single PGF injection 12 d before initiation of Ovsynch improved fertility in repeat service cows (Silva et al., 2007). Presynchronization by administration of GnRH 7 d before initiation of Ovsynch, as a resynchronization protocol, increased P/AI compared with cows not presynchronized before Ovsynch (Dewey et al., 2010; Lopes et al., 2013). Use of a nonbreeding Ovsynch protocol as a presynchronization protocol 7 d before initiation of a TAI Ovsynch (Double Ovsynch) protocol improved P/AI in repeat service lactating dairy cows (Giordano et al., 2012). Bello et al. (2006) reported the G-6-G presynchronization treatment consisting of a GnRH injection preceded 2 d by a PGF injection with an interval of 6 d before initiating Ovsynch resulted in increased ovulatory and luteolytic responses to the first GnRH and PGF of Ovsynch, respectively, and increased synchronization rate to Ovsynch in first-service lactating dairy cows. In first-service lactating dairy cows, presynchronization using a PG3G protocol (PGF injection followed in 3 d by GnRH) 7 d before initiating the Ovsynch protocol increased ovulation rates and luteal function 7 d before Ovsynch, resulting in improved follicular synchrony (Stevenson et al., 2012) and increased P/AI in cows inseminated during summer heat stress (Stevenson and Pulley, 2012). An anovular cow to which a Double Ovsynch, G-6-G, or PG3G protocol is applied provides another opportunity to induce ovulation before initiation of the Ovsynch protocol (Bello et al., 2006; Giordano et al., 2012; Stevenson et al., 2012). Presynchronization improves fertility during resynchronization protocols by increasing synchronization of the estrous cycle in lactating dairy cows during the Ovsynch protocol before TAI (Dewey et al., 2010; Giordano et al., 2012; Bilby et al., 2013; Lopes et al., 2013).

The objective of this review is to present recent data from studies in which contemporary protocols were utilized with or without the addition of a CIDR insert to a 5 d Resynch protocol, and its effects on fertility outcomes and the use of the presynchronization protocols before resynchronization of ovulation in lactating dairy cows.

Period of Follicle Dominance

High-producing dairy cows have more 2 than 3 follicular waves during the estrous cycle compared with heifers (Savio et al., 1988). Interval from follicular emergence to ovulation is increased approximately 3.5 d for cows with 2 follicular waves compared with cows having 3 follicular waves (Bleach et al., 2004). Conception rates linearly decreased as the interval from follicle emergence to ovulation increased (Bleach et al., 2004). Periods of extended follicle dominance associated with observed reduction in fertility were associated with compromised oocytes (Revah and Butler, 1996), decreased embryo quality (Cerri et al., 2009), and impaired embryonic development (Ahmad et al., 1995). Extending emergence to ovulation interval by 12 d resulted in oocytes prematurely resuming meiosis (Mihm et al., 1999), which may have led to the observed decrease in oocyte (Revah and Butler, 1996) and embryo quality (Cerri et al., 2009). As a result of early resumed meiosis, fertilization rates are normal but death of the embryo occurred during early cleavage stages (Ahmad et al., 1995). Bleach et al. (2004) demonstrated that dairy cows that became pregnant to insemination service following spontaneous estrus had a mean interval from follicle emergence to ovulation that was 1-d less than that of cows that failed to become pregnant. Similarly, Cerri et al. (2009) reported the extension of follicular dominance by 1.5 to 2 d reduced embryo quality. Collectively, these studies indicate appropriately implemented TAI protocols may improve embryo quality by regulating the period of follicular dominance.

In an effort to reduce the period of follicle dominance, an Ovsynch protocol with a reduced interval between GnRH and PGF has been developed (Wiltbank and Pursley, 2013). Recent studies have emphasized the importance of reducing the period of follicle dominance to improve embryo quality and fertility outcomes to TAI protocols (Bridges et al., 2008; Cerri et al., 2009; Santos et al., 2010). A new follicular wave is recruited 24 to 48 h after the first GnRH injection of the Ovsynch protocol, thus reducing the interval from the first GnRH to PGF injection from 7 to 5 d also reduces the period of follicle dominance (Nascimento et al., 2014). Bridges et al. (2008) observed increased P/AI by reducing that interval from 7 to 5 d when proestrus (interval from PGF to second GnRH) was extended from 48 to 72 h in beef cows that were subjected to a modified Cosynch protocol (GnRH followed 5 d later by 2 PGF injections 24 h apart with second GnRH given concurrent with TAI at 60 h) including a CIDR insert. A similar study in lactating dairy cows reported encouraging results. By reducing the period of follicle

dominance from 2 d in a 5-d Cosynch-72 protocol [2 PGF injections 24 h apart 5 d after GnRH and a second GnRH 72 h after PGF given at TAI] significantly increased P/AI in lactating dairy cows (Santos et al., 2010). Using a 5-d Ovsynch program with the second GnRH administered 16 h before or concurrent with TAI resulted in no P/AI differences in lactating dairy cows (Bisinotto et al., 2010).

The advantage to P/AI achieved by decreasing the period of follicle dominance also has been reported in cows displaying spontaneous estrus. Bleach et al. (2004) observed increased P/AI in cows when the period of dominance was spontaneously shorter in dairy cows having 3 follicular waves compared with cows having 2 follicular waves, in which the period of dominance was 3.5 d longer. Dairy cows in which the ovulatory follicle originated from the third follicular wave also had increased P/AI compared with cows in which ovulatory follicles originated from the second follicular wave (Townson et al., 2002).

Prostaglandin Administration in 5-d Ovsynch Protocol

A limitation in 5-d programs to manipulate the period of follicle dominance is the ability of PGF to regress a newly formed CL subsequent to the first GnRH injection (Santos et al., 2010; Ribeiro et al., 2012). In the first 5 d, the CL is refractory and less responsive to luteolytic actions of PGF (Tsai and Wiltbank, 1998; Miyamoto et al., 2005). It has been proposed that the early CL has distinct molecular responses to PGF compared with the mid-cycle CL that is sensitive to PGF (Miyamoto et al., 2005). To induce acceptable luteal regression and decrease circulating progesterone concentrations, 2 doses of PGF is required (Nascimento et al., 2014). A single standard dose of PGF (25 mg of PGF or 500 µg of a PGF analog [cloprostenol]) is inadequate to induce luteolysis in the first 4 d of the estrous cycle in beef heifers (Henricks et al., 1974), cows (Rowson et al., 1972), and dairy cows (Momont and Seguin, 1984; Nascimento et al., 2014). A single 25-mg dose was not effective in the regression of a d 5 CL in lactating dairy cows (Santos et al., 2010) and in beef cows (Kasimanickam et al., 2009). An additional PGF injection given 24 h after the initial PGF of the Ovsynch protocol increased luteolysis incidence from 85 to 96% in lactating dairy cows (Brusveen et al., 2009). A double standard dose (300 µg of d-cloprostenol) of PGF induced complete luteolysis more cows having 132 h or 5.5 d CL than in cows with younger (96 to 120 h) CL, which neither the standard (150 µg of d-cloprostenol) or double dose (300 µg) had an effect on progesterone concentrations (Valldecabres-Torres et al., 2012). A

double dose of PGF given on d 5 failed to induce luteolysis equivalent to 2 standard doses given on d 5 and 6 (Riberio et al., 2012a; Riberio et al., 2012b). A double standard dose (50 mg) given 24 h apart on d 5 and 6 or concurrently on d 6 was effective in inducing luteolysis in lactating dairy cows (Stevenson et al., 2013). Valldecabres-Torres et al. (2013) also reported the number of cows with complete luteolysis after 2 administrations of 150 μg of d-cloprostenol on d 5 and 6 was comparable with a single injection of 375 μg of d-cloprostenol on d 6 in nonlactating dairy cows. In contrast, cows receiving 2 standard PGF doses on d 6 had less luteolysis compared with cows administered PGF on d 5 and 6, and P/AI was decreased in in 1 of 2 herds when cows received 2 standard PGF doses (Stevenson et al., 2014). Collectively, administration of 2 standard doses of PGF 24 h apart is the most effective in causing complete luteolysis in protocols with 5 d between the first GnRH and PGF treatments.

Day of Initiation of Resynchronization Protocols

In order for lactating cows to begin an ovulation-synchronization protocol within the optimal interval from d 5 to 12 of a 23 d the estrous cycle (Vasconcelos et al., 1999; El-Zarkouny et al., 2004; Sartori et al., 2004), initiation of the resynchronization protocol between 28 and 33 d post-AI may be advantageous (Sterry et al., 2006; Bilby et al., 2013). Resynchronization programs initiated at 21 d post-AI increased P/AI compared with programs initiation 28 or 42 d post-AI (Chebel et al., 2003). In contrast, initiation of the Ovsynch protocol 19 d post-AI reduced P/AI compared with initiation on d 26 or 33 (Fricke et al., 2003). The P/AI for initiation at d 26 and d 33 was similar; however, pregnancy diagnosis was confounded with protocol initiation and P/AI from the treatments should not be directly compared (Fricke et al., 2003). In a subsequent study in which pregnancy diagnosis for both treatments occurred 33 d post-AI, protocols initiated on d 33 compared with d 26 had increased P/AI after the Ovsynch protocol as a resynchronization program (Sterry et al., 2006). Similarly to the this earlier study, resynchronization protocols initiated at 32 and 39 d post-AI resulted in increased P/AI after the Ovsynch protocol as a resynchronization program (Bilby et al., 2013; Lopes et al., 2013). Resynchronization protocols in nonpregnant cows 33 d post-AI theoretically place most cows at d 5 to 12 of the estrous cycle, which has been shown to improve fertility to the Ovsynch protocol (Vasconcelos et al., 1999; El-Zarkouny et al., 2004; Sterry et al., 2006), but two recent studies

reported similar P/AI when resynchronization protocols were initiated at either 32 or 39 d post-AI (Bilby et al., 2013; Lopes et al., 2013).

Synchronization Protocols Including Progesterone Inserts

Lactating dairy cows treated intravaginally with progesterone inserts induced cyclicity in anovular cows (Rhodes et al., 2003; Chebel et al., 2006), and increased synchronization of the estrous cycle when added to a synchronization protocol (Lima et al., 2009). Inclusion of a CIDR insert to a first-service synchronization protocol increased P/AI (Stevenson et al., 2008; Chebel et al., 2010). Addition of a 7 d CIDR insert to the Presynch-Ovsynch protocol improved P/AI only in first-service cows with high (> 1 ng/mL) progesterone (Bartolome et al., 2009). In contrast, a few studies have reported the addition of a CIDR insert had no effect on first-service P/AI (El-Zarkouny et al., 2004; Galvão et al., 2004; Stevenson et al., 2006). In one study the addition of CIDR inserts to Ovsynch protocol improved conception and embryo survival in the first experiment, but not the second, which is possibly attributable to different proportions of cyclic cows at treatment onset (El-Zarkouny et al., 2004). Treatment with 1 or 2 CIDR inserts did not affect P/AI when added to the Ovsynch protocol that replaced the final GnRH injection with 1 mL of estradiol cypionate (Lima et al., 2009). Reports of the inclusion of a CIDR insert in first-service synchronization protocols have been inconsistent, which may be attributed to the type of protocol utilized, differences in genetics, cycling status, stage of the estrous cycle, and milk production.

Resynchronization Protocols Including Progesterone Inserts

Inclusion of a CIDR insert in a resynchronization protocol in lactating dairy cows has been evaluated in recent studies (Bisinotto et al., 2010; Dewey et al., 2010; Bilby et al., 2013; Chebel et al., 2013). Without the use of presynchronization protocols, cows enrolled into a resynchronization protocol are at random stages of the estrous cycle, and treatment with CIDR insert may improve synchrony and P/AI (Bisinotto et al., 2010; Dewey et al., 2010). Two studies indicated increased P/AI in cows receiving a CIDR insert compared with cows not receiving an insert concurrent with the first GnRH injection of the 7-d Resynch protocol initiated 39 ± 3 d post-AI (Dewey et al., 2010) or 32 vs. 39 ± 3 d post-AI (Bilby et al., 2013). In contrast, no increase in P/AI was reported by Chebel et al. (2013) when injections of either GnRH or PGF were employed as presynchronization treatments 7 or 11 d, respectively, before a Resynch

protocol, with the addition of a CIDR insert concurrent with the first GnRH injection. Regardless of presynchronization protocol used, cows receiving a 7-d CIDR insert between the first GnRH and PGF injections of the Resynch protocol had numerically increased (4.1% units) in P/AI 31 d post-AI, which was insufficient to show a statistical difference (Chebel et al., 2006). Cows without a CL receiving a CIDR insert had similar P/AI compared with cows having a CL at pregnancy diagnosis (El-Zarkouny et al., 2004).

Bisinotto et al. (2010) reported an increase in P/AI in cows receiving a CIDR insert in a 5 d Resynch protocol 34 d after a previous AI service. Progesterone supplementation tended to improve P/AI only in cows having an active CL at pregnancy diagnosis (Bisinotto et al., 2010), which is similar to the report by Bartolome et al. (2009) evaluating the 14 d Presynch before the 7 d Resynch protocol with or without CIDR insert. Bisinotto et al. (2010) is the only study, to the author's knowledge, to evaluate the addition of a CIDR device to the 5 d Resynch protocol, and these results warrant further investigation.

Presynchronization of Estrous Cycles before Ovsynch Protocol

Response to the first GnRH injection of the Ovsynch protocol differs by stage of estrous cycle or follicular wave at protocol initiation, which is the physiological basis for presynchronization protocols before initiation of the Ovsynch (Vasconcelos et al., 1999). A presynchronization treatment (Presynch) consisting of 2 treatments of PGF administered 14 d apart, 12 d (Presynch-12) before initiation of the Ovsynch protocol improved P/AI (42.8% vs. 29.4%) compared with cows only receiving Ovsynch at random stages of the estrous cycle (Moreira et al., 2001). Presynch-12 improved P/AI in cyclic cows, but not in anovular cows, which may be expected in a presynchronization strategy only utilizing PGF treatments (Wiltbank and Pursley, 2013). Subsequent reports confirmed 2 PGF injections 10 to 14 d before Ovsynch improved P/AI compared with Ovsynch alone (Navanukraw et al., 2004; El-Zarkouny et al., 2004). Intervals of 10 d (Stevenson, 2011), 11 d (Galvão et al., 2007), 12 d (Moreira et al., 2001), and 14 d (El-Zarkouny et al., 2004; Navanukraw et al., 2004) between the second PGF injection of Presynch and the onset of Ovsynch have been tested. Decreasing the interval from 14 to 11 d between the second PGF of Presynch and the first GnRH injection of Ovsynch improved P/AI (36.4% vs. 30.2%; Galvão et al., 2007). Improved P/AI resulted from an increased ovulation response to the initial GnRH injection of Ovsynch (Vasconcelos et al., 1999; Galvão et

al., 2007; Stevenson, 2011). Presynchronization consisting of 2 PGF injections improves synchrony and ovulation response to the first GnRH injection of Ovsynch, which is key to coordination of a dominant follicle at PGF and final GnRH injection (Moreira et al., 2001; Bello et al., 2006).

Other presynchronization programs involve the use of GnRH and PGF such as a non-breeding Ovsynch protocol (Double-Ovsynch; Souza et al., 2008) or a combination of PGF and GnRH injections given 2 (G-6-G; Bello et al., 2006) or 3 d (PG-3-G; Peters and Pursley, 2002) apart followed in 6 or 7 d, respectively, by enrollment into Ovsynch. The rationale for development of the Double-Ovsynch program was that GnRH treatment would effectively induce ovulation in anovular cows (Gumen et al., 2003) and synchronize estrous cycles so that the breeding Ovsynch protocol would be initiated on d 7 of the cycle (Wiltbank and Pursley, 2013). Double-Ovsynch improved P/AI compared with Presynch-12 (Souza et al., 2008; Herlihy et al., 2012; Ayres et al., 2013). Souza et al. (2008) reported the improvement in fertility in response to Double-Ovsynch was limited to primiparous, not multiparous, cows compared with Presynch-12. Double-Ovsynch compared with Presynch-12 increased the proportion of cows with elevated progesterone concentrations at PGF and the proportion of cows having a CL at the first GnRH injection (Herlihy et al., 2012; Ayres et al., 2013). In a grazing herd, Double-Ovsynch did not improve P/AI compared with Presynch-10 before a 5 d Cosynch protocol (GnRH and timed AI given concurrently at either 58 or 72 h following PGF treatment; Ribeiro et al., 2012b). The lack of an effect of Double-Ovsynch on fertility compared with Presynch reported by Ribeiro et al. (2012b) may be because of differences in grazing dairy cows compared with those in conventional-TMR conditions or because of the 2-d shorter Presynch program in the grazing study (Presynch-10). The implementation of the Double-Ovsynch protocol as a resynchronization program improved P/AI compared with Ovsynch alone (Giordano et al., 2012).

Another presynchronization protocol consists of a PGF injection followed by GnRH 2 or 3 d later with subsequent initiation of Ovsynch 4, 5, 6, 7, or 8 d later (Peters and Pursley, 2002; Bello et al., 2006; Stevenson et al., 2012; Stevenson and Pulley, 2012). This protocol is similar to Double-Ovsynch except that the first GnRH injection of the nonbreeding Ovsynch is omitted in this protocol. Studies have reported it to be effective in synchronizing cows to a stage of the estrous cycle in which ovulation response to the first GnRH of Ovsynch increases (Peters and

Pursley, 2002; Bello et al., 2006; 2007). Bello et al. (2006) reported the percentage of cows ovulating in response to the first GnRH of Ovsynch increased when the interval was 6 d (G6G, 85%) compared with 4 (G4G, 56%) or 5 d (G5G, 67%). In a similar study, ovulation incidence also was increased with a 6-d interval (94%) compared with a 7- (82%) or 8-d (73%) interval (Pursley et al., unpublished; Wiltbank and Pursley, 2013). The proportion of pregnant cows tended (P = 0.08) to be greater for cows receiving G6G than for controls (50% vs. 27%; Bello et al., 2006).

Another variation to this presynchronization protocol is a 2 d interval between PGF and GnRH and delaying the initiation of Ovsynch to 6 or 7 d (PG-3-G; Peters and Pursley, 2002; Stevenson et al., 2012; Stevenson and Pulley, 2012). The PG-3-G protocol 6 or 7 d before Ovsynch tended to improve P/AI in lactating dairy cows (Peters and Pursley, 2002). Presynchronization with PG-3-G before Ovsynch compared with cows enrolled in Ovsynch at random stages of the estrous cycle increased the proportion of cows having increased serum concentrations of progesterone (≥ 1 mg/mL) at the initial GnRH of Ovsynch (Peters and Pursley, 2002). At PGF treatment of the Ovsynch protocol, PG-3-G + Ovsynch cows had greater proportion of cows with progesterone concentrations ≥ 2 ng/mL than cows enrolled into a standard Ovsynch protocol without presynchronization (Peters and Pursley, 2002). No difference was detected in P/AI between PG-3-G + Ovsynch cows (41.5%) and Ovsynch (38.3%) cows; however, in primiparous cows, P/AI was 52.8% in PG-3-G + Ovsynch treated cows compared with 43.5% in Ovsynch cows (Peters and Pursley, 2002).

Presynchronization using PG-3-G was determined to be superior to Presynch-10 before enrollment of cows into Ovsynch 7 d later (Stevenson et al., 2012). Before Ovsynch initiation, ovulation incidence was increased due to induced ovulations in PG-3-G treated cows (80%) compared with spontaneous ovulation in Presynch-10 (53.3%) presynchronized cows (Stevenson et al., 2012). At the first GnRH of Ovsynch, more PG-3-G cows had at least 1 CL, more average numbers of CL per cow, and more cows had elevated (≥ 1 ng/mL) progesterone concentrations than Presynch-10 cows (Stevenson et al., 2012). Although after the PGF injection of Ovsynch, no further ovarian differences were detected (Stevenson et al., 2012). This indicates the effectiveness of PG-3-G in synchronizing ovarian events before the final GnRH of Ovsynch (Stevenson et al., 2012). Presynchronization with PG-3-G increased ovulation incidence and

luteal function before initiation of Ovsynch, causing improved synchronization and theoretically predisposing lactating dairy cows to increased pregnancy risk per AI (Stevenson et al., 2012).

A subsequent study compared presynchronization of estrous cycles with PG-3-G vs. Presynch-10 before enrolling cows into Ovsynch (Stevenson and Pulley, 2012). Authors reported more P/AI for PG-3-G (35.9%) than Presynch-10 (26.7%) treated cows in the hot or summer months in 4 commercial dairy herds (Stevenson and Pulley, 2012). Pregnancy per AI was numerically increased for PG-3-G (46.8%) than Presynch-10 (44.3%) cows during months without heat stress (Stevenson and Pulley, 2012). In contrast to the study by Peters and Pursley (2002), no parity differences were detected between presynchronization treatment effects on P/AI (Stevenson and Pulley, 2012).

Presynchronization protocols consisting of GnRH in addition to PGF provide at least one additional opportunity to induce ovulation before initiation of Ovsynch, which has been shown to increase synchrony and improve fertility in anovular cows (Bello et al., 2006; Souza et al., 2008; Stevenson et al., 2012). Further investigation into the efficacy of presynchronization with PG-3-G before resynchronization of ovulation via the Ovsynch protocol is warranted given the potential advantages to anovular cows and superior pregnancy risk during the summer in lactating dairy cows.

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Chapter 2 - Five-day Resynch Programs in Dairy Cows Including the CIDR at Two Stages Post-Artificial Insemination

INTRODUCTION

Timed artificial insemination (TAI) protocols enable control of reproductive events and provide efficient options compared to programs that are solely based on detection of estrus in lactating dairy cows. Poor expression and inadequate detection of estrus in cows previously submitted to AI service continue to challenge dairy herd managers (Bilby et al., 2013). Reducing the inter-insemination interval or increasing the AI service rate increases pregnancy risk. Following the first postpartum AI, between 55 and 65% of cows submitted to AI are diagnosed nonpregnant (Bisinotto et al., 2010). Submission of cows into a resynchronization protocol following nonpregnancy diagnosis programs leads to timely rebreeding and increases the AI service rate (Fricke, 2002).

As the Ovsynch protocol (Pursley et al., 1998; GnRH injection 7 d before [GnRH-1] and 2 d after [GnRH-2] PGF$_{2\alpha}$ [PGF] with TAI performed 16 h after GnRH-2) has become a routine first and repeat service TAI program for dairy reproductive management, researchers have modified Ovsynch strategies to enhance fertility in lactating dairy cows (Wiltbank and Pursley, 2013). Such modifications include the addition of a progesterone-releasing controlled internal drug release (CIDR) insert and reduction of the period of follicle dominance.

Reducing the period of follicle dominance by decreasing the interval between GnRH-1 and PGF injections from 7 to 5 d in a 5-d Cosynch program improved pregnancy per AI (P/AI) in lactating dairy cows (Santos et al., 2010). In contrast, comparisons of Ovsynch-56 and Cosynch-72 in a 5-d program yielded no difference in P/AI in first-service lactating dairy cows despite the 16 h difference in the period of follicle development between the treatments (Bisinotto et al., 2010).

Without use of presynchronization protocols, cows enrolled in a resynchronization protocol are more or less at random stages of the estrous cycle, and treatment with CIDR insert may improve synchrony (Chenault et al., 2003; Lima et al., 2009) and P/AI (Bisinotto et al., 2010; Dewey et al., 2010). Addition of a CIDR induces cyclicity in anovular cows (Rhodes et al., 2003). In first-service dairy cows, inclusion of a CIDR improved P/AI in some cows (Stevenson et al., 2008; Chebel et al., 2010), but had no effect (El-Zarkouny et al., 2004; Galvão et al., 2004;

Stevenson et al., 2006) in other studies. Recent studies have evaluated the inclusion of a CIDR insert into a resynchronization program. Resynch (GnRH injection either 5 or 7 d before and 56 to 72 h after PGF, with TAI at 72 h after PGF) initiated 39 d after previous AI increased P/AI when cows received a CIDR insert or an injection of GnRH 7 d before initiation of a 7-d Resynch compared with Resynch alone (Dewey et al., 2010). In contrast, Chebel et al. (2013) did not observe an increase in P/AI with the inclusion of a CIDR insert in a 7-d Resynch protocol initiated 32 d post previous AI. Inclusion of CIDR insert into a 7-d Resynch protocol improved P/AI when cows were not at ideal stages of the estrous cycle by reducing early ovulation incidence, improving cycle synchrony, and synchrony of ovulation (Bilby et al., 2013). Cows lacking a CL or having progesterone < 1 ng/mL at protocol initiation benefited from CIDR treatment as indicated by increased P/AI (Bilby et al., 2013). Addition of a CIDR insert in a 5-d Resynch protocol improved P/AI in repeat service cows with a CL present at program initiation 34 d after previous service (Bisinotto et al., 2010). The hypothesis for the present experiment 1 was that inclusion of progesterone (via a CIDR insert) into a 5-d Resynch protocol initiated 34 d after a previous service would improve P/AI in repeat-service lactating dairy cows. The objectives for experiment one were to determine the effect of progesterone supplementation via CIDR insert on pregnancy outcomes in dairy cows in which ovulation was synchronized after a non-pregnant diagnosis.

Presynchronization of estrous cycles before initiation of first AI services generally improve P/AI and have been used extensively (Chebel et al., 2013). Recent studies have indicated that presynchronization of nonpregnant cows following a previous service improves P/AI after reinsemination (Silva et al., 2007; Dewey et al., 2010). Dewey et al. (2010) utilized a GnRH injection to presynchronize estrous cycles and cows were reinseminated when detected in estrus despite the observation that such GnRH treatments decreased the proportion of cows observed in estrus (Mendonça et al., 2012). Chebel et al. (2013) reported no difference in P/AI when estrous cycles were presynchronized using PGF or GnRH, but the authors suggested presynchronization with PGF may reduce inter-insemination intervals. Presynchronization with GnRH provides an additional opportunity for ovulation before resynchronization protocol initiation (Bello et al., 2006; Souza et al., 2008). This additional opportunity for ovulation may benefit anovular cows (Stevenson et al., 2012). Presynchronization with PG-3-G [PGF and GnRH 3 d later followed with Ovsynch enrollment 7 d later] was a more effective treatment than

other Presynch treatments to synchronize follicle development and increase progesterone before enrollment in Ovsynch (Stevenson et al., 2012) and produced superior P/AI during summer (Stevenson and Pulley, 2012). The objective of experiment 2 was to determine the effect of presynchronization of estrous cycles and progesterone supplementation via a CIDR insert on ovarian characteristics and pregnancy outcomes in dairy cows in which ovulation is synchronized with a 5-d TAI program after non-pregnant diagnosis.

MATERIALS AND METHODS

Experiment 1

Experimental Cows

The current studies were approved by the Kansas State University Institutional Animal Care and Use Committee. Lactating Holstein cows were enrolled between September 2011 and May 2012 in a single commercial dairy herd located in northeast Kansas. Cows were housed in covered, sand bedded, two-row freestall barns, which were equipped with sprinklers above feed lines, fans over freestalls, and grooved concrete floors. Cows were milked thrice daily and fed once daily a diet consisting of alfalfa hay, corn silage, soybean meal, whole cottonseed, corn or milo grain, corn gluten feed, vitamins, and minerals.

Experimental Design

At weekly 34 d post-AI pregnancy diagnosis, nonpregnant, lactating dairy cows (n = 1,122) were clustered into rebreeding groups. The resynchronization program to which cows were systematically assigned was a 5-d Ovsynch protocol (RES; GnRH injection 5 d before [d 0] and 56 h after PGF injections on d 5 and 6, and TAI on d 8] with (RES-CIDR) and without (RES-CON) a 5-d CIDR. Cows with ear tags ending in even digits were enrolled in RES-CIDR: 100-µg i.m. GnRH injection (GnRH-1; 2 mL of Factrel, Pfizer Animal Health, Madison, NJ) was administered concurrent with placement of a new CIDR insert (Eazi-Breed CIDR Cattle Insert, Pfizer Animal Health) for 5 d; plus at insert removal, an 25-mg i.m. injection of PGF2α (PG1; 5 mL of Lutalyse Sterile Solution, Pfizer Animal Health) was administered and a second $PGF_{2\alpha}$ (PGF) i.m. injection on d 6, 32 h later (56 h after PGF-1), a second i.m. GnRH (GnRH-2) injection was administered and TAI occurred 16 h later (Figure 2.1).

Cows with odd digit ear tags were enrolled in RES-CON: same as RES-CIDR but without CIDR insertion for 5 d. All injections and inseminations were performed by onsite trained farm personnel with whom the corresponding author has conducted designed studies for more than 12 years. Seven technicians performed inseminations, with one technician performing approximately 50% of AI services, and multiple sires were used. At each insemination, date, sire, and technician were recorded and full access to herd data was provided.

Of the 1,122 cows originally enrolled in the study, 90 cows were dropped from the study because of culling (n = 31), lameness (n = 10), failure to inseminate (n = 30), or miscellaneous health reasons (n = 19). Final cows per treatment were RES-CON (n = 503) and RES-CIDR (n = 528). At each insemination, date, sire, technician, and breeding codes (chalk or tail paint rub, standing estrus observed, activity monitors, or timed artificial insemination), subsequent post-treatment AI date(s) were entered into PC-DART (Dairy Records Management Systems, Raleigh, NC) software. Post-treatment reinsemination was evidence for failed treatment pregnancy unless contraindicated by subsequent diagnosis. Full access to herd records was provided by the dairy herd manager with weekly herd downloads. Monthly DHI test-day ECM and test-day milk yield, projected 305-d actual milk yield, and projected 305-d mature equivalent milk yield near the onset of the resynchronization protocol was recorded for cows enrolled in the study.

Figure 2.1 Experimental design for experiment 1. At pregnancy diagnosis (PD) 34 d after a previous service, nonpregnant lactating dairy cows were systematically assigned to 2 treatments according to ending ear tag number (even or odd): RES-CON [GnRH injection 5 d before and 56 h after PGF injections on d 5 and 6, and timed artificial insemination (TAI) on d 8] or RES-CIDR [GnRH injection 5 d before and 56 h after PGF injections on d 5 and 6, and timed artificial insemination (TAI) on d 8 with CIDR insert concurrent with GnRH-1 and insert withdrawal at PGF-1]. Pregnancy diagnosis was performed via transrectal palpation 34 and 62 d post-TAI. P4 = Blood sample collection to determine circulation progesterone concentrations; PGF-1 or PGF-2 = $PGF_{2\alpha}$; GnRH-1 or GnRH-2 = Gonadotropin-releasing hormone; CIDR= controlled intravaginal drug releasing insert.
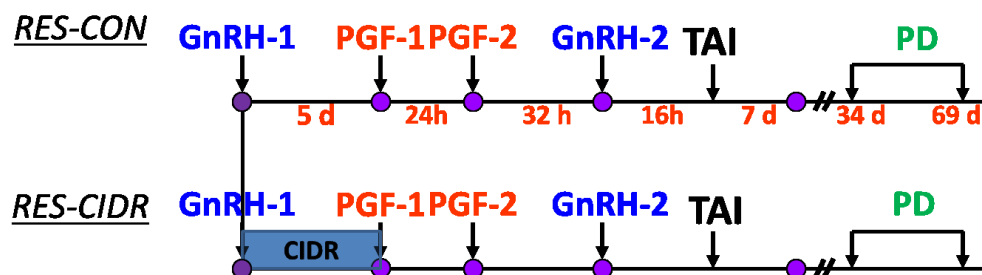
Body Condition Scores, Blood Sampling, and Progesterone Assay

At each weekly farm visit, cows scheduled for pregnancy diagnosis and determined not pregnant were body condition scored (1 = thin and 5 = fat; Ferguson et al., 1994), and a blood sample was collected on d 34 from the coccygeal vein or artery by using evacuated tubes (Vacutainer; Becton, Dickinson and Co., Franklin Lakes, NJ; Figure 1). Blood samples were stored on ice and transported to the laboratory for storage at 5°C until serum was harvested by centrifugation (1,200 x g). Serum samples were assayed for progesterone concentration by direct quantitative (non-extracted) radioimmunoassay using Coat-A-Count progesterone kits (Catalog no. TKPG; Siemens Medical Solutions Diagnostics, Los Angeles, CA) previously validated for bovine serum (Stevenson et al., 2012). Assay sensitivity was 2.47 ± 0.5 pg/mL. Inter- and intraassay coefficients of variation for 13 assays were 8.8 and 5.0%, respectively, for a pooled serum sample that averaged 3.04 ± 0.02 ng/mL. Pregnancy diagnosis was performed by transrectal palpation by the same veterinary practitioner 34 and 69 d post-TAI. Positive pregnancy diagnosis was determined by membrane slip or palpation of the amniotic vesicle.

Statistical Analyses,

Data for pregnancy per AI (P/AI) at 34 and 69 d, and pregnancy loss between the 2 diagnoses were analyzed using the GLIMMIX procedure (SAS Institute Inc., Cary, NC), with fixed effects of treatment (CON or CIDR), parity, BCS (≤ 2.5 vs. > 2.5), season [hot (June through September) vs. cool (October through May)] and all two-way interactions with treatment.

Experiment 2

Experimental Cows

Lactating Holstein cows were enrolled at nonpregnant diagnosis from November 2010 through October 2012 at the Kansas State University Dairy Teaching and Research Center (Manhattan). Cows were housed in covered, sand bedded, two-row freestalls with overhead sprinklers along the feed alley, and shade cloth covering the feeding area and feed bunk during summer. Cows were milked every 8 h in a double-6 herringbone milking parlor and fed twice or thrice (summer) daily a TMR calculated to meet nutrient requirements for lactating dairy cows producing 50 kg of 3.5% milk (NRC, 2001). The diet consisted of alfalfa, corn silage, soybean meal, whole cotton seed, corn or milo grain, corn gluten feed, vitamins, and minerals.

Experimental Design

At a non-pregnancy diagnosis 31 d post-previous AI service, cows were stratified by lactation number (1 vs. 2+) and randomly assigned to 3 resynchronization protocols (Figure 2.2). The first treatment (PG-3-G; n = 101) was initiated at d 31 nonpregnancy diagnosis and consisted of a 25-mg i.m. injection of PG (Pre-PGF; 5mL of Lutalyse Sterile Solution, Pfizer Animal Health) 3 d before a 100-µg i.m. injection of GnRH (Pre-GnRH; 2 mL of Factrel, Pfizer Animal Health) then the 5 d Ovsynch-Resynch (RES) TAI program (GnRH-1 injection 5 d before PGF-1 on d 5 and PGF-2 on d 6 (PGF-2), GnRH-2 56 h after PGF-1,and TAI on d 8) was initiated 7 d after the Pre-GnRH injection. The second treatment (RES-COS) was initiated on d 41 and consisted of the RES described in experiment 1. The third treatment (RES-CIDR) was initiated on d 41 and consisted of the RES-CIDR protocol described in experiment 1.

Of the 448 cows originally enrolled in the study, 64 cows were dropped from the study because of culling (n = 30), health reasons (n = 16), insemination before pregnancy determination (n = 10), failure to inseminate (n = 6), and death (n = 2). Therefore, numbers of cows per treatment were: (1) PG-3-G (n = 120); (2) RES-CON (n = 129); RES-CIDR (n = 140). Furthermore, 77 cows identified in estrus at any time after enrollment, including the day before TAI, were inseminated early before completing the entire experimental protocol to which they had been previously assigned and did not receive any further scheduled injections or blood sampling. The early bred cows included those identified by farm staff by visual detection of standing estrus.

Figure 2.2 Experimental design for experiment 2. At pregnancy diagnosis (PD) 31 d after previous service, nonpregnant lactating dairy cows were randomly assigned to 3 treatments and stratified by lactation number (1 vs. 2+): PG3G [injection of PGF 3 d before an injection of GnRH then the 5 d Ovsynch-Resynch [GnRH injection 5 d before and 56 h after PGF injections on d 5 and 6, and timed artificial insemination (TAI) on d 8]; RES-CON [GnRH injection 5 d before and 56 h after PGF injections on d 5 and 6, and TAI on d 8] or RES-CIDR [GnRH injection 5 d before and 56 h after PGF injections on d 5 and 6, and TAI on d 8 with CIDR insert concurrent with GnRH-1 and insert withdrawal at PGF-1]. Pregnancy diagnosis was performed via transrectal ultrasonography on 31 and 59 d post-TAI. US = Ultrasonography to determine ovarian structure present on ovaries; P4= Blood samples collected to determine circulating concentrations of progesterone.
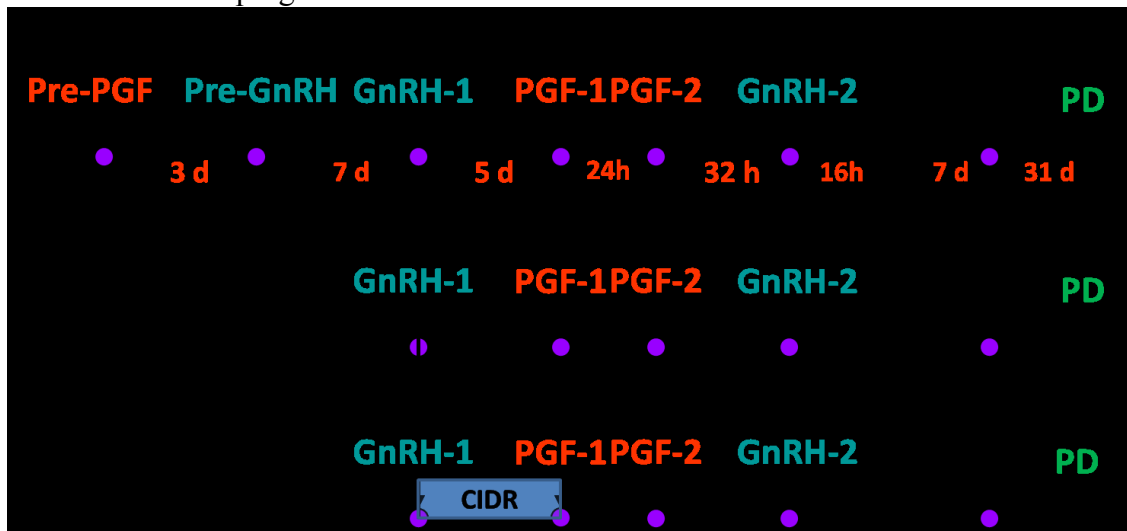
Body condition scores (Ferguson et al., 1994) were assigned and pregnancies were diagnosed 31 d post-AI (Figure 2.2). Four technicians performed inseminations, with one technician performing more than 70%, and multiple sires were used. At each insemination, date, time, sire, and technician were recorded and entered into PC-DART (Dairy Records Management Systems, Raleigh, NC). Pregnancy diagnosis was performed by transrectal ultrasonography (5.0 MHz linear-array transducer, Aloka 500V; Corometrics Medical Systems Inc., Wallingford, CT) 31 and 59 d after TAI. A positive pregnancy was determined by the presence of anechoic uterine fluid and a CL ≥ 25 mm in diameter or anechoic uterine fluid and presence of an embryo with a heartbeat. Pregnancy loss was defined as a confirmed pregnancy at initial pregnancy diagnosis at 31 d post-TAI and diagnosed non-pregnant status at 59 d or if detected in estrus and reinseminated during the interim. Monthly DHI test-day ECM and test-day

milk yield near the onset of the resynchronization protocol was recorded for cows enrolled into the study.

Ovarian Steroids

Blood samples were collected via puncture of the caudal vessels into evacuated tubes (Vacutainer; Becton, Dickinson and Co.) as indicated in Figure 2.2. Blood samples were not collected from any cow inseminated based on detected estrus (EB). Samples were stored on ice and transported to the laboratory for storage at 5°C until serum was harvested by centrifugation (1,200 x g). Serum samples were stored at 15°C until they were assayed for progesterone and estradiol-17β concentrations. Circulating concentrations of progesterone were measured in all blood sera samples as described in experiment 1. Assay sensitivity was 1.49 ± 0.5 pg/mL. Inter- and intraassay coefficients of variation for 13 assays were 7.6 and 6.0%, respectively, for a pooled serum sample that averaged 3.44 ± 0.6 ng/mL.

Concentrations of estradiol-17β were measured by RIA (Stevenson, 2011) in samples collected on d 46 and 48 concurrent with GnRH-1 and PGF-1, respectively. Assay sensitivity was 0.07 ± 0.5 pg/mL in 4 assays. Interassay and intraassay coefficients of variation were 16.0 and 6.8%, respectively, for a pooled serum sample that averaged 7.47 ± 0.3 pg/mL.

Ovarian Sonograms

Ovarian scans were performed by transrectal ultrasonography (Figure 2.2) on d 34, 41, 46, 48, and 55 to measure and map all ovarian follicles > 5 mm. Number and location of CL were mapped and recorded during each scan, except on d 55 when the diameter of each CL was measured. The volume of each d 55 CL (7 d post-TAI) was calculated [$4/3$ x r3 x π, where r = radius (W/2 +H/2)/2, where W = largest width and H= largest height of the structure; and where $\pi = 3.14159$]. When fluid-filled cavity was present in a CL, the volume of the cavity was subtracted from the total CL volume.

Measurements

Luteolysis was assessed in response to injections of PGF given 24 and 48 h after PGF-1. Luteal regression was defined to have occurred when progesterone concentrations were ≥ 1 ng/mL immediately before PGF-1 and < 1 ng/mL 48 h later. The proportion of cows that ovulated and ovulation response to Pre-GnRH, GnRH-1, and GnRH-2 was determined 5 to 7 d

after injections, when a new CL was visualized at the site of a previously mapped and recorded follicle ≥ 10 mm. Preovulatory follicle was determined retrospectively following CL formation on d 55 (7 d post-AI). Growth rate of preovulatory follicle was determined between diameter measurements on d 46 and 48. At the ultrasound ovarian exam on the morning of before GnRH-2 administration (48 h post PGF-1), the presence or absence of anechoic fluid detected in the uterine lumen was recorded (physiological evidence of potential estrus and effects of elevated estradiol).

Statistical Analyses

Binomial ovarian characteristics and responses to hormonal injections (GnRH and PGF$_{2\alpha}$) were analyzed by logistic regression using the LOGISTIC procedure of SAS (SAS Institute Inc.). These characteristics and responses included the proportions of cows having elevated (≥ 1 ng/mL) or decreased (< 1 ng/mL) progesterone concentrations at various time points during the experimental period, single or multiple ovulation responses to GnRH injections, proportions of cows with an ultrasound-detected CL, and occurrence of luteal regression. The initial model included effects of treatment (PG-3-G, RES-CON, and RES-CIDR), lactation number (1 vs. 2+), season of year (hot = June through September vs. moderate and cold = October through May), and 2-way interactions between treatment and lactation number. The final model produced by backward stepwise selection of independent variables entered or retained in the model was based on the Wald statistic ($P < 0.10$).

Continuous variables were analyzed by ANOVA using a general linear model (GLM procedure, SAS Inst. Inc.). Dependent variables included the follicle diameters, number of follicles ovulating, number of CL per cow, CL volume, and concentrations of estradiol and progesterone. The model included the effects of treatment, lactation number, season, test day milk (TDM) yield, BCS, and two-way interactions of treatment and lactation number. Treatment means were separated by F-tests resulting from ANOVA.

Pregnancies per AI at 31 and 59 d as well as intervening losses were analyzed using the GLIMMIX procedure in SAS (SAS Institute Inc., Cary, NC). The model included the effects of treatment, lactation number, season, technician, sire, TDM yield, and BCS. Additional models included the dependent factors of ovulation response to both Pre-GnRH and GnRH-1 or cumulative ovulation responses to all GnRH injections and synchronization rate (progesterone <

1 ng/mL at GnRH-2 and ovulation success to GnRH-2). All continuous variable results are reported as least square means ± SE.

The rate at which cows were reinseminated was analyzed by the Cox proportional hazard ratio using the PHREG procedure in SAS, with the removal of variables by the stepwise backward elimination process based on the Wald statistic criterion when $P > 0.10$. Using the LIFETEST procedure of SAS, survival analysis was used to analyze the interval from enrollment to TAI. Statistical significance was defined as $P \leq 0.05$ and statistical tendencies as $0.05 < P \leq 0.10$.

RESULTS

Experiment 1

Only 76% of 1,023 cows had elevated (≥ 1 ng/mL) progesterone concentrations, indicative of a functional CL, at the d 34 nonpregnant diagnosis. Pregnancy outcome at d 34 post-treatment was affected by an interaction between treatment and progesterone status at GnRH-1. Cows with reduced (< 1 ng/mL) progesterone concentrations at GnRH-1 and assigned to d 34 RES-CIDR treatment had greater ($P = 0.028$) P/AI than d 34 RES-CON cows (39.1 vs. 31.3%; Figure 2.3). Day 34 RES-CIDR treated cows with elevated progesterone concentrations had reduced P/AI compared with cows receiving 34 d RES-CON treatment (28.1 vs. 35.3%). Pregnancy outcome at d 69 was not affected by treatment, progesterone concentration, or their interaction (Figure 2.4).

Figure 2.3 Pregnancy per AI determined 34 d post-insemination (Exp. 1) based on progesterone concentration at the onset of treatment. RES-CON is indicated by solid black bar and RES-CIDR indicated by stippled bars. Number values within bars represent number of cows.
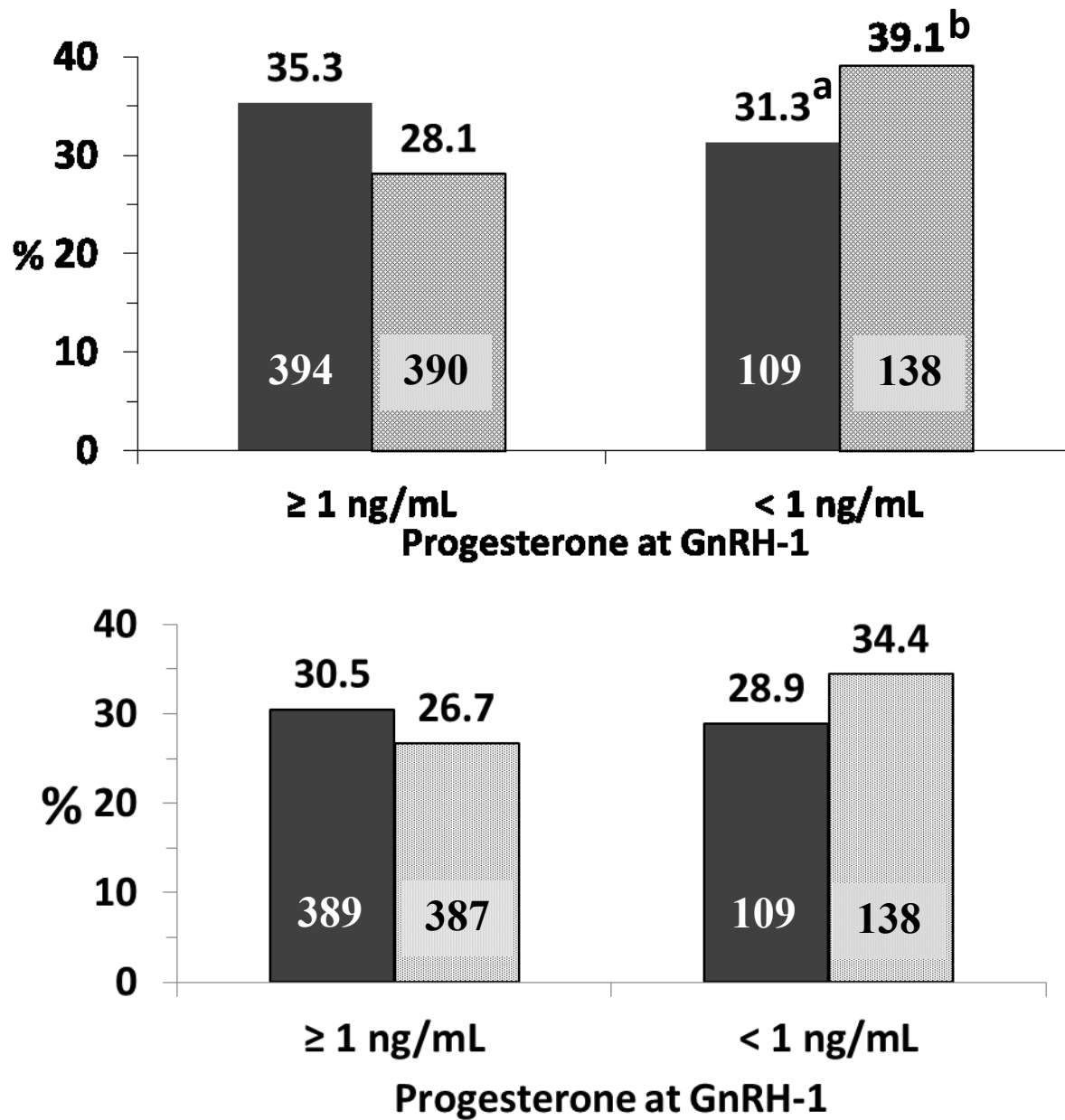
Figure 2.4 Pregnancy per AI determined 69 d post-insemination (Exp. 1) based on progesterone concentration at the onset of treatment. RES-CON is indicated by solid black bar and RES-CIDR indicated by stippled bars. Number values within bars represent number of cows.

Experiment 2

Proportion of cows with elevated progesterone concentrations at non-pregnancy diagnosis on d 31 was 70.3%. Proportion of cows with progesterone concentration ≥ 1 ng/mL at Pre-PGF was similar ($P = 0.16$) between PG-3-G cows and RES-treated (combined RES-CON and RES-CIDR) cows (74.8 vs. 68.1%, respectively). More ($P < 0.001$) PG-3-G cows had

luteolysis after Pre-PGF on d 31 than RES cows. More ($P < 0.001$) cows ovulated after the Pre-GnRH injection on d 34 than spontaneous ovulation in the RES cows. Proportion of cows with increased (≥ 1 ng/mL) progesterone concentrations on d 41 at GnRH-1 tended ($P = 0.08$) to be greater for PG-3-G than for RES treatments. Proportion of cows with CL present at GnRH-1 did not differ ($P = 0.13$) between treatments.

Table 2.1 Ovarian characteristics during presynchronization phase of Experiment 2 treatment periods.

| | Treatment | | |
| | Day 31 | Day 41[1] | |
Item	PG-3-G	RES	*P* value
Pre-GnRH			
Luteolysis[2], %	72.7	9.3	0.001
Ovulation after Pre-GnRH[3],%	62.3[a]	14.1	0.001
GnRH-1			
Progesterone ≥ 1 ng/mL, %	76.6	66.8	0.08
CL present, %	78.5	70.2	0.13

[1] Combination of RES-CON and RES-CIDR cows before treatment.
[2] Luteolysis was determined by changes in progesterone concentration between Pre-PGF (≥ 1 ng/mL) and Pre-GnRH injections 72 h later (< 1 ng/mL).
[3] Ovulation of at least one follicle after GnRH-1.

Incidence of ovulation after GnRH-1 was increased ($P = 0.05$) in PG-3-G treated cows compared those receiving RES-CIDR, and RES-CON treated cows had an intermediate ovulation response (Table 2.2). Double ovulation did not differ ($P = 0.46$) among PG-3-G, RES-CON, and RES-CIDR treatments after GnRH-1. Incidence of luteolysis, ovulation, and double ovulation after GnRH-2 did not differ ($P > 0.18$) among treatments.

Table 2.2 Ovarian characteristics during the synchronization phase of Experiment 2 treatment protocols.

	Treatment			
	Day 31	Day 41	Day 41	
Item	PG-3-G	RES-CON	RES-CIDR	_P_ value
GnRH-1				
Ovulation after GnRH[1], %	42.1[a]	36.3[a,b]	28.3[b]	0.05
Double ovulation after GnRH[2],%	8.4	4.4	5.7	0.46
GnRH-2				
Luteolysis[1], %	96.6	93.5	95.7	0.63
Ovulation after GnRH[2],%	88.6	85.0	84.9	0.67
Double ovulation after GnRH[3],%	19.1	28.3	24.5	0.18

[a,b] Proportions within row with different superscript letters differ ($P < 0.05$).
[1] Luteolysis was determined by changes in progesterone concentration between PGF-1 ($\geq$ 1 ng/mL) and GnRH-2 injections 48 h later (< 1 ng/mL).
[2] Ovulation of one follicle after GnRH-2.
[3] Ovulation of more than one follicle after GnRH-2.

Survival analysis indicate that PG-3-G treatment decreased ($P = 0.003$) days to reinsemination compared with RES-COS and RES-CIDR treatments (Figure 2.5).

Figure 2.5 Survival analysis of the interval from enrollment to TAI according to treatment (Exp. 2). Treatments: PG-3-G = presynchronization with a 25-mg injection of PGF$_{2\alpha}$ 3 d before 100-µg i.m. injection of GnRH 7 d before initiation of a 5 d Ovsynch-Resynch (RES) TAI program [GnRH injection 5 d before (GnRH-1) and 48 h after PGF injections on d 5 (PGF-1) and 6, and TAI on d 8], RES-CIDR = the RES protocol with a 5 d CIDR inserted concurrent with GnRH-1 and device removal at PGF-1, RES-COS = consisted of the RES protocol only.
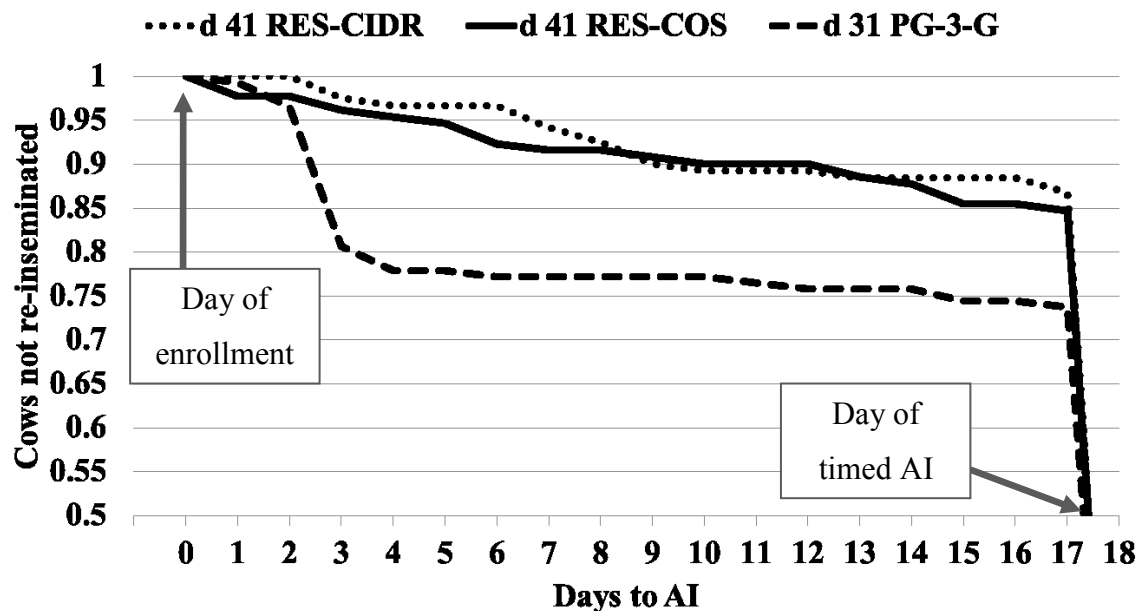
The P/AI was greater in cows starting RES on day 41 when progesterone concentration was decreased (< 1 ng/mL; 44%) than increased (≥ 1 ng/mL; 33%). Only 25.1% (77/307) of cows enrolled were inseminated early (EB) in the present study and approximately half (49.35%; 38/77) of those EB cows received PG-3-G compared with other RES treatments (RES-COS = 25.97%, 20/77; RES-CIDR= 24.68%, 19/77). No treatment differences were detected at 31 d or 59 d after TAI (Table).
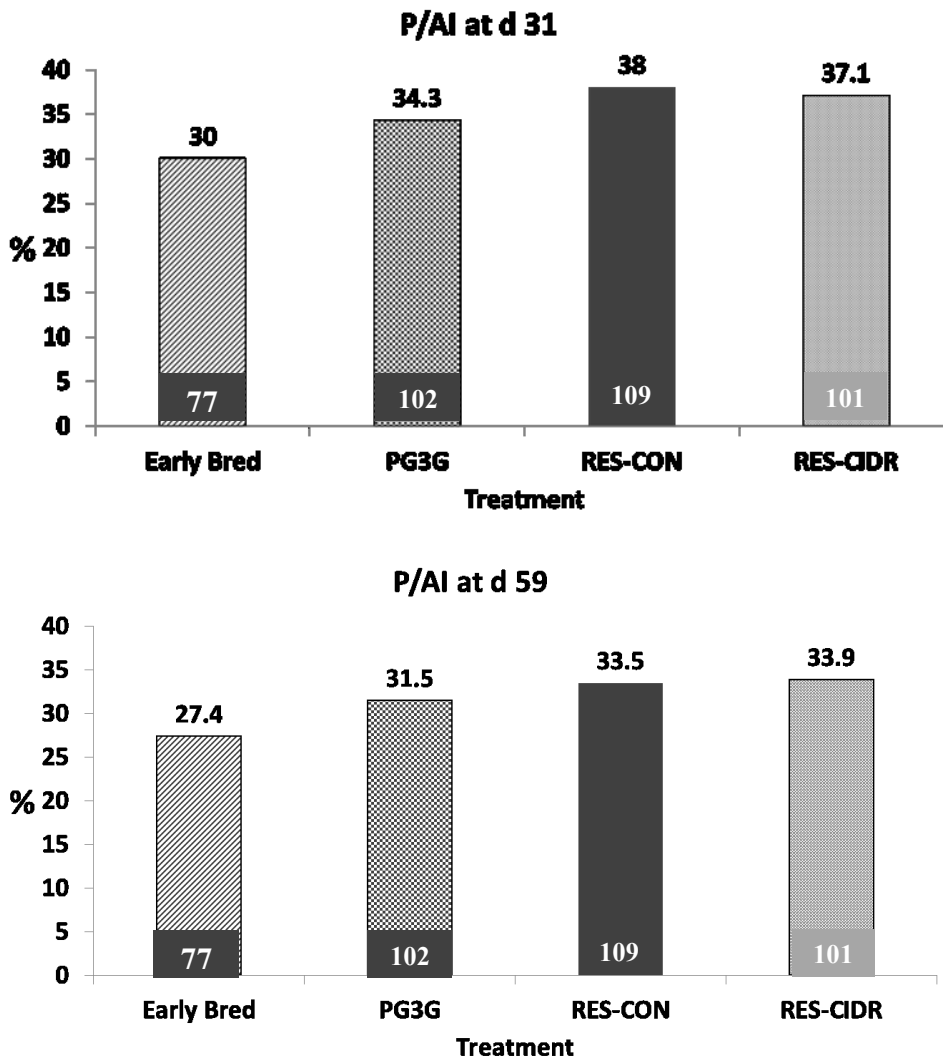
Figure 2.6 Pregnancy per AI 31 and 59 d post-AI for Experiment 2 treatment protocols.

DISCUSSION

Our objectives for Experiment 1 were to determine the effect of progesterone supplementation via the CIDR insert on pregnancy outcomes in dairy cows in which ovulation was synchronized using a 5-d Ovsynch protocol after a non-pregnant diagnosis. Intravaginal inserts previously have been utilized to increased synchrony and P/AI to TAI protocols (Chebel et al., 2006; Lima et al., 2009). Addition of a CIDR insert concurrent with GnRH-1 resulted in improved P/AI when circulating progesterone concentrations were more basal (< 1 ng/mL) and decreased P/AI when progesterone concentrations were increased (≥ 1 ng/mL) at GnRH-1. Bilby et al. (2013) also reported improved d 60 P/AI to a 7-d RES protocol when cows without a CL (27.0%) or decreased progesterone concentration received CIDR inserts (29.4%) compared with cows not receiving CIDR inserts without CLs (19.2%) or decreased progesterone concentrations (15%). In contrast, Bisinotto et al. (2010) reported increased P/AI to a 5-d RES protocol initiated 34 d after a previous AI in cows receiving CIDR with CL present (53.1%) compared with cows lacking a CL and no CIDR insert (43.1%).

Treatment with a CIDR insert when progesterone concentration is < 1 ng/mL at the initiation of a 5-d Resynch protocol may have improved P/AI via suppression of estrus and/or ovulation until TAI. This may have improved synchrony of the estrous cycle in CIDR-treated cows before TAI. Lima et al. (2008) reported improved synchronization of the estrous cycle because of CIDR treatment, which inhibited estrus and ovulation in the event of spontaneous luteolysis. Based on our observations that only 24% of cows had lower or basal progesterone (no CL) at the non-pregnancy diagnosis, only 1 of 4 nonpregnant cows would have improved P/AI when treated with progesterone via the CIDR insert. Furthermore, because of this interaction of treatment and progesterone status, blanket treatment of all cows is contraindicated because cows with elevated progesterone (likely bearing a functional CL) may have poorer P/AI when exposed to the CIDR as determined in experiment 1.

We conclude that improved P/AI by addition of a CIDR insert is progesterone-dependent for cows initiating RES on d 34 such that only cows with near basal concentrations of progesterone had improved fertility after supplemental progesterone.

Our objectives for experiment 2 were to determine the effect of presynchronization of estrous cycles and progesterone supplementation via a CIDR insert on ovarian characteristics and pregnancy outcomes in dairy cows in which ovulation is synchronized after non-pregnant

diagnosis. Presynchronization with PG-3-G resulted in a faster reinsemination rate because the interval from enrollment to AI was reduced. In a similar study, Chebel et al. (2013) reported presynchronization of non-pregnant cows using only PGF led to expedited reinsemination or a reduced insemination interval. Bruno et al. (2013) compared Ovsynch to a GnRH-based or a PGF-based synchronization program, and reported cows receiving PGF-based synchronization had increased proportions of cows submitted to AI after detection of estrus compared with the other programs. In contrast, GnRH-based synchronization reduced the proportion of cows submitted to AI following estrous detection, which resulted in more cows completing the TAI protocol (Bruno et al., 2013). In both studies, improved detection of estrus would have further enhanced the reinsemination rate and reduced the inter-insemination interval to the TAI protocol.

Several studies have reported return to estrus within 7 d following PGF administration (Lauderdale, 1972; Rowson et al., 1972; Louis et al., 1973; Inskeep, 1973) with approximately 50% of cows being detected in estrus on d 3 after PGF (Lauderdale et al., 1974). Recently, Chebel et al. (2006) confirmed these prior findings and reported the majority of cows return to estrus between 2 and 8 d after PGF treatment. Presynchronization using PG-3-G with PGF administration at initiation of the protocol may explain the increased proportion of cows displaying estrus and being reinseminated before TAI compared with the RES-CON and RES-CIDR cows. All cows receiving RES-CIDR treatment received an early insemination before CIDR insertion on d 41. Of cows receiving PG-3-G treatment, the majority (73.7% [28/38]) of cows submitted to AI early were 1 through 3 d post-PGF administration.

Early insemination following visual detection of estrus (EB) resulted in numerically decreased (30.0%) P/AI 31 d post-TAI in the current study compared with the other 3 treatments (PG-3-G= 34.3%, RES-COS= 38.0%, RES-CIDR= 37.1%). This substantiates our previous report (Stevenson and Pulley, 2012) indicating PG-3-G had improved P/AI compared with EB cows in cool-cold months and only numerically increased P/AI in summer months. In general, fertility is superior after detected standing estrus compared with submissions following TAI; however, these results indicate cows enrolled might have increased P/AI if allowed to complete the TAI protocol and resist engaging in so-called cherry picking of enrolled cows.

Giordano et al. (2013) demonstrated that reducing the interval between TAI services resulted in increased economy when models included various levels of estrous detection rates between TAI services. The models simulated Presynch-Ovsynch programs for first-service TAI

and following pregnancy detection via rectal palpation, transrectal ultrasound, or chemical test before enrollment into Resynch for subsequent TAI services in combination with estrous detection between TAI, and programs (Giordano et al., 2013). A decreased interval between AI services increases pregnancy risk closer to the end of the voluntary waiting period, which positively affects the herd dynamics. This may be achieved by increasing the number of cows submitted to AI following detected estrus, as this was reported to have increased economic returns (Giordano et al., 2013). In the present study, approximately 50% of cows enrolled into PG-3-G were inseminated before TAI compared with the other two RES treatments. Presynchronization with PG-3-G provides another opportunity for expression of estrus and ovulation in anovular cows; however, accurate assessment of estrus by farm personnel is important to ensure fertility of early inseminated cows is similar to that at TAI services.

Incidence of luteolysis to Pre-PGF and ovulation in response to Pre-GnRH was increased in cows receiving PG-3-G compared with RES treated cows. Before first service, cows presynchronized with Pre-PGF 2 d before Pre-GnRH had 77% luteolysis occurrence in response to Pre-PGF (Bello et al., 2006). Our results confirm this earlier report, in which luteolysis response to Pre-PGF was 73% for PG-3-G treated cows.

Ovulation incidence in response to Pre-GnRH was increased in cows receiving PG-3-G compared with RES treated cows. Presynchronization protocols including GnRH have reported ovulation incidence of 80% (Bello et al., 2006; Stevenson et al., 2012). In the current study, ovulation incidence was less than previously reported at 62.3% for PG-3-G cows.

Although d 31 PG-3-G increased luteolysis and ovulation rates before RES, no difference in P/ AI was detected compared with RES started on d 41 with or without a CIDR insert (Exp. 2), but PG-3-G cows were inseminated at a faster rate.

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Chapter 3 - LH Study Literature Review

Introduction

Synchronization of ovulation to allow for timed artificial insemination (TAI) has become one of the most utilized reproductive technologies. Various adaptations of this technology are now utilized world-wide in both beef and dairy cattle industries. Discovery and application of exogenous prostaglandin $F_{2\alpha}$ (PGF; Rowson et al., 1972) and gonadotropin-releasing hormone (GnRH; Schally et al., 1971) products have allowed for the development of ovulation synchronization protocols. Induction of a luteinizing hormone (LH) surge by administration of exogenous GnRH can induce ovulation in approximately 32 h (Pursley et al., 1995). Many studies have reported GnRH-induced ovulation responses in ovulation synchronization protocols (Stevenson et al., 1996; Pursley et al., 1997; Vasconcelos et al, 1999; Bello et al., 2006). Ovulatory responses are generally reduced during the luteal phase of the estrous cycle and increased in cows following PGF administration in response to the second GnRH injection before TAI (Vasconcelos et al, 1999). In the luteal phase, ovulation occurrence is thought to be reduced as a result of elevated progesterone concentrations, which subsequently reduce the ovulation response to GnRH by blocking or reducing GnRH-induced LH release. Dias et al. (2010) reported plasma LH concentrations to increase in heifers exposed to low progesterone [no CL + used controlled intravaginal drug release (CIDR); 15.4 ± 2.2 ng/mL] compared with heifers exposed to high progesterone (CL; 9.1 ± 1.2 ng/mL) and were increased in response to treatment with 200 or 100 µg GnRH (14.8 ± 2.2 vs. 9.8 ± 1.4 ng/mL, respectively).

Luteinizing Hormone

Gonadotropin-releasing hormone stimulates the release of follicle stimulating hormone (FSH) and LH from the anterior pituitary (White, 1970). Gonadotropin-releasing hormone is synthesized by nuceli, such as the ventromedial and arcuate nucleus, in the hypothalamus (Silverman, 1987), and is released into the hypophyseal portal blood system. Synthesis and release of GnRH is controlled by neurons located in the fore-, mid-, and hind-brain (Silverman, 1987). Pulsatile release of GnRH occurs daily every 1.5 to 2.0 h throughout the follicular phase and every 4 to 8 h in the luteal phase of the bovine estrous cycle (Stojikovic et al., 1994). Control

mechanisms for tonic pulsatile GnRH secretion has not been fully elucidated (Senger, 2003). Each GnRH pulse begins with simultaneous depolarization on several GnRH neurons, which release a small quantity of GnRH or a GnRH pulse (Senger, 2003). Neural secretion of GnRH is comparatively low (5 pg/mL), and subsequently low amplitude pulses of LH are released (Senger, 2003).

The preovulatory surge of GnRH is regulated by a combination of decreased progesterone and elevated estradiol concentrations, because estradiol in the presence of decreased progesterone exerts a differential effect on GnRH (Senger, 2003). The tonic center of the hypothalamus releases small amplitude pulses of GnRH that cause release of FSH and LH from the anterior lobe of the pituitary (Senger, 2003). The surge center of the hypothalamus releases large amplitude pulses of GnRH resulting in the release of a surge of LH that causes ovulation to occur (Senger, 2003).

Estradiol Effects on LH release

Pulse amplitude and pulse frequency of LH are influenced by steroid hormone concentrations (Rahe et al., 1980). In the follicular phase of the estrous cycle, developing follicles produce increasing estradiol concentrations. Increased production of estradiol by growing follicles, especially the dominant follicle, is responsible for initiating a cascade of events leading to the LH surge (Knobil, 1974; Legan et al., 1975; Fink, 1979). Researchers have demonstrated that increased circulating concentrations of estradiol are required for the endogenous LH surge and subsequent ovulation to occur (Sarkar et al., 1976; Sherwood et al., 1980; and Ching, 1982). During behavioral estrus, circulating estradiol concentrations reach a threshold level or peak concentration, which stimulates the surge center of the hypothalamus to release large quantities of GnRH that stimulate the anterior lobe of the pituitary to secrete the preovulatory surge of LH, which is 10 times greater than the tonic LH pulse (Senger, 2003).

Ovariectomized cows treated with estradiol implants have greater circulating LH concentrations than cows without implants (Crister et al., 1983; Day et al., 1986; Stumpf et al., 1988b; Kinder et al., 1991). Kinder et al. (1991) reported these elevated LH concentrations resulted from increased LH pulse amplitude and estradiol concentrations are critical for LH pulse activity.

During periods of elevated estradiol concentrations, LH pulse frequency is decreased; conversely, when estradiol is decreased, frequency of LH pulses increases. Thus, estradiol enhances the pituitary responsiveness to GnRH. Several laboratories have demonstrated that initially estradiol exerts a negative feedback on GnRH secretion; however, after 8 to 12 h, the effect becomes a positive feedback on GnRH (Vilchez-Martinez et al., 1974; Cooper et al., 1974; Henderson et al., 1977b). Cooper et al. (1974) reported that LH secretion was inhibited in rats treated for 3 h with estradiol. In contrast, after 9 h of continuous estradiol administration, pituitary responsiveness was enhanced. When estradiol was elevated above physiological concentrations, the biphasic effect also was observed in cows (Kesner et al., 1981; Butler et al., 1983). Pulse frequency of LH was enhanced by increasing serum estradiol concentration that stimulated the LH surge before ovulation (Stumpf et al., 1989, 1991; Cupp et al., 1995). Normal physiological concentrations of estradiol during the follicular phase modulated LH secretion via increasing LH pulse amplitude (Day et al., 1986; Stumpf et al., 1988, 1989; Kinder et al., 1991). In contrast, Wolfe et al. (1992) reported exogenous estradiol administration, which increased circulating estradiol concentrations during late gestation, retarded both the frequency and amplitude of LH pulses.

Progesterone Effects on LH release

Progesterone produced by the corpus luteum (CL) exerts a strong negative feedback on the GnRH neurons of the hypothalamus and elevated progesterone concentrations reduce the frequency of basal episodic GnRH secretion by the tonic center of the hypothalamus (Senger, 2003). Therefore, a low frequency, high amplitude LH pulse occurs under progesterone dominance (Rahe et al., 1980). This pattern of LH secretion along with tonic FSH secretion allows for follicle development during the luteal phase of the estrous cycle (Senger, 2003). Elevated concentrations of progesterone prevent the development of preovulatory follicles, estradiol production, behavioral estrus, and preovulatory surge of GnRH and LH (Senger, 2003).

Scaramuzzi et al. (1971) first determined that high concentrations of circulating progesterone hindered the positive feedback effects of estradiol on LH release in ovariectomized ewes. Short et al. (1979) demonstrated that a LH surge similar to the preovulatory surge is unattainable during the luteal phase because of elevated progesterone concentrations. Roberson et al. (1989) demonstrated that cows treated with mid-luteal phase progesterone concentrations

had an extended interval between LH pulses (i.e., reduced pulse frequency). Exogenous treatment with estradiol effectively produced an LH surge when progesterone concentrations were basal during the follicular phase (Bolt et al., 1971) or during anestrus (Symons et al., 1973). Research from several independent laboratories has demonstrated that progesterone blocks the estradiol-induced LH surge in cattle (Bolt et al., 1971; Short et al., 1973; Kesner et al., 1981, 1982). Similarly, Bergfeld et al. (1995) administered large and small doses of progesterone to cattle and observed that when the treatments were reversed, the frequency of LH release was dramatically affected during the first 6 h after treatment.

Luteinizing Hormone Release during Ovulation-Synchronization Protocols

Despite wide application of the Ovsynch protocol in the field and in research settings, relatively little information is known about the characteristics of LH secretion in association with GnRH injections and PGF-induced luteolysis. Induced ovulation responses after GnRH administration during the Ovsynch (Bello et al., 2006; Chebel et al., 2006; Bisinotto et al., 2010; Ayres et al., 2013; Bilby et al., 2013) and Cosynch (Ribeiro et al., 2012a; Stevenson, 2012) protocols have been reported in many studies. Ovulatory responses are generally less during the luteal phase of the estrous cycle and are greater in cows after PGF administration in response to a second GnRH injection administered before TAI (Vasconcelos et al. 1999). Ovulatory responses during the luteal phase are thought to be less because of elevated progesterone concentrations (Ayres et al., 2013) and are increased during the follicular phase when progesterone is at baseline concentrations and estradiol is increasing. Elevated endogenous progesterone concentrations can reduce ovulation response to GnRH, likely by blocking or reducing GnRH-induced LH release. For example, we reported significantly reduced ovulatory responses to the first GnRH injection of Ovsynch in lactating dairy cows having a CL (43.4%) or when a progesterone insert was applied vaginally just before GnRH (47.1%) compared with controls without CL and into which no insert was administered (76.8%; Stevenson et al., 2008).

Ovulation Synchronization

The most widely used and adapted ovulation-synchronization protocol is Ovsynch, which consists of 2 injections of GnRH and a single injection of PGF (Pursley et al., 1995; Wiltbank and Pursley, 2013). Various presynchronization programs initiated before Ovsynch protocols and

numerous modifications of the original protocol have been studied in the attempt to increase reproductive efficiency (Moreira et al., 2001; Stevenson et al., 2005; Yamada et al., 2005; Thatcher et al., 2006; Souza et al., 2008; Stevenson et al., 2012; Wiltbank and Pursley, 2013).

Presynchronization Protocols

To begin a synchronization protocol during the optimal period of a 23-d estrous cycle (day 5 to 12) in lactating dairy cows (Vasconcelos et al., 1999; Moreira et al., 2001; El-Zarkouny et al., 2004; Sartori et al., 2004), synchronization of estrous cycles was attempted. Presynchronization of estrous cycles before the initiation of the Ovsynch protocol generally improves pregnancy per TAI (P/AI) compared with Ovsynch alone (Moreira et al., 2001). The standard presynchronization protocol (Presynch) includes 2 PGF injections given 14 d apart with the Ovsynch protocol beginning 10 to 14 d after the second PGF injection. The interval between Presynch and Ovsynch has been tested with 10 (El Zarkouny et al., 2001), 11 (Galvão et al., 2007), 12 (Moreira et al., 2001), or 14 d (Navanukraw et al., 2004) between the second PGF injection and the onset of Ovsynch. A Presynch program with intervals of 11 d improved P/AI compared with programs with 14 d (Galvão et al., 2007). This improvement is a result of an increased ovulatory response to the initial GnRH injection of Ovsynch, which has been shown to increase P/AI (Vasconcelos et al., 1999; Bello et al., 2006; Chebel et al., 2006; Galvao et al., 2007; Stevenson et al., 2012). Presynch-10 protocol should result in more cows at d 5 to 10 of their estrous cycle when the first follicular wave is present at the initiation of the Ovsynch protocol.

Other presynchronization programs involve the use of a non-breeding Ovsynch protocol (Double-Ovsynch; Souza et al., 2008), or a combination of PGF and GnRH injections given 2 (G-6-G; Bello et al., 2006) or 3 d (PG-3-G; Peters et al., 2002) apart followed in 6 or 7 d, respectively, by enrollment into Ovsynch. Double-Ovsynch improved P/AI in primiparous, but not in multiparous cows compared with Presynch-12 (Souza et al., 2008; Herlihy et al., 2012). The PG3G protocol increased the proportion of cows having at least one corpus luteum (CL), the number of CL per cow, and the proportion of cows having progesterone concentrations ≥ 1ng/mL at GnRH-1 compared with Presynch-10 treatment (Stevenson et al., 2012). Most previously anovular cows ovulate in response to GnRH administration (Gumen et al., 2003), suggesting that incorporation of GnRH into presynchronization protocols may induce cyclicity

and improve fertility (Ribeiro et al., 2012b). The employment of the PG3G protocol is likely a more effective presynchronization program because of its ability to induce ovulation in anovular cows compared with Presynch-10 and merits further investigation.

Ovsynch and Cosynch Protocols

Adaptations to the Ovsynch protocol, such as Ovsynch-56 (GnRH-7 d-PGF-56 h-GnRH-16 h-TAI) have been developed to improve pregnancy rates by optimizing timing of AI at approximately 16 h after final GnRH in relation to ovulation, which occurs between 24 and 32 h after GnRH (Pursley et al., 1998; Brusveen et al., 2008; Wiltbank and Pursley, 2013). Insemination near or after ovulation may provide insufficient time for sperm capacitation and transport resulting in aged oocytes before fertilization (Hunter and Wilmut, 1983, Wilmut and Hunter, 1984, Hawk, 1987). In contrast, an extended interval (>24 h) between insemination and ovulation also reduces fertility and several studies have reported a decline in P/AI (Dransfield et al., 1998; Saacke et al., 2000) in cows inseminated at either the onset of estrus or during the GnRH-induced LH surge (Pursley et al., 1998). Decreased fertilization rate has been reported when cows were inseminated at estrus onset compared with breeding 12 or 24 h later (Dalton et al., 2001). Previous reports collectively indicated the ideal timing of insemination is between 12 and 24 h after estrus to allow for adequate fertilization rates and viable embryos (Hunter and Wilmut, 1983; Wilmut and Hunter, 1984; Dalton et al., 2001). Pursley et al. (1998) reported the greatest P/AI when AI occurred 16 h after the final GnRH injection of Ovsynch. Time required for sustained sperm transport is between 5 and 12 h (Hunter and Wilmut, 1983; Wilmut and Hunter, 1984); therefore, a 4-h interval between AI and anticipated ovulation may be insufficient for sperm transport before oocyte aging (Brusveen et al., 2008). Despite these reported results, many dairies utilize a protocol known as Cosynch, in which the final GnRH is given concurrently with insemination (Geary and Whittier, 1998). The Cosynch protocol requires less handling as it eliminates one cow handling and facilitates once-daily cow restraint for administration of hormone injections and TAI; thereby potentially decreasing labor costs for hormonal injections (Sterry et al., 2007).

Studies have compared Cosynch to the Ovsynch protocol with conflicting results about which protocol produces P/AI similar to protocols with AI closer to the timing of ovulation. Inseminating 24 h after the final GnRH in the Ovsynch protocol improved P/AI compared with

Cosynch at 48 h (Cosynch-48; Vasconcelos et al., 1997). Two recent studies reported no difference in P/AI for cows assigned to either Cosynch-48 or Ovsynch with a 24 h interval between the final GnRH and AI (Portaluppi and Stevenson, 2005; Cornwell et al., 2006). Comparisons between Ovsynch with a 24 h interval between the final GnRH and AI, Cosynch-48 and Cosynch at 72 h (Cosynch-72) showed Cosynch-72 had improved P/AI compared with the other two treatments (Portaluppi and Stevenson, 2005). In contrast, P/AI was not significantly different between Cosynch-48 and Cosynch-72 (Brusveen et al., 2008). First-service P/AI was significantly increased in cows receiving Ovsynch-56 compared with Cosynch-72 treated cows and the difference was attributed to the timing of GnRH administration because AI timing was similar in both treatments (Brusveen et al., 2008). Substantial variation in fertility reported after altered intervals of the final GnRH administration and AI indicates that further research is warranted to determine if the improvement in fertility by using Ovsynch-56 compared with Cosynch-72 might offset increased labor and cow handling.

Collectively, studies in the literature are consistent in reporting an optimal time of AI to be between 8 and 16 h before ovulation (Trimberger and Davis, 1943; Trimberger, 1944; Dransfield et al., 1998; Pursley et al., 1998; Dalton et al., 2001). Improvement in P/AI for the Ovsynch-56 protocol likely occurs because of the 16 h interval between the GnRH-induced LH surge and AI compared with concurrent GnRH administration with AI for the Cosynch-72 option, which results in a more prolonged interval from AI to ovulation (Brusveen et al., 2008). Furthermore, differences in timing of PGF to GnRH injection may affect ovulatory follicle size as administration of GnRH 56 h after PGF likely reduces follicle size compared with Cosynch-72. In addition, the relationship of the characteristics of LH release associated with each GnRH injection of both Ovsynch and Cosynch protocols and the steroid milieu has not been determined. Lucy and Stevenson (1986) treated dairy heifers and cows with 2 PGF injections 11 d apart followed 72 h later by GnRH and detected both induced and spontaneous LH surges in all females. It is not known what proportion of LH surges occur before GnRH administration in an Ovsynch or Cosynch protocol, or whether they have been preceded by the PG3G or Presynch-10 presynchronization protocols. Therefore, further research is warranted to determine if improvement by implementation of the Ovsynch-56 protocol is related to more optimal timing from PGF to GnRH and LH release.

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Chapter 4 - Concentrations of Luteinizing Hormone and Ovulatory Response in Dairy Cows before Timed Artificial Insemination

INTRODUCTION

Synchronization of ovulation to allow for timed artificial insemination (TAI) has become one of the most popular reproductive technologies in dairy herds (Caraviello et al., 2006; Moeller et al., 2010). The most commonly used TAI programs in the dairy industry are variants to the original Ovsynch protocol [Pursley et al., 1998; an injection of GnRH 7 d before and 48 h after PGF$_{2\alpha}$ (PGF) with TAI 16 h following the last GnRH injection]. Presynchronization programs improve pregnancy per AI (P/AI) compared with cows starting Ovsynch at random stages of the estrous cycle (Moreira et al., 2001; El-Zarkouny et al., 2004; Navanukraw et al., 2004). Improved P/AI resulting from presynchronization programs before Ovsynch has been associated with synchronizing the majority of cows' estrous cycles to d 5 through 12, which improved ovulation incidence to the first GnRH injection of Ovsynch and resulting P/AI compared with cows treated at random stages of the estrous cycle (Vasconcelos et al., 1999; Galvao et al., 2007). A standard presynchronization protocol involves 2 PGF injections given 14 d apart (Presynch-14) with initiation of the Ovsynch protocol 14 d later (Navanukraw et al., 2004). The intervals between Presynch and Ovsynch have been investigated with 14 d (Navanukraw et al., 2004), 12 d (Moreira et al., 2001), 11 d (Galvao et al., 2007), and 10 d (Stevenson et al., 2012) between the second PGF injection and the onset of Ovsynch. The Presynch programs with 11 d (Galvão et al., 2007) intervals have improved P/AI compared with 14 d (Galvão et al., 2007). Presynchronization programs including PGF and GnRH tended to improve P/AI (Peters and Pursley, 2002) compared with Ovsynch alone. Use of a non-breeding Ovsynch protocol before the TAI Ovsynch program (Double Ovsynch) improved P/AI in primiparous cows in 1 study (Souza et al., 2008), but in primiparous and multiparous parity groups in a second study (Herlihy et al., 2012).

Presynchronization using G-6-G [PGF injection 2 d before GnRH with 5 d Cosynch protocol initiated 6 d after GnRH (2 PGF injections 24 h apart 7 d after the initial GnRH injection with the breeding GnRH injection administered 72 h after second PGF to coincide with

TAI)] compared with Presynch-11 in grazing dairy cows did not differ in P/AI at 30 or 65 d (Ribeiro et al., 2011). Presynchronization with PG-3-G compared with Presynch-10 improved ovulatory responses before Ovsynch, increased proportion of cows having progesterone concentrations $\geq$ 1 ng/mL at GnRH-1, and increased the number of CL in anovular cows at GnRH-1 (Stevenson et al., 2012). During the summer, PG-3-G produced more P/AI than Presynch-10 in 4 commercial dairy herds (Stevenson and Pulley, 2012). Given the potential advantages to anovular cows and its positive pregnancy outcome during summer, PG-3-G presynchronization seems to be superior to Presynch-10. In an experiment in grazing dairy cows whose estrous cycles were synchronized with Presynch-Ovsynch (5-d Ovsynch program initiated 10 d after the second PGF of Presynch), pregnancy outcome at d 30 tended to be reduced in cows administered with GnRH at 56 vs. 72 h and was reduced at d 65 (Ribeiro et al., 2012). Comparison of Cosynch to Ovsynch with the final GnRH timing at either 56 or 72 following presynchronization with either Presynch-10 or PG-3-G warrants further investigation.

Despite wide application of TAI programs in field, relatively little is known about the characteristics of LH concentrations in association with PGF-induced luteolysis and GnRH injections that are a part of these TAI programs. Concentrations of LH in response to either 50 or 100 µg of different GnRH products have been reported (Souza et al., 2009). One product tended to release less LH after both doses and induce fewer ovulations than the other 3 products in lactating dairy cows treated 7 d after AI (Souza et al., 2009). Similarly, this same GnRH product stimulated fewer ovulations and less LH release in beef heifers compared with another GnRH product (Martinez et al., 2003). Virgin heifers and lactating dairy cows were treated with 2 injections of PGF 11 d apart with GnRH administered 72 h after the last PGF injection (Lucy and Stevenson, 1986). Blood sampled every 4 h from 32 to 108 h after PGF detected LH surges in all females with 39% being induced by GnRH and 61% occurring spontaneously (Lucy and Stevenson, 1986). It is not known what proportion of LH surges occur before or after GnRH administration in the Ovsynch-56 or Cosynch-72 TAI protocols, whether they have been preceded by either the Presynch-10 or PG-3-G presynchronization protocols. Further, the relationship of the characteristics of LH concentrations associated with each GnRH injection and the steroid milieu has not been determined. Thus, this study was designed to determine: (1) the incidence of spontaneous and predictable GnRH-induced LH surges (peak LH magnitude, area under the LH secretion curve, and time to peak LH concentration); (2) size of the largest follicle

and its ovulatory incidence; (3) concentrations of estradiol-17b (estradiol) and progesterone; and (4) spontaneous and ovulatory responses to GnRH in lactating dairy cows enrolled in a TAI program preceded by presynchronization of estrous cycles.

MATERIALS AND METHODS

Cows, Housing, and Diets

The current studies were approved by the Kansas State University Institutional Animal Care and Use Committee. Lactating Holstein cows were enrolled at calving from September 2011 through March 2012 at the Kansas State University Dairy Teaching and Research Center (Manhattan). Enrolled cows were considered to be structurally sound and housed individually in a tie-stall barn equipped with stall mats covered with wood shavings, feed bunks, and automatic waterers. Enrolled cows were moved to a double-six Herringbone parlor and milked thrice daily. Cows were individually fed ad libitum twice daily at 0630 h and 1600 h. A TMR diet calculated to meet nutrient requirements for lactating dairy cows producing 50 kg of 3.5% milk (NRC, 2001), which consisted of alfalfa hay, corn silage, soybean meal, whole cottonseed, corn or milo grain, corn gluten feed, vitamins, and minerals. Enrolled cows were evaluated daily for health status by trained farm personnel.

Experimental Design and Treatments

At calving, 90 cows enrolled in the study were stratified by lactation number (1 vs. ≥ 2) and assigned randomly to receive either of 2 presynchronization treatments (Figure 4.1). The first presynchronization program (PG-3-G; Stevenson et al., 2012, 2013) consisted of a 25 mg i.m. injection of PGF (Pre-PGF; 5 mL Lutalyse Sterile Solution, Zoetis, Florham Park, NJ) 3 d before a 100 μg i.m. injection of GnRH (GnRH-1; 2 mL Factrel, Zoetis). The second treatment (Pre10) was timed so that the second of two 25 mg i.m. injections of PGF (PGF-2; 5 mL Lutalyse Sterile Solution, Zoetis) was administered on the same day as the Pre-PGF injection in the PG-3-G program (Figure 1). A 7-d Ovsynch TAI program was initiated 10 d after PGF-2 injection for Pre10 or 7 d after GnRH-1 of the PG-3-G program. Treatment injections were staggered within cluster so that all cows were inseminated on the same day of the week.
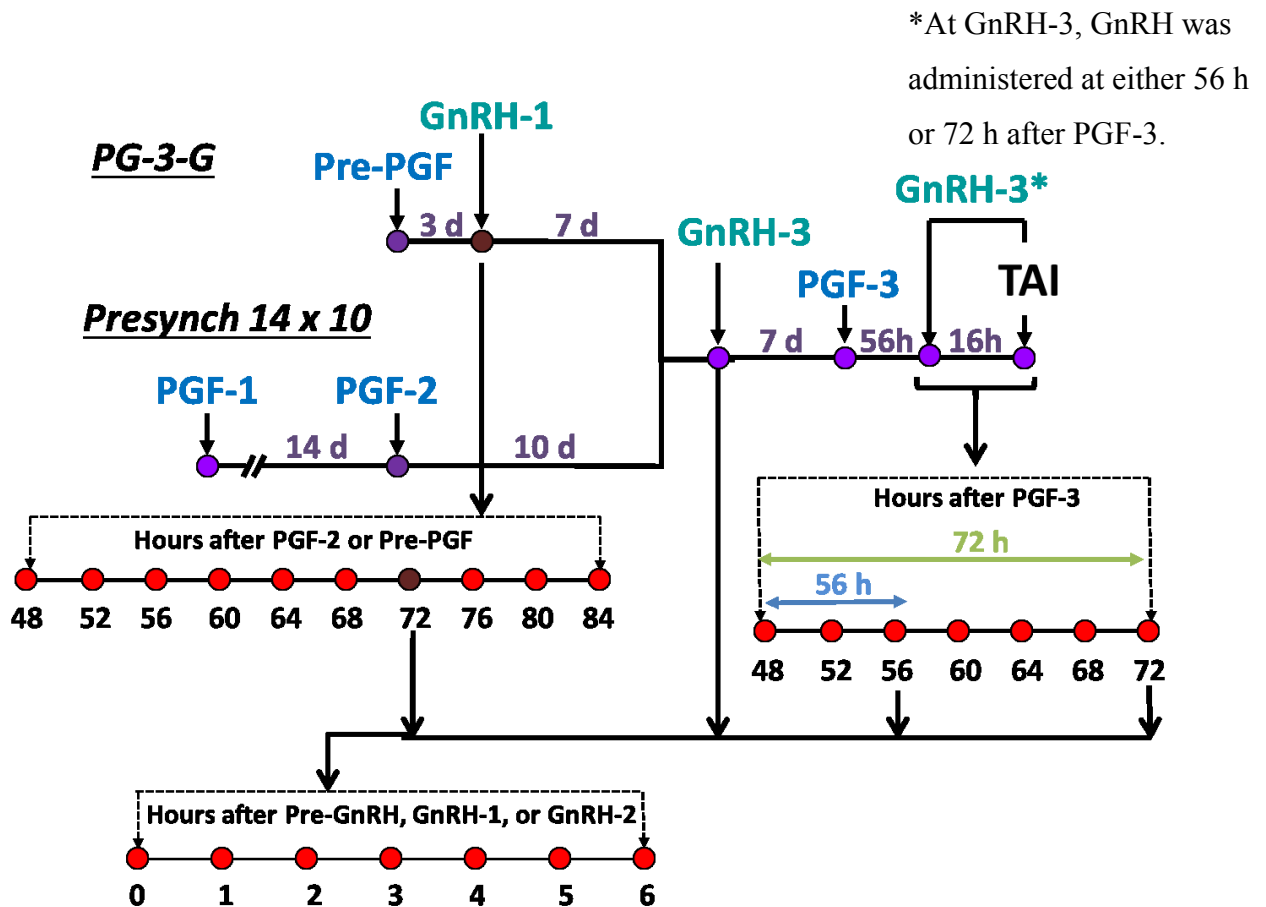
Figure 4.1 Experimental protocol. At calving, lactating dairy cows were assigned randomly to either of 2 presynchronization protocols: PG-3-G [25 mg injection of $PGF_{2\alpha}$ (Pre-PGF) and then 100 µg of GnRH (GnRH-1) 3 d later, followed by enrollment into the Ovsynch program 7 d later] or Pre10 [25 mg injection of $PGF_{2\alpha}$ (PGF-1 and PGF-2) administered 14 d apart, with the Ovsynch protocol initiated 10 d later]. The Ovsynch protocol is initiated with an injection of GnRH (GnRH-2) followed 7 d with an injection of PGF (PGF-3) with a second GnRH injection (GnRH-3) administered at either 56 or 72 h after PGF-3. Blood was collected to determine LH concentrations at: (1) GnRH-1: every 4 h from 0 to 80 h after PGF-2 and hourly from 72 to 78 h (GnRH-1 injected at 72 h); (2) GnRH-2: hourly from 0 to 6 h after GnRH-2; (3) GnRH-3: every 4 h from 0 to 80 h after PGF-3 and either hourly from 56 to 62 h (GnRH-3 injected at 56 h) or hourly from 72 to 80 h (GnRH-3 injected at 72 h).

A new breeding cluster was formed every week. Body condition scores (1 = thin and 5 = fat; Ferguson et al., 1994) were determined and assigned 7 d before the initiation of the Ovsynch program or concurrent with GnRH-1 administration in PG-3-G assigned cows (Figure 4.1). Cows were at a median of 68 DIM (68.4 ± 0.7; mean ± SE) at TAI. All inseminations were performed by 4 onsite trained AI technicians with 1 technician conducting 70% (49/70) of the inseminations. Multiple sires were used.

Ovarian Structures and Ovulation

Ovarian scans in all cows were conducted by transrectal ultrasonography (5.0 MHz linear-array transducer, Aloka 500V; Corometrics Medical Systems Inc., Wallingford, CT) to record diameter of all follicles and map location of follicles and CL present at GnRH-1, GnRH-2, PGF-3, GnRH-3, and 6 d post TAI, when the diameter of all luteal structures was measured (Figure 4.1). A map of each ovary was drawn with the position and size of all follicles ≥ 5 mm and location of each CL, which allowed for evaluation of ovulatory response to GnRH-1, GnRH-2, and GnRH-3. Follicle diameter was determined by averaging the width and height of each follicle using the internal electronic calipers of the ultrasound machine. During the ultrasound exam before administration of GnRH-2, presence of anechoic fluid in the uterine lumen was recorded (bioassay of potential estrus and effects of estradiol).

Pregnancy diagnosis was conducted by transrectal ultrasonography on d 31 and 61 after TAI. A positive pregnancy outcome required the presence of anechoic uterine fluid and a corpus luteum ≥ 25 mm in diameter or anechoic uterine fluid and the presence of a viable embryo with a visible heartbeat. Cows that displayed estrus and reinseminated before the first pregnancy diagnosis were considered non-pregnant to TAI unless later ultrasonography found reinseminated cows to be pregnant to the TAI. Pregnancies per AI were calculated by dividing the number of cows diagnosed pregnant at 31 ± 3 d or 61 ± 3 d after TAI by the number of cows receiving TAI in each treatment.

Procedures and Blood Sample Collections

Blood samples were collected by placement of an indwelling jugular catheter or by puncture of caudal vessels into evacuated tubes (Vacutainer; Becton, Dickinson and Co., Franklin Lakes, NJ), in the event of jugular catheter failure. Cows were fitted with a guide wire-style jugular catheter (Mila International Inc., Erlanger, KY). Under local anesthesia (lidocaine hydrochloride; Agri Laboratories Ltd., St. Joseph, MO), a size 10 scalpel blade was used to make a 0.5 inch incision to initially breech the skin to allow the access the jugular vein. A 45-cm long guide wire was threaded through the needle. The catheter (20 cm) was then strung onto the wire and was guided into a jugular vein. The catheter was flushed twice daily and after each blood collection with 5 mL of a sterile saline solution of 3.5% sodium citrate to prevent clotting. All blood samples upon collection were placed on ice and transported to the laboratory and stored at 5°C for approximately 16 h before serum was harvested by centrifugation at 1,000 x g for 15 min

in a refrigerated centrifuge. Blood sera was frozen and stored at −20°C until progesterone, estradiol, and LH concentrations were determined by radioimmunoassay (RIA).

Hormone Concentrations

Concentrations of LH were measured in the following blood sera samples. Beginning with the 0 h at PGF administrations (Pre-PGF or PGF-2, PGF-3) sample then for samples collected every 4 h from 48 through 84 h. Additional samples were collected hourly (0 to 6 h) after each GnRH injection (GnRH-1, GnRH-2, GnRH-3 at either 56 or 72 h; Figure 4.1). All sera samples for LH concentration were measured in triplicate and analyzed by liquid-phase double-antibody RIA (Atkins et al., 2008). Sera were assayed in triplicate (100 μL) in one assay with an intraassay coefficient of variation of 2.9% for a bovine serum pool that averaged 10.0 ± 0.3 ng/mL. Pooled sera assayed in quadruplicate at 25, 40, 60, 100, 175, 200, and 300 μL averaged 9.72 ng/mL and paralleled the standard curve.

Area under the curve for LH concentration was calculated as the sum of trapezoid areas between days of the experiment where Cp2 was concentration of a LH serum sample taken at time 2 [t2] and Cp1 was the LH serum concentration of a sample taken at time 1 [t1]. The following formula was used to calculate area under the LH curve for the 7 hourly samples after each GnRH injection (0 h):

$$AUC = \Sigma \, [(Cp1 + Cp2) / 2 * (t2 - t1) + \ldots + (Cpn + Cpn) / 2 * (tn - tn)]$$

Concentrations of progesterone in blood serum were measured at PGF-2 (Pre10) or Pre-PGF (PG-3-G) at 0 h, PGF-3 at 0 h, and PGF-3 at 60 h (Figure 4.1). Additional serum samples were pooled during the 7 hourly sampling windows at each GnRH injection (GnRH-1, GnRH-2, GnRH-3; Figure 4.1) by taking 0.1 mL of serum from each sample and combining into a single aliquot, which was then assayed for progesterone concentration. All serum samples for progesterone concentration were measured in duplicate and analyzed by direct quantitative (non-extracted) radioimmunoassay using Coat-A-Count progesterone kits (Catalog no. TKPG; Siemens Medical Solutions Diagnostics, Los Angeles, CA) and validated for bovine serum (Stevenson et al., 2012). Cows having luteolysis (CL regression) must have had serum progesterone ≥ 1 ng/mL at PGF-2 and < 0.5 ng/mL 60 h later and ≥ 1 ng/mL at PGF-3 and < 0.5

ng/mL 72 h later. Assay sensitivity was 4.7 ± 0.4 pg/mL and inter- and intraassay coefficients of variation for 12 assays were 5.1 and 3.4%, respectively, for a pooled serum sample that averaged 2.8 ± 0.04 ng/mL.

Concentrations of estradiol were measured in duplicate by RIA (Stevenson, 2011) in samples collected at 0, 24, 36, 48, 60, and 72 h following PGF-3 administration (Figure 4.1). Assay sensitivity was 1.1 ± 0.4 pg/mL and inter- and intraassay coefficients of variation for 2 assays were 5.5 and 2.6%, respectively, for a pooled serum sample that averaged 6.3 ± 0.3 pg/mL.

Statistical Analyses

The experiment was a completely randomized design with 2 treatments (Pre10 vs. PG-3-G) with cows balanced for lactation number (1 vs. ≥ 2) until 56 h after PGF-3 when the timing of GnRH-3 occurred at either 56 or 72 h after PGF-3. From that point (56 h), the experimental design became a 2 x 2 factorial arrangement of 4 treatments (Pre10-56, Pre10-72, PG-3-G-56, or PG-3-G-72). Cow was the experimental unit. Unless otherwise specified all values are expressed as least square means $\pm$ SEM.

Various binomial characteristics and responses to hormonal injections (GnRH and PGF) were analyzed by logistic regression using the LOGISTIC procedure of SAS (SAS Inst. Inc. Cary, NC, USA). These responses included the proportions of cows having luteolysis (defined previously), LH surge, uterine fluid, ovulation, and double ovulation (ovulation of more than 1 follicle after GnRH administration). The initial model included effects of treatment (PG-3-G vs Pre10), lactation number (1 vs. ≥ 2), and BCS (< 2.25 vs. ≥ 2.25). The final model produced by backwards stepwise selection of independent variables entered or retained in the model was based on the Wald statistic ($P > 0.10$). In general, for variables assessed at 56 h after GnRH-3, the model included permutations of treatment and GnRH timing to form 4 treatments. Those models first examined the main effects of treatment and GnRH timing. When a significant interaction was detected by ANOVA, the model included all 4 permutations of the main effects.

Analyses of serum LH concentrations were performed using the MIXED procedure of the SAS program (SAS Inst. Inc.). The model included treatment, lactation number, and BCS. Analyses of follicle diameter, CL number, LH peak and area under the curve of LH profiles for GnRH-1 and GnRH-2 were performed using the MIXED procedure of the SAS program with models that included treatment (PG-3-G vs. Pre10). The model for the comparisons of area under

the curve of LH profiles for GnRH-3 employed a model including treatment, GnRH-3 timing (56 vs. 72 h), circulating progesterone concentrations (< 0.5 vs. ≥ 0.5 or < 1.0 vs. ≥ 1.0 ng/mL), circulating estradiol concentration (< 2.0 vs. ≥ 2.0 pg/mL), treatment by time, treatment by progesterone concentration cut point, treatment by estradiol concentration cut point, and all three-way interactions.

Repeated measurements, including serum concentrations of estradiol and progesterone were analyzed as a split plot design in the MIXED procedure in SAS. The model used to analyze estradiol (0, 24, 36, 48, 60, and 72 h following PGF-3 administration) and progesterone (PGF-2 [Pre10] or Pre-PGF [PG-3-G] at 0 h, at 0 and 60 h after PGF-3, and pools at GnRH-1, GnRH-2, and GnRH-3 included treatment, day, treatment by day, lactation number, and BCS. Split-plot error term for testing treatment was cow-within-treatment variance.

Pregnancy outcomes at d 31 and 61 post-TAI and pregnancy loss were analyzed by SAS procedure LOGISTIC using the initial model of treatment, lactation number, their interaction, technician, sire, and BCS.

RESULTS

Prostaglandin F-2 (PGF-2)

Before PGF-2 administration, circulating concentrations of progesterone and the proportion of cows having progesterone concentrations ≥ 1 ng/mL did not differ ($P > 0.338$) between treatments. The proportion of cows having luteolysis after PGF-2 also did not differ ($P = 0.740$) between presynchronization treatments (Table 4.1).

Table 4.1 Progesterone concentrations and responses to presynchronization injection of PGF-2.

	Treatment		
Item	PG-3-G	Pre10	*P*-value
Progesterone[1], ng/mL	3.3 ± 0.5[4]	3.3 ± 0.5	0.973
	% (no./no.)		
Progesterone ≥ 1 ng/mL[2], %	72.7 (24/33)	64.9 (24/37)	0.338
Luteolysis[3], %	76.9 (20/26)	80.7 (21/26)	0.740

[1] Progesterone concentrations before PGF-2 (see Figure 4.1).
[2] Proportion of cows having progesterone concentrations ≥ 1 ng/mL before PGF-2.
[3] Luteolysis was determined by changes in progesterone concentration between PGF-2 (≥ 1 ng/mL) and 60 h later (< 1 ng/mL).

Gonadotropin-Releasing Hormone-1 (GnRH-1)

Before GnRH-1 administration, circulating concentrations of progesterone and the proportion of cows having progesterone concentrations < 1 ng/mL did not differ ($P > 0.216$) between treatments (Table 4.2). Number of CL per cow and proportion of cows having CL also did not differ ($P > 0.618$) between treatments. Diameters of the largest follicle (Table 4.2) and second largest follicle (PG-3-G: 11.2 ± 4.2 vs. Pre10: 11.4 ± 4.4 mm) before GnRH-1 administration did not differ ($P > 0.83$) between treatments. The proportion of cows ovulating after GnRH-1 was greater ($P < 0.003$) in cows receiving PG-3-G presynchronization and GnRH-1 administration compared with spontaneous ovulation occurrence in Pre10 cows.

Table 4.2 Ovarian responses before and in response to GnRH-1.

Item	Treatment		P-value
	PG-3-G	Pre10	
Progesterone[1],ng/mL	1.0 ± 0.3^{5}	0.6 ± 0.2	0.216
Progesterone < 1 ng/mL[2], %	$78.8 (26/33)^{6}$	83.8 (31/37)	0.594
Corpora lutea (CL) per cow (no.)	0.5 ± 0.1	0.5 ± 0.1	0.992
Cows with CL, %	45.5 (15/33)	40.5 (15/37)	0.681
Follicle diameter[3], mm	15.8 ± 0.8	15.6 ± 0.8	0.836
Ovulation[4],%	90.9 (30/33)	59.5 (22/37)	0.003

[1] Progesterone concentrations during h 6 LH sampling time period for GnRH-1 (see Figure 4.1)
[2] Proportion of cows having progesterone concentrations < 1 ng/mL after PGF-2.
[3] Diameter of the largest follicle before GnRH-1 injection.
[4] Spontaneous ovulation or ovulation in response to GnRH-1.
[5] Least square mean ± SEM
[6] % (no./no.).

Time to the LH peak after GnRH-1 tended ($P = 0.09$) to be shorter in cows receiving GnRH-1 compared with spontaneous peaks in those not receiving GnRH-1 (Table 4.3). The mean LH peak concentration was less ($P = 0.05$) in PG-3-G treated cows than in Pre10 cows. The proportion of cows with an LH surge was greater ($P < 0.001$) in cows receiving PG-3-G presynchronization and GnRH-1 administration than in Pre10 cows, in which only spontaneous LH surges could occur. No differences were detected in the area under the LH curve between treatments. Progesterone concentrations ($P = 0.47$) and number of CL ($P = 0.58$; 0 vs. 1+) had no effect on LH peak concentrations after GnRH-1.

Table 4.3 Effect of presynchronization treatment on LH secretion after GnRH-1 in lactating dairy cows.

Item	Treatment		P-value
	PG-3-G	Pre10	
Time to LH peak[1], h	1.1 ± 0.3^5	2.1 ± 0.4	0.09
LH peak[2], ng/mL	3.5 ± 0.3	4.5 ± 0.4	0.05
LH surge[3], %	$100 \ (33/33)^6$	$33.3 \ (12/36)$	< 0.001
Area under LH curve[4]	92.8 ± 20.2	94.3 ± 21.2	0.78

[1] Time to peak LH concentration was determined by changes in LH serum concentration from baseline before GnRH administration to maximum LH concentration.
[2] Maximum LH concentrations.
[3] Increase in LH concentration greater than 2 SD above baseline.
[4] Calculated by the trapezoidal method.
[5] Least square mean ± SEM.
[6] % (no./no.).

Patterns of LH concentrations during the 6-h blood collection period are shown in Figure 4.2. As expected, cows receiving the GnRH-1 had greater ($P < 0.001$) peak LH at 1 and 2 h compared with cows receiving no GnRH.
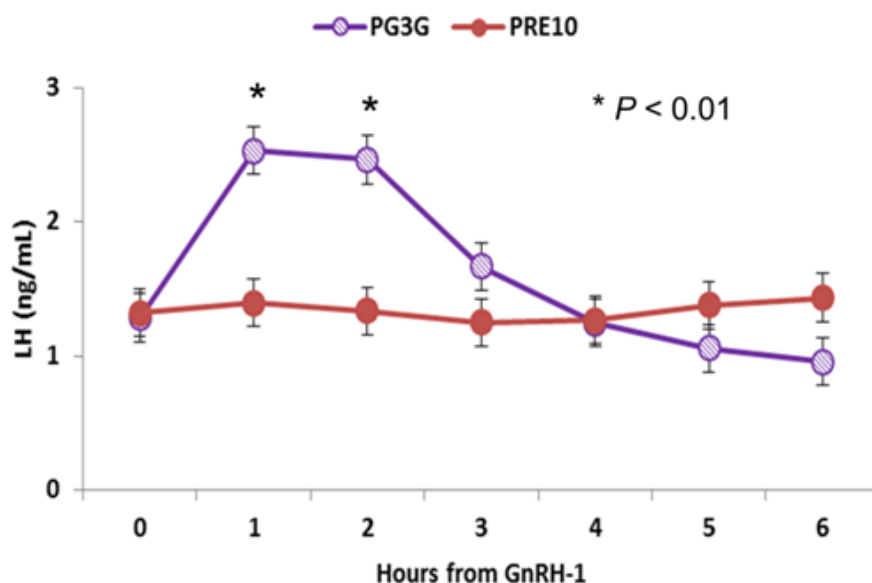
Figure 4.2 Pattern of serum LH concentrations during 6 h after GnRH-1.

Gonadptropin-Releasing Hormone-2 (GnRH-2)

Progesterone concentrations, proportion of cows with progesterone concentrations ≥ 1 ng/mL, number of CL per cow, and proportion of cows with a CL were greater ($P < 0.01$) for PG-3-G than for Pre10 cows at the onset of the Ovsynch protocol (Table 4.4). Diameters of the largest follicle (Table 4.4) and second largest follicles (PG-3-G: 10.7 ± 0.9 vs. Pre10: 9.7 ± 0.9) before GnRH-2 administration did not differ ($P > 0.45$) between treatments. Incidence of single ovulation did not differ, but double ovulation tended ($P = 0.112$) to differ between treatments.

Table 4.4 Ovarian responses before and in response to GnRH-2.

Item	Treatment		
	PG-3-G	Pre10	P-value
Progesterone[1],ng/mL	4.6 ± 0.4[6]	2.7 ± 0.3	< 0.001
Progesterone ≥ 1 ng/mL[2], %	100 (33/33)[7]	80.0 (28/35)	0.007
Corpora lutea (CL) per cow (no.)	1.5 ± 0.1	0.9 ± 0.1	0.005
Cows with CL, %	97.0 (32/33)	70.3 (26/37)	0.003
Follicle diameter[3], mm	15.4 ± 0.9	14.6 ± 0.9	0.584
Ovulation[4],%	51.5 (17/33)	62.2 (23/37)	0.372
Double ovulation[4],%	6.1 (2/33)	18.9 (7/37)	0.112

[1] Progesterone concentrations during 6 h LH sampling time period for GnRH-2.
[2]Proportion of cows having progesterone concentrations ≥ 1 ng/mL before GnRH-2.
[3] Diameter of the largest follicle before GnRH-2 injection.
[4] Proportion of cows with spontaneous ovulation or ovulation in response to GnRH-2.
[5] Proportion of cows with spontaneous ovulation or ovulation of more than 1 follicle in response to GnRH-2.
[6] Least square mean $\pm$ SEM.
[7] % (no./no.).

Time to peak LH concentration after GnRH-2 did not differ ($P = 0.342$) between treatments (Table 4.5). As a result of progesterone concentrations being less in the Pre10 treatment (Table 4.4), peak LH concentration and area under the LH curve were greater ($P < 0.05$) in the Pre10-treated cows compared with PG-3-G cows. Concentrations of LH were greater ($P < 0.01$) from 1 to 3 h after GnRH-2 in Pre10 cows than in PG-3-G-treated cows (Figure 4.5). All cows had LH surges in response to GnRH-2 regardless of presynchronization treatment (Table 4.5). Number of CL did not affect ($P = 0.56$) peak LH concentrations; however, cows with progesterone concentrations ≥ 1.0 ng/mL had decreased ($P = 0.001$) LH peak concentrations after GnRH-2 administration compared with cows having progesterone concentrations ≤ 1.0 ng/mL (2.2 ± 0.2 vs. 4.1 ± 0.4 ng/mL, respectively).

Table 4.5 Effect of presynchronization treatment on LH secretion after GnRH-2 in lactating dairy cows.

Item	Treatment		*P*-value
	PG-3-G	Pre10	
Time to LH peak[1],h	1.25 ± 0.10^5	1.38 ± 0.09	0.342
LH peak[2],ng/mL	2.19 ± 0.23	2.98 ± 0.21	0.014
LH surge[3],%	100 (33/33)[6]	100 (37/37)	--
Area under LH curve[4]	7.62 ± 1.97	10.56 ± 4.94	0.049

[1] Time to peak LH concentration was determined by changes in LH serum concentration from baseline before GnRH administration to maximum LH concentration.
[2] Maximum LH concentrations.
[3] Increase in LH concentration greater than 2 SD above baseline.
[4] Calculated by the trapezoidal method.
[5] Least square mean ± SEM.
[6] % (no./no.).
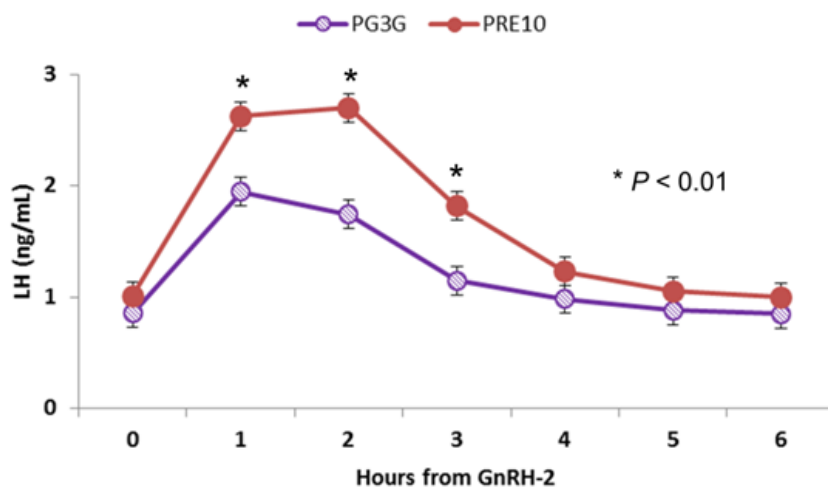
Figure 4.3 Pattern of serum LH concentrations during 6 h after GnRH-2.

Concentrations of LH were greater ($P < 0.01$) at 1, 2, and 3 h after GnRH-2 in Pre10 cows than in PG-3-G-treated cows (Figure 4.3).

Prostaglandin F-3 (PGF-3)

Although the proportion of cows with 1 or more CL was greater ($P = 0.053$) in PG-3-G cows than in Pre10 cows before PGF-3, none of the other ovarian characteristics assessed before

PGF-3 differed ($P > 0.275$) between treatments (Table 4.6). Mean concentrations of progesterone were approximately 5 ng/mL, the proportion of cows with progesterone concentrations ≥ 1 ng/mL exceeded 93%, and number of CL ranged from 1.7 to 1.9 regardless of treatment. No differences ($P > 0.275$) were detected in follicle size or estradiol concentrations before PGF-3. Incidence of luteolysis after PGF-3 also did not differ ($P = 0.367$) between treatments.

Table 4.6 Ovarian responses (means ± SE) before and after the PGF-3 injection.

Item	Treatment		P-value
	PG-3-G	Pre10	
Progesterone[1],ng/mL	5.2 ± 0.4[6]	4.6 ± 0.4	0.313
Progesterone [2], %	93.9 (31/33)[7]	94.6 (35/37)	0.907
Corpora lutea (CL) per cow (no.)	1.9 ± 0.2	1.7 ± 0.1	0.329
Cows with CL, %	100 (33/33)	89.2 (33/37)	0.053
Follicle diameter[3], mm	14.5 ± 0.8	13.3 ± 0.8	0.275
Estradiol[4], pg/mL	1.5 ± 0.6	2.4 ± 0.6	0.303
Luteolysis[5],%	96.8 (30/31)	91.4 (32/35)	0.367

[1] Progesterone concentrations before PGF-3 administration.
[2] Proportion of cows having progesterone concentrations ≥ 1 ng/mL before PGF-3.
[3] Diameter of the largest follicle before PGF-3 injection.
[4] Estradiol concentrations before PGF-3 administration.
[5] Luteolysis was determined by changes in progesterone concentration between PGF-3 (≥ 1 ng/mL) and 60 hours later (< 1 ng/mL).
[6] Least square mean ± SEM.
[7] % (no./no.).

Gonadotropin-Releasing Hormone-3 (GnRH-3)

No differences were detected in luteolysis after PGF-3 administration or incidence of single or double ovulation that were attributable to presynchronization treatment or timing of GnRH-3 (Table 4.7). All cows, except for a single cow in the PG-3-G-72 treatment combination, had an LH surge. All cows receiving GnRH-3 at 56 h had induced LH surges compared with 83.3 % of Pre10 and 82.4 % of PG-3-G cows treated at 72 h; however, no statistical differences were detected ($P = 0.154$) for GnRH-3 timing. Spontaneous LH surges did not occur before GnRH-3 was given at 56 h. In contrast, more cows ($P = 0.025$) treated with GnRH-3 at 72 h had spontaneous LH surges. An interaction ($P = 0.017$) of time and treatment on the diameter of the largest follicle was detected. Incidence of single and double ovulation did not differ ($P > 0.178$) between treatments.

Table 4.7 Luteolysis, incidence of LH surges, and ovulation after GnRH-3.

| | Treatment (T) | | | | P-value | | |
| | PG-3-G | | Pre10 | | | | |
Item	56 h	72 h	56 h	72 h	T	Time	T x time
Luteolysis[1], %	100 (16/16)[5]	82.4 (14/17)	94.7 (18/19)	87.5 (14/16)	0.367	0.250	0.533
LH surge[2], %	100 (16/16)	94.1 (16/17)	100 (19/19)	100 (18/18)	0.289	0.317	0.374
Induced	100 (16/16)	82.4 (14/17)	100 (19/19)	83.3 (15/18)	0.935	0.154	0.568
Spontaneous	0 (0/16)	17.7 (3/17)	0 (0/19)	16.7 (3/18)	0.864	0.025	0.138
Follicle size, mm	17.5 ± 0.9[6]	13.4 ± 0.9	14.1 ± 0.9	14.5 ± 0.9	0.019	0.049	0.017
Ovulation[3],%	87.5 (14/16)	93.3 (14/15)	100 (19/19)	88.9 (16/18)	0.178	0.235	0.347
Double ovulation[4],%	12.5 (2/15)	13.3 (2/15)	5.3 (1/19)	11.1 (2/18)	0.579	0.692	0.883

[1] Luteolysis was determined by changes in progesterone concentration between PG-3 (≥ 1 ng/mL) and 60 h later (< 1 ng/mL).

[2] Increase in LH concentration greater than 2 SD above baseline.

[3] Ovulation of a single follicle after GnRH-2.

[4] Ovulation of more than one follicle after GnRH-2.

[5] % (no./no.).

[6] Least square mean ± SEM.

Cows treated at 56 h had greater ($P = 0.025$) intervals from GnRH-3 administration to peak LH concentrations than cows receiving GnRH-3 at 72 h (Table 4.8). No treatment or treatment by time interaction was detected ($P > 0.348$) for time to peak LH concentration after GnRH-3. Cows with progesterone concentrations ≥ 0.5 ng/mL had smaller ($P = 0.009$) LH peak concentrations after GnRH-3 administration than cows having progesterone concentrations ≤ 0.5 ng/mL (2.5 ± 0.7 vs. 4.3 ± 0.5 ng/mL). Area under the LH curve did not differ ($P > 0.243$) between treatments.

Table 4.8 Effect of presynchronization treatment and GnRH-3 timing on LH secretion in lactating dairy cows.

| | Treatment (T) | | | | P-value | | |
| | PG-3-G | | Pre10 | | | | |
Item	56 h	72 h	56 h	72 h	T	Time	T x time
Time to LH peak[1], h	1.7 ± 0.2[4]	1.4 ± 0.2	1.6 ± 0.1	1.2 ± 0.1	0.35	0.03	0.58
LH peak[2], ng/mL	4.5 ± 0.5	5.2 ± 0.5	5.0 ± 0.5	4.8 ± 0.5	0.87	0.55	0.28
Area under LH curve[3]	15.4 ± 1.8	18.2 ± 1.7	16.5 ± 1.6	15.3 ± 1.7	0.62	0.66	0.24

[1] Time to peak LH concentration was determined by changes in LH serum concentration from baseline before GnRH administration to maximum LH concentration.

[2] Increase in LH concentration greater than 2 SD above baseline.

[3] Calculated by the trapezoidal method.

After GnRH-3 administration, LH concentrations increased from 0 to1 h and peaked at 2 h before steadily decreasing to basal concentrations at 6 h (Figure 4.4). Neither treatment nor time had any detectable effects on LH concentrations during the 6-h sampling period.
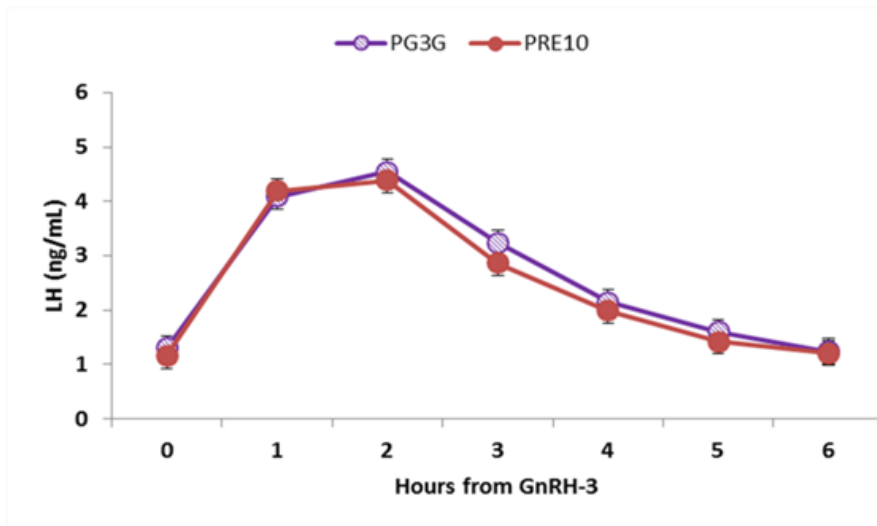
Figure 4.4 Pattern of serum LH concentrations during 6 h after GnRH-3.

No treatment, time of GnRH administration, or treatments by GnRH time interactions were detected for LH concentrations during the 6-h sampling period after GnRH-3 administration (Figure 4.5).
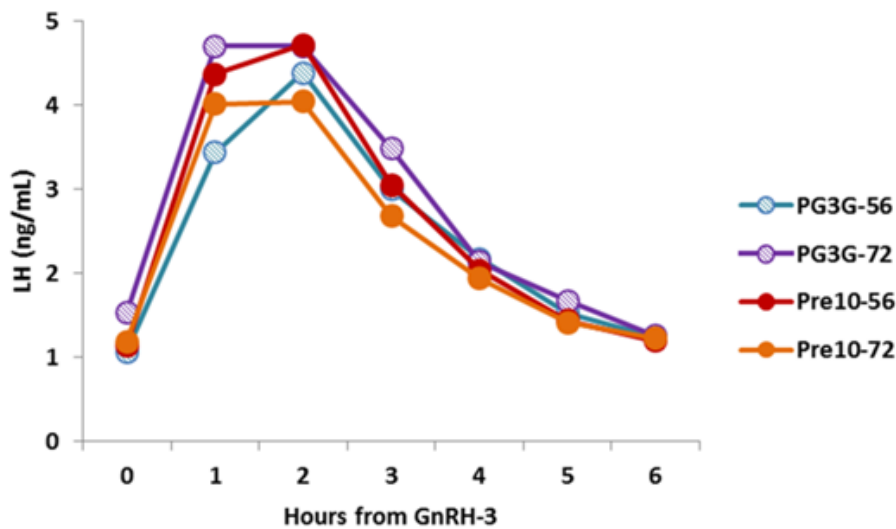
Figure 4.5 Pattern of serum LH concentrations during 6 h after GnRH-3 according to presynchronization treatment and timing of GnRH administration.

Concentrations of estradiol are shown in Figure 4.6. No effects of treatment, GnRH-3 time, or treatment by time interaction were detected at 0, 24, 36, or 48 h. In contrast, at 60 and 72 h, estradiol concentrations were greater ($P < 0.05$) for cows receiving GnRH-3 at 72 h than for cows administered GnRH-3 at 56 h (48 h: 5.0 ± 0.5 vs. 2.8 ± 0.9; 72 h: 5.1 ± 0.6 vs. 1.9 ± 1.3.

Presence of uterine fluid during the ultrasound examination before GnRH-3 administration was not influenced by treatment, time, or treatment by time interaction ($P > 0.272$). Diameter of the largest and second largest follicle did not influence ($P > 0.35$) presence of uterine fluid. Cows with estradiol concentration ≥ 2.0 pg/mL 48 h after PGF-3 had a greater ($P = 0.04$) incidence of uterine fluid than cows having estradiol concentrations ≤ 2.0 pg/m (87.0% (20/23) vs. 17.4% (4/23), respectively).
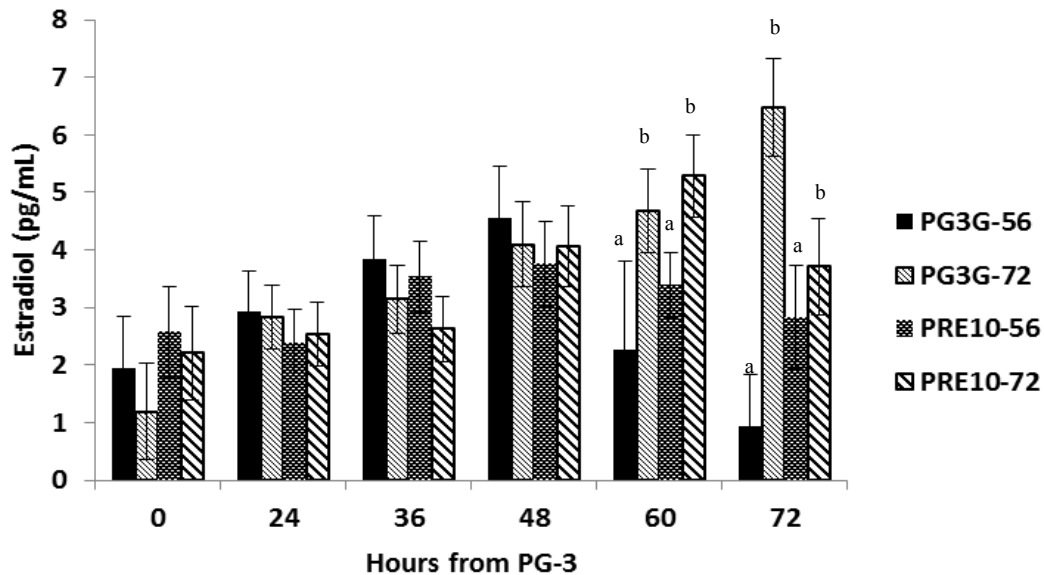
Figure 4.6 Serum estradiol concentrations for Pre10 or PG-3-G cows treated with GnRH-3 at 56 or 72 h after PGF-3. Least square means $\pm$ SEM within hour having different letters (a and b) differ ($P < 0.05$).

Fewer ($P < 0.002$) pregnancies were detected at 31 d post-AI in cows receiving the Pre10-72 treatment combination than in cows receiving Pre10-56, PG-3-G-56, or PG-3-G-72 treatment combinations (Table 4.9). Treatment differences at d 60 post-TAI followed a similar pattern to d 31. Pregnancy loss did not differ among treatments; however, only 1 cow in PG-3-G-72 treatment had pregnancy loss between pregnancy diagnoses.

Table 4.9 Pregnancy per AI after presynchronization with Pre10 or PG-3-G and GnRH administration at 56 or 72 h.

| | Treatment[1] | | | |
| | PG-3-G | | Pre10 | |
Item	56 h	72 h	56 h	72 h
Pregnancy per AI[2], %				
At 31 d	57.1[a] (8/14)	56.3[a] (9/16)	52.6[b] (10/19)	22.2[b] (9/16)
At 61 d	57.1[a] (8/14)	53.3[a] (8/15)	52.6[b] (10/19)	22.2[b] (8/15)
Pregnancy loss[3], %	0 (0/14)	6.3 (1/16)	0 (0/19)	0 (0/18)

[a,b] Different ($P < 0.002$) within row.
[1] See Figure 1.
[2] Determined by transrectal ultrasonography of uterine fluid plus a CL or presence of viable embryo.
[3] Pregnancy losses were calculated between the 2 pregnancy diagnoses.
[4] % (no./no.).

DISCUSSION

This study was designed to determine: (1) incidence of spontaneous and predictable GnRH-induced LH surges (peak LH magnitude, area under the LH secretion curve, and time to peak LH concentration); (2) diameter of the largest follicle and its ovulatory incidence; (3) concentrations of estradiol and progesterone; and (4) ovulatory responses in lactating dairy cows enrolled in a TAI program. This objective was met by using 2 presynchronization protocols, one of which (Pre10) should provide greatest potential for ovulation in response to GnRH-2 of all the PGF Presynch programs, which is based on studies in which administering the first GnRH injection of Ovsynch at various stages of the first follicular wave reported 10- or 11- d intervals from PGF-2 to GnRH facilitated the greatest ovulatory responses to GnRH-2 (Vasconcelos et al., 1999; Galvão et al., 2007). Perhaps equally as important as increasing ovulation response to GnRH of Ovsynch is to produce greater concentrations of progesterone at the onset of Ovsynch, which resulted in greater fertility as well (Stevenson et al., 2012; Ayres et al., 2013).

Improving ovulatory response to GnRH-2 has produced improved embryo quality (Cerri et al., 2009) and increased P/AI (Chebel et al., 2006; Stevenson et al., 2007; Rutigliano et al., 2008). The second protocol (i.e., PG-3-G) was chosen because of its potential to stimulate ovulation in cows before commencement of a TAI protocol (Bello et al., 2006; Souza et al., 2008), particularly in anovular cows (Stevenson et al., 2012). The novel findings of the present

study are the characteristics of LH release associated with each GnRH injection for both Ovsynch and Cosynch protocols.

Incidence of spontaneous LH surges following GnRH-1 was less in Pre10 cows compared with the incidence of LH surges in cows receiving PG-3-G protocol, which included a Pre-GnRH injection. Interestingly, the 43% of Pre10 treated cows that had a spontaneous LH surge had greater LH peak concentrations than the induced LH surge peak concentrations in all of the PG-3-G treated cows. Lucy and Stevenson (1986) reported the incidence of spontaneous LH surges in control cows between 52 and 104 h after a PGF injection. In cows administered GnRH injection at 72 h, induced LH surges occurred between 74.2 and 74.5 h (Lucy and Stevenson, 1986). The findings of current study confirms this earlier report, because time to peak LH concentration tended to be delayed in Pre10 cows compared with PG-3-G cows, which received the GnRH-1 injection, compared with Pre10, which did not.. After GnRH-1 treatment of PG-3-G cows, LH increased from basal concentrations at 0 h and stayed at maximum concentration until after 2 h, at which time they decreased to a baseline, similar to the Pre10 cows. The Pre10 cows had greater LH peak concentrations during spontaneous LH surges compared with PG-3-G cows during induced LH surges. Before GnRH-1, progesterone concentrations were similar at approximately 1 ng/mL and incidence of ovulation in response to GnRH-1 was 91% for PG-3-G and 60% for Pre10 cows, respectively. Previous studies reported ovulation incidence of 80% and 85% , which is similar to the 91% of the current study, following the GnRH-1 injection in the same (Stevenson et al., 2012) or similar treatments [G-6-G (GnRH injection preceded 2 d by a PGF injection before Ovsynch); Bello et al., 2006)]. Progesterone concentrations were significantly increased in PG-3-G treated cows after GnRH-1 because all cows in this treatment ovulated in response to GnRH-1.

Before GnRH-2, progesterone concentrations, proportion of cows with progesterone concentrations ≥ 1 ng/mL, number of CL per cow, and the proportion of cows having a CL were greater in PG-3-G treated cows than in Pre10 cows. These results confirm our earlier findings utilizing the same presynchronization treatment (Stevenson et al., 2012). Progesterone concentrations > 3 ng/mL in cows presynchronized with Double Ovsynch 7 d after GnRH-induced ovulation (Souza et al., 2008; Giordano et al., 2012) or Presynch-Ovsynch (Moreira et al., 2001; Stevenson et al., 2012) have been reported in several studies, and the current study collaborates these previous results. A greater proportion of PG-3-G cows had progesterone

concentrations $\geq$ 1 ng/mL (100% vs. 80%) compared with Pre10 cows, similar to our previous report for the same treatments (90.5% vs. 76.2%; Stevenson et al., 2012). Likewise, the number of CL per cow and proportion of cows having a CL was greater in PG-3-G cows than Pre10 cows in the current study and in our recent report (Stevenson et al., 2012).

After GnRH-2 all cows regardless of treatment had induced LH surges, and no time to peak LH concentration differences were detected; however, the LH peak and the area under the LH curve was greater in Pre10 treated cows than PG-3-G cows. Following GnRH-2 in the presence of functional CL and mean progesterone concentrations of 4.6 ng/mL, only 51.5% of PG-3-G cows ovulated, which is similar to the previous report of ovulation incidence of 55.3% 7 d following a previous GnRH injection (Souza et al., 2009). After GnRH-2, Pre10 cows had lesser mean progesterone concentrations of 2.7 ng/mL, and incidence of ovulation was numerically greater at 62.2 % and double ovulation was significantly increased compared with PG-3-G cows.

Giordano et al. (2012) reported that peak LH concentrations and the area under the LH curve were increased for 12 cows with low (< 1 ng/mL) progesterone compared with 12 cows having high (>1 ng/mL) progesterone concentrations. In the current study, progesterone concentrations, proportions of cows with progesterone $\geq$ 1 ng/mL, number of CL per cow, and proportions of cows with a CL were less in Pre10 cows than in PG-3-G cows, which may explain why peak LH concentrations were greater in Pre10 cows at GnRH-2. The pituitary gland has a decreased responsiveness or attenuation in its response to exogenous GnRH treatment in the presence of high circulating concentrations of progesterone (Giordano et al., 2012). Similar findings have been reported in beef heifers (Dias et al., 2010) and beef cows (Cline, 2002; Colazo et al., 2008). The area under the LH curve was greater in Pre10 cows with lower progesterone concentrations in the current study than in PG-3-G cows having greater progesterone concentrations. Reports of area under the LH curve (39.9 ± 4.5 ng^2) after using the same gonadorelin product 7 d after a previous GnRH injection in lactating dairy cows (Souza et al., 2009) was greater than that detected in the current study after GnRH-2 (PG-3-G: 7.62 ± 1.97 vs. 10.56 ± 4.94 ng^2). Despite the lesser LH concentrations secreted in response to GnRH than those previously reported, follicle diameter and ovulation rates after GnRH-2 administration did not differ between treatments, and incidence of double ovulation was similar.

Before PGF-3, progesterone and estradiol concentrations, proportion of cows with progesterone concentrations ≥1 ng/mL, number of CL per cow, and follicle diameter did not differ. In our previous study of these same presynchronization protocols, we reported PG-3-G tended to increase the proportion of cows with progesterone concentrations ≥ 1 ng/mL, progesterone and estradiol concentrations, and increased the number of CL per cow (Stevenson et al., 2012). In the current study, we were unable to detect any of the previously reported differences at PGF-3, except for the increased proportion of cows with a CL in PG-3-G cows.

More spontaneous LH surges before GnRH-3 administration at 72 h was observed than before GnRH-3 at 56 h, which induced LH surges. By delaying the GnRH-3 injection to 72 h, 17.1% (6/35) of cows regardless of treatment had spontaneous LH surges before GnRH injection. In order to control timing of the LH surge and ovulation, the final GnRH injection should be given before spontaneous a LH surge for TAI to result in pregnancy outcomes similar to breeding based on detected estrus (Peters and Pursley, 2003). Studies have indicated that the earliest a spontaneous LH surges occurs in the range of 36 to 48 h following PGF-induced luteal regression (Momont and Sequin, 1983; Walker et al., 1996). In the present study, administration of GnRH at 56 h after PGF-3 induced a LH surge before a spontaneous surge occurred; however, delaying an additional 16 h resulted in 17% of cows having spontaneous surges before GnRH administration, producing less synchrony between AI and ovulation.

In the current study, LH peak concentrations in response to GnRH treatments ranged from 2.19 to 5.24 ng/mL. These results are similar to those reported by Lucy and Stevenson (1986) with LH peak concentration ranging from 0.6 to 6.7 ng/mL in dairy cows. In contrast to our results, Souza et al. (2009) reported LH peak concentration in response to 50 μg of GnRH of 9.6 ng/mL and 21.6 ng/mL after 100 μg of GnRH in lactating dairy cows. Most of these differences are likely related to employing different LH standards and GnRH products (Souza et al., 2009).

At 60 and 72 h, estradiol concentrations were increased for cows receiving GnRH-3 at 72 h compared with those cows administered GnRH at 56 h. Estradiol concentration was at maximal concentrations (4.55 ± 0.89 and 3.76 ± 0.74) at 48 h for cows receiving GnRH-3 at 56 h. Interestingly, cows that received GnRH-3 at 72 h had maximal estradiol concentrations at either 60 (Pre10: 6.48 ± 0.89) or 72 h (PG-3-G: 5.28 ± 0.72). Previously reported maximal estradiol concentration before ovulation in lactating dairy cows were reported to be 7.3 ± 0.8 pg/mL

(Sartori et al., 2004) and 7.9 ± 0.8 pg/mL (Wiltbank et al., 2006). Cows receiving GnRH-3 at 72 h had an additional 16 h for follicular maturation and estradiol production than cows receiving GnRH at 56 h; however, follicle diameter was decreased in cows receiving PG-3-G treatment with GnRH adminstration at 72 h compared with cows given GnRH at 56 h. These results contradict our hypothesis that administration of GnRH 56 h after PGF would reduce follicle size compared with GnRH injection at 72 h. Diameter of the largest and second largest follicles did not have an effect on the presence of uterine fluid. Nevertheless, cows having estradiol concentrations ≥ 2 pg/mL had an increased probability of uterine fluid compared with cows having lesser estradiol concentrations.

Pregnancy per AI at 31 d post-insemination was less in cows receiving the Pre10-72 treatment compared with other treatment-time permutations. We previously reported that P/AI was numerically greater in PG-3-G (40%) cows than in Pre10 (33.3%) cows at d 32 when GnRH was given 56 h after PGF (Stevenson et al., 2012). In the current study, PG-3-G 56 h treated (57.1%) cows also had P/AI that was numerically greater than cows receiving Pre10-56 h (52.6%) treatment combinations. In another study investigating the same presynchronization protocols in commercial dairy herds, we reported P/AI was numerically increased in PG-3-G (41.2%) treated cows compared to Pre10 (35%) cows at d 32 to 38 (Stevenson and Pulley, 2012). During the summer months, cows presynchronized with PG-3-G had greater P/AI than Pre10 cows, but the results did not differ during cool-cold weather (Stevenson and Pulley, 2012).

Adaptations to the Ovsynch protocol, such as the timing of the final GnRH administration at 56 h after the PGF injection to optimize timing of AI in relation to ovulation, which is occurs approximately 24 to 32 h after GnRH, may explain the improved P/AI in the present study as well as in other reports (Pursley et al., 1998; Brusveen et al., 2008; Wiltbank and Pursley, 2013). Cows receiving the combination of Pre10 and GnRH-3 administered at 72 h had increased incidence of spontaneous LH surges before GnRH-3. Insemination near or after ovulation may provide insufficient time for sperm capacitation and transport in the reproductive tract resulting in aged oocytes before fertilization (Hunter and Wilmut, 1983; Wilmut and Hunter, 1984; Hawk, 1987). A 4-h interval between AI and anticipated ovulation may be insufficient for sperm transport and before oocyte aging (Brusveen et al., 2008). Despite these reports, many dairies utilize the Cosynch-72 protocol, in which the final GnRH injection is given concurrently with TAI at 72 h after PGF, because it eliminates one cow handling period, facilitates once-daily

restraint of cows for administration of hormone injections, and TAI (Sterry et al., 2007). Studies have compared Cosynch to Ovsynch with conflicting results as to which produces the greatest P/AI (Vasconcelos et al., 1997; DeJarnette et al., 2003; Portaluppi and Stevenson, 2005; Cornwell et al., 2006; Brusveen et al., 2008). Reports by Brusveen et al. (2008) of increased first-service and repeat-service P/AI in cows receiving Ovsynch-56 compared with Cosynch-72 have been confirmed by the current study. Brusveen et al. (2008) attributed the differences in P/AI to the timing of the final GnRH injection because time of AI was not different among treatments. Another study reported no difference in P/AI at 30 d in grazing dairy cows treated with either Double Ovsynch or Presynch-10 when assigned to a 5 d Cosynch with the final GnRH and TAI administered at either 58 or 72 h (Ribeiro et al., 2012). The delayed interval between the second GnRH injection and AI is likely to optimize P/AI in ovulation-synchronization protocols (DeJarnette et al., 2003). With regards to timing, the PG-3-G protocol seems to be more flexible in GnRH administration because P/AI was similar regardless of when GnRH administration occurred (56 or 72 h) in the current study.

In summary, presynchronization using the PG-3-G protocol increased the proportion of cows having LH surges and ovulation incidence after GnRH-1. At GnRH-2, cows receiving Pre10 had greater LH peak concentrations and area under the curve for LH compared with PG-3-G; however, single and double ovulation rates did not differ among treatments. Incidence of LH surges after GnRH-2 did not differ because all cows had an LH surge regardless of treatment. At GnRH-3, treatments did not differ in regards to single or double ovulation incidence, incidence of induced LH surges, LH peak concentrations, or area under the LH curve. Administration of GnRH-3 at 72 h decreased the time to peak LH concentration and incidence of spontaneous LH surges compared with 56 h. Pregnancy per AI at 31 d post-insemination was reduced in cows receiving the Pre10-72 treatment compared with those receiving Pre10-56, PG-3-G-56, or PG-3-G-72 treatments. Treatment differences at 60 d post-TAI followed a similar pattern to d 31.

We conclude that PG-3-G increased progesterone concentrations and the number of CL before GnRH-2 or the onset of Ovsynch, thus decreasing LH peak concentrations. Delaying GnRH-3 administration to 72 h after PGF-3 increased the incidence of spontaneous LH surges, and P/TAI was less in cows receiving Pre10-72 treatment compared to Pre10-56, PG-3-G-56, or PG-3G-72. The PG-3-G protocol may be more flexible for the time of GnRH administration

before TAI because P/AI was similar regardless of when GnRH administration occurred (56 or 72 h) in the current study.

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