# Managing the dominant follicle in high-producing dairy cows

MC Wiltbank[1], R Sartori[2], JLM Vasconcelos[3], AB Nascimento[1], AH Souza[4], AP Cunha[1], A Gumen[5], S Sangsritavong[6], JN Guenther[1], H Lopez[7] and JR Pursley[8]

*[1]Department of Dairy Science, University of Wisconsin-Madison, Madison, Wisconsin, USA; [2]Department of Animal Science, USP, Piracicaba, São Paulo, Brazil; [3]Department of Animal Production, UNESP, Botucatu, São Paulo, Brazil; [4]Accelerated Genetics, Baraboo, Wisconsin, USA; [5]Department of Obstetrics and Gynecology, University of Uludag, Bursa, Turkey; [6] Central Research Unit, 113 Thailand Science Park, Prathumthani, Thailand; [7]American Breeders Service, DeForest, Wisconsin, USA; [8]Department of Animal Sciences, Michigan State University, E. Lansing, Michigan, USA*

Reduced reproductive efficiency has been reported in high-producing dairy cows. Sources of reproductive inefficiency include decreased expression of estrus, increased diameter of the ovulatory follicle and reduced fertility when cows are inseminated after estrus, increased incidence of double ovulation and twinning, and increased pregnancy loss. To overcome some of these inefficiencies, reproductive management programs have been developed that synchronize ovulation and enable effective timed artificial insemination (AI) of lactating dairy cows. Effective regulation of the corpus luteum (CL), follicles, and hormonal environment are critical for optimizing these programs. Recent programs, such as the 5-day CIDR program, Double-Ovsynch, G-6-G, and estradiol benzoate-CIDR programs were designed to more effectively control one or more physiological events. These events include synchronization of a new follicular wave at the beginning of the program, optimization of the circulating progesterone (P4) concentrations and duration of follicular dominance, optimized reductions in P4 and increases in circulating estradiol (E2) concentrations during the preovulatory period, and tightly synchronized ovulation of a follicle of optimal size and fertility for implementation of timed AI. The success of these programs has been remarkable, although there is substantial variability in effectiveness due to environmental, management, nutritional, genetic, and disease factors as well as potential variability in some aspects of reproductive physiology among commercial dairy farms. Future programs will optimize the reproductive physiology while simplifying the protocol implementation and also match specific reproductive management protocols to specific farms and even specific cows (for example primiparous vs. multiparous).

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Corresponding author E-mail: wiltbank@wisc.edu

## Distinctive aspects of follicular growth in lactating dairy cows

The high-producing dairy cow has unique physiology related to follicular growth that impacts applied aspects of reproductive management programs. Dynamics and regulation of follicular waves have been reviewed previously (Ginther *et al.* 1996b; Adams *et al.* 2008; Aerts & Bols 2010). This review will focus on observations in lactating dairy cows that are relevant to reproductive management. Specific research citations will be used as examples to illustrate concepts. Due to space limitations, there will be no attempt to provide exhaustive literature citations or comparison of all important research studies during the discussion of each concept.

Follicular waves occur throughout pregnancy with each wave preceded by a surge in FSH. The magnitude of the FSH surge increased as pregnancy progressed. The maximum diameter of the dominant follicle, however, decreased as pregnancy progressed from 11.1 mm in Month 4 to 8.5 mm in Month 9 (Ginther *et al.* 1996b). Emergence of the final follicular wave during pregnancy occurred three weeks before parturition (Ginther *et al.* 1996b) whereas the interval from the last peak of FSH until parturition was approximately 12 days. Following parturition, there was a large increase in FSH with average FSH concentrations being 2-fold greater after parturition than average FSH surge concentrations during pregnancy. The interval from parturition to the day of emergence of the first follicular wave post-partum averaged 4 days (range of 2 to 7 days) (Ginther *et al.* 1996b).

Lactating dairy cows have variable intervals to first ovulation that depend on which post-partum follicular wave is ovulated. The dominant follicle from the first post-partum follicular wave has three potential outcomes: ovulation, atresia, or to become a large anovular follicle such as a follicular cyst. For example, Savio *et al.* (1990) reported that 74% of lactating cows ovulated the dominant follicle of the first follicular wave whereas 21% became cystic. In contrast, Butler *et al.* (2006) reported that 31% of cows ovulated the first dominant follicle post-partum, whereas 44% of cows had atresia of the first wave dominant follicle. The formation of atretic follicles was associated with low circulating E2 concentrations. There were 25.4% of cows that had a large anovular follicle that either became cystic (15%) or produced high E2 but did not ovulate (11%). These two papers illustrate the variability (31% vs. 74%) between studies/herds in the fate of the first-wave dominant follicle. The prevailing LH pulse frequency during the dominance phase of the first follicular wave is likely the major driver for growth and E2 production by the dominant follicle. Insufficient LH causes an atretic first dominant follicle with inadequate E2 production (see Crowe, 2008). It is still not clear what physiological changes result in a large anovular follicle with high E2 production during the first post-partum follicular wave. The lack of an adequate GnRH/LH surge in response to increased E2 may underlie the development of these large anovular follicles (Gumen & Wiltbank 2002). Following the first follicular wave post-partum there continue to be follicular waves every 7 to10 days with multiple potential outcomes such as ovulation, atresia, or cyst development, that depend on the physiological status of the cow.

There are a number of intriguing aspects of follicular wave dynamics that are unique to high production lactating dairy cows (Sartori *et al.* 2004). First, higher producing dairy cattle have lower circulating E2 concentrations than would be expected given their size of dominant follicles. For example, Lopez *et al.* (2005) reported decreasing peak circulating E2 concentrations with increasing milk production. Paradoxically lower circulating E2 was associated with greater follicular diameter when milk production increased. This contradiction could be due to reduced E2 production by the dominant follicle or increased E2 metabolism with increasing milk production in ldairy cows. The hypothesis that follicular E2 production varies with milk production has not yet been adequately tested. Greater E2 metabolism with increasing milk production, however, has been demonstrated (Sangsritavong *et al.* 2002). It seems likely that lesser circulating E2 in cows with greater follicular volume is primarily related to greater E2 metabolism in high milk-producing cows (Lopez *et al.* 2005). Greater E2 metabolism is probably caused by greater blood flow through the gastrointestinal tract and liver associated with the greater feed intake that is required to maintain high milk production (Wiltbank *et al.* 2006).

There also appears to be decreased expression of estrus in dairy cattle (Lopez *et al.* 2004). Lesser expression and detection of estrus in lactating dairy cows may reduce reproductive efficiency in the highest producing cows. Timed AI programs allow high- and low-producing dairy cows to be inseminated at a similar efficiency. This aspect makes these programs particularly appealing for herds with high-producing cows. In addition, the time of AI can be optimized in relation to the time of ovulation. Programs based on the expression of estrus may have more variability in the interval from AI to ovulation. A third intriguing aspect of follicular development in lactating dairy cows is that there is an increasing size of the ovulatory follicle with increasing milk production when cows are inseminated to estrus (Wiltbank *et al.* 2006). These differences may be reduced with timed AI programs because GnRH (or other agent) is used to induce ovulation before expression of estrus (discussed below). When cows are inseminated to estrus there may be a delay in ovulation in the highest-producing cows because greater E2 metabolism delays the attainment of sufficient circulating E2 to induce the GnRH/LH surge. Ovulation of larger follicles in higher producing dairy cows may partially explain the reduction in fertility in cows inseminated to estrus (Santos *et al.* 2010).

A fourth intriguing aspect of lactating dairy cows is the large percentage of cows that are anovular. Surprisingly, there is generally no relationship detected between level of milk production and the percentage of anovular cows. The follicular dynamics and physiology underlying different types of anovulation in cattle have been reviewed (Wiltbank *et al.* 2002; Wiltbank *et al.* 2008a). The most common type of anovular lactating dairy cow ($\sim 60\%$ of anovular dairy cows) had follicles larger than ovulatory size but smaller than the classically defined cystic size (Wiltbank *et al.* 2002; Gumen *et al.* 2003). Anovular cows of this type probably have hypothalamic resistance to the positive feedback effects of E2 (Wiltbank *et al.* 2002; Gumen & Wiltbank 2005). The timed AI programs discussed below generally will induce ovulation in this type of anovular cow as well as most other types of anovular dairy cows (Wiltbank *et al.* 2008a). Nevertheless, cows that are anovular or have low P4 (proestrous period) at the start of the Ovsynch program have lower fertility than ovular cows with elevated P4 at start of the Ovsynch program (Bisinotto *et al.* 2010a).

A fifth intriguing aspect of follicular development in lactating dairy cows is an increase in double ovulation associated with greater milk production (Lopez *et al.* 2005). A comparison of the hormonal concentrations during the first follicular wave in cows that select a single follicle compared with cows that select two or three dominant follicles has been performed (Lopez *et al.* 2005). The main hormonal differences between cows with single vs. multiple dominant follicles are increased circulating FSH and LH during the 24 h before follicular deviation (largest follicle $\geq$ 8.5 mm). A reduction in circulating E2 does not appear to be the underlying cause of selection of multiple follicles. Circulating E2 is actually greater in cows with two or three dominant follicles than in cows with one dominant follicle during this critical period before deviation. A reduction in circulating P4 during this period may partially explain the increases in FSH and LH and increased selection of co-dominant follicles (see below). Differences in other aspects of reproductive physiology in lactating dairy cows have also been reported, including number and dynamics of follicular waves, CL volume, circulating P4, and reproductive diseases (Thatcher *et al.* 2010). The reader is referred to the many other reviews on related topics such as follicular management of anovular cows, patterns of follicular waves and fertility, and nutritional effects on follicular development and fertility in dairy cattle (Thatcher *et al.* 2002; Macmillan *et al.* 2003; Bilby *et al.* 2006; Leroy *et al.* 2008a; Leroy *et al.* 2008b; Wiltbank *et al.* 2008a).

## Management of the follicle for timed AI in lactating dairy cows

Synchronized ovulation combined with timed AI has been a goal of reproductive physiologists for many years (Wiltbank *et al.* 1965; Zimbelman & Smith 1966; Odde 1990; Pursley *et al.* 1995; Thatcher *et al.* 1996; Lauderdale 2009). Obtaining good fertility with timed AI programs requires optimization of at least 5 distinct aspects of reproductive physiology (Fig. 1). First, synchronization of the follicular wave that will produce the dominant follicle for ovulation near the timed AI. Second, optimization of the hormonal environment and length of time during growth of this synchronized follicular wave. Third, optimization of the preovulatory period including synchronized regression of the CL with optimized timing in the reduction in circulating P4 and increases in circulating E2. Fourth, synchronization of ovulation of a dominant follicle with a high fertility oocyte, optimal periovulatory hormonal environment, and optimal timing and placement of high fertility semen in relation to the synchronized ovulation. Fifth, development of an optimal hormonal environment after AI that allows pregnancy establishment and maintenance. All of these physiological aspects of the program, together with management, nutritional, genetic, and disease aspects of the cow and herd, will contribute to the ultimate outcomes from these programs.

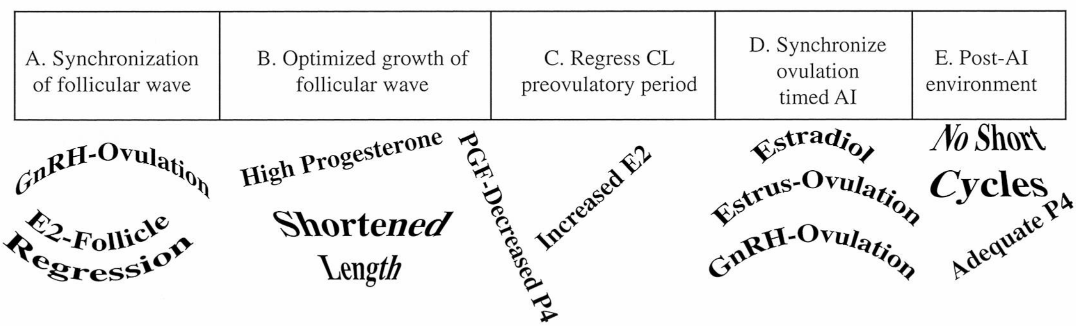


**Fig. 1.** Schematic of characteristic processes and current methods for timed AI programs.

*Initiation of a synchronized follicular wave*

One of the major changes with modern reproductive management protocols is the focus on controlling follicular development in addition to control of CL function and circulating P4 concentrations. Detailed studies of the follicular development during synchronization protocols became practical with the development of commercially-available transrectal ultrasound systems. Early ultrasound studies of bovine ovaries clearly showed the dynamics of follicular growth in cows that included follicular waves emerging every 7 to10 days (Savio *et al.* 1988; Sirois & Fortune 1988; Knopf *et al.* 1989). During emergence of a follicular wave, multiple follicles begin growing with the eventual deviation of a single dominant follicle (normally) from this cohort of follicles (Ginther *et al.* 1996a). The size of the follicle at deviation in Holstein dairy cattle averages about 8.5 mm and is associated with a nadir in circulating FSH concentrations. Continued growth of the dominant follicle is dependent on LH secretion. Ovulation depends on the LH surge. The maximal size of the dominant follicle during the estrous cycle or pregnancy and the timing of loss of function of the dominant follicle (i.e., turnover) depend on the hormonal environment and particularly circulating LH concentrations.

Synchronization of a new follicular wave generally entails removal of the functional dominant follicle either by physical destruction, hormonally-induced ovulation, or hormonal inhibition of gonadotropins so that the follicle regresses (Bodensteiner *et al.* 1996). Following aspiration of the dominant follicle there is a rapid increase in circulating FSH caused by the removal of follicle-derived inhibitors of FSH secretion. This FSH "surge" is followed by emergence of a new follicular wave, usually within 1 day. Similarly, induction of ovulation of the dominant follicle either with LH or hCG is followed by an FSH surge and emergence of a new follicular wave. From a practical standpoint, removal of the dominant follicle is usually performed by ovulation of the follicle using GnRH treatment (Thatcher *et al.* 1993; Pursley *et al.* 1995). Treatment with GnRH induces an LH and FSH surge that peaks within 2 h after treatment and returns to nadir concentrations by about 4 h. If this gonadotropin surge causes ovulation then there is a surge in FSH that peaks at about 24 h after the initial GnRH treatment with coincident emergence of a new follicular wave (Bodensteiner *et al.* 1996). Each of these follicular synchronization methods is primarily effective when the dominant follicle is removed, in other words in cows that ovulate in response to GnRH or gonadotropin treatments. Treatment of dairy cows at random stages of the estrous cycle with GnRH results in ovulation in only 50 to 70% of the cows (Pursley *et al.* 1995; Galvao & Santos 2010). Different stages of the estrous cycle have different ovulatory responses with the greatest response on Days 5 to 9 and reduced response earlier and later in the estrous cycle (Vasconcelos *et al.* 1999; Moreira *et al.* 2000; Bello *et al.* 2006). It is unrealistic, therefore, to expect 100% synchronization of follicular waves by using GnRH alone at the beginning of a synchronized ovulation program. Although ovulation to the initial GnRH is not necessarily a requirement for conception with these programs, there is generally a reduction in fertility in cows that do not ovulate to the initial GnRH treatment (Thatcher *et al.* 2002; Galvao & Santos 2010).

One of the methods that is used to increase ovulation to the first GnRH treatment during Ovsynch is to control the day of the cycle at the initiation of Ovsynch. Generally, the best day to give the first GnRH treatment is Day 6 or 7 of the cycle when there is a large dominant follicle (Vasconcelos *et al.* 1999; Moreira *et al.* 2000; Bello *et al.* 2006). There is a high ovulation rate after GnRH on Day 6 or 7 and therefore a synchronized emergence of the new follicular wave. This is one of the key ideas underlying the development of Presynch-Ovsynch (Moreira *et al.* 2001; Ei-Zarkouny *et al.* 2004), G-6-G (Bello *et al.* 2006), and Double-Ovsynch (Souza *et al.* 2008). During Double-Ovsynch, for example, the first Ovsynch protocol synchronizes the cows so that the second Ovsynch procedure is initiated on Day 7 of the cycle.

An alternative method for removing the functional dominant follicle involves inhibiting the gonadotropins that are necessary to sustain dominance. From a practical stand-point this can be accomplished with E2 treatment in the presence of high P4 (Bo *et al.* 1995; Burke *et al.* 2003). It is necessary to have P4 present during E2 treatment to prevent an E2-induced LH surge and ovulation. The combination of E2 and P4 decreases LH and FSH and this reduction in gonadotropin support results in follicular atresia and the regression of dominant follicles within 36 h after treatment (Burke *et al.* 2003; Martinez *et al.* 2005). After treating Holstein cows with 2 mg of E2-benzoate with or without injectable P4 in a CIDR protocol, circulating FSH and LH reached nadir concentrations by ~0.5 days; a time that corresponded with peak circulating E2. The FSH surge occurred at 3 to 4 d after the initial E2 treatments (Cavalieri *et al.* 2003). The length of time from hormonal treatment to emergence of the new follicular wave has been shown to be independent of the stage of follicular development at E2 treatment (Kim *et al.* 2007a). It does depend, however, on the type (Martinez *et al.* 2005) and dose (Burke *et al.* 2003; Colazo *et al.* 2005) of E2 that is used for treatment. The circulating E2 profiles for different esters of E2 are different in cattle (Martinez *et al.* 2005; Souza *et al.* 2005). Although long half-life estrogens (e.g., E2-cypionate) have been successfully used to synchronize the emergence of follicular waves in beef cattle and heifers (Colazo *et al.* 2005), they seem to be less precise for synchronizing wave emergence when used in lactating dairy cows (Thundathil *et al.* 1998) perhaps because of the rapid E2 clearance rates in the liver of high milk-producing cows. The time to emergence of the new follicular wave is reduced with increasing milk production (Souza *et al.* 2009). For example, treatment of cows with 2 mg of E2-benzoate results in follicular wave emergence at 3.8 d in the highest-producing cows but not until 4.5 d in the lowest-producing cows.

Collectively, E2 + P4 protocols will induce synchronous follicular wave emergence in about 70 to 90% of cattle. Most failures in synchronization are related to lack of dominant follicle regression and late wave emergence (Diskin *et al.* 2002; Souza *et al.* 2009). One of the key benefits of synchronizing the follicular wave by inhibiting follicular growth (compared with ovulating the dominant follicle) is that a new CL is not present during the synchronization protocol. This makes incomplete luteal regression less likely in the E2 + P4 protocol compared with the Ovsynch-like protocols (Kim *et al.* 2007b). A downside of not inducing a new ovulation at the beginning of the synchronization procedures is that circulating P4 could be lower in E2 + P4 protocols than needed for optimal oocyte quality in timed AI protocols (Kim *et al.* 2007b; Rutigliano *et al.* 2008).

*Optimizing growth of the follicular wave*

It is important to optimize the "fertility" of the follicle that will ovulate the oocyte for subsequent fertilization and embryonic development. This optimization involves ovulation of an "optimized" oocyte with production of an "optimized" CL to maintain embryo development and pregnancy. These may be conflicting goals since ovulation of a larger follicle will likely result in a larger CL with greater P4 production. The oocyte from a larger follicle, however, may have reduced fertility (Ahmad *et al.* 1995; Ahmad *et al.* 1996; Revah & Butler 1996). The hormonal environment and duration of growth of this follicle have been found to be key components in optimizing fertility in timed AI programs. Hormonal environment and duration of the follicular wave may impact fertility through its effects on oocyte function, granulosa/thecal cell number or function, oviductal or uterine function, and other potential reproductive functions.

The circulating P4 concentrations have been found to have substantial impact on subsequent fertility. A relationship between pre-AI P4 concentration and subsequent fertility was documented almost 3 decades ago where first service conception rate increased by approximately 10% for every 1 ng/ml increase in average P4 (Fonseca et al. 1983). A study in seasonal dairy herds in New Zealand tested this hypothesis as well (Xu et al. 1997). Cows were synchronized with prostaglandin F2α (PGF) 13 d apart and supplemented or not supplemented with a CIDR for 5 d before the second PGF to increase circulating P4 before AI. There was an increase in percentage of cows that showed estrus after the second PGF (89.6% vs. 82.9%) and percentage pregnant to AI (P/AI) after this estrus (65.1% vs. 59.7%). Analysis of the stage of the estrous cycle at the time of the second PGF demonstrated that the CIDR improved fertility in cows in the earlier (Days 5 to 9; 52.3% vs. 64.8% P/AI) and mid-cycle (Days 10 to13; 59.3% vs. 66.2%) but not later cycle (Days 14 to 19; 71.3% vs. 71.4%). Increasing P4 in cows with lower P4 before PGF synchronization, therefore, improved fertility at the subsequent AI. Indeed, many of the pre-synchronization programs, such as Presynch-Ovsynch, may improve fertility through their effects on P4 during the pre-AI period.

We tested whether P4 concentrations during growth of the preovulatory follicle would alter double ovulation rates (DOV) and fertility in lactating cows (Cunha & Wiltbank, unpublished). Holstein cows (n = 624) were presynchronized before the breeding Ovsynch with an Ovsynch72 protocol (GnRH-7d-PGF-3d-GnRH) but timed AI was not performed (Fig. 2). Cows then began Ovsynch immediately (the 2nd GnRH of the Ovsynch72 was the 1st GnRH of the breeding Ovsynch) (Low-P4; Short Double-Ovsynch) or cows received the first GnRH of the breeding Ovsynch 1 week later (High-P4; Double-Ovsynch; Fig. 2). Ovarian ultrasound and blood sampling were performed in order to assess ovulation, pregnancy status, and circulating P4 concentrations. As expected, cows in the High-P4 group had greater P4 concentrations than cows in the Low-P4 group at the first GnRH of the breeding Ovsynch (1.80 ng/mL vs. 0.38 ng/mL) and at the PGF (4.43 ng/mL vs. 2.51 ng/mL). The DOV was greater in the Low-P4 than in the High-P4 group (20.6% vs. 7.0%; P=0.03). A previous study also reported a reduction in DOV in cows with increased pre-AI P4 concentrations (Rutigliano et al. 2008). Overall P/AI at Day 29 was greater in the High-P4 compared with the Low-P4 group (51.0%, n=292 vs. 37.1%, n=272; P=0.001). Surprisingly, pregnancy loss (between Day 29 to 57) was also less in the High-P4 than the Low-P4 group (6.8% vs. 14.3%; P=0.05). High-P4 during follicular development, therefore, reduced selection of co-dominant follicles and DOV. In spite of ovulating fewer follicles and in spite of a lower P4 concentration after AI, cows treated with the High-P4 protocol had better fertility than those treated with the Low-P4 protocol. These data provide strong evidence for the importance of high P4 during Ovsynch.

A. Double-Ovsynch - High P4 during follicle growth.

| Sun | Mon | Tues | Wednesday | Thursday | Friday | Sat |
|---|---|---|---|---|---|---|
| | | | | | GnRH | |
| | | | | | PGF | |
| | GnRH | | | | | |
| | GnRH | | | | | |
| | PGF | | GnRH-PM | AI-AM | | |

B. Short Double-Ovsynch - Low P4 during follicle growth.

| Sun | Mon | Tues | Wednesday | Thursday | Friday | Sat |
|---|---|---|---|---|---|---|
| | | | | | GnRH | |
| | | | | | PGF | |
| | GnRH | | | | | |
| | PGF | | GnRH-PM | AI-AM | | |

**Fig. 2.** Diagrams of a typical weekly calendar for the protocols used in the experiment to test the effect of P4 concentration during the Ovsynch protocol (growth of ovulatory follicle) on fertility and double ovulation rate.

There are many potential physiological mechanisms that may underlie the effect of high P4 during follicular growth to reduce DOV, increase fertility, and reduce pregnancy loss in timed AI protocols. Greater circulating P4 decreases LH pulses (Stumpf *et al.* 1993). In previous studies, low P4 during follicular growth resulted in development of persistent, lower-fertility follicles (Ahmad *et al.* 1994; Ahmad *et al.* 1996; Revah & Butler 1996). For example, lactating dairy cows had lower fertility after development of a persistent follicle compared with control dairy cows (44% vs. 12%; (Ahmad *et al.* 1996). A recent study (Cerri *et al.* 2009) flushed embryos from cows that started Ovsynch on Day 3 or Day 6 of the estrous cycle. Only 7.1% of cows that started Ovsynch on Day 3 ovulated to the first GnRH, whereas 88.6% of cows ovulated to the first GnRH when Ovsynch was started on Day 6. Fertilization rate was similar for the two groups (85 vs. 86%). The percentage of high quality embryos (Grades 1 and 2), however, was greater for cows that started Ovsynch on Day 6 (83.7%) compared with Day 3 (47.0%).

The period of follicular dominance averaged 8.0 d in cows that started Ovsynch on Day 3, and averaged 5.8 d for Day 6 cows. A 2 d increase in follicular dominance, therefore, reduced embryo quality (78% increase in degenerate embryos). In our experiment that used Double-Ovsynch with high or low P4 concentrations, the age of the follicles were identical in the two groups. The preovulatory follicle from the low P4 group, however, would have been exposed to a greater number of LH pulses and this could underlie the low fertility. Thus, reduced fertility can occur in cows that ovulate even minimally persistent follicles or in cows with follicles that may not be older but are overexposed to LH. This may be a critical concept for fertility in normally-ovulating dairy cows or during synchronized breeding protocols.

In an attempt to reduce the duration of follicular dominance, a shortened Ovsynch strategy has been developed. The interval between GnRH and PGF was reduced from 7 to 5 d along with an increase in the proestrous period from 48 to 56 h (time of second GnRH) until 72 h. This strategy has resulted in improved in fertility in beef cattle (Bridges *et al.* 2008). A similar strategy in dairy cattle produced encouraging results. Santos *et al.* (2010) reported an improvement in fertility in the 5-d compared with the 7-d Ovsynch protocol (5-d: 37.9% vs. 7-d: 30.9%). Two treatments with PGF were required, however, for optimizing the synchronization of cows assigned to the 5-d protocol. For the 5-d and the 7-d protocols, timing of the second GnRH and timed AI were both done at 72 h after the PGF (Cosynch-72). This timing of GnRH and AI has been found to reduce fertility in the 7-d protocol (Brusveen *et al.* 2008) but does not alter fertility in the 5-d protocol (Bisinotto *et al.* 2010b) compared with GnRH treatment at 56 h and AI 16 h later. Additional studies are necessary to evaluate the differences between these protocols and to optimize the protocols. It seems likely that protocols with reduced duration of follicular dominance, combined with a longer proestrous period (discussed below), can increase fertility in lactating dairy cows.

### *Optimizing the hormonal environment during the preovulatory period*

The hormonal environment during the proestrous period is critical for reproductive success. First, lack of complete CL regression leads to elevated P4 during the proestrous period and reduced fertility during timed AI protocols (Souza *et al.* 2007). In one study we found that 15% of cows did not have complete CL regression following the Double-Ovsynch protocol. These cows had greatly reduced fertility to the timed AI (Brusveen *et al.* 2009). Treatment with a second injection of PGF, 24 h after the first, resulted in almost all cows having complete CL regression with a slight (~5%) but not significant improvement in fertility.

A second critical factor is to optimize E2 concentrations before AI. In cows treated with GnRH followed seven days later by PGF and then inseminated to estrus, treatment with 1 mg of E2-cypionate at 24 h after PGF, increased E2 concentrations, and increased fertility to the estrus breeding (Cerri *et al.* 2004). To test the effect of E2 during an Ovsynch protocol, we treated cows with Ovsynch (GnRH - 7d - PGF - 56h - GnRH - 16h - timed AI) with or without treatment with 1 mg of E2 (native estradiol-17ß) at 48 h after PGF (8 h before second GnRH treatment and 24 h before timed AI at 72 h) (Souza *et al.* 2007). As expected, there was an increase in expression of estrus in cows treated with E2 (Ovsynch: 44.4% vs. Ovsynch + E2: 80.2%); however, there was no overall improvement in fertility associated with E2 treatment (Ovsynch: 39.4% vs. Ovsynch + E2: 42.4%). Treatment with E2 improved fertility in low body condition score (BCS $\leq$ 2.5) cows (Ovsynch: 28.1% vs. Ovsynch + E2: 40.0%) such that these cows had similar fertility to cows in high BCS (Ovsynch: 43.7% vs. Ovsynch + E2: 43.9%). A sufficient E2 surge, therefore, may be the key rate-limiting step for obtaining high fertility in low BCS cows during timed AI protocols. Treatment with E2 also tended to increase fertility in cows ovulating medium-sized follicles (15 to 19 mm) but not in cows ovulating smaller or larger follicles. This observation is consistent with the concept that increasing circulating E2 may be important for optimizing the preovulatory hormonal environment during timed AI protocols, at least in cows that ovulate an optimized follicle size. Thus, optimizing circulating E2, along with other follicular/luteal optimizations, may improve fertility in cows inseminated to estrus or in cows that are timed AI (Cerri *et al.* 2004; Souza *et al.* 2007).

A third factor to consider for improving fertility is increasing the length of the proestrous period. The importance of increasing the proestrous period has been demonstrated in beef cattle treated with the 5-day protocol (Bridges *et al.* 2010). It seems likely that changes in the proestrous period may alter fertility by affecting the uterine environment and (or) the follicle and oocyte.

*Synchronization of ovulation*

There are many reasons to synchronize ovulation and perform timed AI rather than wait for estrus. First, higher-producing lactating dairy cows demonstrate less estrous behavior than lower-producing cows (Lopez *et al.* 2004). Timed AI programs, therefore, should result in an increased percentage of higher-producing cows receiving AI compared with programs based on detection of estrus. Second, follicle size could be theoretically optimized during a synchronized ovulation program and this could improve fertility. Third, timing of AI can be optimized in relation to ovulation (one previously designated time in all cows) and this should increase management efficiency and fertility. The first advantage (increased percentage of cows receiving AI) has been reported in direct comparisons of timed AI and estrus programs. Improvements in fertility, however, have not been a consistent result. A meta-analysis done in 2005 (71 trials in 53 research publications with sufficient experimental details for inclusion in the analysis) reported no significant differences in P/AI between Ovsynch compared with various other reproductive management strategies (Rabiee *et al.* 2005).

There are several methods to synchronize the time of ovulation. Any method that will synchronize estrus will also synchronize the time of ovulation; although, synchronization may not be sufficient to allow good success with timed AI. Most timed AI programs use either a GnRH or estradiol treatment to increase the synchrony of ovulation during these programs.

Treatment with GnRH results in an LH surge that reaches a peak by about 2 h and causes ovulation between 24 to 32 h after GnRH (Pursley *et al.* 1995). There are at least 10% of cows that do not ovulate to the second GnRH treatment of Ovsynch (Vasconcelos *et al.* 1999; Bello *et al.* 2006; Souza *et al.* 2007; Brusveen *et al.* 2009; Galvao &v Santos 2010). Two primary reasons explain the lack of synchronized ovulation to the Ovsynch protocol. First, cows may come into estrus before the GnRH treatment and therefore ovulate prematurely because of an endogenous GnRH/LH surge (Vasconcelos *et al.* 1999). Premature ovulation during Ovsynch was only found in cows that started Ovsynch in the later estrous cycle ($\geq$ Day 12) and did not ovulate to the first GnRH treatment of Ovsynch (Vasconcelos *et al.* 1999). Second, cows may not have a dominant follicle at the time of the second GnRH treatment due to initiation of a new follicular wave during the Ovsynch protocol. Immature follicular development at the second GnRH of Ovsynch was found in cows that initiated Ovsynch at various stages of the estrous cycle and in cows that may or may not have ovulated to the first GnRH of Ovsynch (Vasconcelos *et al.* 1999). Obviously, cows that do not ovulate to the second GnRH of Ovsynch have little or no fertility to the timed AI.

Cows that ovulate after Ovsynch may not ovulate an ideal size of follicle. For example, Souza *et al.* (2007) found that only 57.6% cows ovulated a follicle of 14 to 19 mm after Ovsynch.

Twenty percent of cows ovulated a follicle that was too small ($\leq$13 mm) and 22.5% ovulated a follicle that was too large ($\geq$20 mm). Vasconcelos *et al.* (2001) used an aspiration to produce a new follicular wave mid-way through the Ovsynch protocol so that there was ovulation of smaller (~ 11.5 mm) follicles at the second GnRH of Ovsynch compared with non-aspirated controls (~ 14.5 mm). The cows ovulating the small follicles had reduced fertility (12% vs. 45% for control). The reduction in fertility in cows ovulating small follicles may be due to reduced E2 before AI, ovulation of a less-mature oocyte, and/or reduced P4 after AI (due to ovulation of a smaller follicle with a subsequently smaller CL). Ovulation of larger follicles may in some cases produce oocytes with reduced fertility (Ahmad *et al.* 1996; Cerri *et al.* 2009). Improvements in fertility to Ovsynch, therefore, are likely to be obtained by increasing the percentage of cows that ovulate an optimally-sized follicle after the final GnRH treatment.

Many studies have used different estrogens to synchronize the time of ovulation in dairy cows treated with E2+P4 or Ovsynch-like protocols. Estrogens are available for use in synchronization protocols in many countries but are not available in the United States or Europe. Estrogen products are generally sold at a lower price than GnRH products and therefore can be economically attractive for producers. Nevertheless, the increased estrous behavior caused by estrogens is negatively viewed by some producers because of the risk of accidents and the excessive activity observed in the estrogen-treated cows. Estrogens with shorter half-lives (E2 and E2-benzoate) are typically used 24 to 48 h after PGF treatment with AI at about 1 d after E2 treatment. Studies with the Heat-Synch protocol (Pancarci *et al.* 2002; Cerri *et al.* 2004; Stevenson *et al.* 2004) have substituted the longer-acting E2-cypionate 24 h after PGF treatment to synchronize the time of ovulation. Although time of ovulation is shorter after GnRH compared with E2-cypionate treatment, fertility was similar in Heat-Synch vs. Ovsynch protocols. The Heat-Synch protocol has been shown to improve fertility as compared with cows inseminated to estrus (Cerri *et al.* 2004). Interesting, there was improved fertility in cows that showed estrus during the Heat-Synch protocol, although expression of estrus had no effect on fertility in cows that were AI to Ovysnch (Pancarci *et al.* 2002). Some protocols have also used E2-cypionate at the time of PGF/CIDR removal with similar results as observed with synchronized ovulation after a GnRH treatment (Souza *et al.* 2009). Synchronized ovulation occurs around 70 to 75 h after CIDR removal. Data from an earlier trial (Colazo *et al.* 2004) using beef heifers, however, indicated that better fertility is achieved when E2-cypionate was given 24 h after CIDR removal rather than at the time of CIDR removal. Thus, there are a variety of different options for synchronizing ovulation with estrogens. Optimal timing of treatments and of AI can vary based on the half-life of the estrogen, management factors, and the type of protocol used to synchronize follicular/luteal function before the synchronized ovulation.

## Post-AI treatments to regulate follicles

Management of follicles after breeding can also be used to regulate reproduction and potentially improve the efficiency of reproductive management programs. Resynchronization of ovulation (Resynch) in cows that did not become pregnant to the first AI can improve reproductive management programs by reducing the time between AI. The physiological aspects of Resynch programs are similar to what has been discussed above; however, some management considerations are critical because of the need to definitively confirm non-pregnancy in cows before treatment with PGF (Galvao *et al.* 2007; Silva *et al.* 2007; Wiltbank *et al.* 2008b).

The use of hCG or GnRH after AI has been tested as a follicular/luteal management strategy to improve fertility in lactating dairy cows (De Rensis *et al.* 2010). Treatment of cattle with these agents at certain times of the cycle can result in ovulation of the dominant follicle. If a follicle is ovulated there should be increased circulating P4 due to the presence of an accessory CL. In addition, hCG could increase circulating P4 due to direct effects of hCG to stimulate luteal function; although, this theoretical hCG effect has not been confirmed experimentally in cattle. The increases in circulating P4 after treatment with hCG on Days 5 to 7 of the estrous cycle have been shown in many studies. For example, Santos *et al.* (2001) treated cows that had been AI to estrus with 3,300 IU of hCG on Day 5 after AI. Treatment with hCG increased circulating P4 and increased fertility (Control: 38.9% vs. hCG: 45.8%). Our recent studies following timed AI protocols found a smaller but significant effect of treatment on Day 5 with hCG on fertility (Control: 37.3% vs. hCG: 40.8%) with significant hCG effects only in first lactation cows (Nascimento, Souza, Bender, Wiltbank, unpublished). Thus, it appears that hCG treatment on Day 5 improves fertility although the physiological reason for this improvement was not defined in these studies. One possibility is that the hCG-induced increase in circulating P4 improves fertility due to P4 effects on the uterus and (or) embryo. A number of studies are consistent with respect to increased P4 increasing embryonic development but the P4 effect appears to be mainly during the early luteal phase (Days 5 to 9) (Mann *et al.* 2006). Treatment with hCG on Day 5 does not increase circulating P4 until Day 8. Perhaps earlier increases in P4 would produce greater improvements in fertility. Another possible effect of hCG treatment is a change in follicular development pattern that could delay luteolysis and thus improve fertility. Ovulation on Day 5 results in a new follicular wave emerging by Day 6 and likely turnover of the dominant follicle of this wave before luteolysis. Thus, the cow is likely to have 3 rather than 2 follicular waves. This change in follicular wave patterns may improve fertility (Townson *et al.* 2002). For example, an absence of circulating E2 from a dominant follicle near the time of normal luteolysis will delay luteolysis until there is sufficient E2 to increase PGF secretion from the uterus (Araujo *et al.* 2009). Changes in follicular wave patterns and timing of luteolysis, therefore, may be part of the mechanism for increased fertility after hCG-induced ovulation of the dominant follicle of the first follicular wave.

## Closing remarks

One of the biggest changes in reproductive management research during the last 2 decades has been a focus on precise regulation of follicular development. This was possible because of the availability of high-resolution ultrasound technology allowing evaluation of the dynamic processes of follicular emergence, selection, growth, atresia, and ovulation. Evaluation of the dynamics of follicular development in anovular conditions has provided much greater insight into the physiology of these processes (Wiltbank *et al.* 2002). An understanding of the natural processes regulating follicular development is allowing greater insight into how changes in patterns of follicular development as well as management and nutritional factors may be regulating fertility (Thatcher *et al.* 2002; Townson *et al.* 2002; Macmillan *et al.* 2003; Leroy *et al.* 2008a; Leroy *et al.* 2008b). The most revolutionary of the research changes after introduction of ultrasound may be the many variations of synchronization protocols that are focused on synchronized ovulation of an "optimized follicle". Initial programs resulted in fertility that was similar to breeding to estrus but offered the advantage of a timed AI of all cattle on a preselected day (Pursley *et al.* 1997). Recent, optimized synchronization strategies may result in better fertility in lactating dairy cows than breeding to estrus. Additional research is needed to evaluate these programs in various commercial and experimental conditions. For example, some of these programs may be best for cows in specific physiological circumstances as exemplified by the better conception rates in primiparous than multiparous cows following the Double-Ovsynch protocol (Souza *et al.* 2008). Clearly, a great deal of progress is being made in understanding the physiology of these programs with future progress needed to increase consistency, simplicity, and economic value of these programs for dairy producers.

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