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The physiology and impact on fertility of the period of proestrus in lactating dairy cows

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Abstract

In cattle, proestrus begins with the initiation of luteolysis and ends with initiation of estrus and the GnRH/LH surge. This period is marked by a dramatic decrease in circulating progesterone (P4) that reaches a nadir by about 36-48 h in cows undergoing natural or prostaglandin F2α (PGF)-induced luteolysis. Inadequate luteolysis is a cause of reduced fertility particularly in timed AI programs with small elevations in circulating P4 reducing fertility. Increasing circulating estradiol (E2) during proestrus is dependent on presence, size, and function of the dominant follicle and this varies during natural proestrus, due to whether animals have two or three follicular waves, and during PGF-induced proestrus, according to stage of the follicular wave at time of PGF treatment. Inadequate circulating E2 can limit fertility and increase pregnancy loss in some specific circumstances such as in cows with low BCS and in cows during heat stress. Thus, studies to optimize the length of proestrus and the concentrations of E2 and P4 during proestrus could produce substantial improvements in fertility and reductions in pregnancy loss.

Keywords: dairy cows, estradiol, fertility, luteolysis, proestrus, progesterone.

Introduction

Although the term "proestrus" will be used throughout this text, it is a term that is clearly more appropriate for describing the day before estrus in the rat (Long and Evans, 1922). Rodents have cycles that are regulated closely by the light-dark cycle as well as by circulating hormonal concentrations (Chappell, 2005). Reference to the four stages of the estrous cycle (proestrus, estrus, metestrus, and diestrus) of the rat is logical based on the normal 4-5 day cycle of the rat and the close link between time of day and events in the estrous cycle (Gay *et al.*, 1970; Nequin *et al.*, 1979). Nevertheless, for purposes of this review the term proestrus will be defined as the time from the beginning of the P4 decline, using various definitions, until the onset of estrus (Lamond *et al.*, 1971, Chenault *et al.*, 1975). The length of proestrus and the hormonal patterns can vary by breed of cattle and by lactation status of cows (Lamond *et al*., 1971; Sartori *et al.*, 2002, 2004; Sartori and Barros, 2011). The two key hormonal features of proestrus in cattle are: 1) Decreasing circulating P4 associated with regression of the corpus luteum (CL), and 2) Increasing circulating E2 associated with the final stages of growth of the dominant follicle. This review will focus on the proestrous patterns of these hormones during natural estrous cycles as well as during programs for synchronization of ovulation. A review will also be provided on the potential effects of differing hormonal patterns during proestrus on subsequent fertility in lactating dairy cows.

Decreasing progesterone concentrations

Natural luteolysis

The onset of proestrus is marked by the onset of luteolysis. In ruminant, the uterus is responsible for initiation of luteolysis. This was first demonstrated by removal of the uterus (or sham operation) and determination of the prolonged life span of the CL in animals with complete removal of the uterus (Wiltbank and Casida, 1956). Use of hemi-hysterectomized cows and surgical anastomoses of uterine and ovarian blood vessels definitively demonstrated that the uterine luteolytic factor acted in a local and not a systemic manner (Moor and Rowson, 1966; Ginther *et al.*, 1973; Del Campo and Ginther, 1974; Mapletoft *et al.*, 1976). The luteolytic factor derived from the uterus was shown to be prostaglandin F2α (PGF) through multiple types of research involving many different laboratories (McCracken *et al.*, 1981, 1999; Knickerbocker *et al.*, 1988).

In many species, including the cow, luteolysis is due to multiple pulses of PGF released by the non-pregnant uterus (McCracken *et al.*, 1999, Niswender *et al.*, 2000, Schams and Berisha, 2004). There is substantial variability in the frequency and amplitude of PGF pulses associated with ruminant luteolysis but typically there are four to eight discrete pulses that occur at 6-14 h intervals (Kindahl *et al.*; 1976, Silvia *et al.*, 1991; Mann and Lamming, 2006). For example, in heifers evaluated

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at hourly intervals during the 7 days around luteolysis there was complete luteolysis, as defined by a decrease to basal P4, after four distinct PGF pulses that occurred during ~30 h (Kindahl *et al*., 1976; Mann and Lamming, 2006).

At the start of proestrus, a series of hormonal events and cellular changes in the endometrial cells result in secretion of distinct pulses of uterine PGF. Current models of the mechanisms regulating initiation of these PGF pulses in ruminants contain critical roles for changes in expression of endometrial receptors for E2, P4 and oxytocin (Silvia *et al.*, 1991). In these models, oxytocin receptor and estrogen receptor expression are suppressed during the early and mid-luteal phases by the presence of uterine P4 receptors (Wathes and Hamon, 1993; Ivell *et al.*, 2000). A decrease in P4 receptor in the late luteal phase leads to increased estrogen receptor (ESR1) in the uterus (Meyer *et al.*, 1988). Subsequently, activation of estrogen receptor by circulating E2 stimulates synthesis of endometrial oxytocin receptors with subsequent pulses of oxytocin from the posterior pituitary gland inducing secretion of pulses of PGF from the uterus (Silvia *et al*., 1991, Mann *et al.*, 2001). In cattle, the initial rise in oxytocin receptors preceding luteolysis occurs 15-16 days after estrus (Robinson *et al.*, 1999). In this regard, uterine venous PGF first increases 15-16 days after estrus in cows (Thatcher *et al.*, 2001) with increases in circulating concentrations of the PGF metabolite, 13,14-Dihydro-15-keto-PGF2α (PGFM), generally used as a monitor for uterine PGF secretion (Ginther *et al.*, 2007).

Induction of luteolysis with exogenous PGF can occur at any stage of the follicular wave, including early in a follicular wave when the subsequent ovulatory follicle is at a very small size. In contrast, the natural luteolytic cascade generally occurs in the presence of a dominant follicle due to the key role of activation of the estrogen receptor by E2 from the dominant follicle in this cascade. Indeed, ablation of the dominant follicle (Araújo *et al.*, 2009) or treatment with steroid-depleted follicular fluid (Salfen *et al.*, 1999) decreased circulating E2 and delayed the timing of both structural and functional luteolysis. Treatment with E2 in cows with delayed luteolysis due to follicular aspiration (Araújo *et al*., 2009) or in cows expected to have three follicular waves (Salfen *et al*., 1999), led to earlier luteolysis. Thus, there is synchronization of luteolysis with the presence of a dominant follicle (Araújo *et al*., 2009). This is due to the requirement for two independent events in the process of luteolysis. First, there must be E2 responsiveness in the uterus and second, there must be sufficient concentrations of E2 to activate a uterine E2 response. The timing of luteolysis, related to presence of a dominant follicle, primarily explains the longer estrous cycle in heifers with three waves compared to heifers with two waves (Ginther *et al.*, 1989). The day of luteolysis was 17.4 in heifers with two waves compared to 19.1 for heifers with three waves (Sartori *et al*., 2004).

Figure 1 shows the difference between heifers and cows in the decrease in luteal tissue volume and circulating P4 in the natural proestrous period (Sartori *et al*., 2004). The luteal tissue volume is greater in lactating cows than heifers due to ovulation during the previous cycle of a larger follicle (r = 0.67 for relationship between ovulatory volume and subsequent luteal volume on day 7) in lactating cows (Sartori *et al*., 2002). The difference was not due to a greater double ovulation rate in lactating cows because a comparison of maximal luteal tissue volume in females with only one CL was also different (heifers = 7,303 ± 308 *vs*. lactating cows = 11,248 ± 776; P < 0.0001). The luteal tissue volume begins to decrease on the day following the day designated as the beginning of luteolysis, defined as the day before P4 concentrations declined by 50%. The luteal tissue volume declined rapidly to ~30% of original volume by 3 days after the onset of luteolysis. In contrast, the serum P4 was greater in heifers than lactating cows prior to luteolysis (Fig. 1B). The decline in circulating P4 was extremely rapid after the onset of luteolysis with concentrations less than 10% of preluteolysis values within 2 days (Sartori *et al*., 2004). Thus, the decline in circulating P4 during natural proestrus is rapid and generally synchronized to the presence of a dominant follicle.

Luteolysis induced by exogenous PGF

The discovery of PGF as the luteolysin in ruminants (Baird *et al.*, 1976; Knickerbocker *et al*., 1988) allowed the commercial introduction of PGF for induction of CL regression in cattle (Lauderdale, 2009). Most protocols for synchronization of estrus or ovulation include at least one treatment with PGF to induce luteolysis. Early studies demonstrated that treatment with PGF was only effective when given at least 6 days after estrus (Rowson *et al.*, 1972; Lauderdale *et al.*, 1973; Louis *et al.*, 1973; Lauderdale, 1974, 2009). A good deal of research has evaluated the differences in the CL that result in lack of luteolysis in early CL and complete luteolysis in the later CL (Tsai *et al.*, 1997; Tsai and Wiltbank, 1998; Diaz *et al.*, 2011, 2013; Mondal *et al.*, 2011). It is clear that there are receptors for PGF present in the early CL (Wiltbank *et al.*, 1995; Anderson *et al.*, 2001) and that some PGF-induced responses occur, however, complete activation of the luteolytic cascade does not occur in the early CL (Wiltbank and Ottobre, 2003). Treatment of the day 5 CL, even with a double dose of PGF (Santos *et al.*, 2010; Ribeiro *et al.*, 2012), does not cause complete luteolysis, although a dramatic decrease in circulating P4 does occur even in the early CL (Nascimento *et al.*, 2014).

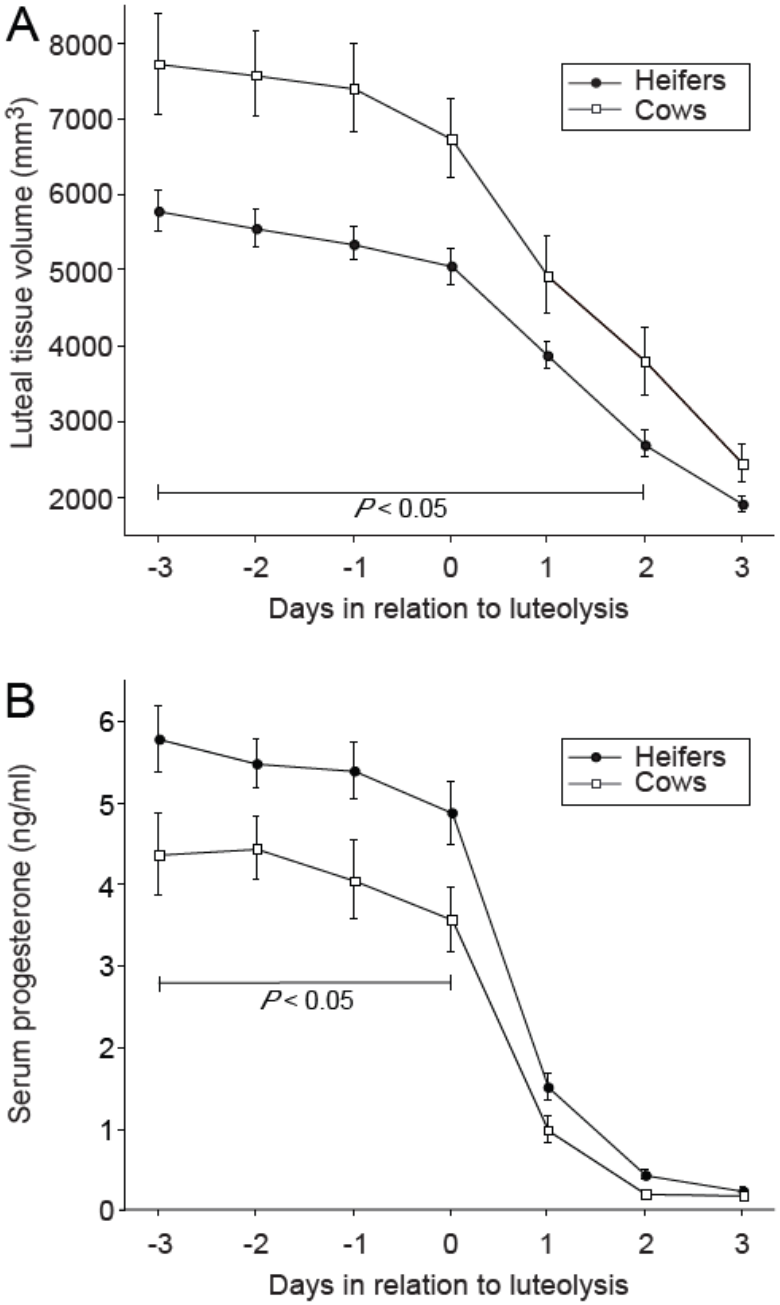

Figure 1. A. Luteal tissue volume and B. Serum P4 concentrations normalized to time of luteolysis in heifers (n = 27) and lactating cows (n = 14). Differences between heifers and cows at each time are shown. From: Sartori *et al*., 2004.

There are two forms of PGF that are available commercially for induction of luteolysis, dinoprost or cloprostenol. There are a substantial percentage of cows that do not have complete luteolysis in response to either product, as will be discussed below. If only cows with complete luteolysis are considered in the analysis, the two products showed slight but significant differences in timing of luteolysis with differences between the two products in P4 concentrations at 3, 4, 8, 9, and 14 h after treatment (Martins *et al.*, 2011b). Overall there were differences between the two products at 0-12 h after treatment but not from 12-24 h, 24-48 h, or 48-90 h after PGF treatment. Mean time from treatment to complete luteolysis varied between animals from 18 to 40 h after treatment but was not different between treatment with dinoprost (29.4 + 1.7 h) and cloprostenol (29.1 + 1.1 h).

Of particular importance to discussion of proestrus, cows administered PGF on different days of the estrous cycle have surprisingly variable times to

estrus and ovulation (Momont and Sequin, 1984; Wiltbank and Pursley, 2014). For example cows treated with PGF on day 7 of the cycle had shorter time and greater synchrony than cows administered PGF at day 10 of the cycle (Momont and Sequin, 1984). This is due to size and functional state of the dominant follicle at the time of PGF treatment. For example, time to ovulation after PGF treatment was highly related to size of the pre-ovulatory follicle at the time of the PGF treatment (R^2 = -0.855; Wiltbank and Pursley, 2014). Thus, variability in time to estrus after treatment with PGF is primarily related to size of the future ovulatory follicle at the time of PGF treatment, rather than the timing of the decrease in P4, or the nature of the PGF product used to induce luteolysis.

Figure 2 shows the pattern of circulating P4 after treatment with cloprostenol or dinoprost. For these studies all cows were presynchronized by using PGF at a random stage of the estrous cycle followed 2 days later by GnRH treatment and then 6 days later cows were given another GnRH treatment, followed 7 days later by PGF and then cows were monitored for estrus, ovulation, and circulating hormone concentrations (Martins *et al*., 2011b). In this model all cows had two CL at the time of PGF administration, a day 7 and a day 13 CL and only cows that had complete luteolysis were included in the analysis. There was an initial 40% decline in P4 during the first hour after PGF, followed by a distinctive rebound in circulating P4 during the next 2 h. This is followed by another decrease until 5 h after PGF with a subsequent rebound that was slightly but significantly more prolonged when dinoprost was utilized rather than cloprostenol. Finally, starting at 6 h after treatment with cloprostenol or 8 h after treatment with dinoprost there is a final decline in P4 until basal concentrations are reached by 36 h after PGF for both products. Thus, initiation of the proestrus by treatment with PGF does not result in a linear decline in circulating P4 but produces a very distinct pattern of decreases and rebounds in circulating P4 that eventually results in basal P4 concentrations by 36 h after PGF treatment.

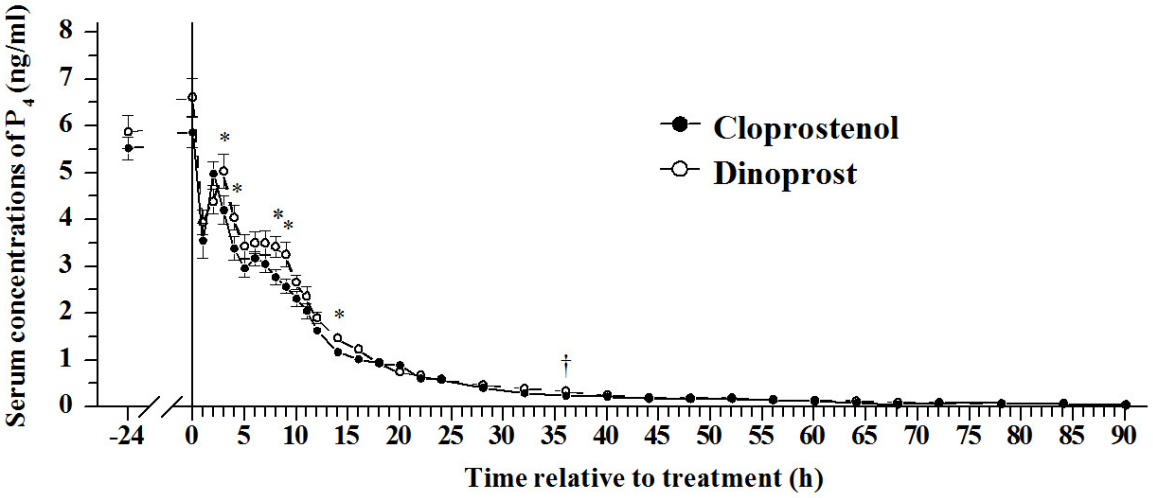

Figure 2. Mean (± SEM) serum P4 concentrations (ng/mL) from -24 to 90 h following treatment with cloprostenol sodium (n = 13) or dinoprost tromethamine (n = 14) in lactating dairy cows. Cows had at least one day 7 CL and one day 13 CL that had undergone complete luteolysis by 56 h after PGF2α and ovulated following a LH surge by 144 h after treatment (27/35). Rate of decrease during this 1st 12 h period was different between treatments (P = 0.025), but not different from 12 to 90 h (P = 0.12) after treatment. From: Martins *et al*., 2011a.

Fertility consequences of inadequate decrease in circulating P4

Lack of complete regression of the CL during the proestrus period has been frequently reported (Souza *et al.*, 2007; Brusveen *et al.*, 2009; Santos *et al*., 2010; Martins *et al.*, 2011a; Giordano *et al.*, 2012). In these studies, cows with a small elevation in circulating P4 near AI, due to lack of complete CL regression, have greatly reduced fertility. This was particularly important in cows treated with the 5-day Ovsynch protocol (Santos *et al*., 2010; Ribeiro *et al*., 2012). Fertility was reduced if only a single PGF was given (Santos *et al*., 2010) or if two PGF treatments were given on the same day (5 days after GnRH) compared to giving one treatment on day 5 and a second on day 6 (Ribeiro *et al*., 2012). In addition, studies on cows that receive AI after detection of estrus, have generally reported that minor elevations in P4 near AI are also detrimental to fertility (De Silva *et al.*, 1981; Waldmann *et al.*, 2001; Ghanem *et al.*, 2006), although some studies did not obtain this result (Erb *et al.*, 1976; Plym Forshell *et al.*, 1991). During Ovsynch, the percentage of cows that do not have complete regression following the PGF treatment before timed AI has been reported to range from 5-30% (Moreira *et al.*, 2000; Gumen *et al.*, 2003; Souza *et al*.,

2007; Brusveen *et al*., 2009; Martins *et al*., 2011a; Giordano *et al*., 2013). A recent extensive study of incomplete luteolysis evaluated multiple blood samples in cows at first postpartum AI (n = 652) and second or greater AI (n = 394; Martins *et al*., 2011a). They defined complete luteolysis and low P4 (<0.5 ng/mL) at 56, 72, and 96 h after PGF. At first AI, 80% of cows underwent complete luteolysis, whereas at second+ AI only 71% underwent complete luteolysis. Surprisingly, greater P4 concentrations at the time of PGF were associated with greater probability of luteolysis after PGF treatment and greater fertility (50 *vs*. 28%). Another study from our research group (Wiltbank *et al*., 2014) evaluated fertility and P4 concentration near the time of AI and reported a dramatic decrease in P/AI as P4 increased above 0.4 ng/mL near the time of the second GnRH of Ovsynch (16 h before AI). Thus, slight elevations in circulating P4 near AI result in dramatic decreases in fertility to the AI. This decrease was observed even when only cows that ovulated to the second GnRH were evaluated for fertility. The decrease that we observed was similar to the decrease described in the pioneering study of De Silva *et al*. (De Silva *et al*., 1981) using visual detection of estrus in lactating cows and heifers.

At times parity effects have been reported. For example, a lower percentage of multiparous (83.9%) than primiparous (89.7%) cows had complete CL regression after treatment with 500 µg cloprostenol in one study (Giordano *et al*., 2012). Other studies have also reported lower rates of luteal regression in multiparous than in primiparous lactating dairy cows (Martins *et al*., 2011a). In contrast, other studies from our laboratory using dinoprost did not observe an effect of parity on percentage of cows with complete CL regression (Souza *et al*., 2005b; Brusveen *et al*., 2009; Wiltbank *et al*., 2014). It is unclear whether differences between products in parity effects can be repeated in direct comparison studies. In addition, the reasons for the parity effects have not yet been determined.

Two methods have been used to increase regression of the CL during timed AI protocols: increasing the dose of PGF or increasing the number of PGF treatments. During the Ovsynch protocol, an increased percentage of cows with complete CL regression (<0.4 ng/mL 56 h after PGF) was observed after two (day 7 and 8; 326/341 = 95.6%) compared to one (day 7; 301/356 = 84.6%) PGF treatment (Brusveen *et a*l., 2009). In a recent study from our laboratory (Giordano *et al*., 2013), increasing the dose of cloprostenol from 500 µg to 750 µg on day 7 of a 7 day Ovsynch protocol, increased CL regression in multiparous (122/154 = 79.2% *vs*. 135/154 = 87.7%; P = 0.025) but not primiparous (131/146 = 89.7% *vs*. 129/139 = 92.8%; P = 0.181) cows. Improved fertility (P = 0.05) was observed at the 39 day pregnancy diagnosis with the 750 (247/544 = 45.4%) compared to the 500 (221/540 = 40.9%) µg dose of cloprostenol (Giordano *et al*., 2013). It has been speculated that the reduction in fertility may be due to changes in gamete transport leading to lack of fertilization, however this has not been definitively tested at this time.

In synchronized ovulation programs using E2 and P4, as used in Brazil, it is also critical to attain low concentrations of P4 near the time of AI (Pereira *et al*., 2013b). In this study, cows with P4 concentrations above 0.1 ng/mL near the time of AI had reduced fertility (Pereira *et al*., 2013b). Part of the reason for reduced fertility was due to a lack of ovulation in response to the estradiol cypionate (ECP) treatment that was used for induction of ovulation. Thus, in Ovsynch protocols, in E2/P4 protocols, and during natural estrus, a small elevation in P4 near AI can reduce fertility. Treatment with an increased dose of PGF or increased number of PGF treatments can alleviate or reduce this problem.

Increasing estradiol concentrations

Natural E2 increase

Figure 3 shows the follicle growth patterns and concentrations of E2 during a natural proestrus in heifers with two or three follicular waves and in lactating cows with two follicular waves (Sartori *et al*., 2004). It is clear that in either heifers or cows with two follicular waves the dominant follicle has been selected well before the time of luteolysis. In contrast in heifers with 3 follicular waves, selection of the dominant follicle is occurring very close to the time of luteolysis. It should be remembered that the timing of luteolysis differs in these three groups with the earliest initiation of luteolysis in two-wave heifers (17.4 ± 0.3 days), slightly delayed in two-wave lactating cows (18.1 ± 0.5 days), and the latest initiation of luteolysis in three-wave heifers (19.1 ± 0.6 days). It is likely in three-wave heifers that the uterus has already attained E2 responsiveness but that the initiation of the new follicular wave delayed the timing of sufficient E2 increase to initiate luteolysis (Araújo *et al*., 2009). The smaller size of the follicle at the time of natural luteolysis causes a longer time from initiation of luteolyis to ovulation in three-wave (5.0 ± 0.2 days) than two-wave (4.3 ± 0.1 days) heifers.

The circulating E2 concentrations during proestrus are shown in Fig. 3D for the heifers and cows. All animals had increased E2 at the time of initiation of luteolysis with E2 rising most rapidly (percentage-wise) between day -1 and 0 for three-wave heifers. The two-wave heifers attained peak E2 by 2 days after initiation of luteolysis followed by a decline, probably due to initiation of estrus and the LH surge in some heifers in this group. Heifers with three waves had a rapid increase in circulating E2 between 2 and 3 days after luteolysis as the follicle attained preovulatory size and increased E2 production. The lactating cows had lower circulating E2 concentrations, in spite of a larger

follicle, probably due to greater E2 metabolism in lactating cows (Sangsritavong *et al.*, 2002; Wiltbank *et al.*, 2006). The peak E2 concentrations have previously been related to level of milk production (Lopez *et al.*, 2004, 2005), with reduced peak circulating E2 concentrations in cows with greater milk production in spite of having greater follicular volume for production of E2. Thus, the length of the proestrous period and the circulating E2 concentrations during this period can vary by whether animals have two or three follicular waves and are related to level of milk production in naturally ovulating cows.

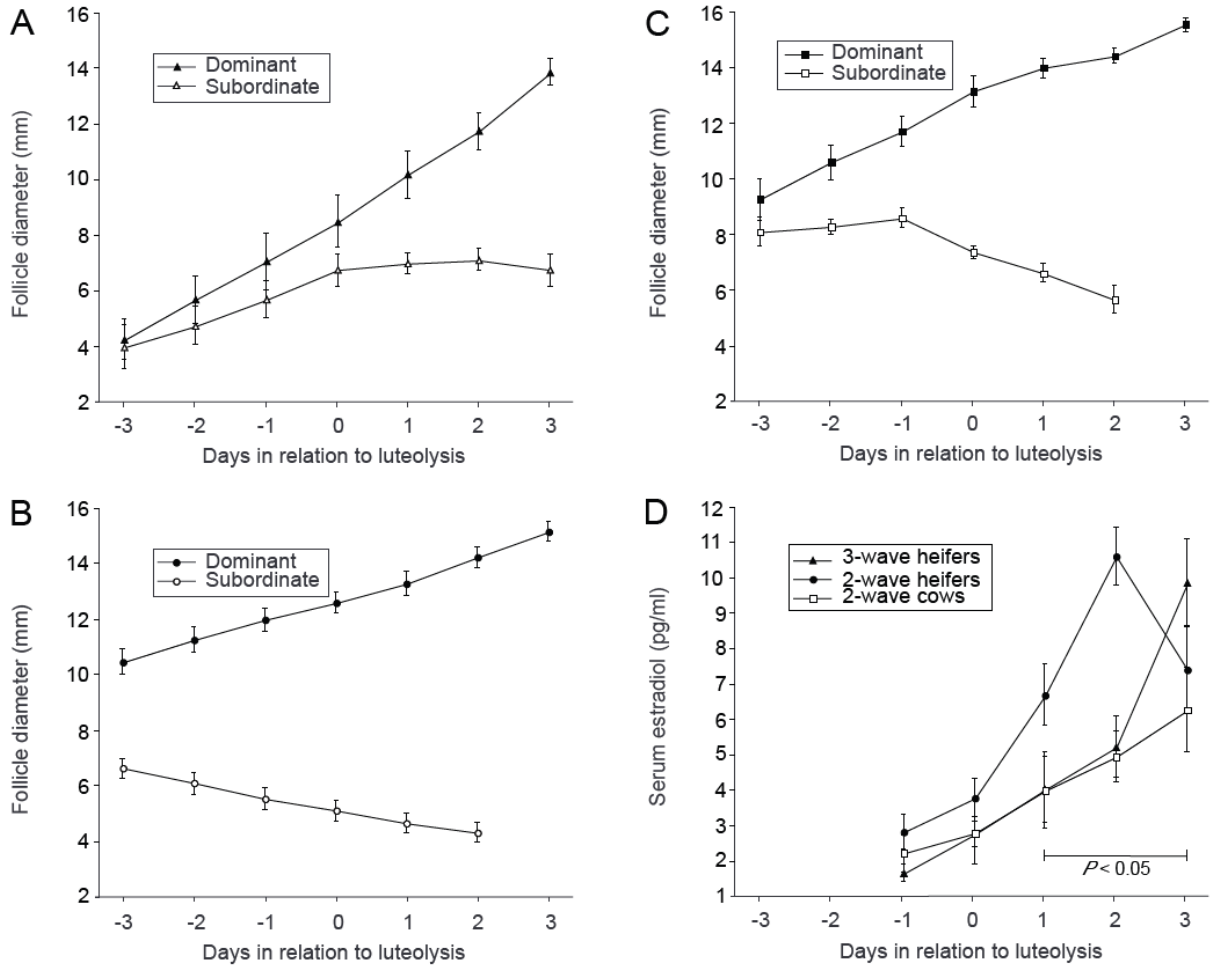

Figure 3. Size of the single dominant and largest subordinate follicle normalized to the time of luteolysis in: A) Heifers with three follicular waves (n = 8), B) Heifers with two follicular waves (n = 15), and C) Lactating cows with two follicular waves (n = 9). D) Serum E2 concentration normalized to time of luteolyis in the three groups. From: Sartori *et al*., 2004.

Increase in E2 using programs that synchronize ovulation

As discussed above, in cows that are treated with exogenous PGF the future ovulatory follicle may be at any stage of the follicular wave and this is a key determinant of time to estrus and ovulation after PGF. Obviously this would also impact the pattern and concentrations of circulating E2 during a PGF-induced proestrus. Circulating E2 was measured in the same experiment discussed above (Martins *et al*., 2011b). Circulating E2 increased from ~0.75 pg/mL at time 0 to ~2 pg/mL by 24 h after PGF treatment. Circulating E2 continued to increase from 24 to 60 h after PGF with a distinctive decrease after 72 h. Thus, the pattern of circulating E2 after PGF treatment is very predictable with increasing E2 until peak E2 is attained and a GnRH/LH surge is induced. This is then followed by decreasing E2 that reaches basal concentrations by 9 h after either a GnRH-induced LH surge or a natural GnRH/LH surge (Haughian *et al.*, 2013).

Thus, the pattern of circulating E2 during a PGF-induced proestrus involves many of the same factors that regulate E2 concentrations during a natural

proestrus. First, the size of the follicle changes circulating E2 due to increased follicular E2 production and there can be more variation in follicular size at the start of a PGF-induced proestrus than a natural proestrus. Second, the high dry matter intake and liver blood flow associated with high milk production causes greatly increased E2 metabolism and therefore will decrease circulating E2. Third, the timing of the GnRH/LH surge will determine the end of the proestrus. During natural proestrus, this timing is determined by the timing of attainment of basal P4 and reaching high circulating E2 concentrations for a sufficient duration to induce the GnRH/LH surge. In timed AI protocols that use GnRH to synchronize ovulation, duration and magnitude of circulating E2 can be lower than during natural proestrus because the GnRH treatment may induce the LH surge and ovulation earlier than would have occurred naturally. In timed AI protocols that synchronize ovulation with E2 treatments the pattern of circulating E2 can be dramatically different based on the timing of E2 treatment and the type of E2 product utilized.

A previous study from our laboratory (Souza *et al.*, 2005a) evaluated the pattern of E2 concentrations following treatment with 1 mg of native E2-17β, estradiol benzoate (EB), or ECP in lactating cows with all follicles aspirated to eliminate endogenous E2 production. As shown in Fig. 4, the control cows remained at about 1 pg/mL or less throughout the experimental period. Treatment with native E2 produced a rapid increase in circulating E2 with peak concentrations observed at the earliest time that was evaluated (4 h). It is likely that even greater E2 concentrations were produced at an earlier time but no earlier measurements were taken. Circulating E2 then declined with basal E2 concentrations attained by 28 h. Treatment with EB reached peak concentrations by 8 h. This was followed by a plateau in circulating E2 at ~6 pg/mL from 12 to 28 h after EB with a decrease to ~4 pg/mL from 32 to 40 h and returning to basal concentrations by 44 h after EB treatment. Thus, treatment with EB reached peak circulating E2 later than treatment with native E2 with a more prolonged plateau after treatment that reaches basal E2 by 2 days after EB treatment. Treatment with 0.5 mg of EB produced a similar pattern but with circulating E2 concentrations of ~50% of those observed after treatment with 1 mg EB (data not shown; Souza *et al.*, 2005a). Treatment with ECP produced a very distinct pattern with little increase in circulating E2 until about 28 h after ECP treatment with peak concentrations less than 4 pg/mL and then a decline back to basal E2 concentrations by 44 h after treatment. These patterns were done in the absence of dominant follicles. In commercial situations, these patterns of E2 produced by the E2 treatment would be combined with the endogenous E2 production by follicles of differing sizes to produce the proestrus circulating E2 pattern that would be observed during the proestrus induced by a timed AI program that uses E2 treatments to synchronize ovulation.

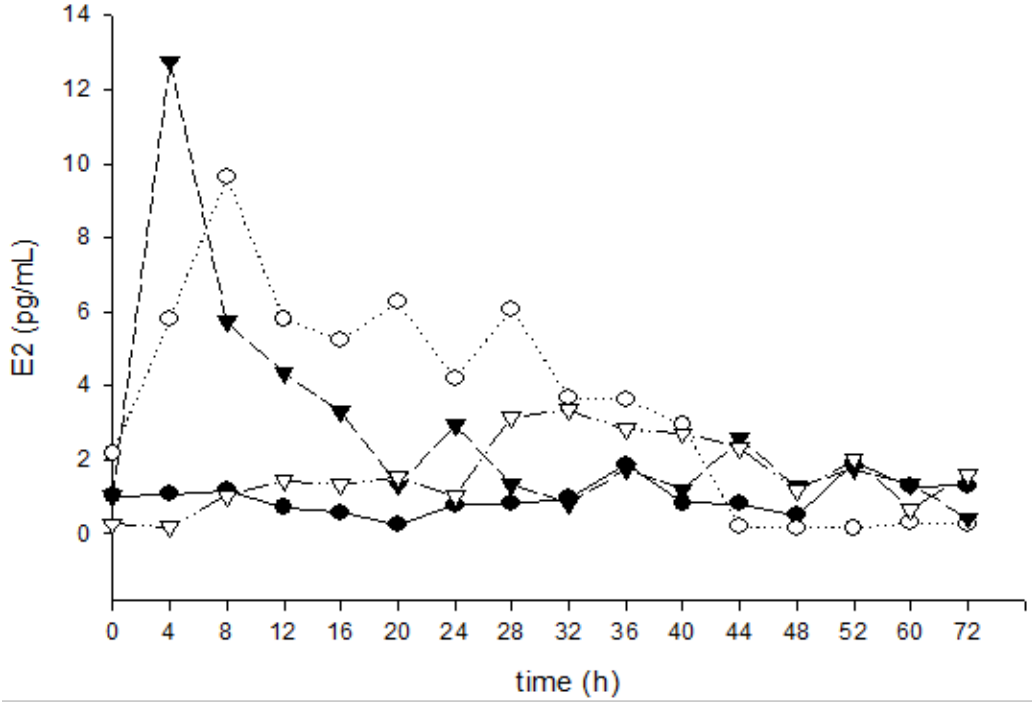

Figure 4. Mean serum E2 concentrations (ng/mL) in cows in the absence of follicle >5 mm for control (dark circle; n = 3), or after treatment with 1 mg E2-17β (dark triangle; n = 3), 1 mg estradiol benzote (EB; open circle; n = 3), or 1 mg of estradiol cypionate (ECP; open triangle; n = 3). Hour 0 is estrogen treatment. From: Souza *et al*., 2005a.

Fertility consequences of inadequate increase in E2

As would be expected, reducing the size of the ovulatory follicle at the time of PGF dramatically alters the pattern of hormone secretion during a PGF-induced proestrus. Cows that were treated with an Ovsynch protocol (GnRH-7d-PGF-2d-GnRH-1d-AI) had the largest follicle aspirated 3 or 4 days after the initial GnRH treatment (4 or 3 days before PGF treatment). As expected this reduced the size of the future ovulatory follicle at time of PGF (8.7 *vs*. 12.5 mm diameter) and at time of second GnRH (11.5 *vs*. 14.5 mm). Circulating E2 was also reduced at time of second GnRH in aspirated compared to non-aspirated cows (2.5 *vs*. 5.7 pg/mL). Thus, this protocol dramatically changed proestrus by inducing a smaller follicle at time of PGF and ovulating this follicle prematurely with a second GnRH. As would be expected, ovulation of a smaller follicle produced a much smaller CL in aspirated than non-aspirated cows (2,862 *vs*. 5,363 mm3 on day 7; 4,652 *vs*. 6,526 mm^3 on day 14). The fertility was also dramatically changed by the alteration in proestrus produced by aspiration of the follicle. The pregnancies per AI (P/AI) were only 10.3% (3/29) for the cows that had follicle aspiration compared to 43.5% (10/23) for the non-aspirated cows (Vasconcelos *et al.*, 2001). Since, fewer of the cows had synchronized ovulation in the aspirated (72.4%) than the non-aspirated (91.3%) group, the P/AI was calculated for only the synchronized cows and the difference in fertility persisted (14.3% *vs*. 47.6%). Thus, altering the pattern of E2 during the proestrus by reducing the size of the ovulatory follicle produced a dramatic reduction in fertility. This could be due to the increase in percentage of cows with short luteal phases (14.3% *vs*. 0%) or reduced P4 concentrations after AI. No studies have differentiated the underlying mechanisms that produce this dramatic reduction in fertility. We speculate that the inadequate circulating E2 during proestrus may have a major role in the fertility reduction.

Programs that synchronize ovulation using GnRH clearly have reduced peak circulating E2 as the final GnRH is given at same time as estrus or prior to estrus. We evaluated whether E2 was limiting to fertility in lactating dairy cows receiving the Ovsynch protocol. A total of 952 high-producing dairy cows were randomized to receive either the Ovsynch protocol (GnRH-7d-PGF-56h-GnRH-16h-Timed AI) or the Ovsynch protocol supplemented with E2 (1 mg native E2-17β at 48 h after PGF; GnRH-7d-PGF-48h-E2-8h-GnRH-16h-Timed AI). Treatment increased expression of estrus from 44.4% in Ovsynch to 80.2% in Ovsynch+E2. However, there was no overall improvement in P/AI with E2 supplementation (Ovsynch = 39.4% [161/409]; Ovsynch+E2 = 42.4% [188/443]). The largest effect of E2 supplementation was in cows with lower BCS (≤2.5). In cows treated with only Ovsynch, the ones with lower BCS had lower P/AI (28.1%) than those with higher BCS (43.7%); however, in cows treated with Ovsynch+E2, cows with lower (40.0%) or higher (43.9%) BCS had similar P/AI. Thus, E2 supplementation increased fertility in cows with low BCS suggesting that in these cows circulating E2 during proestrus may be limiting to fertility. These cows may have insufficient energy intake and therefore inadequate follicle growth and E2 production due to low numbers of LH pulses (Wiltbank *et al.*, 2002). Interestingly, expression of estrus was not associated with P/AI in cows treated only with Ovsynch (Estrus = 45.1% *vs*. No estrus = 40.5%) but was strongly associated with fertility in cows treated with Ovsynch+E2 (Estrus = 53.7% *vs*. No estrus = 22.8%).

In a second study (Brusveen *et al*., 2009), we evaluated a similar concept but supplemented the E2 (0.5 mg E2-17β) at the same time as the second GnRH of Ovsynch (~8 h later than in the previous study). This was done for ease of providing the supplemental E2. Again, treatment with E2 increased expression of estrus (Ovsynch = 37.2% *vs*. Ovsynch+E2 = 87.7%). However, in this study, supplementation tended to decrease P/AI in cows that were synchronized by the protocol (Ovsynch = 54.4% [136/250] *vs*. Ovsynch+E2 = 46.6% [123/264]; P = 0.10). It should be asked why supplemental E2 was somewhat positive in at least a subgroup of cows in the first study but tended to be negative to fertility in the second study. We postulate that this is due to the timing of E2 supplementation. In the first study supplementation at 48 h would lead to the E2 peak at the time of GnRH treatment with decreasing E2 by the time of AI. However, in study 2 supplementation at GnRH treatment would lead to an E2 peak about 8 h after GnRH and continuing high E2 near the time of AI. No study has directly tested this hypothesis by altering the timing of E2 treatment in a GnRH-based protocol.

In Brazil and many other countries, ovulation is synchronized for timed AI using E2/P4-based protocols with final ovulation synchronized using ECP or EB at the end of the protocol (Baruselli *et al.*, 2012). One study compared GnRH and ECP for stimulating ovulation at the end of an E2/P4 protocol (Souza *et al.*, 2009). This study used a 2X2 factorial design with cows also treated with or without equine chorionic gonadotropin (eCG). In cows with ovulation induced by GnRH, the eCG treatment increased P/AI (GnRH-No eCG = 28.9% [56/194] *vs*. GnRH+eCG = 33.8% [67/198]; P < 0.05) but not in cows with ovulation induced by ECP (ECP-No eCG = 30.9% [60/194] *vs*. ECP+eCG = 29.1% [57/196]). This was particularly apparent in cows with low BCS (GnRH = 6.1%; GnRH+eCG = 44.4%; ECP = 21.7%; ECP+eCG = 32.6%). Thus, cows may need supplementation with E2 and/or eCG when they have low BCS and therefore the final stages of follicle growth and circulating E2 are limiting to fertility.

One study evaluated the role of E2

supplementation during the proestrus on fertility in lactating dairy cows in Brazil during the summer (Pereira *et al.*, 2013a). Lactating dairy cows (n = 1,190) received either a 5 day CoSynch protocol (GnRH/CIDR inserted-5d-PGF/CIDR removed-1d-PGF-2d-GnRH+AI) or an E2/P4 protocol (EB/CIDR inserted-7d-PGF-1d-ECP/CIDR removed-2d-AI). The GnRH-based protocol (5 day CoSynch) produced better synchronization (78.2 *vs*. 70.7%; P = 0.02) and had poorer fertility in all cows (16.7 *vs*. 20.7%; P = 0.07) and in synchronized cows (17.7 *vs*. 25.6%; P = 0.03). Much of the problem with the GnRH-based protocol was that it had greater pregnancy loss (21.7%) than the E2-based protocol (6.7%). The fertility and pregnancy loss differences between the GnRH-based and E2-based protocols were only observed in cows that had heat stress (body temperature above 39.1^0C) and not in cows without heat stress. In this study, increasing follicle size always produced increases in fertility and decreases in pregnancy loss in cows treated with the GnRH protocol but this relationship was not observed in cows treated with E2. It seems likely that cows under heat stress have reduced follicle growth and E2 may become limiting for fertility and pregnancy maintenance. Obviously, there are numerous other factors such as fertilization and embryo death that are also major problems for fertility in cows under heat stress (Hackbart *et al.*, 2010; Hansen, 2013; Sakatani *et al.*, 2013).

In conclusion, circulating E2 during the proestrus period may be limiting for fertility in lactating dairy cows in some situations. If a GnRH-based protocol causes ovulation of a smaller follicle, then E2 is likely to be limiting for fertility. In cows with low BCS, follicle growth and circulating E2 are reduced and either stimulators of follicle growth like eCG or treatment with E2 can improve fertility. During heat stress, follicle growth and circulating E2 may be limiting for fertility but the studies to analyze these effects must be very large because of the numerous other effects of heat stress on fertility that are likely to be independent of circulating E2.

Conclusions

In cattle, proestrus begins with the initiation of luteolysis and ends with initiation of estrus and the GnRH/LH surge. This period is marked by a dramatic decrease in circulating P4 that reaches a nadir by about 36-48 h in cows undergoing natural or PGF-induced luteolysis. Inadequate luteolysis is a cause of reduced fertility particularly in timed AI programs with small elevations in circulating P4 reducing fertility. Increasing circulating E2 during proestrus is dependent on presence, size, and function of the dominant follicle and this varies during natural proestrus, due to whether animals have two or three follicular waves, and during PGF-induced proestrus, according to stage of the follicular wave at time of PGF treatment. Inadequate circulating E2 can limit fertility and increase pregnancy loss in some specific circumstances such as in cows with low BCS and in cows during heat stress. Thus, studies to optimize the length and hormonal concentrations during proestrus could produce substantial improvements in fertility and reductions in pregnancy loss.

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