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Positive and negative effects of progesterone during timed AI protocols in lactating dairy cattle

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Abstract

Circulating concentration of progesterone (P4) is determined by a balance between P4 production, primarily by corpus luteum (CL), and P4 metabolism, primarily by liver. The volume of large luteal cells in the CL is a primary factor regulating P4 production. Rate of P4 metabolism is generally determined by liver blood flow and can be of critical importance in determining circulating P4 concentrations, particularly in dairy cattle. During timed AI protocols, elevations in P4 are achieved by increasing number of CL by ovulation of accessory CL or by supplementation with exogenous P4. Dietary manipulations, such as fat supplementation, can also be used to alter circulating P4. Elevating P4 prior to the timed AI generally decreases double ovulation and can increase fertility to the timed AI. This appears to be an effect of P4 during the follicular wave that produces the future ovulatory follicle, possibly by altering the oocyte and subsequent embryo. Near the time of AI, slight elevations in circulating P4 can dramatically reduce fertility. The etiology of slight elevations in P4 near AI is inadequate luteolysis to the prostaglandin F2α (PGF) treatment prior to timed AI. After AI, circulating P4 is critical for embryo growth and establishment and maintenance of pregnancy. Many studies have attempted to improve fertility by elevating P4 after timed AI. Combining results of these studies indicated only marginal fertility benefits of <5%. In conclusion, previous research has provided substantial insight into the effects of supplemental P4 on fertility and there is increasing insight into the mechanisms regulating circulating P4 concentrations and actions. Understanding this prior research can focus future research on P4 manipulation to improve timed AI protocols.

Keywords: fertility, lactating dairy cows, progesterone, timed AI.

Introduction

Reproductive efficiency is dependent upon optimization of management, health, and physiology of cows. The interactions between nutrition, the hormonal systems, and altered reproduction in dairy cattle are increasingly being elucidated (Lucy, 2001; Wiltbank *et al.*, 2006; Chagas *et al.*, 2007; Sartori *et al.*, 2010). This review will focus on the role of progesterone (P4) in reproduction in cattle, with primary emphasis on the lactating dairy cow.

Progesterone is a steroid hormone primarily secreted by the corpus luteum (CL) and placenta. Adequate circulating P4 concentrations are essential for establishment and maintenance of pregnancy. Studies at the turn of the last century showed that pregnant rabbits did not maintain their pregnancy following bilateral ovariectomy (Fraenkel and Cohn, 1901) or electrocautery of all CL (Magnus, 1901). Corner and Allen (1929) determined that the CL factor that was responsible for pregnancy maintenance could be extracted with ethanol but not with saline. By 1934, four different laboratories independently isolated the crystalline hormone that maintained pregnancy and progesterone was selected as the scientific name for the hormone (Allen, 1974).

The receptors for P4 and the intracellular mechanisms of action have been extensively reviewed (Ellmann *et al.*, 2009). The classical P4 receptors (PR) are members of the nuclear receptor superfamily. In other words, these receptors work through actions in the nucleus that regulate expression of specific genes. There are two isoforms of the nuclear PR, termed PR-A and PR-B. These PR are products of the same gene with the key distinction that PR-B contains an additional 165 amino acids at the N-terminal end of the protein. Studies in knockout mice in which PR-A or PR-B are selectively eliminated show that these two receptors are functionally distinct and both are critical for successful reproduction (Arck *et al.*, 2007). The PR-A isoform alone is sufficient for the establishment and maintenance of pregnancy; whereas, PR-B is not sufficient for establishment or maintenance of pregnancy but is essential for fertility, possibly through actions on tissues other than the uterus (Fernandez-Valdivia *et al.*, 2005). Surprisingly, there are also multiple types of plasma membrane P4 receptors that act through activation of intracellular signal transduction systems that are not related to the nuclear PR systems (Peluso, 2006).

The P4 concentration that reaches the receptors within each particular cell is the key determinant of the physiological actions of P4 in an animal. In most tissues the circulating P4 concentration is the primary determinant of P4 concentrations within the cell. Therefore, factors regulating circulating P4 primarily

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determine the magnitude of P4 responses throughout the body.

Circulating P4 concentrations (Fig. 1) represent a balance between the production of P4, primarily by the CL (shown on left), and the metabolism of P4, primarily by the liver (shown on right). If P4 production is increased by an increase in luteal tissue without a change in liver blood flow, then circulating P4 will increase. Conversely, if liver blood flow doubles, from 1000 to 2000 l/h, then there will be a corresponding decrease in circulating P4 even though P4 production has not been altered. Thus, an understanding of regulation of circulating P4 must carefully consider the factors regulating P4 production and P4 metabolism. Over 80% of P4 production during the estrous cycle of the cow is due to constitutive P4 production by the large luteal cell (Niswender *et al.*, 1994; 2000; Diaz *et al.*, 2002). Regulation of constitutive P4 production by the CL does not appear to require stimulatory pathways but can be dramatically decreased during luteolysis induced by exogenous or endogenous PGF. These concepts are more fully developed in a companion manuscript on regulation of the CL (Wiltbank *et al*., 2012). Conversely, although liver enzymes involved in P4 metabolism can be regulated, we speculate in this model that the primary regulation of P4 metabolism in the lactating cow is related to the rate of liver blood flow.

The role of feed intake in regulating P4 metabolism and thus circulating P4 was initially demonstrated in studies with pigs and sheep (Christenson *et al.*, 1985; Parr *et al.*, 1993; Prime and Symonds, 1993). For example, the studies in sheep showed convincingly that as feed intake increased, there was a clear increase in liver blood flow with a corresponding decrease in circulating P4 (Parr *et al*., 1993). Our studies extended these studies to the lactating dairy cow by demonstrating the clear relationship between dry matter intake, liver blood flow, and circulating concentrations of P4 as well as another steroid hormone, estradiol-17ß (Sangsritavong, 2002; Sangsritavong *et al.*, 2002; Wiltbank *et al*., 2006). After matching Holstein cows for weight and age, high producing dairy cows had more than twice the liver blood flow as found in non-lactating cows. Similarly, the metabolic clearance rate of estradiol and P4 were much greater in lactating than non-lactating cows, probably reflecting the increased blood flow to the liver. Thus, when P4 was infused at a constant rate in lactating and non-lactating dairy cows, the concentration of P4 that was reached at steady-state in the blood was much greater in non-lactating than lactating cows due to the high metabolic clearance rate of P4 in the lactating cows. Thus, if there was the same production of P4 from the CL, a lactating cow will have a much lower circulating P4 concentration than a non-lactating cow.

The underlying physiology for the decrease in circulating P4 seems to be relatively simple (Wiltbank *et al*., 2006). If we assume that any blood that flows through the liver is completely cleared of P4, in other words 100% metabolism of P4 in liver blood (Bedford *et al.*, 1974; Freetly and Ferrell, 1994), then any increase in rate of blood flow to the liver would produce a corresponding increase in rate of P4 metabolism. Blood flow to the liver comes from two major blood vessels, the hepatic artery and the portal vein. The portal vein drains the blood from the digestive tract and takes this blood to the liver. Any increase in blood flow to the digestive tract, for example due to an increase is feed intake, will necessarily increase blood flow to the liver through the portal vein. Thus, an increase in feed intake will increase blood flow to the digestive tract, increasing blood flow to the liver and thereby increasing the rate of P4 metabolism solely due to higher amount of P4 entering the liver due to greater volume of liver blood flow (Sangsritavong *et al*., 2002). It is also likely that P4 is metabolized in the tissue of the digestive tract, as well as the liver (Bedford *et al*., 1974; Freetly and Ferrell, 1994). This simple physiological explanation helps to illustrate how various physiological states could produce dramatic changes in circulating P4 solely due to changes in P4 metabolism.

Metabolism of P4 by the liver generally involves enzymatic reactions that have been described as phase I or phase II reactions (Fig. 1). The phase I reactions can either hydroxylate progesterone in multiple positions using the P450 enzymes, primarily CYP3A4 and CYP2C enzymes, as well as other CYP enzymes. A more active pathway for P4 metabolism in bovine involves reduction of the double bond at the 4-5 position to a 5-alpha or 5-beta reduced progesterone and reduction of the ketone at the 3 position to a hydroxyl group. The ketone at the 20 position is also hydroxylated to produce a tetrahydrosteroid from the P4 molecule. The enzymes involved in these reactions are the 5-alpha reductase enzyme producing 5-alpha-reduced progestins and AKR1D1 producing 5-beta reduced progestins. The 5-beta reduced progestins account for about two-thirds of the mass of steroids that are inactivated. Reduction of the 3-ketone to a 3-hydroxy and reduction of the 20-ketone to a 20-hydroxy are mediated by the AKR1C enzymes with AKR1C4 being the most abundant enzyme with the high catalytic efficiency. However other AKR1C isoforms may also be important in metabolisms of P4 in cattle. The most common pathway, based on ovine liver microsome metabolism of P4, is 6-beta hydroxylation (Murray, 1991) or 21-hydroxylation (Murray, 1992). However, almost all P4 metabolites found in blood, urine, or feces are 5-alpha or 5-beta reduced pregnanes (Stupnick and Williams, 1968; Chantilis *et al.*, 1996; Schwarzenberger *et al.*, 1996), indicating that the reductase and AKR1D pathways represent a major part of in vivo P4 metabolism. These pathways are considered the phase I pathways and P4 metabolism by liver microsomes has been found to yield a myriad of different metabolites apparently through multiple liver enzymes, as

summarized by (Sangsritavong, 2002).

Insulin appears to be an inhibitor of gene and protein expression for 2 of the major P450 enzymes, CYP3A and CYP2C, in bovine or ovine liver (Smith *et al.*, 2006; Lemley *et al.*, 2008, 2010a, b). This suggests that future programs to reduce P4 metabolism may focus on increasing circulating insulin. Polyunsaturated fat acids (PUFAs) appear to inhibit the P4 metabolizing activity of the CYP enzymes from bovine liver slices (Piccinato *et al.*, 2010), although, this effect could not be replicated in vivo possibly because the concentrations of PUFAs that were effective *in vitro* were not achieved *in vivo* (Piccinato *et al.*, 2010). Following the phase I reactions there are generally phase II reactions that involve conjugation of hydrophilic groups to P4. The primary circulating P4 conjugates have an attached glucuronide, although, mono- and disulfates can also be detected (Sangsritavong, 2002). These steps inactivate P4 and facilitate excretion of hydrophilic P4 conjugates in urine and feces (Sangsritavong, 2002). The primary enzymes involved in glucuronidation of reduced or hydroxylated pregnane compounds are UGT1A and UGT1B. Although all of these complex pathways are clearly involved in P4 metabolism and are targets for manipulation of P4 metabolism, our current view is that the primary reason for high P4 metabolism in lactating dairy cows is the extraordinary liver blood flow due to extremely elevated feed consumption and gastrointestinal liver blood flow.

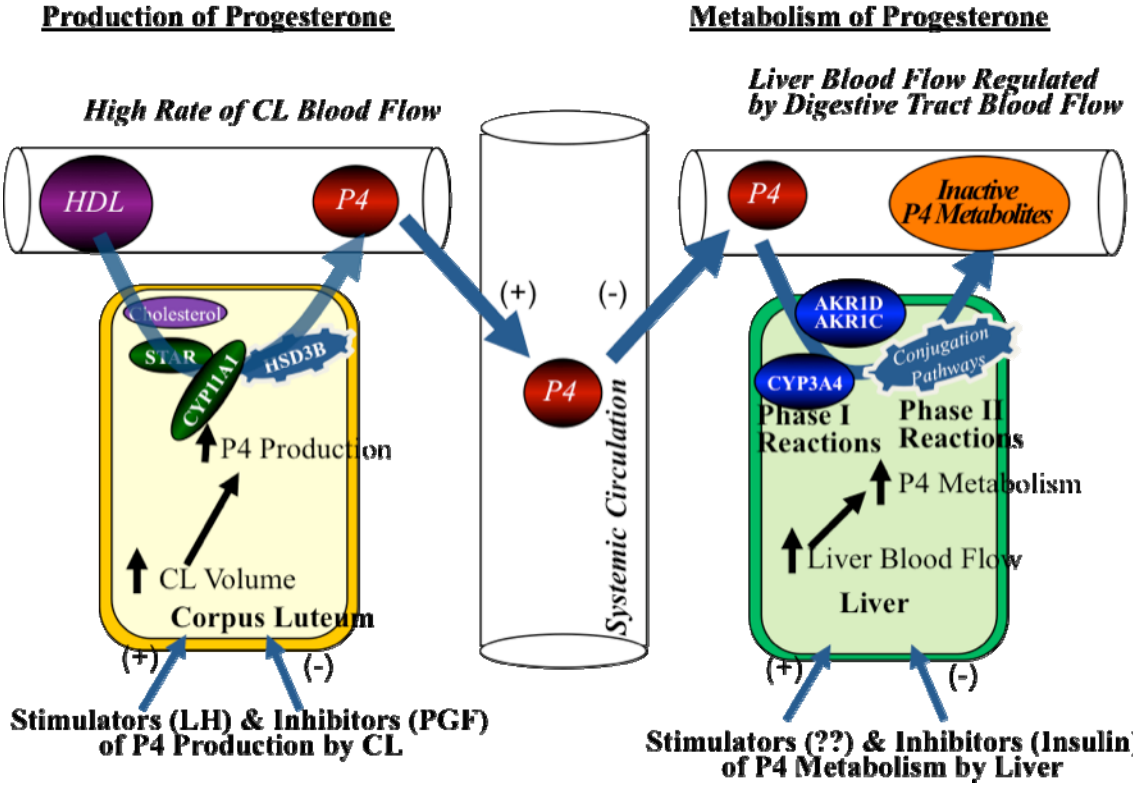

Figure 1. Physiological model of the factors that regulate circulating P4 concentrations in the lactating dairy cow (center of model). Production of P4 (left side of model) is primarily a function of production in the corpus luteum through pathways that convert cholesterol from HDL (high density lipoprotein) into P4. The cholesterol transport protein, StAR, and the 2 steroidogenic enzymes, CYP11A1 and HSD3B, are critical for this conversion. Although stimulators, such as LH, and inhibitors, such as PGF, can regulate P4 production by CL, it is emphasized in the model that the primary factor determining greater P4 production is likely to be CL volume due the constitutive nature of P4 production in the bovine CL. On the right side of the model are pathways involved in P4 metabolism tending to decrease circulating P4. The 2 Phase I pathways are hydroxylation, with CYP3A4 shown as an example, and 5-alpha or 5-beta reduction. Phase II conjugation pathways are also involved in producing hydrophilic P4 metabolites. Although stimulation and inhibition of the P4 metabolizing enzymes has been reported, this model emphasizes the critical role of the elevated liver blood flow in producing the extraordinary rates of P4 metabolism in high producing lactating dairy cows.

Importance of high P4 during follicular growth before AI

Although the effects of P4 on reproduction are numerous, this review will divide these effects into three time periods that will be discussed in the next three sections of this manuscript. These time periods will be in relationship to the time of breeding. In this manuscript, we will briefly discuss this relationship during timed AI programs focusing on the role of P4 concentrations before, during, or after timed AI.

There is a clear association between milk production and double ovulation and it is now clear that this association is, at least partly, related to the circulating P4 concentrations near the time of follicle selection (Wiltbank *et al.*, 2000; Lopez *et al.*, 2005a). From a practical standpoint, high percentage of cows with double ovulation appears to be the underlying cause of increased twinning rate in lactating dairy cows, with 93% of twins being non-identical (del Rio *et al.*, 2006). Numerous factors have been recognized as possible regulators of twining rates, including age of dam, season, genetics, use of reproductive hormones or antibiotics, ovarian cysts, and days open; however, peak milk production is clearly the largest contributor (Kinsel *et al.*, 1998; Wiltbank *et al*., 2000). We have done two studies that evaluated the effect of milk production on double ovulation. In cows synchronized with the Ovsynch protocol (Fricke and Wiltbank, 1999), double ovulation was much greater in cows that were above average milk production, 40.7 kg/day, than below (20.2 *vs.* 6.9%; $P < 0.05$). Similarly, in a study with cows that were evaluated near natural estrus (Lopez *et al*., 2005a), almost 50% of cows that were above 40 kg/day of milk production had double ovulation, whereas less than 10% had double ovulation for cows producing below 40 kg/day. These effects were similar for multiparous or primiparous cows (Fricke and Wiltbank, 1999; Lopez *et al*., 2005a). It should be noted that neither of these studies involved manipulation of milk production and were only performed as an analysis of the percentage of cows with double ovulation compared to different levels of milk production.

The underlying physiology that produces the relationship between milk production and double ovulation is becoming clearer. There is an increase in FSH near the time of follicle selection that is associated with a decrease in circulating P4 concentrations (Lopez *et al.*, 2005b). Manipulative studies have now been performed that have demonstrated a decrease in percentage of cows that double ovulate when circulating P4 is increased (Cunha *et al.*, 2008; Cerri *et al.*, 2011b). It is postulated that the endocrine mechanism that underlies the decrease in double ovulation in the presence of high P4 is related to a decrease in circulating FSH during a critical time of follicle selection, although this idea has not yet been experimentally evaluated.

The key role of greater P4 prior to AI on fertility of lactating dairy cows was first described by (Fonseca *et al.*, 1983). More recent manipulative studies have shown some improvements of ~5-7% in pregnancy by using a P4 vaginal dispositive such as a CIDR during the Ovsynch program prior to AI (Stevenson *et al.*, 2006, 2008; Chebel *et al.*, 2010). Nevertheless, the results from these studies may be somewhat confounded due to improvements in synchronization rate that may accompany the use of CIDR in the Ovsynch protocol. In the study mentioned above we tested the effects of elevated P4 on fertility to a timed AI during the Double Ovsynch program (Cunha *et al*., 2008). Cows (n = 564) were randomly assigned to have either high or low P4 during the Ovsynch protocol. The cows with low P4 had increased double ovulation rate, which would be expected to potentially increase fertility in these cows. Ovulation of more follicles could potentially result in a greater probability of pregnancy. However, cows with lower P4 before AI had much lower fertility (37.1% pregnant at day 29 pregnancy diagnosis) compared to cows with high P4 (51.0%; $P < 0.001$). This indicates that increasing P4 prior to timed AI can result in a substantial improvement in fertility, suggesting that the reason for the lower fertility in lactating dairy may be, at least partly, due to reduced P4 concentrations during the time period prior to AI. Obviously, we produced an artificial reduction in P4 during this study; however, under normal conditions, P4 concentrations may be reduced due to the high feed intake and increased P4 metabolism of lactating dairy cows. In the future, practical programs may be developed that target an elevation in circulating P4 in order to increase fertility.

One further observation of this study was related to pregnancy loss. Pregnancy loss is generally defined as the number of pregnancies that are lost between the pregnancy diagnosis at Day 28 after AI compared to Day 60 after AI. This time period is critical for implantation and development of the embryo. We observed a decrease ($P < 0.05$) in pregnancy loss between day 29 and day 57 when cows had high P4 (6.8% loss) *vs.* low P4 (14.3% loss). Thus, the high P4 group not only had increased number of pregnancies at the day 29 pregnancy diagnosis, but also had less susceptibility to pregnancy loss after this time. This was not due to P4 after AI because circulating P4 concentrations after AI were actually somewhat higher in the cows that had low P4 prior to AI (2.9 ng/ml) compared to the cows with high P4 prior to AI (2.5 ng/ml). Thus, there was a positive effect of elevated P4 prior to AI on subsequent maintenance of pregnancy even after 29 days following AI.

Studies by Bisinotto *et al*. (2010) also demonstrate the importance of higher P4 during the growth of the final follicular wave. Two studies were performed in which the effect of P4 during the Ovsynch protocol was evaluated. In the first study, cows were evaluated for P4 concentration at the time of the first

GnRH of Ovsynch and 7 days prior to this first GnRH. Cows were classified as anovular, or as beginning Ovsynch with high P4 or low P4. Cows that began Ovsynch with high P4 had greater pregnancies per AI (P/AI = 43.0%) than cycling cows that had low P4 (31.3%) or that were anovular (29.7%) at the time of initiation of Ovsynch (Bisinotto *et al*., 2010). In the second study, cows were presynchronized with two PGF treatments and Ovsynch was initiated either 3 days or 10 days after the second PGF. This design would produce cows that ovulate a dominant follicle from either the first or second follicular wave near the timed AI. Similar to the first study, cows that ovulated the second follicular wave in which P4 concentrations are higher had greater P/AI that cows ovulating the first follicular wave that grew during low P4 concentrations (41.7 *vs.* 30.4%; Bisinotto *et al*., 2010). Thus, it seems clear that increasing P4 during growth of the follicular wave increases fertility more than 10% to the subsequent timed AI. The mechanisms that produce this increase in fertility are still being investigated.

An elegant study in superstimulated cows (Rivera *et al.*, 2011) showed that high P4 concentrations during superstimulation increased the subsequent quality of embryos flushed on day 7 after superovulation. Cows began superstimulation during the second follicular wave with high P4, during the first follicular wave with low P4, or during the first follicular wave with P4 supplementation using 2 CIDRs to increase P4 concentrations. Although, total number of embryos/oocytes that were collected was not different between groups, the percentage of structures that were transferable embryos was much less for cows superstimulated during the first follicular wave (55.9%), than during the second follicular wave (88.5%) or during the first follicular wave with P4 supplementation (78.6%). This result is consistent with a positive effect of elevated P4 during follicle growth on embryo development prior to Day 7 of the estrous cycle. It is postulated that effects of P4 during growth of the follicles may allow production of a better oocyte for subsequent fertilization and embryo development (Rivera *et al*., 2011). However, a recent study that flushed embryos from single ovulating cows that had follicles growing during low or high P4 did not find a difference in embryo quality on day 7 (58.3 *vs.* 53.3%; Cerri *et al*., 2011b). A companion study (Cerri *et al.*, 2011a) indicated that although cows with low P4 had increased basal LH concentrations and altered follicular dynamics and follicular fluid composition that could alter oocyte quality, a particularly distinct difference in cows with low P4 was the premature development of pathways leading to uterine PGF secretion. Thus, altered uterine function could also have an important role in reducing fertility in cows that have low P4 concentrations prior to AI. Our laboratory (Wiltbank *et al*., unpublished) has recently completed a study evaluating day 7 embryo quality in single ovulating cows with follicle development occurring in low *vs.* high P4. We found a greater percentage of grade 1 and 2 embryos from cows with high P4 than low P4 prior to AI (86.5 *vs*. 61.5%; $P < 0.02$). Thus, these results are consistent with the idea that low P4 during follicle growth reduces fertility and this fertility decrease is evident in embryo quality at day 7 after AI. This reduction in embryo quality has been observed in single-ovulating cows (our study) and in superovulated cows (Rivera et al., 2011).

At this time it is not possible to provide a definitive explanation for the reason that high P4 prior to AI produces greater fertility. Nevertheless, the fertility enhancing effects are dramatic and consistent in lactating dairy cows. Future studies are needed to define the mechanism for this effect and to design reproductive management programs that optimize these fertility-enhancing effects.

Importance of low progesterone near time of AI

Inadequate luteolysis can result in an elevation in circulating P4 near AI and a reduction in fertility. This is clearly a problem with some cows during timed AI programs (Souza *et al.*, 2007; Brusveen *et al.*, 2008) but also may be a problem in AI programs based on detection of estrus. Studies on cows that receive AI based on estrus, have generally reported that minor elevations in P4 near AI are 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 CL 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.*, 2011). A recent extensive study of incomplete luteolysis evaluated multiple blood samples in cows at first AI (n = 652) and second or greater AI (n = 394; Martins *et al*., 2011). 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 the second AI only 71% underwent regression. 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%).

To study whether P4 near AI had an effect on fertility during timed AI protocols our laboratory (Souza *et al*., 2007; Brusveen *et al*., 2009) used lactating cows synchronized with Ovsynch on three commercial dairy herds. We collected blood samples for P4 measurement near the time of the last GnRH of Ovsynch. Both studies supported the concept that greater circulating P4 near AI decreases fertility. This decrease was observed even when only cows that ovulated to the second GnRH were evaluated for fertility. Combining the data from both studies (Fig. 2) it is clear that there is a dramatic decrease

in pregnancy per AI (P/AI) as P4 increases above 0.4 to 0.5 ng/ml near the time of the second GnRH of Ovsynch. The decrease that we observed was similar to the decrease described in a pioneering study (De Silva *et al*., 1981) using visual detection of estrus in lactating cows and heifers.

There may be multiple physiological mechanisms that result in the reduced fertility when P4 is elevated near AI. First, P4 may alter sperm or oocyte transport by altering uterine or oviductal contractility and thus reduce fertilization (Hunter, 2005). Second, addition of P4 to in vitro fertilization (IVF) media reduced blastocyst rate (Silva and Knight, 2000) suggesting that there may be direct effects of P4 during IVF on subsequent embryo development. The detrimental effect of added P4 was reversed by treatment with the P4 antagonist, RU-486, suggesting a role for P4 receptors in this action. Elevated P4 in vitro also increased total α-inhibin production by the cumulus-oocyte complex, which may reduce embryo development after cleavage (Silva *et al.*, 1999). Further, the reduced endometrial thickness with slight elevations in P4 (Souza *et al.*, 2011) may indicate other major effects of P4 on the uterus that could result in reduced embryo development.

In summary, high circulating concentrations of P4 near AI has been shown by a number of large field studies to be detrimental to fertility in dairy cattle, but the underlying physiological mechanisms that reduce fertility are not well understood. Further, the causes of elevated P4 near AI probably differ for cows bred after natural estrus compared to cows bred following timed AI protocols such as Ovsynch. However, an additional PGF treatment in cows receiving Ovsynch and more precise detection of estrus may help to minimize percentage of cows having somewhat greater circulating P4 near AI.

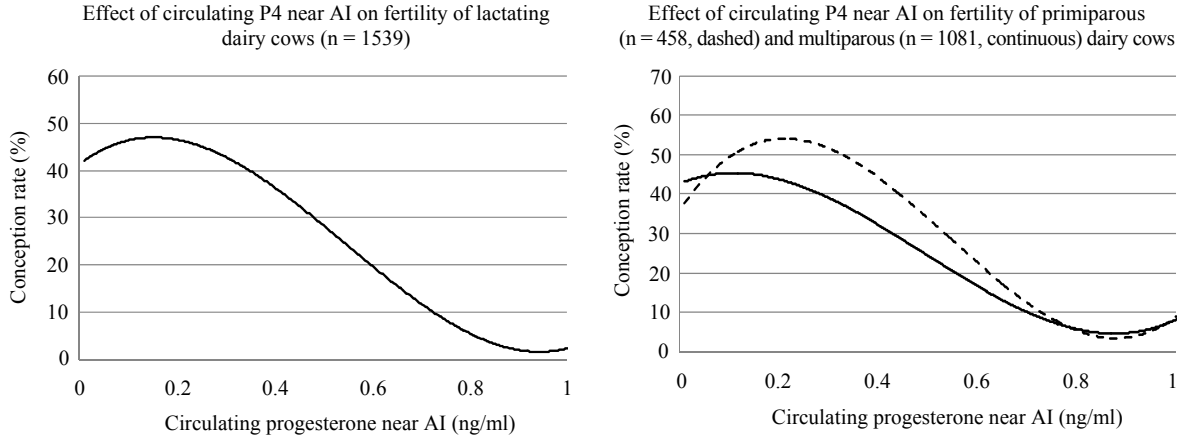

Figure 2. Relationship between circulating P4 near AI and conception results in lactating dairy cows receiving Ovsynch (data from Souza *et al*., 2007 and Brusveen *et al*., 2009 combined).

Importance of high progesterone after AI

The role of the "CL hormone" after ovulation in maintaining pregnancy was described more than 100 yr ago and was recognized well before the chemical structure of P4 was identified (Fraenkel and Cohn, 1901; Magnus, 1901). Recent studies continue to characterize the effects of P4 in inducing the dramatic changes in the uterus, termed progestational changes, that are essential for inducing an environment compatible with embryo growth, implantation, and maintenance of pregnancy (Arck *et al*., 2007).

Although there is unequivocal evidence that an absolute requirement for P4 is needed for pregnancy maintenance, results have been somewhat more equivocal about the relationship between concentrations of circulating P4 after AI and fertility in lactating dairy cows. A number of studies have reported lower P4 in non-pregnant than pregnant cows, whereas, other studies reported no relationship between post-AI P4 and fertility (Bulman and Lamming, 1978; Larson *et al.*, 1997; Mann and Lamming, 1999; Gumen *et al*., 2003; Stronge *et al.*, 2005; Lonergan *et al.*, 2007; Morris and Diskin, 2008). More extensive modeling of P4 concentrations with pregnancy using logistic regression have demonstrated a relationship between circulating P4 on days 5, 6, and 7 after AI with P/AI in dairy cows and a relationship between rate of P4 increase and P/AI (Stronge *et al*., 2005). They reported that 60-85% of dairy cows had sub-optimal circulating P4 for pregnancy, based on absolute P4 concentrations during the early luteal phase or rate of P4 increase (Stronge *et al*., 2005). Many recent studies have attempted to unravel the mechanisms involved in the complex relationship between circulating P4 concentrations and fertility in lactating dairy cows.

Early embryos express different types and concentrations of P4 receptors (Clemente *et al.*, 2009)

raising the possibility that P4 may be acting directly on the embryo to improve embryo development. An elegant series of experiments found no effect of P4 supplementation, in vitro, on blastocyst yield, in the presence or absence of bovine oviductal epithelial cells (Clemente *et al*., 2009). Thus, it appears that development of the early embryo is not directly altered by treatment with P4. In a surprising twist, these researchers treated recipient cows with a intravaginal P4-releasing device (PRID) starting on day 3 after estrus with in vitro produced blastocysts transferred on day 7. Circulating P4 concentrations were elevated in the recipient cows from days 3 to 6 but were not elevated in treated cows after that time. Thus, the rise in P4 concentrations in treated cows occurred prior to the transfer of embryos. Nevertheless, embryos that were transferred into recipients that had received prior P4 exposure from day 3 to 6 with increased P4, were much longer and the embryos had a larger surface area on day 14 than embryos transferred to unsuplemented recipients. (Clemente *et al*., 2009). The authors conclude that "P4-induced changes in the uterine environment are responsible for the advancement in conceptus elongation reported previously in cattle and that, interestingly, the embryo does not need to be present during the period of high P4 in order to exhibit advanced elongation" (Clemente *et al*., 2009). These results are consistent with the studies of Larson *et al.* (2011) that also failed to find a direct effect of P4 during either days 1 to 3 or days 4 to 7 of culture on percentage of embryos that developed to the morula or blastocyst stage; although small differences in glucose metabolism were observed. Further evidence for a lack of P4 effect in early embryo is found in the studies of Carter *et al*. (2008, 2010). In the first experiment, 210 crossbred beef heifers were used to analyze the effects of in vivo supplementation with P4 on embryo development. These researchers observed no difference in early embryo development by day 5 or 7 after AI, however dramatic effects of P4 supplementation on embryonic length could be observed on days 13 and 16 after AI (Carter *et al*., 2008). In an elegant study that continued this research focus (Carter *et al*., 2010), *in vitro* produced embryos were transferred to the oviduct of beef heifers that received or did not receive a PRID on day 3 after estrus. There was no detectable effect of P4 on the proportion of embryos that developed to the blastocyst stage by day 7 when embryos were recovered or during subsequent culture of the embryos in vitro. However, there were subtle but intriguing differences in gene expression, detected by microarray, in the embryos recovered from recipients that received P4 supplementation (Carter *et al*., 2010). Thus, it seems clear that increased P4 during days 3 to 7 induces changes in the uterus that increase embryo elongation by day 14. Whether a P4-induced increase in embryo development can improve fertility in lactating dairy cows continues to be an area of investigation, as discussed below.

Many studies have investigated the P4-induced changes in gene expression that occur in the endometrial tissue and these will not be extensively reviewed in this manuscript. However, it seems clear that there are dramatic differences in endometrial gene expression as the luteal phase progresses and that early supplementation with P4 can induce earlier expression of this P4 program (McNeill *et al.*, 2006; Forde *et al.*, 2008, 2009, 2011). The P4-induced changes in uterine gene expression can have dramatic consequences for the development of the embryos (Forde *et al*., 2011). There have been numerous studies that have evaluated the effects of P4 supplementation on fertility in cattle with the earliest experiments conducted in the 1950s (Herrick, 1953; Wiltbank *et al.*, 1956). Throughout the last 60 yr (Mann and Lamming, 1999), there have been numerous methods to increase P4 including: treatment with exogenous P4 (injectable P4; P4 releasing intravaginal device, PRID; or controlled internal drug release, CIDR) or by treatments attempting to ovulate a follicle and produce an accessory CL using human chorionic gonadotropin (hCG) or gonadotropin releasing hormone (GnRH). These experiments have varied considerably in regard to type of animal (beef *vs.* dairy, heifers *vs.* cows), supplementation/administration day relative to AI, utilization of synchronization prior to AI, and number of animals in the trial. Of the 30 trials that we evaluated, the vast majority (25/30) showed a numeric improvement in fertility with P4 supplementation, although only six of these trials showed significance ($P < 0.05$). Of these six trials, only one (Stevenson *et al.*, 2007) used greater than 100 animals per comparison.

The most extensive trials to increase P4 have been done by inducing formation of an accessory CL with hCG or GnRH treatment. When hCG or GnRH is administered on day 5 after AI, there is generally formation of an accessory CL and increase in P4 by day 9. Previous work has demonstrated that ovulation occurs most often when GnRH is administered between days 5 and 12 of the estrous cycle (Vasconcelos *et al.*, 1999). Our large unpublished trial using hCG treatment on day 5 produced only a 3.5% increase in fertility. Although this result was statistically significant, it was not of a large magnitude. Combining all of the results for hCG indicated a slight improvement in fertility from 38.1% (1511/3963) in control cows to 41.6% (1616/3884) in hCG-treated cows (+3.5%). This result indicates that there is some improvement due to the hCG treatment, however, it is unclear why the effect is not of a larger magnitude. It seems possible that formation of the CL and the increase in P4 following

hCG or GnRH administration is not sufficiently early in the cycle to induce the uterine changes that are needed to optimize fertility. Further research may clarify and improve on these results.

Conclusions

This manuscript has attempted to describe the underlying physiology that produces the changes in circulating P4 in lactating dairy cows and the potential reproductive challenges associated with suboptimal P4 concentrations. Metabolism of P4 appears to be the primary cause of lowered P4 in lactating dairy cows, although changes in P4 production by the CL have not yet been experimentally excluded. This manuscript reviewed the scientific literature on P4 and fertility with clear evidence for effects of P4 at all three time periods that were analyzed. Prior to AI, there were very dramatic effects observed with more than 10% units difference in P/AI observed by increasing P4 concentrations. The lactating dairy cow may have insufficient P4 during this time period and insufficient P4 at this time may, at least partially, underlie the high incidence of double ovulations and low fertility that is characteristic of high-producing dairy cows. Near the time of AI, it is critical that P4 concentrations are below a critical value, which appears to be about 0.4 ng/ml during Ovsynch-type protocols in which ovulation before FTAI is induced with GnRH. Even small increases in P4 near the time of AI were associated with dramatic reductions in fertility, either in cows bred to natural estrus or after timed AI protocols. Following AI, there are dramatic effects of increasing P4 on embryo elongation, however, these dramatic effects have generally not been observed in field trials focused on improving fertility by supplementing P4 after AI. Thus, although substantial research has investigated the role of P4 on fertility in lactating dairy cows for more than 6 decades, it seems clear that future focus in this research area is likely to continue to yield exciting research results related to the physiology and practical management of dairy cows.

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References

Allen W. 1974. Recollections of my life with progesterone. *Gynecol Invest*, 5:142-182.

Arck P, Hansen PJ, Jericevic BM, Piccinni MP, Szekeres-Bartho J. 2007. Progesterone during pregnancy: endocrine-immune cross talk in mammalian species and the role of stress. *Am J Reprod Immunol*, 58:268-279.

Bedford CA, Harrison FA, Heap RB. 1974. Splanchnic, ovarian and adrenal uptake of progesterone and 20-alpha-dihydroprogesterone in pregnant and nonpregnant sheep. *J Endocrinol*, 62:277-290.

Bisinotto RS, Chebel RC, Santos JEP. 2010. Follicular wave of the ovulatory follicle and not cyclic status influences fertility of dairy cows. *J Dairy Sci*, 93:3578-3587.

Brusveen DJ, Cunha AP, Silva CD, Cunha PM, Sterry RA, Silva EP, Guenther JN, Wiltbank MC. 2008. Altering the time of the second gonadotropin-releasing hormone injection and artificial insemination (AI) during Ovsynch affects pregnancies per AI in lactating dairy cows. *J Dairy Sci*, 91:1044-1052.

Brusveen DJ, Souza AH, Wiltbank MC. 2009. Effects of additional prostaglandin F-2 alpha and estradiol-17 beta during Ovsynch in lactating dairy cows. *J Dairy Sci*, 92:1412-1422.

Bulman DC, Lamming GE. 1978. Milk progesterone levels in relation to conception, repeat breeding and factors influencing acyclicity in dairy cows. *J Reprod Fertil*, 54:447-458.

Carter F, Forde N, Duffy P, Wade M, Fair T, Crowe MA, Evans ACO, Kenny DA, Roche JF, Lonergan P. 2008. Effect of increasing progesterone concentration from day 3 of pregnancy on subsequent embryo survival and development in beef heifers. *Reprod Fertil Dev*, 20:368-375.

Carter F, Rings F, Mamo S, Holker M, Kuzmany A, Besenfelder U, Havlicek V, Mehta JP, Tesfaye D, Schellander K, Lonergan P. 2010. Effect of elevated circulating progesterone concentration on bovine blastocyst development and global transcriptome following endoscopic transfer of in vitro produced embryos to the bovine oviduct. *Biol Reprod*, 83:707-719.

Cerri RLA, Chebel RC, Rivera F, Narciso CD, Oliveira RA, Amstalden M, Baez-Sandoval GM, Oliveira LJ, Thatcher WW, Santos JEP. 2011a. Concentration of progesterone during the development of the ovulatory follicle. II. Ovarian and uterine responses. *J Dairy Sci*, 94:3352-3365.

Cerri RLA, Chebel RC, Rivera F, Narciso CD, Oliveira RA, Thatcher WW, Santos JEP. 2011b. Concentration of progesterone during the development of the ovulatory follicle. I. Ovarian and embryonic responses. *J Dairy Sci*, 94:3342-3351.

Chagas LM, Bass JJ, Blache D, Burke CR, Kay JK, Lindsay DR, LucyMC, Martin GB, Meier S, Rhodes FM, Roche JR, Thatcher WW, Webb R. 2007. New perspectives on the roles of nutrition and metabolic priorities in the subfertility of high-producing dairy cows. *J Dairy Sci*, 90:4022-4032.

Chantilis S, Dombroski R, Shackleton CHL, Casey ML, MacDonald PC. 1996. Metabolism of 5 alpha-dihydroprogesterone in women and men: 3 beta- and 3 alpha-,6 alpha-dihydroxy-5 alpha-pregnan-20-ones are major urinary metabolites. *J Clin Endocrinol Metab*, 81:3644-3649.

Chebel RC, Al-Hassan MJ, Fricke PM, Santos JEP, Lima JR, Martel CA, Stevenson JS, Garcia R, Ax RL. 2010. Supplementation of progesterone via controlled internal drug release inserts during ovulation synchronization protocols in lactating dairy cows. *J Dairy Sci*, 93:922-931.

Christenson RK, Ford JJ, Redmer DA. 1985. Metabolic-clearance and production-rates of estradiol and progesterone during pubertal and postpubertal development in gilts. *J Reprod Fertil*, 75:247-253.

Clemente M, de la Fuente J, Fair T, Al Naib A, Gutierrez-Adan A, Roche JF, Rizos D, Lonergan P. 2009. Progesterone and conceptus elongation in cattle: a direct effect on the embryo or an indirect effect via the endometrium? *Reproduction*, 138:507-517.

Corner G, Allen WM. 1929. Physiology of the corpus luteum. II. Production of a special uterine reaction (progestational proliferation) by extracts of the corpus luteum. *Am J Physiol*, 88:326-399.

Cunha AP, Guenther JN, Maroney MJ, Giordano JO, Nascimento AB, Bas S, Ayres H, Wiltbank MC. 2008. Effects of high *vs.* low progesterone concentrations during Ovsynch on double ovulation rate and pregnancies per AI in high producing dairy cows. *J Dairy Sci*, 91(suppl 1):246. (abstract).

De Silva AWMV, Anderson GW, Gwazdauskas FC, McGilliard ML, Lineweaver JA. 1981. Interrelationships with estrous behavior and conception in dairy-cattle. *J Dairy Sci*, 64:2409-2418.

del Rio NS, Kirkpatrick BW, Fricke PM. 2006. Observed frequency of monozygotic twinning in Holstein dairy cattle. *Theriogenology*, 66:1292-1299.

Diaz FJ, Anderson LE, Wu YL, Rabot A, Tsai SJ, Wiltbank MC. 2002. Regulation of progesterone and prostaglandin F-2 alpha production in the CL. *Mol Cell Endocrinol*, 191:65-80.

Ellmann S, Sticht H, Thiel F, Beckmann MW, Strick R, Strissel PL. 2009. Estrogen and progesterone receptors: from molecular structures to clinical targets. *Cell Mol Life Sci*, 66:2405-2426.

Erb RE, Garverick HA, Randel RD, Brown BL, Callahan CJ. 1976. Profiles of reproductive hormones associated with fertile and nonfertile inseminations of dairy cows. *Theriogenology*, 5:227-242.

Fernandez-Valdivia R, Mukherjee A, Mulac-Jericevic B, Conneely OM, DeMayo FJ, Amato P, Lydon JP. 2005. Revealing progesterone's role in uterine and mammary gland biology: insights from the mouse. *Semin Reprod Med*, 23:22-37.

Fonseca FA, Britt JH, Mcdaniel BT, Wilk JC, Rakes AH. 1983. Reproductive traits of holsteins and jerseys - effects of age, milk-yield, and clinical abnormalities on involution of cervix and uterus, ovulation, estrous cycles, detection of estrus, conception rate, and days open. *J Dairy Sci*, 66:1128-1147.

Forde N, Carter F, Fair T, Crowe M, Evans A, Spencer T, Bazer F, O'Gaora P, McBride R, Boland M, Lonergan P, Roche J. 2008. Effect of pregnancy and progesterone on gene expression in the uterine endometrium of cattle. *Biol Reprod*, 60-61.

Forde N, Carter F, Fair T, Crowe MA, Evans ACO, Spencer TE, Bazer FW, McBride R, Boland MP, O'Gaora P, Lonergan P, Roche JF. 2009. Progesterone-regulated changes in endometrial gene expression contribute to advanced conceptus development in cattle. *Biol Reprod*, 81:784-794.

Forde N, Beltman ME, Duffy GB, Duffy P, Mehta JP, O'Gaora P, Roche JF, Lonergan P, Crowe MA. 2011. Changes in the endometrial transcriptome during the bovine estrous cycle: effect of low circulating progesterone and consequences for conceptus elongation. *Biol Reprod*, 84:266-278.

Fraenkel L, Cohn F. 1901. Experimentelle untersuchungen des corpus luteum auf die insertion des eies (Theorie von Born). *Anat Anz*, 20:294-300.

Freetly HC, Ferrell CL. 1994. Net uptakes of estradiol-17-beta and progesterone across the portal-drained viscera and the liver of ewes. *J Endocrinol*, 141:353-358.

Fricke PM, Wiltbank MC. 1999. Effect of milk production on the incidence of double ovulation in dairy cows. *Theriogenology*, 52:1133-1143.

Ghanem ME, Nakao T, Nakatani K, Akita M, Suzuki T. 2006. Milk progesterone profile at and after artificial insemination in repeat-breeding cows: effects on conception rate and embryonic death. *Reprod Domest Anim*, 41:180-183.

Gumen A, Guenther JN, Wiltbank MC. 2003. Follicular size and response to Ovsynch versus detection of estrus in anovular and ovular lactating dairy cows. *J Dairy Sci*, 86:3184-3194.

Herrick JB. 1953. Clinical observation of progesterone therapy in repeat breeding heifers. *Vet Med*, 48:489-490.

Hunter RHF. 2005. The Fallopian tubes in domestic mammals: how vital is their physiological activity? *Reprod Nutr Dev*, 45:281-290.

Kinsel ML, Marsh WE, Ruegg PL, Etherington WG. 1998. Risk factors for twinning in dairy cows. *J Dairy Sci*, 81:989-993.

Larson JE, Krisher RL, Lamb GC. 2011. Effects of supplemental progesterone on the development, metabolism and blastocyst cell number of bovine embryos produced in vitro. *Reprod Fertil Dev*, 23:311-318.

Larson SF, Butler WR, Currie WB. 1997. Reduced fertility associated with low progesterone postbreeding and increased milk urea nitrogen in lactating cows. *J Dairy Sci*, 80:1288-1295.

Lemley CO, Butler ST, Butler WR, Wilson ME. 2008. Short communication: insulin alters hepatic progesterone catabolic enzymes cytochrome P4502C and 3A in dairy cows. *J Dairy Sci*, 91:641-645.

Lemley CO, Vonnahme KA, Tager LR, Krause KM, Wilson ME. 2010a. Diet-induced alterations in hepatic progesterone (P(4)) catabolic enzyme activity and P(4) clearance rate in lactating dairy cows. *J Endocrinol*,

205:233-241.

Lemley CO, Wilmoth TA, Tager LR, Krause KM, Wilson ME. 2010b. Effect of a high cornstarch diet on hepatic cytochrome P450 2C and 3A activity and progesterone half-life in dairy cows. *J Dairy Sci*, 93:1012-1021.

Lonergan P, Woods A, Fair T, Carter F, Rizos D, Ward F, Quinn K, Evans A. 2007. Effect of embryo source and recipient progesterone environment on embryo development in cattle. *Reprod Fertil Dev*, 19:861-868.

Lopez H, Caraviello DZ, Satter LD, Fricke PM, Wiltbank MC. 2005a. Relationship between level of milk production and multiple ovulations in lactating dairy cows. *J Dairy Sci*, 88:2783-2793.

Lopez H, Sartori R, Wiltbank MC. 2005b. Reproductive hormones and follicular growth during development of one or multiple dominant follicles in cattle. *Biol Reprod*, 72:788-795.

Lucy MC. 2001. ADSA Foundation Scholar Award - Reproductive loss in high-producing dairy cattle: where will it end? *J Dairy Sci*, 84:1277-1293.

Magnus V. 1901. Ovariets betydning for svangerskabet med soerligt hensyn til corpus luteum. *Norsk Mag Loegevidensk* 62:1138-1142.

Mann GE, Lamming GE. 1999. The influence of progesterone during early pregnancy in cattle. *Reprod Domest Anim*, 34:269-274.

Martins JPN, Policelli RK, Neuder LM, Raphael W, Pursley JR. 2011. Effects of cloprostenol sodium at final prostaglandin F-2 alpha of Ovsynch on complete luteolysis and pregnancy per artificial insemination in lactating dairy cows. *J Dairy Sci*, 94:2815-2824.

McNeill RE, Sreenan JM, Diskin MG, Cairns MT, Fitzpatrick R, Smith TJ, Morris DG. 2006. Effect of systemic progesterone concentration on the expression of progesterone-responsive genes in the bovine endometrium during the early luteal phase. *Reprod Fertil Dev*, 18:573-583.

Moreira F, de la Sota RL, Diaz T, Thatcher WW. 2000. Effect of day of the estrous cycle at the initiation of a timed artificial insemination protocol on reproductive responses in dairy heifers. *J Anim Sci*, 78:1568-1576.

Morris D, Diskin M. 2008. Effect of progesterone on embryo survival. *Animal*, 2:1112-1119.

Murray M. 1991. Microsomal cytochrome-P450-dependent steroid metabolism in male sheep liver - Quantitative importance of 6-beta-hydroxylation and evidence for the involvement of a P450 from the IIA subfamily in the pathway. *J Steroid Biochem Mol Biol*, 38:611-619.

Murray M. 1992. Participation of a cytochrome P450 enzyme from the 2C subfamily in progesterone 21-hydroxylation in sheep liver. *J Steroid Biochem Mol Biol*, 43:591-593.

Niswender GD, Juengel JL, Mcguire WJ, Belfiore CJ, Wiltbank MC. 1994. Luteal function - the estrous-cycle and early-pregnancy. *Biol Reprod*, 50:239-247.

Niswender GD, Juengel JL, Silva PJ, Rollyson MK, McIntush EW. 2000. Mechanisms controlling the function and life span of the corpus luteum. *Physiol Rev*, 80:1-29.

Parr RA, Davis IF, Miles MA, Squires TJ. 1993. Liver blood-flow and metabolic-clearance rate of progesterone in sheep. *Res Vet Sci*, 55:311-316.

Peluso JJ. 2006. Multiplicity of progesterone's actions and receptors in the mammalian ovary. *Biol Reprod*, 75:2-8.

Piccinato CA, Sartori R, Sangsritavong S, Souza AH, Grummer RR, Luchini D, Wiltbank MC. 2010. In vitro and in vivo analysis of fatty acid effects on metabolism of 17 beta-estradiol and progesterone in dairy cows. *J Dairy Sci*, 93:1934-1943.

Plym Forshell K, Andersson L, Pehrson B. 1991. The relationships between the fertility of dairy cows and clinical and biochemical measurements, with special reference to plasma glucose and milk acetone. *Zentralbl Veterinarmed A*, 38:608-616.

Prime GR, Symonds HW. 1993. Influence of plane of nutrition on portal blood-flow and the metabolic-clearance rate of progesterone in ovariectomized gilts. *J Agric Sci*, 121:389-397.

Rivera FA, Mendonca LGD, Lopes G, Santos JEP, Perez RV, Amstalden M, Correa-Calderon A, Chebel RC. 2011. Reduced progesterone concentration during growth of the first follicular wave affects embryo quality but has no effect on embryo survival post transfer in lactating dairy cows. *Reproduction*, 141:333-342.

Sangsritavong S. 2002. *Studies of steroid metabolism in dairy cattle*. Madison, WI: University of Wisconsin. Dissertation.

Sangsritavong S, Combs DK, Sartori R, Armentano LE, Wiltbank MC. 2002. High feed intake increases liver blood flow and metabolism of progesterone and estradiol-17 beta in dairy cattle. *J Dairy Sci*, 85:2831-2842.

Sartori R, Bastos MR, Wiltbank MC. 2010. Factors affecting fertilisation and early embryo quality in single- and superovulated dairy cattle. *Reprod Fertil Dev*, 22:151-158.

Schwarzenberger F, Mostl E, Palme R, Bamberg E. 1996. Faecal steroid analysis for non-invasive monitoring of reproductive status in farm, wild and zoo animals. *Anim Reprod Sci*, 42:515-526.

Silva CC, Groome NP, Knight PG. 1999. Demonstration of a suppressive effect of inhibin alpha-subunit on the developmental competence of in vitro matured bovine oocytes. *J Reprod Fertil*, 115:381-388.

Silva CC, Knight PG. 2000. Effects of androgens, progesterone and their antagonists on the developmental competence of in vitro matured bovine oocytes. *J Reprod Fertil*, 119:261-269.

Smith DL, Stinefelt BM, Blemings KP, Wilson ME. 2006. Diet-induced alterations in progesterone clearance appear to be mediated by insulin signaling in hepatocytes. *J Anim Sci*, 84:1102-1109.

Souza AH, Gumen A, Silva EPB, Cunha AP, Guenther JN, Peto CM, Caraviello DZ, Wiltbank MC. 2007. Supplementation with estradiol-17 beta before the last gonadotropin-releasing hormone injection of the ovsynch protocol in lactating dairy cows. *J Dairy Sci*, 90:4623-4634.

Souza AH, Silva EPB, Cunha AP, Gumen A, Ayres H, Brusveen DJ, Guenther JN, Wiltbank MC. 2011. Ultrasonographic evaluation of endometrial thickness near timed AI as a predictor of fertility in high-producing dairy cows. *Theriogenology*, 75:722-733.

Stevenson JS, Pursley JR, Garverick HA, Fricke PM, Kesler DJ, Ottobre JS, Wiltbank MC. 2006. Treatment of cycling and noncycling lactating dairy cows with progesterone during Ovsynch. *J Dairy Sci*, 89:2567-2578.

Stevenson JS, Portaluppi MA, Tenhouse DE, Lloyd A, Eborn DR, Kacuba S, DeJarnette JM. 2007. Interventions after artificial insemination: Conception rates, pregnancy survival, and ovarian responses to gonadotropin-releasing hormone, human chorionic gonadotropin, and progesterone. *J Dairy Sci*, 90:331-340.

Stevenson JS, Tenhouse DE, Krisher RL, Lamb GC, Larson JE, Dahlen CR, Pursley JR, Bello NM, Fricke, PM, Wiltbank MC, Brusveen DJ, Burkhart M, Youngquist RS, Garverick HA. 2008. Detection of anovulation by heatmount detectors and transrectal ultrasonography before treatment with progesterone in a timed insemination protocol. *J Dairy Sci*, 91:2901-2915.

Stronge AJH, Sreenan JM, Diskin MG, Mee JF, Kenny DA, Morris DG. 2005. Post-insemination milk progesterone concentration and embryo survival in dairy cows. *Theriogenology*, 64:1212-1224.

Stupnick R, Williams KI. 1968. Urinary metabolites of 4-14C-progesterone in ewes. *Steroids*, 12:581-587.

Vasconcelos JLM, Silcox RW, Rosa GJM, Pursley JR, Wiltbank MC. 1999. Synchronization rate, size of the ovulatory follicle, and pregnancy rate after synchronization of ovulation beginning on different days of the estrous cycle in lactating dairy cows. *Theriogenology*, 52:1067-1078.

Waldmann A, Reksen O, Landsverk K, Kommisrud E, Dahl E, Refsdal A, Ropstad E. 2001. Progesterone concentrations in milk fat at first insemination - effects on non-return and repeat-breeding. *Anim Reprod Sci*, 65:33-41.

Wiltbank JN, Hawk HW, Kidder HE, Black WG, Ulberg LC, Casida LE. 1956. Effect of progesterone therapy on embryos survival in cows of lowered fertility. *J Dairy Sci*, 39:456-461.

Wiltbank MC, Fricke PM, Sangsritavong S, Sartori R, Ginther OJ. 2000. Mechanisms that prevent and produce double ovulations in dairy cattle. *J Dairy Sci*, 83:2998-3007.

Wiltbank M, Lopez H, Sartori R, Sangsritavong S, Gumen A. 2006. Changes in reproductive physiology of lactating dairy cows due to elevated steroid metabolism. *Theriogenology*, 65:17-29.

Wiltbank MC, Salih SM, Atli MO, Luo W, Bormann CL, Ottobre JS, Vezina CM, Mehta V, Diaz FJ, Tsai SJ, Sartori R. 2012. Comparison of endocrine and cellular mechanisms regulating the corpus luteum of primates and ruminants. *Anim Reprod*, 9:242-259.