The Effect of Progesterone Concentrations during Follicular Development in Cattle on Luteinizing Hormone Secretion, Follicular Development, Oocyte Competence and Fertility

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Graduate School of The Ohio State University

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2015

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Abstract

Synchronization programs aim to effectively create an ideal hormonal environment to successfully induce the development of a dominant follicle containing a healthy oocyte that is capable of being fertilized and developing to term. It has been repeatedly suggested that greater gonadotropin stimulation for a finite period during follicular development may enhance fertility in beef cattle. It was hypothesized that decreased progesterone (P4) concentrations during early follicular development would result in increased luteinizing hormone (LH) pulse frequency, leading to oocytes of greater developmental capacity, ultimately increasing pregnancy rate to AI in beef cattle.

In the first experiment, decreased P4 concentration during early follicular development and its effect on pregnancy rate to timed-AI and estrus-AI were investigated in multiparous beef cows in Ohio (Experiment 1.1) and Montana (Experiment 1.2). In both experiments, P4 concentrations at the end of P4 treatment were, as designed, greater in the high P4 (HiP4) than the low P4 (LoP4) treatment. The administration of prostaglandin F2α (PGF) to induce luteolysis prior to emergence of the new follicular wave, in conjunction with either insertion (Experiment 1.1) or maintenance (Experiment 1.2) of a previously used CIDR, resulted in decreased P4 concentrations during emergence and early development of the ovulatory follicle in the LoP4 treatment. It was demonstrated that decreased P4 concentrations during follicular development resulted in
greater estradiol (E2) concentrations and increased follicle diameter at termination of P4 treatment (Experiment 1.1), but did not influence pregnancy rates attained at the subsequent synchronized ovulation/estrus in beef cows (Experiments 1.1 and 1.2).

In the second experiment, the effect of P4 concentrations during early follicular development on LH secretion and characteristics of oocytes that reflect developmental competence were investigated in primiparous beef cattle. The animal model used was similar to Experiment 1.1. Concentrations of P4 were lesser and E2 were greater in the LoP4 than HiP4 treatment throughout follicular development. LH pulse frequency was greater in the LoP4 than HiP4 treatment, however, LH mean concentration and pulse amplitude did not differ between treatments. Among treatments, follicles aspirated per cow, total oocytes recovered per cow, recovery rate, percentage of oocytes graded 1 to 3, oocyte diameter, percentage of oocyte stained for BCB (BCB+), and the relative expression of oocyte mRNA for FST did not differ.

In conclusion, decreased P4 concentrations during early follicular development increased LH pulse frequency and enhanced E2 concentrations in both early antral and dominant follicles. However, the parameters used to investigate developmental competence in oocytes in the current experiment did not differ between HiP4 and LoP4 treatments. In addition, differences in P4 concentrations do not impact pregnancy rates in beef cows, when adequate intervals of proestrus are afforded.

**Keywords:** Progesterone, Used CIDR, Fertility, Beef Cows, LH, Oocyte Developmental Capacity
This work is dedicated to all those that help me make this journey come to the end!

Family, friends, and mentors.

In lovely memory of my aunt Nicinha.
Acknowledgements

I read once that “the man who moves a mountain begins by carrying away small stones” (Confucius). With that being said, I would like to really thank all the ones that help me carry all the stones (some small, and some big enough) that were on my away until now. Thank you mom and dad, thank you sisters, and thank you all my family! You were definitely essential for the first years of my life, for all the early accomplishments, all the stones carried, even carrying some stones for me sometimes!!! I am sure that you still are and will keep being more than fundamental to my life and success!! Love you all!! Leandro, thank you for the amazing, and crazy 10 years together and partners in crime! As painful as it is for me to assume (just kidding!), you were more than vital for many of the accomplishments I had until now; and although it feels good to your ego act as you don’t care, I know you are always on the first row cheering and hoping the best for me! Thank you for helping me with the stones on my way and holding yourself for not throwing any on me!! I know that I deserved it few times…… And as people use to say, the boyfriend does not come alone! And I could not be more thankful for that! Thank you Aguinaldo, thank you Denize, and thank you Gika for being my second family! You are priceless! Dr. Vasconcelos (Zequinha), thank you for not only teaching me how to deal with the unexpected stones that showed up so early in my professional carrier, but thank you also for keeping supporting me until now!! Thank you for the opportunity to get to
know your family, and be able to call them my friends now!! Carla, Gabi and Rafinha, thank you! Dr. Geary (Tom), thank you for giving me the chance to explore the stones that were, at somehow, out of my league!! Thank you for the unique opportunity offered in 2008!! That stone was definitely a fun and important one to carry! Thank you for being there for me since then!! Thank you Maggie, Hugh, Elle, Sam and Kevin! You made Montana feels like home for me!! Dr. da Silva (Marco), thank you for everything throughout these years in Columbus! Thank you for being a friend, thank you for being a family, thank you for accepting me as your student, and thank you for being my advisor!! Thank you for having such an awesome family and letting them be my family too!!! Tati, Arthur and Lala, thank you for the comfort during these years!! Dr. Day (Mike), thank you for everything I am and achieved! Thank you for the, at first glance, risked opportunity given in 2009!! At that point you didn’t know me, and wasn’t sure if that could be or not a good idea….. I hope I proved to you that it was!!! Thank you for being on my side every time I had trouble with some stones. Thank you for teaching me how to deal with any type of stone that showed up on my way. And more important, thank you for even carry some of the “heavy” stones for me! I have no words to express all the gratitude I have for all the years I was under your mentoring, and for all the ones that are still to come! Or you thought you were done with me?!??!! Thank you for supporting me throughout my entire journey!!! Thank you for sharing your lovely family with me! Thank you for being my American family! Thank you Toni, Trevis, Leslie and Miss Nancy!! Thank you, thank you, thank you!!! Thank you Dr. Bridges, Dr. Schuenemann, and Dr. Paivi for being in my committee, for helping every time I needed, and for being
understandable when requested!! You were definitely important pieces to successfully solve this puzzle!!! Thank you Lucas, Martin, Matt and Bo for being such great lab mates and friends throughout these years!! Thank you for keeping the insanity of the third floor, for having fun with any stone carried, even when it looks like an avalanche!!! Thank you Luciana, Ronaldo, Felipe and Rafaela. Thank you Santiago, Valeria and Pedro. Thank you Bruno and Rosangela. Thank you Aclecia and Bruna. Thank you Rafitcha and Gabi!! You were all essential for all these years in Columbus! Thank you for being more than friends! Thank you for being a family!!! Thank you all at THE OSU for all the support throughout these years, especially Marty and Gregg!!! No words would be enough to express how thankful I am!!!! Thank you very much!!!

“And my heart is sinking like a setting sun, setting on the things I wish I'd done. Oh the last goodbye's the hardest one to say, and this is where the cowgirl rides away”.

(George Strait)
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PUBLICATIONS

Journal Articles


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Abstracts


cycling heifers. In: XIX Congresso de Iniciação Científica, Ilha Solteira, SP, Brasil


FIELDS OF STUDY

Major Field: Animal Sciences

Reproductive Physiology
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CHAPTER 1
LITERATURE REVIEW

1.1 Introduction

With the estimation of the world population to be approximately nine billion in 2050 (Dahlen et al., 2013), the Food and Agriculture Organization of the United Nations (2009) reported the necessity to double food supply in the next 40 years. As a quest to increase beef production, one of the primary source of dietary protein for humans, various reproductive technologies to control the reproductive cycle of cattle have been developed. Application of these technologies has been demonstrated to improve reproductive efficiency of cattle. Furthermore, these technologies promote the use of artificial insemination (AI) in beef cattle which, in itself, through its impact on genetic improvement, is the most powerful tool available to increase overall production efficiency. Gains from combination of these technologies continue to accelerate with time as a consequence of the large investment and discoveries in cattle reproductive physiology research in the last decades. However, there are many physiology questions that are not presently understood for which understanding could hasten the improvement in efficiency of reproduction of this species. This review addresses important topics related to the reproductive physiology in beef cattle, aiming to provide an extensive background that connects to the research contained within this dissertation.
1.2 The hormone Progesterone

Progesterone (P4) is a steroid hormone that was first isolated from the corpus luteum (CL) in rabbits in 1929 (Corner and Allen, 1929). At that time, it was identified as a substance capable of transforming the endometrium with characteristics of early pregnancy (e.g. proliferation of the endometrium), and was at that time named “progestin” (Allen, 1930). After its discovery, many investigations were conducted in order to isolate the “new” hormone (Butenandt and Westphal, 1934; Slotta et al., 1934; Hartmann and Wettstein, 1934; Wintersteiner and Allen, 1934). Finally, in 1935, at the Health Organization of the League of Nations Conference, a consensus was reached to use the name “progesterone” for the CL’s hormone in scientific literature.

As all steroid hormones, P4 is derived from cholesterol, which is hydrolyzed to free cholesterol in the liver and later transported, as a lipoprotein, to all steroidogenic tissues such as adrenal cortex, ovarian follicles, CL, and placenta (when applicable; Salles and Araujo, 2010). Once in these tissues, cholesterol is transported from the cytoplasm to the mitochondria and then, through the action of the enzyme cytochrome P450 side-chain cleavage (P450scc), it is converted into pregnenolone. Later, pregnenolone is converted into P4 by the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) in the endoplasmic reticulum, and immediately released in the blood stream after its synthesis.

In cattle, the main source of P4, in a non-pregnant female is the CL that is present during the luteal phase of the estrous cycle. The luteal phase is characterized by the formation of the CL from the just ovulated dominant follicle, resulting in increased
peripheral P4 that is synthesized by the luteinized granulosa and theca cells of the ovulatory follicle (Niswender, 1981); designated as large and small luteal cells, respectively (Ursely and Leymarie, 1979; Koos and Hansel, 1981; Alila and Hansel, 1984; Chegini et al, 1984; Rodgers et al, 1986; Weber et al, 1987). Approximately 4 days after ovulation, P4 concentration starts to rise gradually, reaching its highest values between days 14 and 16 of the estrous cycle (Erb and Stormshak, 1961; Mares et al., 1932; Gomes et al., 1963).

During diestrus, P4 acts by negatively controlling the release of gonadotropin release hormone (GnRH) in the hypothalamus, and directly affecting luteinizing hormone (LH) pulse frequency release by the pituitary gland (Kinder et al., 1996). As a consequence, growth and development of the dominant follicle (Taft et al., 1996) will also be negatively related to P4 concentrations in circulation. Moreover, the sustained high concentration of P4 during the luteal phase prepares the endometrium to sustain and to recognize the pregnancy (Vincent and Inskeep, 1986; Vincent et al., 1986), by altering the pattern of genes expression in the uterus (Forde et al., 2009), as well as inhibiting mitosis (Padykula et al., 1989) and stimulating glandular secretion in the endometrium (Maslar et al., 1986).

Around d 16 of the estrous cycle in the absence of a conceptus or if the conceptus fails to induce the maternal recognition of pregnancy, luteolysis (regression of the CL) is induced by prostaglandin F2α (PGF) that is secreted by the uterine endometrium (Shemesh et al., 1968; Stabenfeldt et al., 1969, Pope et al., 1969; Donaldson et al., 1970, Lauderdale, 1972; Hixon and Hansel, 1974; Goding, 1974; Horton and Poyser, 1976;
On approximately d 17, P4 concentration decreases sharply and remains at basal levels (0.1-0.4 ng/ml; Robertson, 1972) until ovulation of the newly formed dominant follicle and formation of a new CL (Dobrowolski et al., 1968). However, if a conceptus is present the early embryo secretes interferon tau that inhibits the endometrial cells to synthesize receptors of oxytocin. This action inhibits PGF release by the endometrium, which enables the CL to maintain P4 secretion, consequently maintaining the pregnancy (Bazer et al., 2012).

1.3 The role of Progesterone in regulating Luteinizing Hormone secretion

LH and P4 are two important hormones that regulate the estrous cycle in cattle. The P4 secreted by the CL, acts on the hypothalamus via the integration between endocrine and neural systems (Skinner et al., 1998) to modulate GnRH secretion. The hormone GnRH is synthesized by neurons located in the hypothalamus and upon release into the hypophyseal portal circulation, reaches the gonadotropes located in the anterior pituitary to regulate the release of LH and follicle stimulating hormone (FSH; Conn et al., 1986; Fink et al., 1988). Philips (2005) demonstrated that while LH pulse frequency is intimately related to GnRH pattern release, FSH secretion relies more on the stimulation or inhibition by estradiol (E2), inhibin, activin and follistatin. Throughout the estrous cycle, the pulse frequency of GnRH changes according to the circulating concentrations of steroid hormones (P4 and E2; Karsch et al., 1987). During the luteal phase, P4 concentrations are elevated, which results in fewer pulses of GnRH (0 to 2 pulses/6 h; Barrel et al., 1992; Clarke, 1995) that results in less frequent release of LH
pulses from the anterior pituitary (6-8 pulses/24 h; Rahe et al., 1980). On the hand, throughout follicular phase, P4 concentrations are low and E2 levels are high, resulting in increased pulse frequency of GnRH (Moenter et al., 1991; Clarke, 1995) up to 8 pulses/6 h (Barrel et al., 1992). Consequently, the increased frequency of GnRH release stimulates LH pulses (20-30 pulses/24 h; Rahe et al., 1980). In turn, LH stimulates follicular growth and maturation, and E2 secretion (Dalkin et al., 1989; Motler-Gerard et al., 1999).

The rise in E2 secretion is due greater frequency of LH pulses enabling a greater amount of substrates (androgens) by the theca cells for E2 production in the granulosa cells; mainly by the dominant follicle (Sirois and Fortune, 1990). The increased androgen and E2 production results from the “two-cell two-gonadotropin theory” in which LH receptors present in the theca cells, once bound to LH, triggers androgen synthesis; while FSH binds to its receptors in the granulosa cells, inducing aromatase expression, consequently augmenting the conversion of androgen to estrogens (Hiller et al., 1980).

1.4 Follicular Development

Follicular growth during the bovine estrous cycle occurs in a wave-like pattern (Rajakoski, 1960; Pierson and Ginther, 1987) characterized commonly by the development of two or three follicular waves during each estrous cycle (Sirois and Fortune, 1988; Savio et al., 1988; Knopf et al., 1989; Adams et al., 1992; Bo et al., 1994; Binelli et al., 2001). Each wave of follicular growth is divided into 4 stages of development: recruitment, selection, deviation, and dominance, that progress continuously (Peters et al., 1975; Diskin et al., 2002).
Preceding the emergence of each wave of follicular development, there is an increase in the concentration of FSH (Adams et al., 1992; Ginther et al., 2000), resulting in the recruitment of a cohort of small antral follicles (2 to 4 mm) designated to grow beyond 5 mm in diameter (Ginther et al., 2001a). Gong et al. (1996) demonstrated that at recruitment, follicular growth is dependent upon FSH. In this experiment, FSH and LH were suppressed by GnRH antagonist infusion, and follicular development beyond 4 mm in diameter was inhibited. However this suppression in follicular development was overcome when FSH, but not LH, was present (Gong et al., 1996). It was reported that at recruitment, the granulosa and theca cells of the recruited follicles contain, respectively, FSH and LH receptors (Richards, 1994; Xu et al., 1995). However, Sirard et al. (2000) reported that mRNA expression of LH receptor was detected in the granulosa cells of follicles that were 5 mm or greater in diameter, suggesting that LH receptor may be present in the granulosa cells early during antral follicular development. Moreover, in an experiment in which heifers were immunized against GnRH, it was demonstrated that constant supplementation of FSH and LH 92 d after immunization (h 0) resulted in greater peripheral E2 concentrations at h 24 and beyond in comparison with heifers that received either FSH or LH alone (Crowe et al., 2001).

Approximately 3 days after the emergence of the new follicular wave, the selection stage is characterized by the presence of the largest follicle in the cohort measuring about 8.5 mm in diameter (Ginther et al., 1996; 1997; Boa and Garverick, 1998; Kulick et al., 1999), followed by the deviation stage, in which the selected follicle continues to grow, while the subordinate follicles undergo atresia (Adams et al., 1993; Ko...
et al., 1991; Gong and Webb, 1996; Ginther et al., 2001a). While still not fully understood, it has been suggested that the follicle from the follicular wave that is selected to be dominant acquires LH receptors in the granulosa cells prior to the subordinate follicles (Ireland and Roche, 1982, 1983; Xu et al., 1995), allowing it to continue its growth utilizing the basal levels of LH available (Kulick et al., 2001). This statement is based on data that demonstrated that LH receptor mRNA expression was only detected in the granulosa cells of follicles ≥ 8 mm in diameter, and absent in granulosa cells of subordinate follicles and follicles with less than 8 mm in diameter (Bao et al., 1997), contradictory to what was aforementioned in this section. The discrepancy between findings relative to detection of LH receptor mRNA expression is suspected to be related to the sensitivity of the assays utilized (Beg et al., 2001). The reason for the cessation of growth of the subordinate follicles is more fully understood and appears to be due to the decrease in FSH shortly after emergence of each wave of follicular development (Ginther, 2000). It has been demonstrated that the decrease in circulating concentrations of FSH is due to the negative feedback of E2 and inhibin (Kaneko et al., 1997; Tohei et al., 2001) that is highly produced by the dominant follicle (reviewed by Fortune et al., 2001). Indeed, when ablation of the largest follicle present in the ovaries was performed, dominance was acquired by the remained largest follicle in the ovaries (Ginther et al., 2001b), reinforcing that the dominant follicle is the one inhibiting development of any other follicle in the ovaries. Findings of this report also indicate that follicles besides the one selected to be dominant is capable to continue its development if the inhibitory actions of the initial dominant follicle are removed.
Following deviation, the selected follicle continues to develop (Sirois and Fortune, 1988; Savio et al., 1988), characterizing the dominance stage (Bo et al., 1994). Under the presence of an active CL and high P4 concentrations, the dominant follicle undergoes atresia and emergence of a new follicular wave occurs (Mapletoft et al., 2008). However, after luteolysis, P4 concentrations decrease, consequently increasing the pulses frequency of LH. Therefore, in this endocrine environment, the dominant follicle rather than undergo atresia, enters the final stages of maturation leading to ovulation. The constant increase in E2 concentrations secreted from the dominant follicle during this time culminates in induction of the GnRH surge by E2 (Evans et al., 1997) followed by the preovulatory LH surge, and final maturation and ovulation of the dominant follicle (Karsh et al., 1997). Moreover, two FSH surges have been demonstrated to occur in cattle around estrus. The first FSH surge occurs simultaneously with the LH surge, while the second FSH surge 18-24 h later induces emergence of the first follicular wave of the subsequent estrous cycle (Dobson, 1978; Walters and Schallenberger, 1984; Quirk and Fortune, 1986; Kaneko et al., 1991).

1.5 Oocyte Growth, Capacitation, and Maturation

A commonly used definition for oocyte competence is its ability to resume meiosis, be fertilized, cleave after fertilization, develop to a blastocyst and bring a healthy pregnancy to term (Eppig et al., 2002; Matzuk et al., 2002; Sirard et al., 2006). In order for this to happen, the oocyte needs to properly develop with and within the follicle. This concomitant development occurs with the follicle growing from the primordial to the
tertiary/antral stage, reaching approximately 2-3 mm in diameter; and the oocyte growing from 30µm to more than 120 µm in diameter (Hyttel et al., 1997). The continued parallel growth between the follicle and the oocyte is dependent upon the bidirectional communication between the oocyte and the cumulus cells thru gap junctions (Bettegowda et al., 2008a; Lechniak et al., 2008). This communication is essential for adequate oocyte maturation (Eppig et al., 2002; Matzuk et al., 2002) as a determinant step to ensure successful embryonic development (Krisher et al., 2004).

As the oocyte increases in diameter (oocyte growth phase), there is further modification, proliferation and redistribution of the cytoplasmic organelles that are required for building up the meiotic and developmental competence of the gamete (Szollosi, 1993). There is also the completion of the majority of the RNA synthesis (Hyttel et al., 1997), and accumulation of proteins (Schultz and Wassarman, 1977) and substrates that are essential for maturation, fertilization and early embryonic development (Eppig, 1994; Sirard, 2001). Increasing meiotic competence during the oocyte growth has been previously demonstrated in cattle (Fuhrer et al., 1989), swine (Motlik et al., 1984; Motlik and Fulka, 1986) and goats (de Smedt et al., 1994). Transcriptional activity and accumulation of mRNAs, ribosomes and polypeptides by the oocyte occurs early during the growth phase, beginning in oocytes within secondary follicles until they reach the early tertiary stage (~2-3 mm in diameter) and the oocytes being approximately 110 µm in size (Crozet et al., 1986; Fair et al., 1995, 1996). The transcription activity was reported to decrease gradually during oocyte growth (De La Fuente and Eppig, 2001), however mRNA synthesis remain detectable until germinal vesicle breakdown occurs
This stage is crucial to later development as bovine oocytes that have not completed their growth phase at the time of submission to the IVF system fail to develop to the blastocyst stage (Pavlok et al., 1992, 1993; Lonergan et al., 1994; Fair and Hyttel, 1997). Once meiotic competence is acquired (~ 120 µm in diameter; Fair et al., 1995), the oocyte diameter plateaus, while the follicle continues to grow up to 15–20 mm in diameter before ovulation (Fair, 2003).

As ovulation approaches, further modifications, prior to oocyte maturation, occur in the oocyte, known as “capacitation” (Hyttel et al., 1997). Throughout this period, there is an increase of lipid content, the Golgi complexes decrease in size, and there is the transition of the cortical granules to a more superficial location (Hyttel, et al., 1997; Fair 2003). Moreover, the nuclear envelop exhibit some undulation, the perivitelline space increases, and there are vacuoles present in the nucleolus, which may indicate either the functional activity of the nucleolus, or the progression of the oocyte to the prophase I (Hyttel, et al., 1997; Fair 2003). Following capacitation, the fully competent oocyte undergoes nuclear and cytoplasmic maturation, stimulated by the LH surge in vivo (Hyttel et al., 1997), or by removal from the dominant follicle into the maturation media in vitro, aiming to be fertilized and bring a healthy pregnancy to term.

1.5.1 Nuclear Maturation

Nuclear maturation precedes the developmental competence (cytoplasmic maturation) acquisition (Blondin and Sirard, 1995; Fair et al., 1995; Arlotto et al., 1996)
and is triggered by the preovulatory LH surge (Kruip et al., 1983) concurrent with a sharp decrease in E2 concentration in the follicular fluid (Dieleman et al., 1983). The process of nuclear maturation is characterized by the reactivation of the nuclear division of the oocyte arrested at the prophase I of metaphase I, resulting in the GVBD and consequently resumption of meiosis for the oocyte to achieve the metaphase (MII) stage (Driancourt and Thuel, 1998). Pavlok et al. (1992) concluded that bovine oocytes of 115 µm in diameter are capable of undergoing GVBD, however the capacity to develop into an embryo is only acquired at a diameter of 120 µm. Moreover, it was demonstrated that the GVBD in the cows occurs between 4 to 8 h after the LH surge (Kruip et al., 1983).

The meiotic block that keeps the oocyte arrested at the metaphase I and avoids GVBD is maintained due to the high levels of cyclic adenosine monophosphate (cAMP) produced by the granulosa cells and transported to the oocyte thru the gap junctions (Kawakura et al., 2004). The pre-ovulatory LH surge decreases the production of cAMP by the granulosa cells and induces the rupture of the gap junctions between the granulosa cells and the oocyte, resulting in diminished concentration of cAMP in the ooplasm (Kawakura et al., 2004). The lower cAMP concentration triggers the activation of the maturation promoting factor (MPF) that leads to GVBD, chromatin condensation and cessation of transcription (Miller and Russel, 1992).

The GVBD is characterized by the completion of metaphase I by the primary oocyte, resulting in the extrusion of the first polar body and the formation of the secondary oocyte. At this point, the chromosomes are aligned in the metaphase plate characterizing the MII stage (Sirard et al., 1992), which will only be completed after
fertilization, resulting in the release of the second polar body (Moore and Presaud, 2004). Moreover, oocytes at the GVBD stage are not able to produce mRNA by transcription until the maternal-zygotic transition (MZT). This transition occurs around the 8-16 cell stage in cattle (Brevini-Gandolfi and Gandolfi, 2001). Therefore, until the embryo genomic activation (EGA), the fertilized oocyte/early embryo growth is dependent upon the maternal mRNA and protein stored during the early oocyte development (Kopecny et al., 1989; Brevini-Gandolfi and Gandolfi, 2001). However, it is important to highlight findings that suggest that embryonic transcription might be active as early as the 4- (Barnes and First, 1991) or 2-cell stage (Viuff et al., 1996; Hyttel et al., 1996).

It has been demonstrated that steroids are required during oocyte maturation and subsequent development of the embryo (Hunter et al., 1976; Moor and Trounson, 1977; Moor et al., 1980). Osborn and Moor (1983) reported that sheep oocytes within follicles that were cultured for 24 h in the presence of 17α hydroxylase-inhibitor (therefore, decreasing steroid production), successfully underwent GVBD; however the oocytes did not advance beyond the metaphase I stage. It was not clear which steroid was the major factor causing the inability of the oocyte to complete maturation. In humans, it has been demonstrated that oocytes that developed in follicles with decreased E2 concentration are unable to undergo nuclear maturation and to reach the MII stage (Botero-Ruiz et. al., 1984; Itskovitz et al., 1991; Artini et al., 1994). On the other hand, Moor and colleagues (1977, 1978, 1980) demonstrated that higher concentrations of E2 6 to 8 h after the LH surge is of greater benefit for the cytoplasmic than for the nuclear maturation in sheep oocytes.
1.5.2 Cytoplasmic Maturation

The capacity of an oocyte to acquire developmental competence relies not only in the ability to undergo nuclear maturation, but also on the ability to undergo cytoplasmic maturation (Fair et al., 1995). It is only after acquiring the ability to resume meiosis (~120 µm) that the oocyte become able to undergo cytoplasmic maturation (Fair et al., 1995). Cytoplasmic maturation is characterized by changes that enable the oocyte to be fertilized and develop to a blastocyst (Driancourt and Thuel, 1998). These modifications are responsible to avoid polyspermy, to trigger the sperm chromatin decondensation and to induce the formation of the male and female pro-nuclei after fertilization (Liu et al., 2009). Before GVBD, transcription and translation take place, but after GVBD, during the process of cytoplasm maturation, transcription strongly declines (Tomek et al., 2002); and proteins modifications and organelle relocation occurs. It is suggested that, due to the condensation of the chromatin, the transcription system is almost inactive (Curtis et al., 1995).

The oocyte cytoplasm maturation involves the continued storage of lipids, decrease in the size of the Golgi complex, the alignment of the cortical granules resulting in a barrier against the polyspermy, and the increased amount of ribosomes near the chromosomes (Kruip et al., 1983; Hyttel et al., 1997). These changes result in a mature oocyte with the adequate cell biological machinery to undergo fertilization and early embryonic development. It was demonstrated that the ability of an oocyte to develop into an embryo depends on the accumulation of specific information in the form of messenger RNA (mRNA) before GVBD or proteins (Sirard et al., 2001).
According to Kruip et al. (1983), during the first 8 h after the LH surge the mitochondria aggregates and move towards the cortical region. Between 8 and 19 h after the LH surge, the fusion of the membrane-bound vesicles occurs and the number of smooth endoplasmic reticulum (SER) increases. Moreover, the authors reported the presence of the clusters containing mitochondria, SER, and lipids, and those clusters were designated as “metabolic units”. The increased amount of clusters was interpreted as an indicator of high metabolic activity in agreement with others (Thibault 1977; Golbus and Stein, 1978; Moor and Warnes, 1978) that reported the importance of the increased synthesis of proteins for a successful cytoplasmic maturation. Approximately 19 h after the LH surge, these metabolic units travel to the center of the cell resulting in the disaggregation of the organelles (Kruip et al. 1983). Failure to complete cytoplasmic maturation results in an oocyte incapable to become an embryo (Krisher, 2004).

1.6 Estradiol influence on Oocyte Competence

As previously mentioned, the steroids play an important role during oocyte maturation. Indeed, E2 concentrations in the follicular fluid has been reported as a possible determinant key of oocyte maturation and competence (Driancourt and Thuel, 1998; van de Leemput et al., 1998; Oussaid et al., 1999), since it enhances mitosis of the granulosa cells (Goldenberg et al., 1972), stimulates gap junction formation within COCs (Merk et al., 1972), and promotes expression of LH and FSH receptors in the granulosa cells (Richards et al., 1976).
Berisha et al. (2002) demonstrated that E2 actions occur through the coordination of mRNA transcription of E2 nuclear receptors α and β (\textit{ERα} and \textit{ERβ}, respectively), being positively correlated with follicular development and diameter, as well as circulating concentrations of E2 in cattle. The actions of E2 are mediated through the binding of E2 and its intracellular receptors, which are members of the nuclear receptor family, resulting in induction of functional changes in the structure of the receptor forming the ligand-receptor complex. This complex will than act on its specific target genes culminating in regulation of their transcription (Conneely, 2001). Expression of both receptors is present in the cumulus cells, while \textit{ERβ} is also expressed in bovine oocytes (Beker-van Woudenberg et al. 2004). Therefore, it is conceivable that E2 may act through its receptors present in the cumulus cells and/or oocytes during oocyte development (Su et al. 2009). Couse et al. (2005) demonstrated that knockout mice in the absence of \textit{ERα} had none or minimal effects on granulosa cells differentiation and ovulation, and gene expression. On the other hand, knockout mice in the absence of \textit{ERβ} were demonstrated to be crucial to FSH-induced differentiation of granulosa cells. Moreover, \textit{ERβ}-knockout mice had impaired cellular organization in preovulatory follicles, were deficient in convert androgens in E2, and had inadequate expression of LH receptor that negatively impacted the response to the LH surge to release the oocyte (Couse et al., 2005).

The G-coupled protein estradiol receptor (\textit{GPER/GPR30}) was also demonstrated to be present in the mice oocyte membrane (Li et al., 2013). The GPER, when activate, acts via stimulation of cyclic adenosine monophosphate (cAMP) production, culminating
in the activation of MAPK and PI3K/Akt pathways that can either result in induce additional rapid (nongenomic) effects, or genomic effects regulating gene transcription.

Li et al. (2013) demonstrated that GPER expression is increased in the membrane of oocytes in a more advanced stage of maturation than oocytes at the germinal vesicle stage (before GVBD), as well as in oocytes matured in vivo in comparison to oocytes that were matured in vitro. This finding suggests that GPER might be involved in oocyte maturation and competence, since it has been demonstrated that oocytes that mature in vitro are of greater competence than oocytes that develop in vitro (Sirard and Blondin, 1996). To my knowledge, GPER has not yet been identified in oocytes of other species.

Van de Leemput et al. (1998) demonstrated that greater intrafollicular E2 concentrations prior to ovulation increased the chances of an oocyte developing into a blastocyst following in vitro production. Moreover, increased levels of aromatase activity were also representative of increased probability of an oocyte developing to the blastocyst stage (Driancourt et al., 1998). In sheep, decreased E2 production that resulted from the inhibition of LH pulse with a GnRH antagonist (Antarelix) resulted in lower oocyte fertilization and the number of blastocysts recovered on day 8 of gestation (Oussaid et al., 1999). A recent study evaluating the effect of supplementing E2 and androstenedione in the culture media of denuded oocytes and COCs recovered from early antral follicles, demonstrated enhanced granulosa cell and oocyte development, as well as nuclear maturation (Endo et al., 2013). Moreover, it was concluded that E2 affects the gene expression profile of granulosa cells to support the development of COC during the
in vitro procedure (Endo et al., 2013). However the specific actions of E2 on oocyte maturation still requires further investigation.

1.7 Oocyte Competence

Oocyte competence was demonstrated to be affected by the size of the follicle from which it is retrieved. Fuhrer et al., (1989) reported that only 1% of oocytes recovered from follicles smaller than 1 mm in diameter achieved the MII stage in cattle. Moreover, Pavlok et al. (1992) demonstrated that bovine oocytes recovered from follicles smaller than 2 mm in diameter were unable to reach the blastocyst stage. On the other hand, it was demonstrated that oocytes recovered from follicles 2-4 and 4-8 mm in diameter are of similar developmental capacity. This similarity in developmental capacity was due to the comparable proportion of hatched blastocysts that developed from all blastocysts, as well as the similar pregnancy rates that resulted between them following transfer (Pavlok et al., 1992). These findings are in agreement with Pavlok et al. (1993) and Tan and Lu (1990) that also reported no difference in oocyte developmental capacity within follicles of 2 mm in diameter and beyond in cattle.

Oocyte competence was also well demonstrated to be greater in oocytes that were recovered after the LH surge *in vivo* (mature *in vivo*) than oocytes that are retrieved at any antral stage but before the LH surge (mature *in vitro*; Sirard and Blondin, 1996). Previous reports demonstrated that oocytes that matured either *in vivo* or *in vitro* did not show any evident differences related to nuclear maturation, fertilization and cleavage rates. However, oocytes matured *in vivo* were reported to have greater blastocyst rate
and/or to yield more hatched blastocysts than oocytes matured in vitro (Leibfried-Rutledge et al., 1987; Marquant Le Guienne et al., 1989). Moreover, it was demonstrated that in vitro maturation and resulting capacity to be fertilized and develop normally was associated to the presence of FSH, LH, estrogens, and other molecules in the maturation media (Leibfried-Rutledge et al., 1986; Critser et al., 1986; Sirard et al., 1988; Brackett et al., 1989; Younis et al., 1989), that may, or may not, mimic the real follicular environment throughout in vivo maturation.

Oocyte competence is reported to be affected by the age of the female donor. For example, oocytes recovered from prepubertal females were demonstrated to have decreased developmental capacity when compared to oocytes retrieved from sexually mature females (Quirke and Hanrahan, 1977; Revel et al., 1995; Damiani et al., 1996; O’Brien et al., 1997; Ledda et al., 2001). Decreased blastocyst rate formation has been demonstrated in prepubertal oocytes in cattle (Revel et al., 1995), pigs (Grupen et al., 2003) and sheep (O’Brien et al., 1997). Moreover, porcine oocytes from prepubertal females that reached the blastocyst stage resulted in embryos with decreased trophectoderm and total cells in comparison with embryos from adult females (Grupen et al., 2003) and lower birth rate in sheep (Quirke and Hanrahan, 1977) when compared to oocytes from mature females. The decreased oocyte competence from prepubertal females is reported to be related to the incomplete cytoplasmic maturation that consequently impairs sperm penetration, and failure in the formation of the pronuclei, and to avoid polyspermy. In addition, early cleavage failure and inability to overcome the
maternal-embryonic transition have also been demonstrated (reviewed by Armstrong, 2001).

It was also demonstrated, that aged females have impaired reproductive efficiency. This field has been extensively investigated in women, but data available in the literature demonstrates that reproductive inefficiency also occurs in other aged female species. In women, it was demonstrated a decrease in antral follicle counts and oocyte competence (Dias et al., 2014), which yield less blastocyst formation during the in vitro embryo production (Janny and Menezo, 1996). Moreover, there is a 20 percentage points increase in pregnancy losses in women >40 years old than women that are under 35 years old (Gunby and Daya, 2006). In cattle, a study evaluating the effect of age on ovarian morphology, detected a decrease in the number of follicles, however did not detect any differences in oocyte quality thru evaluation of visual and subjective oocytes morphology in cows up to 17 years of age (Katska and Smorag, 1984). However, recent discoveries have demonstrated that embryonic development is decreased in cows older than 12 years (Dias et al., 2014). Malhi et al. (2005) detected a decrease in number of follicles recruited per follicular wave, increased circulating E2 concentrations during the follicular phase, and a tendency to have decreased CL diameter and P4 concentrations in aged cows compared to young cows. In addition, the diameter of the ovulatory follicle in cows having 2 follicular waves cycles, were decreased in aged cows (Malhi et al., 2005). A study using a superovulatory animal model, demonstrated that aged cows had approximately 8 less ovulations than young cows, due to decreased number of follicles recruited and their capability to successively develop to the larger size categories (Malhi
et al., 2006, 2008). Oocyte competence was also reported to be impaired in aged cows, resulting in decreased number of embryos, and a greater proportion on unfertilized oocytes than young cows (38% vs. 71%, respectively; Malhi et al., 2007). Oocytes retrieved from aged cows also had decreased development during in vitro embryo production, with lesser capability to successfully undergo maturation and be fertilized (Iwata et al., 2011). Takeo et al. (2013) reported a negative relationship between age of the cow and cleavage status. The combination of all these findings greatly supports the idea that oocyte competence is lessened in aged cattle.

Much remains to be elucidated about oocyte quality/developmental capacity assessment, as well as the effect of the environment in which the oocyte develops. Therefore, the continuous advancement in research will help to better understand the physiological mechanism for oocyte maturation, hopefully leading to the discovery of new approaches to improve both in vivo and in vitro matured oocytes, consequently increasing fertility.

1.8 Oocyte characteristics of Developmental Competence

The primary visual criterion of oocyte quality and developmental capacity in the bovine is the evaluation of subjective morphological parameters such as shape, homogeneity of cytoplasm, presence of polar body, and compactness of cumulus cells (Armstrong, 2001; Lonergan et al., 2001, 2003; Cottichio et al., 2004; Krisher et al., 2004). However, it is well known that morphological criteria alone are insufficient to
distinguish oocytes that differ in competence (Lonergan et al., 2003; Cottichio et al., 2004; Krisher et al., 2004).

1.8.1 Non-invasive Methods applied to evaluate Oocyte Competence

Non-invasive methods for evaluation of oocyte quality and developmental capacity are typically preferred since they usually do not compromise further oocyte development. A variety of subjective morphological parameters have been extensively used as the primary criterion for selection of oocytes to undergo in vitro maturation, fertilization and culture.

Blondin and Sirard (1995) developed a cumulus-oocyte complex (COC) grading system, in which the oocytes were evaluated by the texture of the ooplasm, as well as by the expansion and number of layers of granulosa cells surrounding the oocyte (Table 1.1). They demonstrated that oocytes from class 3 (Table 1.1) had greater in vitro developmental capacity in comparison with the oocytes from all the other classes. Oocytes from classes 1 and 2, although less competent than class 3, also demonstrated high developmental rates (Blondin and Sirard, 1995). Moreover, classes 4 to 6 represent oocytes with clear signs of degeneration and significant decrease in blastocyst formation (Blondin and Sirard, 1995). Polar body morphology has also been analyzed as a predictor of oocyte quality in humans, in which the shape (ovoid or round), size, integrity (intact or fragmented), and surface (smooth or rough) of the first polar body are evaluated (Balaban et al., 1998; Kahraman et al., 2000). Moreover, size of the periviteline space (De Sutter et al., 1996), and the thickness and organization of the zona pellucida (Talevi et al., 1997;
Gabrielsen et al., 2001) have also been used as a parameter to subjectively estimate oocyte developmental capacity in humans. In the bovine, it has been reported that color of the ooplasm could also be used as a predictor of oocyte quality, in which oocytes with darker ooplasm were demonstrated to have greater amount of mitochondria and increased developmental capacity in comparison with oocytes presenting pale ooplasm (Leroy et al., 2005, Jeong et al., 2009).

Another non-invasive parameter used as an indicator of oocyte development is oocyte size; as the intensive synthesis of RNA during oocyte development causes an increase in oocyte diameter (Crozet et al., 1981; Lazzari et al., 1994). The association between the oocyte diameter and its ability to resume and complete meiotic division during in vitro maturation has been described in humans (Durinzi et al., 1995), cattle (Otoi et al., 1997), buffalo (Raghu et al., 2002), pigs (Motlik and Fulka, 1986), and the dog (Otoi et al., 2000). Bovine oocytes were demonstrated to acquire meiotic competence at a diameter of 115 µm, but full developmental competence is only achieved at 120 µm (Otoi et al., 1997).

Time of first-cleavage is a post-fertilization measurement that can also be linked to the intrinsic quality/competence of the oocyte (Patel et al., 2007), in which the oocytes that cleave earlier after in vitro fertilization are the ones with the greatest chances to reach the blastocyst stage in comparison with the oocytes that cleave later (Dinnyes et al., 1999; Lonergan et al., 1999).
Although the use of non-invasive parameters to evaluate oocyte competence is commonly classified as subjective, they still can be used to preselect COC before *in vitro* procedures as an approach to enhance the outcomes of *in vitro* embryo production.

**Table 1.1. COC grading system**.

<table>
<thead>
<tr>
<th>Class of oocytes</th>
<th>Number of cumulus layers</th>
<th>Expansion of cumulus</th>
<th>Texture of ooplasm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>≥ 5</td>
<td>Compact</td>
<td>Homogeneous</td>
</tr>
<tr>
<td>2</td>
<td>≥ 5</td>
<td>Compact</td>
<td>Dark zone around periphery</td>
</tr>
<tr>
<td>3</td>
<td>≥ 5</td>
<td>Slight expansion in outer layers</td>
<td>Slight granulations</td>
</tr>
<tr>
<td>4</td>
<td>≥ 5</td>
<td>Full expansion with dark clumps</td>
<td>Heavy granulations</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>Only corona radiata</td>
<td>Variable</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>No cumulus</td>
<td>Variable</td>
</tr>
</tbody>
</table>

Adapted from Blondin and Sirard, (1995).

### 1.8.2 The use of Brilliant Cresyl Blue to assess Oocyte Competence

The Brilliant Cresyl Blue (BCB) stain has been used as an additional non-invasive method to indirectly predict the developmental competence of the oocyte. The primary purpose of BCB is to identify oocytes that are either growing or that have already
completed their growth phase. The BCB stain assesses the activity of the enzyme glucose-6-phosphate dehydrogenase within the oocyte (G6PDH; Pujol et al., 2004; Alm et al., 2005). This enzyme is involved in the pentose phosphate pathway to supply energy to cells (Aster et al., 2010), and can degrade the BCB stain. Thus for oocytes in which the enzyme is active, G6PDH activity will reduce the blue compound of the BCB stain, and as a result, their ooplasm will be colorless (BCB-). On the other hand, when the oocyte stops growing, and the G6PDH activity ceases, it becomes unable to degrade the blue compound of the stain, and the ooplasm will in turn exhibit a blue color (BCB+; Mangia and Epstein, 1975; Wassarman, 1988; Roca et al., 1999; Rodriguez-Gonzalez et al., 2002; Alm et al., 2005; Opiela et al., 2008).

The credibility and functionality of the BCB stain varies between and within species. In cattle, a variety of studies have reported significantly greater developmental capacity of BCB+ oocytes compared to BCB- oocytes (Pujol et al., 2004; Alm et al., 2005; Bhojwani et al., 2007; Silva et al., 2011; Janowski et al., 2012). However, Opiela et al. (2008) did not detect any significant difference in blastocyst formation between BCB+ and BCB- oocytes. In goats, contradictory results have also been reported, in which increased developmental capacity of BCB+ oocytes was (Rodriguez-Gonzalez et al., 2002) or was not detected (Katska-Ksiazkiewics et al., 2007). It was also demonstrated in sheep that BCB+ oocytes have increased oocyte diameter and blastocyst rate (Mohammadi-Sangcheshmeh et al. 2012; Wang et al., 2012), greater number of cells (Catalá et al., 2011), and higher maturation rate (Mohammadi-Sangcheshmeh et al., 2012) in comparison to BCB- oocytes. In porcine oocytes, Egerszegi et al. (2010)
demonstrated greater meiotic progression, and ability to be fertilized in BCB+ than BCB-
oocytes. However, although there are no evidence that BCB stain is harmful to bovine,
 goat and sheep oocytes, the BCB stain seems to be detrimental to porcine oocytes.
Indeed, results from porcine oocytes showed that oocytes that were negative to BCB
 immediately after retrieval and submitted to BCB stain again after in vitro maturation
 (“double staining”; Wongsrikeao et al., 2006; Kempisty et al., 2011) or 90 min incubation
during the BCB test is harmful for the oocytes, either resulting in decreased amount of
 RNA and protein for fertilization or in the occurrence of abnormal number of
 chromosomes, respectively.

1.9 Markers of Oocyte Competence

Recent research has focused on identifying molecular markers in both oocytes and
 cumulus cells that more accurately define oocyte quality in order to complement the
 assessment by non-invasive parameters. Identification of molecular markers enabling
 more precise evaluation of oocyte competence and its capacity to develop into a quality
 embryo has vast implication for reproductive techniques in humans and farm animal
 species (Bettegowda et al., 2008b). Comparison of oocytes from adult females to less
 competent oocytes collected from prepubertal heifers, as previously mentioned in this
dissertation (see section 1.6; Revel et al., 1995; Damiani et al., 1996) has served as a
 useful model in the process to discover these markers of oocyte competence. The oocyte
 transcriptome has been compared between mature and prepubertal cattle to begin to
 understand the differences in gene expression that may serve as determinants of
developmental competence. In cattle, 193 genes were more abundantly displayed in adult-derived vs. prepubertal heifer-derived oocytes, and 223 were more abundantly displayed in prepubertal than adult oocytes (Patel et al., 2007). In this experiment, greater amount of mRNA for follistatin (FST) was detected by real-time PCR in germinal vesicle stage oocytes of mature cows compared to prepubertal heifers (Patel et al., 2007). In addition, the author revealed a positive correlation between follistatin mRNA abundance and time of first cleavage (oocyte competence). FST is currently being investigated as a potential marker of oocyte competence and it is further discussed in a subsequent section.

Another set of molecular markers that are also being further investigated as a predictor of oocyte competence is the cathepsin family of lysosomal cysteine proteinases. This marker resides in the cumulus cells, and their expression was negatively related to oocyte developmental competence (Bettegowda et al., 2008b). Through real-time PCR, Bettegowda et al. (2008b) identified that Cathepsin B (CTSB), cathepsin Z (CTSZ) and cathepsin S (CTSS) were more expressed in the cumulus cells surrounding poor quality oocytes (Bettegowda et al., 2008b). In fact, when using E-64 (cathepsin inhibitor), Bettegowda et al. (2008b) reported a decreased number of apoptotic nuclei in the cumulus layer and increased development of in vitro-fertilized oocytes to the blastocyst stage. However, effects of E-64 during meiotic maturation on subsequent embryonic development were not observed in maturation of denuded oocytes, indicating that cathepsins present in the cumulus cells are the likely functional marker of oocyte competence. Similarly, Balboula et al. (2010) demonstrated that inhibition of CTSB using E-64 significantly decreased the number of apoptotic nuclei in the cumulus layers of
matured oocytes and blastocysts, improving the development and total cell number of embryos. Although still unclear how the cathepsins specifically act in the follicles, it has been demonstrated a clear relationship between cathepsins and the occurrence of apoptosis (Stoka et al., 2001) enforcing the negative relationship existent between the amount of cathepsin mRNA in the granulosa cells and oocyte competence.

Evidence that other oocyte secreted factors may also function as markers of oocyte competence continue to accumulate. For example, growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) are members of the transforming growth factor beta (TGF-β) superfamily, and their mRNA were first identified in oocytes (Elvin et al., 1999; Erickson and Shimasaki, 2001; Hosoe et al., 2011). Recently, Hosoe et al. (2011) demonstrated that BMP15 and GDF9 mRNA expression is also present in the cumulus cells. BMP15 and GDF9 were reported to regulate cumulus cells functions (Matzuk et al., 2002; Gilchrist et al., 2008; Su et al., 2009), such as steroidogenesis (Miyoshi et al., 2007; Spicer et al., 2008), proliferation (Gilchrist et al., 2004; McNatty et al., 2005), differentiation (Kathirvel et al., 2013), metabolism (Otsuka et al., 2011), and apoptosis (Hussein et al., 2005); throughout oocyte development. Hosoe et al. (2011) demonstrated that GDF9 and BMP15 mRNA expression did not differ between oocytes that differed in developmental competence, but was greater in the cumulus cells from mature cows vs. prepubertal heifers. Hussein et al. (2006) reported increased blastocyst rate when exogenous GDF9 and BMP15 were supplemented during the in vitro maturation process. Moreover, GDF9 and BMP15 were demonstrated to be crucial to control the development of granulosa cells and the metabolism in the cumulus cells (Su et
Homozygous BMP15 knockout ewes were reported to fail to continue folliculogenesis beyond the primary stage (Galloway et al., 2000; Hanrahan et al., 2004). Mutation in the BMP15 gene in humans was reported to inhibit normal ovarian development; however the exact mechanism remains unclear (Di Pasquale et al., 2004). It was demonstrated that homozygous GDF9 knockout female mice were identified to be sterile, due to the absence of follicular development beyond the primary stage (Dong et al., 1996). Additionally, Joyce et al. (2000) suggested that GDF9 in the mouse may suppress growth of the oocyte by down-regulation of the oocyte growth factor, kit ligand, in the granulosa cells. GDF9 may also play a role in regulating the expression of the genes for steroid acute regulatory protein (StAR) and hyaluronic acid synthase 2 (HAS2), apparently regulating P4 production and cumulus cells expansion, respectively (Elvin et al., 1999).

Hyaluronic acid (HA) is produced by the cumulus cells which are stimulated by the enzyme HAS2. HA acts by facilitating the extrusion of the oocyte upon ovulation, by maintaining the surrounding cells together, and may also play a role in fertilization and sperm capacitation. HAS2 mRNA expression in the cumulus cells has been reported to be positively correlated to the HA synthesis during oocyte maturation (Fulop et al., 1997). Additionally, HAS2 expression may also be required to induce the HA-mediated effects at the time of oocyte final maturation, as well as for the interaction between the oocyte and the sperm (Schoenfelder and Einspanier, 2003).

A novel protein (JY-1) that promotes early embryonic development, and for which expression is restricted to the oocyte, was recently identified as another possible
marker of oocyte competence (Bettegowda et al., 2007). It was demonstrated that JY-1 activity is essential before and after fertilization to ensure progress of embryonic development beyond fertilization (Bettegowda et al., 2007). The authors reported a decreased rate of developmental capacity to both 8-16 cell and blastocyst stage in JY-1 knockdown zygotes in comparison to the controls (Bettegowda et al., 2007). The authors propose that JY-1 protein in bovine is oocyte-specific with multiple distinct actions for a gene specific in the oocyte that is related to folliculogenesis and early embryonic development. However, the mechanisms of how JY-1 protein supports early embryonic development remain unclear and require further investigation.

1.9.1 Follistatin

FST is a cysteine-rich monomeric glycoprotein (Knight and Glišter, 2001), and was first detected in granulosa cells of secondary follicles, becoming more abundant as the follicle forms an antrum (Findlay, 1993). The strongest signals were found in preovulatory follicles and newly formed corpora lutea (Findlay, 1993). FST acts as a binding protein through the common β subunit of inhibin and activin (Shimonaka et al., 1991). Different reports have pointed to the presence of FST mRNA not only in the follicular fluid, but also in the ovary, kidney, brain (Shimazaki et al., 1989), testis, adrenal gland, thymus, gut, uterus, heart, pancreas, lung, skeletal muscle (Michel et al., 1990) and pituitary (Gospodarowicz and Lau, 1989; Michel et al., 1990), suggesting that FST actions are not limited only to the reproductive system. Shukovski et al. (1992) demonstrated that, in the bovine, FST transcripts present in granulosa cells are decreased in early antral follicles (< 7 mm), increased in growing follicles, and greatest in the
preovulatory follicles (> 11 mm); in agreement with others (Shimasaki et al., 1989; Nakatami et al., 1991). Moreover, this finding supports the earlier findings that hypothesized that FST supports follicular luteinization or atresia through a local mechanism (Findlay et al., 1990; Shukovski et al., 1991). It was demonstrated that FST during granulosa cells differentiation decreases synthesis of estrogen and inhibin, and stimulates P4 production (Gore-Langton and Armstrong, 1988). These actions characterize the changes that occur during luteinization and atresia (Braw and Tsafriri, 1980). It was hypothesized in rats that FST may act by inactivating the 20 α-hydroxysteroid oxidoreductase, which will then inhibit the reduction of P4 to 20 α-hydroxy P4 in luteinized granulosa cells, thereby increasing production of P4 (Gore-Langton and Armstrong, 1988). Lindsell et al. (1994) demonstrated in pigs that the amount of FST mRNA in the granulosa cells increases concomitant with follicle diameter. Furthermore, the authors also demonstrated that FST concentrations in the granulosa cells increased in response to FSH in a dose and time-dependent manner. Nevertheless, FSH stimulation also enhanced P4 secretion, indicating differentiation of the granulosa cells; suggesting a proportional relationship between granulosa cells differentiation and FST mRNA expression. Indeed, Klein et al. (1991) demonstrated a 2 to 3 fold increase in FST secretion in bovine granulosa cells when FSH was added to the culture media, but no effect of LH supplementation was detected in FST secretion.

FST has also been identified in oocytes and used as a marker of oocyte quality. Patel et al. (2007) demonstrated using real-time PCR that oocytes from adult females and of greater developmental capacity, had a greater abundance of mRNA for FST in
comparison to oocytes of lower developmental capacity (prepubertal females), which is in agreement with previous reports (Revel et al., 1995; Damiani et al., 1996). Moreover, a positive relationship between FST and time of first cleavage has also been detected, suggesting that this molecule may affect early embryonic development (Patel et al., 2007; Lee et al., 2009), and that time of first cleavage may be linked to the intrinsic quality/competence of the oocyte (Patel et al., 2007). Subsequently, Lee et al., (2009) demonstrated that supplementation of FST for 72 h after fertilization resulted in an increased number of oocytes cleaving earlier and reaching the 8-16 cell and blastocyst stage. Similar results have been reported in other species, such as in the rhesus monkey, in which FST supplementation significantly increased the amount of embryos cleaving earlier, reaching the blastocyst stage, and resulted in increased cell numbers and trophectoderm cells (Van deVoort et al., 2009). The role of FST on early embryonic development is further supported by the additional findings by Lee et al. (2009) that demonstrated that siRNA-mediated FST knockdown resulted in less fertilized oocytes reaching the 8-16 cell and blastocyst stage, with decreased trophectoderm cells numbers. However, this effect was diminished with addition of FST to the culture medium (Lee et al., 2009). Collectively, the results support the existence of a positive relationship between the levels on FST present in the oocyte and its competence. Data available in the literature suggests that FST is involved in early and also in the progression of the embryonic development. Moreover, it was demonstrated that although FST has a high-affinity to bind activin, FST can also bind other molecules with a lower affinity (e.g. bone morphogenic proteins; Otsuka et al. 2001; Balemans and Van Hul 2002; Lin et al.; 2003).
Therefore, current data from FST in early embryonic developmental seems paradoxical and requires further investigation.

1.10 Influence of Progesterone on Fertility

Synchronization programs used to facilitate AI in the beef cattle industry rely on the manipulation of estrus and/or ovulation by the administration of hormones that mimics the phases of the estrous cycle. The use of progestins (synthetic progestogens) has been applied in synchronization programs since the 60's (Hansel and Malven, 1960; Zimbelman, 1963; Hansel et al., 1966; Zimbelman and Smith, 1966). In current synchronization programs, such as the 7-d CO-Synch + CIDR (Lamb et al., 2001) and the 5-d CO-Synch + CIDR programs (Bridges et al., 2008), P4 is delivered via an intravaginal device that releases P4 constantly while implanted in the animal. The aim of P4 in synchronization programs is to mimic luteal phase and to group animals to a similar stage of the estrous cycle by preventing ovulation. Administration of P4 was also demonstrated to induce ovulatory estrus in pre-pubertal heifers (Hall et al., 1997; Rodrigues et al., 2012) and anestrous cows (Odde, 1990; Meneghetti et al., 2009; Sa Filho et al., 2009).

It is known that P4 regulates the pulse frequency of LH, with frequency of pulses less when P4 is high (6-8 pulses/24 h; Rahe et al., 1980), and greater when P4 concentrations are low (20-30 pulses/24h; Rahe et al., 1980). Moreover, it is also known that LH is needed for growth of the ovulatory follicle (Savio et al., 1993; Kulick et al., 2001) and for the final maturation of the oocyte (Savio et al., 1993; Gong et al., 1995),
which is one of the determinant step to ensure successful embryonic development (Krisher, 2004). Therefore, alternatives in estrus synchronization programs to ensure adequate gonadotropin stimulation of the oocyte and the dominant follicle have been studied with the aim to provide a better environment for oocyte and follicular development and to maximize reproductive efficiency in beef cattle.

The combined results from a series of individual studies in *Bos taurus* cattle conducted by Mussard et al. (2002, 2003a, 2003b, 2007) demonstrated that proestrus length (period from luteolysis to either a spontaneous or GnRH-induced LH surge) could be a major factor affecting follicle maturity and fertility in beef cattle. This concept was directly investigated by Bridges et al. (2010), in which proestrus was lengthened by 1 day (1.25 vs. 2.25 days). With this animal model, greater pregnancy rates were detected in the cows that experienced long than short proestrus (73.0% vs 14.3%, respectively). These findings are supported indirectly by Bridges et al. (2008) who detected a 10.5 percentage point increase in timed-AI pregnancy rate by modifying the standard 7 d CO-Synch + CIDR to a 5 d program. In this study, the proestrus length, in a timed-AI program, (referred to as the interval from PGF administration and P4 device withdrawal to induction of the LH surge with GnRH), was increased by 12 h (60 h vs 72 h). As a result of these findings, prolonging proestrus length has been one of the approaches used to enhance gonadotropin stimulation of the ovulatory follicle and improve fertility in *Bos taurus* cattle (Geary et al., 2010; Dadarwal et al., 2013). Enhanced preovulatory E2 concentrations are a primary result of lengthened proestrus (Bridges et al., 2008, 2010, 2012, 2014). Indeed, Bridges et al. (2010) reported that cows that experienced longer
proestrus prior to GnRH-induced ovulation had enhanced preovulatory E2 concentrations and greater pregnancy rate to timed-AI than cows that experienced shorter proestrus. These findings agree with those from Perry et al. (2005) that associated lower fertility rates with decreased E2 concentrations prior to insemination in a different animal model.

Taking into consideration the findings in *Bos taurus* cattle, alternative approaches in synchronization programs with *Bos indicus* beef cattle were developed. The primary approach taken to enhance gonadotropin stimulation of the growing follicle was to use a device that releases less P4, such as an intravaginal drug release (CIDR®) that has been previously used to synchronize estrus (Martínez et al., 2003). The resultant peripheral concentrations of P4 from the used device, in the absence of a CL, are less than the P4 concentrations during the luteal phase; therefore minimizing the negative effect of high P4 concentrations on LH secretion during follicular development. It was demonstrated that the decreased P4 concentrations in response to the insertion of the used device enhanced ovulatory follicle diameter (Dias et al., 2009; Claro Jr. et al., 2010; Martins et al., 2014) and increased pregnancy rate to AI (Dias et al., 2009; Claro Jr. et al., 2010).

The other approach used in *Bos indicus* cattle was to hasten PGF administration in order to induce luteolysis earlier during the synchronization program. With this approach, the dominant follicle is allowed to develop for a longer period under low P4 concentration (P4 from the device only). As a result, females that received PGF injection earlier had lower P4 concentrations at CIDR withdrawal, greater follicle diameter at AI (Mantovani et al., 2010), and increased pregnancy rate to timed-AI (Peres et al., 2009). Similarly, Dias et al., (2009) demonstrated that administration of PGF on d 9 instead of d
7, in a 9 day-CIDR program resulted in decreased pregnancy rate to timed-AI. However, the negative effects associated with delaying PGF to d 9 (e.g. decreased gonadotropin stimulation) was compensated by the administration of an exogenous gonadotropin stimuli (equine chorionic gonadotropin; eCG) on d 9. Since eCG has gonadotropin properties, its administration at the end of the program overcome apparent negative effects of high P4, promoting the gonadotropin stimulus needed for the ovulatory follicle.

Conversely, in Bos taurus cattle, the levels of P4 concentrations during synchronization programs were reported to either have no impact (Pfeifer et al., 2009; Hill et al., 2014, Mercadante et al., 2014a; 2014b) or to have only minimal effects (Sparks et al., 2012; Mellieon et al., 2012) on pregnancy rates to AI either after spontaneous or induced ovulation. So while decreased P4 concentrations during follicular development were clearly demonstrated to enhance fertility in Bos indicus cattle, its benefit in Bos taurus cattle remains unclear. Therefore, we deemed it is necessary to further explore the effect of P4 concentrations during early follicular development in Bos taurus beef cattle. Specifically, to investigate the site of action of a follicular environment with increased LH stimulus, looking at the implications of reducing P4 concentrations on oocyte quality, developmental competence, and reproductive efficiency.

1.11 Statement of the Problem

Development of timed-AI programs by necessity requires manipulation of the normal levels of hormones during the estrous cycle. Although current programs are successful, it is without question that manipulations result in concentrations of hormones
that exceed or are less than those typically present; at least in some females that are being synchronized. There are many methods to synchronize estrous cycles and a variety of hormones used, but in beef cattle, the central component of all programs is the administration of exogenous P4 through an intravaginal insert. Due to increasing use of these P4-based programs, evidence continues to emerge to indicate that the concentrations of P4 that are attained during a synchronization program, throughout emergence and growth of the ovulatory follicle, may impact reproductive performance in beef cattle. This response appears to vary between *Bos indicus* and *Bos taurus* cattle with clear indications in *Bos indicus* but less so in *Bos taurus* cattle. The potential to increase fertility seems to be specific to beef cattle or dairy heifers, since most evidence suggests that high producing dairy cows may well be deficient in P4 even during a spontaneous estrous cycle. Furthermore, data that has demonstrated a positive effect of lower P4 on pregnancy rates, presume that this improvement in reproductive outcome is due to the greater gonadotropic stimulation of the eventual ovulatory follicle throughout its development. The resulting ovulatory follicles of greater diameter, increased ovulation rate, and enhanced preovulatory E2 concentrations indirectly indicates that greater LH stimulation may be the mechanisms by which decreased P4 improves fertility. This theory is supported by the knowledge that LH pulse frequency is inversely related to P4 concentrations in cattle. Furthermore, it is also known that elevated LH secretion is necessary for maturation of oocytes and the dominant follicle destined to ovulate and plays an important role in embryonic development. Therefore, it is conceivable that the reduction of peripheral P4 concentrations during the synchronization programs that
coincides with the follicular growth may increase LH secretion and enhance fertility in beef cattle.

The overall objective of this work is to address the role of P4 concentrations during early follicular development in *Bos taurus* beef cows. It was hypothesized that lesser P4 concentrations would enhance LH pulse frequency, that in turn would provide a better follicular environment for the oocyte; therefore resulting in an oocyte of greater developmental competence, increased follicular growth, and enhanced conception rate to AI after either spontaneous or induced ovulation.
CHAPTER 2

The role of progesterone concentrations during early follicular development on ovulatory follicle growth and pregnancy rates in beef cattle

Abstract

Two experiments were conducted to investigate the role of decreased progesterone (P4) concentrations throughout follicular development on ovulatory follicle growth and pregnancy rate in beef cattle. In Experiment 1, ovulation was synchronized with the 5 d CO-Synch + CIDR program in multiparous cows (n = 241). Six days after the 2nd GnRH injection of the pre-synchronization program (d 0), ablation of all visible follicles in the ovaries was performed and cows were assigned to receive either a previously used CIDR and two-25 mg PGF doses 8 h apart (LoP4), or a new CIDR (HiP4). On d 5, CIDR were removed from all cows, two-25 mg PGF were administered, and estrus detection tail paint was applied. Timed artificial insemination (TAI) was performed coupled with GnRH-induced ovulation on d 8. Specific objectives were to compare follicle diameters at d 5 and 8, and the respective follicular growth rate; P4 and estradiol (E2) concentrations on d 5; estrus response; proestrus length; and pregnancy rate to AI. On d 5, P4 concentrations were greater ($P < 0.01$) in the HiP4 (4.91 ± 0.13 ng/mL) than LoP4 (0.99 ± 0.06 ng/mL). Conversely, d 5 E2 concentrations and follicular diameter were greater ($P < 0.01$) in the LoP4 (5.00 ± 0.23 pg/mL and 8.9 ± 0.20 mm).
than HiP4 (1.54 ± 0.12 pg/mL and 7.38 ± 0.15 mm) treatment. Follicular diameter at TAI (12.0 ± 0.12 mm) did not differ between treatments. In Experiment 2, a new follicular wave was induced with estradiol benzoate on d -7, and cows (n = 275) were assigned on d 0 to receive either 25 mg PGF and maintain the previously inserted CIDR (presynchronization; LoP4) or to replace the previously inserted CIDR with a new CIDR (HiP4). Moreover, all cows received GnRH on d 0. CIDR were removed from all cows on d 5 concomitant with two-25 mg of PGF. Estrous detection coupled with AI 12 h later (Estrus-AI) was performed for 60 h after CIDR removal (d 5) with TAI coupled with GnRH administration at 72 h if estrus was not exhibited. P4 concentrations on d 5 were greater ($P < 0.01$) in the HiP4 (2.81 ± 0.10 ng/ml) than LoP4 (1.73 ± 0.05 ng/ml) treatment. Within cows that were detected in estrous after PGF administration, estrus response (83.5%) and interval to estrus (55.0 ± 0.5 h) did not differ between treatments. Synchronized pregnancy rate (Estrus-AI + TAI) was similar between treatments (79.7%). In conclusion, differences in P4 concentrations during early follicular development do not impact synchronized pregnancy rate in beef cows when afforded adequate and similar intervals of proestrus.

**Keywords:** Progesterone, Used CIDR, Conception Rate, Beef Cows

**Introduction**

Reproductive efficiency is a major limiting factor in cattle production efficiency, and estrous/ovulation synchronization programs have been used as a powerful tool to enhance reproductive efficiency. It is known that luteinizing hormone (LH) pulse
frequency is regulated by progesterone (P4) concentrations throughout the estrous cycle (Rahe et al., 1980; Schallenberger et al., 1985), and increasing evidence suggests that greater gonadotropin stimulation throughout follicular development positively impacts fertility in beef cattle. Bridges et al. (2010) demonstrated that by prolonging the period of follicular development under low progesterone (P4) concentration, presumably by increasing the duration of LH stimulation, resulted in greater preovulatory estradiol (E2) concentration and increased pregnancy rate to artificial insemination (AI). Similarly, hastening prostaglandin F2α (PGF) administration at the end of the synchronization program resulted in greater ovulatory follicle diameter (Carvalho et al., 2008), and increased reproductive efficiency (Dias et al., 2009; Meneghetti et al., 2009).

In Bos indicus influenced cattle, an alternative commonly applied to enhance gonadotropin stimulation during follicular development is the use of a previously used intravaginal drug release (CIDR®) to synchronize estrus, which results in lesser circulating concentrations of P4 when used (Martinez et al., 2003). Dias et al. (2009) reported increased follicle diameter and greater pregnancy rate to timed-AI (TAI) in Nelore heifers that received a twice-used CIDR in comparison to a new CIDR. Similarly, Claro Jr. et al. (2010) demonstrated that providing less P4 during a synchronization program resulted in greater ovulatory follicle diameter, greater estrus behavior after CIDR removal, and increased conception and pregnancy rates. The cumulative interpretation of research suggests that P4 concentration during follicular development is a significant source of variation in fertility, most notably in Bos indicus cattle, and it has not been adequately investigated in Bos taurus cattle. Therefore, the objective of the
present study was to investigate the effect of decreased P4 concentrations during follicular development on pregnancy rates to TAI and estrus-AI. It was hypothesized that decreasing circulating concentrations of P4 during early follicular development would enhance pregnancy rates to AI in beef cattle.

**Material and Methods**

*Animals and treatments*

All procedures involving animals used in this study were approved by The Ohio State University Agricultural Animal Care and Use Committee (Experiment 1) or the Fort Keogh Livestock and Range Research Laboratory Animal Care and Use Committee (Experiment 2).

**Experiment 1**

Multiparous postpartum (64.9 ± 0.9 d) Angus and Angus crossbred cows (n = 241) from 3 locations (BV, n = 98; COL, n = 68; and JK, n = 75) were used. Ovulation was pre-synchronized with the 5 d CO-Synch + CIDR program (Figure 1; CIDR [Zoetis, New York, NY, USA] + GnRH [Cystorelin®, 100 µg, i.m., Merial, Inselin, NJ, USA] on d -14, followed 5 d later with CIDR removal and 2 injections of PGF [Lutalyse®, 25 mg each, i.m., Zoetis; d – 9] and GnRH [100 µg, i.m.] 3 d after PGF; d - 6). Cows with a corpus luteum (CL) present (6 d following synchronized ovulation) were stratified by age and days post-partum within location and assigned to one of two treatments. Transvaginal ultrasound-guided aspiration (ablation) was also performed on d 0 of the experiment to
remove the dominant follicle of the first wave and other follicles with a diameter \( \geq 5 \) mm. This approach synchronizes emergence of a new follicular wave 1 to 2 days later (Mussard et al., 2007; Bridges et al., 2010). For cows in the high P4 (HiP4) treatment, a new CIDR was inserted (d 0) to supplement P4 being produced by the CL. In the low P4 (LoP4) treatment, on d 0 cows received a CIDR that had been used previously for 5 days and two-25 mg injections of PGF 8 h apart. On d 5, the CIDR was removed and 2 doses of PGF (25 mg, i.m. each) were administered and ail paint was applied to all cows for estrus detection. On d 8, TAI and administration of 100 µg of GnRH were performed (72 h after CIDR removal; Figure 2.1); and tail paint score (TPS; 1 = paint completely removed; 2 = paint partially removed; 3 = paint largely undisturbed and no evidence of mounting) was evaluated.

Transrectal ultrasonography (US; Figure 1) was performed using a 7.5 MHz linear array transducer (Aloka 500V; Aloka, Wallingford, CT) to characterize ovarian structures in all cows on d 0, d 5 and d 8. Pregnancy diagnosis was conducted using a 5.0 MHz transrectal linear array transducer (Aloka 500V) approximately 30 to 40 d after TAI in all locations. Blood samples were collected (BS; Figure 2.1) via jugular venipuncture into 10 ml EDTA vacutainer tubes (BD Vacutainer®, Franklin Lakes, NJ) on d 5 (immediately at CIDR insert removal, thus P4 concentrations include P4 contributed by the CIDR insert) and d 8 to assess circulating P4 and E2 concentrations.
Experiment 2

A total of 275 crossbred (Angus x Hereford) multiparous postpartum cows (3 to 14 yrs old; 56.51 ± 0.83 d) were used in Miles City, MT. All cows received estradiol benzoate (EB; 1 mg/500 kg BW, i.m. [beta-Estradiol 3-Benzoate (Sigma-Aldrich Co. LLC, Milwaukee, WI, USA) in 10% Benzyl Alcohol (Sigma-Aldrich Co. LLC) 90% sesame oil]) and a previously used (for 7 d) intravaginal P4 device (CIDR®; Zoetis, New York, NY, USA) on d -7, to induce emergence of a new follicular wave approximately 3 days later (d -4; Burke et al. 2001). On d 0, all cows received 100 μg of GnRH (i.m., Factrel®, Zoetis, New York, NY, USA) and were randomly assigned to one of the two treatments (Figure 2.2). In the high P4 (HiP4; n = 136) treatment, the previously inserted CIDR was replaced with a new CIDR on d 0. In the low P4 (LoP4; n = 139) treatment, 25 mg prostaglandin F2α (PGF; Lutalyse®, Pfizer Animal Health, New York, NY, USA) was administered on d 0, and the CIDR previously inserted on d -7 remained in the vagina. On d 5, all cows received 2 PGF (25 mg, i.m. each) the CIDR was removed and all cows received an Estrotec heat detector (Rockway Inc., Estrotec™, Spring Valley, WI) patch on their tailhead. Estrous detection was performed at least twice daily for 60 h with artificial insemination (AI) approximately 12 h after estrus detection (Estrus-AI). Cows not detected in estrus within this period received 100 μg GnRH and were bred by TAI at 72 h (Figure 2). Blood samples were collected (Figure 2.2) via tail venipuncture into 10 ml Vacutainer tubes (Fisher Scientific, Pittsburg, PA) on d 5 to assess circulating P4 concentrations.
Blood Samples Handling and Radioimmunoassay (RIA)

In Experiment 1, samples were stored on ice immediately after collection and then centrifuged at 1,500 x g at 4°C for 20 min. Plasma was removed and stored in cryovials at -20°C until analyses. In Experiment 2, samples were placed in ice immediately after collection, incubated for 24 h at 4°C followed by centrifugation at 1,200 x g for 25 min. Serum was collected and stored at -20°C until analyses.

Plasma concentrations of E2 were analyzed using a double antibody assay previously validated in our lab (Cruppe et al., 2014 submitted). Average intra-assay CV was 6.2%, inter-assay CVs (three assays) for low (2.3 pg/ml), medium, (7.6 pg/ml), and high (16.3 pg/ml) pools were 27%, 15.4%, and 13%, respectively. The sensitivity of the assay was 2.1 pg/ml.

Circulating concentrations of P4 were determined using a Coat-a-Count® RIA kit (Siemens, Los Angeles, CA) as previously described (Bellows et al., 1991; Burke et al., 2001). Average intra-assay CV was 6.1%, inter-assay CV (two assays) for pooled plasma samples containing 0.29, 1.30, and 3.50 ng/ml of P4 were 6.7%, 9.2% and 9.2%, respectively, and the average sensitivity of the assay was 0.072 ng/mL.

Used CIDR Sanitation

After removal from the cows, CIDR were immediately washed in water followed by a final rinse with diluted chlorhexidine solution (Novalsan®, Zoetis, New York, NY, USA). The devices were then allowed to air dry, and later packaged and stored at room temperature for future use. At time of insertion, CIDRs were first submerged either in a
diluted povidone iodine solution (Betadine®, Purdue Frederick, Norwalk, CT, USA) in Experiment 1 or again in diluted chlorhexidine solution in Experiment 2, followed by regular procedure upon insertion.

**Statistical Analyses**

**Experiment 1**

Cows identified without a CL at ablation (n = 11), with a CL on d 5 if on the LoP4 treatment (n = 1), or without a CL on d 5 if on the HiP4 treatment (n = 1) were excluded from the analyses. Final analyses were performed on a total of 228 cows. Data were analyzed using a model that included treatment, location and their interaction. Concentrations of P4 and E2, follicle diameters on days 5 and 8, and follicular growth rate (d 5 to d 8) were compared using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA, version 9.3). Pregnancy rates were compared with the GLIMMIX procedure of SAS. Tail paint score distribution between treatments was compared using the FREQUENCY procedure (CHI-SQUARE test) of SAS. Data are expressed as the mean ± SEM.

**Experiment 2**

All cows that were missing a CIDR on d 0 (n = 10) or d 5 (n = 4) were removed from further analyses, resulting in 261 cows included in all analyses (HiP4, n = 131; LoP4, n = 130). Data were analyzed using a model that included treatment only. Estrous response (proportion of cows detected in estrus during the 60 h period after CIDR
removal) and pregnancy rates (estrus-AI, TAI and overall) were analyzed with the GLIMMIX procedure of SAS (SAS Institute Inc., Cary, NC, USA, version 9.3). The MIXED procedure of SAS was used to analyze interval to estrus (from PGF administration; proestrous interval) and serum concentrations of P4 on d 5. Distribution of estrus was compared using the FREQUENCY procedure (CHI-SQUARE test) of SAS. Serum concentrations of P4 and either estrous response or length of proestrus were analyzed with the CORR procedure of SAS. Data are expressed as the mean ± SEM.

Results

Experiment 1

Across location, P4 concentrations at CIDR withdrawal (d 5) were greater ($P < 0.01$) in the HiP4 than LoP4 (Table 2.1) treatment. A treatment by location interaction ($P < 0.01$) was detected and predominantly due to elevated P4 concentrations in one location. Concentrations of E2 on d 5 were greater ($P < 0.01$) in the LoP4 than HiP4 (Table 2.1) treatment. Somewhat elevated concentrations of E2 in the LoP4 treatment at one location resulted in detection of a treatment by location interaction ($P < 0.05$). Diameter of the ovulatory follicle was greater ($P < 0.05$) in the LoP4 than HiP4 (Table 2.1) treatment on d 5, but did not differ between treatments on d 8 when TAI was performed (Table 2.1). Accordingly, follicle growth from d 5 to 8 was greater ($P < 0.05$) in the HiP4 than in the LoP4 (Table 2.1) treatment. Distribution of TPS (Figure 2.3) differed ($P < 0.05$) between treatments with a majority of cows in the HiP4 treatment having TPS 3 (56%) and a majority in the LoP4 with TPS 1 (54%). Cows with TPS 1 or 2

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had a greater \((P < 0.05)\) PR than cows with a TPS 3. However, pregnancy rate did not differ between the HiP4 and LoP4 (Table 2.1) treatment. Ovulation before the moment of TAI (determined by disappearance of the largest follicle between d 5 and d 8) occurred in 20\% of the cows in the LoP4 and no cows in the HiP4 treatment (Table 2.1). Pregnancy rate to TAI of cows that had ovulated was 56.5\% (13/23). Pregnancy rate to TAI did not differ between the LoP4 and HiP4 treatments; regardless of whether the cows that prematurely ovulated were included in this comparison (Table 2.1).

**Experiment 2**

P4 concentrations at CIDR withdrawal (d 5) were greater \((P < 0.01)\) in the HiP4 than LoP4 (Table 2.2) treatment. Estrous response within the 60 h after PGF administration (Table 2.2), interval from PGF to estrus (Table 2.2) and estrus distribution (Figure 2.4) did not differ between the HiP4 and LoP4 treatments. Synchronized pregnancy rate (combination of Estrus-AI and TAI) was similar between HiP4 and LoP4 treatments (Table 2.2). Across treatments, pregnancy rates were greater \((P < 0.01)\) with Estrus-AI (82.9\%) than TAI (63.6\%). Regardless of treatment, concentrations of P4 on d 5 were negatively related \((P < 0.01; r = -0.19)\) with estrous response and positively related \((P < 0.01; r = 0.2)\) to interval to estrus.

**Discussion**

The influence of altering P4 concentrations during follicular development on pregnancy to AI in multiparous beef cows was evaluated in these experiments. In both
experiments, P4 concentrations at CIDR withdrawal (and luteal regression in the HiP4 treatment; d 5) were, as designed, greater in the HiP4 than the LoP4 treatment. The administration of PGF on d 0 to induce luteolysis prior to emergence of the new follicular wave, in conjunction with either insertion (Experiment 1) or maintenance (Experiment 2) of a previously used CIDR, resulted in decreased P4 concentrations during emergence and early development of the ovulatory follicle in the LoP4 treatment. It was demonstrated that decreased P4 concentrations during follicular development resulted in greater E2 concentrations and increased follicle diameter at CIDR withdrawal on d 5 (Experiment 1), but did not influence synchronized pregnancy rate in beef cows (Experiments 1 and 2).

The different concentrations of P4 were used with the aim to enable different gonadotropin stimulation of the ovulatory follicle throughout its development. It is known that frequency of LH varies throughout the estrous cycle and is mainly regulated by circulating concentrations of P4 (Rahe et al., 1980; Schallenberger et al., 1985); being decreased during luteal phase (6-8 pulses/24 h; Rahe et al., 1980), and increased after luteal regression (20-30 pulses/24 h; Rahe et al., 1980). In Experiment 1, follicle diameter and E2 concentrations at cessation of P4 treatment were greater in the LoP4 than HiP4 treatment and in agreement with others (Pfeifer et al., 2009; Cerri et al., 2011). It is presumed that this acceleration in growth of the dominant follicle and E2 secretion was the result of increased LH pulse frequency. Although LH pulses were not measured in the current experiments, it is speculated that an increase in LH pulse frequency in the LoP4 treatment occurred in response to the sub-luteal P4 concentrations during follicular
development (Ireland and Roche, 1982; Roberson et al., 1989) and enhanced growth and E2 production of the dominant follicle (Stegner et al., 2004). Indeed, 20% of the cows in the LoP4 had already ovulated at TAI.

Estrous response and interval to estrus did not differ between treatments in Experiment 2. The relative difference in P4 concentrations between the LoP4 and HiP4 treatment was much greater in Experiment 1 than Experiment 2 and may explain the apparent inconsistency for time of estrus between the two experiments. With the animal model used in Experiment 2, it is likely that some cows in the HiP4 treatment did not have a functional CL at the time of insertion of the new CIDR. Hence, the only source of P4 in some cows in the HiP4 was the new CIDR. Conversely, all cows in the HiP4 treatment in Experiment 1 had a CL present at insertion of the new CIDR. The absence of an endogenous source of P4 would result in lesser concentrations of P4 in these animals in the HiP4 treatment. Furthermore, lack of the CL as an endogenous source of P4 may interfere in the pattern of release of LH (Roberson et al., 1989). In these cows this may have changed gonadotropin stimulation of the growing follicle; leading to follicles that grew faster and with greater steroidogenic capacity than others in the same treatment with a CL.

Increased follicle diameter at AI in response to decreased P4 concentrations during follicular development has been reported in lactating dairy cows (Cerri et al., 2011), beef heifers (Carvalho et al., 2008; Dias et al., 2009; Pfeifer et al., 2009; Mantovani et al., 2010; Martins et al., 2014) and mature cows (Pfeifer et al., 2009). Although follicle diameter at CIDR withdrawal was greater in the LoP4 than HiP4 on d 5,
diameter of the ovulatory follicle did not differ between treatments at TAI 72 h later. It appears that 72 h of very low P4 from d 5 to 8 allowed follicles that developed under different endocrine environments to reach an adequate level of competence by the time of ovulation. Cessation of P4 treatment resulted in an increased follicular growth rate during proestrus in the HiP4 treatment and may have corrected for any functional differences in follicular development created by differences in P4 during earlier development.

The idea that decreased P4 concentrations during early follicular development may be beneficial for fertility is supported by previous data from our laboratory (Abreu et al., 2013) and work with Nelore cattle in Brazil. In our previous study, oocytes were recovered from follicles by ovum pick-up after they developed either under high or low P4 concentrations. Follicular development under low P4 concentrations resulted in a greater number of follicles aspirated, increased oocyte recovery rate and enhanced oocyte quality compared to follicles developing under high P4 concentrations (Abreu et al., 2013). Moreover, in vitro fertilization of oocytes collected from cows with decreased P4 concentrations yielded blastocysts with greater numbers of cells than oocytes from cows in the high P4 treatment (Kruse et al. 2014). Studies conducted with Bos indicus beef cattle have established a clear inverse relationship of P4 concentrations and pregnancy rate to TAI (Dias et al., 2009; Peres et al., 2009; Claro Jr. et al., 2010). However, in the current experiments and others using Bos taurus beef cattle, pregnancy rates to AI, either after spontaneous or induced ovulation, did not differ (Pfeifer et al., 2009; Hill et al., 2014, Mercadante et al., 2014a; 2014b) with P4 concentrations or only resulted in marginal differences (Sparks et al., 2012; Mellieon et al., 2012). Perhaps in Bos taurus
cattle the sensitivity to elevated P4 concentrations is less than in Nelore females and sufficient gonadotropin stimulation of follicular development occurs across a wider range of P4 concentrations. Indeed, Cerri et al. (2011) reported no difference in the proportion of good quality embryos and oocytes, proportion of degenerated embryos and unfertilized oocytes recovered 6 d after AI in lactating dairy cows exposed to different P4 concentrations during follicular development. The reasons for the different response between Bos indicus and Bos taurus beef cattle regarding P4 concentrations and fertility remain unclear and require further investigation.

In summary, decreased P4 concentrations during early follicular development resulted in greater E2 concentrations and increased follicle diameter at the end of the P4 treatment. In groups with the greatest differences in P4, this altered timing of estrus and ovulation during the period after cessation of P4 treatment. However, the modifications in follicular development and time of estrus did not impact pregnancy rate to either estrus-AI or TAI. It is suggested that if an appropriate interval of time in the absence of P4 is provided after cessation of a period of differential P4 treatment, differences in early developmental rate of follicles do not influence fertility of Bos taurus cows.
Figure 2.1. Diagram of treatments (high P4; HiP4 and low P4; LoP4), blood sample (BS) and ultrasonography (US) performed in Experiment 1. CIDR = intravaginal progesterone device (new = never used; used = previously used for 5 d). PGF = prostaglandin F2α. TAI = timed-AI.
Figure 2.2. Diagram of treatments (high P4; HiP4 and low P4; LoP4) and blood sample (BS) in Experiment 2. EB = estradiol benzoate. NFW = new follicular wave emergence. CIDR = intravaginal progesterone device (new = never used; used = previously used for 5 d). PGF = prostaglandin F2α. TAI = timed-AI.
Figure 2.3. Distribution of Tail Paint Score (TPS) on d 8 (Timed-AI) in Experiment 1; (1 = paint completely removed; 2 = paint partially removed; 3 = paint largely undisturbed and no evidence of mounting).
**Table 2.1. Effect of treatments on response variables (Mean ± SE) in Experiment 1.**

<table>
<thead>
<tr>
<th></th>
<th>HiP4 (n = 113)</th>
<th>LoP4 (n = 115)</th>
<th><em>P</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone concentration on d 5, ng/ml</td>
<td>4.91 ± 0.13</td>
<td>0.99 ± 0.06</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Estradiol concentration on d 5, pg/ml</td>
<td>1.54 ± 0.12</td>
<td>5.00 ± 0.23</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Follicle diameter on d 5, mm</td>
<td>7.38 ± 0.15</td>
<td>8.92 ± 0.20</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Follicle diameter at TAI (d 8), mm</td>
<td>11.81 ± 0.15</td>
<td>12.14 ± 0.19</td>
<td>&gt; 0.1</td>
</tr>
<tr>
<td>Follicle growth rate (d 5 to d 8), mm/d</td>
<td>1.52 ± 0.06</td>
<td>1.28 ± 0.06</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Ovulation rate at TAI (d 8), % (n)</td>
<td>0 (0/113)</td>
<td>20 (23/115)</td>
<td>&gt; 0.1</td>
</tr>
<tr>
<td>Pregnancy rate of ovulated cows, % (n)</td>
<td>-</td>
<td>56.5 (13/23)</td>
<td>na</td>
</tr>
<tr>
<td>Pregnancy rate without ovulated cows, % (n)</td>
<td>67.3 (76/113)</td>
<td>70.7 (65/92)</td>
<td>&gt; 0.1</td>
</tr>
<tr>
<td>TAI pregnancy rate with all cows, % (n)</td>
<td>67.3 (76/113)</td>
<td>67.8 (78/115)</td>
<td>&gt; 0.1</td>
</tr>
</tbody>
</table>
Table 2.2. Effect of treatments on response variables (Mean ± SE) in *Experiment 2*.

<table>
<thead>
<tr>
<th></th>
<th>HiP4 (n = 131)</th>
<th>LoP4 (n = 130)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone concentration on d 5, ng/ml</td>
<td>2.81 ± 0.10</td>
<td>1.73 ± 0.05</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Estrus response within 60 h after PGF, %</td>
<td>81.7</td>
<td>85.4</td>
<td>&gt; 0.1</td>
</tr>
<tr>
<td>Interval from PGF to estrus, h</td>
<td>56.1 ± 0.7</td>
<td>54.0 ± 0.7</td>
<td>&gt; 0.1</td>
</tr>
<tr>
<td>Synchronized pregnancy rate, % (n)</td>
<td>77.1 (101/131)</td>
<td>82.3 (107/130)</td>
<td>&gt; 0.1</td>
</tr>
</tbody>
</table>
Figure 2.4. Distribution of estrus within 60 h after PGF administration on d 5 ($P = 0.0991$) in Experiment 2.
CHAPTER 3
The role of progesterone concentrations during early follicular development on characteristics of developmental competence in oocytes, and LH secretion in beef cattle

Abstract

The objective was to investigate the impact of decreased progesterone (P4) concentrations during early follicular development on luteinizing hormone (LH) secretion and oocyte characteristics in beef cows. Primiparous cows (n = 24) were presynchronized with the 5 d CO-Synch + CIDR program and received transvaginal ultrasound-guided follicular aspiration 6 d following the synchronized ovulation (ablation; d 0 of the experiment). Cows were stratified by days-postpartum and assigned to either: 1) high P4 treatment (HiP4; a new CIDR was inserted to supplement P4 from the existing corpus luteum [CL]), or 2) low P4 treatment (LoP4; a CIDR that had previously been used for 5 d was inserted and two doses of prostaglandin F2α [PGF] 8-12 h apart were given at CIDR insertion to induce luteal regression). Blood samples were collected on days 0, 1.5, 2.5, and 3.5 for P4, and on days 2.5, and 3.5 for estradiol (E2) concentrations. A serial blood sample collection period (15 min intervals for 12 h)
for LH was performed on d 2.5 in a subset of cows (n = 13). On d 4, CIDR were removed and ovum pick-up (OPU) was performed through transvaginal aspiration to collect oocytes from all follicles ranging from 3 to 8 mm in diameter. Oocytes recovered were graded (1 to 6 scale), stained for brilliant cresyl blue (BCB), and diameter was determined before being snap frozen for later subsequent mRNA analysis of follistatin (FST). Concentrations of P4 were greater (treatment x d, P < 0.01) in the HiP4 than LoP4 treatment on days 1.5, 2.5, and 3.5. Peripheral concentrations of E2 were greater (treatment x d, P < 0.05) in the LoP4 than HiP4 on days 2.5 and 3.5. Frequency of LH pulses was greater (P < 0.05) in the LoP4 than HiP4 treatment on d 2.5; LH mean concentration and pulse amplitude did not differ between treatments. Among treatments, follicles aspirated per cow, total oocytes recovered, recovery rate, percentage of oocytes graded 1 to 3, oocyte diameter, percentage of oocytes that were BCB+, and the relative expression of oocyte mRNA for FST did not differ. In conclusion, decreased P4 concentrations during early follicular development increased LH pulse frequency and E2 concentrations, but did not affect characteristics of developmental competence in oocytes.

**Keywords:** Progesterone, Used CIDR, Luteinizing Hormone, Oocyte competence

**Introduction**

Luteinizing hormone (LH) stimulates and regulates the final stages of follicular growth and development (Savio et al., 1993; Gong et al., 1995), and it is crucial for ultimate oocyte maturation (Gong et al., 1995), which is required for successful
embryonic development (Krisher et al., 2004). Studies, in which presumably a finite period of greater gonadotropin stimulation was afforded during follicular development preceding the LH surge, have shown increased fertility in cattle. For example, when proestrus length was increased ahead of a GnRH induced ovulation and timed-AI, enabling a longer period of low progesterone (P4) concentration for the dominant follicle (DF) to develop, pregnancy rates were increased both in dairy (Peter and Pursley, 2003) and beef (Bridges et al., 2008, 2010) cattle. Similarly, decreased P4 concentrations afforded during synchronization programs throughout follicular development (Dias et al., 2009; Claro Jr. et al., 2010), or administration of equine chorionic gonadotropin (eCG; Baruselli et al., 2004; Peres et al., 2009) at the end of the synchronization program, resulted in greater follicular diameter at artificial insemination (AI) and increased pregnancy rates in Nelore beef cattle.

The mechanism by which increased LH stimulation during follicular development enhances reproductive performance in cattle is considered to be multifactorial. The actions of LH to promote competence of the oocyte to be fertilized (Savio et al., 1993; Gong et al., 1995) and maturity of the ovulatory follicle, which contains and nurtures the oocyte, are putative mechanisms for successful fertilization. Indeed, it has been demonstrated that LH up regulates expression of its receptor and other genes and proteins in follicles that are required for proliferation and function of cells and for steroidogenesis (Luo et al., 2011). Another potential mechanism is that the resulting increase in E2 concentrations enhanced the capacity of the uterus to sustain embryo development (Bridges et al., 2012, 2013), and increased the likelihood of pregnancy success (Bridges
et al., 2010; Jinks et al., 2013; Atkins et al., 2013; Perry et al., 2014). Furthermore, within the follicle, E2 was demonstrated to enhance mitosis (Goldenberg et al., 1972), formation of gap junction (Merk et al., 1972), and to promote mRNA expression of FSH and LH receptors (Richards et al., 1976) in the granulosa cells. Therefore, it is conceivable that increased LH pulse frequency throughout follicular development may improve oocyte competence in beef cattle. This concept is supported by findings that demonstrated enhanced blastocyst formation following in vitro embryo production of oocytes recovered from follicles with increased intrafollicular E2 concentrations (van de Leemput et al., 1998). Therefore, the objective of the present study was to investigate the impact of decreased P4 concentrations on LH secretion, follicular development and oocyte characteristics in beef cows. It was hypothesized that decreasing circulating concentrations of P4 during early follicular development would increase LH pulse frequency and, consequently, increase E2 concentration and enhance oocyte competence.

**Material and Methods**

**Animals and Treatments**

All procedures involving animals used in this research were approved by The Ohio State University Agricultural Animal Care and Use Committee. Angus and Angus Crossbred primiparous cows (n = 24) were pre-synchronized with the 5 d CO-Synch + CIDR program (Figure 3.1; CIDR [Zoetis, New York, NY, USA] + GnRH [Cystorelin®, 100 µg, i.m., Merial, Inselin, NJ, USA] followed 5 d later with CIDR removal and 2
doses of PGF [Lutalyse®, 25 mg each, i.m., Zoetis] and GnRH [100 µg, i.m.] 3 d after PGF). Cows received transvaginal ultrasound-guided follicular aspiration 6 d following the synchronized ovulation (ablation; d 0 of the experiment). Cows were stratified by d postpartum (85.1 ± 1.0 d), and assigned to receive either: 1) high P4 treatment (HiP4): a new CIDR was inserted to supplement P4 from the existing corpus luteum (CL) or 2) low P4 treatment (LoP4): a CIDR that had previously been used for 5 d was inserted and two doses of prostaglandin F2α (PGF; 8 to 12 h apart) were given to induce luteal regression. Blood samples were collected on days 0, 1.5, 2.5, and 3.5 for P4 and on days 2.5, and 3.5 for E2 concentrations. A serial blood sample collection period (15 min intervals for 12 h) for LH was performed on d 2.5 in a subset of cows (n = 13). On d 4, CIDR were removed, and ovum pick-up (OPU) was performed. Immediately after OPU, samples were retrieved and transported to the laboratory.

**Used CIDRs sanitation**

Previously used CIDR (in a 5 d CO-Synch + CIDR program) were immediately washed after removal in water followed by a final cleaning with a Novalsan® Solution (Zoetis, New York, NY, USA). The devices were then allowed to air dry, and later packaged and stored at room temperature for future use. At time of insertion, CIDR were first submerged in a Betadine® Solution (Purdue Frederick, Norwalk, CT, USA) followed by regular procedure upon insertion.
**Ultrasonography**

Transrectal ultrasonography (US; Figure 1) was performed using a 7.5 MHz linear array transducer (Aloka 500V; Aloka, Wallingford, CT) to characterize ovarian structures in all cows on the following d: 1) d 0 (ablation): to verify the response of the presynchronization program through the presence of a CL; 2) d 1.5: to identify emergence of the new follicular wave; 3) d 2.5: to assess absence of CL in the LoP4 treatment to confirm luteolysis; assess emergence of the new follicular wave in cows that were not identified on d 1.5; and to assess the size of the largest follicle in the ovaries as well as follicular development; 4) d 3.5: assess the number of follicles present in the ovaries and to verify if follicular deviation (DF ≥ 8.5 mm; Ginther et al., 2001) had occurred.

**Ablation Procedure**

Prior to ablation, cows were properly restrained in a cattle squeeze-chute, epidural anesthesia was performed with 5 ml of 2% (20 mg/ml) of lidocaine (Sparhawk Laboratories, Inc., Lenexa, TX), and the rectal and vaginal area were cleaned and sanitized using water and a mild disinfectant. Ablation was performed with a 17-gauge needle, by using the ultrasonography-guided transvaginal approach (Bergfelt et al., 1994) with a 5-MHz convex array transducer (Aloka 500V, Corometrics Inc. Wallingford, CT). The procedure was performed to remove the DF of the first wave, and any other follicles with a diameter equal or greater than 5 mm, in order to synchronize the emergence of the new follicular wave (Mussard et al., 2007; Bridges et al., 2010).
Blood Collection and Radioimmunoassay (RIA)

Blood samples were collected (BS; Figure 3.1) via jugular venipuncture into 10 ml EDTA Vacutainer tubes (BD Vacutainer®, Franklin Lakes, NJ) on days 0, 1.5, 2.5, and 3.5 for P4 and on days 2.5 and 3.5 for E2 concentrations. Samples were stored on ice immediately after collection and then centrifuged at 1,500 x g at 4°C for 20 min. Plasma was decanted and stored at -20°C until analyses. Plasma concentrations of P4 were determined using a Coat-a-Count® RIA kit (Siemens, Los Angeles, CA) as previously described (Burke et al., 2001). Average intra-assay CV was 7.1%, inter-assay CV (two assays) for pooled plasma samples containing 1.3 and 3.4 ng/ml of progesterone were 9.7% and 5.4%, respectively, and the average sensitivity of the assay was 0.06 ng/ml. Plasma concentrations of E2 were analyzed using a double antibody assay previously validated in our lab (Cruppe et al., 2014 unpublished). The intra-assay CV was 4.3% and inter-assay for low (2.6 pg/ml), medium (7.7 pg/ml) and high (16.1 pg/ml) pools were 19.6%, 17.5%, and 16.6%, respectively. The sensitivity of the assay was 2.3 pg/ml.

Serial blood samples were collected to assess patterns of LH secretion on day 2.5 of the experiment in a subset of randomly selected cows from each treatment (HiP4, n = 6; LoP4, n = 7). Indwelling jugular catheters were placed in the cows, and blood samples were collected at 15 min intervals for 12 h. Blood samples for LH analysis were allowed to clot for 48 h at 4°C, and then centrifuged at 2,785 x g for 20 min. Serum were stored at -20°C until analyses were performed. Serum concentrations of LH were determined using a double-antibody system previously validated in our laboratory (Anderson et al., 1996). The intra-assay CV was 8.2% and the inter-assay CV for low and high pools was 16.7
and 12.8, respectively. The sensitivity of the assay was 0.17 ng/ml. An LH pulse was defined as an increased concentration that occurred within two samples of the previous nadir that exceeded three standard deviations of the assay, and with a decline of two or more samples before the subsequent nadir. Amplitude of LH pulses was defined as the concentration of LH at the peak minus the concentration at the previous nadir, and mean LH concentrations was determined as the average of the LH concentration during the 12 hours of sampling.

**OPU Procedure and Cumulus-Oocyte Complex Handling**

**OPU Procedure**

Prior to OPU, cows were properly handled as previously described in the ablation procedure. OPU was performed with an 17-gauge needle (one per cow) by using the ultrasonography-guided transvaginal approach (Bergfelt et al., 1994) with a 5-MHz convex array transducer (Aloka 500V, Corometrics Inc. Wallingford, CT) to collect oocytes from all follicles measuring between 3 to 8 mm in diameter. The aspiration tubing was filled with flush media (Vigro Flush Media; Bioniche Life Sciences, Belleville, ON, Canada) supplemented with 50 USP heparin/mL (H3393; Sigma 101 Aldrich, St. Louis, MO). The follicular aspirate was collected via the aspiration tubing attached to the needle passed through a rubber stopper in a 50 mL conical tube (Falcon; Thermo Scientific, Waltham, MA) that was kept warm at 35 ± 2 °C in a heating block (Multi-Blok Heater; Lab-Line Instruments, Melrose Park, IL). The aspiration was performed using a foot-operated pump (V-MAR-5100; Cook Veterinary Products,
Queensland, Australia) set at 80 mmHg at a flow rate of 15 ml/min. The aspirate from the OPU was immediately transported to the laboratory.

*Cumulus-Oocyte Complex Handling*

Cumulus-oocytes complexes (COCs) were identified using a zoom stereomicroscope (Nikon® SMZ 475T, Philadelphia, PA), and graded on a 1 to 6 scale (1 = ≥ 5 layers compact cumulus and homogenous cytoplasm, 6 = denuded) according to Blondin et al. (1995). Brilliant cresyl blue (BCB) staining was used to identify oocytes in the growing phase. COCs were washed 3 times into Vigro-complete flush media (Bioniche Life Sciences, Belleville, ON, Canada), exposed to 26µm of BCB diluted into PBS, and incubated for 90 min at 38.5°C in a humidified atmosphere. The oocytes were then washed again in Vigro-complete flush media and cytoplasm color evaluation was performed under the stereomicroscope. Those oocytes detected with blue ooplasm were designated as **BCB+**, and the oocytes without blue coloration in the cytoplasm were designated as **BCB-**. The concentration of 26µM has been previously used for goat (Rodriguez-Gonzalez et al., 2002), pig (Roca et al., 1998) and bovine (Alm et al., 2005) oocytes, without any later detrimental effect on *in vitro* maturation of oocytes. Following BCB staining, cumulus cells were removed by continuous pipetting of the COCs, and denuded oocytes were measured from the inside zona diameter using a micrometer attached to the stereomicroscope. Oocytes were evaluated using the 5x magnification, in which 1 division was equivalent to 13.3 µm. All subjective parameters were performed blind to treatments, to avoid any bias of the data. Cumulus cells and oocytes from
individual cows were pooled and stored in 100µl of lysis buffer (RNAqueous micro kit, Ambion®) at -80 °C until further analyses.

**Follistatin expression**

Total RNA was extracted from each pool of denuded oocytes from a single cow, and residual genomic DNA was removed by DNase I digestion by using the RNAqueous micro kit (Ambion) according to the manufacturer’s instructions (Patel et al., 2007). Preceding RNA extraction, samples were individually spiked with 250 g of Green Fluorescent Protein (GFP) synthetic RNA as an exogenous control and 50 µg tRNA as a carrier (Bettegowda et al., 2005; Patel et al., 2007). The Oligonucleotide primers for bovine FST were obtained from Dr. George Smith (Michigan State University) and can be found in GenBank (GenBank accession number for FST: BF774514). Each reaction mixture consisted of 1 µl of cDNA, 0.75 µl each of forward and reverse primers, 3.75 µl of nuclease-free water, and 6.25 µl of SYBR Green PCR Master Mix into a total reaction volume of 12.5 µl. Reactions were performed in duplicate and changes in mRNA expression were determined by real-time PCR using the CFX96 touch real time PCR machine (Biorad). The amount of mRNA of interest was normalized relative to the abundance of the endogenous control 18s rRNA in cumulus cells and GFP in oocytes (Patel et al., 2007; Bettegowda et al., 2008b).
**Statistical Analyses**

Data were analyzed using a model that included treatment. Concentrations of P4 and E2 over time were analyzed as repeated measures using the MIXED procedure of SAS (SAS Institute Inc. Cary, NC, USA, version 9.3). Frequency of LH pulses, mean LH concentration and pulse amplitude, P4 and E2 concentrations on d 2.5 were analyzed in the subset group of cows that received the serial bleeding (n = 13) using the MIXED procedure of SAS. For variables related to ovarian activity, such as number of follicles aspirated, size of the largest follicle at d 2.5 and at OPU, number of oocytes recovered, oocyte recovery rate (total oocytes/total follicles), and the relative abundance of mRNA for FST, experimental unit was cow (n = 24) within treatment and analyzed using the MIXED procedure of SAS; and occurrence of follicular deviation at OPU (DF ≥ 8.5mm at OPU) was analyzed using the GLIMMIX procedure of SAS. Oocyte was the experimental unit for percentage of good quality oocytes (grade 1 to 3), and percentage of oocytes that were positive for BCB (n = 49) and were analyzed by treatment using the GLIMMIX procedure of SAS; and oocyte size was analyzed using MIXED procedure of SAS. Mean values were analyzed using the MEANS procedure of SAS. Data are expressed as the mean ± SEM.

The relationships between 1) P4 concentrations and E2 concentrations on d 2.5 and d 3.5; 2) E2 and follicular diameter on d 3.5; 4) E2 and occurrence of follicular deviation on d 3.5 were assessed in a separate analysis across treatments. Regression analysis using the general linear models procedure of SAS (PROC GLM) was used to determine if the relationship was linear, quadratic, or cubic. Individual equations for each
parameter were generated based on the intercept and slope estimates values according to maximum likelihood estimates from each significant continuous order effect.

**Results**

Concentrations of P4 differed (treatment x d, \( P < 0.01 \)) between HiP4 and LoP4 treatments, being lesser in the LoP4 than HiP4 on days 1.5, 2.5, and 3.5 (Figure 3.2). Concentrations of E2 were greater (treatment x d, \( P < 0.05 \)) in the LoP4 than HiP4 on days 2.5 and 3.5 (Figure 3.3). In the subset group of cows sampled for LH (n = 13), P4 and E2 differences were consistent with the overall group, with P4 being decreased and E2 being greater in the LoP4 than HiP4 treatment (Table 3.1). LH pulse frequency was greater (\( P < 0.05 \)) in the LoP4 than HiP4 treatment; mean LH concentration and pulse amplitude did not differ between treatments (Table 3.1).

Between treatments, follicles aspirated per cow, size of the largest follicle at d 2.5 and at OPU, number of oocyte recovered per cow and oocyte recovery rate did not differ (Table 3.2). Percentage of occurrence of follicular deviation at OPU tended to be greater in the LoP4 than in HiP4 treatment (Table 3.2). Regarding characteristics of developmental competence in oocytes, percentage of oocytes graded 1 to 3, percentage of oocytes positive for BCB (BCB+), oocyte size, and the relative expression of oocyte mRNA for FST (pooled by cow) were similar between treatments (Table 3.3).

A negative linear relationship was detected between P4 and E2 concentrations on d 2.5 (\( P = 0.03 \); Figure 3.5), and on d 3.5 (\( P = 0.05 \); Figure 3.6). A positive linear relationship was detected between E2 on d 3.5 and follicle diameter on d 3.5 (\( P = 0.03 \);
Figure 3.7); and between E2 on d 3.5 and probability of follicular deviation occur by d 3.5 ($P = 0.02$; Figure 3.8).

**Discussion**

The influence of decreased P4 concentrations during early follicular development on LH and E2 secretion, follicular development, and oocyte characteristics that predict developmental competence were investigated in primiparous beef cows in the present experiment. Administration of PGF at ablation (d 0) in conjunction with insertion of a CIDR that was previously used for 5 d resulted in substantially lesser concentrations of P4 throughout follicular development as compared to the combination of P4 from the CL and a new CIDR. It was demonstrated that decreased P4 concentrations during early follicular development resulted in increased LH pulse frequency during early follicular growth. Additionally, follicles subjected to low P4 and increased LH were more estrogenic based on the increased E2 concentrations throughout early follicular development in the LoP4 treatment, as well as the negative relationship between P4 and E2 concentrations on d 2.5 and 3.5. However, no differences in indicators of developmental competence of oocytes were detected.

In the current experiment, LH pulse frequency in the LoP4 was almost twice that of the HiP4 treatment, in agreement with previous reports (Rahe et al., 1980; Schallenberger et al., 1985). Previous studies demonstrated that an extended period of decreased P4 concentrations resulted in a DF that grew larger than the normal ovulatory diameter and for a longer period (Sirois and Fortune, 1990, Stock and Fortune, 1993;
Anderson and Day, 1994), negatively impacting fertility (Savio et al., 1993; Mihm et al., 1994; Ahmad et al., 1995). In the present study, decreased P4 concentrations were afforded only in the period between follicular emergence and deviation (~ 3 d). Therefore the endocrine environment in the LoP4 treatment, although it resulted in increased gonadotropin stimulation during early follicular development, did not prolong the lifespan of the DF since the oocytes were retrieved either before or only one d after follicular deviation have occurred.

Decreased P4 concentrations during early follicular development have been previously demonstrated to increase follicle diameter in Bos indicus (Carvalho et al., 2008; Dias et al., 2009; Martins et al., 2014), Bos taurus (Pfeifer et al., 2009; Cerri et al., 2011b) and crossbred (Mantovani et al., 2010) cattle. In the current experiment, diameter of the largest follicle present in the ovaries approximately 2.5 d after emergence of the new follicular wave (OPU) did not differ, but was numerically greater in the LoP4 than in HiP4 treatment. Also, follicular diameter of the largest follicle in the ovaries was positively related with E2 concentrations on d 3.5. It is speculated that a longer period of sub-luteal P4 concentration and increased follicular gonadotropin stimulation would result in a DF of greater diameter in cows with lesser P4. In fact, using a similar animal model, we recently demonstrated that decreased P4 concentrations during early follicular development for a 5 d period from ablation to P4 removal, resulted in a DF of significantly greater diameter and increased E2 concentrations at P4 withdrawal; and an increased percentage of cows in estrus before timed-AI (Abreu et al., 2014). The postulate that LH pulse frequency augmented follicular growth rate in the present
experiment is further reinforced by the observation that on the d of OPU, follicular deviation had occurred in 69% of the LoP4 and 30% of the HiP4 treatment. Further, it was demonstrated that increased E2 concentrations on d 3.5 were related to the probability of follicular deviation to have occurred by OPU. Greater mean peripheral E2 concentrations as early as 2.5 d after follicular ablation (~ 1 d after new follicular emergence) further emphasizes the actions that the endocrine environment in the LoP4 treatment had on follicular function. This is perhaps not surprising and in agreement with previous report, in which was demonstrated that heifers that were GnRH-immunized, when supplemented with FSH and LH 92 d after immunization (h 0), had greater peripheral E2 concentrations at h 24 and beyond than heifers that received either FSH or LH alone (Crowe et al., 2001). Moreover, only heifers that received FSH and LH had medium (5-9 mm in diameter) and large (≥ 10 mm) follicles that were estrogen active (Crowe et al., 2001).

Stewart and colleagues (1995) reported that LH in conjunction with insulin-like growth factor I (IGF-I) stimulates steroidogenesis in bovine theca cells. Moreover, data from Kojima et al. (2003) suggested that frequency of LH release in response to varying P4 concentrations may control peripheral and intrafollicular steroids concentrations. Indeed, Cerri et al. (2011a) reported increased E2 concentrations in both circulation and follicular fluid of follicles that developed under low P4 concentrations. Although hormonal concentrations in the follicular fluid were not assessed in the current experiment, it is likely that intrafollicular E2 concentrations of follicles in the LoP4 were greater than in the HiP4 treatment. Van de Leemput et al. (1998) demonstrated that
greater intrafollicular E2 concentrations of preovulatory-sized follicles increased the chances of an oocyte to develop into a blastocyst following *in vitro* production. Moreover, levels of aromatase activity were detected to be greater in oocytes capable to develop to the blastocyst stage (Driancourt et al., 1998). In view of the aforementioned benefits of E2, it is somewhat surprising that oocytes that developed under low P4 and increased E2 concentrations in the present experiment did not have greater indicators of developmental competence than oocytes from the HiP4 treatment (i.e. FST and BCB+). This finding is in disagreement with previous findings in our laboratory and others that reported that lower P4 concentrations during early follicular development resulted in more oocytes grade 1 to 3 (Abreu et al, 2013), and yielded blastocysts with increased numbers of cells (Kruse et al. 2014) than oocytes recovered from follicles that developed in the presence of greater P4 concentrations.

It was postulated that oocytes acquire fully capacity to complete maturation and be fertilized at a diameter of ≥ 120 µm (Fair et al., 1995); this diameter is normally achieved when follicles are approximately 2-3 mm in diameter (Hyttel et al., 1997). In the present study, in which oocytes were collected only from follicles ≥ 3 mm, oocyte diameters ranged from 133 to 173 µm, indicating that all oocytes had surpassed this threshold, and that treatments did not differ in this characteristic. Surprisingly, only 44.5% of oocytes, across treatments stained positively for BCB, suggesting more than half of the oocytes were still growing at the time of collection. However, it is unsure whether BCB stain accurately indicates the stage of oocyte development since previous reports have shown that BCB+ oocytes were either of greater (Pujol et al., 2004;; Silva et al.,
2011; Janowski et al., 2012) or similar (Opiela et al., 2008) developmental capacity of BCB- oocytes. Oocytes collected in the present study were not submitted to the in vitro embryo production but rather analyzed for FST.

Oocyte derived FST has been identified as a marker of oocyte competence. Patel et al. (2007) demonstrated that abundance of mRNA for FST was greater in oocytes of increased developmental capacity in comparison to oocytes of lower developmental capacity. Additionally, a positive relationship between FST and time to first cleavage has also been detected, suggesting that this molecule may affect early embryonic development (Patel et al., 2007; Lee et al., 2009). It was speculated that the greater gonadotropin stimulation afforded during follicular development in the LoP4 treatment would enhance developmental competence that could be identified through mRNA expression for FST. However, in the current experiment, the relative abundance of oocyte mRNA for FST did not differ between oocytes recovered from follicles developing either under high or low P4 concentrations.

In conclusion, decreased P4 concentrations afforded greater gonadotropin stimulation during early follicular development, resulted in antral follicles that were more estrogenic, and apparently had enhanced development than follicles developing under increased P4 concentrations. The negative linear relationship between P4 and E2 concentrations, and the positive linear relationship between E2 concentrations and follicle diameter suggest that decreased P4 concentrations during early follicular development augmented the follicular environment for the oocyte to develop. However, the parameters
used in the current experiment to investigate oocyte developmental competence did not differ between oocytes retrieved either from LoP4 or HiP4 treatments.
Figure 3.1 Diagram of treatments (HiP4 = high progesterone; LoP4 = low progesterone), ultrasonography (US) and blood sample (BS). CIDR = intravaginal progesterone device (new = never used; used = previously used for 5 d). GnRH = gonadotropin release hormone. PGF = prostaglandin F2α. CL = corpus luteum. LH = luteinizing hormone. OPU = ovum pick-up. *LH serial blood collection was performed every 15 min for 12 h.
Figure 3.2 Mean (±SEM) progesterone concentrations on days 0, 1.5, 2.5, and 3.5 of the experiment between high progesterone (HiP4) and low progesterone (LoP4) treatments. *treatments differ within days ($P < 0.01$). Treatment*day: $P < 0.01$. 
**Figure 3.3** Mean (±SEM) estradiol concentrations on days 2.5 and 3.5 of the experiment between high progesterone (HiP4) and low progesterone (LoP4) treatments.

*treatments differ within days (P < 0.01). Treatment*day: P = 0.01.
<table>
<thead>
<tr>
<th>Variable</th>
<th>HiP4 (n = 6)</th>
<th>LoP4 (n = 7)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH pulse frequency, pulses/h</td>
<td>0.27 ± 0.05</td>
<td>0.46 ± 0.06</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Mean LH concentration, ng/ml</td>
<td>1.17 ± 0.15</td>
<td>1.58 ± 0.28</td>
<td>&gt; 0.1</td>
</tr>
<tr>
<td>Mean LH pulse amplitude, ng/ml</td>
<td>0.93 ± 0.15</td>
<td>0.95 ± 0.14</td>
<td>&gt; 0.1</td>
</tr>
<tr>
<td>Progesterone concentration, ng/ml</td>
<td>3.80 ± 0.95</td>
<td>1.29 ± 0.16</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Estradiol concentration, pg/ml</td>
<td>0.16 ± 0.08</td>
<td>1.36 ± 0.50</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

Table 3.1 Characteristics of LH, and progesterone and estradiol concentrations on d 2.5 of the experiment (mean ± SEM).
Figure 3.4. Representative LH secretion pattern throughout the serial bleeding; **A**: high progestesterone (HiP4) treatment; **B**: low progesterone (LoP4) treatment.
Table 3.2 Follicular characteristics and oocytes recovery between HiP4 and LoP4 treatments (mean ± SEM).

<table>
<thead>
<tr>
<th></th>
<th>HiP4 (n = 11 cows)</th>
<th>LoP4 (n = 13 cows)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follicles aspirated per cow, n</td>
<td>4.55 ± 0.49</td>
<td>5.23 ± 0.46</td>
</tr>
<tr>
<td>Size of the largest follicle at d 2.5, mm (range)</td>
<td>5.8 ± 0.3 (4.2 to 7.3)</td>
<td>6.3 ± 0.3 (4.4 to 8.5)</td>
</tr>
<tr>
<td>Size of the largest follicle at OPU, mm (range)</td>
<td>7.4 ± 0.5 (5.0 to 9.6)</td>
<td>8.9 ± 0.7 (6.2 to 11.1)</td>
</tr>
<tr>
<td>Follicular deviation, %</td>
<td>30(^b)</td>
<td>69(^a)</td>
</tr>
<tr>
<td>Total oocytes recovered per cow, n</td>
<td>1.82 ± 0.48</td>
<td>2.23 ± 0.34</td>
</tr>
<tr>
<td>Oocyte recovery rate, %</td>
<td>40</td>
<td>43</td>
</tr>
</tbody>
</table>

\(^{ab}\) Values tend to differ (P = 0.08)
Table 3.3. Characteristics of developmental competence in oocytes (mean ± SEM)

<table>
<thead>
<tr>
<th></th>
<th>HiP4 (n = 20 oocytes)</th>
<th>LoP4 (n = 29 oocytes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oocytes graded 1 to 3, %</td>
<td>40</td>
<td>38</td>
</tr>
<tr>
<td>Oocyte size, µm</td>
<td>155.4 ± 2.1</td>
<td>154.0 ± 1.8</td>
</tr>
<tr>
<td>BCB + staining, %</td>
<td>45</td>
<td>38</td>
</tr>
<tr>
<td>FST, relative expression¹</td>
<td>1.0 ± 0.51</td>
<td>0.34 ± 0.11</td>
</tr>
</tbody>
</table>

¹Oocyte relative expression of mRNA for follistatin (FST) was analyzed by cow (pool of oocytes)
**Figure 3.5.** Relationship between progesterone (P4) concentrations (ng/ml) on d 2.5 and estradiol (E2) concentrations (pg/ml) on d 2.5 in all cows regardless of treatment. Across treatments, decreased P4 concentrations on d 2.5 were related ($P = 0.03$) to increased E2 concentrations on d 2.5. The linear relationship was generated with the following equation: $[E2\ on\ d\ 2.5] = -0.1928 \times [P4\ on\ d\ 2.5] + 1.1776; \ r^2 = 0.191$
Figure 3.6. Relationship between progesterone (P4) concentrations (ng/ml) on d 3.5 (ovum pick-up) and estradiol (E2) concentrations (pg/ml) on d 3.5 in all cows regardless of treatment. Across treatments decreased P4 concentrations on d3.5 was related ($P = 0.05$) to increased E2 concentrations on d 3.5. The linear relationship was generated with the following equation: \[ E2 \text{ on d 3.5} = -0.2574 \times [P4 \text{ on d 3.5}] + 3.4609; r^2 = 0.1646 \]
Figure 3.7. Relationship between estradiol (E2) concentrations (pg/ml) on d 3.5 (ovum pick-up) and follicle diameter (mm) on d 3.5 in all cows regardless of treatment. Across treatments increased E2 concentrations on d3.5 were related ($P = 0.03$) to greater follicle diameter on d3.5. The linear relationship was generated with the following equation:

Follicle diameter on d 3.5 = 0.3522 x [E2 on d 3.5] + 6.9690; $r^2 = 0.2016$
Figure 3.8. Relationship between estradiol (E2) concentrations (pg/ml) on d 3.5 (ovum pick-up) and probability of follicular deviation (%) in all cows regardless of treatment. Across treatments increased E2 concentrations on d3.5 were related \( (P = 0.02) \) to increased probability of follicular deviation occur by d 3.5. The linear relationship was generated with the following equation: Probability of follicular deviation = 0.1271 x [E2 on d 3.5] + 0.1992; \( r^2 = 0.2474 \)
CHAPTER 4
GENERAL DISCUSSION

It is clear that P4 influences on LH and E2 concentrations even in early stages of follicular development. The effects of P4, and its influence in other hormones are major endocrine events determinant for fertilization, maintenance and establishment of pregnancy. Both the oocyte and the environment for the oocyte to develop can be directly and indirectly affected by P4 during the recruitment of follicles, their maturation and development. Several reports attempted to describe the effects of P4 on oocyte development and fertility; however the great variability of factors such as species (Bos taurus vs. Bos indicus), breed, class (heifers, primiparous and multiparous), and production type (beef or dairy) creates difficulties and misinterpretations in both in vivo and in vitro experiments. Nutritional status and age (prepubertal and aged cows) are factors that must also not be ignored. The models used to study the effects of P4 vary among researchers in the area; therefore the comparisons between studies have to be carefully interpreted. Basic experiments that deepen the molecular characteristics of the P4 actions are of great value; in contrast, the ultimate outcome for fertility, pregnancy rates, is only achieved and significantly meaningful when satisfactory number of animals is inseminated. Meta analyses of the literature are of little importance in this case due to the huge range of differences in experimental conditions. Therefore, the speculations
have to be created based predominantly on conclusions made throughout the reports available in this subject. Terms recently used such as differences in sensibility to P4 between heifers and cows, beef and dairy, and Bos indicus and Bos taurus probably has some sense, but no one has demonstrated such a phenomenon exists. In addition, the endocrine environment that the complex follicle/oocyte/adjacent cells experience locally within the ovary is a challengeable measurement. In order to make a long story short, as probably everything in science, more comprehensive studies are needed. The main variables in the initial model of such experiments will either be related to or actually be: E2, LH, FSH, ........proestrus length, animal class, d postpartum, age at puberty, follicle size, follicle age........number of receptors, time for responding for exogenous hormones (genomic and non-genomic actions), mRNA content.......uterine environment, maternal recognition of pregnancy, corpus luteum lifespan and subsequent estrous cycle hormone production, embryo size/weight, uterine tonus.......pregnancy establishment, maintenance.........live calf born........until the profit somehow is trapped in the producer pocket.
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BMP15 are associated with both increased ovulation and sterility in Cambridge and Belclare sheep (Ovis aries). Biol. Reprod. 70:900-909.


