University of Alberta

Effect of porcine luteinizing hormone on intrafollicular milieu and gene expression in granulosa cells and oocytes in dairy cows

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science

in

Animal Science

Department of Agricultural, Food and Nutritional Science

Spring 2014
Edmonton, Alberta

Dedication

This thesis is dedicated to my parents and my uncle. Thank you for your unconditional encouragement, enthusiasm, love and support.

Abstract

In previous research, the use of porcine luteinizing hormone (pLH) in lieu of gonadotropin-releasing hormone (GnRH) for synchronizing ovulation in a fixed-timed artificial insemination protocol in dairy cows improved pregnancy rates (42 vs 28%) without increasing progesterone concentrations. The mechanisms associated with this increase in pregnancy rates are not known. We hypothesized that exposure to pLH alters intrafollicular protein/gene expression compared to GnRH treatment, particularly the oocyte-expressed members of the TGF-β superfamily, and EGF-like growth factor, which regulate oocyte competence. To address this, two studies were conducted to determine whether replacing pLH with GnRH to synchronize ovulation in dairy cows altered intrafollicular milieu and gene expression in mural granulosa cells and oocyte cumulus complexes. The results indicated that the relative abundance of BMP-15, GDF-9 and TGF-β1 were greatly increased in pLH-treated cows. Since these factors promote cumulus expansion and oocyte competence, they may have contributed to the pregnancy increase previously reported.

Acknowledgements

It has been a great pleasure to spend the past several years in the Department of Agricultural, Food and Nutritional Science at the University of Alberta. The memories of these years, and the kindness and helpfulness of its members, will always remain dear to me. I still remember my first meeting with Dr. Divakar Ambrose when I was looking for an advisor for my Master's thesis. My first and foremost debt of gratitude goes to Dr. Ambrose who agreed to supervise my thesis. I deeply appreciate his generous patience, encouragement and guidance. He has taught me how to think critically and scientifically, and as a result, helped me grow as a researcher. I am grateful to him for all his support while I was in the Master's program and I will always feel indebted to him. I also express my gratitude to my supervisory committee member Dr. Michael Dyck for his valuable and constructive comments and suggestions which helped me to fine-tune this project. I also thank Dr. John Kastelic, for serving in the capacity of the external examiner on my defense committee.

It is with immense gratitude that I acknowledge the support and help of Dr. Marcos Colazo. Knowing that I could rely on his vast knowledge and experience in the field of bovine reproductive physiology, I always felt confident during my field studies. I have learned several reproductive technologies in dairy cattle from him, and this has definitely improved my understating of reproductive physiology. I owe my deepest gratitude to Dr. Ana Ruiz-Sanchez for her assistance with processing the collected samples in our IVF lab. I am beyond grateful for her moral support in every step of the lab analysis, and also for sharing her lab

experiences with me. I cannot find words to express my gratitude to Mr. Reza Salehi and Mr. Mohammad Ali Fakheri for their willingness to help me during my sample collection days at the Dairy Research and Technology Center. I also extend my thanks to Mr. Farshad Hassanzadeh for making the Western Blot training available to me. I also take this opportunity to acknowledge the support, help, and encouragement of Dr. Majid Behrouzi, Dr. Mojtaba Yegani and Dr. Ghader Manafiazar.

This thesis would not have been possible without financial support from the following institutions and organizations: the Department of Agricultural, Food and Nutritional Science of the University of Alberta, Dairy Research and Technology Centre Ignition Fund, Alberta Agriculture and Rural Development and Alberta Milk. Product donations by Bioniche Animal Health Canada Inc. (Folltropin-V® and Lutropin-V®), Vetoquinol Canada Inc. (Estroplan®, Fertiline® and PRID®) are gratefully acknowledged. I express my gratitude to all of them.

And finally, God (Tanrim) who is always there for me and empowered me to finish this invaluable research program.

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List of Abbreviations

AI	Artificial insemination
AREG	Amphiregulin
BS	Blood sample collection
bLH	Bovine luteinizing hormone
BMP-15	Bone morphogenetic protein-15
BSA	Bovine serum albumin
BTC	Betacellulin
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxy-ribonucleic acid
CL	Corpus luteum
COC	Cumulus oocyte complex
C_T	Cycle threshold
DF	Dominant follicle
DPBS	Dulbecco's phosphate buffered saline
d	Day
EGF	Epidermal growth factor
EREG	Epiregulin
E_2	Estradiol
FA	Follicle aspiration
FSH	Follicle stimulating hormone
GAPDH	Glyceraldehyde-3-phosphate-dehydrogenase
GDF9	Growth differentiation factor-9

GnRH	Gonadotropin releasing hormone
GOI	Gene of interest
GPCR	G protein coupled receptors
h	Hour
hCG	Human chorionic gonadotropin
IGFBP	Insulin-like growth factor-binding protein
IGF-I	Insulin-like growth factor I
im	Intramuscular
IU	International units
L	Litre
LH	Luteinizing hormone
LHr	Luteinizing hormone receptors
LS means	Least squares means
min	Minute
mEQ	Milli-equivalents
mg	Milligram
ml	Millilitre
mRNA	Messenger ribonucleic acid
NEB	Negative energy balance
NEFA	Non-esterified fatty acids
ng	Nanogram
PBS	Phosphate buffered saline
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$

PGF	Prostaglandin $F_{2\alpha}$
PGr	Progesterone receptors
P_4	Progesterone
pLH	Porcine luteinizing hormone
PRID	Progesterone-releasing intravaginal device
RT-PCR	Real-time quantitative polymerase chain reaction
SAS	Statistical Analysis System
SBS	Sequential blood sample collection
SEM	Standard error of the mean
SDHA	Succinate dehydrogenase complex
TAI	Timed artificial insemination
TGF-β	Transforming growth factor family beta
US	Ultrasonographic examinations

Chapter 1. General Introduction

1.1. Introduction

Reproductive efficiency in dairy cattle has been decreasing over the past few decades, despite genetic selection, better management, and improved feeding practices. According to the CanWest Dairy Herd Improvement (DHI), Western Herd Improvement Report, the number one reason for culling of cows from a dairy herd was reproductive failure. During 2012, the number of cows culled for reproductive problems was 5,071 cows (29%) in Alberta, whereas mastitis or high somatic cell count and low milk production accounted for 17 and 15% of cows' culled reasons. In a 23-herd study in Alberta (Ambrose and Colazo, 2007) undetected estrus, low artificial insemination submission rates, and long inter-breeding intervals were major contributors to poor reproductive efficiency.

Furthermore, high embryonic mortality, poor estrous behavior, low blood concentrations of reproductive hormones, and increased metabolic and reproductive disorders are some of the important problems that are prevalent in high producing dairy cows (Lucy, 2001). Time artificial insemination (TAI) is a key strategy that can improve reproductive efficiency without estrus detection. The Ovsynch protocol (Pursley et al., 1995) has been successfully used to synchronize ovulation in dairy cattle; the protocol consists of two treatments of 100 µg gonadotropin-releasing hormone (GnRH) given 9 days apart, with 500 µg prostaglandin $F_{2\alpha}$ (PGF) given 7 days after first GnRH, and FTAI 16-20 h after the second GnRH treatment. The first GnRH treatment of Ovsynch protocol is intended to trigger emergence of a new follicular wave within approximately 2 days and

induce ovulation if a dominant follicle is present (Macmillan and Thatcher, 1991; Twagiramungu et al., 1995). Although the protocol works well, administration of GnRH resulted in a shorter LH surge than that of a spontaneously occurring preovulatory LH surge (Colazo et al., 2008). Use of porcine luteinizing hormone (pLH) in lieu of the second GnRH treatment resulted in plasma LH concentrations elevated for a considerably longer interval than those after GnRH treatment (Ambrose et al., 2005; Ree et al., 2009). Furthermore, in a recent study by our research group (Colazo et al., 2009), replacing the second GnRH treatment with pLH in dairy cows significantly improved pregnancy rates (42 vs 28%), yet peripheral plasma progesterone concentrations after 12 d post ovulation were not increased, suggesting that the pregnancy improvement occurred independent of a progesterone increase.

1.2. Research hypothesis

The mechanisms associated with this increase in pregnancy rates are still not known. We hypothesized that exposure to pLH alters the intrafollicular environments including follicular fluid factors, expression of genes regulating preovulatory follicular function and oocyte competence. Overall, this thesis aimed to identify follicular fluid factors and target genes in mural granulosa cells and the cumulus oocyte complex that may be differentially regulated after pLH versus GnRH treatment.

1.3. Literature cited

Ambrose, J. D., J. P. Kastelic, R. Rajamahendran, M. Aali and N. Dinn. 2005. Progesterone (CIDR)-based timed AI protocols using GnRH, porcine LH or estradiol cypionate for dairy heifers: Ovarian and endocrine responses and pregnancy rates. Theriogenology. 64:1457-1474.

Colazo, M. G., M. B. Gordon, R. Rajamahendran, R. J. Mapletoft and D. J. Ambrose. 2009. Pregnancy rates to timed artificial insemination in dairy cows treated with gonadotropin-releasing hormone or porcine luteinizing hormone. Theriogenology. 72:262-270.

Colazo, M. G., J. P. Kastelic, H. Davis, M. D. Rutledge, M. F. Martinez, J. A. Small and R. J. Mapletoft. 2008. Effects of plasma progesterone concentrations on LH release and ovulation in beef cattle given GnRH. Domestic Animal Endocrinology. 34:109-117.

Lucy, M. C. 2001. Reproductive loss in high-producing dairy cattle: Where will it end? Journal of Dairy Science. 84:1277-1293.

Macmillan, K. L. and W. W. Thatcher. 1991. Effects of an agonist of gonadotropin-releasing hormone on ovarian follicles in cattle. Biology of Reproduction. 45:883-889.

Pursley, J. R., M. O. Mee and M. C. Wiltbank. 1995. Synchronization of ovulation in dairy cows using PGF2alpha and GnRH. Theriogenology. 44:915-923.

Ree, T. O., M. G. Colazo, A. G. Lamont, J. P. Kastelic, M. K. Dyck, R. J. Mapletoft, B. N. Ametaj and D. J. Ambrose. 2009. The effect of porcine luteinizing hormone in the synchronization of ovulation and corpus luteum development in nonlactating cows. Theriogenology. 72:120-128.

Twagiramungu, H., L. A. Guilbault and J. J. Dufour. 1995. Synchronization of ovarian follicular waves with a gonadotropin-releasing hormone agonist to increase the precision of estrus in cattle: A review. Journal of Animal Science. 73:3141-3151.

Chapter 2. Review of Literature

2.1. Oogenesis and folliculogenesis

Ovarian follicles are classified as primordial, primary, secondary, tertiary, and antral follicles in the ovarian cortex. This classification is based on the size of the oocyte, granulosa cell morphology and number of layers of granulosa cells surrounding the oocyte, presence of antrum, etc. (Fair, 2003; Aerts and Bols, 2010b). The ovaries comprise large reserves of non-growing primordial follicles in mammals. Each primordial follicle contains an immature quiescent egg cell or oocyte (Fair, 2003). The primordial follicles are isolated from a basal lamina and have a single layer of 4–8 pre-granulosa cells; the oocyte is surrounded by these flattened granulosa cells (Fair, 2003; Adams et al., 2008).

After appearance of the second layer of the granulosa cells, oocyte growth induces formation of a zona pellucida (Bilodeau-Goeseels, 2008; Aerts and Bols, 2010b). Deposition of the zona pellucida material around the oocyte is characterized by formation of gap junctions between granulosa cells and individual theca cells (Bilodeau-Goeseels, 2008; Aerts and Bols, 2010b). The somatic cells in the follicle are classified into two major types: the more internal granulosa cells that surround the oocyte before antrum formation, and theca cells, which are separated from the granulosa cells by a basement membrane (Albertini and Anderson, 1974). The cumulus oophorus is differentiated granulosa cells that surround the oocyte after antrum formation (Bilodeau-Goeseels, 2008). The cumulus cells are attached to the oocyte with gap junctions (Bilodeau-Goeseels, 2008; Aerts and Bols, 2010b). Consequently, all essential nutrients for the growth of oocyte such as glucose

metabolites, amino acids and nucleotides are transferring through the gap junctions to the oocytes (Eppig, 1991). An antral follicle is classified based on a fluid-filled cavity (antrum); the fluid inside it is called follicular fluid (Fair, 2003). The development of antral follicles is initiated during fetal life and the first antral follicles can be visible approximately on day 230 of the gestation in cattle (Russe, 1983). A dominant preovulatory follicle is called the Graafian follicle (Figure 2.1). A Graafian follicle has three distinct layers which are: the theca externa, the theca interna and the granulosa cell layer (Fair, 2003; Aerts and Bols, 2010b).

The developmental process from primordial to preovulatory follicle takes about 180 days in the cow (Lussier et al., 1987; Fair, 2003). The main part of this development occurs in the pre-antral stage; as a result, development from primordial to antral follicle requires approximately 42 days in cows (approximately two estrous periods; Aerts and Bols, 2010b). The incessant activation of primordial follicles is in marked contrast to the cyclic recruitment of antral follicles during each estrous cycle. The continuous primordial follicle activation and growth of antral follicles result in elevation of follicle stimulating hormone (FSH) during the estrous cycle (Ginther et al., 1998; Findlay and Drummond, 1999). This step is known to be critical for follicular development and reproductive viability. Follicle stimulating hormone receptors (*FSHr*) are located only in granulosa cells, which can supply extra FSH stimulus during follicular wave and participate in regulating oocyte growth (Ginther et al., 1998; Adams et al., 2008).

Luteinizing hormone receptors (*LHr*) are present in the both theca cells and granulosa cells of antral follicles (Adams et al., 2008); furthermore, *LHr* are more

likely located in granulosa cells of the dominant follicle than in subordinate follicles. The dominant follicle shifts its gonadotropin dependence to the *LHr* when FSH secretion is suppressed; consequently, the growth phase of dominant follicle continues while the subordinate follicles regress (Adams et al., 2008). In addition, *LHr* expression on cumulus granulosa cells changes only slightly during follicular development, whereas this expression on mural granulosa cells increases as the follicle matures (Goudet et al., 1999).

2.2. Effects of LH exposure on pre-antral follicular development

The transition of pre-antral to antral follicle occurs via development of meiotic competence (Aerts and Bols, 2010b). The resumption of meiosis in the oocyte is re-initiated when it reaches its full size in the growing follicle (Bilodeau-Goeseels, 2008). Preovulatory LH surge triggers expansion of the cumulus cover and disruption of the contact between oocyte membrane and corona radiata that prepare the oocyte for final maturation and competence (Fair, 2003). The resumption of meiosis initiates breakdown of the large oocyte nucleus and release of its nucleoplasm into the cytoplasm (Aerts and Bols, 2010b). The process is termed germinal vesicle breakdown (Bilodeau-Goeseels, 2008; Aerts and Bols, 2010b). The oocyte chromosomes condense and progress through final stage of meiosis I and arrest at metaphase of meiosis II (Fair, 2003).

In addition, a preovulatory LH surge also induces alterations in the follicle and cumulus cells, which results in secretion of hyaluronic acid, a non-sulfated glycosaminoglycan that can be bound to the cumulus cells by linking proteins (Aerts and Bols, 2010b). Mural and cumulus granulosa cells synthesize hyaluronic

acid and cumulus expansion depends on synthesis and secretion of the hyaluronic acid by cumulus cells. (Aerts and Bols, 2010b). When the hyaluronic acid becomes hydrated, the hydration develops spaces between cumulus cells and forms mucified matrix (Eppig, 2001). The process of cumulus expansion is an important feature of the ovulation mechanism; which is instrumental in facilitating fertilization (Zhuo and Kimata, 2001).

2.2.1. Function of LH during oocyte maturation

Oocyte maturation is defined at three levels: meiotic maturation, cytoplasmic competence and molecular maturation (Sirard and Assidi, 2013). As previously discussed, the LH surge triggers differentiation of follicular cells as well as oocyte maturation in mammals; moreover, it can start meiotic maturation and a cascade of nuclear events (Adams et al., 2008). Cytoplasmic maturation of oocyte is initiated with modification of ooplasm during pre-competence prior to the LH surge when the oocyte waits for the ovulatory signal (Sirard and Coenen, 2006).

Cytoplasmic competence is initiated by terminating the oocyte preparation phase (RNA and protein synthesis) and re-distribution of the cortical granules and mitochondria at metaphase stage close to the LH surge (Sirard et al., 2006). Cyclic AMP (cAMP) is synthesized by adenylyl cyclases (AC) and is degraded by phosphodiesterases (PDE) (Bornslaeger et al., 1986). It is hypothesized that activation in the degradation of cAMP by PDE induces LH stimulation; subsequently, oocyte activation is initiated (Bilodeau-Goeseels, 2008).

Epidermal growth factor (EGF) is a polypeptide growth factor that stimulates cell growth, proliferation, and differentiation by binding to the EGF

receptor (Bilodeau-Goeseels, 2008). The LH induces the rapid and transient expression of EGF family members including amphiregulin *(AREG)*, epiregulin *(EREG)*, and β-cellulin *(BTC)*, which can induce resumption of meiosis in oocytes (Conti et al., 2012; Figure 2.3). These molecules can be up-regulated in granulosa cells in response to LH (Park et al., 2004). In addition, these molecules and LH are involved in stimulating meiotic resumption of follicle- and cumulus-enclosed oocytes (Bilodeau-Goeseels, 2008). Furthermore, it has been reported that LH stimulation can be phosphorylated by the EGF receptor and pharmacological inhibition of phosphorylation of EGF (Park et al., 2004). Phosphorylation of the EGF receptor and increases in *AREG* and *EREG* mRNAs in reaction to LH stimulation precede oocyte germinal vesicle breakdown by at least 2 h (Panigone et al., 2008).

The transforming growth factor β-family is involved in regulation of early follicular development. Bone morphogenetic protein-15 (BMP-15) and growth differentiation factor-9 (GDF-9) that are secreted by the oocyte, induce granulosa cell mitosis and proliferation (Orisaka et al., 2006) and inhibit granulosa cell apoptosis and follicular atresia (Moore et al., 2003), respectively,

2.2.2. Role of LH during the estrous cycle

The majority (i.e., >95%) of bovine estrous cycles consists of either two or three follicular waves (Adams, 1998; Figure 2.2). In both two- and three-wave estrous cycles, the first growth wave of the cycle emerges on the day of ovulation (Day 0) (Adams et al., 2008; Aerts and Bols, 2010a). The next wave (second wave) in a two-wave cycle starts on day 9–10, whereas the second and third waves emerge

on days 8 to 9 and days 15 to 16, respectively, in three-wave cycles (Mapletoft et al., 2002; Aerts and Bols, 2010a). Consequently, the dominant follicle (DF) ovulates in the last wave of either the second or third wave (Adams et al., 2008).

Preovulatory LH pulses are extremely important to follicle maturation and ovulation in mammals. The LH receptors appear in granulosa cells 8 h before the beginning of deviation in future dominant follicle in heifers (Ginther et al., 200b). It is generally accepted that LH receptors emerge in the follicle when it attains 8 mm diameter; furthermore, the change in dependence from FSH to LH results in DF establishment (Mihm et al., 2006). Although, basal FSH concentrations remain critically important during dominance, the DF still is able to mature and survive with low circulating FSH concentrations (Aerts and Bols, 2010a). The functional changes in DF result in a decline in the intrafollicular ratio of estradiol-17β (E_2) to progesterone (P_4) during dominance that can initiate preparation for ovulation (Mihm et al., 2006). In other words, there is a decrease in intrafollicular estradiol-17β concentrations during the LH-induced maturation of the dominant ovulatory follicle. Gonadotropin-releasing hormone (GnRH) pulse frequency is acutely inhibited by progesterone; subsequently, progesterone is regarded as the primary regulator of LH (McCartney et al., 2007). In addition, the positive effect of GnRH pulse frequency can increase peripheral estradiol concentrations thus resulting in elevated LH secretions (Aerts and Bols, 2010a).

Corpus luteum (CL) is a structure in the ovary and secretes progesterone that prevents the animal from returning to estrus. Formation of CL widely depends on episodic LH pulses, whereas LH pulses are not essential for maintenance of

luteal function (Aerts and Bols, 2010a). Progesterone secretion from an active CL can negatively affect LH pulse frequency, decreasing the LH pulse frequency in the mid-luteal phase and resulting in atresia of the LH dependent DF (Aerts and Bols, 2010a). The DF will only benefit from increasing LH pulse frequencies in the follicular phase, which will promote maturation and finally ovulation (Mihm and Austin, 2002).

2.3. Fertility in lactating dairy cattle

Global demand for dairy products is continuing to grow and in order to meet this growing demand, the size of dairy herds has increased and individual cows now produce more milk. For instance, milk production per Holstein cow in Alberta has increased from 7,717 kg/year on average in 1991 to 9,666 kg/year in 2012 based on published records (Can West DHI 2012). Increased herd size and milk production dramatically affect herd reproductive efficiency. This could happen because the lactation period is initiated and renewed by the pregnancy and milk production; that is, it largely depends on a cow's ability to become pregnant. Fertility is an important economic indicator for evaluation of dairy herd productivity and is defined as the ability of a cyclic animal to establish pregnancy (Plaizier et al., 1998). Declines in fertility and poor reproductive efficiency in lactating dairy cows are still the critical limiting aspects in sustainability and profitability of dairy production (Lucy, 2001). Although reproductive performance is dependent on a combination of management factors and physiological pathways in lactating dairy cows, service rate and maternal fertility can greatly influence reproductive performance (Pursley et al., 1997).

2.4. Management strategies to improve fertility in dairy cows

Changes in lactating dairy cattle physiology, improvements in nutrition, genetics and management approaches can all influence dairy herd fertility. Improved nutritional management, increased estrus detection followed by artificial insemination (AI) and timed artificial insemination are feasible approaches to improve reproductive efficiency in dairy cattle.

2.4.1. Nutrition

Nowadays, high-producing postpartum cows are fed more dry matter to match requirements for milk production. Feeding high-energy rations or providing specific nutrients is one strategy to increase the establishment and maintenance of pregnancy (Butler, 2003). Nevertheless, high-producing postpartum dairy cows enter a state of negative energy balance (NEB) and body weight loss 1 to 2 weeks after calving when the energy demands for lactation and maintenance exceeds that of energy intake (Bauman and Currie, 1980) and they may remain in a state of anestrus for varying periods of time. The resumption of ovarian activity and the initiation of a new follicular wave typically occurs during the NEB period (Beam and Butler, 1999). Consistently, lower pregnancy rates and high pregnancy losses occurred more often in cows with NEB and low body condition scores (Thatcher et al., 2006). Negative energy balance can suppress pulsatile LH secretion and insulin-like growth factor I (IGF-I) (Lucy, 2000). Furthermore, ovarian response to LH stimulation can be reduced during NEB (Butler, 2003). Suppressing LH pulse frequency has deleterious effects on the sufficient estradiol secretion by dominant follicle which is needed to induce a preovulatory gonadotropin surge (Roche et al.,

1999). Both IGF-I and LH are required for fully functional development of an estrogenic follicle and the combined effect of a reduction of both hormones can suppress follicular growth and ovulation (Lucy, 2001).

2.4.2. Artificial insemination

Artificial insemination is known as the first feasible biotechnological approach to improve reproduction and genetics of farm animals. The technique has been used commercially since the 1940s. Artificial insemination can increase the efficiency of male usage and is a safe procedure for both animals and farmers (Gallagher and Senger, 1989). Artificial insemination provides a potential for using progeny-tested sires that can facilitate rapid improvement in genetics, animal production and conformation (Overton and Sischo, 2005). It also minimizes costs of maintaining male animals, risk of disease transmission, inbreeding depression, and injury hazards to farm workers. The inbreeding depression is one of the contributing factors that may cause the decline in fertility (Funk, 2006). In addition, AI can improve selection intensity by the use of superior males and enables management of breeding and parturition dates (Vishwanath, 2003; Johnson and Jones, 2008).

Although AI provides numerous benefits, inseminator experience can influence the efficiency of AI. Technician skill, imperfections in the handling process of semen and human-animal interactions during insemination can affect the probability of satisfactory results (Pfeifer et al., 2009). Indeed, failure to detect cows in estrus is the biggest and most important challenge for AI success in the most herds (Senger, 1994; Leblanc, 2005; Ambrose and Colazo, 2007).

2.4.3. Estrus detection

Since AI is extensively used in dairy cattle, estrus detection is the most important component of a successful and effective reproductive management program. Traditionally, visual observation has been used for estrus detection in dairy herds, which is tedious, incurs high labor costs and requires diligent attention (At-Taras and Spahr, 2001). Increased physical activity (mounting and walking), physiological changes (increased vaginal mucus resistance, slightly elevated body and milk temperature and reduced milk production) and behavioral signs (sniffing the vagina and flehmen) are some methods that have been used to facilitate estrus detection (At-Taras and Spahr, 2001; Firk et al., 2002). Estrus detection can be improved by the use of chalking the tail head (Halsey D, 1978), heat mount detectors (At-Taras and Spahr, 2001), pedometers (Felton et al., 2012), Heat Watch^{TM} (Ambrose et al., 2010) and progesterone test (Delwiche et al., 2000). Prostaglandin $F_{2\alpha}$ (PGF) is released naturally from the uterus around 16 to 18 days after estrus in the absence of an embryo. Prostaglandin $F_{2\alpha}$ treatment can be used effectively to regress the CL. The corpus luteum regression results in rapid reduction in progesterone concentrations (Hittinger et al., 2004), subsequently; the cow returns to estrus. The most common estrus synchronization protocol is to treat the cows with PGF and breed those that are detected in estrus over the next 5 to 7 days (Folman et al., 1990). Cows that are not detected in estrus will receive a second PGF treatment 11 to 14 days later and again observed for estrus for up 5 days and bred at estrus. The administration of an intravaginal progesterone device 7 d prior to PGF followed by a single injection of PGF is another method to improve

synchronization of estrus (Macmillan and Peterson, 1993). Administration of an exogenous progesterone device could prevent premature estrus and ovulation in cattle that naturally undergo CL regression (Roche et al., 1999; Kim et al. 2003). The use of PGF is a simple, affordable and easy-to-administer method to synchronize estrus in cattle. Regardless, it is ineffective on animals that do not possess a CL, cows in days 1 to 5 of the estrous cycle, prepubertal heifers and postpartum anestrous cows.

Accurate estrus detection is the main factor in herds using artificial insemination to improve reproductive efficiency. Estrus detection rate is the major index that can influence fertility. However, the average estrus detection rate (eligible cows submitted for AI), conception rate and pregnancy rate were 33, 38 and 13%, respectively, in Canadian dairy herds (Leblanc, 2005), consistent with a recent study in Alberta (Ambrose and Colazo, 2007). Based on both studies, it can be concluded that a very high proportion of estrus events go undetected. For instance, type of flooring (concrete vs. dirt; Britt et al., 1986) and housing system (tie-stall vs free-stall; Kiddy, 1977) can influence the estrus detection rate. The accurate of the estrus detection rate can vary within visual observation or assisted detection techniques such as tail chalk, Heat Watch ^{TM}, pedometers or other electronic aids and duration, interval and frequency of the observation (Xu et al., 1998; Ambrose et al., 2010; Felton et al., 2012).

2.5. Timed artificial insemination protocols

Timed artificial insemination (TAI) programs can overcome deficiencies in estrus detection and can improve pregnancy rate. At the present time, use of GnRH

in combination with PGF followed by TAI is recommended for synchronization of estrus. Timed AI programs can eliminate estrus detection, improve pregnancy rates and reduce the interval from calving to first service (Ambrose et al., 2010). Moreover, the protocol is a feasible method to get cystic and anestrous cows pregnant and is viewed as an applicable and important protocol to enhance optimal reproductive management (Ambrose et al., 2010).

2.5.1. Synchronization of ovulation (Ovsynch)

Hypothalamic neurosecretory neurons secrete GnRH (Mellon et al., 1990) that can then stimulate secretion of the follicle stimulating hormone (FSH) and LH from the anterior pituitary gland (Senger, 2003). Regression of the CL results in reduction of progesterone concentrations in the bloodstream and CL regression and progesterone decline result in elevated estradiol concentrations which triggers the LH surge (Colazo et al., 2008). A combination of GnRH and PGF were used to develop synchronization protocols for estrus (Thatcher et al., 1989) and ovulation (Pursley et al., 1995) when the fundamental role of GnRH in follicular growth and ovulation were established.

Ovsynch (Pursley et al., 1995) is the common Timed-AI protocol that consists of two injections of GnRH given 9 d apart, with an intervening injection of PGF given 7 d after the first GnRH treatment, followed by AI, 16-20 h later (Figure 2.4). Select-synch, Heat-synch, Pre-synch and Co-synch are some modified versions of Ovsynch protocol (Patterson et al., 2003). For instance, Co-synch is the most common modification that involves increasing the interval from PGF to

second GnRH to 56 h, or combining the second GnRH with AI, usually 56 to 66 h after PGF (Ambrose et al., 2010).

The first GnRH treatment is performed at a random stage of estrous cycle in the Ovsynch protocol. Administration of the initial GnRH initiates a new follicular wave and ovulates a dominate follicle, if present (Macmillan and Thatcher, 1991; Wolfenson et al., 1994); as well, first GnRH treatment may also increase the response to the PGF treatment. The CL regression will be initiated by PGF treatment 7 day after first GnRH treatment; meanwhile, the inhibitory action of progesterone will be eliminated. Thus, growth and maturation phase of follicle in preparation for ovulation will be started. The second GnRH treatment stimulates LH secretion and increases the synchrony of ovulation by inducing ovulation of the preovulatory follicle (Chenault et al., 1975; Ambrose et al., 2005).

2.6. Use of porcine luteinizing hormone (pLH) in Ovsynch protocol

2.6.1. Introduction

The administration of GnRH at random stages of the estrous cycle may not synchronize emergence of a new wave and induce the ovulation of the dominant follicle; for instance, only 50% of dairy heifers ovulated in response to 100 µg GnRH treatment (Ambrose et al., 2005). This is because the effectiveness of treatment is dependent on the stage of follicular development at the time of (Pursley et al., 1995). In another study, heifers were treated with 25 mg pLH or 100 µg GnRH on days 3, 6 or 9 after ovulation (Martinez et al., 1999). The proportion of heifers that ovulated after pLH treatment were higher compared to GnRH treatment in the Martinez et al. (1999) study; ovulation rates were 78 and 56% for pLH- and

GnRH-treated heifers, respectively. Lutropin-V® (Bioniche Animal Health, Belleville, ON, Canada) is the only commercially available porcine LH product in Canada; nonetheless, the product is rarely used for ovulation synchronization in cattle. The higher cost of pLH relative to GnRH may be a limiting factor.

2.6.2. Previous research findings in cattle treated with pLH

2.6.2.1. LH profile

In a preliminary study, dairy heifers were treated with 12.5 or 25 mg pLH or 100 mg GnRH in a modified Ovsynch protocol (Ambrose et al., 2005) in which a controlled internal drug [progesterone] release (CIDR®) device was placed for 7 days in all heifers. Heifers given GnRH had an acute LH response, and LH reached peak concentrations 2 h after basal concentrations of <0.5 ng/mL to a mean peak of 6.2 ng/mL and returned to basal concentrations within 5 h. However, LH concentrations in pLH-treated cows peaked at approximately 2 ng/mL 2 h after treatment (Ambrose, personal communication) and it remained elevated for up to 20 h. In addition, progesterone concentrations 9 d after pLH treatment were higher than that of those treated with GnRH (Ambrose et al., 2005) suggesting that pLH-treated heifers had increased P_4 production at the ovarian level.

In a follow-up study, the rise in LH occurred 15 min after giving 100 µg GnRH, reached peak concentrations after 2 h, and returned to pre-treatment concentrations after 5 h in dairy cows during proestrus (Ambrose, unpublished), whereas, LH concentrations remained elevated for 12 h in 25 mg pLH-treated cows.

2.6.2.2. Improvement in pregnancy rate

Colazo et al. (2009a) compared the effects of using pLH in lieu of GnRH on ovulatory response and pregnancy rate after Timed-AI (Ovsynch) in a study involving 605 lactating dairy cows. Pregnancy rate to Timed-AI was significantly ($P < 0.01$) improved by 14-percentile points in the GnRH/PGF/pLH group of cows (42%) compared to cows in the GnRH/PGF/GnRH group (28%). Colazo et al. (2009a) reported that the improvement in pregnancy rates of pLH-treated cows occurred without increasing progesterone concentrations 12 d after ovulation or significantly reducing embryonic losses. Therefore, it was concluded that this improvement in pregnancy rate must have occurred through a mechanism other than elevated progesterone.

2.6.2.3. Corpus luteum structure and function

Follicles and CL diameter were assessed by transrectal ultrasonography after administration of 12.5 or 25 mg pLH or 100 μg GnRH and no treatment (control) during proestrus in non-lactating Holstein cows (Colazo et al., 2009b). Preovulatory follicle diameter was greater in control cows ($P < 0.05$); regardless, CL area did not differ among treatments. As previously reported, overall mean plasma P_4 did not differ ($P > 0.05$) between both groups, although pLH-treated cows had prolonged LH concentrations. In a follow-up study, cows were ovariectomized 12 days after either 12.5 or 25 mg pLH, or 100 μg GnRH treatment and CL structure and function were studied (Ambrose et al., 2009); the CL weight and number of small luteal cells per unit area did not differ among the treatments in that study.

2.6.2.4. In vivo and in vitro progesterone production

The *in vitro* and *in vivo* production of progesterone induced by LH was studied in cows treated with 12.5 or 25 mg pLH, or 100 μg GnRH during proestrus (Ambrose et al., 2009). The *in vitro* progesterone production (ng/mL) was not significantly different between *in vivo* treatments. Conversely, GnRH-treated cows had higher plasma P_4 concentrations on day 12 ($P < 0.05$) than those given 12.5 mg pLH.

2.7. Knowledge gap

As reviewed, based on previous studies, we know that giving pLH to induce ovulation in an Ovsynch protocol results in an extended period (several hours) of elevated LH concentrations prior to ovulation (Ree et al., 2009). In addition, replacing the second GnRH treatment in the Ovsynch/timed AI protocol with pLH significantly improved pregnancy rates by up to 14 percentile points (Colazo et al., 2009a). Nevertheless, pregnancy improvement was not associated with plasma progesterone concentrations or ovulation rates. It remains unclear how improvement in pregnancy occurs in cows given pLH; the purpose of the current research presented in this Master's thesis is to determine some of the factors that may be contributing to this increase in pregnancy rates.

2.8. Hypothesis and objectives

The hypothesis of the present research is that the improvement in pregnancy rate reported previously (Colazo et al., 2009) when GnRH was replaced with pLH to synchronize ovulation in dairy cows, occurred through alterations in

intrafollicular milieu or gene expression in granulosa cells or the cumulus-oocyte-complex.

The objectives were to identify:

1) if preovulatory follicular fluid factors, IGF-I, IGFBP-3, BMP-15, GDF-9 and TGF-β1 differed in response to pLH or GnRH-induced bLH exposure (Chapter 3)

2) target genes that may be differentially regulated in granulosa cells of preovulatory follicle in response to pLH or GnRH-induced bLH exposure (Chapter 3)

3) target genes that may be differentially regulated in the cumulus-oocyte-complex in response to pLH or GnRH-induced bLH exposure (Chapter 4).

2.9. Literature cited

Adams, G. 1998. Control of ovarian follicular wave dynamics in mature and prepubertal cattle for synchronization and superstimulation. Proceedings of the XX Congress of the World Association for Buiatrics, Sydney, Australia. 595-605.

Adams, G. P., R. Jaiswal, J. Singh and P. Malhi. 2008. Progress in understanding ovarian follicular dynamics in cattle. Theriogenology. 69:72-80.

Aerts, J. and P. Bols. 2010a. Ovarian follicular dynamics. A review with emphasis on the bovine species. part II: Antral development, exogenous influence and future prospects. Reproduction in Domestic Animals. 45:180-187.

Aerts, J. M. and P. E. Bols. 2010b. Ovarian follicular dynamics: A review with emphasis on the bovine species. part I: Folliculogenesis and pre-antral follicle development. Reproduction in Domestic Animals. 45:171-179.

Albertini, D. F. and E. Anderson. 1974. The appearance and structure of intercellular connections during the ontogeny of the rabbit ovarian follicle with particular reference to gap junctions. Journal of Cell Biology. 63:234-250.

Ambrose, J. D., M. G. Colazo and J. P. Kastelic. 2010. The applications of timed artificial insemination and timed embryo transfer in reproductive management of dairy cattle. Revista Brasileira De Zootecnia. 39:383-392.

Ambrose, J. D., M. G. Colazo, T. O. Ree, J. P. Kastelic, M. K. Dyck, P. Ponce Barajas and A. G. A. 2009. Lamont. In vitro assessment of corpus luteum function in cows induced to ovulate with porcine LH, GnRH or estradiol benzoate. Journal of Dairy Science. 92 (Suppl 1):266.

Ambrose, D. J. and M. G. Colazo. 2007. Reproductive status of dairy herds in Alberta. Advances in Dairy Technology. 19: 227-244.

Ambrose, J. D., J. P. Kastelic, R. Rajamahendran, M. Aali and N. Dinn. 2005. Progesterone (CIDR)-based timed AI protocols using GnRH, porcine LH or estradiol cypionate for dairy heifers: Ovarian and endocrine responses and pregnancy rates. Theriogenology. 64:1457-1474.

At-Taras, E. E. and S. L. Spahr. 2001. Detection and characterization of estrus in dairy cattle with an electronic heatmount detector and an electronic activity tag. Journal of Dairy Science. 84:792-798.

Bauman, D. E. and W. B. Currie. 1980. Partitioning of nutrients during pregnancy and lactation: A review of mechanisms involving homeostasis and homeorhesis. Journal of Dairy Science. 63:1514-1529.

Beam, S. W. and W. R. Butler. 1999. Effects of energy balance on follicular development and first ovulation in postpartum dairy cows. Journal of reproduction and fertility. Supplement. 54:411-424.

Bilodeau-Goeseels, S. 2008. Review: The regulation of meiotic maturation in bovine oocytes. Canadian Journal of Animal Science. 88:343-349.

Bornslaeger, E. A., P. Mattei and R. M. Schultz. 1986. Involvement of cAMP-dependent protein kinase and protein phosphorylation in regulation of mouse oocyte maturation. Developmental Biology. 114:453-462.

Britt, J. H., R. G. Scott, J. D. Armstrong and M. D. Whitacre. 1986. Determinants of estrous behavior in lactating holstein cows. Journal of Dairy Science. 69:2195-2202.

Butler, W. R. 2003. Energy balance relationships with follicular development, ovulation and fertility in postpartum dairy cows. Livestock Production Science. 83:211.

Chenault, J., W. Thatcher, P. Kalra, R. Abrams and C. Wilcox. 1975. Transitory changes in plasma progestins, estradiol, and luteinizing hormone approaching ovulation in the bovine. Journal of Dairy Science. 58:709-717.

Colazo, M. G. and D. J. Ambrose. 2013. New research in controlled breeding programs for dairy cattle. Advances in Dairy Technology. 25:75-95.

Colazo, M. G., M. B. Gordon, R. Rajamahendran, R. J. Mapletoft and D. J. Ambrose. 2009a. Pregnancy rates to timed artificial insemination in dairy cows treated with gonadotropin-releasing hormone or porcine luteinizing hormone. Theriogenology. 72:262-270.

Colazo, M. G., T. O. Ree, A. G. A. Lamont, J. P. Kastelic, R. J. Mapletoft and D. J. Ambrose. 2009b. Plasma LH concentrations and CL function in Holstein cows given porcine LH, GnRH, or estradiol benzoate. Journal of Dairy Science. 92 (Suppl 1):443.

Colazo, M. G., J. P. Kastelic, H. Davis, M. D. Rutledge, M. F. Martinez, J. A. Small and R. J. Mapletoft. 2008. Effects of plasma progesterone concentrations on LH release and ovulation in beef cattle given GnRH. Domestic Animal Endocrinology. 34:109-117.

Conti, M., M. Hsieh, A. Musa Zamah and J. S. Oh. 2012. Novel signaling mechanisms in the ovary during oocyte maturation and ovulation. Molecular and Cellular Endocrinology. 356:65-73.

Custer, E., W. Beal, S. Wilson, A. Meadows, J. Berardinelli and R. Adair. 1994. Effect of melengestrol acetate (MGA) or progesterone-releasing intravaginal device (PRID) on follicular development, concentrations of estradiol-17 beta and progesterone, and luteinizing hormone release during an artificially lengthened bovine estrous cycle. Journal of Animal Science. 72:1282-1289.

Delwiche, M., E. Cox, B. Goddeeris, C. Van Dorpe, J. De Baerdemaeker, E. Decuypere and W. Sansen. 2000. A biosensor to detect penicillin residues in food. Transactions of the ASAE-American Society of Agricultural Engineers. 43:153-160.

Eppig, J. J. 2001. Oocyte control of ovarian follicular development and function in mammals. Reproduction. 122:829-838.

Eppig, J. J. 1991. Intercommunication between mammalian oocytes and companion somatic cells. Bioessays. 13:569-574.

Fair, T. 2003. Follicular oocyte growth and acquisition of developmental competence. Animal Reproduction Science. 78:203-216.

Felton, C. A., M. G. Colazo, P. Ponce-Barajas, C. J. Bench and D. J. Ambrose. 2012. Dairy cows continuously-housed in tie-stalls failed to manifest activity changes during estrus. Canadian Journal of Animal Science. 92:189-196.

Findlay, J. K. and A. E. Drummond. 1999. Regulation of the FSH receptor in the ovary. Trends in Endocrinology and Metabolism. 10:183-188.

Firk, R., E. Stamer, W. Junge and J. Krieter. 2002. Automation of oestrus detection in dairy cows: A review. Livestock Production Science. 75:219-232.

Folman, Y., M. Kaim, Z. Herz and M. Rosenberg. 1990. Comparison of methods for the synchronization of estrous cycles in dairy cows. 2. effects of progesterone and parity on conception. Journal of Dairy Science. 73:2817-2825.

Funk, D. A. 2006. Major advances in globalization and consolidation of the artificial insemination industry. Journal of Dairy Science. 89:1362-1368.

Gallagher, G. R. and P. L. Senger. 1989. Concentrations of spermatozoa in the vagina of heifers after deposition of semen in the uterine horns, uterine body or cervix. Journal of Reproduction and Fertility. 86:19-25.

Ginther, O., M. Beg, D. Bergfelt, F. Donadeu and K. Kot. 2001. Follicle selection in monovular species. Biology of Reproduction. 65:638-647.

Ginther, O., D. Bergfelt, L. Kulick and K. Kot. 1998. Pulsatility of systemic FSH and LH concentrations during follicular-wave development in cattle. Theriogenology. 50:507-519.

Goudet, G., F. Belin, J. Bezard and N. Gerard. 1999. Intrafollicular content of luteinizing hormone receptor, alpha-inhibin, and aromatase in relation to follicular growth, estrous cycle stage, and oocyte competence for in vitro maturation in the mare. Biology of Reproduction. 60:1120-1127.

Halsey D. 1978. Every morning ed fletcher locks ‘emup and chalks ‘em. Dairy Herd Management. 15:20-24.

Johnson, S. K. and R. D. Jones. 2008. A stochastic model to compare breeding system costs for synchronization of estrus and artificial insemination to natural service. The Professional Animal Scientist. 24:588-595.

Kiddy, C. A. 1977. Variation in physical activity as an indication of estrus in dairy cows. Journal of Dairy Science. 60:235-243.

Leblanc, S. 2005.Overall reproductive performance of Canadian dairy cows: challenges we are facing. Advances in Dairy Technology. 17:137-147.

Lucy, M. C. 2001. Reproductive loss in high-producing dairy cattle: Where will it end? Journal of Dairy Science. 84:1277.

Lucy, M. C. 2000. Regulation of ovarian follicular growth by somatotropin and insulin-like growth factors in cattle. Journal of Dairy Science. 83:1635-1647.

Lulai, C., I. Dobrinski, J. Kastelic and R. Mapletoft. 1994. Induction of luteal regression, ovulation and development of new luteal tissue during early pregnancy in heifers. Animal Reproduction Science. 35:163-172.

Lussier, J., P. Matton and J. Dufour. 1987. Growth rates of follicles in the ovary of the cow. Journal of Reproduction and Fertility. 81:301-307.

Macmillan, K. and W. Thatcher. 1991. Effects of an agonist of gonadotropin-releasing hormone on ovarian follicles in cattle. Biology of Reproduction. 45:883-889.

Macmillan, K. L., & A. J. Peterson. 1993. A new intravaginal progesterone releasing device for cattle (CIDR-B) for oestrous synchronisation, increasing pregnancy rates and the treatment of post-partum anoestrus. Animal Reproduction Science. 33(1): 1-25. (Abstract).

Mapletoft, R. J., K. B. Steward and G. P. Adams. 2002. Recent advances in the superovulation in cattle. Reproduction Nutrition Development. 42:601-611.

Martinez, M., G. Adams, D. Bergfelt, J. Kastelic and R. Mapletoft. 1999. Effect of LH or GnRH on the dominant follicle of the first follicular wave in beef heifers. Animal Reproduction Science. 57:23-33.

McCartney, C. R., S. K. Blank and J. C. Marshall. 2007. Progesterone acutely increases LH pulse amplitude but does not acutely influence nocturnal LH pulse frequency slowing during the late follicular phase in women. American Journal of Physiology-Endocrinology and Metabolism. 292:E900-E906.

Mellon, P. L., J. J. Windle, P. C. Goldsmith, C. A. Padula, J. L. Roberts and R. I. Weiner. 1990. Immortalization of hypothalamic GnRH by genetically targeted tumorigenesis. Neuron. 5:1-10.

Mihm, M. and E. Austin. 2002. The final stages of dominant follicle selection in cattle. Domestic Animal Endocrinology. 23:155-166.

Mihm, M., P. Baker, J. Ireland, G. Smith, P. Coussens, A. Evans and J. Ireland. 2006. Molecular evidence that growth of dominant follicles involves a reduction in follicle-stimulating hormone dependence and an increase in luteinizing hormone dependence in cattle. Biology of Reproduction. 74:1051-1059.

Moore, R. K., F. Otsuka and S. Shimasaki. 2003. Molecular basis of bone morphogenetic protein-15 signaling in granulosa cells. Journal of Biological Chemistry. 278:304-310.

Orisaka, M., S. Orisaka, J. Jiang, J. Craig, Y. Wang, F. Kotsuji and B. K. Tsang. 2006. Growth differentiation factor 9 is antiapoptotic during follicular development from preantral to early antral stage. Molecular Endocrinology. 20:2456-2468.

Overton, M. and W. Sischo. 2005. Comparison of reproductive performance by artificial insemination versus natural service sires in California dairies. Theriogenology. 64:603-613.

Panigone, S., M. Hsieh, M. Fu, L. Persani and M. Conti. 2008. Luteinizing hormone signaling in preovulatory follicles involves early activation of the epidermal growth factor receptor pathway. Molecular Endocrinology. 22:924-936.

Park, J., Y. Su, M. Ariga, E. Law, S. C. Jin and M. Conti. 2004. EGF-like growth factors as mediators of LH action in the ovulatory follicle. Science. 303:682-684.

Patterson, D., F. Kojima and M. Smith. 2003. A review of methods to synchronize estrus in replacement beef heifers and postpartum cows. Journal of Animal Science. 81:E166-E177.

Plaizier, J. C., K. D. Lissemore, D. Kelton and G. J. King. 1998. Evaluation of overall reproductive performance of dairy herds. Journal of Dairy Science. 81:1848-1854.

Pursley, J., M. Mee and M. Wiltbank. 1995. Synchronization of ovulation in dairy cows using $PGF_{2\alpha}$ and GnRH. Theriogenology. 44:915-923.

Pursley, J. R., M. C. Wiltbank, J. S. Stevenson, J. S. Ottobre, H. A. Garverick and L. L. Anderson. 1997. Pregnancy rates per artificial insemination for cows and heifers inseminated at a synchronized ovulation or synchronized estrus. Journal of Dairy Science. 80:295-300.

Ree, T. O., M. G. Colazo, A. G. Lamont, J. P. Kastelic, M. K. Dyck, R. J. Mapletoft, B. N. Ametaj and D. J. Ambrose. 2009. The effect of porcine luteinizing hormone in the synchronization of ovulation and corpus luteum development in nonlactating cows. Theriogenology. 72:120-128.

Roche, J. F., E. J. Austin, M. Ryan, M. O'Rourke, M. Mihm and M. G. Diskin. 1999. Regulation of follicle waves to maximize fertility in cattle. Journal of Reproduction and Fertility. Suppl. 54:61-71.

Russe, I. 1983. Oogenesis in cattle and sheep. Bibliotheca Anatomica. 24:77-92.

Senger, P. L. 2003. Pathways to Pregnancy and Parturition. 2nd ed. Pulman, WA:

Current Conceptions, Inc.

Senger, P. L. 1994. The estrus detection problem: New concepts, technologies, and possibilities. Journal of Dairy Science. 77:2745-2753.

Sirard, M. and K. Coenen. 2006. In vitro maturation and embryo production in cattle. Nuclear Transfer Protocols. Methods in Molecular Biology™. 348:35-42.

Sirard, M., F. Richard, P. Blondin and C. Robert. 2006. Contribution of the oocyte to embryo quality. Theriogenology. 65:126-136.

Thatcher, W., K. Macmillan, P. Hansen and M. Drost. 1989. Concepts for regulation of corpus luteum function by the conceptus and ovarian follicles to improve fertility. Theriogenology. 31:149-164.

Thatcher, W. W., T. R. Bilby, J. A. Bartolome, F. Silvestre, C. R. Staples and J. E. P. Santos. 2006. Strategies for improving fertility in the modern dairy cow. Theriogenology. 65:30-44.

Vishwanath, R. 2003. Artificial insemination: The state of the art. Theriogenology. 59:571-584.

Wolfenson, D., W. W. Thatcher, J. D. Savio, L. Badinga and M. C. Lucy. 1994. The effect of a GnRH analogue on the dynamics of follicular development and synchronization of estrus in lactating cyclic dairy cows. Theriogenology. 42:633-644.

Xu, Z. Z., D. J. McKnight, R. Vishwanath, C. J. Pitt and L. J. Burton. 1998. Estrus detection using radiotelemetry or visual observation and tail painting for dairy cows on pasture. Journal of Dairy Science. 81:2890-2896.

Zhuo, L. and K. Kimata. 2001. Cumulus oophorus extracellular matrix: Its construction and regulation. Cell Structure and Function. 26:189-196.

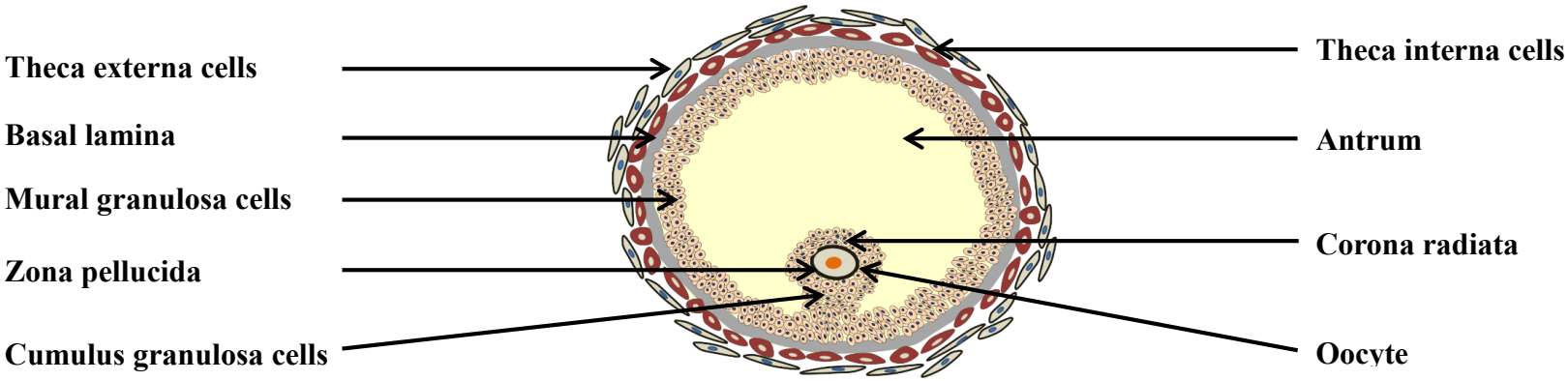

Figure 2.1. Schematic diagram showing the structure of a bovine ovarian follicle (modified from Aerts and Bols, 2010b).

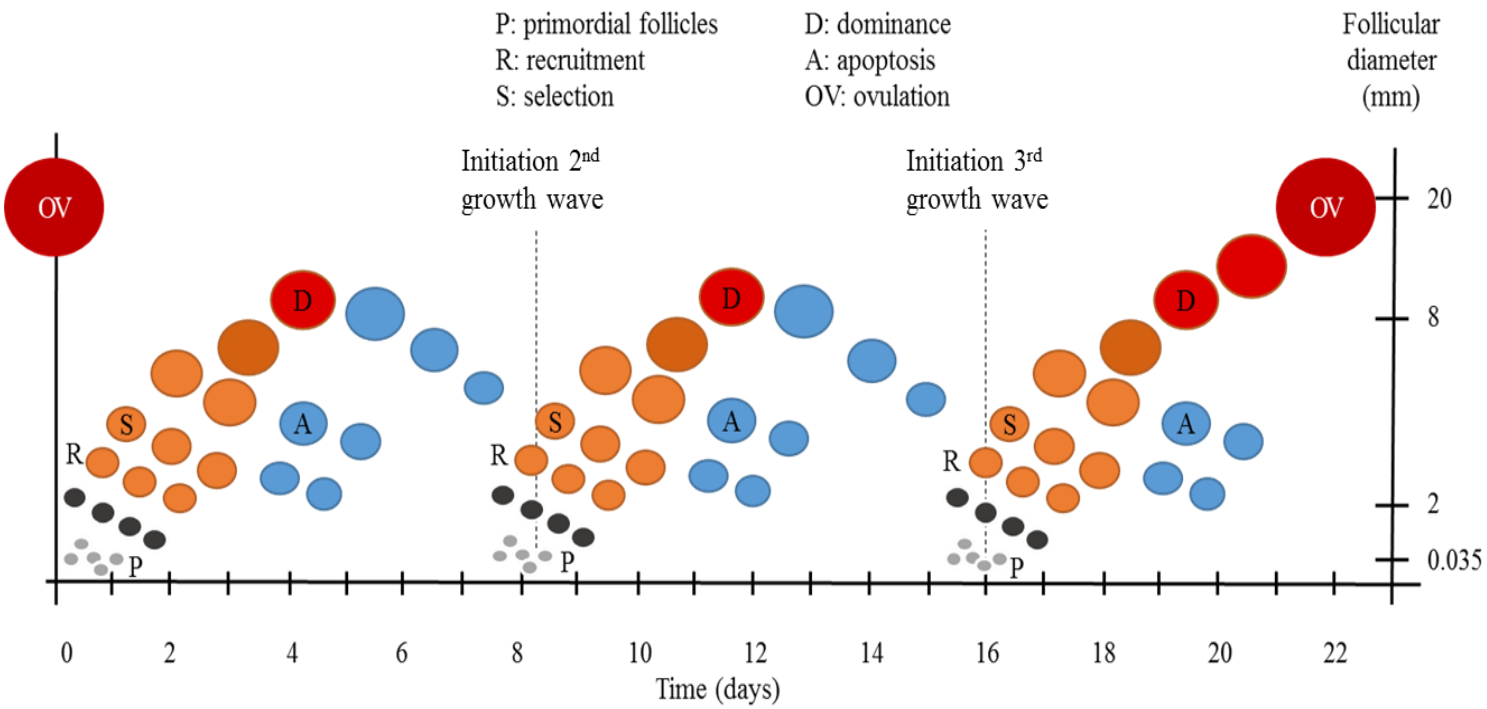

Figure 2.2. Recruitment, selection, and dominance phase in a two-wave and three-wave growth pattern during a cattle estrous cycle (modified from Adams et al., 2008).

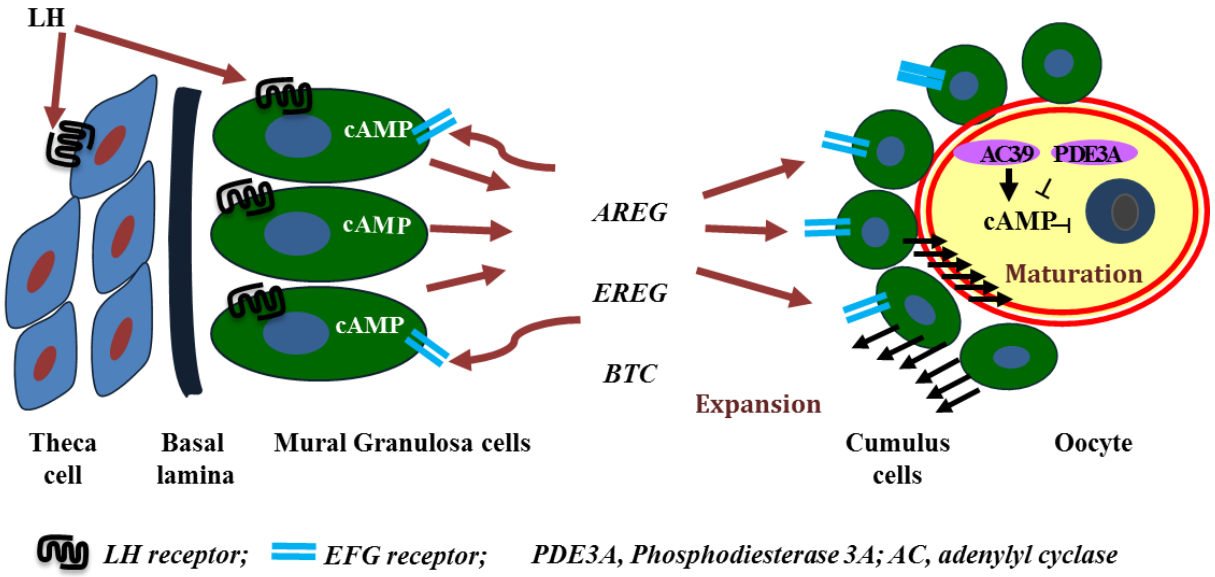

Figure 2.3. Model for LH regulation of the EGF network in ovarian follicle during a preovulatory LH surge (modified from Conti et al., 2006).

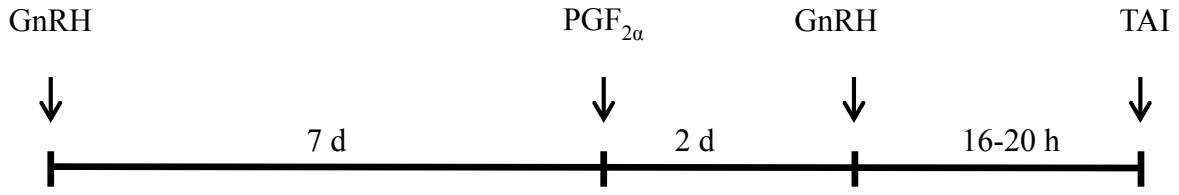

Figure 2.4. Ovulation synchronization (Ovsynch) protocol for timed artificial insemination (TAI). In Ovsynch protocol, two treatments of GnRH are administered 9 d apart with a single treatment of prostaglandin F2α given 7 d after first GnRH treatment; TAI is performed 16-20 h after second GnRH treatment.

Chapter 3. Follicular Fluid Factors and Gene Expression in Granulosa Cells of Preovulatory Follicle Exposed to Bovine or Porcine LH

3.1. Introduction

In a previous study by our research group in lactating dairy cows, replacing the second GnRH treatment in the Ovsynch/timed AI protocol with exogenous porcine luteinizing hormone (pLH) significantly improved pregnancy rates by up to 14 percentage points with no increase in peripheral progesterone concentrations (Colazo et al., 2009a). The underlying mechanism associated with this increase in pregnancy rates is not known. In addition, LH profiles differed between GnRH- and pLH- treatments (Ambrose et al., 2005; Ree et al., 2009). The mean plasma LH concentration was higher and LH remained elevated for an extended period of time in pLH- than in GnRH-treated cows.

Recently, the importance of oocyte and embryo quality in the final fertility outcome has been highlighted (Sirard et al., 2006). It has been hypothesized that the follicular fluid provides a very important microenvironment for the development of oocytes; furthermore, it can have a critical role in oocyte competence to achieve high fertilization and embryo development (Revelli et al., 2009). Follicular fluid is a product of the transfer of blood plasma constituents that cross the blood-follicular-barrier and of the secretory activity of granulosa and theca cells (Gosden et al., 1988).

High concentrations of estradiol and LH in the bovine intrafollicular environment are associated with good follicular growth and have anti-atretic effects

(Dieleman et al., 1983). The cytoplasmic maturation of oocytes can be regulated by estradiol (E_2) elevation via a direct non-genomic action (Tesarik and Mendoza, 1997). In addition, elevated estradiol concentrations and a high estradiol, progesterone (E_2:P_4) ratio in follicular fluid can be used to distinguish healthy growing follicles from atretic follicles (Guzeloglu et al., 2001); whereas, a ratio ≥ 1 indicates a highly estrogenic follicle at the preovulatory stage (Christenson et al., 2013).

Insulin-like growth factors I and II (IGF-I and -II) are polypeptides that have important roles in follicular cell proliferation and differentiation by stimulating the action of gonadotropins, e. g., LH and follicle stimulating hormone (FSH) (Armstrong et al., 2002). The biological availability of both IGF-I and II to granulosa and theca cells is regulated by a family of IGF-binding proteins (from IGFBP-1 to IGFBP-6) (Spicer and Echternkamp, 1995). In general, IGFBPs are inhibitory to the action of IGF-I (Spicer and Echternkamp, 1995) and IGFBP-3 is the most abundant member of IGBPs in large estradiol-active follicles (Echternkamp et al., 1994). It has been reported that the IGF-I has synergistic actions on LH-induced steroidogenesis via bovine theca cells; consequently, this can increase the number of LH receptors in theca cells (Stewart et al., 1995).

Members of the transforming growth factor-β (TGF-β) superfamily, expressed by oocytes and ovarian somatic cells, can regulate cell proliferation and differentiation. Bone morphogenetic protein-15 (BMP-15) and growth differentiation factor-9 (GDF-9) belong to of TGF-β superfamily and are secreted by oocytes. Secretion of an adequate amount of BMP-15 and GDF-9 by oocytes of

the preovulatory follicles during pre- and post-LH surge promoted cumulus expansion (Su et al., 2004). Growth differentiation factor 9 is essential for granulosa cell proliferation and oocyte growth (Dong et al., 1996). Moreover, GDF-9 has an inhibitory action on follicular atresia and cell apoptosis (Orisaka et al., 2006). Granulosa cell mitosis and proliferation are initiated by BMP-15; furthermore, BMP-15 can restrict FSH-induced granulosa cell differentiation (Moore and Shimasaki, 2005).

The LH surge is associated with activation of the epidermal growth factor (EGF) signaling network. The role of EGF in inducing oocyte maturation and improving oocyte competence is well demonstrated (De La Fuente et al., 1999). Luteinizing hormone induces the release of EGF-like growth factors amphiregulin (*AREG*), epiregulin (*EREG*) and betacellulin (*BTC)* in somatic cells of the preovulatory follicle (Ashkenazi et al., 2005). It is also hypothesized that the LH induces the release of *AREG* and *EREG* from the surface of mural granulosa cells (Park et al., 2004); consequently, stimulation of the EGF receptor of cumulus cells promotes oocyte meiotic resumption and cumulus expansion.

The increased pregnancy rate previously reported by Colazo et al. (2009), when GnRH was replaced by pLH to induce ovulation in dairy cows, possibly occurred through alterations in the intrafollicular environment. Therefore, in the present study it was hypothesized that replacing GnRH with pLH to synchronize ovulation in dairy cows will alter the intrafollicular milieu and gene expression in granulosa cells.

The specific objectives were to:

1. confirm previous findings of differences in LH profiles following GnRH or pLH treatments
2. compare LH profiles in response to pLH using bovine- and porcine-LH-specific standard curves
3. determine if preovulatory follicular fluid factors, IGF-I, IGFBP-3, BMP-15, GDF-9 and TGF-β1 differed in response to pLH or GnRH-induced bLH exposure
4. detect differences in the expression level of target genes that may be differentially regulated in cumulus oocyte complex of the preovulatory follicle in response to pLH or GnRH-induced bLH exposure
5. detect differences in the expression level of target genes that may be differentially regulated in granulosa cells of the preovulatory follicle in response to pLH or GnRH-induced bLH exposure.

3.2. Materials and methods

The study was conducted with non-lactating Holstein cows at the Dairy Research and Technology Centre (DRTC), University of Alberta, Edmonton, Alberta (53°4' N, 113°31' W). Animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care. Experimental procedures were approved by the University of Alberta's Animal Care and Use Committee (Protocol 2012-176). Cows were housed in an outdoor pen with adequate shelter and straw bedding, fed *ad libitum* alfalfa hay and had unrestricted access to water.

3.2.1. Animals and experimental design

Cyclic non-lactating Holstein cows were used in a crossover design replicated over time with two experimental periods (Periods 1 and 2; Figure 3.1). At the start of Period 1, twelve cows were treated with prostaglandin $F_{2\alpha}$ (PGF; 500 μg, cloprostenol; Estroplan®, Vetoquinol N.A Inc., Lavaltrie, QC, Canada) im twice (14 d apart) to induce estrus. Estrus detection was performed for 30 to 60 min three times a day for up to 3 days after second PGF treatment by visual observation. The first eight cows detected in estrus were allocated into two equal groups of four cows each. Cows were given gonadotropin releasing hormone (GnRH; 100 μg, gonadorelin acetate; Fertiline®, Vetoquinol N.A. Inc.) im treatment 6 days after estrus (Day 0), followed by PGF on Day 7. On Day 9, cows were randomly allocated to one of the two treatment groups to receive 100 μg GnRH or 25 mg porcine luteinizing hormone (pLH; Lutropin-V®, Bioniche Animal Inc., Health, Belleville, ON, Canada).

Period 2 was started 4 days after Period 1, which is identified as Day -5 (Figure 3.2; ablation of dominant follicle and all subordinate follicles ≥ 10 mm in diameter). Cows received a 1.55g progesterone releasing intravaginal device (PRID®, Vetoquinol N.A. Inc.) at Day -1. Cows were treated with 100 μg GnRH 24 h after PRID treatment (Day 0) and the device was removed at the time of 500 μg PGF injection. The follow up experimental design in all treatment groups was similar to Period 1.

3.2.2. Ovarian ultrasonography

Transrectal ultrasonography (Aloka-500 V scanner equipped with a 7.5-MHz linear transducer; Aloka Co., Tokyo, Japan) was performed by one individual on Days 0 and 7. Additional ultrasound examinations were performed on Day 9 (at pLH or GnRH injection) and Day 10 (at follicular aspiration). The diameter and location of follicles and corpora lutea (CL) were recorded. Ultrasonography on Day 0 confirmed the start of the new cycle, and that on Day 7 determined the ovulatory response to the GnRH treatment on Day 0.

Ovulatory response to the GnRH treatment was determined retrospectively based on location, and size of follicles and number of CL on Day 0, and comparing these data to ovarian structures on Day 7. Ultrasonography on Day 9 was performed to determine the response to PGF treatment, the diameter of the preovulatory follicle and to detect any early ovulations. Additional ultrasonography on Day 10 was also used to determine the ovulatory response to pLH or GnRH treatment on Day 9, the diameter of the preovulatory follicle and early ovulations.

3.2.3. Blood sample collection

To determine luteinizing hormone concentrations on Day 9, 15 sequential blood samples were collected by indwelling jugular catheter, according to the following schedule: 30 min before and immediately before administration of the GnRH or pLH treatment, at 30 min intervals during the first 2 h after the treatment, hourly until 6 h after the treatment, every 2 h for the next 6 h, and every 4 h thereafter up to 20 h after GnRH or pLH treatment.

Blood samples to determine progesterone concentrations were collected from coccygeal vessels on Days 0, 7, 8 and 10 (at time of aspiration). The response to PGF was confirmed if the plasma progesterone concentrations had decreased from Day 7 to Day 8. To determine estradiol concentrations, samples were collected at 14 and 2 h before GnRH or LH treatment and at 12 and 20 h after GnRH or LH treatment. Samples were collected into evacuated Vacutainer® tubes containing sodium heparin (Becton Dickinson and Co., Franklin Lakes, NJ, USA) as an anti-coagulant. After collecting, samples were immediately placed on ice and centrifuged within 1 h at 4°C for 20 min at 1500 × *g*. Plasma was separated and stored in a -20°C until LH, progesterone and estradiol concentrations were determined by radioimmunoassay.

3.2.4. Follicular fluid collection

Ultrasound-guided transvaginal follicular aspiration was performed 21 ± 1 h after the administration of GnRH or pLH to collect follicular fluid. An epidural injection (3-4 ml) of 2% lidocaine hydrochloride (Bimeda-MTC®, Animal Health Inc., Cambridge, ON, Canada) was given immediately before the procedure to reduce straining and facilitate ovarian manipulation.

Follicular aspiration was performed using an ultrasound machine (Aloka-500 V scanner; Aloka Co., Tokyo, Japan) equipped with a 5.0 MHz curvilinear transvaginal transducer and needle guide, a 60 cm long 17-gauge, single-lumen needle fitted with a 19-gauge sterile disposable needle tip. The distal end of the long needle was connected to a vacuum aspiration pump (Cook Aspiration Unit; Model K-MAR-5200; Cook Medical, Inc., Bloomingdale, IN, USA) set to aspirate at 80

mm Hg of vacuum pressure. The preovulatory follicle and any suspected co-dominant follicle, if present, were punctured individually. The follicular contents (follicular fluid, granulosa cells, and possible oocyte) of each preovulatory follicle were collected in separate RNase-free tubes. Immediately after each collection, the tube containing the follicular contents was labeled and transferred to the laboratory.

3.2.4.1 Sample processing

The collected contents of the follicular fluid were searched for cumulus-oocyte complexes (COC) within 5 minutes after collection. Subsequently, the COC-free follicular fluid samples were centrifuged 2000 × *g* for 3 min at 4°C. The supernatant was transferred to cryo-vials (Nalgene®, New York, USA) kept frozen in a -80°C freezer until further analysis. The granulosa cell pellets were washed with 1 ml ice-cold RNase-free PBS (phosphate buffer saline, 0.01M PBS, pH=7.5), re-suspended and centrifuged at and centrifuged at 2000 × *g* for 3 min at 4°C. The supernatant was discarded and the pellet was re-suspended in as little fluid as possible (≤ 20 µl) and stored in a -80ºC freezer until further RNA extraction to determine the differential expression of genes.

3.2.5. Laboratory analyses

3.2.5.1. Radio-immunoassays for reproductive hormones

3.2.5.1.1. Plasma LH concentrations

Plasma samples were sent to the Endocrine Laboratory at the Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine (Saskatoon, SK, Canada) for measuring LH concentrations. Plasma LH

concentrations were measured by radioimmunoassay using an anti-bovine LH monoclonal antibody (518B7; Quidel Corporation, San Diego, CA, USA) by an established radioimmunoassay using iodinated tracers (Rawlings et al., 1984). Plasma LH concentrations were expressed in terms of NIDDK-bLH4 and the sensitivity of the assay, assessed as the lowest concentration LH capable of displacing labeled LH from the antibody (0.1 ng ml^{-1}). Intra and inter-assay coefficients of variation for LH were 7.6% and 4.3%, respectively, for a high reference serum (mean = 1.26 ng/ml^{-1}) and 6.0% and 13.0%, respectively, for a low reference serum (mean = 0.61 ng/ml^{-1}). The monoclonal antibody cross-reacts equally with both bovine and porcine LH (Matteri et al., 1987). To obtain porcine-specific LH profiles, the LH concentrations in pLH-treated cows were calculated using a porcine-specific standard curve in addition to calculations based on the bovine standard curve.

3.2.5.1.2. Plasma estradiol and progesterone concentrations

Solid phase radio-immunoassay kits (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA, USA) were used to determine estradiol and progesterone in triplicates. An aliquot (100 µl) of plasma was added to an antibody-coated tube followed by 1 mL of a buffered ^{125}I-labeled estradiol and progesterone solution. Tubes were incubated for 3 h at room temperature and consequently fluid was aspirated. The sensitivity of the assay and the inter- and intra-assay coefficients of variations for plasma estradiol and progesterone were 0.1 pg/ml, 9.7 and 5.9%; and 0.1 ng/ml, 6 and 3.8%, respectively.

3.2.5.1.3. Follicular fluid estradiol and progesterone concentrations

The concentrations of estradiol and progesterone in follicular fluid were measured by solid phase radio-immunoassay kits (Coat-A-Count, Diagnostic Products Corporation) for each individual as described in section 3.2.5.1.2. In an active dominant follicle, the estradiol-to-progesterone ratio is greater than 1 (Kruip and Dieleman, 1982); whereas, a ratio less than 1 indicates inactive or subordinate follicles. The follicular fluid samples were thawed at room temperature and then centrifuged at 3000 × *g* for 5 min at 4ºC. To determine estradiol concentrations, follicular fluid samples were diluted 1:600 and 1:1200 in phosphate buffer saline (0.01 M PBS, pH 7.5) with 0.1% gelatin as reported by Guzeloglu et al. (2001). For determining the follicular fluid progesterone, samples were diluted 1:100 with zero standards provided with the kit as previously reported by Thangavelu et al. (2008).

3.2.5.2. Determination of NEFA, Glucose and IGF-I in follicular fluid

Follicular fluid nonesterified fatty acids (NEFA) were analyzed using a commercial NEFA-HR (2) kit (Wako Chemicals, Inc., Richmond, VA, USA) in triplicate, in 5 µl samples of follicular fluid. Glucose concentrations were also determined in triplicate in 10 µl follicular fluid samples by using glucose oxidase/peroxidase enzyme and O-dianisine-dihydrochloride (Sigma, Inc., St. Louis, MO, USA). Follicular fluid IGF-I concentrations were determined using an ELISA kit (Cedarlane Laboratories, Hornby, ON, Canada); the assay was run with 20 µl of follicular fluid in duplicate.

3.2.5.3. Western blotting for protein expression in follicular fluid

Expression levels of BMP-15, IGFBP-3, GDF-9 and TGFβ1 proteins were determined by Western blot analysis. The follicular fluid was diluted 1:64, 1:128 and 1:256 with the PBS. Protein concentration was measured with Pierce BCA protein assay kit (Thermo Scientific, Inc., Waltham, MA, US) to ensure equal sample loading. The total protein content of the samples was normalized by using PBS and was diluted 1:1 with lysis buffer (65.8 mM Tris-HCl, pH 6.8, 2.1% SDS, 26.3% glycerol, 0.01% bromophenol blue and 1M β-mercaptoethanol). The mixture was heated to 95 ºC for 5 min and 75 µg protein of FF was loaded onto a 10% sodium dodecyl sulfate gel electrophoresis. The gel was electrophoresed under non-reducing conditions for 30 to 45 min.

The separated proteins were transferred onto polyvinylidene difluoride membranes (PVDF; Amersham™ Hybond™ Blotting Membranes, GE Healthcare, QC, Canada) by applying a semi-dry transfer method. The transfer was carried out at 65 mA for 1.5 h with a transfer buffer containing 30.3 g Tris Base, 14.4 glycine and 20% methanol. Transfer conditions were optimized to ensure complete transfer of proteins. The blots were blocked for 1 h at room temperature or overnight at 4°C in Tris-buffered saline (TBST 0.1%, 0.2 M NaCl, 0.1% Tween 20, 10 mM Tris, pH 7.4) containing 5% non-fat dry milk or 3% bovine serum albumin (BSA; Sigma, Inc.). After blocking, the blots were washed three times for 5 min in TBST 0.05%. Subsequently, the blots were incubated overnight at 4°C with the primary antibody solution composed against BMP-15 (GDF-9B [H-83], sc-28911, Santa Cruz Biotechnology, Santa Cruz, CA, USA), IGFBP-3 (IGFBP-3 [H-98], sc-9028, Santa

Cruz Biotechnology), GDF-9 (GDF-9 [C-20], sc-7407, Santa Cruz Biotechnology) and TGF-β1(TGF-β1 [C-16], sc-31609, Santa Cruz Biotechnology) were diluted 1:300 in a solution containing 5 % non-fat dry milk in TBST (0.1%). The blots were washed 3 to 6 times for 15 min with TBST (0.1%). The membranes were incubated with either a 1:5000 dilution of anti-rabbit horseradish peroxidase (NA934V, Amersham, Inc., GE Healthcare) for BMP-15 and IGFBP-3 or 1:20,000 dilution of anti-goat horseradish peroxidase (HRP) (sc-2020, Santa Cruz Biotechnology) for GDF-9 and TGF-β1 around 1 h at room temperature. The blots were washed 3 times for 15 min with TBST (0.1%) and followed TBS for 10 min. The membranes were developed using chemiluminescence substrate reagent (ECL-Plus Western blotting detection kit, Amersham, Inc., GE Healthcare) with exposure to X-ray film (Carestream® Kodak® BioMax® XAR Film Sigma, Inc.). The exposure time was adjusted from a few seconds to 1 or 2 min. The relative densities of the BMP-15, IGFBP-3, GDF-9 and TGF-β1 bands were analyzed by ImageJ software (version 1.46r; National Institutes of Health, Washington, DC, USA).

3.2.5.4. Gene expression analysis

3.2.5.4.1. RNA isolation, quantitation and reverse transcription

Total RNA was extracted from 10 to 40 mg of the granulosa cell pellets using the Absolutely RNA Miniprep kit (Agilent Technologies, Inc., Palo Alto, CA, USA) following the modified protocol for small samples. Pellets were homogenized with lyses buffer for protein denaturation, followed by a pre-filtration step to remove particles and to reduce DNA amount. In the third step, low salt buffer and DNase were added to remove the remaining DNA. Series of washes were

applied to remove the DNase and protein. Finally, highly pure RNA was eluted in elution buffer. The integrity and quantity of RNA were evaluated using an Agilent 2100 Bioanalyzer and the RNA 600 Pico Chips assay kit (Agilent Technologies, Inc.). Reverse transcription of 38 ng of total RNA was performed with SuperScript III (Invitrogen Life Technologies, Carlsbad, CA, US) according to manufacturer's instructions.

3.2.5.4.2. PCR primers, probe, and amplification efficiency

Primer sequences and Taqman-MGB probes for each gene were designed with Express® software v3.0 (Applied Biosystems Inc., Foster City, CA, USA) based on species-specific sequences reported in GENBANK (Table 3.1). Real-Time PCR was performed in triplicates in 96-well plates using the Taqman® Universal PCR Master Mix (Applied Biosystems Inc.) and the ABI 7900HT thermocycler (Applied Biosystems., Inc.). The amplification efficiency for all the genes was determined by serial dilution (1:1, 1:10, 1:100; 1:1000, 1:10,000 and 1: 100,000). The amplification efficiency for all candidate genes was above 95 % and the slope was close to -3.32.

3.2.5.4.3. Real-time PCR

The real-time PCR program parameters were as follows: 95ºC for 20 min; then 40 cycles of 95°C for 10 min and 60ºC for 60 s. The comparative cycle threshold (C_T) method was used to calculate accurate and reproducible data for relative gene expression (Schmittgen and Livak, 2008). Briefly, relative gene expression of the targeted genes were normalized against the housekeeping genes (β-actin, glyceraldehyde-3-phosphate dehydrogenase; GAPDH, and succinate dehydrogenase; SDHA) per individual. In this method, PCR efficiency of the

housekeeping genes (internal control) and target genes included in the equation and consequently differences in the efficiency between internal control (reference gene; RG) and targeted genes (gene of interest; GOI) were used for final calculation (Pfaffl, 2001). Consequently, the relative changes in gene expression were analyzed using $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

3.3. Statistical Analyses

Two GnRH-treated cows were removed from the study and data of these cows were not included in the statistical analyses. One cow had insufficient follicular fluid and total RNA for assays, and another cow ovulated before the scheduled time for follicle aspiration. The LH profile of one pLH-treated cow that had a late endogenous LH surge was also not included in the LH profile analysis.

Reproductive hormone data (plasma LH, progesterone and estradiol) were analyzed by repeated measures using the MIXED procedure of SAS (9.3; SAS Institute Inc., Cary, NC, USA) with an autoregressive covariance structure and time of blood sample collection as the repeated effect.

The statistical model included:

$$Y_{ijk} = \mu + T_i + P_j + C\,(T)ik + \varepsilon_{ijk}$$

where Y_{ijk} is the individual observation, μ is the overall mean, T_i is the effect of treatment (i = 1, 2), P_j is the effect of time period (j = 1, 2, 3 … and 15). The term $C\,(T)_{ik}$ was included as a random effect (k = 1, 2, 3… and 8; treated as a random effect), and ε_{ijk} is the residual error term. Cow effect was included in the model as a random effect. Statistical difference was declared at $P < 0.05$.

Follicular fluid data IGF-1, glucose, NEFA, reproductive hormones and Western Blot relative densities, the individual fold change for each GOI data were analyzed using ANOVA PROC MIXED (SAS 9.3, Institute Inc.) procedure assuming a crossover design according to the model:

$$Y_{ijk} = \mu + T_i + P_j + G_k + C\,(G)_{kl} + \varepsilon_{ijkl}$$

where Y_{ijk} is the individual observation, μ is the overall mean, T_i is the effect of treatment (i = 1, 2), P_j is the effect of period (j = 1, 2), G_k is the effect of group (k = 1, 2) and ε_{ijk} is the residual error. The term C $(G)_{kl}$ was included as a random effect (l = 1, 2, 3… and 8; treated as a random effect), term. Cow effect was included in the model as a random effect. The subject of the repeated statement was the effect of period.

Correlations were determined between follicular fluid metabolites, target proteins, aspirated follicles diameter, progesterone and estradiol concentration using Pearson correlation in SAS. Pearson correlation was determined between follicular fluid estradiol concentration and mRNA expression of each GOI in granulosa cells. Statistical difference was declared at $P < 0.05$.

3.4. Results

3.4.1. Reproductive hormones in plasma and follicular fluid

3.4.1.1. Plasma LH

There were effects ($P < 0.0001$) of treatment, time, and the treatment by time interaction for plasma LH concentrations in serial blood samples from 30 min before to 20 h after treatment (Figure 3.3). The GnRH-treated cows had an acute

response, with LH surging from 0.4 ± 1.3 ng/mL to a mean peak of 14.3 ± 1.3 ng/mL by 1.5 h after treatment and returning to basal concentrations by 8 h (0.5 ± 1.3 ng/mL; Figure 3.4). In pLH-treated cows, plasma LH concentrations were determined with both bovine- and porcine-specific standard curves. The LH concentrations in bovine-specific standard curve increased from basal concentrations (0.2 ± 0.2 ng/mL) to a mean peak of 2.1 ± 0.2 ng/mL by 1.5 h after pLH treatment and remained higher than basal concentrations up to 20 h after treatment (0.08 ± 0.2 ng/mL; $P < 0.00$; Figure 3.5). Likewise, LH peaked from 0.8 ± 2.9 ng/mL to 20.4 ± 2.9 ng/mL ($P < 0.0001$) by 1.5 h after pLH treatment in porcine-specific standard curve LH profile and never returned to basal levels until 20 h (6.7 ± 3.3 ng/mL, $P \leq 0.05$).

3.4.1.2. Plasma estradiol and progesterone

Least squares means of plasma estradiol concentrations (pg/mL) did not differ at 14 and 2 h before, and 12 and 20 h after either pLH or GnRH treatment (Figure 3.5). In period 1, least squares means of plasma progesterone concentrations on Days 0, 7, 8 and 10 were not different among cows given pLH or GnRH (Figure 3.6). In Period 2, mean P_4 concentrations did not differ between treatment groups at the time of PRID insertion (Day -1), Days 8 or ultrasound-guided transvaginal follicular aspiration (Day 10; Figure 3.7). However, plasma progesterone concentrations were higher at 24 h after PRID insertion in pLH group cows compared to GnRH cows (Day 0; 7 ± 0.5 ng/mL vs. 3.3 ± 0.6 ng/mL; $P < 0.0001$). The pLH-treated cows still had higher plasma progesterone concentrations than GnRH-treated cows at time of PRID removal and PGF treatment (Day 7; 7.7 ± 0.5

ng/mL vs. 3.2 ± 0.6 ng/mL; $P < 0.0001$). Despite this difference, both groups of cows responded equally to the PGF treatment on Day 7 as evidenced by a sharp decline in progesterone concentrations on Day 8 in both Period 1 and Period 2 (≤ 1 ng/mL; $P > 0.90$).

3.4.1.3. Follicular fluid estradiol and progesterone

Least squares means of follicular fluid estradiol and progesterone concentrations did not differ at time of follicular aspiration (Table 3.2) and there was no significant correlation between follicular fluid and plasma estradiol and progesterone concentrations at time of follicular aspiration (20 ± 1 h after pLH or GnRH treatment).

3.4.1.4. Metabolite profiles in follicular fluid

Treatment, period and group did not influence IGF-1, glucose and NEFA concentrations in follicular fluid of preovulatory follicle (Table 3.2). No correlations were observed between follicular fluid concentrations for insulin, IGF-1, glucose, NEFA, estradiol, progesterone and diameter of preovulatory follicle in GnRH-treated cows. However, there was a positive correlation between follicular fluid estradiol concentrations and diameter of preovulatory follicle in pLH-treated cows ($R^2 = 0.99$, $P = 0.0001$).

3.4.2. Protein expression in follicular fluid (Western Blot)

Western blotting assay was performed to confirm the specificity of the antibodies directed against BMP-15, IGFB-3, GDF-9 and TGF-ß1 in follicular fluid and results are presented in the Table 3.3 and Figure 3.8. There was no statistical

difference in mean normalized optical density (OD) level of the IGFBP-3 between treatments. No correlations were observed between follicular fluid concentrations for IGF-1 and OD of IGFBP-3 between treatments. Because BMP-15 and GDF-9 are glycosylated proteins and have both pro-peptide and mature forms, the BMP-15 and GDF-9 antibody recognized two bands of about at 50 and 25 kDa, respectively; which represented the pro-peptide and mature forms of GDF-9 and BMP-15, respectively (Yan et al., 2001). The least squares means of normalized optical density levels of the mature form of BMP-15, GDF-9 and TGF-β1 were significant in pLH-treated cows compared to GnRH-treated cows. The abundance of mature forms of BMP-15, GDF-9 and TGF-β1 were approximately 2-fold higher in pLH- than GnRH-treated cows. Moreover, higher pro-peptide form of BMP-15 was detected in pLH-treated cows compared to GnRH-treated cows. A positive correlation existed between OD levels of mature form of GDF-9 and TGF-β1 (R^2 = 0.9, *P* =0.002) of pLH-treated cows and mature form of BMP-15 and TGF-β1 (R^2 = 0.97, *P* = 0.0006) of GnRH-treated cows. However; no relationship was found between the pro-peptide and mature forms of BMP-15 and GDF-9. No correlations were observed between follicular fluid estradiol concentration, follicle diameter, follicular fluid estradiol and progesterone concentrations and abundance of both pro-peptide and mature forms of GDF-9 and BMP-15.

3.4.3. Gene expression

In this study only one cumulus-oocyte-complex was collected from the 15 cows, which was not used for gene expression analysis. The expression levels of five target GOI (Table 3.4) in the granulosa cells of preovulatory follicles 20 ± 1 h

post treatments were measured by RT-PCR. The relative abundance of mRNA for *AREG* was approximately 2-fold higher in the granulosa cells of GnRH-treated cows versus pLH-treated cows ($P < 0.05$). No effect of treatment, period or group was observed for changes in expression of the *BTC*, *EREG*, *LHr* and *PGr* in the granulosa cells of preovulatory follicles. However, the expression of the *LHr* tended ($P = 0.07$) to be higher in cows subjected to pLH treatment. No correlation was observed between follicular fluid estradiol concentration and expression of targeted GOI in granulosa cells of preovulatory follicle.

3.5. Discussion

The present study was conducted to determine if replacing GnRH with pLH to synchronize ovulation in non-lactating Holstein cows altered intrafollicular milieu and gene expression in granulosa cells of preovulatory follicles 20 ± 1 h after either treatment.

In the present work, the second injection of 100 μg GnRH was replaced with 25 mg pLH (Lutropin-V®) in dairy cows subjected to an Ovsynch protocol because these are the manufacturer-recommended dosages in cattle. Earlier studies (Ambrose et al. 2005; Ree et al. 2009) used similar doses (100 μg GnRH and 25 mg pLH). Furthermore, reduced doses (5.0, 8.0 or 12.5 mg) of pLH have not been as effective as 25 mg pLH in inducing ovulations or in maintaining LH profiles (Ambrose et al., 2005; Ree et al., 2009)

Cows given 25 mg pLH had a significantly longer period of elevated plasma LH concentration than GnRH-treated cows, lasting from 1.5 h after treatment to at least 20 h post treatment and LH concentrations had not returned to basal levels up

to the end of the collection period, as evident by both bovine and porcine standard curves. To our knowledge, this is the first study that reports pLH concentrations in cattle calculated based on both bovine- and porcine-LH specific standard curves. Ambrose et al. (2005) and Ree et al. (2009) used only a bovine-specific standard curve for measuring exogenously administered pLH in pLH-treated cows. Therefore, to make direct comparisons between LH profiles of the current study and previous studies (Ambrose et al., 2005 and Ree et al., 2009) the LH profile of pLH-treated cows was calculated using a bovine-specific standard curve. Furthermore, this study is unique in that a porcine-LH specific standard curve was also used to measure pLH as it was considered appropriate to use a standard curve specifically developed to measure porcine LH.

Since the porcine- and bovine-LH specific standard curves have very different slopes (Susan Cook, personal communication), it was considered appropriate to calculate pLH concentrations with both standard curves (in the pLH-treated cows) and make a direct comparison.

Ambrose et al. (2005) previously reported that the area under the LH curve was about 50% greater in dairy heifers treated with 25 mg pLH compared to those given either 100 µg GnRH or 12.5 mg pLH and LH remained elevated for a significantly longer period than in GnRH-given heifers when treatments were applied during the proestrous period. The LH concentrations reported in the study by Ambrose et al. (2005) were based on a bovine standard curve, and is in agreement with the LH profile of the present study based on a bovine standard curve. In an earlier study, Ambrose et al. (2005) inferred that the endogenous LH

surges were suppressed by pLH treatment, based on their observation that pLH-treated heifers did not have LH surges with peaks >5 ng/mL. Although it cannot be confirmed whether exogenous pLH administration suppressed endogenous LH, the finding of Ambrose et al (2005) is in agreement with the current study. Furthermore, the LH profiles in pLH-treated cows based on bovine and porcine standard curves had very similar shapes, although the magnitude of LH concentrations was considerably higher when fitted with a porcine-specific standard curve.

In another study (Ree et al., 2009), non-lactating dairy cows were treated with either 25 mg pLH or 100 µg GnRH during diestrus. The mean plasma LH concentration was greatest ($P < 0.01$) in cows given 25 mg pLH (4.3 ± 0.4 ng/mL) compared to GnRH-treated cows (1.8 ± 0.4 ng/mL). In the study by Ree et al. (2009), the LH peak was higher in pLH- than in GnRH-treated cows. Similar to the findings of Ambrose et al. (2005) in dairy heifers, the LH profile was elevated and prolonged above-basal (1 ≥ ng/mL) in pLH-treated cows than in GnRH-treated cows for up to 10 h in the study by Ree et al. (2009), and had not returned to basal levels up by the end of the collection period.

As mentioned previously, plasma LH concentrations were higher during proestrous in GnRH-treated cows in the current study and the LH profile in pLH-treated cows (bovine standard curve) was similar to that reported by Ambrose et al. (2005), but the overall concentrations of LH in pLH-treated cows remained lower than that reported by Ree et al. (2009). The inconsistency in LH concentrations among studies was more likely due to differences in the biological efficacy of different batches of pLH (Lutropin-V®) used among the cited studies, and not likely

due to assay differences because the LH assays in all three studies were performed in the same laboratory using the same antibody under similar assay conditions.

Plasma estradiol concentrations did not differ between GnRH- and pLH-treated cows; moreover, there was no correlation between plasma and follicular fluid estradiol concentrations at the time of follicular aspiration (20 h post-treatment). High estradiol concentration triggers preovulatory surge of GnRH and indirectly stimulates both FSH and LH in the peripheral circulation (Haughian et al., 2004). In the current study, mean plasma estradiol concentrations did not differ between treatments. As high progesterone concentrations can have a negative effect on pituitary LH release (Colazo et al., 2008), we also documented progesterone concentrations during the study period. Progesterone concentrations rapidly declined after PGF treatment on Day 7 dropping to below 1.0 ng/mL on Day 8. Mean plasma and follicular fluid progesterone concentrations did not differ between treatments at 20 ± 1 h after treatment (follicular aspiration time).

The estradiol and progesterone in follicular fluid did not differ between treatments 20 ± 1 h post treatment. High (> 1.0) E_2:P_4 ratio is indicative of dominant follicles that are actively producing estradiol; hence, this ratio is used as an indicator of follicular estrogenic status. Follicular fluid was collected at 20 ± 1 h after GnRH or pLH treatment in the present study; as a result, the E_2:P_4 ratio was < 1 in many of the treated cows (three of six in GnRH-treated cows; five of eight in pLH-treated cows). It must be noted that the follicles had already been exposed to LH for an extended period of time (> 20 h) potentially contributing to E_2:P_4 ratios lower than 1. Concentrations of estradiol, progesterone and E_2:P_4 ratio in preovulatory follicles

were investigated in beef cattle at 0, 8.5 and 20.5 h post GnRH administration (Funston et al., 1996). In the study by Funston et al. (1999), concentrations of estradiol and E_2:P_4 ratio were higher at 0 or 8.5 h than 20.5 h after GnRH injection. In current study, the overall mean of estradiol concentrations in follicular fluid was higher than progesterone concentrations in both GnRH- and pLH-treated cows; whereas, progesterone concentrations were about 3-fold higher than estradiol concentrations in preovulatory follicles 20.5 h post GnRH treatment in the study by Funston et al. (1996). In the current study, preovulatory follicle diameters were assessed by transrectal ultrasonography at treatment administration and 20 h post treatment (before follicle aspiration) and the mean diameter did not differ between the time of treatment and 20 h post treatment, indicating that neither GnRH nor pLH treatment affected preovulatory follicle diameters.

Both BMP-15 and GDF-9 are pre-proteins that are converted to a preform and the turned into an active form by posttranslational modification (Alberts et al., 2002). Thus, GDF-9 and BMP-15 proteins are exclusively oocyte-secreted and have been localized to the oocytes of growing follicles in most species, suggesting that these growth factors most likely play a key role in regulation of follicular development (Juengel and McNatty, 2005; Knight and Glister, 2006). In this study, both pro-peptide (larger) and mature (smaller) forms of GDF-9 and BMP-15 were detected. The relative abundance of mature forms of GDF-9 and BMP-15 was higher in pLH- than in GnRH-treated cows; whereas, only the pro-peptide form of BMP-15 was higher in pLH-treated cows compared to those given GnRH. The expression level of pro-peptide and mature forms of BMP-15 and GDF-9 in

monkey's follicular fluid during the preovulatory period before and after administration (0, 12, 24 and 36 h) of an ovulatory dose of human chorionic gonadotropin (hCG) were detected by Western blotting (Duffy, 2003). Duffy (2003) reported that the pro-peptide and mature form of BMP-15 expression did not change across the preovulatory interval; in contrast, different levels of expression of pro-peptide and mature form of GDF-9 during preovulatory interval were observed. Expression level of pro-peptide form was higher than mature form at time of the hCG administration; nonetheless, the level of the pro-peptide form of protein expression was reduced at 24 h after hCG treatment. The relative abundance of mature form of GDF-9 increased 12 h after t hCG treatment and was still higher up to 36 h compared to treatment administration time (Duffy, 2003). This is in agreement with the current study suggesting that GDF-9 protein expression is associated with preovulatory LH surge.

Both BMP-15 and GDF-9 play a key role in the induction of normal cumulus expansion at two different stages of follicular development (pre- and post-LH surge; Su et al., 2004). Before initiation of the LH surge, BMP-15 and GDF-9 promote differentiation of the cumulus cell and increase the cumulus cell response to the LH surge. Consequently, after induction of the LH surge, BMP-15 and GDF-9 promote the actual processes involved in cumulus expansion (Su et al., 2004). Different expression of BMP-15 and GDF-9 proteins in follicular fluid of preovulatory women has recently been reported (Otsuka et al., 2011). Follicles $\geq$ 18 mm were aspirated with transvaginal ultrasound-guided procedure 36 h after hCG injection. Only the pro-peptide forms of the BMP-15 and GDF-9 were

detected in all follicular fluid samples, whereas, the mature form- of BMP-15 and GDF-9 were only detected in 60.5 and 95.1% of samples, respectively. In the current study, both pro-peptide and mature forms of BMP-15 and GDF-9 were detected with different intensity in all GnRH- and pLH-treated cows. The expression levels of both proteins did not correlate with follicular fluid estradiol concentrations in Otsuka et al. (2011) study; likewise, no correlation was detected between follicular fluid estradiol concentrations and BMP-15/ GDF-9 abundance in the current study. A negative correlation between follicular fluid progesterone concentration and mature form of GDF-9 was reported by Otsuka et al. (2011); in contrast, no such correlation was observed in the current study. In addition, there was no correlation between pro-peptide and mature forms of BMP-15 and GDF-9 by treatment; whereas, Otsuka et al. (2011) reported a positive correlation between mature form of BMP-15 and GDF-9.

The relative abundance of TGF-ß 1 was higher in pLH- than in GnRH-treated cows in current study. The transforming growth factor-β superfamily (TGF-β1, TGF-β2 and TGF-β3) and their possible actions as local regulators of ovarian function and fertility in mammals have been investigated in recent years (Ouellette et al., 2005). The TGF-β1, as well as TGF-β2 and TGF-β3, have been detected in oocytes of primary follicles and in theca and granulosa cells of antral follicles (Juengel and McNatty, 2005). First detection of TGF-β1 was reported in oocytes and some granulosa cells of pre-antral follicles of rat (Teerds and Dorrington, 1992). In cattle, TGF-β1, TGF-β2 and TGF-β3 proteins were localized to oocytes of all sizes of follicles (Juengel and McNatty, 2005). Both theca and

granulosa cells produce TGF-β; however, in sheep, cows and pigs, it is mainly secreted by theca cells (Knight and Glister, 2006). In this study, TGF-β1 was chosen as an oocyte-secreted marker. The TGF-β1 is reported to have either inhibitory or stimulatory action in estradiol and progesterone synthesis in granulosa cells of antral follicles in various species (Ouellette et al., 2005). For instance, TGF-β1 counteracts estradiol and stimulates progesterone secretion in bovine granulosa cells cultured in the absence of FSH (Zheng et al., 2009); whereas, there was no correlation between estradiol and progesterone concentrations with TGF-β1 expression level in follicular fluid in the present study.

Relative abundances of IGFBP-3 and IGF-I concentration in follicular fluid were not affected by the differences in LH profile of GnRH- and pLH-treated cows. The action and expression of IGF-I on the antral follicle development have been reported in many studies. Insulin-like growth factor-I and binding proteins are regulated in bovine follicular fluid, granulosa and theca cells (Echternkamp et al., 1994). The proliferation of follicular cells enhances gonadotropin-stimulated steroidogenesis in both follicular and luteal cells, and are stimulated by IGF-I in mammalian ovarian tissue (Adashi et al., 1985), including the cattle (Spicer and Echternkamp, 1995).

The IGFBPs either stimulate (Elgin et al., 1987) or inhibit (Clemmons et al., 1986) the action of IGF-I in experimental animals. However, the predominant and inhibitory effects of IGFBPs have been reported on ovarian follicular cells (Adashi et al., 1985; Giudice, 1992). The developmental differences of IGFBPs existing in follicular fluid before and after LH surge have been investigated in some species

(Echternkamp et al., 1994; Stanko et al., 1994; Funston et al., 1996). Funston et al (1996) have demonstrated that concentrations of IGF-I and relative abundance of IGFBP-3 in preovulatory follicle did not differ at 8.5 and 20.5 h after GnRH injection in cattle; in fact; their results were in agreement with current study. A positive correlation between IGFBP-3 and both estradiol and IGF-I concentrations in follicular fluid of preovulatory follicle was reported by Funston et al (1996); nonetheless, no correlation was observed in the current study. Funston et al (1996) also observed a positive correlation between IGFBP-3 in follicular fluid with serum IGF-I concentrations; as well, concluded the circulating IGF-I and IGFBP-3 might be associated with LH surge. The relative abundance of IGFBP-3 in follicular fluid of the preovulatory and non-ovulatory follicles remained constant pre and post LH surge; indeed, it is hypothesized that IGFBP-3 may be derived from vascularization and has a key role in transport of IGF-I from the circulation into the follicle (Funston et al., 1996).

Relative expression of amphiregulin (*AREG*) was greater in granulosa cells of preovulatory follicles 20 ± 1 h post-treatments in GnRH-treated cows compared to pLH-treated cows. No differences were observed for expression of the epiregulin (*EREG*), betacellulin (*BTC*), LH receptor (*LHr*) and progesterone receptor (*PGr*) genes in granulosa cells of preovulatory follicles of both treatment groups. The LH surge triggers a number of cellular signaling cascades within the preovulatory granulosa cells (Bachelot and Binart 2005). It has been reported that the LH surge induction initiates the activation of the epidermal growth factor (EGF)-like ligand

and subsequently the expression of *AREG*, *EREG* and *BTC* are important for oocyte maturation and cumulus expansion (Park et al., 2004; Conti et al., 2006).

Microarray gene expression analysis has been used to rapidly identify LH (hCG) -induced genes expressed in murine granulosa cells at various time points after treatment (Carletti and Christenson, 2009); ovaries were collected at 0 up to 12 h after hCG treatment for microarray analysis. The *AREG* had the most up-regulated transcript of all genes present on the array expression 1 h post hCG; whereas, the *EREG* was the third highest up-regulated gene. Carletti and Christenson et al. (2009) confirmed the relative expression *AREG* and *EREG* by qRT-PCR. The expression of *AREG* and *EREG* were, respectively, 21-fold and 5.5-fold 30 min after hCG treatment, while at 1 h post hCG *AREG* and *EREG* expression increased to 133-fold and 27.5-fold, respectively. The findings of Carletti and Christenson et al. (2009) are in agreement with that of Park et al. (2004) in that *AREG*, *BTC* and *EREG* have rapid and transient expression after hCG treatment within 2 h.

Carletti and Christenson et al. (2009) reported that the expression of *AREG* and *EREG* in mouse granulosa cells did not differ between 1 and 12 h after hCG treatment. To the best of our knowledge, the present work is the first study that reports differential expression of epidermal growth factor family in the presence of higher and acute LH surge (GnRH-treated cows) versus lower, but prolonged above-basal LH profile (pLH-treated cows). We speculate that an acute and elevated LH peak in GnRH-treated cows at 2 h would have stimulated the *AREG*

expression in the bovine granulosa cells; indeed, this assumption needs to be corroborated.

One of the most confusing aspects of LH surge action on the follicle, though, is the restricted expression of the *LHr* in mural granulosa cells. In the current study, pLH treatment tended to increase the relative expression of *LHr* in granulosa cells of the preovulatory follicles 20 ± 1 h post-treatment. We speculate that this positive tendency might have been associated with the elevated LH profile of pLH-treated cows for a prolonged period. The LH surge can directly and indirectly affect the expression level of *LHr* in mural granulosa cells and cumulus cells (few or no *LHr*; Peng et al., 1991). The *LHr* is a member of G protein coupled receptors (GPCR) and LH effects are mediated by GPCR superfamily (Panigone et al., 2008). Each G protein is defined via its specific α subunit (Gsα) and *LHr* expression is dependent on interaction with Gsα and elevated second messenger cyclic AMP (cAMP) generated by adenylyl cyclase (Richard, 2001). It is well documented that elevated concentrations of cAMP mediates the biological effects of LH and induces meiotic arrest of oocyte (Conti et al., 2012). In our study, no correlation was observed between follicular fluid estradiol and progesterone concentrations and mRNA expression levels of *LHr* and *PGr*. A positive correlation was observed between diameter of the dominant follicle and expression of *LHr* mRNA by Beg et al. (2001), while no correlation was observed in the current study.

No differences were observed for estradiol, progesterone, glucose, IGF-I and NEFA in follicular fluid of GnRH- and pLH-treated cows. Moreover, no correlations were observed between evaluated follicular fluid components and

preovulatory follicle diameter. In the current study, estradiol and IGF-I did not differ in follicular fluid of cows with either acute/high or blunted but prolonged elevation of LH concentrations. Spicer and Echternkamp (1995) have reported the production of estradiol was not regulated consistently by IGF-I in bovine follicles; furthermore, their finding is in agreement with the current study that no correlation was observed between follicular fluid IGF-I and estradiol.

Gene expression in the cumulus-oocyte-complex (COC) of the GnRH- and pLH-treated cows was an objective of the present study. Because only one cumulus-oocyte-complex was recovered in this experiment, the objective was not fulfilled due to the lack of sufficient research material for gene expression. Therefore, gene expression in COC that may be differentially regulated after pLH versus GnRH treatment was detected in FSH-stimulated cows (Chapter 4).

3.6. Conclusion

This study demonstrated that prolonged and elevated LH concentrations in pLH-treated cows increased relative abundance of BMP-15 and GDF-9, factors that have important roles in improving oocyte competence, fertilization, and pre-implantation embryogenesis. It is likely that BMP-15 and GDF-9 contributed to the increased pregnancy rates reported in pLH-treated cows in Colazo et al. (2009) study. Further studies are required to improve the understanding of different LH profiles on intrafollicular milieu, their effects on oocyte competence and embryonic development leading to higher pregnancy rates in dairy cattle.

3.7. Literature cited

Adashi, E. Y., C. E. Resnick, a. J. D'ercole, m. E. Svoboda and J. J. Van Wyk. 1985. Insulin-like growth factors as intraovarian regulators of granulosa cell growth and function. Endocrine Reviews. 6:400-420.

Alberts, B., A. Johnson, J. Lewis, M. Raff, K. Roberts and P. Walter. 2002. Membrane structure. Molecular Biology of the Cell. Chapter 10, 4th edition. New York: Garland Science

Ambrose, J. D., J. P. Kastelic, R. Rajamahendran, M. Aali and N. Dinn. 2005. Progesterone (CIDR)-based timed AI protocols using GnRH, porcine LH or estradiol cypionate for dairy heifers: Ovarian and endocrine responses and pregnancy rates. Theriogenology. 64:1457-1474.

Armstrong, D., J. Gong, J. Gardner, G. Baxter, C. Hogg and R. Webb. 2002. Steroidogenesis in bovine granulosa cells: The effect of short-term changes in dietary intake. Reproduction. 123:371-378.

Ashkenazi, H., X. Cao, S. Motola, M. Popliker, M. Conti and A. Tsafriri. 2005. Epidermal growth factor family members: Endogenous mediators of the ovulatory response. Endocrinology. 146:77-84.

Carletti, M. Z. and L. K. Christenson. 2009. Rapid effects of LH on gene expression in the mural granulosa cells of mouse periovulatory follicles. Reproduction. 137:843-855.

Clemmons, D., R. Elgin, V. Han, S. Casella, A. D'ercole and J. Van Wyk. 1986. Cultured fibroblast monolayers secrete a protein that alters the cellular binding of somatomedin-C/insulinlike growth factor I. Journal of Clinical Investigation. 77:1548.

Colazo, M. G., J. P. Kastelic, H. Davis, M. D. Rutledge, M. F. Martinez, J. A. Small and R. J. Mapletoft. 2008. Effects of plasma progesterone concentrations on LH release and ovulation in beef cattle given GnRH. Domestic Animal Endocrinology. 34:109-117.

Colazo, M. G., M. B. Gordon, R. Rajamahendran, R. J. Mapletoft and D. J. Ambrose. 2009. Pregnancy rates to timed artificial insemination in dairy cows treated with gonadotropin-releasing hormone or porcine luteinizing hormone. Theriogenology. 72:262-270.

Conti, M., M. Hsieh, J. Park and Y. Su. 2006. Role of the epidermal growth factor network in ovarian follicles. Molecular Endocrinology. 20:715-723.

De La Fuente, R., M. J. O'Brien and J. J. Eppig. 1999. Epidermal growth factor enhances preimplantation developmental competence of maturing mouse oocytes. Human Reproduction. 14:3060-3068.

Dieleman, S. J., T. A. M. Kruip, P. Fontijne, W. H. R. de Jong and G. C. van der Weyden. 1983. Changes in oestradiol, progesterone and testosterone concentrations in follicular fluid and in the micromorphology of preovulatory

bovine follicles relative to the peak of luteinizing hormone. Journal of Endocrinology. 97:31-NP.

Dong, J., D. F. Albertini, K. Nishimori, T. R. Kumar, N. Lu and M. M. Matzuk. 1996. Growth differentiation factor-9 is required during early ovarian folliculogenesis. Nature. 383:531-535.

Duffy, D. M. 2003. Growth differentiation factor-9 is expressed by the primate follicle throughout the periovulatory interval. Biology of Reproduction. 69:725-732.

Echternkamp, S. E., H. J. Howard, A. J. Roberts, J. Grizzle and T. Wise. 1994. Relationships among concentrations of steroids, insulin-like growth factor-I, and insulin-like growth factor binding proteins in ovarian follicular fluid of beef cattle. Biology of Reproduction. 51:971-981.

Elgin, R., W. Busby and D. Clemmons. 1987. An insulin-like growth factor (IGF) binding protein enhances the biologic response to IGF-I. Proceedings of the National Academy of Sciences. 84:3254-3258.

Funston, R., G. Seidel, J. Klindt and A. Roberts. 1996. Insulin-like growth factor I and insulin-like growth factor-binding proteins in bovine serum and follicular fluid before and after the preovulatory surge of luteinizing hormone. Biology of Reproduction. 55:1390-1396.

Giudice, L. C. 1992. Insulin-like growth factors and ovarian follicular development. Endocrine Reviews. 13:641-669.

Gosden, R. G., R. H. F. Hunter, E. Telfer, C. Torrance and N. Brown. 1988. Physiological factors underlying the formation of ovarian follicular fluid. Journal of Reproduction and Fertility. 82:813-825.

Guzeloglu, A., J. D. Ambrose, T. Kassa, T. Diaz, M. J. Thatcher and W. W. Thatcher. 2001. Long-term follicular dynamics and biochemical characteristics of dominant follicles in dairy cows subjected to acute heat stress. Animal Reproduction Science. 66:15.

Haughian, J., O. Ginther, K. Kot and M. Wiltbank. 2004. Relationships between FSH patterns and follicular dynamics and the temporal associations among hormones in natural and GnRH-induced gonadotropin surges in heifers. Reproduction. 127:23-33.

Juengel, J. and K. McNatty. 2005. The role of proteins of the transforming growth factor-β superfamily in the intraovarian regulation of follicular development. Human Reproduction and Embryology. 11:144-161.

Kim IH, Suh GH, Son DS. 2003. A progesterone-based timed AI protocol more effectively prevents premature estrus and incomplete luteal regression than an Ovsynch protocol in Holstein cows. Theriogenology. 60:809–17.Knight, P. G. and C. Glister. 2006. TGF-β superfamily members and ovarian follicle development. Reproduction. 132:191-206.

Kruip, T. A. and S. J. Dieleman. 1982. Macroscopic classification of bovine follicles and its validation by micromorphological and steroid biochemical procedures. Reproduction Nutrition Development. 22:465-473.

Livak, K. J. and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. Methods. 25:402-408.

Matteri, R., J. Roser, D. Baldwin, V. Lipovetsky and H. Papkoff. 1987. Characterization of a monoclonal antibody which detects luteinizing hormone from diverse mammalian species. Domestic Animal Endocrinology. 4:157-165.

Moore, R. K. and S. Shimasaki. 2005. Molecular biology and physiological role of the oocyte factor, BMP-15. Molecular and Cellular Endocrinology. 234:67-73.

Orisaka, M., S. Orisaka, J. Y. Jiang, J. Craig, Y. Wang, F. Kotsuji and B. K. Tsang. 2006. Growth differentiation factor 9 is antiapoptotic during follicular development from preantral to early antral stage. Molecular and Cellular Endocrinology. 20:2456-2468.

Otsuka, F., K. J. McTavish and S. Shimasaki. 2011. Integral role of GDF-9 and BMP-15 in ovarian function. Molecular Reproduction and Development. 78:9-21.

Park, J. Y., Y. Q. Su, M. Ariga, E. Law, S. L. Jin and M. Conti. 2004. EGF-like growth factors as mediators of LH action in the ovulatory follicle. Science. 303:682-684.

Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research. 29:e45.

Rawlings, N.C., I. A. Jeffcoate and D. L. Rieger. 1984. The influence of estradiol 17-b and progesterone on peripheral serum concentrations of luteinizing hormone and follicle stimulating hormone in the ovariectomized ewe Theriogenology. 22 473–488

Ree, T. O., M. G. Colazo, A. G. Lamont, J. P. Kastelic, M. K. Dyck, R. J. Mapletoft, B. N. Ametaj and D. J. Ambrose. 2009. The effect of porcine luteinizing hormone in the synchronization of ovulation and corpus luteum development in nonlactating cows. Theriogenology. 72:120-128.

Revelli, A., L. Delle Piane, S. Casano, E. Molinari, M. Massobrio and P. Rinaudo. 2009. Follicular fluid content and oocyte quality: From single biochemical markers to metabolomics. Reproductive Biology and Endocrinology. 7:4330-4337.

Richards, J.S., 2001. Perspective: the ovarian follicle–a perspective in 2001.

Endocrinology 142 (6), 2184–2193.

Roche, J. F. and D. O'Callaghan. 1987. Follicular Growth and Ovulation Rate in Farm Animals. Springer.

Schmittgen, T. D. and K. J. Livak. 2008. Analyzing real-time PCR data by the comparative CT method. Nature Protocols. 3:1101-1108.

Sirard, M., F. Richard, P. Blondin and C. Robert. 2006. Contribution of the oocyte to embryo quality. Theriogenology. 65:126.

Spicer, L. J. and S. E. Echternkamp. 1995. The ovarian insulin and insulin-like growth factor system with an emphasis on domestic animals. Domestic Animal Endocrinology. 12:223-245.

Stanko, R., W. Cohick, D. Shaw, R. Harvey, D. Clemmons, M. Whitacre and J. D. Armstrong. 1994. Effect of somatotropin and/or equine chorionic gonadotropin on serum and follicular insulin-like growth factor I and insulin-like growth factor binding proteins in cattle. Biology of Reproduction. 50:290-300.

Stewart, R. E., L. J. Spicer, T. D. Hamilton and B. E. Keefer. 1995. Effects of insulin-like growth factor I and insulin on proliferation and on basal and luteinizing hormone-induced steroidogenesis of bovine thecal cells: Involvement of glucose and receptors for insulin-like growth factor I and luteinizing hormone. Journal of Animal Science. 73:3719-3731.

Thangavelu, G., Colazo, M. G., Oba, M., Dyck, M. K., Okine, E. K., and Ambrose, D. J. 2008. Fecal and urinary lignans, intrafollicular estradiol, and endometrial receptors in lactating dairy cows fed diets supplemented with hydrogenated animal fat, flaxseed or sunflower seed. Journal of Reproduction and Development 54:439-446.

Tesarik, J. and C. Mendoza. 1997. Direct non-genomic effects of follicular steroids on maturing human oocytes: Oestrogen versus androgen antagonism. Human Reproduction Update. 3:95-100.

Yan, C., P. Wang, J. DeMayo, F. J. DeMayo, J. A. Elvin, C. Carino, S. V. Prasad, S. S. Skinner, B. S. Dunbar and J. L. Dube. 2001. Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. Molecular Endocrinology. 15:854-866.

Zheng, X., D. Boerboom and P. D. Carrière. 2009. Transforming growth factor-β1 inhibits luteinization and promotes apoptosis in bovine granulosa cells. Reproduction. 137:969-977.

Table 3.1. Details of primers used for RT-PCR analysis.

Gene Symbol	Gene Bank accession no	Forward primer	Reverse primer
AREG	NM_001099092	TGGTCGACAGCGATTTATCAAA	TCATGGCAGAGACGAAAGCA
BTC	NM_173896	GCCCCAAGCAGTACAAGCAT	GCTCGGCCACCACGAA
EREG	XM_002688367	CCTCTCCCCATCACTCACTTCT	GACGGGTTTTGTGGAAGACAA
LHr	U20504.1	CGGAAGGCGTCGTTGTG	CACGCCTGGAGAAGA
PGr	NM_001205356	CGGGCACTGAGTGTTGAATTT	TCTTGGGTAACTGTGCAGCAA
GAPDH	U85042.1	TGCCGCCTGGAGAAACC	CGCCTGCTTCACCACCTT
SDHA	NM_174178.2	ACTTCACCGTTGATGGCAATAA	CGCAGAAATCGCATCTGAAA
B-Actin	NM_173979.3	CCTGCGGCATTCAGAA	GCGGATGTGGACGTCACA

Table 3.2. Least squares means (±SEM) concentrations of estradiol, progesterone, IGF-1, glucose and NEFA in preovulatory follicular fluid at 21 ± 1 h after administration of 100 µg GnRH or 25 mg porcine LH (pLH). Ultrasound-guided transvaginal follicular aspiration was used to collect follicular fluid.

	Treatment		***P* value**
	GnRH (n=6)	**pLH (n=8)**	
Estradiol (ng/ml)	847.4 ± 316.9	326.7 ± 267.8	0.23
Progesterone (ng/ml)	384.52 ± 111.8	150.4 ± 94.5	0.14
IGF-1 (ng/ml)	71.0 ± 5.0	64.2 ± 4.1	0.32
Glucose (mg/dl)	53.9 ± 4.5	60.2 ± 3.8	0.28
NEFA (m EQ/l)	247.1 ± 69.3	186.7 ± 55.7	0.51

Table 3.3. Least squares means (±SEM) normalized optical density level of BMP-15, IGFBP-3, GDF-9 and TGF-ß1 in preovulatory follicular fluid at 21 ± 1 h after administration of 100 µg GnRH or 25 mg porcine LH (pLH). Ultrasound-guided transvaginal follicular aspiration was used to collect follicular fluid.

	Treatment		***P* value**
	GnRH (n=6)	**pLH (n=8)**	
Pro-peptide BMP-15	4.2 ± 1.2	8.6 ± 1.2	0.004
Mature BMP-15	4.4 ± 2.3	8.4 ± 2.2	0.02
IGFBP-3	4.9 ± 2.2	8.8 ± 1.8	0.27
Pro-peptide GDF-9	4.9 ± 2.2	7.8 ± 1.9	0.27
Mature GDF-9	4.9 ± 1.8	8.7 ± 1.7	0.04
TGF-β1	5.6 ± 2.0	8.6 ± 2.0	0.04

Table 3.4. Summary of statistical analysis showing the fold changes in the expression of *AREG*, *BTC*, *EREG*, *LHr* and *PGr* genes in granulosa cells of preovulatory follicular fluid at 21 ± 1 h after the administration of 100 µg GnRH or 25 mg porcine LH (pLH). Ultrasound-guided transvaginal follicular aspiration was used to collect granulosa cells.

	Treatment		***P* value**
	GnRH (n=6)	**pLH (n=8)**	
AREG	1.4 ± 0.2	0.8 ± 0.2	0.05
BTC	0.7 ± 0.3	0.6 ± 0.2	0.73
EREG	2.0 ± 2.5	3.2 ± 2.0	0.71
LHr	0.2 ± 1.2	3.0 ± 1.0	0.07
PGr	0.5 ± 0.6	1.3 ± 0.5	0.38

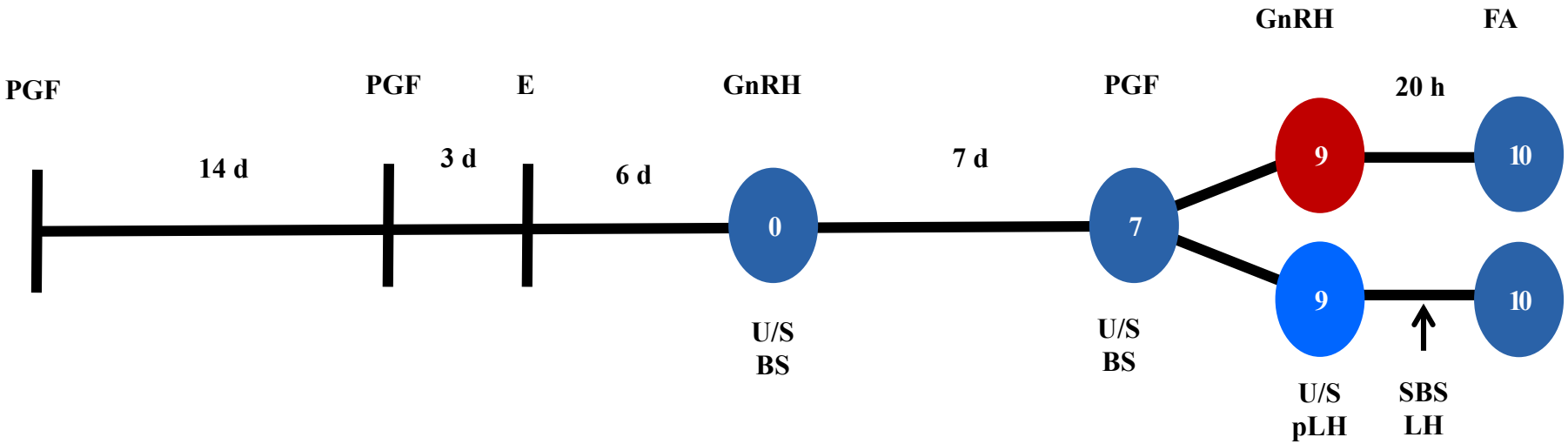

Figure 3.1. Schematic representation of the timeline of Period 1. Cyclic, non-lactating, Holstein cows (n = 8) were given 500 µg cloprostenol (PGF) twice (14 d apart) to synchronize estrus (E). All cows received 100 µg GnRH 6 day after estrus (Day 0) and PGF treatment 7 d after GnRH (Day 7). On Day 9, cows were randomly allocated to one of two treatments to receive either 100 µg GnRH or 25 mg porcine LH (pLH). Ultrasonographic examinations (U/S) of ovaries were performed on Days 0, 7 and 9 to determine response to treatments and dominant follicle diameter. Blood samples (BS) were collected on Days 0, 7, 8 and 10 to determine progesterone (P_4) concentrations and 14 and 2 h before and 12 and 20 h after either GnRH or pLH treatment to determine estradiol concentrations. Sequential blood samples (SBS) were collected after either GnRH or pLH treatment to determine LH concentrations. Ultrasound-guided transvaginal follicular aspiration (FA) was performed 21 ± 1 h after administration of GnRH or pLH to collect follicular fluid and granulosa cells.

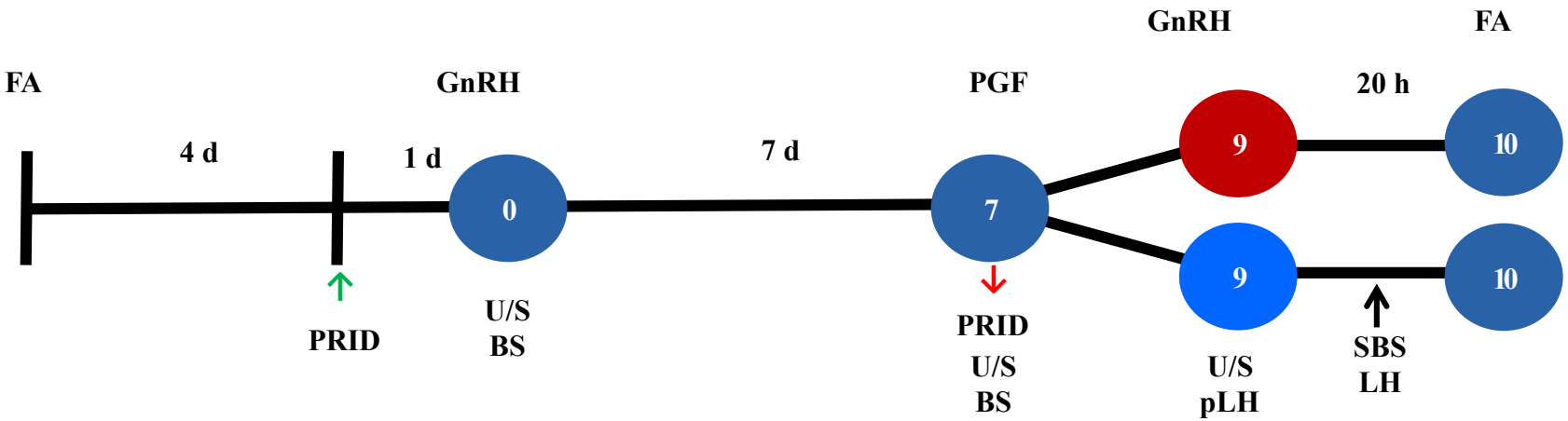

Figure 3.2. Schematic representation of the timeline of Period 2. Non-lactating Holstein cows (n=8) assigned to Period 1 received a progesterone (P_4) intravaginal device (PRID; 1.55g P4) 4 day after ultrasound-guided transvaginal follicular aspiration (FA) in Period 1, and 100 µg GnRH treatment 24 h later (Day 0). Cows were given 500 µg cloprostenol (PGF) on Day 7 and were assigned to receive either 100 µg GnRH or 25 mg porcine LH (pLH). Ultrasonographic examinations (U/S) were performed on Days 0, 7 and 9 to determine response to treatments and dominant follicle diameter. Blood samples (BS) were collected on Days -1, 0, 7, 8 and 10 to determine P_4 concentration. Additional blood samples to determine estradiol (E_2) concentration were collected 14 and 2 h before and 12 and 20 h after either GnRH or pLH. Sequential blood samples (SBS) were collected after either GnRH or pLH to determine LH concentrations. FA was performed 21 ± 1 h after administration of GnRH or pLH to collect follicular fluid and granulosa cells.

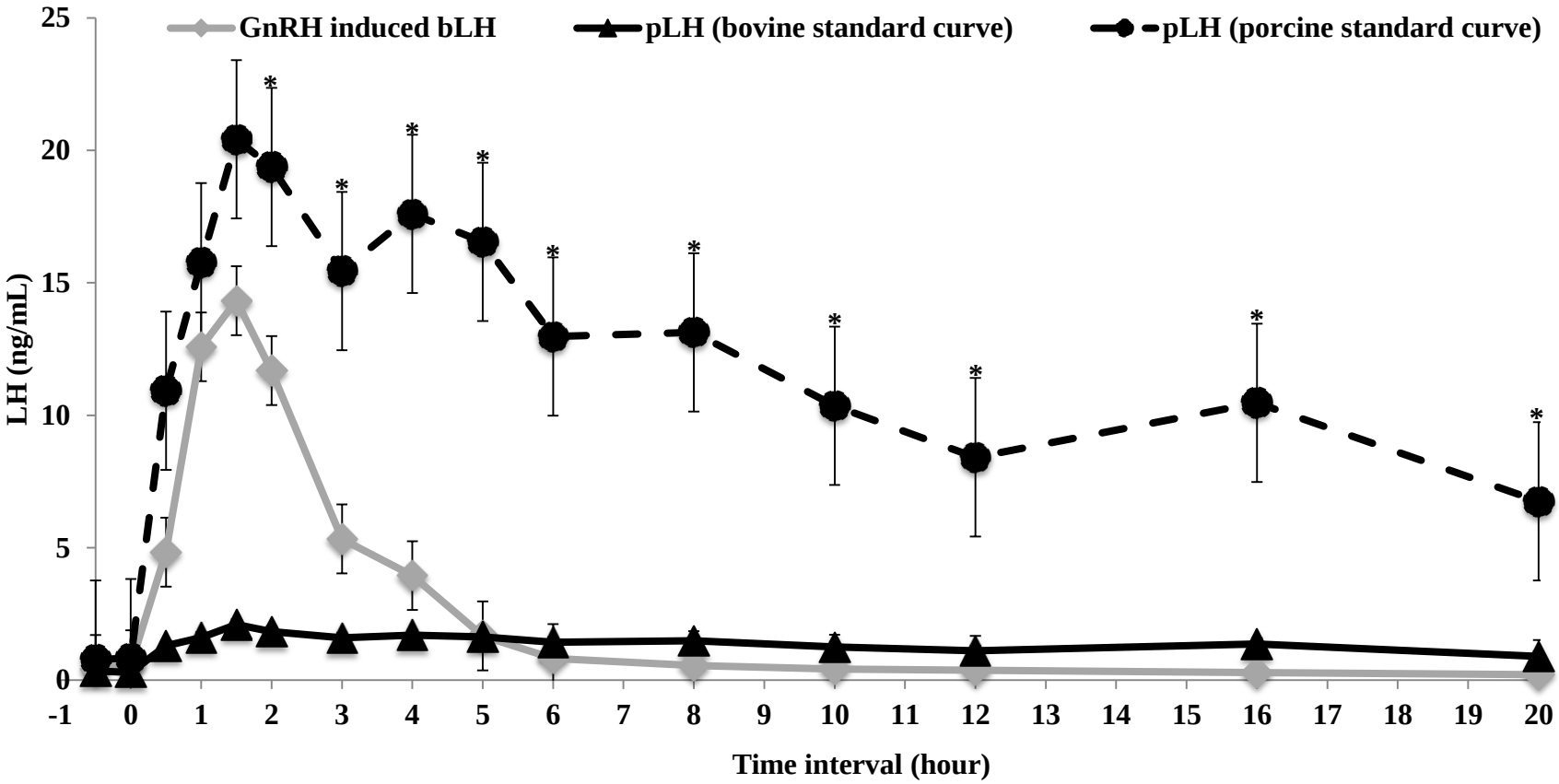

Figure 3.3. Plasma LH concentrations in a subset of 13 non-lactating cows (GnRH n=6; pLH n=7) after treatment with 100 µg GnRH or 25 mg pLH during proestrus. Concentrations of plasma bLH in GnRH-treated cows were calculated using a bovine-LH-specific standard curve, whereas in pLH-treated cows, LH concentrations were calculated using both bovine- and porcine-LH-specific standard curves. The LH concentrations in GnRH-treated cows reached basal levels by 8 h post-treatment. However, LH concentrations calculated using bovine-LH-specific standard curve differed from that of GnRH-treated cows from 8 to 20 h (see Figure 3.4). Porcine-LH measured using a pLH-specific standard curve remained elevated and differed from that of bLH concentrations of GnRH-treated cows from 1.5 h up to 20 h after treatment ($P \leq 0.05$).

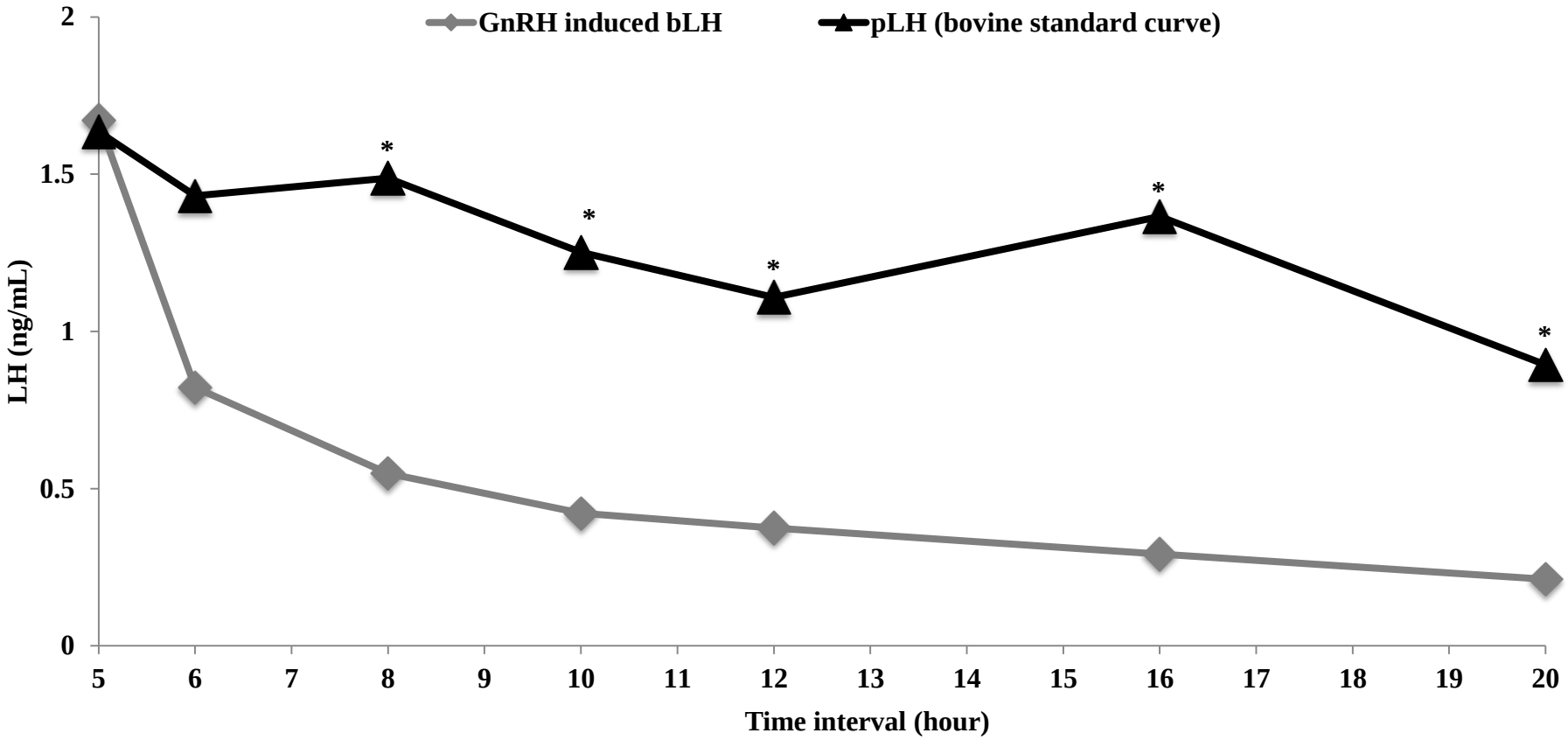

Figure 3.4. Magnified graph for plasma LH concentrations in a subset of 13 non-lactating cows (GnRH n=6; pLH n=7) from 5 h after treatment with either 100 µg GnRH or 25 mg pLH during proestrus up to 20 h (*$P < 0.05$).

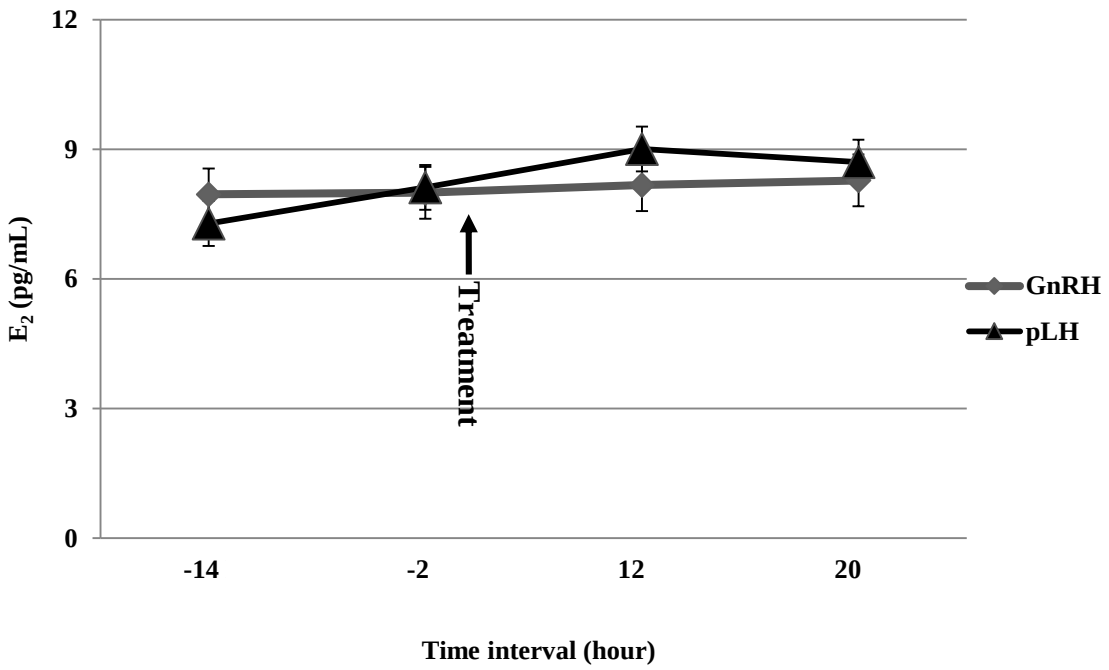

Figure 3.5. Least squares means (±SEM) plasma estradiol (E_2) concentrations (pg/mL) before (-14 and -2 h) and after (12 and 20 h) administration of 100 µg GnRH (n=6) or 25 mg porcine LH (pLH; n=8). Means of plasma estradiol concentrations did not differ at any time point ($P > 0.05$).

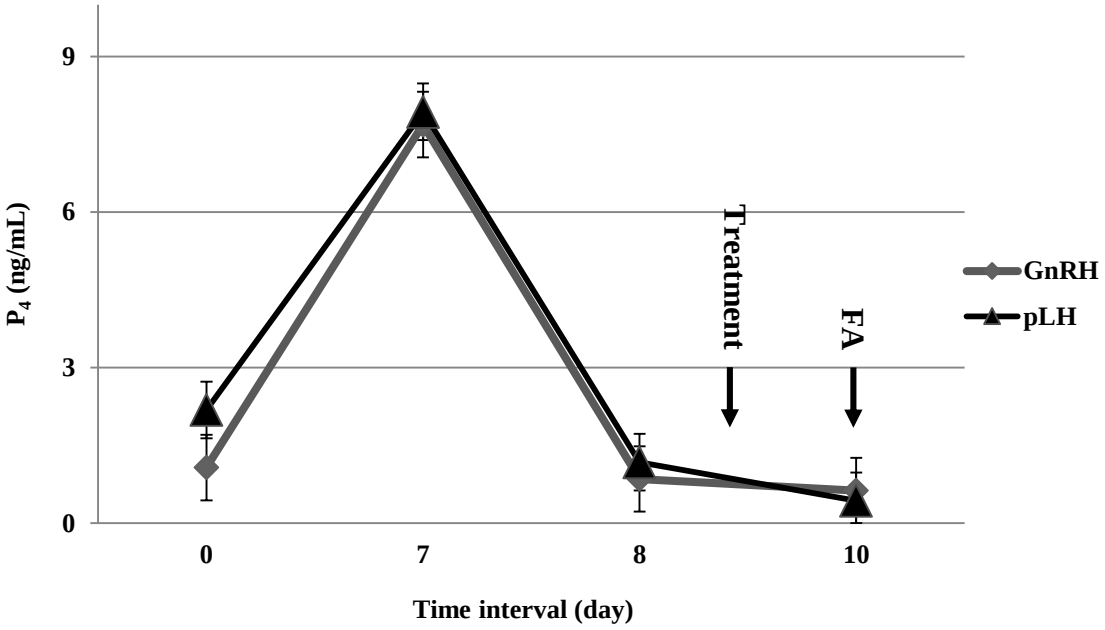

Figure 3.6. Least squares means (±SEM) plasma progesterone (P_4) concentrations (ng/mL) in100 µg GnRH- (n=3) or 25 mg porcine LH- (pLH; n=4; Period 1) over the collection period (Day 0: GnRH treatment, Day 7: PGF treatment, Day 8: one day after PGF treatment and Day 10: Ultrasound-guided transvaginal follicular aspiration [FA]). Means of plasma progesterone concentrations did not differ over the collection period between pLH- and GnRH-treated cows ($P > 0.05$).

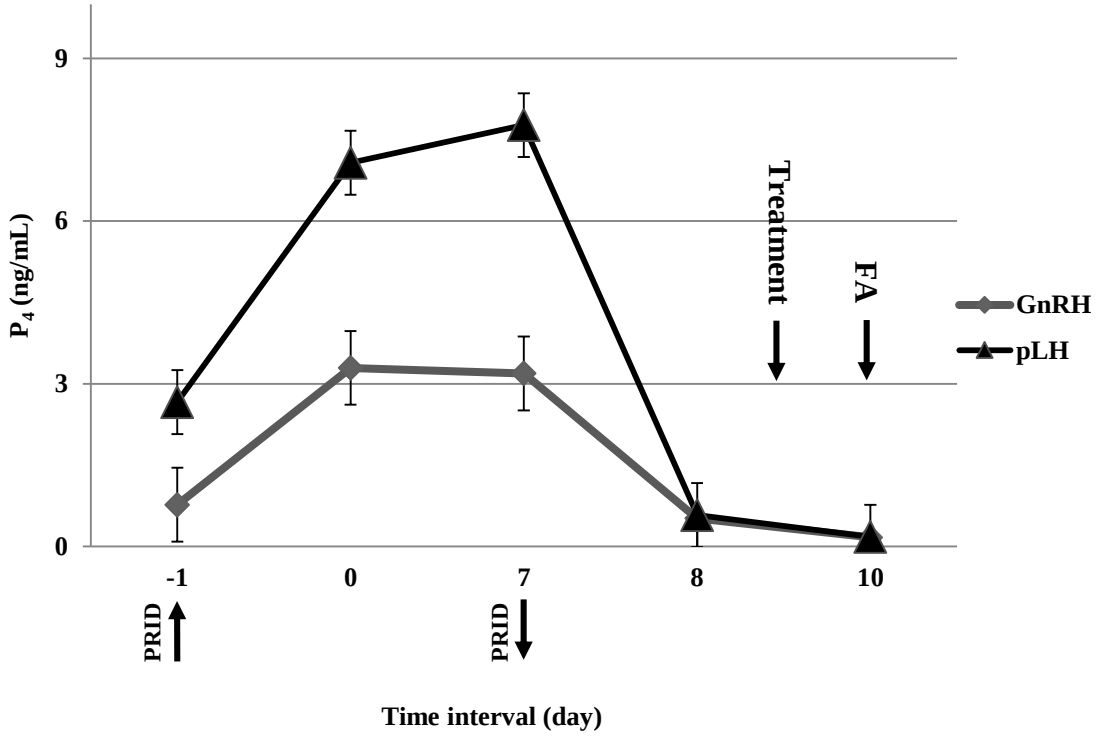

Figure 3.7. Least squares means (±SEM) plasma progesterone (P4) concentrations (ng/mL) in 100 µg GnRH- (n=3) or 25 mg porcine LH- (pLH; n=4; Period 2) over the collection period (Day -1: PRID insertion, Day 0: GnRH treatment, Day 7: PGF treatment and PRID removal, Day 8: one day after PGF treatment and Day 10: Ultrasound-guided transvaginal follicular aspiration). Means of plasma progesterone concentrations were higher in pLH group cows compared to GnRH cows at Days 0 and 7 ($P < 0.05$).

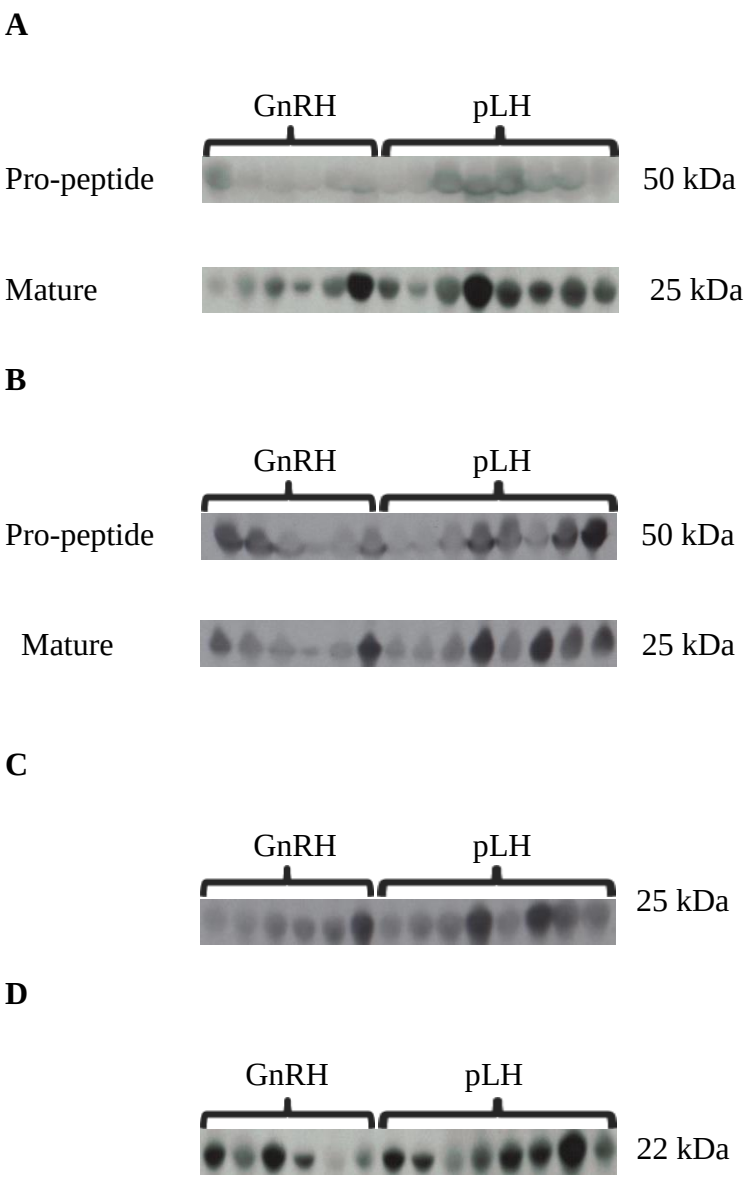

Figure 3.8. Western blot analysis of preovulatory follicular fluid at 21 ± 1 h after administration of 100 µg GnRH or 25 mg porcine LH (pLH). Ultrasound-guided transvaginal follicular aspiration was used to collect follicular fluid. The least squares means of normalized optical density levels of the mature form of BMP-15, GDF-9 and TGF-β1 differed in pLH-treated cows compared to GnRH-treated cows ($P < 0.05$). A) Pro-peptide and mature forms of bone morphogenetic protein-15 (BMP-15). B) Pro-peptide and mature forms of growth differentiation factor-9 (GDF-9). C) Transforming growth factor-beta 1 (TGF-β1). D) Insulin-like growth factor binding protein-3 (IGFBP-3).

Chapter 4. Gene Expression Profiles in Granulosa Cells and Cumulus Oocyte Complexes of FSH-Stimulated Follicles Exposed to Bovine or Porcine LH

4.1. Introduction

The present study was designed to test the main hypothesis that replacing GnRH with pLH to synchronize ovulation in dairy cows alters the expression of target genes in cumulus oocyte complexes.

Luteinizing hormone induces activation of the epidermal growth factor (EGF); consequently, EGF network signaling induces oocyte competence (De La Fuente et al., 1999) and oocyte maturation in vitro (Dekel and Sherizly, 1985). The activation of the EGF-like growth factors, amphiregulin (*AREG*), epiregulin (*EREG*) and Betacellulin (*BTC*) by an LH surge, induces activation of EGF receptors on the surface of somatic cells and promotes cumulus expansion and oocyte maturation (Conti et al., 2012). In addition, expression of the progesterone receptors (*PGr*) and LH receptors (*LHr*) are induced by LH surge during the preovulatory period in mural and cumulus granulosa cells (Sirard and Assidi, 2013). The key role of the *LHr* and *PGr* in the final differentiation of the dominant follicle and oocyte maturation have been well documented (Sirard and Assidi, 2013). The first study of this thesis (Chapter 3) was conducted to assess changes in the intrafollicular environment, including follicular fluid factors and gene expression in the granulosa cells of dairy cows, approximately 20 h after treatment with either GnRH or pLH in an Ovsynch protocol. Since cows are mono-ovulatory animals, oocyte recovery

from a mature preovulatory follicle by transvaginal follicle aspiration is not always possible. In the previous experiment (Chapter 3), for example, only one cumulus-oocyte-complex (COC) was collected from the 15 cows.

Due to the lack of sufficient research material (COC) obtained from the previous study (Chapter 3) to characterize gene expression in COC, the present experiment was designed with cows that were subjected to FSH treatment, to increase the number of preovulatory follicles and oocytes recovered.

The objectives were to:

1. characterize LH profiles in FSH-stimulated dairy cows in response to GnRH or pLH administration, and compare LH profiles after pLH administration using bovine- and porcine-LH specific standard curves
2. detect target genes that may be differentially regulated in COC of the preovulatory follicle in response to pLH or GnRH-induced bLH exposure in FSH-stimulated cows
3. detect target genes that may be differentially expressed in granulosa cells of the preovulatory follicles in response to pLH or GnRH-induced bLH exposure in FSH-stimulated cows.

4.2. Materials and methods

The study was conducted with eight cyclic non-lactating Holstein cows at the Dairy Research and Technology Centre (DRTC), at the University of Alberta, Edmonton, Alberta (53°4' N, 113°31' W). Animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care. The University of Alberta's Animal Care and Use Committee approved the experimental protocols

used is this study (Protocol 2012-176). Cows were housed in an outdoor pen with adequate shelter and straw bedding, and had unrestricted access to alfalfa hay and water.

4.2.1. Animals and experimental design

Cows (n=8) were subjected to ovarian stimulation with follicle stimulating hormone (FSH) (Folltropin-V®, Bioniche Animal Health Inc., Belleville, ON, Canada) to maximize the number of collected oocytes. To synchronize the follicular wave emergence, the dominant follicle and all subordinate follicles ≥ 10 mm in diameter were ablated in all cows 36 h before FSH treatment (Day -1) by a transvaginal ultrasound-guided procedure. The protocol for ovarian stimulation used decreasing doses of 40, 30, 20, and 10 mg of FSH twice daily (07:00 and 19:00 h) over a 4-d period with two prostaglandin $F_2\alpha$ treatments (PGF; 500 μg, cloprostenol; Estroplan®, Vetoquinol N.A Inc., Lavaltrie, QC, Canada) im at 12 h intervals given in conjunction with the sixth and seventh treatments of FSH. At the first injection of FSH on Day 0 (pm), all cows received a 1.55 g progesterone releasing intravaginal device (PRID®, Vetoquinol N.A. Inc.) and the device was removed at last injection of FSH on Day 4 (am). On Day 5 (24 h after last portion of FSH and PRID removal) all cows were assigned to one of the two treatment groups to receive either gonadotropin releasing hormone (GnRH; 100 μg, gonadorelin acetate; Fertiline®, Vetoquinol N.A. Inc.) or 25 mg porcine luteinizing hormone (pLH; Lutropin-V®, Bioniche Animal Health Inc.; Figure 4.1).

4.2.2. Ovarian ultrasonography

Transrectal ultrasonography was performed on Days 0 and 5. Ultrasonography on Day 0 was to determine the ovarian status, and that on Day 5 was to determine response to PGF and FSH treatment, diameter of the preovulatory follicles, and detect any early ovulations. Additional ultrasonography on Day 6 was also used to determine ovulatory response to GnRH or pLH treatment on Day 5, early ovulations and diameter of the preovulatory follicles.

4.2.3. Blood samples collection

Blood samples were collected from coccygeal vessels on Days 0, 2, 4 (10 h after PRID removal) and 6 (at aspiration time) to determine progesterone concentrations. Blood samples for estradiol concentrations were collected 14 and 2 h before GnRH or pLH treatment, as well as 12 and 20 h after GnRH or pLH. Sequential blood samples (SBS) were collected by indwelling jugular catheter to determine LH concentrations on Day 5. Blood samples were collected into heparinized tubes (Vacutainer) and were immediately processed until further solid phase radio-immunoassays were done as described in Chapter 3. Response to PGF was identified with reduction of progesterone concentrations 10 h after PRID removal.

4.2.4. Cumulus oocyte complexes collection

Ultrasound-guided transvaginal follicle aspiration was performed 21 ± 1 h after the administration of GnRH or pLH to collect follicular fluid as described in Chapter 3. Briefly, follicular aspiration was performed using an ultrasound machine (Aloka-500 V scanner; Aloka Co., Tokyo, Japan) equipped with a 5.0 MHz

curvilinear transvaginal transducer and needle guide, a 60 cm long 17-gauge single lumen needle fitted with a 19-gauge sterile disposable needle tip. To maximize oocyte recovery, needles and tubes were washed with 0.4% bovine serum albumin (BSA; BioLife Bovine Serum Albumin; Agtech Inc., Manhattan, KS, USA) in phosphate buffered saline (PBS; phosphate buffer saline, 0.01 M PBS, pH=7.5; Sigma Inc.) and preovulatory follicles ≥ 10 were punctured individually. Summary of the aspirated follicles per treatment is presented at Table 4.1. The follicular contents (follicular fluid), granulosa cells, and cumulus oocyte complexes of each individual were collected in separate RNase-free tubes. The tube containing the follicular fluid was labeled and transferred to the laboratory immediately after each aspiration.

4.2.4.1. Sample processing

The contents of the tube were searched for possible COC immediately after collection. Cumulus oocyte complexes from each individual were sorted and washed with 1 % Dulbecco's phosphate buffered saline (BioLife D-PBS; Agtech Inc.). Subsequently, cumulus oocyte complexes were transferred to cryo-vials (Nalgene®, New York, USA) for storage in a -80°C until RNA extraction to determine differential expression of genes. After COC isolation, follicular fluid samples were centrifuged 2000 × *g* for 3 min at 4°C. Since the needles were washed with PBS-BSA before and between follicular punctures, the supernatant (PBS + follicular fluid mixture) was not used for any further analyses. The granulosa cell pellets were washed with 1 ml ice-cold PBS-RNase free and centrifuged 2000 × *g* for 3 min at 4°C. The supernatant was discarded and the pellet was suspended in as

little fluid as possible and stored in the -80º C freezer until further gene expression analysis.

4.2.5. Laboratory Analyses

4.2.5.1. Radio-immunoassays for reproductive hormones

4.2.5.1.1. Plasma LH concentrations

Plasma LH concentrations were measured by radioimmunoassay using an anti-bovine LH monoclonal antibody (518B7; Quidel Corporation, San Diego, CA, USA) which has a high cross reactivity with both bovine and porcine LH as described in Chapter 3 (Matteri et al., 1987). Intra and inter-assay coefficients of variation for LH were 7.6 and 4.3%, respectively, for a high reference serum (mean = 1.26 ng/ml^{-1}) and 6.0 and 13.0%, respectively, for a low reference serum (mean = 0.61 ng/ml^{-1}). The LH concentrations in pLH-treated cows were calculated based on both bovine and porcine standard curves

4.2.5.1.2. Plasma estradiol and progesterone concentrations

Plasma estradiol and progesterone concentrations were measured by solid phase radio-immunoassay kits (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA) in triplicate as described in Chapter 3. Briefly, 100 µl plasma and 1 mL of a buffered 125^{I}-labeled estradiol or progesterone was added to an antibody-coated tube. After 3 h incubation at room temperature, fluid was aspirated. The radioactivity bound to the antibody coated tubes were counted and standardized by standard curve. The sensitivity of the assay and the intra- and inter-assay and coefficients of variation, respectively, were 0.1 pg/ml, 9.7 and 5.9%, for estradiol and were 0.1 ng/ml, 6 and 3.8%, for progesterone.

4.2.5.2. Gene expression analysis

4.2.5.2.1. RNA isolation and quantitation from COC

Total RNA extraction from pools of COC was achieved using the PicoPure RNA isolation kit (Applied Biosystems Inc., Carlsbad, CA, USA), with DNase treatment on the purification column according to the manufacturer's instructions. Briefly, 100 µl extraction buffer was added to COC pool (1 to 3) and incubated 42º C for 30 min. After incubation, an equal volume of 70% ethanol was then added to mixture. Consequently, the mixture was added to the RNA purification column that it was preconditioned with a 250 µl conditioning buffer for 5 min. The RNA purification columns were followed by with DNase treatment, two washings and RNA elution in 10 µl of elution buffer. The integrity and quantity of RNA were evaluated using an Agilent 2100 Bioanalyzer and the RNA 600 Pico Chips assay kit (Agilent Technologies, Inc., Palo Alto, CA, USA).

4.2.5.2.2. Amplification and reverse transcription of RNA from COC

RNA was amplified using the RiboAmp® HS RNA amplification kit (Applied Biosystems, Inc.). The kit produced RNA from our very small samples by using two successive rounds of *in vitro* T7 RNA transcription. Quantity of aRNA was determined using a Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). Reverse transcription of 900 ng of total RNA was performed with SuperScript III (Invitrogen Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions.

4.2.5.2.3. RNA isolation and quantitation from granulosa cells

Total RNA were extracted from 100 to 200 mg of pool of the granulosa cell pellets using the Absolutely RNA Miniprep kit (Agilent Technologies, Inc.) following the modified protocol for small samples as described in Chapter 3. Briefly, the lyses buffer was added to granulosa cell pellets and mixture was homogenized. The mixture was filtrated to remove particles. Columns were incubated with DNase treatment and low salt buffer, then washed to remove the DNase and protein; consequently, highly pure RNA was eluted in elution buffer. The integrity and quantity of RNA were evaluated using an Agilent 2100 Bioanalyzer and the RNA 600 Pico Chips assay kit (Agilent Technologies, Inc.). Reverse transcription of 900 ng of total RNA was performed with SuperScript III (Invitrogen Life Technologies) according to manufacturer's instructions

4.2.5.2.4. PCR primers, probe, amplification efficiency and Real-time PCR

The Express® software v3.0 (Applied Biosystems, Inc.) was used to design primer sequences and Taqman-MGB probes for each candidate gene based on species-specific sequences reported in GENBANK (Table 4.2). Real-Time PCR was performed in triplicates in 96-well plates using the Taqman® Universal PCR Master Mix (Applied Biosystems, Inc.) and the ABI 7900HT thermocycler (Applied Biosystems, Inc.). The serial dilution of cDNA (1:1, 1:10, 1:100; 1:1000, 1:10,000 and 1: 100,000) were used to evaluate the amplification efficiency of target genes. The amplification efficiency for all candidate genes were above 97% and slope was close to -3.44.

The real-time PCR program parameters were as follows: 95ºC for 20 min; then 40 cycles of 95°C for 10 min and 60ºC for 60 s. The comparative C_T method ($2^{-\Delta\Delta C_T}$ method) was used to detect fold-induction of each sample as described in Chapter 3. Briefly, the average C_T of the GOI (gene of interest) is normalized to the average C_T of internal control (housekeeping genes) for the same sample to calculate the normalized C_T for the gene of interested (Schmittgen and Livak, 2008).

4.3. Statistical analyses

The reproductive hormones data (plasma LH, progesterone and estrogen) were analyzed by repeated measures using the MIXED procedure of SAS (9.3; SAS Institute Inc., Cary, NC, USA) with an autoregressive covariance structure and time of blood sample collection as the repeated effect.

The statistical model included:

$$Y_{ij} = \mu + T_i + P_j + C\,(T)i_j + \varepsilon_{ij}$$

where Y_{ij} is the individual observation, μ is the overall mean, T_i is the effect of treatment (i = 1, 2), P_j is the effect of time period (j = 1, 2, 3 … and 14). The term C $(T)_{ik}$ was included as a random effect (k = 1, 2, 3 and 4; treated as a random effect), and ε_{ijk} is the residual error term. Cow effect was included in the model as a random effect. Statistical difference was declared at $P < 0.05$.

Treatment effects were determined for comparisons assessed of each GOI using MIXED procedure of SAS and $P < 0.05$ was considered significant.

The statistical model included:

$$Y_{ij} = \mu + T_i + C\,(T)i_j + \varepsilon_{ij}$$

where Y_{ij} is the individual observation, μ is the overall mean, T_i is the effect of treatment (i = 1, 2). The term C $(T)_{ik}$ was included as a random effect (k = 1, 2, 3 and 4; treated as a random effect), and ε_{ij} is the residual error term.

4.4. Results

4.4.1. Reproductive hormones in plasma and follicular fluid

4.4.1.1. Plasma LH

The GnRH-treated cows had an acute LH response, with concentrations surging from 0.2 ± 0.8 ng/mL to a mean peak of 6.3 ± 0.8 ng/mL by 1.5 h after treatment returning to basal concentrations by 8 h (0.3 ± 0.8 ng/mL; $P > 0.05$; Figure 4.2). Plasma LH concentrations were determined with bovine- and porcine-LH specific standard curves in pLH-treated cows. When GnRH-induced bLH concentrations were compared with pLH concentrations calculated using the bovine LH-specific standard curve, overall treatment differences were not evident. However, time and time-by-treatment interactions were detected ($P < 0.001$), with GnRH-induced bLH being higher than pLH concentrations from 30 min post-treatment until 4 h post treatment. When GnRH-induced bLH concentrations were compared with pLH concentrations calculated using the porcine LH-specific standard curve, both treatment and time effects were significant ($P < 0.05$), whereas no treatment-by-time interaction was evident. In pLH-treated cows, LH concentrations calculated with bovine-specific standard curve were elevated from basal concentrations (0.1 ± 0.18 ng/mL) to a mean peak of 1.3 ± 0.1 ng/mL by 3 h. Similarly, LH concentration peaked from 0.1 ± 1.6 ng/mL to 9.7 ± 1.6 ng/mL in

porcine-LH specific standard curve by 3 h. The pLH concentrations measured using both bovine and porcine standard curves remained higher than the mean basal concentration for up to 10 h after treatment (0.5 ± 0.1 and 2.2 ± 1.6 ng/mL; $P < 0.05$, respectively).

4.4.1.2. Plasma estradiol and progesterone

Least squares means of plasma estradiol concentrations (pg/mL) did not differ at 14 and 2 h before, and 12 and 20 h after either pLH or GnRH treatment (Figure 4.3). Least squares means of plasma progesterone concentrations (ng/mL) on Days 0, 2, 4 and 6 were not different among cows given pLH or GnRH (Figure 4.4). Both groups of cows responded equally to the PGF treatments during FSH stimulation treatments and progesterone concentrations decreased by 10 h after PRID removal on Day 4 ($P = 0.41$).

4.4.2. Gene expression

The expression levels of candidate genes were measured by RT-PCR in the amplified RNA of cumulus oocyte complexes and granulosa cells (Table 4.2). The relative expressions of candidate genes were not different between treatments.

4.5. Discussion

The results of the present study did not support the main hypothesis that replacing GnRH with pLH to synchronize ovulation in dairy cows alters gene expression in COC and granulosa cells of FSH-stimulated cows 20 ± 1 h after treatment.

The LH concentrations in serial blood samples from 30 min before to 20 h after treatment were determined in cows that had received FSH stimulation. Similar to the non-stimulated study (Chapter 3), GnRH-treated cows had an acute response to treatment and LH concentrations returned to the basal level by 8 h (Figure 4.5). Although, the LH concentrations peaked by 1.5 h in both non-stimulated and FSH-stimulated studies for GnRH-treated cows, the shape of the curve and peak LH concentrations in the FSH-stimulated GnRH cows were significantly lower compared to non-stimulated GnRH cows.

In the present study, plasma LH concentrations in pLH-treated cows peaked 3 h after treatment, whereas non-stimulated pLH cows had an LH peak by 1.5 h. When calculated using the bovine standard curve, the LH concentrations decreased to lower than 1 ng/ml by 4 h in FSH stimulated pLH cows; nevertheless, LH concentrations remained significantly higher than basal concentrations for up to 10 h. The LH profile based on the bovine standard curve for FSH-stimulated pLH cows was not in agreement with that reported previously for non-stimulated pLH cows (Chapter 3; Ambrose et al. 2005). The LH concentration had a greater area under curve and LH was $\geq$ 1 ng/ml until 20 h after treatment in non-stimulated pLH cows compared to FSH-stimulated pLH cows.

As stated earlier, the main objective of the current study was to determine whether target genes were differentially expressed in the cumulus oocyte complexes of preovulatory follicles after either GnRH or pLH treatments 20 ± 1 h post treatment. To increase recovery of COC, the aspiration needle and collection tubes were washed with PBS containing BSA before, during and after the

aspirations; as a result, follicular fluid obtained in the present study was mixed with PBS, and hence not used for either hormone assay or Western blotting. The oocyte is surrounded with highly specialized follicular cells called cumulus cells. Removal of the cumulus cells from the oocyte will disrupt intercellular communications between the oocyte and cumulus cells, which can reduce or terminate oocyte developmental competence (Tanghe et al., 2002; Matzuk et al., 2002; Tanghe et al., 2003). For this reason, we measured gene expression in intact COC rather than either cumulus cells or oocytes separately.

Furthermore, EGF-like peptides are oocyte-secreted factors that can influence cumulus functions and expansion during the preovulatory LH surge (Park et al., 2004; Panigone et al., 2008). The effect of *AREG*, *EREG* and *BTC* on oocyte maturation and expansion is well documented (Conti et al., 2006). Park et al. (2004) reported the rapid and transient expression of *AREG*, *EREG* and *BTC* mRNA in the first 3 h after hCG treatment in the mouse granulosa and cumulus cells. Furthermore, Park et al. (2004) made the interesting observation that *AREG* and *EREG* promote oocyte maturation and cumulus expansion faster than LH, and that LH was not effective when COC were isolated from mural granulosa of preovulatory follicle. According to this observation, *AREG* and *EREG* acted downstream of LH and stimulation of these factors in cumulus cells initiate a meiotic resumption of the oocyte (Conti et al., 2006). Although *AREG*, *EREG* and *BTC* mRNA expression in cumulus oocyte complexes and granulosa cells did not differ between treatments in COC and granulosa cells did not differ between treatments in present study, the predominant EGF-like growth factors' mRNA were

expressed at different levels (Table 4.3 and 4.4) in granulosa and cumulus cells 21 ± 1 h post LH surge.

The relative expression of the *AREG* was higher in granulosa cells of the GnRH- than pLH-treated cows in non-stimulated cows (Chapter 3); however, no differences in *AREG* expression was observed in FSH-stimulated cows after GnRH or pLH treatment. As stated earlier, cows were subjected to FSH treatments in the present study (Chapter 4); thus, two different physiological approaches were used in Chapters 3 and 4. In addition, different quantities of RNA were used for gene expression analyses in Chapters 3 and 4. As a consequence, expression levels of the target genes in granulosa cells of non-stimulated study (Chapter 3) were not directly comparable with FSH-stimulated study (Chapter 4). Either of two possibilities might have contributed to the high *AREG* abundance in granulosa cells of the non-stimulated GnRH cows. First, the acute and higher mean LH concentration in non-stimulated GnRH cows might have resulted in late or extended expression of *AREG* post 20 h after GnRH treatment. The possible association between plasma LH concentrations and EGF-like growth factor expression has apparently not been reported in the literature. However, a positive association was detected between *AREG* abundance and LH concentrations in the follicular fluid of the mature follicle in women 36 h post human chorionic gonadotropin (hCG) stimulation (Zamah et al., 2010). The second possibility is that FSH treatment used for ovarian stimulation might have resulted in follicles with low competence to reach the preovulatory stage. Sirard et al. (2006) documented that a significant proportion of the stimulated oocytes did not reach to the blastocyst stage 7 days post fertilization. It is assumed

that the ovary may produce some late growing follicles under stimulation protocol and the oocytes produced in natural non-stimulated condition are more fully competent compared to stimulated oocytes (Sirard et al., 2006).

The LH receptors mediate actions of LH and are G protein-coupled receptors (McFarland et al., 1989). The *LHr* appearance on granulosa cells is a fundamental stage for folliculogenesis from the achievement of follicular dominance until ovulation (Ginther et al., 2001). Both low and no expression of *LHr* in the rat and mouse cumulus cells and oocyte have been reported (Peng et al., 1991). Although the relative abundance of *LHr* in both cumulus oocyte complexes and granulosa cells did not differ between GnRH- and pLH-treated cows, the *LHr* were detected in the cumulus cells of the both treatments. The expression level of *LHr* in follicles of super-stimulated Holstein heifers with either FSH (FolltropinV®, 225 mg) or equine chorionic gonadotropin (Folligon®, 2,500 UI) were investigated by Soumano et al. (1998). They observed that the *LHr* abundance was reduced in the equine chorionic gonadotropin-treated heifers compared to FSH treatments. This observation would suggest that the *LHr* expression in this study might not have been influenced by FSH stimulation protocol.

Armstrong et al. (1996) have reported that progesterone is secreted by cumulus cells during *in vitro* meiotic maturation of cumulus oocyte complexes and stimulation of LH can increase the progesterone secretion. In addition, a positive association between the progesterone concentrations and germinal vesicle breakdown has been reported by Yamashita et al (2003). Expressions of progesterone receptors are initiated by the LH surge (Sirard and Assidi, 2013). The

expression of *PGr* play an important role in cumulus cell differentiation and avoiding the granulosa cells apoptosis; consequently, the *PGr* facilitate progression of the dominant follicle to ovulation (Shimada et al., 2004; Sirard et al., 2013). In the current study, there were no significant differences in the abundance of *PGr* in both cumulus oocyte complexes and granulosa cells between GnRH- and pLH-treated cows.

4.6. Conclusion

In the present study, FSH-stimulated cows treated with either 100 µg GnRH or 25 mg pLH during the preovulatory period had lower mean plasma LH concentrations than that of non-stimulated GnRH- or pLH-treated cows. The abundance of *AREG*, *EREG*, *BTC*, *LHr* and *PGr* mRNA in COC and granulosa cells did not differ between GnRH- and pLH-treated cows at approximately 20 h post treatment. Since preovulatory follicles were aspirated approximately 20 h after treatment in this study, the experimental design of the study did not allow us to run gene expression at earlier times after treatments. This study could not provide evidence that EGF signaling cascade was activated by LH 20 h post treatment.

4.7. Literature cited

Ambrose, J. D., J. P. Kastelic, R. Rajamahendran, M. Aali and N. Dinn. 2005. Progesterone (CIDR)-based timed AI protocols using GnRH, porcine LH or estradiol cypionate for dairy heifers: Ovarian and endocrine responses and pregnancy rates. Theriogenology. 64:1457-1474.

Baruselli, P. S., de Sá Filho, Manoel F, C. M. Martins, L. F. Nasser, M. F. Nogueira, C. M. Barros and G. A. Bó. 2006. Superovulation and embryo transfer in Bos indicus cattle. Theriogenology. 65:77-88.

Conti, M., M. Hsieh, A. Musa Zamah and J. S. Oh. 2012. Novel signaling mechanisms in the ovary during oocyte maturation and ovulation. Molecular and Cellular Endocrinology. 356:65-73.

Conti, M., M. Hsieh, J. Park and Y. Su. 2006. Role of the epidermal growth factor network in ovarian follicles. Molecular Endocrinology. 20:715-723.

De La Fuente, R., M. J. O'Brien and J. J. Eppig. 1999. Epidermal growth factor enhances preimplantation developmental competence of maturing mouse oocytes. Human Reproduction. 14:3060-3068.

Dekel, N. and I. Sherizly. 1985. Epidermal growth factor induces maturation of rat follicle-enclosed oocytes. Endocrinology. 116:406-409.

Ginther, O., D. Bergfelt, M. Beg and K. Kot. 2001. Follicle selection in cattle: Role of luteinizing hormone. Biology of Reproduction. 64:197-205.

Hendriksen, P., P. Vos, W. Steenweg, M. Bevers and S. Dieleman. 2000. Bovine follicular development and its effect on the in vitro competence of oocytes. Theriogenology. 53:11-20.

Matteri, R., J. Roser, D. Baldwin, V. Lipovetsky and H. Papkoff. 1987. Characterization of a monoclonal antibody which detects luteinizing hormone from diverse mammalian species. Domestic Animal Endocrinology. 4:157-165.

McFarland, K., R. Sprengel, H. S. Phillips, M. Kohler, N. Rosemblit, K. Nikolics, D. L. Segaloff and P. H. Seeburg. 1989. Lutropin-choriogonadotropin receptor: An unusual member of the G protein-coupled receptor family. Science. 245:494-499.

Panigone, S., M. Hsieh, M. Fu, L. Persani and M. Conti. 2008. Luteinizing hormone signaling in preovulatory follicles involves early activation of the epidermal growth factor receptor pathway. Molecular Endocrinology. 22:924-936.

Park, J. Y., Y. Q. Su, M. Ariga, E. Law, S. L. Jin and M. Conti. 2004. EGF-like growth factors as mediators of LH action in the ovulatory follicle. Science. 303:682-684.

Peng, X., A. J. Hsueh, P. S. Lapolt, L. Bjersing and T. Ny. 1991. Localization of luteinizing hormone receptor messenger ribonucleic acid expression in ovarian cell types during follicle development and ovulation. Endocrinology. 129:3200-3207.

Schmittgen, T. D. and K. J. Livak. 2008. Analyzing real-time PCR data by the comparative CT method. Nature Protocols. 3:1101-1108.

Sirard, M., L. Picard, M. Dery, K. Coenen and P. Blondin. 1999. The time interval between FSH administration and ovarian aspiration influences the development of cattle oocytes. Theriogenology. 51:699-708.

Sirard, M. and M. Assidi. 2013. Screening for oocyte competence. In Krisher R.L. (Ed.), *Oocyte Physiology and Development in Domestic Animals (pp 191-206).* 1st ed. John Wiley & Sons, Inc., Hoboken, NJ. USA.

Sirard, M., F. Richard, P. Blondin and C. Robert. 2006. Contribution of the oocyte to embryo quality. Theriogenology. 65:126-136.

Zamah, A., M. Hsieh, J. Chen, J. Vigne, M. Rosen, M. Cedars and M. Conti. 2010. Human oocyte maturation is dependent on LH-stimulated accumulation of the epidermal growth factor-like growth factor, amphiregulin. Human Reproduction. 25:2569-2578.

Table 4.1. Least squares means (±SEM) number of preovulatory follicles, aspirated follicles and cumulus oocyte complexes collected in 100 µg GnRH- (n=4) and 25 mg porcine LH (pLH; n=4) treated FSH stimulated Holstein cows.

	GnRH	**pLH**	***P* value**
No. cows	**4**	**4**	
Preovulatory follicles	11.5 ± 3.1	8 ± 3.1	0.45
Range	4-24	6-10	
Aspirated follicles	7.5 ± 1.3	6.5 ± 1.3	0.62
Range	4-10	5-8	
Cumulus oocyte complexes	2.2 ± 1.3	2.5 ± 1.3	0.89
Range	2-3	0-8	

Table 4.2. Details of primers used for RT-PCR analysis.

Gene Symbol	Gene Bank accession no	Forward primer	Reverse primer
AREG	NM_001099092	TGGTCGACAGCGATTTATCAAA	TCATGGCAGAGACGAAAGCA
BTC	NM_173896	GCCCCAAGCAGTACAAGCAT	GCTCGGCCACCACGAA
EREG	XM_002688367	CCTCTCCCCATCACTCACTTCT	GACGGGTTTTGTGGAAGACAA
LHr	U20504.1	CGGAAGGCGTCGTTGTG	CACGCCTGGAGAAGA
PGr	NM_001205356	CGGGCACTGAGTGTTGAATTT	TCTTGGGTAACTGTGCAGCAA
GAPDH	U85042.1	TGCCGCCTGGAGAAACC	CGCCTGCTTCACCACCTT
SDHA	NM_174178.2	ACTTCACCGTTGATGGCAATAA	CGCAGAAATCGCATCTGAAA
B-Actin	NM_173979.3	CCTGCGGCATTCAGAA	GCGGATGTGGACGTCACA

Table 4.3. Summary of statistical analysis showing the fold changes in the expression of *AREG*, *BTC*, *EREG*, *LHr* and *PGr* genes in cumulus oocyte complexes of preovulatory follicular fluid at 21 ± 1 h after the administration of 100 µg GnRH or 25 mg porcine LH (pLH) in FSH stimulated Holstein cows. Ultrasound-guided transvaginal follicular aspiration was used to collect granulosa cells.

	Treatment		***P* value**
	GnRH (n=3)	**pLH (n=3)**	
AREG	1.5 ± 0.9	0.009 ± 0.9	0.34
BTC	1.2 ± 0.7	1.0 ± 0.7	0.91
EREG	1.5 ± 1.2	0.8 ± 1.2	0.71
LHr	2.6 ± 1	0.1 ± 1	0.23
PGr	1.5 ± 0.9	0.2 ± 0.9	0.38

Table 4.4. Summary of statistical analysis showing the fold changes in the expression of *AREG*, *BTC*, *EREG*, *LHr* and *PGr* genes in granulosa cells of preovulatory follicular fluid at 21 ± 1 h after the administration of 100 µg GnRH or 25 mg porcine LH (pLH) in FSH stimulated Holstein cows. Ultrasound-guided transvaginal follicular aspiration was used to collect granulosa cells.

	Treatment		***P* value**
	GnRH (n=3)	**pLH (n=3)**	
AREG	1.3 ± 0.3	0.7 ± 0.3	0.22
BTC	0.8 ± 0.3	0.7 ± 0.3	0.90
EREG	0.3 ± 0.2	0.6 ± 0.2	0.41
LHr	0.1 ± 0.2	0.5 ± 0.2	0.19
PGr	1.5 ± 0.6	1.0 ± 0.6	0.57

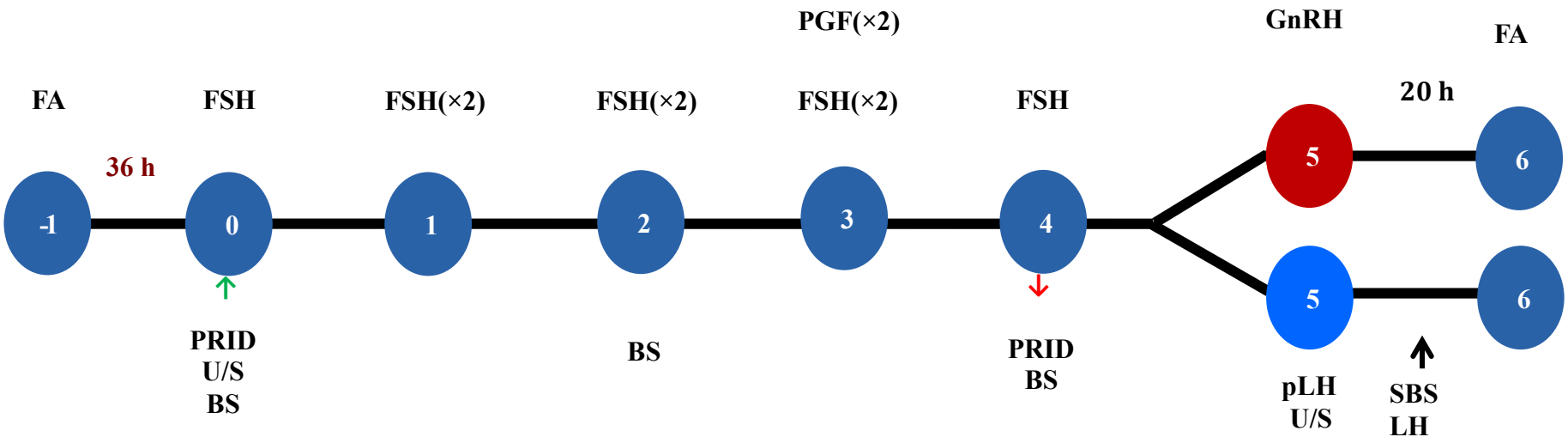

Figure 4.1. Schematic representation of the timeline of FSH-stimulation study. Cyclic non-lactating Holstein cows (n = 8) Cows received a progesterone (P_4) intravaginal device (PRID; 1.55g P_4) 36 h after follicular aspiration. Ovarian stimulation protocol was applied with decreasing doses of 40, 30, 20, and 10 mg of FSH twice daily (07:00 and 19:00 h) over a 4-d period, with injection of prostaglandin $F_{2\alpha}$ PGF (at 12 h intervals on Days 3). Ultrasonographic examinations (U/S) were performed on Days 0, 5 and 6 to determine response to treatments and dominant follicle diameter. Blood samples (BS) were collected on Days 0, 4 and 6 to determine P_4 concentration. Additional blood samples to determine estradiol (E_2) concentration were collected 14 and 2 h before and 12 and 20 h after either GnRH or pLH. Sequential blood samples (SBS) were collected after either GnRH or pLH to determine LH concentrations. FA was performed 21 ± 1 h after the administration of GnRH or pLH to collect follicular fluid and granulosa cells.

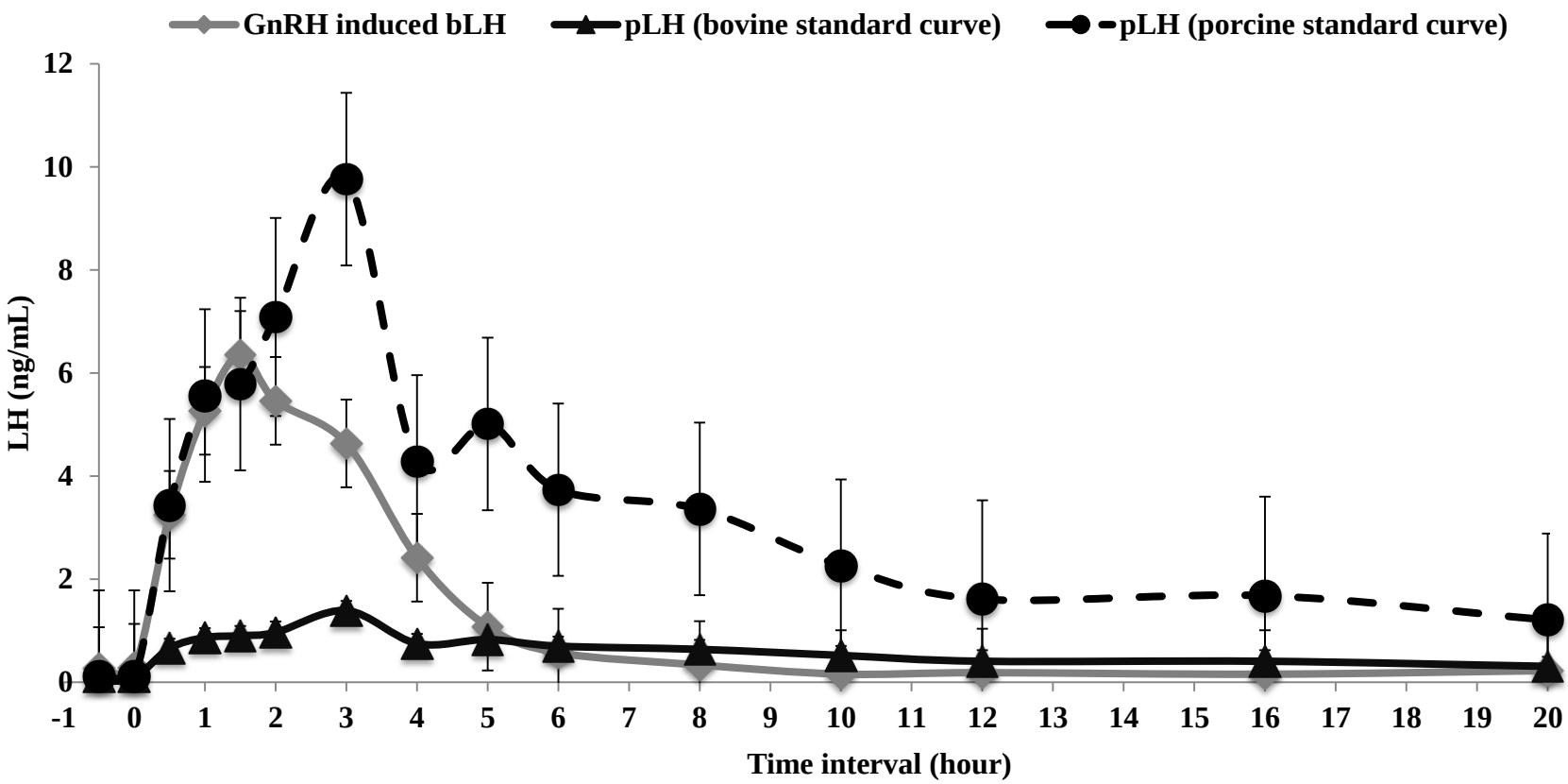

Figure 4.2. Plasma LH concentrations in a subset of 8 non-lactating FSH-stimulated cows (GnRH n=4; pLH n=4) after treatment with 100 µg GnRH or 25 mg pLH. Concentrations of plasma bLH in GnRH-treated cows were calculated using a bovine-LH-specific standard curve, whereas in pLH-treated cows LH concentrations were calculated using both bovine-LH- and porcine-LH-specific standard curves. The LH concentrations in GnRH-treated cows reached basal levels by 8 h post-treatment. However, LH concentrations calculated using bovine-LH-specific standard curve returned to basal level. However, the LH concentrations in both bovine and porcine standard curves were statistically different up to 10 h after treatment *(P* < 0.05).

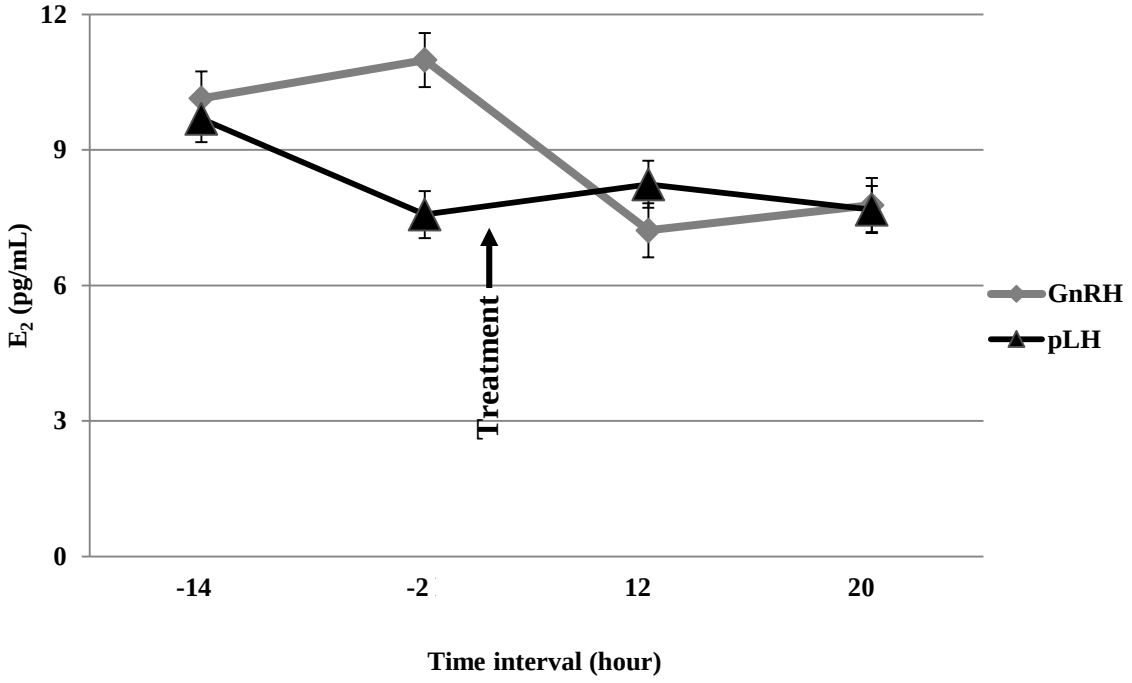

Figure 4.3. Least squares means (±SEM) plasma estradiol (E_2) concentrations (pg/mL) before (-14 and -2 h) and after (12 and 20 h) administration of 100 µg GnRH (n=4) or 25 mg porcine LH (pLH; n=4) in FSH stimulated Holstein cows. Means of plasma estradiol concentrations did not differ over the collection periods between GnRH- and pLH-treated cows ($P > 0.05$).

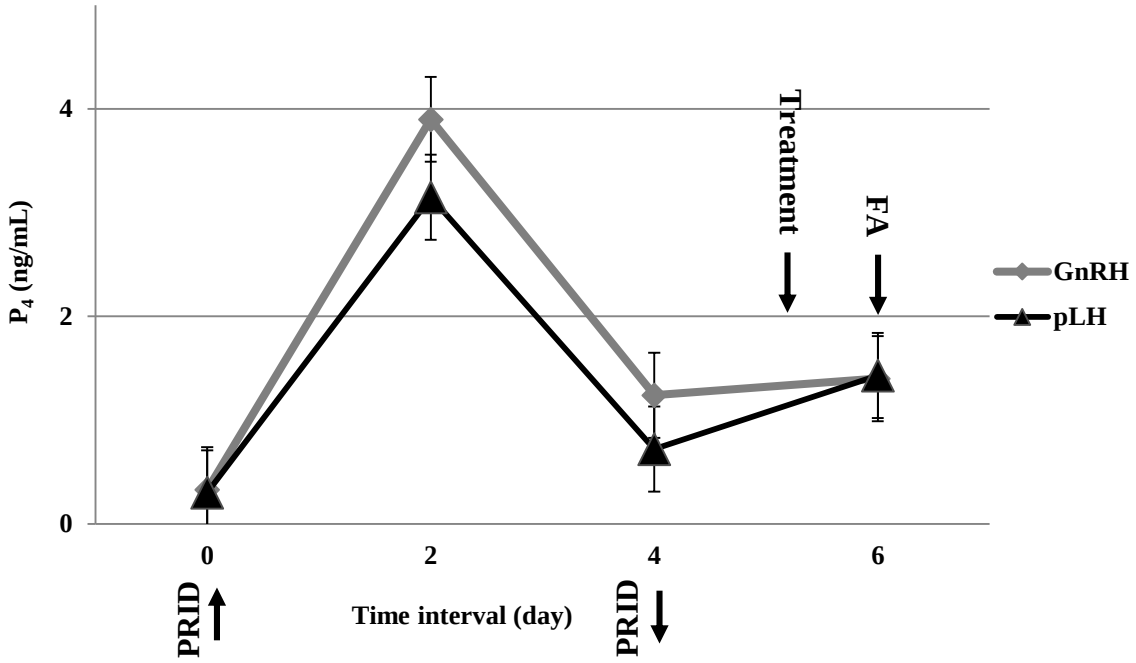

Figure 4.4. Least squares means (±SEM) plasma progesterone (P_4) concentrations (ng/mL) in 100 µg GnRH- (n=4) and 25 mg porcine LH- (pLH; n=4) treated FSH-stimulated Holstein cows over the collection period (Day 0: PRID insertion, Day 2, Day 4: 10 h after PRID removal, and Day 6: Ultrasound-guided transvaginal follicular aspiration, FA). Means of plasma progesterone concentrations did not differ over the collection periods between GnRH- and pLH-treated cows ($P > 0.05$).

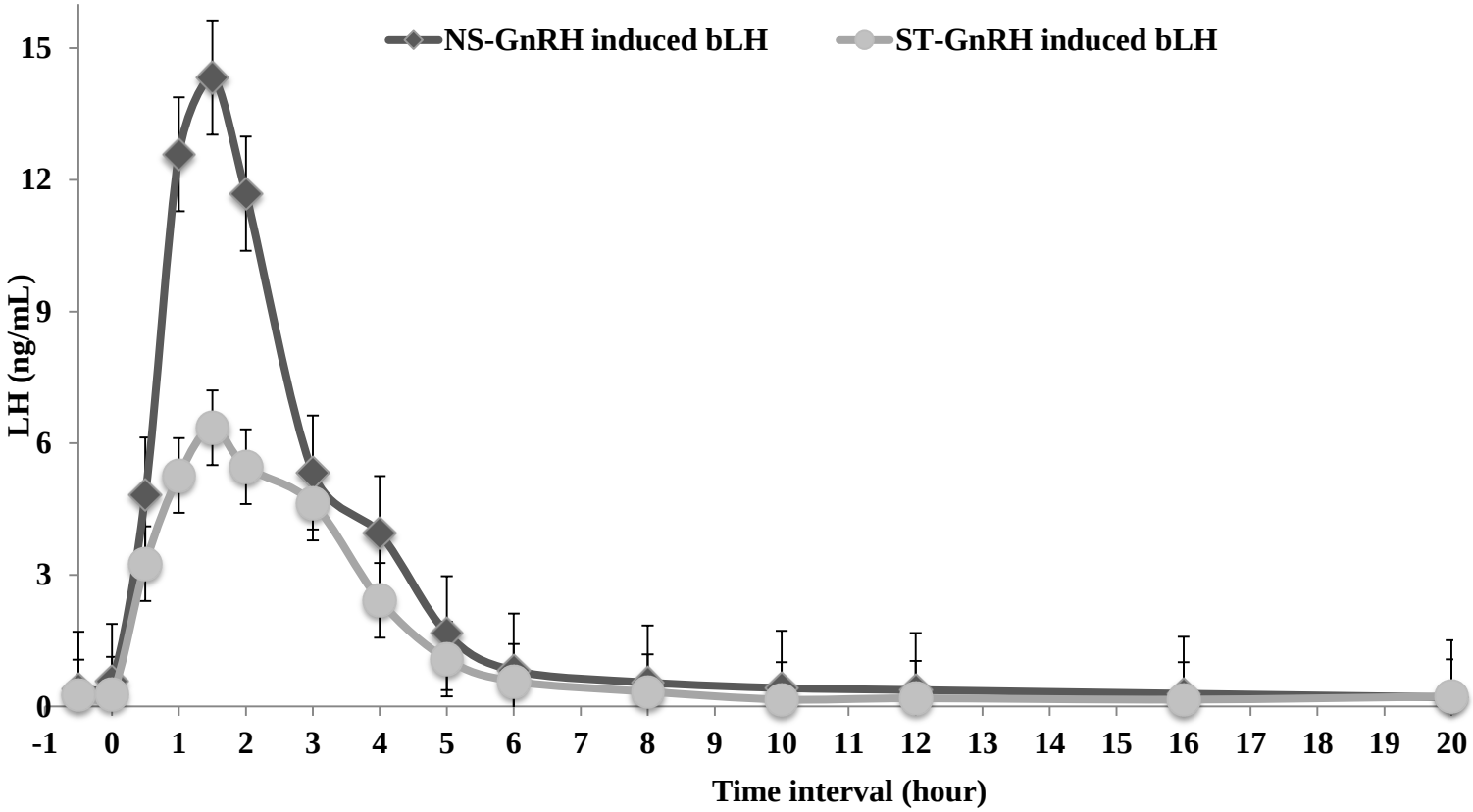

Figure 4.5. Plasma LH concentrations in a subset of non-stimulated (NS-GnRH induced bLH; n=6) versus FSH-stimulated (ST-GnRH induced bLH; n=4) non-lactating Holstein cows after treatment with 100 µg GnRH.

Chapter 5. General Discussion

5.1. Main findings

Two studies were conducted to identify the follicular fluid characteristics and the target genes that may be differentially regulated in bovine granulosa cells and cumulus-oocyte-complexes after pLH or GnRH treatment. The main objectives of the first study were to: 1) detect expression of *AREG*, *EREG*, *BTC*, *LHr* and *PGr* genes in granulosa cells and cumulus oocyte complexes of the preovulatory follicle in response to pLH or GnRH-induced bLH exposure; 2) compare porcine and bovine LH profiles in pLH-treated cows versus GnRH-treated cows; and 3) determine if preovulatory follicular fluid factors, IGF-I, IGFBP-3, BMP-15, GDF-9 and TGF-β1 differed in response to porcine LH or GnRH-induced bovine LH exposure. The relative abundance of the BMP15, GDF9 and TGF-β1 were higher in the pLH-treated cows compared to GnRH- treated cows in the follicular fluid of preovulatory follicle 21 ± 1 h after treatments. The expression of all the target genes was detected in granulosa cells of the preovulatory follicle 20 h ± 1 after either pLH or GnRH treatments. However, only *AREG* expression differed between treatments and the relative abundance of *AREG* was higher in GnRH- than in pLH-treated cows Therefore, a prolonged above-basal (≥1 ng/mL) LH profile, characteristic of pLH-treated cows, might have been associated with the higher expression of BMP-15, GDF-9 and TGF-β1 proteins. In other words, these factors might have been secreted more abundantly by oocytes of the pLH-treated cows under an extended exposure to elevated LH concentrations. Both BMP-15 and GDF-9 are essential for oocyte maturation and competence, and also for fertilization and pre-implantation

embryogenesis (Su et al., 2004); therefore, it is very likely that these factors contributed to improved oocyte competence in pLH-treated cows resulting in the higher pregnancy rates previously reported (Colazo et al., 2009).

The second study was conducted to identify potential differences in the expression level of the target genes in the cumulus oocyte complexes. The main objectives of the second study were to: 1) detect *AREG, EREG, BTC, LHr* and *PGr* genes expression in cumulus oocyte complexes of the preovulatory follicles in response to pLH or GnRH-induced bLH exposure; 2) compare LH profiles in the ovarian stimulated cows with FSH treatment; and 3) identify the differential expression of target genes in the granulosa cells of the preovulatory follicles, in response to pLH or GnRH-induced bLH exposure in the FSH-stimulated cows. Cows were subjected to ovarian stimulation protocol with FSH treatment in this study to increase the proportion of COC recovery rate. No treatment differences were observed for gene expression profiles in the cumulus oocyte complexes and granulosa cells of the preovulatory follicles. Although FSH-stimulated cows also had an acute response to GnRH treatments, their mean LH concentration was lower than that of non-stimulated cows used in Chapter 3 and by Ambrose et al. (2005). The LH concentrations in the FSH-stimulated pLH cows was elevated $\geq$ 1 ng/mL until 4 h and decreased sooner than what had been reported previous studies (Ambrose et al., 2005; Ree et al., 2009).

5.2. Limitations

Although the fundamental questions about the oocyte competence and embryo quality can be investigated with non-surgical collection techniques in

cattle, the response of the treated individuals to the treatments often varies. In accordance with this view, a brief explanation will be provided about the main limitations encountered during the current research. Taken together, the cows' responses to Ovsynch and FSH stimulation were satisfactory in both studies and only one GnRH-treated cow ovulated prematurely before aspiration of the preovulatory follicle in the non-stimulated cows group.

Although collection of follicular fluid, cumulus oocyte complexes and the granulosa cells by ultrasound guided transvaginal follicular aspiration offers great advantages and possibilities for studying ovarian physiology, it also presents some difficulties and limitations. Ultrasound-guided transvaginal follicular aspiration is the most frequently used method for *in vivo* collection of follicular contents (Čech et al., 2011). Rupture of follicles (De Castro e Paula, LA et al., 2008) and blood contamination in the follicular fluid (Ginther et al., 1997) have resulted in unsuccessful aspirations. In the current study, all preovulatory follicles ≥ 13 mm diameter in the non-stimulated cows and preovulatory follicles ≥ 10 mm diameter in FSH- stimulated cows were aspirated successfully.

Working with live animal species, such as cattle, is often difficult. Cow movements during aspiration result in needle movements. Consequently, injuries can be caused by the needle movement while attempting to reach the targeted follicle. Visible blood contamination in the follicular fluid limits the number of the follicles collected (Arashiro et al., 2013). Arashiro et al. (2013) reported that around 11.4% of the follicular fluid samples collected from Holstein cows were excluded during the *in vivo* aspiration due to the visible blood contamination. They explained

that granulosa cells might be unusable for gene expression analysis when visible blood contamination occurs. Blood contamination may result in miscount of the cell concentration and could also interfere with the efficiency of the total RNA extraction using the commercial kit (Arashiro et al., 2013). Fortunately, no follicular fluid samples were excluded in our study with non-stimulated cows (Chapter 3) due to the visible blood contamination. However, one GnRH-treated cow was excluded due to the low follicular fluid volume and poor RNA quantity in granulosa cells. In the FSH-stimulated study (Chapter 4), several aspirations were performed in the same ovary. Follicular aspiration was only stopped occasionally when a visible blood contamination was observed in the extension tube. Subsequently, the contaminated fluid from the last aspiration was not mixed with the tube contents.

The bovine cumulus oocyte complex obtained in mature condition is sticky and tends to adhere to the needle, extension tubes, etc. (Samper et al., 2007). To keep COC from sticking, the aspiration needle and tubes were washed with 0.4% bovine serum albumin in phosphate buffered saline to increase the recovery rate in the second study. Although the cows' responses to FSH stimulation were satisfactory, the recovery rates for COC were lower than expected. Subsequently, the total RNA extracted from the COC did not provide enough mRNA for analysis by RT-PCR, and total RNA was amplified using a commercial kit.

The main objective of the current work was to study the intrafollicular environment, including follicular fluid factors, genes expression in granulosa cells and COC approximately 20 h after GnRH or pLH administration. Although this

study did detect all target genes in the granulosa cells and the COC 21 ± 1 h after GnRH or pLH administration, the study design did not allow us to obtain follicular contents at various times after treatments. Thus, we speculate that only the late expression of target genes was detected in the present study. As stated in Chapters 3 and 4, LH induces the initial activation of the EGF network as early as 30 min after stimulation in mouse (Conti et al., 2012). Likewise, the rapid and transient expression of *AREG* and *EREG* were maximal 2 h after LH stimulation with hCG treatment (Park et al., 2004; Conti et al., 2012). In mouse, the preovulatory LH surge duration is around 2 to 4 h and ovulation most often occurs approximately 12 h after LH stimulation (Robertson et al., 2009). In view of the above, investigating activation of the EGF network in first hours after the LH surge in GnRH- and pLH-treated cows would provide a better understanding of the action of different LH profiles (exogenous porcine LH and GnRH-induced endogenous bovine LH) on the mural and cumulus granulosa cells.

5.3. Further research

The two studies discussed in this thesis have provided valuable information regarding the intrafollicular environment changes 20 h after either GnRH or pLH administration in dairy cattle which were subjected to an Ovsynch protocol. As stated earlier, future research may be warranted to determine differential regulation of EGF-like growth factor, genes and proteins required for oocyte maturation in response to pLH and GnRH administration earlier than the 20 h.

There have been a few *in vivo* studies that have investigated EGF-like factor expressions starting from immediately after hCG administration to 4-6 h post

treatment in rodents (Park et al., 2004; Panigone et al., 2008; Carletti and Christenson, 2009). However, it should be pointed out that most of the current data in the literature on EGF-like growth factor actions in the ovary were derived from *in vitro* studies.

As stated earlier, non-surgical follicular aspiration technique is a frequently-used method to investigate *in vivo* oocyte maturation. In this technique, cattle are usually subjected to synchronization treatments for 1 or 2 weeks. Most often the recovery rate is much lower than the expected rates in the ultrasound guided transvaginal follicular aspiration (Cech et al., 2002). On the contrary, *in vitro* oocyte maturation studies can investigate the specific molecules produced during oocyte competence in any developmental stage. Consequently, an *in vitro* oocyte culture system may be a good alternative to *in vivo* studies. The culture systems for *in vitro* maturation of bovine oocytes are well-established and can provide the appropriate environment for growth and maturation of the oocyte that can lead to a better understanding of factors directly involved in regulation of oocyte competence and early embryogenesis.

Therefore, future studies could be designed to detect the signaling cascades that are activated by LH at the time of oocyte maturation and ovulation in the cultured oocyte using either follicular fluid or serum of GnRH- and pLH-treated cows. For instance, involvement of the EGF network in the mural and cumulus granulosa cells of the mouse have been investigated in some *in vivo* and *in vitro* studies. Exogenous *AREG* or *EREG* induces oocyte maturation as efficiently as LH in the cultured preovulatory follicles of mice; whereas, the *BTC* is only partially

effective (Park et al., 2004). The physiological processes such as *in vivo* oocyte maturation and ovulation in either *AREG*- or *EREG*-null-mice have been investigated by Hsieh et al. (2008) that activation of EGF receptors were initiated by LH stimulation. Moreover, the role of the combination BMP-15 and pLH (Lutropin-V) on the COC expansion and differential expression of the *AREG* and *EREG* has been investigated in the recent bovine *in vitro* maturation study (Caixeta et al., 2013). In spite of these data, evidence for a physiological role and possible association between LH-induced signaling networks with the LH concentration in either *in vitro* or *in vivo* studies are lacking. The early transactivation of the EGF network and mechanism regulating meiotic oocyte resumption and critical signals for oocyte maturation can be investigated in small multi-ovulatory ruminants, either sheep or goat, as experimental models for cattle species. In addition, the mechanisms of BMP-15 and GDF-9 in the cumulus expansion during preovulatory stage and embryo survival in the cattle can be tested by adding recombinant doses of pLH, BMP-15 and GDF-9 to *in vitro* culture media during oocyte maturation. Following in vitro fertilization, blastocysts can be transferred to recipients to test hatched embryo survival per treatment.

Our findings have important implications on our understanding of the role of LH exposure time on the oocyte competence and embryo development. In addition, further studies may shed new light on conditions that improve embryo development and could help in the development of practical methods to increase fertility in dairy cows.

5.4. Literature cited

Ambrose, J. D., J. P. Kastelic, R. Rajamahendran, M. Aali and N. Dinn. 2005. Progesterone (CIDR)-based timed AI protocols using GnRH, porcine LH or estradiol cypionate for dairy heifers: Ovarian and endocrine responses and pregnancy rates. Theriogenology. 64:1457-1474.

Arashiro, E., M. Palhao, S. Wohlres-Viana, L. Siqueira, L. Camargo, M. Henry and J. Viana. 2013. In vivo collection of follicular fluid and granulosa cells from individual follicles of different diameters in cattle by an adapted ovum pick-up system. Reproductive Biology and Endocrinology. 11:73.

Carletti, M. Z. and L. K. Christenson. 2009. Rapid effects of LH on gene expression in the mural granulosa cells of mouse periovulatory follicles. Reproduction. 137:843-855.

Cech, S., V. Havlicek, M. Lopatarova, M. Vyskocil and R. Dolezel. 2002. Effects of superstimulation with FSH on follicular population and recovery rate of oocytes in the growing phase of the first and the second follicular wave. Veterinarni Medicina-Praha-. 47:33-37.

Čech, S., E. Indrová, M. Lopatářová, J. Malá, A. Pechová and R. Doležel. 2011. Limitations of ultrasound guided follicular aspiration for analysis of ovarian follicular fluid in dairy cattle. Journal Acta Veterinaria Brno. 80:179-184.

Conti, M., M. Hsieh, A. Musa Zamah and J. S. Oh. 2012. Novel signaling mechanisms in the ovary during oocyte maturation and ovulation. Molecular and Cellular Endocrinology. 356:65-73.

De Castro e Paula, LA, J. Andrzejewski, D. Julian, L. Spicer and P. Hansen. 2008. Oxygen and steroid concentrations in preovulatory follicles of lactating dairy cows exposed to acute heat stress. Theriogenology. 69:805-813.

Ginther, O., K. Kot, L. Kulick and M. Wiltbank. 1997. Sampling follicular fluid without altering follicular status in cattle: Oestradiol concentrations early in a follicular wave. Journal of Reproduction and Fertility. 109:181-186.

Panigone, S., M. Hsieh, M. Fu, L. Persani and M. Conti. 2008. Luteinizing hormone signaling in preovulatory follicles involves early activation of the epidermal growth factor receptor pathway. Molecular Endocrinology. 22:924-936.

Park, J. Y., Y. Q. Su, M. Ariga, E. Law, S. L. Jin and M. Conti. 2004. EGF-like growth factors as mediators of LH action in the ovulatory follicle. Science. 303:682-684.

Ree, T. O., M. G. Colazo, A. G. Lamont, J. P. Kastelic, M. K. Dyck, R. J. Mapletoft, B. N. Ametaj and D. J. Ambrose. 2009. The effect of porcine luteinizing hormone in the synchronization of ovulation and corpus luteum development in nonlactating cows. Theriogenology. 72:120-128.

Samper, J. C., J. F. Pycock and A. O. McKinnon. 2007. Current Therapy in Equine Reproduction. Elsevier Health Sciences.