THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF ANIMAL SCIENCE

EXPRESSION OF NR5A2 DURING THE LIFECYCLE OF THE BOVINE CORPUS LUTEUM

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ABSTRACT

Genetic selection for increased productivity in dairy cattle in the United States has unfortunately coincided with a decline in fertility among these animals. Decreased conception rates and prolonged calving intervals lead to significant economic losses for dairy farmers. Pregnancies in cattle are often lost during the period between fertilization and day 24 as a result of insufficient progesterone secretion from the corpus luteum (CL). Orphan nuclear receptor NR5A2 has been shown to be necessary for proper ovulation as well as CL formation and function, but its expression profile in the CL has yet to be described. Therefore, the goal of this study was to examine the abundance of NR5A2 in the CL during early cyclicity, early pregnancy, and luteal regression. Luteal NR5A2 mRNA abundance decreased by 8 hr after PGF2α injection ($p<0.05$) while NR5A2 protein tended to decrease by 0.5hr and decreased by 2 hours after PGF2α ($p<0.05$). NR5A2 mRNA increased between day 4 and day 6 of the estrous cycle ($p<0.05$) but protein abundance between these days was not different. During early pregnancy, luteal NR5A2 mRNA was lesser on days 20 and 23 compared to day 14 ($p<0.05$); none of these days differed from day 17. Protein abundance during early pregnancy did not change. Discrepancies between mRNA and protein abundance in each group give strong evidence to potential micro RNA (miRNA) regulation of NR5A2 translation. Further work can be done to investigate specific miRNA that target NR5A2 during these times in the CL lifecycle.

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It takes a village to raise a child, and I certainly would not be who I am today if it wasn't for the incredibly strong and noble village that raised me. My mother, one of the most resilient and determined people I know, has truly been by biggest fan and most trusted confidant; for that I am eternally grateful. Thank you for never letting me settle, for making sure I don't lose my focus, and for helping me be the best version of myself. I could not have accomplished all that I have without your guidance and love. To my father and brother, thank you for your endless support, interest, and level- headedness that guided me through some of the more challenging aspects of my college career. All that I am is because of all of you and I wouldn't want it any other way.

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Chapter 1

Literature Review

Dairy Cattle Fertility

As dairy farmers select for cows with exceptional milk production, the negative relationship between high production and fertility has become apparent. Over the last 40 years, milk output has more than doubled in the high producing dairy cow (Dobson et al., 2007), but common measurements of herd fertility have not seen such improvements. Lower conception rates, defined as the ratio of successful pregnancies to the number of insemination services, as well as prolonged calving intervals, or the time between calvings for one cow, have been key markers of this infertility phenomenon (Pryce et al., 2004). Economically, declining fertility is detrimental to dairy farmers; losses commonly accrue from increased artificial insemination costs, increased labor and resources for proper cow management, and decreased milk revenue from prolonged return to milking (Inchaisri et al., 2010). Cows are commonly culled from herds due to poor reproductive performance. In fact, about one third of culled dairy cows are removed from the herd due to infertility, the most prevalent cause of involuntary culling among farms today (Ansari- Lari et al., 2010). High culling rates can have further economic impacts for farmers because of the costs associated with purchasing and managing replacement heifers. The average estimated cost of each pregnancy loss in a dairy cow is $555, which is a significant loss for farmers especially when experienced repeatedly (DeVries, 2006).

While genetic selection for improved milk production does correlate with decreased fertility, elevated milk production itself does not appear to be the direct cause of infertility. Instead, high milk production has downstream effects that have been shown to alter fertility. It is well known that lactation is an energetically demanding process that cannot be met by feed intake alone due to the limitations of rumen capacity and appetite. Therefore, cows must mobilize their body reserves to meet the energy demands of lactation, placing them in a negative energy balance (NEB). This process is characteristic to all lactating dairy cattle regardless of the level of production. It is typically the most severe during early lactation, and eventually the cow returns to a positive energy balance. However, the high milk production that has been observed in recent decades requires cattle to mobilize more reserves than are required to sustain a growing embryo or fetus. Thus, even more nutrients are partitioned towards milk production as opposed to reproductive processes, presenting as lowered pregnancy rates and increased time between calving and conception (Berry et al., 2014).

Mechanistically, NEB affects the efficiency of the hypothalamic- pituitary- ovarian axis in high producing dairy cattle (Leroy et al., 2008). The pulse frequency and amplitude of luteinizing hormone (LH) is altered during NEB, resulting in prolonged time to ovulation and impaired oocyte maturation (Lucy, 2003). It is generally accepted that endocrine disruption as a result of NEB results in anovulation and atresia of the dominant follicle. In the event of successful ovulation, it is likely that the oocyte quality will be compromised. The mobilization of body fat during NEB results in elevated non esterified fatty acids (NEFA); high concentrations of NEFA have been shown to slow oocyte maturation rate, leading to lower fertilization and cleavage rates (Leroy et al., 2005c).

A severe negative energy balance is by no means the only cause of infertility in high producing dairy cattle; in fact, nutrition's role in fertility can often times be overestimated (Berry et al., 2014). However, the relationship has certainly become more prominent in recent years given the emphasis on genetic selection to maximize milk output. Proper management of cows both pre and post- partum is essential to minimizing the extent of NEB in these elite cows to give them the best chance of reproducing successfully and thus being profitable for farmers.

Attention need also be paid to early embryonic mortality, specifically, embryonic losses that occur between fertilization and day 24 of gestation. Most embryonic losses in dairy cattle occur during this time and are economically detrimental to farmers. These types of embryo deaths can delay the animal from returning to estrus in 21 days after breeding, which would normally be expected for a non pregnant cow. Therefore, farmers may incorrectly assume the animal is actually pregnant, causing the cow to be open for more days and effectively postponing her return to milking. Early embryonic mortalities tend to be more prevalent in higher producing dairy cattle. Embryonic and fetal losses occur in about 56% of high yielding dairy cattle compared to 40% of moderate yielding cattle (Diskin et al., 2012; Walsh et al., 2014). A well-documented cause of these pregnancy losses is insufficient circulating progesterone concentrations. Progesterone plays an important role in preparing the animal for pregnancy. It acts on the hypothalamus to inhibit GnRH release and the anterior pituitary to inhibit LH release, effectively suppressing ovulation. Progesterone also increases uterine receptivity to pregnancy by suppressing smooth muscle contractions and inducing glandular secretions that support pregnancy (Lonergan et al., 2013). Given the hormone's importance in supporting pregnancy, developing methods to enhance progesterone production during early gestation is an extremely plausible solution to decreasing early embryonic mortalities in dairy cattle.

Role of the Corpus Luteum in Pregnancy Maintenance

The corpus luteum (CL) is a transient endocrine organ whose primary function is to secrete progesterone to establish and maintain early pregnancy. The surge of LH from the anterior pituitary gland initiates ovulation as well as the process of luteinization, in which the thecal and granulosa cells of the ovulated follicle differentiate into small and large luteal cells, respectively. Successful luteinization also includes extensive remodeling of the extracellular matrix, rapid angiogenesis, changes in expression of hormone receptors, and an alteration in the steroidogenic pathway to produce primarily progesterone instead of estradiol. Progesterone is a product of cholesterol, which is derived mainly from circulating high- and low-density lipoproteins (HDL and LDL). Cholesterol enters luteal cells via cell- mediated endocytosis and is transported to the mitochondria for steroidogenesis. The rate- limiting step of progesterone synthesis is the movement of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane; this is mediated by steroidogenic acute regulatory protein (StAR). Next, cholesterol is cleaved by the P450 side chain cleavage enzyme to form pregnenalone, which is converted to progesterone by the enzyme 3β-hydroxysteroid dehydrogenase (3βHSD) in the smooth endoplasmic reticulum. Both of these enzymes are upregulated during luteinization (Niswender et al., 2000). Following ovulation, progesterone levels begin to rise by day 4, and peak around days 8 to 10 (Robinson et al., 2008).

In the event that the ovulated oocyte is not fertilized, the CL will be signaled to regress. In the cow, progesterone from the corpus luteum suppresses the actions of estrogen on the uterus until about day 10 post- ovulation. At this time, progesterone downregulates its own receptors in the hypothalamus and uterine endometrium, restoring estrogen influence on these tissues. Estrogen induces the formation of oxytocin receptors in the uterus; these receptors will bind

oxytocin released from the hypothalamic oxytocin pulse generator, which is also stimulated by estrogen. It is the binding of oxytocin in the uterus that signals the pulsatile secretion of prostaglandin F2α (PGF2α) around days 18-19 after ovulation (McCraken et al., 1999). The hormone travels to the CL through the utero-ovarian vein, crossing into the ovarian artery and binding to receptors on large lluteal cells to initiate lueolysis. Interestingly, the bovine CL does not respond to PGF2α before day 5 of the estrous cycle despite expressing the appropriate receptors. Luteolysis occurs in two phases which are not entirely temporally distinct; the first is functional regression in which progesterone production drastically declines. Structural regression follows, characterized by apoptosis of luteal and vascular cells and a subsequent decrease in the size and weight of the CL (Stocco et al., 2007).

If the ovulated oocyte is fertilized, the fate of the corpus luteum is quite different. The CL will be spared from luteolysis when maternal recognition of pregnancy occurs. During this process, the bovine embryo secretes the hormone interferon tau (IFNT) into the uterine lumen around days 13 to 15 of pregnancy; production declines sharply around days 19 to 21 (Ealy and Yang, 2009). The precise mechanism by which IFNT rescues the CL is still disputed, but three models have gained the most support. The first model is that IFNT prevents the expression of oxytocin receptors in the uterus, effectively preventing the secretion of PGF2α and maintaining the presence of the CL. The second model describes how IFNT stimulates increased secretion of prostaglandin E_1 and E_2 (PGE_1 and PGE_2) from the uterine endothelial cells. These luteotropic prostaglandins block the action of PGF2α on large luteal cells, rescuing the CL from luteolysis. The final model for maternal recognition of pregnancy is that IFNT escapes from the uterine lumen, enters the systemic circulation and acts directly on the CL to rescue it. Regardless of which model is correct or even predominant, it is evident that the IFNT signal must be present in

sufficient quantity by day 16 of gestation for the successful establishment of pregnancy (Wiltbank et al., 2016).

Adequate progesterone production by the corpus luteum is essential for viability of the early embryo. Progesterone acts indirectly on the embryo by stimulating the uterine endometrium to create an environment that is optimal for embryonic growth, elongation, and implantation. In response to progesterone, the uterine endometrial glands secrete elevated amounts of specific amino acids, growth factors, cytokines, and ions necessary for embryonic survival. Certain genes that increase uterine receptivity to pregnancy are upregulated in response to luteal progesterone as well (Spencer et al., 2015). Delayed or decreased progesterone secretion from the CL delays genetic changes in the endometrium, compromising blastocyst development and conceptus elongation (Forde et al., 2009). In fact, Green et al. (2005) demonstrated that the embryos from cattle with greater serum progesterone as early as day 5 of pregnancy were more developed than the embryos from cattle with lesser serum progesterone, further highlighting the importance of adequate progesterone early in gestation.

Supplementation with progesterone during early gestation would seem like a logical approach to ensure sufficient levels of the hormone for improved conception and embryonic development; however, the effects of supplementation are varied. It appears that exogenous supplementation of progesterone to pregnant cattle, specifically from days 5 to 9 of gestation, improves embryonic elongation and increases IFNT production (Mann et al., 2006). Progesterone supplementation later than this seems to have no effect on embryonic survival or development. Furthermore, a meta analysis of 53 publications found that progesterone supplementation proved beneficial for embryo survival rates when administered between days 3

and 7 of gestation, but specifically in cattle with poor fertility that were bred on natural estrus (Yan et al., 2016).

Interestingly, other studies have reported that progesterone supplementation during the early luteal stage has no effect on early embryonic survival when compared to a lack of supplementation (Arndt et al., 2009; Beltman et al., 2009), with one study even demonstrating decreased conception rates when progesterone supplementation was given on days 4 to 9 (Parr et al., 2014). In high producing dairy cattle, the effects of supplementation are largely negligible, possibly due to increased metabolism of progesterone by the liver and subsequently decreased circulating progesterone in these animals (Leroy et al., 2008). Given these varied results, devising a new solution to ensure adequate progesterone post ovulation would be beneficial; enhancing luteal function through genetic manipulation has promising potential.

Role of NR5A2 in Fertility

Proper formation and function of the CL depends on the activity of several genes, transcription factors, and signaling pathways; of recent interest is the potential role of nuclear receptor subfamily 5 group A member 2 (NR5A2) in the CL. NR5A2 was first identified in the liver, hence its original name, liver receptor homologue- 1 (LRH-1). In mammals, NR5A2 expression is mainly concentrated in tissues of endodermal origin such as the liver, pancreas, and intestine, yet it has also been identified in ovarian granulosa cells and luteal cells. Like all nuclear receptors, NR5A2 acts as a transcription factor and is activated when its ligand binds to it. However, it is classified as an orphan nuclear receptor because its ligand has yet to be identified. NR5A2 has a variety of tissue- dependent functions, but mainly appears to be related

to cholesterol metabolism and homeostasis. In the liver, this nuclear receptor has been implicated in reverse cholesterol transport by mediating the activity of HDL- remodeling enzyme cholesteryl- ester- transfer protein (CETP) (Fayard et al., 2004).

In the ovary, NR5A2 plays an essential role in successful ovulation. In mice with granulosa cell- specific NR5A2 mutation, the lack of NR5A2 was detrimental to ovulation of antral follicles. Cumulus cell expansion, a crucial process for ovulation, was absent in antral follicles of NR5A2 null mice. This failure was traced to defective prostaglandin signaling from decreased expression of prostaglandin synthase 2 (PTGS2). Downregulation of this gene was attributed to elevated estradiol concentrations in the follicular fluid of mutant mice as a result of upregulation of CYP19, a gene responsible for converting androgens to estrogens. Interestingly, NR5A2 absence was not directly responsible for this increased activity. Nitric oxide synthase 3 (NOS3), a target of NR5A2, has been shown to suppress CYP19 activity in the ovary. Therefore, lack of NR5A2 expression removed this regulation and led to impaired ovulation fertility via the aforementioned process (Duggavathi et al., 2008).

Previous studies have demonstrated the role of NR5A2 in luteal formation and function. Bertolin et al. (2014) created three NR5A2 knockdown models in mice, effectively eliminating NR5A2 activity at different times of follicular development. The nuclear receptor was excised in primary follicles, in antral follicles prior to the LH surge, and in antral follicles immediately following the LH surge. The results of the knockdown varied depending on the stage of the follicle. When NR5A2 was absent in primary follicles, folliculogenesis was maintained, but cumulus expansion, ovulation, and luteinization were absent. Knockdown of NR5A2 in antral follicles just prior to the LH surge resulted in normal folliculogenesis and cumulus expansion but faulty ovulation. “Luteal- like structures” were found in these mice, comprised of luteinized

thecal cells surrounding undifferentiated granulosa cells; these structures often contained a trapped oocyte. Additionally, progesterone production from these structures following gonadotropin stimulation was significantly lesser than that found in control mice. Finally, in mice in which granulosal NR5A2 activity ceased after LH release, folliculogenesis, cumulus expansion, and ovulation all proceed normally to generate a CL. However, the identified CL were smaller than in the control mice and produced lower circulating levels of progesterone. Using this same knockdown, Zhang et al. (2013) found that embryos in NR5A2 null mice were less developed than embryos of control mice. Additionally, when progesterone releasing implants were placed in mutants to achieve appropriate developmental rates, embryos were crowded together in the uterus, which led most of these pregnancies to fail prior to parturition. The variety of negative downstream effects as a result of NR5A2 elimination in the ovary provides strong evidence that NR5A2 regulates several pathways necessary for female fertility.

Given the reports of impaired progesterone production in the absence of NR5A2, it is logical to investigate a potential role of NR5A2 in regulating luteal function and steroidogenesis. Several studies in mice have found that mRNA for StAR, CYP11A1, which codes for the P450 side chain cleavage enzyme, and scavenger receptor B1 (SCARB1), which is responsible for selective uptake of cholesterol from HDL, are significantly decreased in NR5A2 null mice (Duggavathi et al., 2008; Zhang et al., 2013; Bertolin et al. 2014). Each of these genes has also been identified as a target of NR5A2 in mice and human cells in vitro (Schoonjans et al., 2002; Sirianni et al., 2002). Expression of 3βHSD in the absence of NR5A2 appears to be dependent on when NR5A2 is rendered inactive. Typically, 3βHSD expression increases in response to gonadotropin stimulation (Oktem et al., 2017); in this case 3βHSD mRNA decrease following gonadotropin stimulation (Duggavathi et al., 2008; Bertolin et al., 2014). In human granulosa

cells, NR5A2 was found to enhance the transcription of 3βHSD in a dose dependent manner. Cells were transfected with either 3βHSD luciferase reporter construct only or with the construct and expression vectors containing NR5A2. When compared to the control, addition of NR5A2 at doses of 0.1 and 0.5μg increased luciferase reporter activity. Furthermore, cells transfected with the 3βHSD construct and NR5A2 expression vector displayed higher 3βHSD reporter activity in the presence of a cyclic adenosine monophosphate (cAMP) analogue when compared to cells that lacked the cAMP analogue, indicating the potential involvement of the protein kinase A (PKA) pathway (Peng et al., 2003). The extensive interactions between NR5A2 and the expression of vital luteal steroidogenic genes further adds evidence for the importance of NR5A2 in proper luteal formation and function.

Purpose of this Study

Because of the importance of NR5A2 in the CL of other mammalian species, its presence in the bovine corpus luteum was of interest. Understanding the expression profile of the gene during early cyclicity (days 4 and 6), early pregnancy (days 14, 17, 20, 23), and PGF2α- induced regression could allow for better comprehension of how the gene is regulated and when it is most needed in the CL. It was hypothesized that both NR5A2 mRNA and protein concentrations in luteal tissue would increase as the CL developed during cyclicity and pregnancy, and would decrease during luteal regression. Knowledge of NR5A2 expression patterns can be useful for future work to determine potential sources of regulation.

Chapter 2

Materials and Methods

Luteal Tissue Collection

Tissue collection protocols were approved by the Institutional Animal Care and Use Committee at The Pennsylvania State University or West Virginia University. Normally cyclic Holstein heifers were observed for estrous behavior (day 0). CL were removed transvaginally on day 11 (mid-cycle, MC). CL from luteal regression were collected at 0.5, 1, 2, 4, 8, 12, and 24 hours after a luteolytic dose of prostaglandin (PG) F2α (25 mg of Lutalyse) (0.5hr PPG, etc) was administered on days 10-11 of the estrous cycle. Early cyclic (EC) CL were collected from cows that had been estrous synchronized with a controlled internal drug release device (CIDR), which is placed intravaginally and slowly releases progesterone to mimic the luteal phase. After 6 days, cows were given a 25mg injection of Lutalyse, and CIDRs were removed one day later. Two days later, cows were observed for heat, and follicle presence was determined by ultrasound. An ovulatory dose of GnRH (100μg Factrel) was administered to the cows, and CL were collected either 4 or 6 days after the injection. All CL were snap frozen in liquid nitrogen immediately following collection and frozen at -80°C until used for RNA and protein isolations.

Corpora lutea were collected from pregnant from cows that had been synchronized with one injection of lutalyse, followed by observation for behavioral signs of estrus. Upon observation of estrus, cows were bred via artificial insemination (AI). CL were removed transvaginally from the animals at day 14, 17, 20, and 23 after AI; potential pregnancies were

maintained with CIDRs, which were changed every 5 days until pregnancy could be confirmed. Pregnancy was confirmed on day 27-32 after AI via observation of a conceptus by ultrasound and by detection of interferon stimulated genes (ISG) in peripheral blood lymphocytes (PBL) with polymerase chain reaction (PCR; data not shown).

RNA Isolation and cDNA Synthesis

All reagents and supplies were ordered from ThermoFischer Scientific unless otherwise specified. Portions (20-30mg) of luteal tissue samples and bovine muscle, a negative control, were homogenized in Lysis Solution (Qiagen, Germantown, MD) and total cellular RNA was isolated per the manufacturer's instructions. RNA concentration was determined using a NanoDrop Microvolume UV-Vis Spectrophotometer, and RNA quality was assessed with an Experion Automated Electrophoresis System (Bio- Rad, Hercules, CA); all RQI values were greater than 9. RNA was then treated with DNase (Bioline, Taunton, MA) according to the manufacturer's protocol. All reactions used 2μg of RNA except for samples from luteal regression, which used 1μg of RNA. 318 ng of DNased RNA was reverse transcribed to cDNA per instructions of the DyNAmo cDNA Synthesis Kit. A pooled, non- reverse transcribed control was used to confirm lack of genomic DNA contamination. All cDNA samples were diluted 1:5 in nuclease free water prior to amplification in qPCR.

Polymerase Chain Reaction and Gel Electrophoresis

Forward and reverse primers directed against *Bos taurus* NR5A2 were designed using NCBI's Primer Basic Local Alignment Search Tool (BLAST). Primer sequences are shown in

Table 1. MC CL and muscle (negative control) cDNA were combined with Go Taq master mix per the manufacturer's protocol and the designed primers (0.9 μM each primer), and amplified using PCR under the following conditions: 95°C for 5 minutes, 40 cycles of 95°C for 30 seconds, 60°C for 45 seconds, 72°C for 1minute, and an additional 5 minutes at 72°C for a final extension. The amplified luteal and muscle cDNA products were purified through gel electrophoresis on a 1.5% agarose gel with 25μM ethidium bromide, ran for about 1.5 hours at 90V. The gel was imaged under UV light using a ChemiDoc XRS System (Bio-Rad, Hercules, CA) to confirm amplification of a single product from luteal tissue at the expected size. The negative control yielded no detectable amplification. The suspected NR5A2 amplicon was excised from the gel, extracted with the QiaQuick gel extraction kit, and sequenced. The resulting sequence was aligned to the genome using NCBI BLAST to confirm its identity as an NR5A2 transcript.

Table 1. Designed and Validated Primer for *Bos taurus* NR5A2 mRNA

GenBank Accession no.	Sequence	Amplicon Size
XM_005217382	FWD: 5'-GTCCTGCCCAAGGCTTCAAA-3' REV:5'- AGCCCGTAATGGTACCCAGA-3'	259

Quantitative Real- Time Polymerase Chain Reaction (qRT-PCR)

Twelve serial dilutions of the purified NR5A2 PCR product (starting with 1ng/μL) were used to generate a standard curve. NR5A2 standards were combined with SYBR™ Green Mix (Bioline, Taunton, MA) and the NR5A2 primer pair (250μM), and subjected to qPCR under the following conditions: 95°C for 5 minutes, 40 cycles of 95°C for 30 seconds, 60°C for 45

seconds, 72°C for 1 minute, and an additional 5 minutes at 72°C. After an optimal standard curve was generated, the aforementioned protocol was used to measure relative steady state concentrations of NR5A2 in luteal samples from EC, pregnancy, and regression; RPL19 was amplified as a constitutively expressed gene. Controls of water and a non reverse- transcribed sample of cDNA was used to confirm genomic DNA was not a subject of amplification.

Western Blot Analysis

To verify antibody specificity, protein was isolated from MC CL, bovine spleen (negative control), and bovine liver (positive control) by homogenizing samples (80-110mg) in either CHAPS lysis buffer or urea lysis buffer (7M urea, 2M thiourea, 5mM dithithreitol (DTT), 2% weight/ volume CHAPS). Recombinant human NR5A2 protein (60μg/mL, Abcam, Cambridge, MA) was also used as a positive control to confirm antibody specificity. Homogenates were centrifuged at 15,000xg for 15 minutes to remove cellular debris, and the supernatant was collected for analysis. A Bradford assay with a standard curve generated from serial dilutions of bovine serum albumin was used to determine total cellular protein concentration (Bradford, 1976; Bio- Rad Protein Assay, Bio-Rad Laboratories).

Isolated protein (30μg) from each tissue was combined with sample buffer (250μM Tris-base, 8% sodium dodecyl sulfate (SDS), 40% glycerol, 20% β-mercaptoethanol, and 1.3% of 2% bromophenol blue in 100% ethanol) and incubated at 95°C for 5 minutes. Denatured protein samples were electrophoresed through Mini-PROTEAN TGX Precast Gels (Bio-Rad, Hercules, CA) for 75 minutes at 120V. Separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane using an iBlot Dry Blotting System (Invitrogen, Rockford, IL). The residual

gel was incubated at room temperature in Coomassie Brilliant Blue dye for 1 hour to evaluate efficiency of protein transfer. The membrane was blocked at room temperature for 90 minutes with 5% non- fat dry milk in 1X Tris Buffered Saline with 0.05% Tween (TBST). Blocking was followed by a 1 hour room temperature incubation with rabbit anti- NR5A2/ LRH-1 primary antibody (1mg/mL, Abcam, Cambridge, MA), diluted 1:1000 (1μg/mL). Prior to incubation, the antibody was pre-adsorbed with BSA (5mg/mL) for 30 minutes at room temperature. The membrane was washed with 1X TBST before a 2 hour incubation with donkey anti- rabbit IgG horseradish peroxidase secondary antibody (concentration not shown, GE Healthcare, Pittsburgh, PA) at a 1:10,000 dilution in 5% non fat dry milk. After 1X TBST washes, SuperSignal West Femto Maximum Sensitivity Substrate was applied to the membrane for 6 minutes in the dark. Blots were visualized using a ChemiDoc XRS System (Bio-Rad, Hercules, CA) to confirm the specific detection of NR5A2 in the appropriate tissues. The membrane was incubated in 1X TBST at 4°C overnight and stripped with Restore Stripping Buffer for 10 minutes the following morning. After washing with 1X TBST, the membrane was reprobed with mouse anti- β-actin primary antibody for one hour in 2% non- fat dry milk. The membrane was washed again with 1X TBST and incubated in sheep anti mouse IgG horseradish peroxidase secondary antibody for two hours at room temperature. After 1X TBST washes, SuperSignal West Femto Maximum Sensitivity Substrate was applied to the membrane for 3 minutes in the dark. Blots were visualized with a ChemiDoc XRS System to visualize B- actin in each sample. The same protocol was used for EC, pregnancy, and regression samples; however, the primary antibody was diluted 1:2000 (0.5μg/mL) for samples from EC and pregnancy.

Statistical Analysis

All statistical analyses were performed using the mixed model of SAS 9.4 (Statistical Analysis System Institute, Cary, NC). An ANOVA test with RPL19 as the covariate was used to analyze mRNA expression for EC, pregnancy, and regression samples. Dunnett's Test compared each time of regression to the control (MC CL,0 hr PPG) to determine how NR5A2 mRNA changes during luteolysis relative to a functional CL. A Tukey test was used to compare the times of early pregnancy to each other. Optical densities from each NR5A2 protein signal were divided by those of β- actin to generate a ratio. These ratios were subjected to a two- way ANOVA using panel (membrane) as a block for experiments in which not all samples could be run on one membrane. All experiments were replicated using CL from four separate animals. Data are reported as least square means ± pooled SEM and differences were considered significant when $p < 0.05$.

Chapter 3

Results

NR5A2 Primer and Antibody Validation

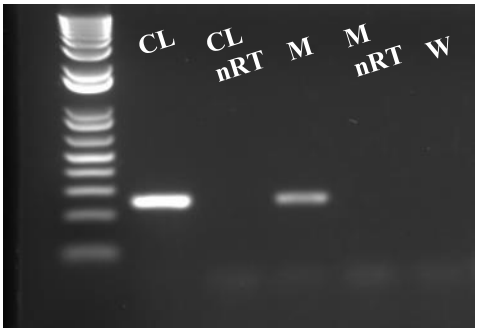

Figure 1. Amplification products of PCR. Successful amplification of NR5A2 at the expected size was achieved with some amplification in the negative control tissue. CL= CL reverse transcribed, CL nRT= CL non- reverse transcribed, M= muscle reverse transcribed, M nRT= muscle non- reverse transcribed, W= water.

PCR yielded an amplicon from luteal tissue of the predicted size of NR5A2, confirming accuracy of the primer pair. qPCR assay using the validated primers and purified NR5A2 cDNA product yielded the standard curve in Figure 2. Specific binding of the mouse anti –NR5A2 primary antibody was confirmed with the western blot shown in Figure 3. A signal at the expected size (61 kDa) was observed strongly from CL tissue, with fainter bands observed in the liver and spleen tissues. Non- specific binding was seen in all tissues, and was addressed in later blots with stronger blocking solution and more dilute primary antibody. Human recombinant NR5A2 protein produced a very strong band of the expected size and thus was used as the preferred positive control (blot not shown).

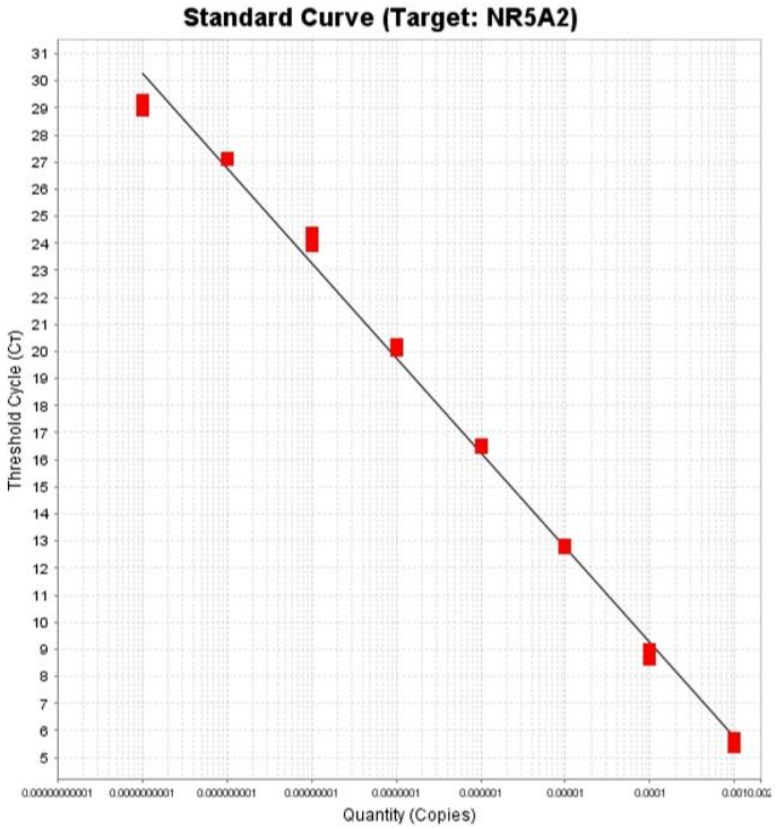

Figure 2. Standard curve developed from qPCR assay of purified NR5A2 cDNA product.
Slope= -3.5, R^2= 0.994, Efficiency= 93%

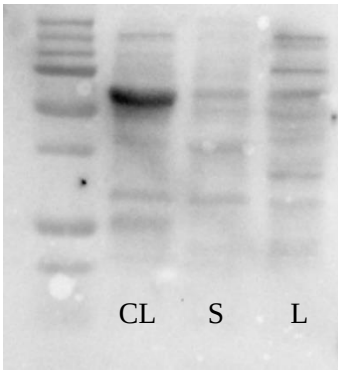

Figure 3. Western blot with CL, negative control, and positive control tissue samples.
Blocked in 2% non- fat dry milk in 1X TBST. Primary antibody diluted 1:500 (2µg/mL). Blot exposed for 30 seconds. Negative control= spleen (S), positive control= liver (L).

NR5A2 Expression During Early Cyclicity

Abundance of NR5A2 mRNA was greater on day 6 than on day 4 of the estrous cycle as depicted in Figure 4. Protein abundance surprisingly did not follow this observation; no difference was observed.

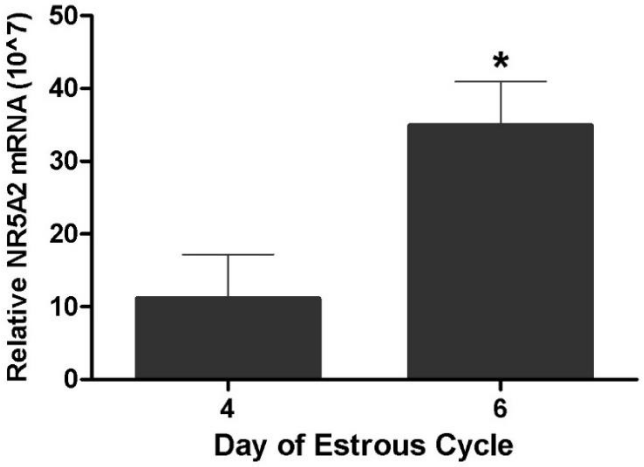

Figure 4. Steady state NR5A2 mRNA concentrations during early cyclicity. Asterisk denotes the significant difference between the days.

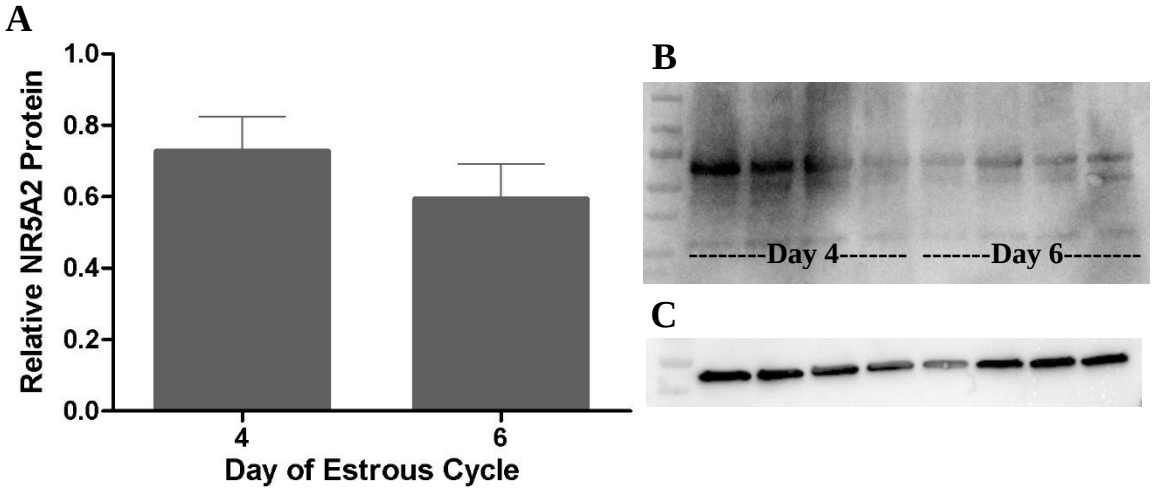

Figure 5. Relative NR5A2 protein abundance during early cyclicity. A, no significant differences were observed between the different days. B, the corresponding Western blot after 7.5 second exposure, is shown to the right. C, respective β- actin signals are shown after a 1 second exposure.

NR5A2 Expression During Luteal Regression

Abundance of NR5A2 mRNA was decreased at 8 and 12 hr after prostaglandin. While mRNA concentrations at 24 hr after prostaglandin were included in Figure 2, they were not used in the statistical analysis because one sample was not detectable within the limits of the qPCR assay.

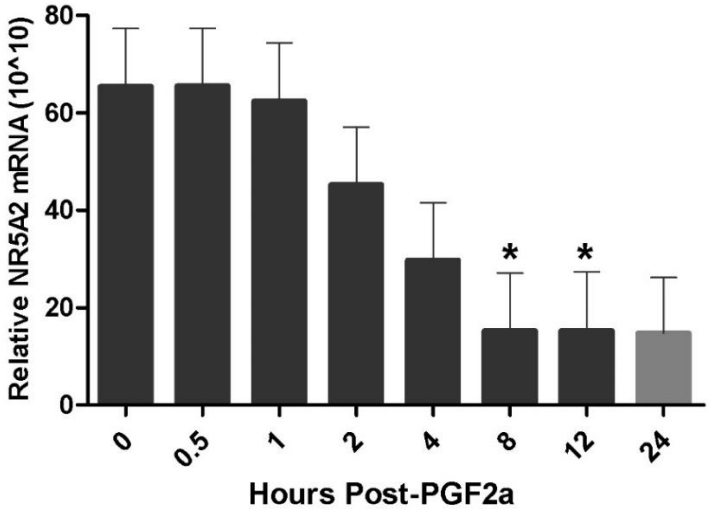

Figure 6. Steady state NR5A2 mRNA concentrations during luteal regression. Asterisks indicate a significant difference from 0 hr PPG. mRNA concentrations at 24 hr PPG were not statistically compared to the control.

Interestingly, abundance of NR5A2 protein during regression dropped much more rapidly following PGF2α exposure. Differences were seen as early as 2 hours after prostaglandin, with this observation persisting for 4, 8, 12, and 24 hr after prostaglandin. The rather immediate decline in abundance at 0.5 hr after PGF2, while striking, merely tended towards significance ($p=0.10$).

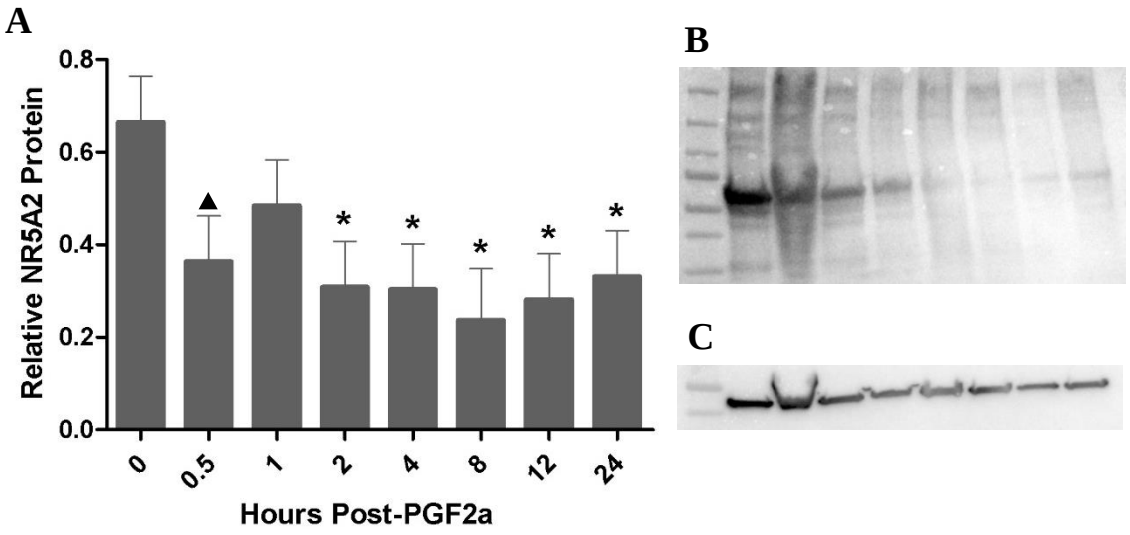

Figure 7. Relative NR5A2 protein abundance during luteal regression. A, asterisks indicate significant differences from the control (0 hr PPG); ▲ denotes data that approached significance (p=0.10). B, a representative western blot of samples from regression is shown. The blot was exposed for 7.5 seconds; samples are arranged from left to right as they appear on the graph. C, corresponding β- actin signals are shown; this blot was exposed for 1 second.

NR5A2 Expression During Early Pregnancy

Discrepancies between NR5A2 mRNA and protein abundance continued in samples from early pregnancy. Surprisingly, mRNA decreased as pregnancy progressed such that abundance on day 20 and 23 was lesser than on day 14. Protein concentrations did not follow this pattern; no differences were found among any points of pregnancy.

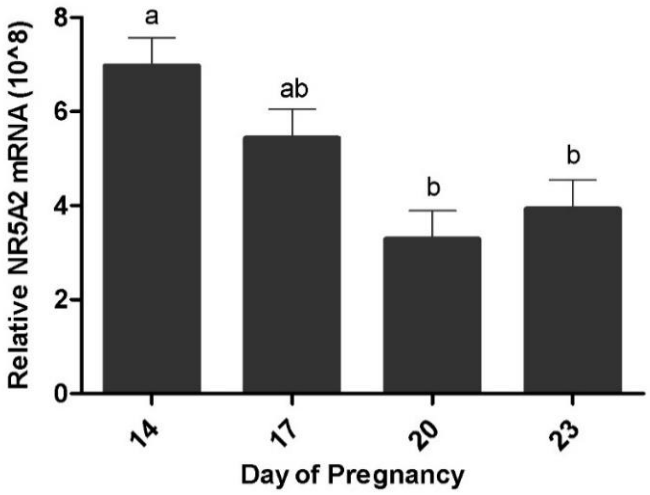

Figure 8. Steady state NR5A2 mRNA concentrations during early pregnancy. Different letters denote significant differences.

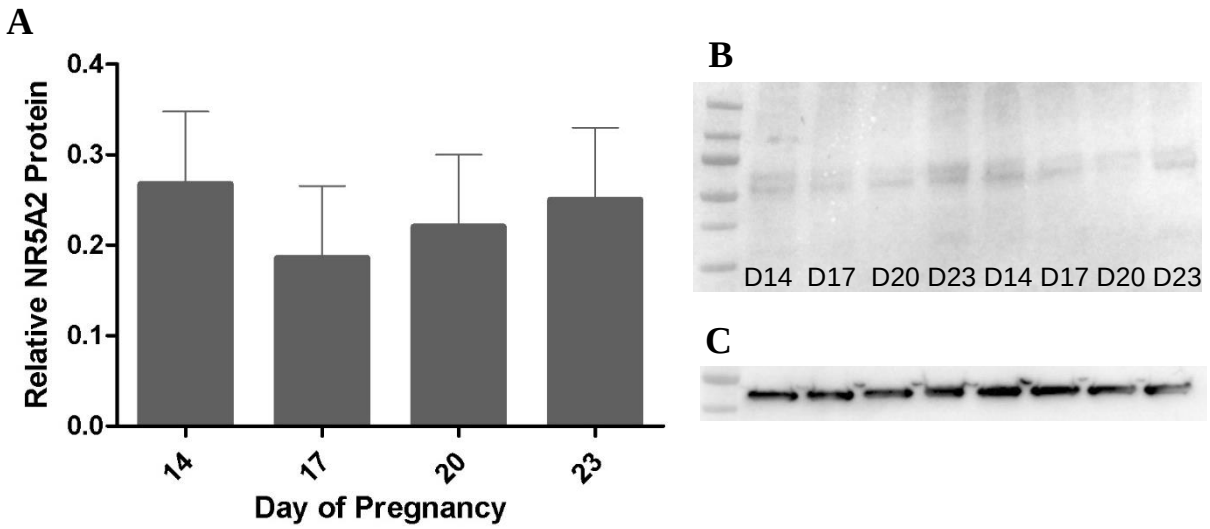

Figure 9. Relative NR5A2 protein abundance during early pregnancy. A, no differences were observed between any time points. B, a representative western blot, exposed for 7.5 seconds, is shown. D14= day 14 of pregnancy, etc. C, corresponding β- actin signals are shown; blot was exposed for 1 second

Chapter 4

Discussion

Recent work in this lab has investigated potential regulators of luteal regression or rescue with specific interest being paid to NR5A2. Using RNAseq data detailing differentially expressed mRNA between CL from day 17 of pregnancy and the estrous cycle, NR5A2 was found to be more abundant in the CL of pregnancy. Furthermore, microarray data from Mondal et al. (2011), accessed with the NCBI Gene Expression Omnibus Database, compared mRNA abundance between day 4 and day 11 CL to day 4 and day 11 CL 4 hr after prostaglandin. In this dataset, NR5A2 was among the genes that displayed lower mRNA abundance in day 11 CL 4hr PPG but not in day 4 CL 4hr PPG. These findings were the rationale for conducting this study, as they implicate NR5A2 as a potential regulator of luteal rescue or regression (Hughes et al., 2018; in preparation).

Other laboratories have investigated changes in luteal NR5A2 abundance as well, with results often aligning with this study. Taniguchi et al. (2009) examined the expression profile of NR5A2 in midcycle (days 10-12), late cycle (days 15-17), and regressing (days 19-21) CL. mRNA concentrations were unchanged between mid and late cycle, but declined in the regressing CL. After luteal regression was induced with intrauterine (IU) pulsatile infusions of PGF2α, Ochoa et al. (2018) reported a downregulation of luteal NR5A2 compared to control cows infused with saline. In accord with these studies, NR5A2 mRNA also declined during luteal regression 8 hours after PGF2α in the current study. NR5A2 protein declined in regression

as well, but it was intriguing to find the abundance had significantly decreased by 2 hours after PGF2α, a much more rapid decline than seen with mRNA.

NR5A2 expression during pregnancy produced the same surprise. While mRNA abundance was lesser on days 20 and 23 of pregnancy compared to day 14, no changes in abundance were observed in NR5A2 protein. This contrasts with the prediction that NR5A2 would be upregulated during early pregnancy due to its importance in luteal function. Furthermore, the differences between NR5A2 mRNA and protein provides evidence for the theory that NR5A2 is regulated post transcriptionally. Recent advances in technology have shown that steady- state mRNA concentrations are only a partial predictor of protein abundance; post transcriptional, translational, and degradation regulation can all play a role in determining protein abundance. (Vogel and Marcotte, 2012). Often, this regulation is carried out by micro RNA (miRNA). miRNA have been shown to suppress protein synthesis, destabilize mRNA, and induce mRNA degradation, typically producing modest changes in protein expression rather than large scale ones (Baek et al., 2008).

Translational repression by miRNA could theoretically explain the observed NR5A2 patterns during luteal regression. Specific miRNA could target NR5A2 transcripts in response to PGF2α and block translation, causing sharper decline in protein concentration while leaving mRNA abundance relatively unchanged. Although mRNA destabilization has been identified as the dominant mode for miRNA- mediated repression at a steady state (Eichorn et al., 2014), translational repression accounts for roughly 10-25% of overall repression and is still a plausible theory for decreased protein expression in mammals (Guo et al., 2010).

miRNA action could also account for the decline in NR5A2 mRNA and unchanged NR5A2 protein concentrations during early pregnancy. Here, the regulation would likely occur

via mRNA destabilization, explaining the lower mRNA abundance in days 20 and 23 of pregnancy compared to day 14. This regulation could leave protein concentrations unchanged if NR5A2 has a slower turnover rate, delaying the effects of mRNA destabilization from being observed in steady state protein. It may be worthwhile to examine NR5A2 mRNA and protein beyond day 23 of pregnancy to see if mRNA continues to decline or is constant, and if the decline in mRNA is reflected in protein abundance at later days of pregnancy.

The timing of the observed mRNA decline is somewhat shocking based on the implications of previous work. The downregulation of NR5A2 mRNA as pregnancy progressed was unexpected given its differential expression during pregnancy versus cyclicity. However, genetic action is ultimately carried out by proteins, which were unchanged as pregnancy progressed. While this result is perhaps not as noteworthy as an increase in protein abundance, maintenance of protein still supports the potential role of NR5A2 in supporting luteal function. In addition to pulsatile PGF2α IU infusions, Ochoa et al. (2018) infused PGE_1 and PGF2α simultaneously in a similar fashion to mimic the uterine environment during early pregnancy. Unlike what was observed after PGF2α infusion, luteal NR5A2 mRNA abundance did not decline after PGF2α + PGE_1 infusion, and the CL did not undergo regression. This maintenance of NR5A2 transcript abundance when luteolysis was blocked further supports the necessity of NR5A2 action during early pregnancy.

In EC CL, NR5A2 mRNA significantly increased from day 4 to day 6; however, protein remained constant. The stark increase in mRNA could simply be due to the rapid growth and development of the CL early in the estrous cycle; transcription of genetic material would naturally increase during this time. An increase in protein abundance would typically be expected as well, especially since proteins are produced at a much higher rate than mRNA

(Vogel and Marcotte, 2012). Again, this discrepancy between mRNA and protein abundance could be attributed to regulation by miRNA, specifically ones that are differentially expressed between day 4 and day 6 of the estrous cycle.

Based on the discrepancies between abundance of mRNA and protein in all sample groups, investigation into miRNA that target NR5A2 is a logical next step for future projects. Identifying factors that regulate NR5A2 action in cells could prove beneficial for developing methods to exogenously regulate NR5A2. If NR5A2 could be upregulated in the CL of pregnant cattle, perhaps early pregnancy losses from insufficient progesterone concentrations would effectively decline.

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- Worked with fourth year veterinary student on their Large Animal Medicine-Surgery rotation by assisting with patient care and observing surgeries

Select Breeder Services, Chesapeake City, Maryland
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- Worked extensively with the facility veterinarian to palpate and ultrasound mares, breed client mares, and assist with foalings throughout the breeding season.

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