The Effects of Fish Meal Supplementation on Gene Expression in the Bovine Corpus Luteum Following Low Dose Administration of Prostaglandin F2A

Jessica Claire Cedillo
jessica.c.cedillo@gmail.com

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THE EFFECTS OF FISH MEAL SUPPLEMENTATION ON GENE EXPRESSION IN THE BOVINE CORPUS LUTEUM FOLLOWING LOW DOSE ADMINISTRATION OF PROSTAGLANDIN F2A

A Thesis Submitted in Partial Fulfillment of the requirements for the Degree of Master of Science

Jessica Claire Cedillo

College of Natural and Health Sciences
School of Biological Sciences

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This Thesis by: Jessica Claire Cedillo

Entitled: The Effects of Fish Meal Supplementation on Gene Expression in the Bovine Corpus Luteum Following Low Dose Administration of Prostaglandin F2α

has been approved as meeting the requirement for Degree of Master of Science in College of Natural and Health Sciences in School of Biological Sciences

Accepted by the Thesis Committee:

Patrick Burns, Ph.D. Research Advisor

Nicholas Pullen, Ph.D. Committee Member

Ann Hawkinson, Ph.D. Committee Member

James Haughian, Ph.D. Committee Member

Accepted by the Graduate School

Linda L. Black, Ed.D.
Associate Provost and Dean
Graduate School and International Admissions
Research and Sponsored Projects
ABSTRACT

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The corpus luteum is a transient endocrine gland that develops from the remnants of the ovulatory follicle and secretes the steroid hormone progesterone. Progesterone is essential in the establishment and maintenance of pregnancy in all mammalian species. In the non-pregnant bovine, uterine prostaglandin F2α is secreted in a series of pulses between days 15 and 17 after ovulation, allowing the corpus luteum to undergo functional and structural regression and an opportunity for mating. During early pregnancy, trophoblastic cells of the developing embryonic placenta secrete interferon-tau, which attenuates uterine prostaglandin F2α secretion allowing for maintenance of the corpus luteum during the period referred to as maternal recognition of pregnancy. Inadequate secretion of interferon-tau or a delayed signal from trophoblastic cells can lead to luteal regression and loss of the pregnancy. Thus, altering luteal responsiveness to prostaglandin F2α during maternal recognition of pregnancy may prevent early embryo loss.

Inclusion of omega-3 fatty acids into the diet may be a novel approach to regulate luteal function during early pregnancy. Supplementation with omega-3 fatty acids in the diet has been shown to incorporate into luteal tissue, resulting in
altered membrane ultrastructure and mobility of the prostaglandin FP receptor. However, the effects of omega-3 fatty acids on luteal sensitivity to prostaglandin F2α is lacking. It was hypothesized that omega-3 fatty acid supplementation from fish meal may have a luteoprotective effect on the expression of immediate early genes (NR4A1 and FOS), key luteotropic (STARD1, CYP11A1, 3βHSD, and LDLR), luteolytic (PGHS2 and PTGFR), and apoptotic genes (BAX, BCL-2, and CAS3) that regulate luteal ability to produce progesterone and the longevity of the gland. Administration of prostaglandin F2α resulted in downregulation of luteotropic genes and PTGFR, upregulation of immediate early genes and no change in apoptotic genes and PGHS2 regardless of luteal function. Supplementation with fish meal resulted in a decrease in BAX expression as compared to corn gluten meal supplementation. Outcomes from proposed studies bridge our current gap in knowledge regarding the influence of dietary supplementation of omega-3 fatty acids on luteal function and gene expression in response to prostaglandin F2α. The dietary supplementation of omega-3 fatty acids may be a strategy to improve reproductive performance in breeding females.
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CHAPTER I
INTRODUCTION AND LITERATURE REVIEW

History of the Corpus Luteum

The corpus luteum is a transient endocrine gland necessary for pregnancy in all mammalian species. The corpus luteum was first anatomically described by Renier de Graaf in 1672 and the name, meaning “yellow body”, was coined by Marcello Malpighi in 1689. Original research concluded that the corpus luteum played a role in endocrine function and secreted its products directly into the blood (1). Studies conducted in rabbits supported the notion that the corpus luteum was a secretory gland involved in maintenance of pregnancy, as ablation of the corpora lutea from pregnant rabbits resulted in abortion of all embryos (2, 3). However, in pregnant rabbits, removal of the ovary and subsequent injection of luteal extract allowed for the maintenance of pregnancy (4). The main secretory product of the corpus luteum was subsequently purified and named progesterone in 1934 (5, 6, 7).

Progesterone is produced in copious amounts by the corpus luteum during the luteal phase of the estrous cycle. The hormone acts on various tissues, and must be present in adequate amounts in order to establish and maintain pregnancy. Reported functions of progesterone include the mitigating effects on uterine contraction, regulation of gene expression and protein secretion in the uterus, ovary and oviduct, effects on gonadotrophin releasing hormone and
prevention of follicle stimulating hormone and luteinizing hormone secretion (8, 9, 10). Approximately 6-13% of progesterone is transported in the blood plasma unbound and “free” while the rest is transported bound to plasma proteins including albumin, sex hormone binding globulins, or corticosteroid binding globulins (11, 12). Adequate levels of progesterone are essential for early maintenance and establishment of pregnancy (13).

**Introduction to Problem**

Each year, millions of dollars are lost in the U.S. dairy and beef industries due to poor reproduction in the bovine. A majority of these lost pregnancies occur between approximately days 14-30 of gestation (14). There are several possible avenues that can lead to early embryonic mortality in the bovine: chromosomal abnormalities, failure of egg fertilization, failure of placental attachment, and a failure of maternal recognition of pregnancy (15). A significant amount of early pregnancies lost are due to failure in maternal recognition of pregnancy (16). The event of maternal recognition of pregnancy is denoted by the successful signaling between a conceptus and the ability to secrete adequate amounts of the cytokine interferon-τ to act on the uterus and prevent intrauterine pulsatile release of prostaglandin F2α and subsequent regression of the corpus luteum. If the release of interferon-τ is too weak or secreted too late, prostaglandin F2α will be secreted from the uterus and result in luteolysis (regression of the corpus luteum), loss of adequate progesterone needed for maintenance of pregnancy and subsequent embryo loss. Interferon-τ also acts on uterine epithelium to inhibit expression of estrogen receptors and subsequent
oxytocin receptors. This prevents oxytocin mediated release of the luteolysin prostaglandin F2α (17). There must be adequate amounts of the cytokine interferon-τ secreted from trophoblastic cells of the conceptus to trigger successful maternal recognition of pregnancy (18, 19). Sufficient release of interferon-τ inhibits the release of prostaglandin F2α from the uterus and prevents regression of the corpus luteum, thus triggering maternal recognition of pregnancy.

The goal of this research study was to determine the effects of polyunsaturated fatty acids found in fish meal on luteal sensitivity and gene expression in response to low dose administration of prostaglandin F2α. While administration of interferon-τ has been shown to improve pregnancy in some studies, this method requires individual animal attention at least twice a day between days 14-17 of pregnancy and is impractical with large dairy operations or range cows (20). Dietary supplementation with omega-3 fatty acids may allow for a greater time period for an embryo to signal maternal recognition of pregnancy and prevent regression of the corpus luteum. A small, slow developing embryo may not secrete adequate amounts of interferon-τ to prevent luteolytic pulses of prostaglandin F2α. By reducing luteal sensitivity and responsiveness to prostaglandin F2α, this may supply a developing conceptus adequate time to secrete a strong enough signal of interferon-τ to successfully prevent regression of the corpus luteum and trigger maternal recognition of pregnancy.
Additionally, incorporating omega-3 fatty acids into the diet is a non-invasive and cost effective way to potentially increase viable pregnancies in the bovine. Supplementing with polyunsaturated fatty acids and allowing for incorporation into reproductive tissues may be a way to preserve the lifespan of the corpus luteum and prevent failure in maternal recognition of pregnancy.

**Bovine Estrous Cycle**

The estrous cycle of a mature cow is denoted by four distinct phases and is approximately 21 days in length (Figure 1). The beginning of the cycle (day 0) is denoted as estrus, or “standing heat”, a 12-18 hour period during which the female will demonstrate sexual behavior. During this time, the cow will mount other females and stand to be mounted by herd mates or the bull. During estrus, a dominant Graafian follicle will secrete high amounts of estradiol which stimulates the hypothalamus to release a surge of gonadotrophin releasing hormone and is also responsible for driving estrous behavior. Subsequently, the release of gonadotrophin releasing hormone will stimulate the anterior pituitary gland to secrete a surge of luteinizing hormone and bring about ovulation. Ovulation, brought about by a surge in luteinizing hormone and rupture of the present Graffian follicle, will take place roughly 24-30 hours following the onset of estrus. Concurrently, granulosa cells of ovarian follicles will secrete inhibin and prevent secretion of follicle stimulating hormone and development of new follicles.

Following estrus, metestrus (days 1-5) is characterized by the initial development of the corpus hemorrhagicum and rising production of
progesterone. Theca and granulosa cells are luteinized and differentiate into small and large luteal cells, respectively. Levels of progesterone rise steadily while the corpus luteum matures and undergoes rapid angiogenesis. Additionally, a surge of follicle stimulating hormone occurs and begins the initial cohort of follicle development.

Diestrus spans from days 6-17 of the estrous cycle and is designated by the presence of a mature corpus luteum. During this phase, copious amounts of progesterone are secreted, and rapid growth and hypertrophy of cells within the corpus luteum continues.

The last phase of the estrous cycle is called proestrus (days 18-24) and is characterized by uterine prostaglandin F2α induced regression of the corpus luteum and rapid decline of progesterone. During proestrus, lack of progesterone allows for an increase in frequency and amplitude of follicle stimulating hormone and luteinizing hormone. These hormones support development of a pre-ovulatory Graffian follicle and subsequent production of estrogen. Once levels of estradiol reach a threshold concentration in the blood, this triggers a surge of luteinizing hormone from the anterior pituitary gland, resulting in ovulation and the beginning of the next estrous cycle.
Figure 1: Diagram of phases of estrus cycle and fluctuations of progesterone, estradiol, luteinizing hormone, and follicle stimulating hormone concentrations. Image adapted from (21).

The Corpus Luteum

Formation of the corpus luteum occurs following ovulation. Secretion of gonadotrophin releasing hormone from the hypothalamus will act on the anterior pituitary gland causing a surge of luteinizing hormone release. Following the surge of luteinizing hormone, rupture of the Graafian follicle will occur and initiate the rapid cell reprogramming of granulosa and theca cells into small and large luteal cells.

The corpus luteum is composed of several major cell types including small and large luteal cells (steroidogenic cells), endothelial cells, fibroblasts, pericytes, and various immune cells. By volume, large luteal cells account for approximately 40% of the gland, while small luteal cells comprise approximately 30% (22). By cell number, large luteal cells account for approximately 4% of the
cells and small luteal cells account for nearly 20% of total cells (23). The corpus luteum is highly vascularized with up to 50% of the gland’s cells composed of endothelial cells that form blood vessels (24). Pericytes, fibroblasts and various immune cells comprise the rest of the gland.

Following ovulation, the process of luteinization begins. Granulosa cells and theca cells reprogram into large and small luteal cells (25). Prior to ovulation, granulosa cells that were once part of the dominant Graffian follicle predominantly produced estradiol (26). After luteinization takes place, mid-cycle large luteal cells will secrete up to 80% of total progesterone (27). Many genes once responsible for biosynthesis of estradiol remain constitutively expressed and are instead utilized for progesterone secretion (28). While the transition from estradiol to progesterone synthesis takes place, luteal cells will migrate from the remnants of the ovulatory follicle to form the corpus luteum. Additionally, extracellular matrix remodeling, activation of matrix metalloproteins 2 and 9, rapid neovascularization and breakdown of basement membrane occur to allow for the migration of cells forming the corpus luteum (29). The developing corpus luteum is a hypoxic environment, with hypoxia-inducible factor 1 highly upregulated, causing stimulation of vascular endothelial growth factor expression and subsequent vascular growth (30). The hypoxic environment during luteinization has been shown to increase progesterone output and increase protein expression levels of STARD1 and 3βHSD (31, 32). During the hypoxic conditions while following ovulation, highly expressed levels of fibroblast growth
factor 2 and platelet derived growth factor in endothelial cells and pericytes drive neovascularization (33, 34).

The growth of the corpus luteum is due to both hypertrophy and hyperplasia of luteal cells (35). Additionally, rapid angiogenesis occurs via expression of vascular endothelial growth factors and fibroblast growth factors. While the cells do increase in size as they grow from the beginning to mid luteal stage, mostly the growth of the corpus luteum is due to high upregulation of genes promoting the cell cycle, which causes an increase in cell number (36). A significant amount of growth of the corpus luteum is due to an upregulation of the fibroblast growth factors heparin binding growth factors 1 and 2 (37). These growth factors directly result in angiogenesis. Upregulation of mRNA for insulin-like growth factors is also necessary for establishment of the corpus luteum and results in downregulation of luteolytic factors (38).

In the bovine, small luteal cells, large luteal cells, collagen, and fibroblasts will cohabitate the corpus luteum. These cells become intermixed upon their formation into the mature corpus luteum (39). Small luteal cells have a high number of receptors for luteinizing hormone with fewer number of these receptors found on large luteal cells (40). Small luteal cells will respond to luteinizing hormone and will begin an intracellular signaling cascade leading to an increase in progesterone biosynthesis. The luteinizing hormone receptor is a seven-helix transmembrane G-protein coupled receptor that initiates the G\alpha_S intracellular signaling cascade. Adenylate cyclase is activated upon binding of ligand and will cause an increase in cAMP within the cells and ultimate activation
of protein kinase A (41). Further downstream, the MAP (mitogen activated
protein) kinase pathway will upregulate production of transcription factors c-fos
and c-jun that promote growth within the corpus luteum (42).

**Progesterone Biosynthesis**

The precursor to all steroid hormones is cholesterol (43). While
cholesterol can be synthesized de novo from acetate within most steroidogenic
cells, the main source of cholesterol is from blood lipoproteins or cholesterol
esters stored as lipid droplets within the cell. Cholesterol is transported in the
blood bound to either low or high density lipoprotein receptors. These
lipoproteins are internalized into the cell using specific low density lipoproteins or
scavenger B1 receptor. Once internalized, cholesterol is sorted from the
lipoprotein and stored as lipid droplets or shuttled to the inner mitochondrial
membrane by the transporter steroidogenic acute regulatory protein (STARD1),
the rate limiting step in steroid biosynthesis. Cholesterol is then converted to
pregnenolone by the P450 side chain cleavage enzyme, CYP11A1.
Pregnenolone is transported to the smooth endoplasmic reticulum where it is
converted to progesterone by the enzyme 3βHSD. The comprehensive pathway
is depicted in Figure 2.

A mid-cycle corpus luteum will produce large quantities of the steroid
hormone progesterone. The surge of luteinizing hormone that induced ovulation
also acts to stimulate luteal cells to produce progesterone (44). Progesterone
primarily acts on the endometrium to prepare the uterus for a potential
pregnancy. Progesterone also targets the mammary gland, brain, and is
considered luteoprotective and helps to prevent apoptosis of the corpus luteum (45, 46). Additionally, progesterone prevents secretion of gonadotrophin releasing hormone from the hypothalamus and luteinizing hormone from the anterior pituitary (47). Progesterone levels are detectable in the blood at approximately day 5 of the estrous cycle and will remain elevated until the beginning of luteolysis. As the corpus luteum ages, steroid production drastically decreases at approximately day 17-20 following ovulation in the non-pregnant cow in response to the luteolytic effects of prostaglandin F2α (48).

Figure 2: Representation of the steroidogenic pathway that takes place in luteal cells to produce progesterone. Cholesterol sources include the bloodstream via low or high density lipoproteins or from cholesterol esters within the cell. The rate limiting step of this pathway is transport of cholesterol into the mitochondrion inner leaflet by StAR protein, where it will then be cleaved to form pregnenolone by P450scc, and finally into progesterone by 3βHSD.
In the bovine, adequate secretion of progesterone from the corpus luteum is necessary for 200 days of a 280 day pregnancy (49). Upon fertilization and prior to implantation, the dam’s body must maternally recognize the pregnancy in order to keep the corpus luteum from regressing. Adequate growth of the corpus luteum is crucial to prevent loss of early pregnancy. Interferon tau is produced by trophoblastic cells of the developing conceptus. Interferon tau will target the uterus and prevent pulsatile release of prostaglandin F2α, preventing the regression of the corpus luteum. However, if the signal is too late or not strong enough, prostaglandin F2α will be released from the uterus causing the death of the corpus luteum, a subsequent drop in progesterone and termination of the pregnancy. However, in the event of a successful pregnancy, the corpus luteum remains well vascularized and secretes copious amounts of progesterone. The vascular endothelial growth factors presence helps to ensure proper perfusion of the corpus luteum of pregnancy until its function is replaced by the placenta (50, 51).

**Luteolysis**

When no pregnancy occurs, the corpus luteum will undergo regression. The primary instigator of luteal regression is caused by prostaglandin F2α. However, the detailed pathway controlling the onset of luteal regression is unknown. There are two mechanisms of luteal regression: structural and functional. Structural regression is denoted as a decrease in size of the gland while functional regression is the decrease and ultimate cessation of progesterone biosynthesis (52). In the event no pregnancy occurs, a series of
pulses of prostaglandin F2α will be released from the uterus leading to regression of the corpus luteum (53).

The mechanism by which prostaglandin F2α acts on the corpus luteum is through binding of the FP receptor and activation of the seven helix G-protein coupled receptor and subsequent signaling cascade. Upon binding to its receptor on large luteal cells, phosphatidylinositol phospholipase C (PLC) is activated leading to the cleavage of phosphatidylinositol 4, 5 bisphosphate (PIP$_2$) and production of inositol 1, 4, 5 triphosphate (IP$_3$) and diacylglycerol (DAG). The IP$_3$ present will diffuse to the smooth endoplasmic reticulum. Binding of the IP$_3$ receptor allows for an intracellular release of Ca$^{2+}$. Increased Ca$^{2+}$ within the cell and DAG located at the plasma membrane stimulate the activity of Ca$^{2+}$ dependent protein kinase C (PKC) (54). The actions of PKC mediate multiple luteolytic actions of prostaglandin F2α in large luteal cells. Protein kinase C can negatively impact steroidogenesis (55), cholesterol liberation (56), and maintenance of the extracellular matrix (57).

Functional luteolysis is defined as the inability of the corpus luteum to secrete adequate amounts of progesterone. Following intramuscular administration of prostaglandin F2α, decline in progesterone can take place within four to eight hours (58). There are many mechanisms by which prostaglandin F2α may bring about a loss in luteal ability to secrete progesterone. Prostaglandin F2α signaling and downstream activation of PKC can alter posttranslational modification of key steroidogenic proteins (59, 60). During luteolysis, downregulation of genes involved with cholesterol uptake takes
place while genes involved in production of prostaglandin F2α increased (61). Several studies indicate that prostaglandin F2α can autoregulate its synthesis by affecting liberation and conversion of membrane phospholipids into arachidonic acid, the precursor molecule of prostaglandin F2α (62). Additionally, a decrease in uptake of cholesterol or transport of cholesterol into the cell or across the mitochondrial membrane may also negatively impact progesterone biosynthesis. Prostaglandin F2α causes a decrease in mRNA expression of the low density lipoprotein receptor (LDLR) yet functionally the corpus luteum may still remain capable of adequate progesterone secretion (63). This suggests that prostaglandin F2α may not affect uptake of cholesterol during spontaneous luteolysis. However, prostaglandin F2α may impact transport of cholesterol across the mitochondrial membrane and causes a decrease in STARD1 mRNA and translated protein levels (64, 65). During luteolysis, there is also a loss in the mRNA expression level of other key players involved in progesterone biosynthesis such as 3βHSD and CYP11A1. Loss of these enzymes may result in a mitigated output in progesterone biosynthesis.

Structural luteolysis is characterized by the onset of programmed cell death, emigration of various white blood cells, loss of gland structure and vascularization. The phagocytosis by leukocytes occurs and dead luteal cells are removed. Immune cell presence within the corpus luteum fluctuates throughout the estrous cycle and is an important driver in luteolysis (66). Various cytokines are secreted by white blood cells driving ultimate regression of the gland and additional attraction of other leukocytes such as macrophages and T-
lymphocytes (67). Expression of the major histocompatibility complex (MHC) class II molecules are important in mediating emigration of T-lymphocytes during luteolysis (68). T-lymphocytes further drive apoptosis of luteal cells through activation of Fas ligand mediated programmed cell death (69). The presence of macrophages increases drastically during the transition from an early to late stage corpus luteum, leading to an increase in cytokine production (70, 71). Secretion of pro-inflammatory cytokines such as tumor necrosis factor α, interferon γ, interleukin-6, and interleukin-1b play a major role in attracting immune cells to the corpus luteum during luteolysis leading to structural regression of the gland (72, 73).

Luteal exposure to prostaglandin F2α will cause an intracellular signaling cascade that leads to intrinsically mediated apoptosis (74). In a healthy cell, the protein B cell lymphoma-2 (BCL-2) will bind to the BCL-2 associated X protein (BAX), preventing apoptosis. However, prostaglandin F2α will cause an increase in BAX expression, altering the ratio of BAX/BCL-2 proteins. BAX protein will then act on the mitochondrial membrane and cause an increase in membrane permeability and subsequent release of cytochrome C leading to apoptosis (75).

Prostaglandin F2α is a potent vasoconstrictor and will cause a decrease in vasculature diameter within the corpus luteum, leading to ischemia and ultimately tissue death (76). During the late stages of the luteal phase, the expression rates of insulin-like growth factors are upregulated, suggesting that these growth factors (once involved in luteotropic activity) play a role in regression of this gland (77). During regression of the corpus luteum, high levels of local cortisol
secretion from adrenocortical are observed and can play a role in driving the
process of structural luteolysis. High levels of cortisol cause a drastic loss in 11
beta-hydroxysteroid dehydrogenases, enzymes necessary for luteal maintenance
and prevention of apoptosis (78). Upon the onset of luteal regression, genes
once involved in extracellular matrix establishment (gelatinases and
metalloproteinases) become downregulated, leading to a loss in structure
surrounding luteal cells (79). In a corpus luteum that is undergoing regression,
the mRNA of key steroidogenic genes needed to properly synthesize
progesterone are drastically downregulated (80). Even though no clear
pathways have been linked to the onset of luteal regression, many individual
genes that concurrently become downregulated lead to death of this gland.

Gene Expression During
Luteal Regression

Immediate-Early gene expression. Regression of the corpus luteum is
directly correlated to exposure of luteal tissue to the endogenous luteolysin
prostaglandin F2α. The luteolytic effects of prostaglandin F2α will bring about a
rapid change in immediate early gene signaling and expression. There are a
multitude of downstream affects mediated by immediate early gene activation
(81, 82). The transcription factors c-FOS and NR4A1 have been suggested to be
critical mediators of apoptotic signaling pathways, while also negatively affecting
downstream steroidogenic gene expression (83, 84). The activator protein-1 (AP-
1), a transcription factor complex activated by c-FOS and JUN proteins, has been
shown to be involved in prostaglandin F2α induced luteolysis (85). Both
pharmacological and physiological doses of prostaglandin F2α administered to animals cause an increase in immediate early gene mRNA (86).

NR4A1 is a part of the NR4A family, and is one of three homologues. The NR4A family has been shown to be involved in metabolism, survival, apoptosis, and differentiation in several cell types (87, 88). In the pregnant rat, NR4A1 (Nur77 protein) was shown to be involved in conversion of progesterone into its non-active form, 20α-hydroxyprogesterone (89). Luteal regression induced by prostaglandin F2α causes rapid increases in NR4A1 mRNA expression levels (90). Increases in NR4A1 transcript levels and subsequent Nur77 protein levels may be important drivers of apoptosis in steroidogenic cells. Nur77 has been shown to interact with the retinoid X receptor (RXR) and further downstream members of the BCL-2 family to bring about apoptosis (91).

The immediate early gene c-FOS belongs to a family with four other members that encode for proteins that interact with the JUN family of proteins and together form the AP-1 transcription factor complex. The AP-1 transcription factor complex regulates the transcription of several genes following its activation. Prostaglandin F2α is known to activate the MAP kinase signaling cascade, which directly regulates activation of the AP-1 complex. Activation of c-FOS by prostaglandin F2α occurs via activation of the PKC-dependent MAP kinase signaling pathway (92). Influences of the luteolysin prostaglandin F2α cause changes in immediate early gene expression, ultimately leading to induction of apoptosis and luteal regression.
**Luteotropic gene expression.** The expression of any gene that is directly involved in cholesterol transport, progesterone biosynthesis or perpetuation of the lifespan of the corpus luteum is considered “luteotropic”. Luteotropic genes are upregulated at the beginning of the luteal phase and their expression is maintained with stimulation of luteinizing hormone and the luteoprotective effect of progesterone (93, 94).

The low density lipoprotein receptor (LDLR) and scavenger B1 receptor (SR-B1) work concurrently to bind the respective lipoprotein in the blood and result in the internalization of cholesterol. Most cholesterol is internalized via clathrin mediated endocytosis of the LDLR or selective uptake of the SR-B1 receptor (95). Upon exposure to prostaglandin F2α, expression of LDLR and SR-B1 decrease, resulting in a reduction in cholesterol uptake in steroidogenic cells and subsequent loss in progesterone secretion.

STARD1 is a protein responsible for shuttling cholesterol into the mitochondrial membrane and is the rate limiting step in progesterone biosynthesis. The STARD1 protein contains a StAR-related lipid transfer (START) domain involved in the binding of cholesterol (96). Recent reports in the literature suggest the mechanism by which cholesterol is shuttled into the mitochondrial matrix does not involve the translocator protein (TSPO). Instead, a newly proposed model suggests that cholesterol is transported into the inner mitochondrial membrane mediated mainly by STARD1 protein while being regulated by voltage-dependent anion-selective channel protein 2 (VDAC2) (97). STARD1 expression is crucial in maintaining progesterone secretion within luteal
steroidogenic cells. Luteolytic pulses of prostaglandin F2α will drastically decrease STARD1 mRNA and protein levels, further slowing the rate-limiting step in progesterone biosynthesis and decreasing progesterone output.

The cytochrome p450 scc enzyme (CYP11A1) is responsible for conversion of cholesterol into the progesterone precursor molecule, pregnenolone. Pregnenolone travels to the smooth endoplasmic reticulum where it is converted to progesterone by 3βHSD protein. Reports in the literature suggest that mitochondrial docking with the endoplasmic reticulum is necessary for progesterone biosynthesis (98, 99). Knockdowns in genes such as cytoskeletal polymers or those facilitating mitochondrial fusion were critical to prevent unimpaired steroidogenesis (100).

Expression of the aforementioned genes and subsequent proteins are critical in driving cholesterol uptake and progesterone biosynthesis within the corpus luteum. These proteins work together to promote longevity of the corpus luteum.

**Luteolytic gene expression.** Prostaglandin F2α is synthesized and released from endometrial cells in the uterus. During the late stages of the estrus cycle, prostaglandin F2α is transported to the ovary via the extensive vascular network of the uterio-ovarian plexus and the prostaglandin transport protein (101). The prostaglandin F receptor (FP) is a G-protein coupled receptor and is responsible for binding prostaglandin F2α. Upon binding of this hormone, an intracellular signaling cascade is triggered that ultimately leads to both functional and structural regression of the corpus luteum. The precursor molecule of
prostaglandin F2α is arachidonic acid, an omega-6 fatty acid. This fatty acid is converted to prostaglandin F2α by the enzymes PGHS1 and PGHS2. Prostaglandin F2α has been reported to increase prostaglandin-endoperoxide synthase 2 (PGHS2) expression in the corpus luteum, acting as a positive feedback that drives further regression of the corpus luteum (102).

**Apoptotic gene expression.** Following the induction of luteolysis, expression of the apoptotic genes BAX, BCL-2, and CAS3 are altered in response to prostaglandin F2α signaling, bringing about an intrinsic mechanism of apoptosis in a late stage corpus luteum. In the primate, a corpus luteum of early pregnancy will have a high expression level of BCL-2, a pro survival gene, and a low expression level of BAX, a pro-apoptotic gene (103, 104). However, a regressing corpus luteum will conversely have a low expression of BCL-2 but a high expression level of BAX. A regressing corpus luteum will express increasing levels of CAS3 mRNA during prostaglandin F2α induced luteolysis (105, 106). Bovine luteal cells exposed to prostaglandin F2α will have a significant increase of p53 protein, BAX, and CAS3 mRNA in the subsequent 24 h period following exposure to this luteolysin (107). Increase in p53 activity can lead to an abundance of reactive oxygen species within the cells, further driving apoptosis (108). It is hypothesized that fish meal supplementation will help prevent a decrease in BCL-2 expression and attenuate BAX and CAS3 expression following exposure to low doses of prostaglandin F2α.

Immune cell populations will fluctuate throughout the lifespan of the corpus luteum. During luteolysis, there is an emigration of macrophages and
other phagocytic white blood cells. A non-functioning cell will be phagocytized during this immigration of immune cells. Newly present immune cells will secrete cytokines such as interleukin-1β and tumor necrosis factor α, causing recruitment of more immune cells to the corpus luteum (109).

Apoptosis can be brought about by an extrinsic mechanism most likely mediated by Fas ligand activation (110, 111). Immune cell emigration during a late stage corpus luteum and their secretion of tumor necrosis factor α and interferon γ can activate Fas mediated apoptosis (112). Activated Fas will bind to the Fas associated death domain protein (FADD) and will lead to downstream activation of caspase 8 and further cleavage of effector caspases leading to apoptosis.

**Omega-3 Polyunsaturated Fatty Acids**

Polyunsaturated fatty acids (PUFA) have been shown to positively influence reproduction in the bovine (113, 114). Nutrition, specifically energy availability, are essential to reproductive success in the bovine (115). There are two essential fatty acids that must be acquired through the diet, the omega-6 fatty acid linolenic acid (LA) and the omega-3 α-linolenic acid (ALA). Omega-3 fatty acids are characterized by the presence of a double bond at carbon three from the methyl end of the acyl chain of each molecule. Long chain omega-3 fatty acids can be synthesized from the short chain ALA through the action of elongases and desaturases (116). Several omega-3 fatty acids are potentially beneficial for reproduction including ALA, eicosapentanoic acid (EPA) and docosahexanoic acid (DHA). Both EPA and DHA have been shown to
incorporate into reproductive tissues as well as benefit luteal, ovarian, and uterine function in the bovine. (117, 118, 119).

At the level of the uterus, supplementation with PUFA has been shown to affect prostaglandin F2α secretion (120, 121). A higher ratio of omega-3 to omega-6 fatty acids in cultured bovine endometrial cells resulted in attenuated secretion of prostaglandin F2α (122). Simultaneously affecting prostaglandin F2α secretion at the uterus in addition to altering sensitivity of the corpus luteum to the luteolytic effects of prostaglandin F2α may be an effective way to ensure maternal recognition of pregnancy and prevent early embryonic mortality.

Fish oils found in cold water fishes are rich in the omega-3 fatty acids EPA and DHA. Previous studies in our laboratory have shown these fatty acids to alter mobility of the FP receptor as well as the structure of lipid microdomains in bovine luteal tissue both in vitro and in vivo (123). Therefore, dietary supplementation of omega-3 fatty acids could decrease luteal sensitivity to prostaglandin F2α (124, 125). Maintaining functional production of progesterone in addition to a loss in sensitivity to the luteolytic effects of prostaglandin F2α can lead to a prolonged lifespan of the corpus luteum following intrauterine infusion of prostaglandin F2α.

**Specific Aims**

This study examined dietary fish meal supplementation on changes in luteotropic, luteolytic, and apoptotic gene expression in the bovine corpus luteum following low dose administration of PGF2α. Luteal tissue from mid-cycle corpus luteum of non-pregnant, cycling cows supplemented with either corn gluten meal
or fish meal was biopsied following treatment with saline or low doses of PGF2α. It was hypothesized that omega-3 fatty acids in fish meal may lead to a global upregulation of luteotropic genes as well as an attenuated expression of luteolytic and apoptotic genes compared to those observed in corn gluten meal supplemented animals following intrauterine infusion of PGF2α. Furthermore, immediate early gene expression was expected to be attenuated in animals supplemented with fish meal following treatment with PGF2α compared to those supplemented with corn gluten meal. 

Long-term goals of this study were to mitigate the effects of PGF2α on the corpus luteum by supplementing animals with omega-3 fatty acids in order to prevent premature luteolysis during early pregnancy. This may be achieved from dietary supplementation of omega-3 fatty acids found in fish meal and subsequent incorporation of these fatty acids into the biological membranes of reproductive tissues.

**Aims, Research Questions and Hypotheses**

**A1** Examine steady-state mRNA expression in the bovine corpus luteum obtained from animals supplemented with fish meal or corn gluten meal.

**Q1** What are the effects of omega-3 fatty acids from fish meal on luteotropic gene expression (STARD1, CYP11A1, 3βHSD, LDLR)?

**H1** Cows supplemented with fish meal will exhibit a higher expression level of steroidogenic mRNA expression than animals fed corn gluten meal.

**Q2** What are the effects of omega-3 fatty acids from fish meal on steady-state expression of apoptotic (BAX, BCL-2, CAS3) and luteolytic (PGHS2, PTGFR)?

**H2** Luteal tissue obtained from cows supplemented with fish meal will have reduced steady-state levels of apoptotic and luteolytic mRNA.
A2 Examine luteotropic, luteolytic, apoptotic, and early immediate steady-state mRNA levels following intrauterine infusion of PGF2α in animals supplemented with fish meal to those supplemented with corn gluten meal.

Q1 What are the effects of omega-3 fatty acids from dietary supplementation of fish meal on luteotropic gene expression (STARD1, CYP11A1, 3βHSD, LDL)?

H3 Cows supplemented with fish meal will have a higher steady-state level of STARD1, CYP11A1, 3βHSD, and LDLR mRNA following intrauterine infusion of PGF2α as compared to cows supplemented with corn gluten meal.

Q2 What are the effects of dietary omega-3 fatty acid supplementation on luteolytic gene expression (PGHS2, PTGFR)?

H4 Animals supplemented with fish meal will have an attenuated level of luteolytic mRNA expression of PGHS2 and PTGFR following intrauterine infusion of PGF2α compared to animals supplemented with corn gluten meal.

Conclusion

Early pregnancy termination in the bovine contributes significantly to substantial monetary losses in the beef and dairy industries every year. Within the first 30 days of pregnancy, up to 30% of embryos die, impacting milk and meat production due to a decrease in healthy births (126). There are several causes of early embryonic death and one potential cause is a miscommunication between the developing embryo and maternal uterine environment, resulting in regression of the corpus luteum and termination of the pregnancy.

The lifespan of the corpus luteum, from its formation to its eventual demise is controlled by a plethora of changes in gene expression. It is crucial to analyze the gene expression rates and changes over the entire lifespan of the gland and properly cluster groups of gene expression. In order to shed light on
pathways that are not known, such as luteolysis, transcriptome analysis and proteomic analysis must be monitored as well as levels of hormones and their actions on each type of cell. It is necessary to ensure that gene expression differences between small and large luteal cells are taken into consideration throughout the lifespan of the corpus luteum as well as during pregnancy. Since there are different mechanisms to keep the corpus luteum in a luteotropic state both during the secretory phase as well as during pregnancy, the mechanisms individually controlling the regression of the corpus luteum during these separate events could differ. This study examined the effects of omega-3 fatty acid supplementation on bovine luteal cell sensitivity to repeated intrauterine doses of prostaglandin F2α. Understanding the direct mechanisms involved in luteolysis and early embryonic mortality may provide insight for non-invasive, cost effective interventions to increase pregnancy rate in the bovine. Incorporation of omega-3 fatty acids from fish meal into luteal tissue of breeding females may increase the chance of successful pregnancy.
CHAPTER II

METHODOLOGY

Animal Husbandry and Luteal
Biopsy Collection

All animal procedures included in this study were approved by the Colorado State University institutional animal care and use committee (Approval # 13 - 4440A). Beef cows of mixed breed were purchased from a local sale barn and housed at the Colorado State University Animal Reproduction and Biotechnology Laboratory in Fort Collins, Colorado. Transrectal ultrasonography was used to scan reproductive organs for pregnancy and anatomical abnormalities. Any cow with anatomical abnormalities or pregnant was removed from the study.

Cows were stratified by body weight and randomly assigned to either corn gluten or fish meal supplementation. Cows were individually fed an isocaloric and isonitrogenous ration consisting of 95% mixed-grass hay and 5% supplement. Animals were supplemented for approximately 60 days, providing adequate time for the omega-3 fatty acids found in fish meal to become incorporated into blood and reproductive tissues. Each week, body weights were recorded and jugular blood samples were collected to monitor changes in plasma fatty acid composition during the supplemental period. Gas chromatography-
mass spectroscopy was used to monitor these changes in plasma fatty acid composition and data have been reported elsewhere (127).

Figure 3: Schematic diagram of dietary supplementation and experimental design. Cows were supplemented with corn gluten meal or fish meal for approximately 60 days. Cows were administered injections of prostaglandin F2α on day 35 and 50 synchronize estrous cycles. On day 10–12 following estrus, cows were treated with intrauterine infusions of saline (four doses at 6-h intervals) or prostaglandin F2α (two doses at 12 h intervals). Intrauterine infusion and luteal biopsies performed at 30 min post-infusion.
Table 1: Dietary supplementation ingredients, chemical composition, and fatty acid profile.

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<td>Docosahexaenoic Acid</td>
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Estrous cycles were synchronized using a 25 mg intramuscular dose of prostaglandin F2α (Lutalyse®) at day 35 and again at day 50. Cows were observed for estrous behavior at dawn and dusk for a minimum of 30 min. Approximately 10 – 12 days following observed estrus after the second prostaglandin F2α injection, cows were randomly assigned to receive either four doses of 0.25 mL saline or two doses of 0.5 mg of prostaglandin F2α in 0.25 mL saline. Intrauterine infusions were administered into the uterine horn ipsilateral to the ovary bearing the corpus luteum. Animals assigned to prostaglandin F2α treatment received two intrauterine infusions of prostaglandin F2α at the first (0 h) and third (12 h) time points and saline given at the second (6 h) and fourth (18 h) time points.

Luteal biopsies were taken from the corpus luteum to determine steady-state mRNA levels of luteotropic, luteolytic, apoptotic, and early immediate genes. Luteal biopsies were collected 30 min post-intrauterine infusion using transvaginal ultrasonography. Prior to each luteal biopsy, a 3.5 mL epidural injection of 2% lidocaine hydrochloride was administered to induce a local anesthesia. An Aloka 500 V ultrasound machine equipped with 5 MHz convex-array transducer was used to visualize the ovary and the corpus luteum. The ovary containing the corpus luteum was extracted to the vaginal vault and the gland was positioned along a projected needle path. Luteal samples were collected using an 18-guage needle that passed through the vaginal wall and punctured the corpus luteum, taking 3-7 mg of tissue upon triggering the spring-loaded needle. Collected luteal samples were washed with sterile PBS to
remove blood or ovarian tissue and placed in a 1.7 mL Eppendorf tube. Tissue samples were snap frozen in liquid nitrogen and stored at -80°C until subjected to RNA isolation.

**Total Ribonucleic Acid Extraction and Complimentary Deoxyribonucleic Acid Synthesis**

Total RNA was isolated from frozen tissue using the TRIzol™ Plus RNA Purification Kit. In brief, tissue was homogenized on ice in 100 µL of TRIzol reagent. Following homogenization, an additional 900 µL of TRIzol was added and allowed to incubate for 5 min at room temperature before adding 200 µL of chloroform. Samples were vortexed and incubated for an additional 3 min. Samples were then subjected to centrifugation at 12,000 x g for 15 minutes at 4°C. The aqueous phase was collected and transferred to a new 1.7 mL Eppendorf tube. An equal volume of 70% ethanol was added to each sample and vortexed. Samples were loaded onto spin columns and centrifuged at 12,000 x g for 15 sec to bind RNA to membranes. After binding, a series of washing were performed. A volume of 700 µL of Wash buffer I was added to spin column and centrifuged at 12,000 x g for 15 sec. Next, 500 µL of Wash Buffer II was used to wash each sample. Samples were then centrifuged at 12,000 x g for 15 sec at room temperature. The 500 µL wash step with Wash Buffer II and subsequent centrifugation at 12,000 x g was repeated. Bound RNA to membranes was dried for 2 min at room temperature and then eluted into a collection tube with 30 µL of nuclease free water for 2 min. RNA quantification was completed using Qubit™ RNA HS Assay Kit purchased from ThermoFisher.
Scientific. Samples were stored at -80°C until subjected to reverse transcription reaction.

Following RNA extraction and quantification, 1 µg total RNA was reverse transcribed into cDNA using QuantiTect Reverse Transcription kit supplied by Qiagen. Genomic DNA was removed from each sample by adding 2 µL genomic DNA wipeout buffer. RNA was diluted using nuclease free water to a final volume of 14 µL. Complimentary DNA was synthesized using a Bio-Rad T100 PCR Thermal Cycler. Samples were then incubated for 2 min at 42°C. Next, 1 µL of RT enzyme, 4 µL of 5x RT buffer, and 1 µL of RT primer mix was added to the RNA samples. Samples were incubated at 42°C for 30 min and QuantiScript reverse transcriptase enzyme inactivated by incubating at 95°C for 3 min. Following synthesis of cDNA, samples were stored at -80°C until used in qPCR.

**Primer Design and Optimization**

Quantitative PCR was completed for each gene using bovine specific primers designed from mRNA sequences accessed from the NCBI nucleotide database. Primer3 was used to generate primer pairs for each gene. All gene names, primer sequences, product sizes, and efficiencies are listed in Table 2. Primer optimization was completed for each gene by using 10-fold serial dilutions of template and a standard curve generated to determine primer efficiency. Primer efficiency standard curves are shown in Appendix B.
Table 2: Gene name, primer sequences, accession numbers, product length and efficiencies.

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<th>Reverse Sequence</th>
<th>Accession No.</th>
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<td>CATGATGTTTGGGATGC</td>
<td>GCGAATTCAACTTTCCATC</td>
<td>NM_174445</td>
<td>154</td>
<td>2.2</td>
</tr>
<tr>
<td>PTGFR</td>
<td>Prostaglandin F Receptor</td>
<td>TGACAGTGGGAATCTATCGA</td>
<td>CTAGTCCATTGAGGATGAG</td>
<td>NM_181028</td>
<td>153</td>
<td>1.7</td>
</tr>
<tr>
<td>BAX</td>
<td>BCL-2 Associated X</td>
<td>TCTGACGGAATCTTACT</td>
<td>TCAAGGACATGAGAATTG</td>
<td>NM_173894.1</td>
<td>135</td>
<td>2.1</td>
</tr>
<tr>
<td>BCL-2</td>
<td>B-cell lymphoma 2</td>
<td>TTTGCTTCAGGTTTCATCC</td>
<td>ATCTCTGCAGCTCCATGTT</td>
<td>NM_001166486.1</td>
<td>147</td>
<td>2.0</td>
</tr>
<tr>
<td>CAS3</td>
<td>Caspase 3</td>
<td>TCAGTCAGTTGGGCAGCTTG</td>
<td>CACACCAGTAGGTGAAGA</td>
<td>NM_001177840.1</td>
<td>144</td>
<td>1.9</td>
</tr>
</tbody>
</table>
Quantitative Polymerase Chain Reaction

All qPCR reactions were completed in duplicate for each gene of interest, along with the reference gene β-Actin. In brief, 5 µL of QuantiTect SYBR Green, 100 nM of each primer, 5 ng of cDNA, and nuclease free water to a total volume of 10 µL were combined on a 384 well PCR plate. Reaction conditions were carried out using a Bio Rad CFX384 real-time PCR system using the following reaction conditions. Assays were carried out beginning with an enzyme activation at 95˚C for 15 min followed by 40 cycles of denaturation (94˚C for 15 sec), annealing (60˚C for 30 sec) and amplification (72˚C for 30 sec).

Dissociation curves for each target product were generated and melting temperatures were noted to ensure single amplification. A negative control that did not include cDNA template was completed for each gene and included in each assay.

Statistical Analysis

Statistical analysis of steady-state mRNA data was completed using the 2^(-ΔCq) method as described by Livak (128) to determine fold change of each gene in relation to the reference gene β-Actin. Data was transformed using the Shapiro-Wilk test for normality prior to statistical analysis. Statistical model included treatment, time, treatment x time, and cow considered as a random variable in the model. Data was analyzed using a 2-way ANOVA and calculations completed in SAS using PROC MIXED with repeated measures.
CHAPTER III

RESULTS

Immediate-Early Genes Steady-State Messenger Ribonucleic Acid Expression

Luteal biopsies were taken 30 min following intrauterine infusions of prostaglandin F2α or saline to determine the effects of corn gluten meal or fish meal supplementation on steady-state mRNA levels of immediate early genes of interest. Figure 4 illustrates changes in immediate early gene expression of FOS and NR4A1. There was a main effect of treatment and time on steady-state mRNA expression of these genes ($P < 0.05$). Dietary supplementation had no effect on FOS and NR4A1 expression in animals treated with saline ($P > 0.05$). In corn gluten meal supplemented animals that received two doses of prostaglandin F2α, there was a significant increase in both FOS and NR4A1 at the 0 and 12 h time points compared to animals that received saline ($P < 0.05$). However, at 6 and 18 h time points where prostaglandin F2α was not administered, there were no differences in FOS or NR4A1 steady-state mRNA ($P > 0.05$). Within fish meal supplemented animals, there was no difference in immediate early steady-state mRNA levels following intrauterine infusion with prostaglandin F2α in either regressed or non-regressed corpora lutea ($P > 0.05$). However, there was an increase in FOS and NR4A1 following prostaglandin F2α administration ($P < 0.05$).
Figure 4: Effect of corn gluten meal or fish meal supplementation on immediate early steady-state mRNA expression following low dose administration of prostaglandin F2α. Cows were administered four intrauterine infusions of 0.25 mL saline or two intrauterine infusions of 0.5 mg prostaglandin F2α between day 10 and 12 following a synchronized estrus. Cows assigned to prostaglandin F2α treatment were administered prostaglandin F2α at the first (0 h) and third (12 h) infusion and saline at the second (6 h) and fourth infusion (18 h). Steady-state mRNA expression of FOS and NR4A1. Animals supplemented with corn gluten meal (open bar; n = 6) or fish meal (closed bar; n = 5) and infused with four doses of saline. Animals supplemented with corn gluten meal (regressed; dotted bar; n = 10) or fish meal (regressed; diagonal bar; n = 5; non-regressed gray bar; n = 6) following intrauterine infusions of prostaglandin F2α. Within time, means with different lettersabc differ significantly within treatment (P < 0.05)
Biopsies taken at each time point were used to determine steady-state mRNA levels of luteotropic gene expression following intrauterine infusions of either saline of prostaglandin F2α. Dietary supplementation did not affect relative gene expression in LDLR, STARD1, CYP11A1, 3βHSD regardless of supplementation ($P > 0.05$) in cows that were administered four doses of saline.

Changes in steady-state mRNA expression for LDLR are denoted in Figure 5. There was a main effect of treatment and time on steady-state mRNA expression for LDLR ($P < 0.05$). Steady-state expression of LDLR mRNA was decreased in corn gluten meal supplemented animals in response to prostaglandin F2α at the 0 h time point when compared to saline treated animals ($P < 0.05$). At each subsequent time point (6, 12, and 18 h), there was a consistent decrease in LDLR ($P < 0.05$). There was a decrease in steady-state LDLR mRNA levels at both 6 and 18 h time points for cows supplemented with fish meal that had a regressed corpus luteum when compared to saline treated animals ($P < 0.05$). Additionally, cows supplemented with fish meal that retained a non-regressed corpus luteum had a decrease in LDLR steady-state mRNA levels at 6, 12, and 18 h time points compared to saline infused animals ($P < 0.05$).

Changes in luteal steady-state STARD1 mRNA expression in response to saline and prostaglandin F2α are shown in Figure 6. There was a main effect of treatment and time on steady-state mRNA expression for STARD1 ($P < 0.05$).
Luteal steady-state mRNA expression of STARD1 was decreased in corn gluten meal supplemented cows treated with two doses of prostaglandin F2α as compared to saline treated control cows. Fish meal supplemented cows that retained a non-regressed corpus luteum following administration of prostaglandin F2α had a decrease in STARD1 mRNA expression at the 6 h time point compared to other treatment groups (P < 0.05). However, there were no differences in STARD1 mRNA expression in cows supplemented with fish meal that maintained a non-regressed or regressed corpus luteum at 0, 12, or 18 h (P > 0.05).

Changes in CYP11A1 luteal steady-state mRNA in response to saline and prostaglandin F2α are shown in Figure 7. There was a main effect of treatment and time on steady-state mRNA expression on CYP11A1 (P < 0.05). In animals that were supplemented with corn gluten meal and administered two doses of prostaglandin F2α, steady-state mRNA levels of CYP11A1 decreased at 6, 12, and 18 h time points when compared to saline treated animals (P < 0.05). Additionally, there was no difference in CYP11A1 mRNA expression between fish meal or corn gluten meal supplemented animals treated with prostaglandin F2α (P > 0.05). In fish meal supplemented animals there was no change in CYP11A1 steady-state mRNA following administration of prostaglandin F2α in animals that maintained a non-regressed corpus luteum or animals that had a regressed gland. (P > 0.05). In fish meal supplemented animals that had a regressed corpus luteum, CYP11A1 steady-state mRNA levels were decreased at both 6 and 18 h time points (P < 0.05). However, CYP11A1 mRNA expression
decreased at the 12 h time point in fish meal supplemented animals that maintained a non-regressed corpus luteum ($P < 0.05$).

Changes in luteal steady-state mRNA expression of 3βHSD throughout the experimental period are shown in Figure 8. There was a main effect of treatment and time on steady-state mRNA expression for 3βHSD ($P < 0.05$). Steady-state mRNA of 3βHSD decreased in corn gluten meal supplemented animals that were treated with two doses of prostaglandin F2α compared to saline treated animals and continued to decline throughout the experimental period ($P < 0.05$). At 0 h time point there was no difference in 3βHSD steady-state mRNA levels in fish meal supplemented animals that received two infusions of prostaglandin F2α, regardless of luteal function ($P > 0.05$). In fish meal supplemented animals that received two doses of prostaglandin F2α there was a decrease in 3βHSD steady-state mRNA levels at the 6 h time point for cows with a regressed corpus luteum ($P < 0.05$). The steady-state mRNA levels of 3βHSD in cows with a regressed corpus luteum continued to decrease throughout the experimental period ($P < 0.05$). However, in fish meal supplemented animals that received two doses of prostaglandin F2α and maintained a non-regressed corpus luteum, the steady-state mRNA levels of 3βHSD did not decrease following treatment with prostaglandin F2α ($P > 0.05$).
Figure 5: Effects corn gluten meal or fish meal supplementation on steady state mRNA of LDLR following low dose administration of prostaglandin F2α. Effects of corn gluten meal or fish meal on steady-state gene expression of enzymes involved in progesterone biosynthesis following intrauterine infusions of prostaglandin F2α. Cows were administered four intrauterine infusions of 0.25 mL saline or two intrauterine infusions of 0.5 mg prostaglandin F2α between day 10 and 12 following a synchronized estrus. Cows were administered prostaglandin F2α at the first (0 h) and third (12 h) infusion and saline at the second (6 h) and fourth infusion (18 h). Steady-state mRNA expression of LDLR. Animals supplemented with corn gluten meal (open bar; n = 6) or fish meal (closed bar; n = 5) and infused with four doses of saline. Animals supplemented with corn gluten meal (open bar; n = 6) or fish meal (closed bar; n = 5) and infused with four doses of saline. Animals supplemented with corn gluten meal (regressed; dotted bar; n = 10) or fish meal (regressed; diagonal bar; n = 5; non-regressed gray bar; n = 6) following intrauterine infusions of prostaglandin F2α. Within time, means with different letters a, b, c differ significantly within treatment (P < 0.05)
Figure 6: Effects of corn gluten meal or fish meal supplementation on steady state mRNA of STARD1 expression following low dose intrauterine infusion of prostaglandin F2α. Effects of corn gluten meal or fish meal on steady-state gene expression of enzymes involved in progesterone biosynthesis following intrauterine infusions of prostaglandin F2α. Cows were administered four intrauterine infusions of 0.25 mL saline or two intrauterine infusions of 0.5 mg prostaglandin F2α between day 10 and 12 following a synchronized estrus. Cows were administered prostaglandin F2α at the first (0 h), third (12 h) infusion, saline at the second (6 h), and fourth infusion (18 h). Steady-state mRNA expression of luteal STARD1. Animals supplemented with corn gluten meal (open bar; n = 6) or fish meal (closed bar; n = 5) and infused with four doses of saline. Animals supplemented with corn gluten meal (regressed; dotted bar; n = 10) or fish meal (regressed; diagonal bar; n = 5; non-regressed gray bar; n = 6) following intrauterine infusions of prostaglandin F2α. Within time, means with different letters differ significantly within treatment (P < 0.05)
Figure 7: Effects of corn gluten meal or fish meal supplementation on steady-state mRNA levels of CYP11A1 in response to low dose intrauterine infusions of prostaglandin F2α. Effects of corn gluten meal or fish meal on steady-state gene expression of enzymes involved in progesterone biosynthesis following intrauterine infusions of prostaglandin F2α. Cows were administered four intrauterine infusions of 0.25 mL saline or two intrauterine infusions of 0.5 mg prostaglandin F2α between day 10 and 12 following a synchronized estrus. Cows were administered prostaglandin F2α at the first (0 h) and third (12 h) infusion and saline at the second (6 h) and fourth infusion (18 h). Steady-state mRNA expression of luteal CYP11A1. Animals supplemented with corn gluten meal (open bar; n = 6) or fish meal (closed bar; n = 5) and infused with four doses of saline. Animals supplemented with corn gluten meal (regressed; dotted bar; n = 10) or fish meal (regressed; diagonal bar; n = 5; non-regressed gray bar; n = 6) following intrauterine infusions of prostaglandin F2α. Within time, means with different letters^abc differ significantly within treatment (P < 0.05)
Figure 8: Effects of corn gluten meal or fish meal supplementation on steady-state mRNA levels of 3βHSD following low dose intrauterine infusion of prostaglandin F2α. Effects of corn gluten meal or fish meal on steady-state gene expression of enzymes involved in progesterone biosynthesis following intrauterine infusions of prostaglandin F2α. Cows were administered four intrauterine infusions of 0.25 mL saline or two intrauterine infusions of 0.5 mg prostaglandin F2α between day 10 and 12 following a synchronized estrus. Cows were administered prostaglandin F2α at the first (0 h) and third (12 h) infusion and saline at the second (6 h) and fourth infusion (18 h). Steady-state mRNA expression of luteal 3βHSD. Animals supplemented with corn gluten meal (open bar; n = 6) or fish meal (closed bar; n = 5) and infused with four doses of saline. Animals supplemented with corn gluten meal (open bar; n = 6) or fish meal (closed bar; n = 5) and infused with four doses of saline. Animals supplemented with corn gluten meal (regressed; dotted bar; n = 10) or fish meal (regressed; diagonal bar; n = 5; non-regressed gray bar; n = 6) following intrauterine infusions of prostaglandin F2α. Within time, means with different letters differ significantly within treatment (P < 0.05).
Data regarding steady-state mRNA expression of the PTGFR and PGHS2 enzyme are shown in Figure 9. There was a main effect of treatment and time on steady-state mRNA expression of these genes ($P < 0.05$). Biopsies of the corpus luteum were taken 30 min post intrauterine infusion of either saline or prostaglandin F2α to determine the effect of corn gluten meal or fish meal supplementation on steady-state mRNA levels of two luteolytic genes. In saline treated animals, there were no differences in PTGFR steady-state mRNA level between supplementation groups ($P > 0.05$). However, following administration of prostaglandin F2α, there was a decrease in PTGFR steady-state mRNA levels in corn gluten meal supplemented animals. ($P < 0.05$). There were no differences in PTGFR expression in fish meal supplemented animals with a non-regressed corpus luteum when compared to animals with a regressed gland ($P > 0.05$).

Steady-state mRNA expression of PGHS2 did not differ between control animals treated with saline, regardless of fish meal or corn gluten meal supplementation ($P > 0.05$). Additionally, there were no changes in PGHS2 expression between saline treated animals and prostaglandin F2α treated animals ($P > 0.05$). Levels of PGHS2 expression did not differ between animals with a regressed corpus luteum and animals that retained a non-regressed corpus luteum ($P > 0.05$).
Figure 9: Effects of corn gluten meal or fish meal supplementation on steady-state mRNA levels of luteolytic genes (PTGFR and PGHS2) following low dose intrauterine infusion of prostaglandin F2α. Effects of corn gluten meal or fish meal on steady-state gene expression of enzymes involved in progesterone biosynthesis following intrauterine infusions of prostaglandin F2α. Cows were administered four intrauterine infusions of 0.25 mL saline or two intrauterine infusions of 0.5 mg prostaglandin F2α between day 10 and 12 following a synchronized estrus. Cows were administered prostaglandin F2α at the first (0 h) and third (12 h) infusion and saline at the second (6 h) and fourth infusion (18 h). Steady-state mRNA expression of luteal PTGFR and PGHS2. Animals supplemented with corn gluten meal (open bar; n = 6) or fish meal (closed bar; n = 5) and infused with four doses of saline. Animals supplemented with corn gluten meal (regressed; dotted bar; n = 10) or fish meal (regressed; diagonal bar; n = 5; non-regressed gray bar; n = 6) following intrauterine infusions of prostaglandin F2α. Within time, means with different lettersabc differ significantly within treatment (P < 0.05)
Apoptotic Genes Steady-State Messenger Ribonucleic Acid Expression

Cows were supplemented for 60 days with either corn gluten meal or fish meal and luteal biopsies were taken every 6 h for an 18 h treatment period to determine steady-state mRNA expression for three target apoptotic genes of interest: BAX, BCL-2, and CAS3 (data shown in Figure 10).

In animals treated with saline, there were no differences in BAX steady-state mRNA levels in animals supplemented with corn gluten meal or fish meal (P > 0.05). There were no changes in BAX expression following administration of prostaglandin F2α in animals supplemented with fish meal (P > 0.05). However, BAX steady-state mRNA was elevated at the 18 h time point in animals supplemented with corn gluten meal compared to those supplemented with fish meal following infusion of prostaglandin F2α (P < 0.05).

In animals treated with four doses of saline, steady-state mRNA expression of BCL-2 differed between supplementation groups. Levels of BCL-2 steady-state mRNA expression was increased in fish meal supplemented animals (P < 0.05). Animals administered prostaglandin F2α, regardless of supplementation group, had a decrease in BCL-2 expression (P < 0.05). There were no changes in BCL-2 expression in both subpopulations of fish meal supplemented animals following administration of prostaglandin F2α. (P > 0.05).

Steady-state mRNA expression of CAS3 did not differ between saline treated animals supplemented with either corn gluten meal or fish meal supplemented (P > 0.05). Steady-state mRNA expression of CAS3 was elevated
at the 18 h time point in corn gluten meal supplemented animals treated with prostaglandin F2α (P < 0.05). However, in fish meal supplemented animals treated with prostaglandin F2α, expression of CAS3 did not increase and did not differ from saline treated animals (P > 0.05).
Effects of corn gluten meal or fish meal on steady-state gene expression of enzymes involved in progesterone biosynthesis following intrauterine infusions of prostaglandin F2α. Cows were administered four intrauterine infusions of 0.25 mL saline or two intrauterine infusions of 0.5 mg prostaglandin F2α between day 10 and 12 following a synchronized estrus. Cows were administered prostaglandin F2α at the first (0 h) and third (12 h) infusion and saline at the second (6 h) and fourth infusion (18 h). Steady-state mRNA expression of luteal BAX, BCL-2 and CAS3. Animals supplemented with corn gluten meal (open bar; n = 6) or fish meal (closed bar; n = 5) and infused with four doses of saline. Animals supplemented with corn gluten meal (regressed; dotted bar; n = 10) or fish meal (regressed; diagonal bar; n = 5; non-regressed gray bar; n = 6) following intrauterine infusions of prostaglandin F2α. Within time, means with different lettersabc differ significantly within treatment (P < 0.05).
CHAPTER IV
DISCUSSION

Nutrition and specifically energy availability can effect reproduction in the bovine (129, 130). A reduction in energy intake has been reported to decrease luteinizing hormone secretion, diameter of the corpus luteum and progesterone biosynthesis (131). Often, fat is added to the diet of breeding cows to compensate for lowered energy levels. Clearly, inclusion of fat in the diet overcame energy deficits and improved reproductive performance (132). Additionally, omega-3 fatty acids found in some sources of dietary fats, such as fish products, may have a direct effect on reproduction.

Reports in the literature have shown that omega-3 fatty acids in fish meal and oil escape ruminal hydrogenation (133) and are incorporated into the blood, making it available for incorporation into reproductive tissues (134). Indeed, recent reports in the literature show that omega-3 fatty acids from fish products influence endometrial oxytocin-induced prostaglandin F2α secretion (135, 136) and luteal function in response to uterine infusion of prostaglandin F2α in the bovine (137).

Regarding luteal function, it was shown that uterine infusion of prostaglandin F2α caused regression (serum progesterone <1 ng/mL within 48h) in approximately 90% of the cows supplemented with corn gluten meal but only in 50% of cows receiving fish meal. This study sought to elucidate the effects of
fish meal supplementation on luteal gene expression in response to low-doses of 
prostaglandin F2α administration in cows that have a regressed or non-regressed 
corpora lutea.

There were no changes in expression of target genes throughout the 
experimental period in response to saline administration for cows supplemented 
with corn gluten meal. This agrees with previous studies showing that repeated 
luteal biopsies did not affect gene expression (137). Therefore, any changes in 
gene expression were due to prostaglandin F2α treatment or dietary 
supplementation and not due to reproductive tract manipulation or inflammatory 
responses from repeated luteal biopsies. In addition, there was no influence of 
dietary supplementation of fish meal on global expression of genes of interest.

Regardless of luteal function, there was a reduction in LDLR, STARD1 
and CYP11A1 steady-state mRNA levels in all fish meal supplemented animals 
treated with prostaglandin F2α. Interestingly, steady-state mRNA levels for 
3βHSD remained unchanged in fish meal supplemented animals that were 
infused with prostaglandin F2α and maintained a non-regressed corpus luteum. 
However, animals with a regressed corpus luteum had a significant decrease in 
3βHSD expression. This could indicate that 3βHSD expression or upstream 
regulatory pathways may play a critical role in altering luteal function and 
progesterone output in response to prostaglandin F2α.

Previous studies in the literature show a decrease in luteotropic gene 
expression in response to prostaglandin F2α (59, 137). Dietary fish meal 
supplementation may be adequate to maintain luteotropic gene expression,
particularly of 3βHSD, and prevent critical loss of progesterone output, thus retaining a non-regressed corpus luteum following prostaglandin F2α administration. The maintenance of 3βHSD steady-state mRNA could be adequate to prevent functional regression of the corpus luteum following exposure to prostaglandin F2α. Additionally, despite the decrease in LDLR, STARD1, and CYP11A1 mRNA levels, this may not be indicative of protein abundance or activity in luteal tissue and requires further investigation. Also, it is possible a rebound in steady state mRNA for LDLR, STARD1, and CYP11A1 may occur between 18 and 48 h of the experimental period in animals that retained a non-regressed corpus luteum following administration of prostaglandin F2α.

Secretion of uterine prostaglandin F2α late in the estrous cycle initiates an intracellular signaling cascade that leads to the functional and structural regression of the corpus luteum. It has been postulated that luteal prostaglandin F2α synthesis is required for complete luteolysis (138). Reports in the literature show that administration of intrauterine prostaglandin F2α leads to a 5-10 fold increase in PGHS2 mRNA expression, supporting the hypothesis that luteal production of prostaglandin F2α may be needed for regression of the corpus luteum. In contrast to recent reports in the literature, there was no increase in luteal PGHS2 mRNA expression following administration of prostaglandin F2α in the current study. Additionally, there was no difference in luteal PGHS2 expression between corn gluten meal and fish meal supplemented animals. Discrepancies between the current study wherein there was no change in mRNA
expression of PGHS2 compared to reports in the literature where prostaglandin F2α increased expression of PGHS2 could be due to physiological status of animals studied. Non-lactating beef cows were used in the current study as opposed to the use of Holstein heifers used in previous studies reported in the literature (139). Doses of prostaglandin F2α, timing and delivery routes were similar between studies. However, it is possible that body weight (cow vs heifer) or maturity (growing heifer vs mature cow) could influence transfer of prostaglandin F2α from the uterus to the luteal vasculature and or clearance of prostaglandin F2α. Additional studies are required to determine the influence of fish products on luteal prostaglandin metabolism and vascular transfer.

There were no differences in steady-state mRNA levels of PTGFR between animals supplemented with corn gluten meal or fish meal that were administered intrauterine infusions of saline. Steady-state mRNA of PTGFR decreased in response to prostaglandin F2α treatment, regardless of dietary supplementation or luteal function. Previous reports in the literature show a decrease in PTGFR expression following treatment with prostaglandin F2α (139). In contrast to recent reports in the literature, there was no difference in PTGFR expression in prostaglandin F2α treated animals that had a regressed corpus luteum compared to those that retained a non-regressed gland. It is possible that no change in PTGFR mRNA was due to biopsy collection time post-intrauterine infusion and resulted in a lack of detectible differences in steady-state mRNA levels.
The effects of fish meal supplementation on the apoptotic gene expression of BAX, BCL-2, and CAS3 steady-state mRNA levels were examined. Interestingly, BCL-2 steady-state mRNA levels in fish meal supplemented animals treated with saline was elevated compared to saline treated corn gluten supplemented animals and animals treated with prostaglandin F2α. Typically, BCL-2 expression remains the same during luteolysis, while an increase in the ratio of BAX to BCL-2 expression can be used as a biomarker to identify apoptotic cells (140). In the present study, there were no differences in BAX or BCL-2 mRNA levels, regardless of dietary supplementation group, following exposure to prostaglandin F2α. The experimental period of 18 hours may not be long enough to detect significant increase in BAX mRNA expression. An extended biopsy collection may be necessary to determine when BAX expression may become elevated within the corpus luteum. Additionally, both BAX and BCL-2 proteins are associated with the intrinsic pathway of apoptosis. It is possible that lack in observable change in BAX and BCL-2 expression following administration of prostaglandin F2α could be due to apoptosis being signaled through an extrinsic pathway of apoptosis instead. Extrinsic apoptosis could be initiated by FAS/FASL, TNFα, or TRAIL signaling, leading to downstream activation of effector caspases, resulting in apoptosis (141). Apoptotic signaling via an extrinsic pathway could explain a lack in change of BAX and BCL-2 and warrants further investigation.

Surprisingly, there was no change in CAS3 expression in either corn gluten meal or fish meal supplemented animals following administration of
prostaglandin F2α. Previous studies in the literature show an increase in CAS3 mRNA expression following exposure to PGF2α (142, 143). The activation of CAS3 will lead to activation of caspase activated DNases, leading to DNA fragmentation and apoptosis. CAS3 is an effector caspase that is activated downstream of both intrinsic and extrinsic apoptotic pathways and it was expected that CAS3 expression would increase in luteal tissue following exposure to prostaglandin F2α regardless of apoptotic signaling pathway. However, dosage of prostaglandin F2α and its effects on gene expression must be taken into consideration. A physiological dose of prostaglandin F2α (0.5mg) was administered in the current study; whereas reports in the literature that showed an increase in CAS3 expression administered pharmacological doses (20mg or greater) of prostaglandin F2α (144). Differences in doses of prostaglandin F2α could explain the lack of change in CAS3 steady-state mRNA levels. Additionally, an alternative signaling pathway leading to apoptosis may explain why CAS3 steady-state mRNA levels remained unchanged. A novel apoptotic pathway was recently reported in the literature describing an endoplasmic reticulum stress-mediated pathway for apoptosis. Endoplasmic stress was shown to activate caspase 12 protein (145) and downstream activation of CAS3 expression. It is possible that a CAS3 activation by caspase 12 occurs after 48 h of initial prostaglandin F2α administration, resulting in non-detectable changes in CAS3 steady-state mRNA in the present study. An extended biopsy period may be necessary to detect an increase in CAS3 expression.
The effects of dietary fish meal supplementation on the expression of FOS and NR4A1 were examined. These two genes are classified as immediate early genes and have been shown to play a role in luteal function and expression of luteotropic genes (83, 146). A previous study conducted in the cow showed that both FOS and NR4A1 expression increases following administration of prostaglandin F2α (147).

In the present study, there was no change in either FOS or NR4A1 expression following saline administration between cows supplemented with either corn gluten meal or fish meal. This indicates that fish meal supplementation did not influence expression of these immediate early genes. However, cows receiving two doses of prostaglandin F2α had a significant increase in FOS and NR4A1 expression within 30 min following treatment, which agrees with a previous study using a similar animal model (139). More experiments are necessary to determine differences in gene expression between FOS and NR4A1 and downstream signaling following administration of prostaglandin F2α.
CHAPTER V

CONCLUSION

Previous studies in our laboratory show the successful incorporation of omega-3 fatty acids found in fish oils into bovine luteal tissue. Supplementation with omega-3 fatty acids affects prostaglandin F2α signaling and luteal sensitivity. Additionally, reports in the literature show that fish oil supplementation of bovine luteal cells altered internalization of the PTGFR and endosomal trafficking of PTGFR protein (148). Fish oil supplementation has been shown to cause a disruption in lipid microdomains and lateral mobility of the PTGFR, thereby affecting prostaglandin F2α induced signaling and luteal sensitivity (149). Increase in PTGFR mobility will result in a lower likelihood of prostaglandin F2α binding to its receptor and initiating intracellular signaling cascades that cause luteal regression. Therefore, it was expected that fish meal supplementation affects luteal gene expression and signaling following administration of prostaglandin F2α.

Responsiveness to prostaglandin F2α was expected to be reduced in fish meal supplemented animals that retained a non-regressed corpus luteum. Surprisingly, the incorporation of omega-3 fatty acids into luteal tissue did not seem to affect immediate early or luteolytic gene expression when compared to animals supplemented with corn gluten meal. Despite changes in PTGFR signaling in previous reports in the literature, the lack in change of immediate
early or luteolytic gene expression indicates an alternative pathway ultimately leading to regression of the corpus luteum. Additionally, expression of 3βHSD may be an important biomarker in regressed or non-regressed corpora lutea following administration of prostaglandin F2α. More research must be completed to investigate differences in animals that retained a non-regressed corpus luteum following administration of prostaglandin F2α compared to animals that had a regressed corpus luteum.
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APPENDIX A

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL
**Protocol Title:** Effect of omega-3 fatty acids on bovine luteal cell lipid microdomains and PGI2/PGF2a Signaling

**Protocol ID:** 16-6761AA (Bruenmer, Jason)

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**COLORADO STATE UNIVERSITY INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE**

**ANIMAL USE APPLICATION**

IACUC approval of this completed form is necessary prior to animals being obtained, housed or manipulated for research, testing or teaching purposes; performed at CSU or by CSU at other locations.

When you have completed all applicable sections of the protocol, you must also complete the certifications section and then click “Submit Form” link on the left-hand column.

All individuals listed on the protocol must have certified completion of the online CSU Animal Care and Use Training. Additionally, a “Training Record” should be uploaded in the Attachments section for the PI, Co-PI, and each person who will handle animals as a part of this study. Also, all individuals working with animals must be enrolled in the CSU Occupational Health and Safety Program (OHSP) via annual submission of a Risk Assessment Form to the OHSP.

Please contact an IACUC Coordinator if you have any questions.

---

### Principal Investigator

<table>
<thead>
<tr>
<th>Name</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bruenmer, Jason</td>
<td>Professor</td>
</tr>
<tr>
<td>Email</td>
<td>EID</td>
</tr>
<tr>
<td></td>
<td>(970) 491-3455</td>
</tr>
<tr>
<td>Department</td>
<td>Mail Code</td>
</tr>
<tr>
<td>1171 Animal Sciences</td>
<td>1171</td>
</tr>
</tbody>
</table>

Will PI work with animals as part of this project?  
Yes  [ ]  No  [ ]  

Upload a “Training Record” for the PI under the “Attachments” section of this protocol.

---

### Co-Principal Investigator

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>Engle, Terry</td>
<td>Professor</td>
</tr>
<tr>
<td>Email</td>
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<tr>
<td></td>
<td>(970) 491-3597</td>
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<tr>
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<td>1171</td>
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</tbody>
</table>

Will Co-PI work with animals as part of this project?  
Yes  [ ]  No  [ ]  

Upload a “Training Record” for the Co-PI under the “Attachments” section of this protocol.

---

### Department Head

<table>
<thead>
<tr>
<th>Name of Department Head</th>
<th>Degree</th>
<th>Title</th>
</tr>
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<tbody>
<tr>
<td>Pond, Kevin</td>
<td></td>
<td>Professor</td>
</tr>
<tr>
<td>Email</td>
<td></td>
<td>Phone</td>
</tr>
<tr>
<td><a href="mailto:Kevin.Pond@colostate.edu">Kevin.Pond@colostate.edu</a></td>
<td></td>
<td>(970) 491-7295</td>
</tr>
<tr>
<td>Department Name</td>
<td>Campus Delivery Code</td>
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</tr>
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</table>

Will the Department Head work with animals as a part of this project?  
Yes  [ ]  No  [ ]  

If this person will work with animals as a part of this protocol, upload a “Training Record” for this individual under the “Attachments” section of this protocol.
### Administrative Contact

<table>
<thead>
<tr>
<th>Name</th>
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<tbody>
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</table>

Will Administrative Contact work with animals as part of this project? [ ] Yes [ ] No

If this person will work with animals as a part of this protocol, upload a "Training Record" for this individual under the "Attachments" section of this protocol.

### Other Submitter

<table>
<thead>
<tr>
<th>Name</th>
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</tr>
</thead>
<tbody>
<tr>
<td>pdburns, pdburns</td>
<td>UNC</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Email</th>
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<th>Phone</th>
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<tbody>
<tr>
<td><a href="mailto:pdburns@co.edu">pdburns@co.edu</a></td>
<td></td>
<td>970 351 2696</td>
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<table>
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<tbody>
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</table>

Will this person be working with animals as a part of this project? [ ] Yes [ ] No

If this person will work with animals as a part of this protocol, upload a "Training Record" for this individual under the "Attachments" section of this protocol.

### Other Personnel

<table>
<thead>
<tr>
<th>Name</th>
<th>Email</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>Graham, Peter</td>
<td><a href="mailto:Peter.E.Graham@co.edu">Peter.E.Graham@co.edu</a></td>
<td></td>
</tr>
<tr>
<td>Jessica Cidello</td>
<td><a href="mailto:cedi7207@bears.unco.edu">cedi7207@bears.unco.edu</a></td>
<td></td>
</tr>
<tr>
<td>Michele Plewes</td>
<td><a href="mailto:micheleplewes@gmail.com">micheleplewes@gmail.com</a></td>
<td></td>
</tr>
<tr>
<td>Hansen, Thomas</td>
<td><a href="mailto:Thomas.Hansen@ColoState.EDU">Thomas.Hansen@ColoState.EDU</a></td>
<td></td>
</tr>
<tr>
<td>Sinedino, Leticia</td>
<td><a href="mailto:leticia.sinedino@co.edu">leticia.sinedino@co.edu</a></td>
<td></td>
</tr>
<tr>
<td>Mandujano, Julio</td>
<td><a href="mailto:Julio.mandujano@unco.edu">Julio.mandujano@unco.edu</a></td>
<td></td>
</tr>
<tr>
<td>Brian Krum</td>
<td><a href="mailto:krum9141@bears.unco.edu">krum9141@bears.unco.edu</a></td>
<td>Graduate Student</td>
</tr>
</tbody>
</table>
APPENDIX B

PRIMER OPTIMIZATION CURVES
NR4A1

\[ y = -3.8525x + 38.452 \]
\[ R^2 = 0.99954 \]

FOS

\[ y = -3.28x + 36.27 \]
\[ R^2 = 1 \]

LDLR

\[ y = -3.26x + 35.43 \]
\[ R^2 = 0.9998 \]