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Mechanisms Behind the Luteoprotective Effects of Fish Meal Supplementation in Bovine: Cytokine Sensitivity and Luteal Blood Flow

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MECHANISMS BEHIND THE LUTEOPROTECTIVE EFFECTS OF FISH MEAL SUPPLEMENTATION IN BOVINE: CYTOKINE SENSITIVITY AND LUTEAL BLOOD FLOW

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ABSTRACT

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Early pregnancy loss continues to be problematic in the cattle industry. A large proportion of the pregnancy loss occurs during the maternal recognition of pregnancy (MRP) phase of gestation. During the MRP, a developing conceptus must secrete sufficient interferon tau to inhibit release of the uterine-derived and luteolytic mediator, prostaglandin F2 alpha (PGF2α). If successful, the progesterone-secreting corpus luteum (CL) will be maintained, which is necessary for establishment and maintenance of pregnancy. Pregnancy loss at the point of MRP may be due to a slow developing conceptus that does not effectively block PGF2α. Therefore, reducing the influence of PGF2α on the CL may lengthen the window of time to establish pregnancy. Fish meal (FM) supplementation, rich in the omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), serves as a luteoprotective agent following PGF2α exposure; an effect we repeated here. However, the mechanistic actions of FM supplementation are not yet apparent. Here, we postulated two mechanisms by which FM supplementation (and components) reduces rates of luteal regression: 1) EPA and DHA protect cells against the cytotoxic effects of TNFα and IFNγ and (or) 2) FM supplementation maintains luteal blood flow in functional CL 48 hrs following exposure to PGF2α. We found that EPA and DHA did not protect cells from the cytotoxic effects of TNFα and IFNγ exposure, although omega-3 fatty acids altered transcriptional regulation of necroptotic cell death in cells of an early and middle-stage CL. Moreover, we demonstrated that cells of a middle stage CL are less sensitive to the cytotoxic effects of TNFα and IFNγ, which may be due to reduced expression of *TNFRI*. Exposure to TNFα and IFNγ increased expression of

TNFα and *NFkB*, and reduced expression of steroidogenic genes *STARD1* and *CYP11A1*. With respect to our second postulated mechanism, we found no change in luteal blood flow 48 hrs following PGF2α exposures, as detected with power Doppler ultrasonography. However, we did find that FM-PGF2α infused cows had a significant reduction in luteal hyperemia when compared to the control supplementation, indicating FM supplementation may be altering the acute blood flow response. In conclusion, the differing micro-environments of each luteal stage pre-conditions cellular sensitivity to TNFα and IFNγ exposure. Moreover, FM supplementation may maintain functional CL by blunting the acute changes in luteal blood flow during luteal regression.

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CHAPTER I

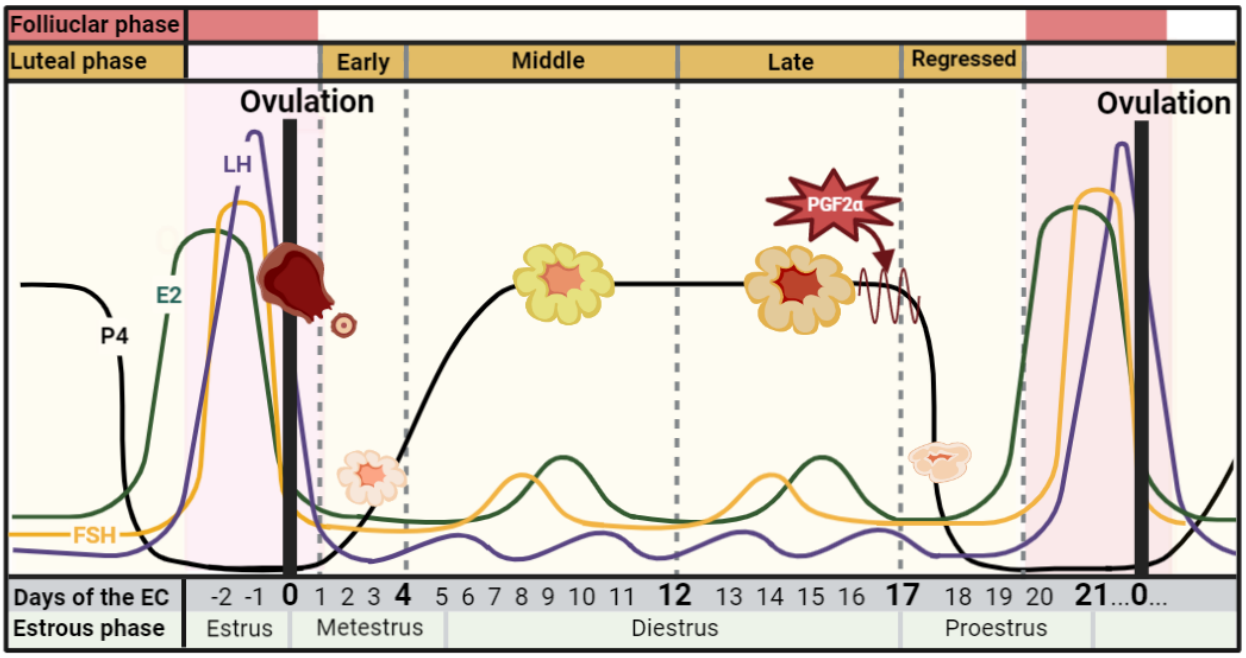
REVIEW OF THE LITERATURE

The Estrous Cycle

The estrous cycle (EC) of the female bovid (cow) encompasses the endocrine and physiological changes that occur from one bout of estrus to the next. In healthy, non-pregnant cattle, the length of the estrous cycle is approximately 18-24 days (Hammond, 1927). The function of the EC, like all reproductive cycles, is to provide the cow with an opportunity for pregnancy through the production and release of an oocyte. The EC is divided into four distinct behavioural phases: estrus (day 0), metestrus (day 1-5), diestrus (day 6-17), and proestrus (day 18-24; figure 1.1). The EC can further be divided into two subcategories with respect to the dominating ovarian structure; the follicular phase precedes when a dominant follicle is the primary ovarian structure (day 18-0 [-4 to 0] of the EC), and the luteal phase precedes when a corpus luteum (CL; to be further defined) is the primary ovarian structure (days 1-17 of the EC).

Figure 1.1

The Bovine Estrous Cycle



Note. Changes in endocrine hormones— luteinizing hormone (LH), Follicle stimulating hormone (FSH), estradiol (E2), and progesterone (P4)— during each phase of the estrous cycle. If no pregnancy is detected, prostaglandin F2 alpha (PGF2α) is released from the uterus and induces regression of the corpus luteum.

Estrus

Estrus— also termed standing estrus, standing heat, or heat—begins on day 0 of the EC and persists for approximately 12 hrs. Ovulation will occur approximately 24 to 32 hrs following onset of estrus. Estrus behaviour is stimulated by elevated circulation of the sex hormone estradiol. This hormone stimulates behavioural and physiological changes, namely: standing to be mounted (lordosis), mounting other cows, agitation, anorexia, increased vocalization, licking, mucus secretions from the vulva, vulva enlargement and increased rectal temperatures (Bane & Rajakoski, 1961).

The hypothalamic-pituitary-gonadal axis is an endocrine feedback loop responsible for the regulation of the EC. At the onset of estrus, a peak in circulating estradiol, derived from the

developing ovulatory follicle on the ovary, triggers a surge of gonadotropin-releasing hormone (GnRH) from the hypothalamus. Subsequently, a pre-ovulatory surge of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) is released from the anterior pituitary (Schoenemann et al., 1985; Turzillo & Nett, 1999). The pre-ovulatory LH surge targets the Graafian (dominant) follicle and initiates the process of ovulation: follicular edema and weakening of the follicular extracellular matrix. The resulting increase in pressure on the weakened follicular wall leads to a rupture of the pre-ovulatory follicle. The antral contents, including the oocyte are captured by the fimbriae of the infundibulum of the oviduct.

Metestrus

Metestrus begins following ovulation and is defined as days 1-5 of the EC. A small amount of uterine-derived blood can be observed exiting the vulva (metestrus bleeding) and residual vaginal mucus are often observed during this stage of the EC (Bane & Rajakoski, 1961; Weber et al., 1948).

The LH surge responsible for ovulation also initiates differentiation and re-organization of the theca, granulosa, endothelial, fibroblast, and immune cells of the ovulatory follicle. These cells will collectively form the CL. The development of the CL occurs during metestrus, wherein the gland rapidly gains the capacity to produce and secrete the sex steroid hormone progesterone. Progesterone is essential for the maintenance and establishment of pregnancy in the event the released ovum becomes fertilized.

During metestrus, GnRH, LH and FSH are released in a tonic release pattern (low amplitude and frequency of secretions). The secretion pattern allows for sufficient luteotropic (pro-CL survival) signal, thereby promoting normal CL function—namely steroidogenesis (Donaldson & Hansel, 1965; Summons & Hansel, 1964).

Diestrus

Diestrus is defined as days 6-17 of the EC. During this phase of the cycle, metestrus bleeding ceases, and hyperplasia and hypertrophy of the CL begins to plateau as the gland reaches full maturity.

Maximal production of progesterone is achieved by day 7-10 of the EC and is maintained until the end of diestrus, or late into pregnancy. Negative feedback of progesterone on the hypothalamus and anterior pituitary results in continued tonic secretion of LH and FSH during diestrus. However, pulses of LH shift to a low frequency/high amplitude pattern due to this negative feedback. This pulse pattern is essential to maintain progesterone production by the diestrus CL.

Proestrus

Proestrus is defined as approximately days 17-24 of the EC. Around day 17 of the EC, the lack of a maternally recognized pregnancy leads to pulsatile endometrial secretions of prostaglandin F2 alpha (PGF2α) from the uterus (Fortier et al., 1988). PGF2α synthesis and secretion initiates when luteal progesterone output is reduced as an outcome of progesterone receptor downregulation within the hypothalamus and endometrium. As a result, the progesterone block on uterine and follicular estradiol synthesis and secretion is removed (McCracken et al., 1999). The elevation in circulating estradiol, from the dominant follicle, induces increased expression of estrogen receptor alpha and subsequent transcriptional regulation in uterine epithelial cells (Bartol et al., 1981; Louis et al., 1977; Meyer et al., 1988; Roberts et al., 1976). Oxytocin G-protein receptor numbers increase due to the action of estradiol on uterine epithelial. Oxytocin, from the posterior pituitary, binds to its receptor leading to the activation of a complex intracellular signaling pathway allowing for the increase in activity of cytosolic

phospholipase A2. Once activated, phospholipase A2 cleaves arachidonic acid from the sn-2 position of membrane glycerophospholipids. Prostaglandin H2 synthase converts the free arachidonic acid to PGH2. The culmination of cell death and changes in the microenvironment of the CL during luteal regression reduces luteal progesterone output such that the hypothalamic surge of GnRH is no longer inhibited. Removal of the negative GnRH feedback generates sufficient FSH for the selection of secondary and eventually dominant follicle. The estrous cycle will then recommence as the dominating follicle produces adequate estradiol to induce the GnRH/LH surge required for ovulation, as previously mentioned. Proestrus can also be induced via administration of exogenous PGF2α after day 4 of the estrous cycle, which is commonly used for estrus synchronization (Inskeep, 1973; Odde, 1990).

Pregnancy

In the event the ovum does become fertilized, the estrus cycle will only be halted if the developing conceptus (embryo and associated extra-embryonic structures) successfully initiates a maternal recognition of pregnancy (MRP). The initiation of an MRP is triggered by trophoblastic-derived interferon tau (IFNτ; previously termed trophoblastic protein-1) secretion between days 16-24 post fertilization (Roberts et al., 1992). IFNτ inhibits estrogen receptor expression and oxytocin binding to endometrial epithelial cells, thereby blocking the pulsatile oxytocin-PGF2α cascade that would otherwise induce luteolysis (Arosh et al., 2004; Helmer et al., 1989; Mirando et al., 1993; Spencer et al., 1995). As a result, the CL is maintained and the progesterone secretion is continued, therein allowing for the establishment and further maintenance of the pregnancy. The luteal derived progesterone specifically targets the uterus, effectively aiding in implantation and embryonic support both directly, and indirectly through stimulation of histotrophic secretions—a culmination of ions and nutrients (Clemente et al.,

2009; Spencer et al., 2006). IFNτ may also support pregnancy via maternal immune cell modulation (Hansen et al., 2010).

Pregnancy Loss

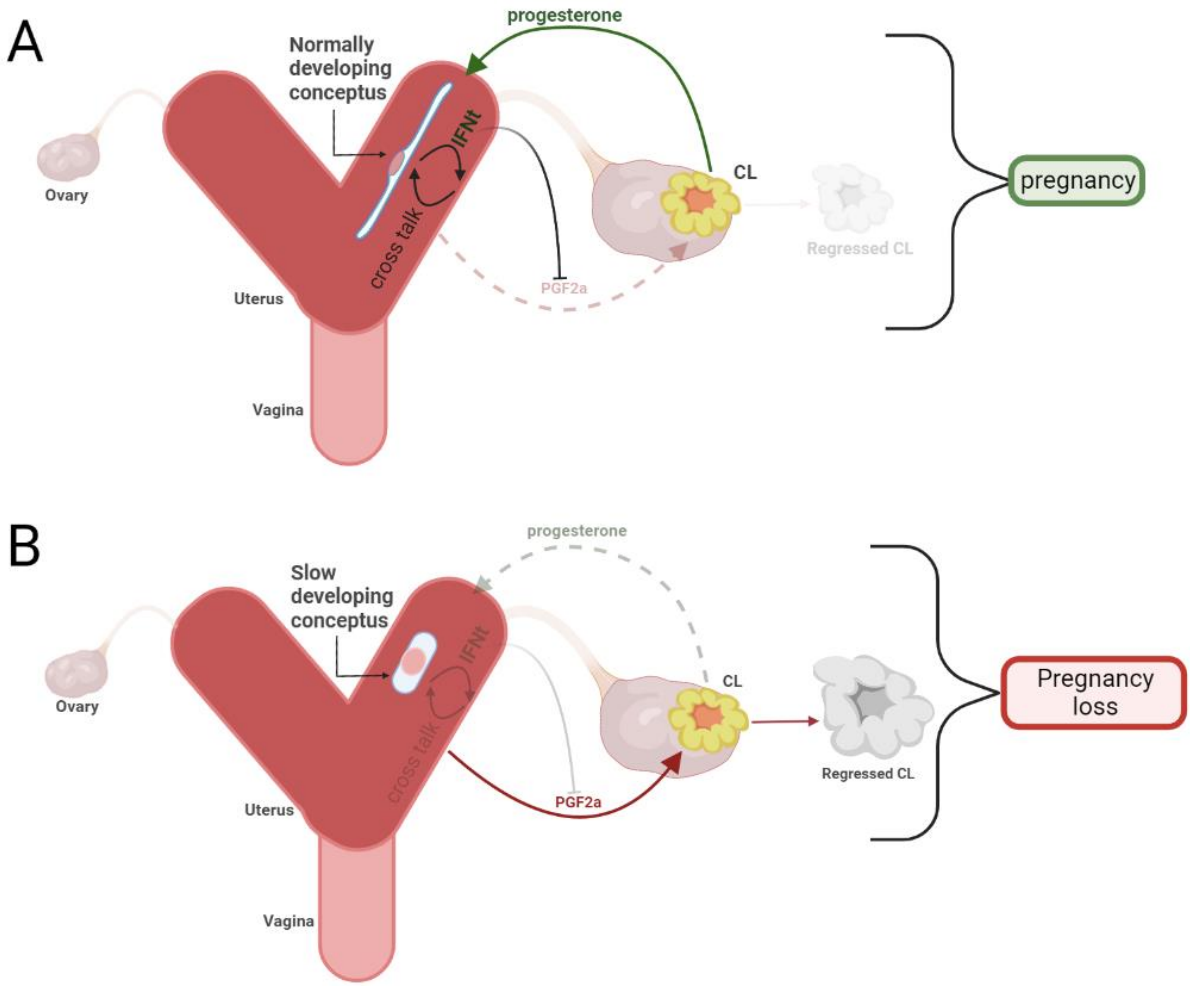
Pregnancy loss in the beef cattle industry continues to be problematic, wherein only 46% of artificial inseminations will result in a successful pregnancy (Reese et al., 2020). A report from the Organisation for Economic Co-operation and Development and the Food and Agricultural Organization (OECD/FAO) predicted that global beef and veal consumption will continue to rise (albeit at a slower rate compared to previous years) world-wide, predominantly in developing nations such as sub-Saharan Africa (2021). Therefore, increasing pregnancy rates is crucial to sustain demand. Moreover, improving pregnancy rates by just one percent equates to a 291-million-dollar economic boon to the industry (Davis & White, 2020). Additionally, findings derived from the pursuit of improved fertility and reproductive health in cattle have already provided key insights into human, female reproductive health (Langbeen et al., 2015; Malhi et al., 2005). As such, it is plausible that the resulting scientific findings from the pursuit of pregnancy interventions in cattle may further contribute to women's reproductive health.

Most pregnancy loss occurs in the first month of gestation, as reported in a recent meta-analysis (Reese et al., 2020). Within the first month of pregnancy, most pregnancy loss occurs before day 7 following fertilization (28.4%), followed by days 16-32 (15.6%; the critical window for a successful MRP), and finally days 7-16 (3.9%). Depending on the breeding method (natural vs artificial insemination), causes for the cessation of pregnancy vary and are specific to the time of gestation (Bellows & Short, 2021; Farin et al., 2006; Moore et al., 2021; Pope, 1988). However, independent of the breeding method utilized, within the vulnerable first 100 days of gestation (average 283-day gestational period) many critical biological processes must be

executed by the fetus and maternal environment, therein providing sources for potential failure. For example, one theorized cause for pregnancy loss between day 16-32 of gestation is due to a slower developing conceptus. Clearly, progesterone regulates development of the early conceptus and as such, a delay in initial progesterone secretion by the early CL leads to a slower-developing conceptus. As a result, the slower developing, albeit viable, conceptus may not secrete sufficient IFNτ to successfully establish an MRP (Godkin et al., 1997; Lafrance et al., 1989; Mann & Lamming, 2001; figure 1.2). Consequentially, the uterine-PGF2α release is no longer suppressed, resulting in luteolysis (luteal regression) and subsequent pregnancy loss (figure 1.2). Multiple interventions have been explored to inhibit the pulsatile secretions of PGF2α, namely the administration of IFNτ or PGF2α synthase inhibitors. However, these methods prove as only partially effective, are inefficient and labor intensive for long-term usage, are highly debated with regards to pregnancy success rates, or only prove beneficial for particularly agitated or abnormally presenting cattle (Besbaci et al., 2021; Geary et al., 2010; Jaśkowski et al., 2021; Karasahin et al., 2021; Merrill et al., 2007; Meyer et al., 1996; Purcell et al., 2005). Therefore, additional interventions and approaches for increasing pregnancy rates are warranted. One approach, which we explore later in more detail, may be reducing luteal sensitivity to PGF2α with use of fish meal dietary supplementation. In doing so, the slower developing conceptus would have a larger window of time to establish an MRP, and sufficiently inhibit PGF2α; therein improving pregnancy rates during days 16-32 of gestation.

Figure 1.2

Maternal Recognition of Pregnancy in a Normal vs Slow Developing Conceptus



Note. A) A normally developing conceptus, shown as a filamentous structure, secretes sufficient interferon-tau (IFNτ) to establish a successful cross talk, and an eventual maternal recognition of pregnancy. The secreted IFNτ effectively inhibits prostaglandin F2 alpha (PGF2α) release from the uterus, therein preserving the lifespan of the corpus luteum (CL). The CL will then continue to secrete progesterone, therein supporting the developing conceptus. B) A slow developing conceptus, shown as an oval-like structure, does not secrete sufficient IFNτ, therein allowing for PGF2α release from the uterus. The secreted PGF2α will then induce luteal regression, and prematurely terminate the pregnancy.

The Corpus Luteum

The term corpus luteum (Latin translation: bodies yellow) was coined by Marcello Malpighi in the early 1600s and further delineated by Regnier de Graaf in the late 1600s (Niswender et al., 2000). As previously described in brief, the CL is an ephemeral gland that is essential for the establishment and maintenance of pregnancy, the function of which was first established in rabbits in the early 1900s (Fraenkel & Cohn, 1901; Magnus, 1901). Following ovulation, the corpus luteum will swiftly develop from the remnants of the ovulatory follicle, and begin its primary function, the production of the sex steroid hormone progesterone. The influence of progesterone is multifactorial; the steroid acts as a luteotropic and pro-survival signal for the gland itself via paracrine signaling (Liszewska et al., 2005; Rekawiecki & Kotwica, 2007; Rekawiecki et al., 2005). The CL lifespan encompasses the luteal phase of the estrous cycle and can further be subdivided into four distinct stages: early, middle, late, and regressing CL. As the gland progresses through these luteal stages, considerable change occurs within the glandular microenvironment, vascularization and blood flow, cell types and phenotypes, immunological responses and regulation, and gross anatomy.

Early-Stage Corpus Luteum: Day 1-4 of the Estrous Cycle

The development of the early-stage CL begins following ovulation and is defined as day 1-4 of the EC (Ireland et al., 1980). The early-stage CL undergoes significant growth and neovascularization, along with extensive cellular infiltration and differentiation.

Gross Anatomy

As a result of the elevated mitogenic and neovascularization factors, luteal cell differentiation and steroidogenesis, and rapid tissue construction and remodeling, the gland undergoes a doubling time of 65 hrs; a trophic rate comparable only to the fastest-growing

tumors (Reynolds & Redmer, 1999). On day 4 of the EC, the glandular mass averages 0.95 g (Reynolds et al., 1994). The gross anatomy of the early CL is as follows: red exterior, non-epithelialized point of ovulation (i.e., where the crown will develop following epithelialization), cells are disorganized and loosely assembled, red/white interior, may contain a blood clot (name's sake of corpus hemorrhagicum, an early luteal structure), avascular, 0.5-1.5 cm in diameter, and the ovary surrounding the CL will have no follicles larger than 10 mm in diameter (Ireland et al., 1980). ***Vascularization and Blood Flow***

The early CL develops in a hypoxic environment, due to localized hemorrhaging resulting in reduced efficiency of gas exchange, decreased ovarian blood flow in the peri-ovulatory period, and lack of established vasculature supplying the readily dividing cells (Wise et al., 1982). Although oxygen perfusion to the developing CL has not been established, hypoxia-inducible factor 1 is highly expressed in the early developing CL (Nishimura & Okuda, 2010). The hypoxic environment is essential for the formation of new (vasculogenesis), and extension of previous (angiogenesis), vasculature to the differentiating gland (Nishimura & Okuda, 2010). Directly before ovulation, and (or) following the LH surge, mass neovascularization (angiogenesis and vasculogenesis) occurs in the ruptured follicle due to the potent angiogenic and mitogenic gene and protein expression; fibroblast growth factor and receptor, vascular endothelial growth factor (VEGFA), VEGFA receptors (VEGFR), matrix metalloproteinases, and tissue plasmogen activator (Berisha et al., 2000, 2006, 2008; Robinson et al., 2007; Woad & Robinson, 2016). Woad and Robinson (2016) review a more comprehensive list of the reported elevated neovascularization factors. Some anti-angiogenic factors are prevalent during luteal development, though most likely play a role in the optimization and remodeling of the vasculature (Woad & Robinson, 2016). Cells associated with the vasculature (endothelial cells,

pericytes, and vascular smooth muscle cells) account for 85% of all cellular divisions in the early CL (Reynolds & Redmer, 1999). The increase in matrix metalloproteinase (a result of the LH surge) results in degradation of the follicular basement membrane allowing for the migration of endothelial cells and pericytes into the developing CL. The number of capillaries per cross-sectional area of the CL is the largest in an early and middle-stage CL (Hünigen et al., 2008). As a result of the metabolic demands from the extensive remodeling, most of the blood flow to the CL-bearing ovary is allocated to the CL, as demonstrated in sheep (Niswender et al., 1976). Major blood supply to the CL-containing ovary comes from the ovarian artery and the ipsilateral uterine artery (Ford & Chenault, 1981). In cattle, by day 4 of the EC, the area of blood flow to the CL already comprises 15% of the gland, which is comparable to a middle-cycle CL (Acosta et al., 2002).

Cellular Composition

Concurrent with the rapid neovascularization, the two predominant steroidogenic cell types of the ovulated follicle, theca and granulosa, undergo LH-stimulated differentiation (luteinization) into small and large luteal cells, respectively. In the dominant follicle, the theca and granulosa cells primarily produced androgens and estradiol, respectively, from unesterified cholesterol, the precursor to all steroid hormones. However, following luteinization, aromatase activity in granulosa cells is inhibited, therein preventing estradiol synthesis (LaVoie, 2016). Additionally, the LH surge further increases mRNA expression and activation of enzymes involved in progesterone synthesis, namely, steroidogenic acute regulatory protein (STAR; encoded from *STARD1*), cholesterol side-chain cleavage enzyme (P450scc; encoded from *CYP11A1*), and 3-beta-hydroxysteroid dehydrogenase (3β-HSD; encoded from *3β-HSD*) as reviewed by King and LaVoie, (2012). Finally, during luteinization, the large luteal cells gain the

ability to synthesize an abundance of progesterone, de-novo, and the small luteal cells maintain their ability to do so from their thecal origin (LaVoie, 2016). Another cell type in the early-stage CL includes fibroblasts, which infiltrate the developing CL and aid in the establishment of the gland (Melmed et al., 2015).

Progesterone Output

Progesterone synthesis and secretion from the large and small luteal cells begin as early as day 1 of the EC, and steadily increases into the next stage of the CL; the middle phase CL (Robinson et al., 2005). By day 4 of the EC, the average blood progesterone concentration reaches 1 ug/g in luteal tissue and 0.6 ng/ml in serum (Tsai & Wiltbank, 1998).

Immunological Regulation

Immune cells play a significant role in early luteal function. Following the LH surge, immune cells begin to infiltrate the ovary and follicular region due to elevated chemokine secretions and elevated expression of immune cell-attracting ligands (adhesion molecules and selectins) on endothelial cells (Rohm et al., 2002; Walusimbi & Pate, 2013). A study demonstrated that in mice, $F4/80^{+}$ macrophages infiltrate from the bone marrow during luteal development (Kizuka et al., 2012). Moreover, in rats, $CD11b/c^{+}$ macrophages and $CD3^{+}$ lymphocytes infiltrate from the spleen (Oakley et al., 2010). An elevated presence of interleukin 8 (IL-8; a neutrophil chemoattractant and pro-angiogenic cytokine) accounts for the prevalence of polymorphonuclear neutrophils in the early (1-4 of the EC) CL (Jiemtaweeboon et al., 2011). *In vitro* studies demonstrated that IL-8 promotes luteal-derived endothelial cell proliferation and augments progesterone output in luteinized granulosa cells, indicating a potential role of the cytokine in angiogenesis and steroidogenesis during luteal development (Shimizu et al., 2012). In the developing bovine CL, T cell populations are nearly undetectable, and begin to increase

only once the gland is established in the middle CL phase (Bauer et al., 2001). The role of T cells in luteal development is not well established, though in certain species (no evidence in bovine), T lymphocytes increased progesterone production in granulosa-luteinized cells—indicating a potential role in steroidogenesis later in development (Walusimbi & Pate, 2013). Furthermore, macrophage-monocytes, eosinophils, and MHC II expression are all elevated in the early CL on day 1-5 of the EC (Penny et al., 1999; Reibiger & Spanel-Borowski, 2000). Macrophages are theorized to transition to an M2- type macrophage in the developing CL, in addition to N2- type neutrophils—both of which are typically located in wound healing and tissue repair environments, thereby aiding in the process of angiogenesis, and are indicative of a more anti-inflammatory environment (Shirasuna & Miyamoto, 2016). The precise role eosinophils play in the early CL is not entirely clear, though evidence suggests a potential role in supporting steroidogenesis, as inhibition of eosinophil infiltration reduced progesterone output (Kliem et al., 2013). Additionally, regulatory cytokines (secreted by local leukocytes and luteal cells) such as tumor necrosis factor-alpha (TNFα), interferon-gamma IFNγ), and interleukin-1 beta (Il-1β) are all expressed in the early CL, along with later luteal stages (Petroff et al., 1999; Sakumoto et al., 2000; Terranova & Rice, 1997). Pate et al. (2010) and Shirasuna and Miyamoto (2016) further explore cytokines and their roles in the early CL. While it is apparent immune regulation is heavily intertwined with the process of neovascularization, much is to be explored.

Middle Stage Corpus Luteum:
Day 5-12 of the Estrous
Cycle

The middle stage CL is defined as day 5-12 of the EC and is a transitionary phase wherein luteal blood supply plateaus (despite continuous angiogenesis), cellular infiltration and cellular differentiation begin to slow, and cellular trophy and steroidogenesis become the

primary functions. Additionally, the proliferation of all luteal-located cells decreases, except for cells associated with vascularization, which continue to increase.

Gross Anatomy

On day 8 of the EC, the gland averages 3.41 g (Reynolds et al., 1994). The gross anatomy of the middle CL is as follows: the point of rupture (crown) is fully epithelialized, is red or brown in color both internally and externally while the body of the gland is orange, vasculature mostly encompasses the periphery of the gland, the CL diameter ranges from 1.6-2 cm, and ovarian follicles greater than 10 mm in diameter will be present (Ireland et al., 1980).

Vasculature and Blood Flow

From day 1-12 of the EC, the CL vasculature increases roughly seven-fold, despite a lack of significant change in the total area of glandular blood flow from day 4 of the EC (Acosta et al., 2002). The absence of a quantified change in luteal blood flow, despite rapidly increasing progesterone levels, and increasing abundance of arterioles may be because Doppler ultrasonography is not sensitive enough to pick up changes in capillary blood flow, as evident by pseudo coloration only displayed on the periphery of the gland, despite the extensive intra luteal capillary network in the middle CL (Hünigen et al., 2008). The number of capillaries per cross-sectional area of the CL is largest in a mature CL on day 8-17 EC (Hünigen et al., 2008). Additionally, extensive angiogenesis continues in the middle CL stage, as evident by the elevated rate of apoptosis in middle CL endothelial cells (Hünigen et al., 2008).

Cellular Composition

While the glandular vascularization continues to expand, by the end of the middle (entering the late phase) luteal phase, luteal cell composition establishes a ratio of 4% large luteal cells (38 um diameter), 27% small luteal cells (17 um diameter), 53% endothelial cells and

pericytes (11 um diameter), 6% fibroblasts (15 um diameter) and 2% immune cells (O'shea et al., 1989). The remaining 8% of space is allocated to vessel lumens and intracellular space. While large luteal cells make up the smallest proportion of total glandular cells, their large size occupies 40% of the volume, and small luteal cells occupy 28% of the volume. In the middle CL, luteal cells increase LH (predominantly in small luteal cells) and non-LH (predominantly in large luteal cells) stimulated progesterone output, and large luteal cells continue to increase in size (Robinson et al., 2006). A genome-wide mRNA expression analysis uncovered potential roles of small and large luteal cells branching beyond the classical function of steroidogenesis, effectively underlining the necessity for exploring the physiology of luteal cells in addition to steroidogenesis (Baddela et al., 2018). Notably in small luteal cells, genes associated with extracellular matrix construction and recruitment of immune cells were elevated, whereas, in large luteal cells, mitochondrial metabolism and luteal regression associate pathways were elevated.

Progesterone Output

Progesterone output reaches heightened concentrations on day 7-12 of the EC (4 ng/ml serum; day 11 of the EC), which is equivalent to $3.41x10^{17}$ molecules of progesterone per minute in the average middle CL (Robinson et al., 2005; Tsai & Wiltbank, 1998). Progesterone output from the corpus luteum is required to maintain pregnancy for the first 215 days of gestation, wherein the placenta and adrenal glands will take over progesterone synthesis, or until luteal regression (Wendorf et al., 1983).

Immunological Regulation

The immune phenotype continues to shift in the middle CL compared to the early CL. Both macrophage and monocyte populations are elevated in the middle CL when compared to

early CL—and do not significantly differ in a late CL (Townson et al., 2002). The T lymphocyte population is comprised of 25% $CD4^+$ T helper cells, 45% $CD8^+$ cytotoxic T cells, and 30% gamma delta T cells. Of the three T cell types, the gamma delta T cell population was the only one activated in cell culture by middle-stage CL, though their role in tissue management is unclear, as both IFNγ and interleukin 10 (apposing pro- and anti-inflammatory molecules, respectively) were secreted (Davis & Pate, 2007). However, more recently, gamma delta T cells are proposed to be regulatory in the middle CL, as only IL-10 mRNA was present in middle phase luteal cells, and IFNγ was downregulated (Walusimbi & Pate, 2014). The immune phenotype of a middle CL is theorized to be regulatory, due to the loss of regulatory cells during luteal regression (Poole & Pate., 2009). Additionally, progesterone has been postulated to play an immunosuppressive role in the middle CL, due to the presence of progesterone receptors in luteal T cells (Ndiaye et al., 2012). Messenger RNA transcripts for select cytokines and their receptors (TNFα, TNFR1, TNFR2, IFNγ) are all present, though are non-differentially expressed when compared to other CL stages (Petroff et al., 1999; Sakumoto et al., 2011). The role of IFNγ and TNFα in the middle CL has not been made apparent, as the middle CL is generally regarded as an anti-inflammatory environment once established.

Late-Stage Corpus Luteum: Day 13-17 of the Estrous Cycle

The late CL is defined here as day 13-17 of the EC. The late luteal stage is a determining phase for the CL, as life or death depends on the success of the MRP, as previously discussed. While the fate of the gland teeters, minimal documented changes occur in the vasculature and blood flow from the middle to the late luteal stage (save for gross vesicle organization) or changes to luteal cell types and steroidogenic capacity, and so will not be discussed as different from the middle stage CL.

Gross Anatomy

On day 14 of the EC, the gland averages 4.45 g, therein reaching the maximal mass (Reynolds et al., 1994). The gross anatomy of the late CL is as follows: the crown of the gland is epithelialized, external color is tan or orange, internal color is orange, diameter ranges from 1.6-2 cm (consistent with the middle CL), vasculature jackets the crown while maintaining the peripheral arterioles observed in the middle CL, and follicles larger than 10 mm in diameter may or may not be present (Ireland et al., 1980).

Immunological Regulation

Within late CL, $CD5^+$ B-cells (which are prevalent during all luteal stages) were reported as the most prevalent immune cell, followed by monocyte and macrophage (Penny et al., 1999). The latter two cell types do not significantly differ from middle-stage CL but do from early CL but the $CD5^+$ B-cells nearly triple in abundance in the late CL (Penny et al., 1999; Townson et al., 2002). Protein expression for the cytokine TNFα is highest during this stage, yet TNFR1 receptor mRNA expression is at its lowest or is unchanged relative to the early and middle CL (Friedman et al., 2000; Sakumoto et al., 2000). Interestingly, subsets of luteal cells begin to express class II MHC markers during the late-stage CL, which is conversely dampened by pregnancy or a pregnancy-like environment (Benyo et al., 1991; Pate, 1995; Penny et al., 1999). Although class II MHC markers are typically reserved for professional antigen-presenting cells (i.e., B-cells, Macrophages, dendritic cells), the presence of luteal cells may indicate a mechanism enhancing the specificity of the T cell response required for luteal regression (Pate et al., 2010; Petroff et al., 1997).

Regressing Corpus Luteum: Day 18-21 of the Estrous Cycle

Luteal regression is temporally defined as day 18 up until day 24 of the EC. In the event of an unsuccessful MRP, the corpus luteum will be targeted for regression, commencing with uterine PGF2α release delivered to the CL via the uterine vein and artery countercurrent network. The mechanisms by which PGF2α is released from the uterus have previously been discussed. How PGF2α influences luteolysis will be reviewed in this section.

Gross Anatomy

On day 18 of the EC, the gland averages 4.12 g and will continuously decrease in mass until the formation of the fibrous corpus albicans (Reynolds et al., 1994). The gross anatomy of the regressing CL includes epithelization of the crown, light yellow to white external, orange to yellow internal pigment, avascular, less than 1 cm in diameter (and progressively decreasing), and follicles larger than 10 mm in diameter will be present on the ovary (Ireland et al., 1980).

Structural vs Functional Regression

Luteal regression is further analyzed with respect to functional or structural regression. Functional regression precedes structural regression and consists of a rapid decrease in serum progesterone below 1 ng/ml, without evidence of rebounding. In cattle, serum progesterone decreases as early as four hrs following exogenous PGF2α exposure (Tsai & Wiltbank, 1998). Gene and protein expression for molecules involved in steroidogeneses such as STARD1/STAR and 3β-HSD are reduced during luteal regression and most likely contribute, in part, to the loss of steroidogenic capacity (Juengel et al., 1998; Pescador et al., 1996). Moreover, structural regression defines the loss in cellular viability and subsequent decline in glandular mass. The CL may begin to structurally regress as early as 8 hrs following exposure to exogenous PGF2α (Acosta et al., 2002), and will continue to do so for multiple days, well into the following estrous

cycle. Only a white scar (corpus albicans) will remain as an indication of the gland's once-lived glory.

Prostaglandin F2 Alpha: The Endogenous Luteolysin

It has been well established that PGF2α is the luteolytic agent in ruminants, as reviewed by Meidan et al. (2016) and Bishop et al. (2022). However, the processes occurring between uterine-PGF2α release and luteal regression are continuously being investigated. Despite PGF2α receptor expression on small and large luteal cells and endothelial cells, the cellular outcomes of ligand-receptor binding are far more convoluted than just direct inhibition of steroidogenesis and cell death (Mamluk et al., 1998). For example, *in vitro* culture of luteal cells exposed to PGF2α generates contradictory results depending on the composition of the culture. Some studies demonstrated reduced progesterone output and cell viability following PGF2α exposure, as was expected given the luteolytic capabilities of PGF2α, and yet other laboratories report an increase in progesterone output and cell viability, as reviewed by Meidan et al. (2016). Some laboratories report that by co-culturing endothelial cells, steroidogenic luteal cells, and immune cells, the expected progesterone knockdown was observed (Girsh et al., 1995; Korzekwa et al., 2008). As a result of such findings, it was theorized that PGF2α, in the appropriate microenvironment, illicit changes in gene expression and cellular outputs (i.e., elevated secretion of pro-inflammatory cytokines) that in turn alter steroidogenesis and cellular viability (Meidan et al., 2016). In other words, PGF2α is merely an initiator of luteal regression, by which the complete abrogation of the gland would not be possible without the collective changes in all luteal cell types. This is concurrent with other theories, outlined and summarized by Talbott et al. (2017), suggesting that PGF2α activates a cascade of alterations in CL gene expression, changes in immune cell phenotypes, and cytokine expression and release, resulting in a knockdown in

steroidogenesis and mass cellular death; all events culminating in luteal demise. In addition, a PGF2α-induced rapid decline in luteal blood flow and loss of vascularization is a well-supported contributor to luteal regression (Nett et al., 1976). Despite some aspects of luteal regression being well-defined and robustly supported by independent works, the true order and complexity of proceedings remains obscure due to the assumed complexity of the event.

Changes in Vasculature and Blood Flow

Alterations in luteal vasculature and blood flow collectively represent key physiological changes that occur during regression. During early luteal regression (day 18-21 of the EC), all cell types undergo similar rates of apoptosis until late regression (day 22-24 of the EC), wherein luteal cells are targeted for cell death more rapidly and robustly than the vasculature (Hünigen et al., 2008). The rate of apoptotic endothelial and luteal cells is highest in a regressing CL (Hünigen et al., 2008). During luteolysis in other species, VEGFA and VEGFR are reduced (Fraser et al., 2005). However, Hünigen et al. (2008) demonstrated that VEGFA and VEGFR were elevated in smooth muscle cells in a late-regressing CL; aligning with a previous finding that a late-regressing CL is predominantly composed of endothelial networks. Additionally, angiotensin 2, which plays a role in vasculature destabilization, was elevated during early regression (Hünigen et al., 2008; Yancopoulos et al., 2000). In accompaniment to the changes in vasculature, alterations in luteal blood flow similarly occur. Following both induced and natural luteolysis, the percentage of luteal blood flow (percent of luteal area) doubles 30 mins following exogenous PGF2α exposure, or during the time of natural uterine-PGF2α release on day 16-17 of the EC (Acosta et al., 2002; Miyamoto et al., 2005). The area of luteal blood flow then drops steadily after the acute surge until the time of regression (Acosta et al., 2002; Miyamoto et al., 2005). Additionally, the time-averaged maximum velocity of a main luteal artery did not

increase, and instead steadily decreased over 24 hrs (Acosta et al., 2002). Furthermore, in sheep, decreased ovarian blood flow coupled with CL-arterial shunting and decreased vasculature occurs during induced and natural luteolysis (Niswender et al., 1976).

While much remains to be explored, some mechanisms and molecules involved in the vascular control of the regressing gland have been identified in ruminants. Endothelial nitric oxide synthase and inducible nitric oxide synthase, both enzymes that synthesize the potent vasodilator nitric oxide, are elevated in luteal tissue during the late CL stage and early luteal regression (Skarzynski et al., 2003a). Inhibition of nitric oxide synthase inhibited the luteolytic capacity of PGF2α, indicating the importance of nitric oxide in luteolysis (Skarzynski et al., 2003b). Interestingly, a middle CL exposed to nitric oxide undergoes a very similar acute increase in luteal blood flow (percent area of blood flow) followed by a dramatic decline, as observed following PGF2α administration (Shirasuna et al., 2008). Shirasuna et al. also demonstrated that administration of NO lead to functional luteolysis just over an hour following exposure (2008). A review by Miyamoto et al. (2005) summarized that mRNA transcripts and proteins of potent vasoconstrictive factors and respective receptors (endothelin-1, endothelin type B receptor, angiotensin-converting enzyme, and angiotensin II) were all elevated at different time points within 0.5-24 hrs following PGF2α administration. Findings were similar in cows with a naturally regressing CL (Miyamoto et al., 2005). Additional mechanisms by which PGF2α may induce changes in luteal blood flow include the early degradation of capillaries and endothelial cells during the time of luteolysis. How and why the CL would undergo an acute increase in blood flow, when destabilization of the vasculature has already begun, remains unclear. It is worth noting that while Doppler ultrasonography remains a powerful tool, it may not have the capacity to detect small changes in centrally located capillaries, as no group has

visually displayed central glandular blood flow to the expect degree given capillary density. It therefore remains plausible that blood flow is not maintained at the level of the central capillaries due to capillary destabilization. Evidently, much is to be explored. Taken together, elevated nitric oxide may be the cause for the acute increase in luteal blood flow, followed by elevation in vasoconstrictive factors and cell death in vasculature cells that collaboratively reduce glandular blood flow.

An additional theory involves a pooling effect, where PGF2α initially acts as a vasoconstrictor, therein clamping off blood supply to the gland and displaying an apparent increase in blood flow, as per the increase in the percentage of luteal blood flow following PGF2α exposure (Acosta et al., 2002). Such an event may expedite a pro-inflammatory and pro-death environment required for regression, as does occur in any tissue deprived of blood profusion (i.e., ischemic events). This theory is based upon two notions: 1) PGF2α in other systems is a known vasoconstrictor and 2) timed average flow velocity of main luteal arteries steadily decreases following administration of PGF2α, despite an increase in percentage area of luteal blood flow (Still & Greiss, 1978; Yamamoto et al., 1972). However, this discrepancy in timed average flow velocity could be due to vasodilation (nitric oxide induce) and increased profusion. Regardless of the mechanism at play, the reduction in glandular blood flow may be limiting the supply of cholesterol to the CL, therein reducing glandular capacity for progesterone synthesis, as cholesterol is primarily supplied to luteal cells through the blood. Additionally, the loss or reduction of nutrient and gas exchange to any tissue causes extreme cellular stress, therein amplifying the pro-death environment of regression. We propose that the maintenance of luteal blood following exposure to low-dose intra-uterine infusions of PGF2α may be a mechanism by which the CL remains resistant to the induction of luteal regression. Although the reduction in

blood flow to an early-developing CL does not influence progesterone output, the degree and speed by which blood flow declines during regression may be more harmful to cells and the process of steroidogenesis (Robinson et al., 2006). Additionally, a reduction in luteal blood flow is always accompanied by a reduction in blood progesterone in regressing CLs, though whether that is a result of loss in blood flow versus repression of steroidogenesis by the changing luteal environment (or both) has yet to be defined (Ford & Chenault, 1981).

Immunological Regulation

During both natural and induced luteolysis, immune cell infiltration begins as early as five mins post-infusion with the predominate cell types being neutrophils and macrophages (Penny et al., 1999; Shirasuna et al., 2012; Townson et al., 2002). P-selectin activity is enhanced following exposure to PGF2α in endothelial cells, indicating increased capability for leukocyte attachment and eventual diapedesis during luteal regression (Mamluk et al., 1998). Broadly, leukocyte proliferation increases 3.5-fold (20%-70%) from a middle to regressing CL (Bauer et al., 2001). The immune profile also shifts during early regression, leading to a significant increase in $CD2^+$ T cells and natural killer cells, $CD8^+$ cytotoxic T-cells, $CD5^+$ B cells, $CD4^+$ helper T cells $CD14^+$ monocytes and macrophages cells (Bauer et al., 2001; Penny et al., 1999; Smith & Meidan, 2014; Townson et al., 2002). During late regression, the previously mentioned cell types increase in abundance, as well as $CD3^+$ T cells, and $CD18^+$ leukocytes; all of which are present in a pro-inflammatory tissue phenotype (Penny et al., 1999). In culture, luteal cells previously exposed to PGF2α elicit greater T cell proliferation than those not exposed to PGF2α (Cannon & Pate., 2003). However, multiple research groups have found no change in either $CD8^+$ cytotoxic T cells or $CD4^+$ helper T cells during regression (Penny et al., 1999; Townson et al., 2002). The regulatory T cell marker, Foxp3, is downregulated during regression, along with a

shift in gamma delta T cells from WC1- (pro-regulatory) to $WC1^{+}$ (pro-inflammatory) (Cannon & Pate, 2003; Hedges et al., 2003; Meissner et al., 2003; Walusimbi & Pate, 2014). Additionally, populations of luteal cells express class II MHC molecules during regression, which like the late-stage CL, may be an attempt to amplify the T cell response (Benyo et al., 1991; Petroff et al., 1997). Changes in cytokine expression also represent a pro-inflammatory environment during regression (Talbott et al., 2017). Expression of monocyte chemoattractant protein-1 mRNA, which is known to play a role in monocyte, macrophage, and T lymphocyte recruitment, was highest during regression when compared to an early CL (Kliem et al., 2009; Townson et al., 2002). Expression of TNFα protein was greatest in the regressing CL, following the drop in progesterone output (Shaw & Britt, 1995). Additionally, following induced regression, TNFα,TNFR1, IL-1β, IFNγ, and interleukin 8 mRNA transcripts were all elevated (Friedman et al., 2000; Jiemtaweeboon et al., 2011; Neuvians et al., 2004). Pate et al. propose that immune cells may have one of two roles during luteal regression (Pate et al., 2012; Pate & Keyes, 2001). The first hypothesis, and the more generally supported hypothesis in the literature, proposes that immune cells and cytokines aid in the reduction of steroidogenesis and promote cell death, therein propagating luteal demise. The second hypothesis states that immune cells have a regulatory role to ensure the remaining luteal cells are continuously supported and nurtured until induction of death. A third hypothesis would entail both proposed mechanisms, wherein a population of immune cells propagate cell death, while another regulates the degree of the inflammatory event to ensure a timely resolution.

Types of Programmed Cell Death

Extensive cellular death occurs during luteal regression, as previously discussed. The most abundantly reported form of programmed cell death during luteal regression includes

apoptosis. Apoptosis signaling can occur through two primary routes: internal and (or) external, both of which are caspase-dependent mechanisms (Cohen, 1997). When the intracellular environment is no longer optimal for cell survival (i.e., unrepairable DNA damage or hypoxia) the intrinsic apoptotic pathway will be activated. In the event of DNA damage, stabilization of the tumour suppressive protein P53 activates BCL2 Associated X (BAX) and other pro-apoptotic factors that propagate cytochrome C release from the mitochondria. B-cell lymphoma 2 (BCL-2) is typically present in a pro-survival cell, inhibiting cytochrome C release, but is overridden and suppressed by prominent upstream apoptotic signals. Cytochrome C, apoptotic peptidase activating factor 1 (apaf-1) and caspase 8 (Casp8) together form the apoptosome, which cleaves the effector caspase 3 (Casp3). Caspase 3 then actuates caspase-activated DNase (CAD), which in turn fragments DNA, a hallmark of apoptosis. The extrinsic apoptotic pathway occurs when the extracellular environment of a cell targets it for cellular death. The pathway begins when a peripheral cell (commonly a killer lymphocyte) binds an FS-7-associated surface (FAS) antigen to the FAS ligand (FAS L) on the recipient cell. The binding event recruits a FAS-associated death domain (FADD) which recruits and activates caspase 8, which in turn galvanizes the executor caspases (Casp3, 6 and 7). A similar series of events can occur upon TNF-related apoptosis-inducing ligand (TRAIL) binding to death receptor 4/5, or TNFα binding to TNFR1 and subsequent recruitment of tumor necrosis factor receptor type 1-associated death domain (TRADD). Pro-apoptotic (intrinsic and extrinsic pathways) and inflammatory factors such as Fas L, Fas, P53, BAX, TNFα, and IFNγ protein and mRNA expression are all significantly elevated following PGF2α administration (Jonczyk et al., 2019; Kim et al., 2019; Kliem et al., 2009). However, one research group found no significant difference in TNFα mRNA expression throughout the luteal phases and found no difference in TNFR1 expression in the late and

regressing CL (Sakumoto et al., 2000). The lack of differential expression could, in theory, be due to a shifting phenotype that prepares a CL for regression by synthesizing and storing receptors in Golgi-associated vesicles for later exocytosis if no pregnancy is detected, therein expediting time until the following ovulation. Anti-apoptotic and pro-survival mRNA transcripts are also significantly elevated, such as TNFR2 and BCL-extra-large (BCL-XL) (Kliem et al., 2009; Sakumoto et al., 2011). and may be attributed to cellular efforts to stave off cell death, and (or) an effort to control the pace of cell death to ensure it remains a controlled, localized event. One group proposes that the external apoptotic pathway is the primary route of apoptosis during luteal regression (Kliem et al., 2009). Additionally, it has been postulated that luteal regression causes a localized elevation in reactive oxygen species (ROS) that in turn further propagates luteal regression by way of increased cell death (namely apoptosis) and reduced steroidogenesis (Kato et al., 1997). A ROS is a molecule with a free radical-containing oxygen, which when accumulated in abundance, can cause irreparable damage to genetic material and proteins therein triggering programmed cell death. In multiple species, including bovine, concentrations of luteal ascorbate (the primary antioxidant in the CL) plummet following PGF2α exposure or during natural luteolysis (Aten et al., 1992; Endo et al., 1993; Petroff et al., 1998; Tsai & Wiltbank, 1998). Interestingly, pre-treatment of a potent ROS scavenger (superoxide dismutase/catalase) in luteal cell culture following exposure to cytotoxic doses of TNFα and IFNγ significantly reduced loss in cell viability, furthering the notion that TNFα and IFNγ stimulate cell death by way of ROS production (Petroff et al., 2001).

Evidence that an additional form of programmed cell death, necroptosis, occurs during luteal regression has begun to emerge (Jonczyk et al., 2019). Necroptosis, like almost all cellular signaling, is a complex network involving multiple inputs and outputs that depend on external

stimuli and the internal cellular environment. Necroptotic signaling can be instigated through multiple plasma membrane receptors, namely toll-like receptors, death receptors, and TNFR1. A well-established pathway includes the TNFR1, wherein ligation via TNFα recruits a TNF-receptor-associated death domain (Galluzzi et al., 2017; Linkermann & Green, 2014). Through a series of ubiquitination and deubiquitinating steps, receptor-interacting protein kinase 1 (RIPK1) transforms from a pro-survival to a pro-death activated state. A pro-death activated RIPK1, in combination with inhibited caspase 8 and (or) FLICE inhibitory proteins (FLIP), activates and associates with receptor-interacting protein kinase 3 (RIPK3) to form the necrosome. The necrosome will then target and activate mixed lineage kinase domain-like (MLKL) protein, which instigates an influx of calcium ions, effectively disrupting the osmotic gradient and lysing the cell (Gong et al., 2017). The rupture of the cell resembles the phenotypic damage of necrosis. Hojo et al. (2019) demonstrated that TNFα and IFNγ synergistically increased cell death via necroptotic signaling in bovine endothelial cells. Since both TNFα and IFNγ are elevated during luteal regression, it is plausible that through TNFR1 signaling, necroptosis occurs in bovine luteal cells.

Progesterone Biosynthesis

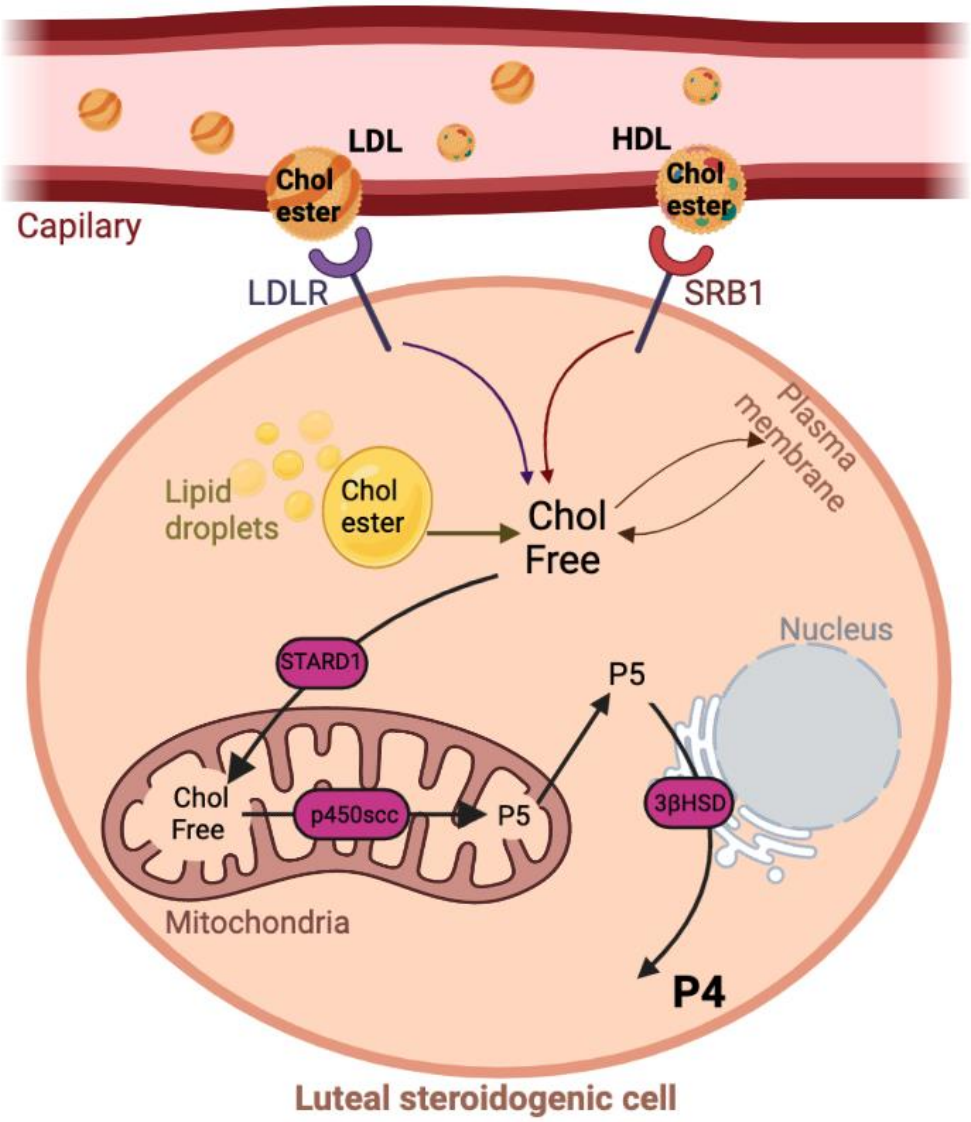
Progesterone synthesis in small and large luteal steroidogenic cells beings with the precursor cholesterol. Steroidogenic cells utilize esterified cholesterol from circulating low-density lipoproteins via the low-density lipoprotein receptor or from high-density lipoproteins via the scavenger receptor B1 as the primary source of cholesterol (Miller & Bose, 2011). Cells can also isolate cholesterol from lipid bilayers of biological membranes or esterified cholesterol from lipid droplets (Steck & Lange, 2010). Esterified cholesterol is removed from lipid droplets via hormone-sensitive lipase and subsequently converted to free cholesterol (Miller & Bose,

2011). While it remains the least common source, most likely due to the inefficiency, cells can synthesize cholesterol *de novo* from acetate in the endoplasmic reticulum (Porter & Herman, 2011; Steck & Lange, 2010).

Progesterone synthesis begins with free, unesterified cholesterol, which is transported across the mitochondrial membranes. While the detailed mechanisms of transport are unknown, STAR plays a pivotal role in the mitochondrial transport of cholesterol, which serves as the rate-limiting step in the enzymatic synthesis of progesterone. Additionally, studies have demonstrated that lipid droplets may be trafficked and doc with the mitochondria to facilitate cholesterol transport (Miller & Bose, 2011). Within the inner mitochondria membrane, P450scc enzymatically converts cholesterol to pregnanolone through cleavage of the 6-carbon acyl side chain of cholesterol (Miller & Bose, 2011). Pregnanolone is then shuttled out into the cytosol by an unknown mechanism. Within the cytosol or the endoplasmic reticulum, pregnanolone is converted to progesterone by way of an alkene rearrangement from carbon 5 to carbon 4, catalyzed by 3β-HSD. A summary of the steroidogenic pathway can be visualized in figure 1.3. Miller and Bose (2011) and Bishop et al. (2022) provide a more extensive review of proteins and pathways involved in cholesterol trafficking and progesterone synthesis.

Figure 1.3

Progesterone Production in Bovine Luteal Steroidogenic Cells



Note. Luteal **s**teroidogenic cells sequester cholesterol esters (Chol ester) from low density lipoproteins (LDL) and high-density lipoproteins (HDL) circulating in blood, from lipid droplets, the plasma membrane, or de novo from the endoplasmic reticulum (not shown). Free, unesterified cholesterol (Chol free) is transported into the mitochondria by steroidogenic acute regulatory protein (STARD1), where it is converted to pregnenolone (P5) by cholesterol side-chain cleavage enzyme (p450scc). P5 is then converted in the endoplasmic reticulum (or cytoplasm) to the final product progesterone (P4).

Influence of Cytokines on Bovine Luteal Cells

Tumor Necrosis Factor Alpha

The term TNFα was first used and reported in 1975 by Carswell et al. to describe the molecule responsible for the hemorrhagic necrosis observed in tumors following endotoxin exposure. While TNFα was first described as a cytotoxic and pro-inflammatory polypeptide, it has been well established that the cytokine is pleiotropic depending on abundance, receptor signaling, and other extracellular immune factors (Aggarwal, 2003). For example, with regards to differential TNFα concentrations a low dose (1 ug) of systemically administered TNFα significantly reduced progesterone output, whereas a high dose (10 ug) increased progesterone output, though not significantly (Korzekwa et al., 2008). Sources of TNFα in the bovine CL include immune cells, large and small luteal cells, and endothelial cells (Sakumoto et al., 2011). TNFα is a part of the TNF superfamily, which comprises upwards of 19 ligands and 29 TNFRs (Aggarwal, 2003). The TNFRs can be further subdivided into two categories: death domain-associated receptors (death receptors) and those without (survival receptors). TNFα specifically binds to TNFR1 and TNFR2 (Aggarwal, 2003). TNFR1 receptors have been identified in bovine luteal endothelial cells and steroidogenic cells, whereas TNFR2 receptor expression is predominantly associated with endothelial cells and immune cells (Aggarwal, 2003; Hojo et al., 2010; Kim et al., 2019). While TNFR1 is generally regarded as a death receptor due to the intracellular FADD binding domain, and TNFR2 as a proliferative survival receptor due to the TNF receptor-associated factor (TRAF) binding domain, potential crosstalk may occur between receptors (Faustman & Davis, 2013; Fotin-Mleczek et al., 2002; Yan et al., 2018). Additionally, the abundant expression of TNFR2 during luteal regression may be indicative of a pro-death cascade, though could be pro-survival and homeostatic in conjunction with Pate et al.'s (2012)

previously discussed theory that immune cells aid to reduce luteal cell death during regression (Pate & Keyes, 2001; Sakumoto et al., 2011).

The TNFR1 signalling cascade commences with the ligation of TNFα to TNFR1. Upon receptor activation, TRADD is recruited which can in turn activate multiple different signalling pathways, depending on additional stimuli (Aggarwal, 2003). Both apoptotic and necroptotic signaling pathways are potential outcomes of TNFR1 activation, as previously discussed. Additionally, TNFR1 stimulation can trigger nuclear factor kappa B activation, which is a known transcription factor for genes of an inflammatory descent (Liu et al., 2017).

The TNFR2 receptor recruits and binds TNF receptor-associated factors (TRAF) and cellular inhibitors of apoptosis (CIAP), which collectively and predominantly promote cell survival (Faustman & Davis, 2013). Following exposure to TNFα, middle-cycle luteal cells in culture increased PGF2α and PGF1α, but not progesterone output (Benyo & Pate, 1992). Additionally, TNFα exposure alone increased the expression of class I MHC molecules. Exposure to TNFα for 24 hrs reduced luteal endothelial cell culture viability by 40%, which was entirely inhibited by a potent antioxidant, glutathione, indicating TNFα may be increasing cellular death via ROS production (Pru et al., 2003). Additionally, TNFα alone reduces the inhibitor of nuclear factor kappa B, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha, in cultured bovine luteal cells (Suter et al., 2001).

Interferon Gamma

Like TNFα, IFNγ is a pleiotropic cytokine, influencing cellular death and cellular differentiation. IFNγ was first reported in 1965 by Wheelock as a cytokine released by immune cells in response to a virus (Wheelock, 1965). IFNγ belongs to the IFN class of cytokines, which is subdivided into Type 1 or Type 2 IFN. Type 1 IFN, such as IFN alpha, IFN beta, and IFNτ are

mainly produced in response to viral infections. Type 2 IFN, IFNγ being the sole member, is produced in response to a pro-inflammatory environment. IFNγ is secreted by an array of immune cells present in the four luteal stages of the corpus luteum, such as helper T cells, cytotoxic T cells, B cells, natural killer cells, and monocytes and macrophages. Production and secretion of IFNγ are regulated by both interleukin 12 and interleukin 18, as reviewed by Schroder et al. (2004). On the contrary, IFNγ is negatively regulated by interleukin 10 and 4 and transforming growth factor-beta, and vice versa (Neuvians et al., 2004). The IFNγ receptor (IFNGR) consists of two ligand-binding domains (IFNGR1) and two signal-transducing regions (IFNGR2). The IFNGR2 serves as a control point for IFNγ signalling, as the gene expression is highly regulated (Bach et al., 1997). Activation of the IFNGR initiates a JAK-STAT signalling cascade resulting in transcriptional regulation of interferon-stimulated genes (Schroder et al., 2004). Expression of the IFNγ receptor is not well defined in the bovine corpus luteum, however, exposure of the cytokine to steroidogenic luteal cells, endothelial cells, and immune cells alters cellular functionality and mRNA expression of IFNγ signalling, indicating a regulatory role of IFNγ on these cells (Suter et al., 2001). Furthermore, across species, IFNγincreases the activity of cellular immunity through the activation of macrophages, natural killer cells, and T cells (Schroder et al., 2004). IFNγ has also been shown to independently increase class II MHC in bovine luteal cells (Fairchild & Pate, 1989). Following prolonged IFNγ exposure (greater than 24 h), cultured bovine luteal cells increase PGF2α and PGF1α production, and reduce progesterone synthesis in a PGF2α-independent manner (Fairchild & Pate, 1991). Despite the influence of IFNγ on prostaglandins and progesterone synthesis, one group demonstrated that IFNγ on its own marginally reduces the cellular viability of cultured luteal cells (Pru et al., 2003). However, Hojo et al. (2010) demonstrated that a low dose of IFNγ

(2.5 nM) reduced cell viability by a near 40%. Moreover, a group exhibited that IFNγ increased indoleamine 2,3 dioxygenase, which is involved in tryptophan degradation, and potentially serves as a mechanism by which IFNγstimulates luteal apoptosis (Cannon & Pate, 2006; Fallarino et al., 2002).

Synergistic Influence of Tumor Necrosis Factor Alpha and Interferon Gamma

Both TNFα and IFNγ independently alter luteal cell functionality. However, a synergistic effect is observed when cytokine treatments are combined. For example, TNFα and IFNγ exposure to middle-cycle cultured luteal cells reduced luteal cell viability by 70-80% (Benyo & Pate, 1992; Hojo et al., 2010). The combination of cytokines also synergistically reduced cultured luteal endothelial cells (Pru et al., 2003). How the cytokines synergistically increase cell death is not well known. However, IFNγ increases expression of TNFR1 receptor expression in bovine luteal endothelial cells, and so it has been hypothesized that IFNγincreases the sensitivity of cells to TNFα (Hojo et al., 2010). Petroff et al. demonstrated that TNFα and IFNγ-induced cell death was significantly reduced by pre-treatment with two potent antioxidants, indicating a role of ROS in the induced cellular death, as previously mentioned (Petroff et al., 2001). Notably, ascorbate is depleted from cells over time in culture and may leave cells more susceptible to ROS generation (Musicki et al., 1996). Moreover, apoptosis and necroptosis occur following TNFα and IFNγ exposure, as previously discussed (Hojo et al., 2016, 2019).

While TNFα and IFNγ are certainly cytotoxic to middle-phase bovine luteal cells, how the cytotoxicity changes over the course of the luteal phases (early, middle, and late) has not yet been explored. In consideration of the differential expression of TNFR1 through the luteal phases, as previously discussed, we postulate that mixed luteal cells from the early, middle, and

late luteal stages will undergo differential losses in cell viability following exposure to low doses of TNFα and IFNγ. Specifically, late and regressed luteal phases will have a higher percentage loss in viability when compared to cells isolated during the early and middle luteal phases.

Omega-3 Fatty Acids in Reproductive Biology

Lipids are defined as a class of organic compounds that are soluble in organic solvents, which are essential in the diet. The feed industry uses crude fat as an estimate of lipid content in a feedstuff. Crude fat is a mixture of lipids in a sample that has been extracted using the Soxhlet method. This mixture contains fatty acids, mono- di- and triglycerides, steroids, and fat-soluble vitamins.

Cows bred in negative energy balance or poor body condition have decreased pregnancy rates. A cow ration contains approximately 2 to 3% crude fat and fats are often supplemented in the diet to improve energy balance and body condition. However, there are conflicting reports in the literature regarding fat supplementation and impacts on reproduction, some showing improved rates of pregnancy, while others show no change (Funston, 2004; Hess et al., 2008). The class of fat incorporated into the diet may differentially support reproductive performance.

Lipids or fats can be further defined as simple, compound or lipid derived. Simple lipids include long-chain fatty acids (saturated, unsaturated, polyunsaturated), triglycerides and waxes. Long-chain fatty acids are named by number of atoms carbon in the acyl chain, number of C=C bonds and the position of these bonds in the acyl chain. There are two systems used in the naming of long-chain fatty acids that have C=C bonds – omega and delta numbering system. The delta nomenclature has been adopted by biochemists and number the carbon atoms starting from the carboxy terminal, while the omega nomenclature has been adopted by nutritionists and number the carbon atoms starting from the methyl end. Omega-3 fatty acids are long-chain fatty

polyunsaturated acids with the first C=C bond between the third and fourth carbon atom. There are three common omega-3 fatty acids found within animal and plant cells. The first of the three, alpha-linoleic acid (ALA; shorthand C18:3n-3) is an essential fatty acid required in the diet as mammals lack enzymes required for de novo synthesis. However, mammals do express desaturase and elongase enzymes that allow for the synthesis of two other common omega-3 fatty acids—eicosapentaenoic acid (EPA; shorthand C20:5n-3) and docosahexaenoic acid (DHA; shorthand C22:6n-3)—from ALA. However, the conversion efficiency of ALA to the longer chain omega-3 fatty acids EPA and DHA is low in cattle, and thus dietary supplementation of EPA and DHA has been recommended (Kairenius et al., 2015). Omega-3 fatty acids from fish meal supplementation significantly surpasses *de novo* blood plasma concentrations (Petit et al., 2002; Plewes et al., 2018).

Fish products are a rich source of EPA and DHA and have been selectively utilized in both the beef and dairy industry for over a century to optimize meat and dairy production (Avramis et al., 2003; Bimbo & Crowther, 1992; Wistuba et al., 2006). In addition to improved meat and milk production, dietary supplementation with fish meal or oil has shown potential for improving reproductive biology functions and pregnancy in cattle. In dairy cows, supplementation has shown to reduce PGF2α release from the endometrium (Coelho et al., 1997; Mattos et al., 2004). However, additional groups found no reduction in PGF2α metabolite (one group found increased output) nor found any difference in follicular activity or progesterone output in cattle supplemented with fish meal (Childs et al., 2008; Moussavi et al., 2007). Burke et al. (1997) demonstrated that fish meal supplementation increased pregnancy rates by 29.5% (9.4% difference), increased luteal progesterone, and altered CL responsiveness to PGF2α in dairy cattle. Furthermore, a combination of omega-6 fatty acids, followed by omega-3 fatty acids

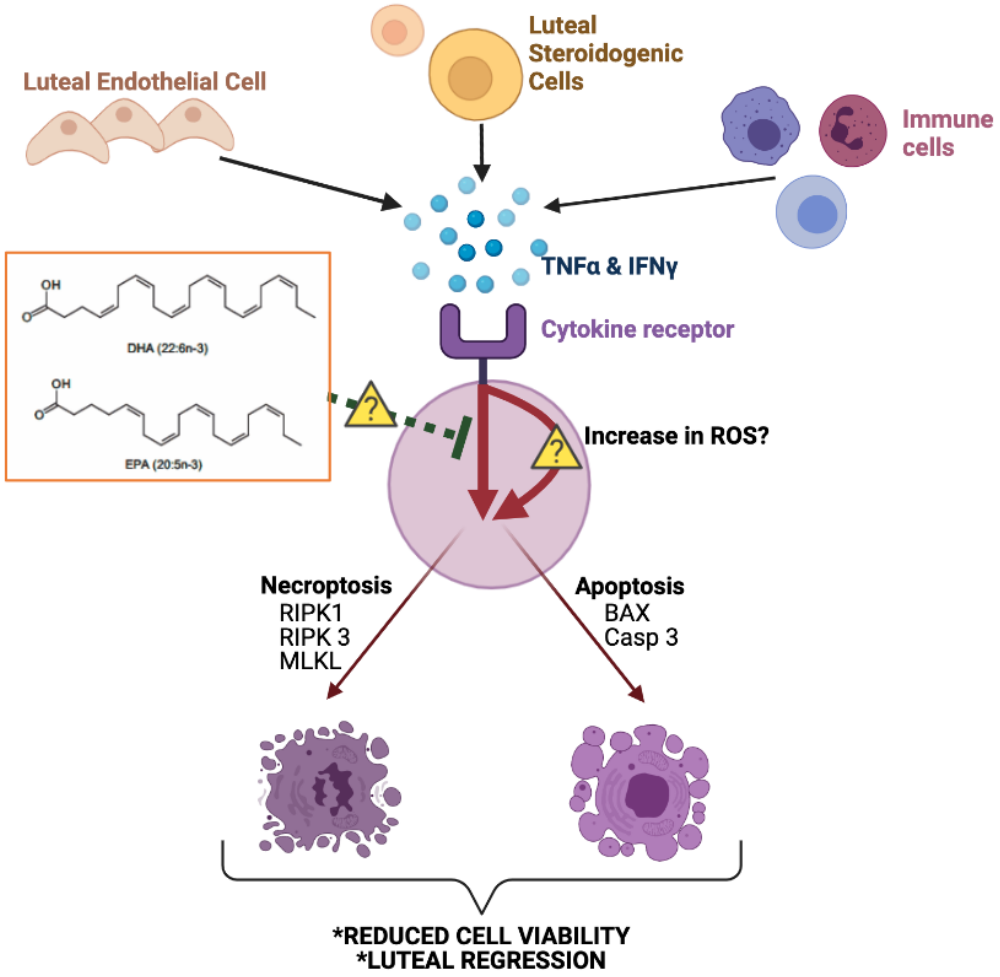
on day 40 of gestation increased pregnancy rates in dairy cows (Dirandeh et al., 2013). A study by Burns et al. (2002) furthered the notion that fish meal supplementation increased pregnancy rates by 22.6 % (14% difference), specifically in primiparous lactating beef cows.

Fish meal supplementation has also been demonstrated to reduce rates of luteal regression following physiological intra-uterine infusions of PGF2α (Plewes et al., 2018). While the mechanisms involved are not well elucidated, it is evident that the omega-3 fatty acids derived from fish meal or oil are altering reproductive function in cattle (Gulliver et al., 2012). Generally, mechanisms explored regarding omega-3 fatty acids and their role in overall health optimization pertain to the anti-inflammatory qualities of the lipids and subsequent metabolites (Serhan et al., 2008). This idea is paralleled in studies where the production of the pro-inflammatory lipid, PGF2α, is underproduced and secreted in the bovine endometrium (as previously mentioned). Additionally, the observed reduction in luteal regression and increased output in progesterone following fish meal or oil supplementation may be in part due to a repressed intra-luteal inflammatory response, though requires further exploration. As discussed in previous chapters, the event of luteal regression is a localized pro-inflammatory event, wherein up-regulation of pro-inflammatory cytokines and cell phenotypes are expressed and contribute to the demise of the gland. We postulate that the luteoprotective effect of omega-3 fatty acid supplementation is in part due to a reduction in cytokine-induced cellular cytotoxicity on bovine luteal cells (figure 1.4). As previously mentioned, cytokines TNFα and IFNγ may reduce cellular viability by way of increased generation of ROS (Petroff et al., 2001; Pru et al., 2003). Although the role of omega-3 fatty acids, specifically EPA and DHA, in ROS management is heavily debated and often dependent on cell type, dosage, and cellular environmental conditions (Al-Gubory, 2012; Giordano & Visioli, 2014; Hsu et al., 2014; Jones

et al., 2013; Lee et al., 2015; Oono et al., 2020; Sakai et al., 2017; Wu et al., 2009). We propose that if EPA and DHA do indeed maintain luteal cell viability, that it is by way of ROS repression. An additional proposed mechanism by which fish meal supplementation maintains luteal functionality includes the preservation of luteal blood flow following low-dose intra-uterine PGF2α exposure. Omega-3 fatty acids have been shown across systems and models to maintain and influence blood flow, indicating an influential role of these lipids in tissue vascularization (Howe et al., 2018; Lescano de Souza Junior et al., 2017; Morgan et al., 2006; Schwarz et al., 2018; Sinn & Howe, 2008; Walser et al., 2006). Prolonging CL blood flow could, in theory, maintain subsequent cholesterol delivery and nutrient and gas exchange to the gland, therein prolonging luteal functionality.

Figure 1.4

Hypothesized Inhibitory Effects of Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) on the Cytotoxicity of Tumor Necrosis Factor Alpha (TNFα) and Interferon Gamma (IFNγ)



Note. During luteal regression, luteal steroidogenic cells, endothelial cells, and immune cells produce the cytokines TNFα and IFNγ, both of which propagate luteal regression and are cytotoxic to bovine luteal cells. Here, we hypothesized that both EPA and DHA reduce the cytotoxic effects of both cytokines (yellow triangle with question mark), and if so, could allude to a potential mechanism by which fish meal supplementation is luteal protective. If EPA and DHA do reduce cytotoxicity, we hypothesize it is through reduction of reactive oxygen species (ROS) production.

Specific Aims, Research Questions and Hypotheses

A1 Examine differences in cytokine sensitivity of mixed bovine luteal cells isolated from the early (day 1-4), middle (day 5-12), and late (day 13 17) luteal phases of the estrous cycle.

Q1 Is the degree of TNFα and IFNγ-induced cytotoxicity dependent on the luteal stage from which mixed bovine luteal are isolated?

H Mixed bovine luteal cells isolated from the late luteal phase will die more (higher percent loss in cellular viability) when compared to cells isolated during the early and middle luteal phase following TNFα and IFNγ exposure.

Q2 Are biomarkers of apoptotic (*BAX, CASP3, BCL2*) and (or) necroptotic (*RIPK1, RIPK3, MLKL*) signaling pathways both elevated following TNFα and IFNγ exposure across all luteal stages?

H2 The abundance of steady state mRNA for necroptotic signaling (*RIPK1, RIPK3, MLKL*) will be more abundant in the late luteal phase, and apoptotic gene expression (*BAX, CASP3, BCL2*) will not differ throughout stages.

Q3 How does TNFα and IFNγ exposure influence pro-inflammatory (programmed cell death related) gene expression (*TNFR1, TNF*α, and *NF*κ*B*) in bovine luteal cells, and does it vary across luteal stages?

H3 Bovine luteal cells exposed to TNFα and IFNγ will undergo increased expression of *TNFR1, TNFa* and *NF*κ*B*, though will be disproportionately elevated in cells isolated from the late luteal phase compared to those of early and middle stage CL.

Q4 What is the influence of TNFα and IFNγ exposure on steroidogenic gene expression (*STARD1* and *CYP11A1*) in bovine luteal cells?

H4 Bovine luteal cells exposed to TNFα and IFNγ will undergo a reduction in *STARD1* and *CYP11A1* steady state mRNA expression.

A2 Explore the influence of EPA and DHA pre-treatment on the TNFα and IFNγ induced cytotoxicity in mixed bovine luteal cells.

Q1 Does pre-treatment of bovine luteal cells with EPA and DHA influence the cytotoxicity of TNFα and IFNγ exposure?

H1 Pre-treatment of mixed bovine luteal cells with EPA and DHA will reduced the cytotoxic effects of TNFα and IFNγ exposure.

Q2 Independent of changes in cellular viability, does pre-treatment of bovine luteal cells with EPA and DHA differentially alter gene expression for apoptotic (*BAX, CASP3, BCL2*) or necroptotic (*RIPK1, RIPK3, MLKL*) signaling following exposure to TNFα and IFNγ?

H2 EPA and DHA pre-treatment will not differentially influence gene expression for apoptotic or necroptotic gene expression following exposure to TNFα and IFNγ

Q3 Does pre-treatment of bovine luteal cells with EPA and DHA influence expression of steroidogenic genes (*STARD1* and *CYP11A1*) following TNFα and IFNγ exposure?

H3 Bovine luteal cells pre-treated with EPA and DHA will undergo a smaller reduction in steroidogenic gene expression following TNFα and IFNγ exposure.

A3 Generate preliminary data on the effects of fish meal supplementation on luteal blood flow following PGF2α induced luteolysis.

Q1 Does luteal blood flow differ in fish meal supplemented cows that maintained a functional CL 48 hrs following two low dose intra-uterine infusions of PGF2α?

H1 Cows that were supplemented with fish meal, and that retained a function CL, will have elevated and prolonged luteal blood flow following two low doses intra-uterine infusions of PGF2α.

Conclusion

For decades, the loss of pregnancy in cattle has plagued the beef and dairy industry. An avenue of popular scientific exploration, in pursuit of a solution, includes alterations in dietary intake. Dietary supplementation with fish meal, a rich source of the omega-3 fatty acids EPA and DHA, has shown promise across multiple studies to positively influence pregnancy rates. However, much is to be gleaned as to how fish meal is a reproductively enhancing supplement and how it alters the physiology of reproductive structures. Past studies from our lab demonstrate that fish meal supplementation reduces the rate of luteal regression following low dose infusions

of PGF2α. Despite some insight as to how fish meal supplementation influences CL-PGF2α sensitivity, such as increased PGF2α receptor mobility and subsequent reduction in downstream signaling, the presumed vastness of the physiological changes has yet to be delineated. Also, exploring and outlining the mechanisms at play will further our understanding of luteal regression, which is imperative for further development of pregnancy rate- augmenting interventions.

During luteal regression, the pro-inflammatory cytokines TNFα and IFNγ appear to contribute to the functional and structural demise of the gland, and as such are particularly cytotoxic to bovine luteal cells. The cytokines may be inducing cellular death by elevating production of ROS, and both EPA and DHA have demonstrated a capacity to reduce damage associated with, and production of, ROS in cells and tissue. Furthermore, changes in luteal blood flow, specifically the decline following PGF2α exposure, are postulated to play a significant role in the cessation of luteal function. Omega-3 fatty acids have demonstrated a capacity to maintain tissue blood flow, despite damage or restriction to said tissue. Taken together, we hypothesized two potential mechanisms by which fish meal supplementation alters rates of luteal regression: 1) omega-3 fatty acids reduce the cytotoxic effects of TNFα and IFNγ and 2) fish meal supplementation maintains luteal blood flow following exposure to PGF2α.

CHAPTER II

METHODOLOGY

The Influence of Eicosapentaenoic Acid and Docosahexaenoic Acid on Tumor Necrosis Factor-Alpha and Interferon Gamma-Induced Cytotoxicity on Mixed Bovine Luteal Cells

Bovine Corpus Luteum Enzymatic Digest and Cellular Preparation

Bovine Holstein and Angus ovaries were retrieved from Double J Meatpacking Inc in Pierce, Colorado. Reproductive tracts were palpated for absence of an embryo/fetus and both ovaries were assessed for the presence and size of follicles for later staging of the corpus luteum. Ovaries were transported to the University of Northern Colorado laboratory on ice in 1× Dulbecco's phosphate-buffered saline (dPBS) within 1-hr following slaughter. Ovaries were briefly sterilized in 70% ethanol and subsequently rinsed in ice-cold calcium, magnesium, and phenol red-free Hanks' Balanced Salt Solution (HBSS; pH 7.34; ThermoFisher 14175095). To determine the stage of the CL, gross anatomical structures on the ovary were used as identifiers, as previously defined by Ireland et al. (1980). The CL was then dissected from the ovary, along with the removal of external connective tissue, and measurements of glandular diameter and coloration were recorded for use in luteal staging. The corpus luteum was further dissected into 500 µm thick slices, using a handheld microtome. Throughout the dissection process, tissue slices were placed on ice in a petri dish containing Ham's F-12 Nutrient Mix (pH 7.34; ThermoFisher 11765062) supplemented with 1× Antibiotic-Antimycotic (ThermoFisher 15240062). Tissue slices were then transferred to a sterile glass Pyrex bottle containing 20 mL of

Ham's F-12 Nutrient Mix and 1× Antibiotic-Antimycotic, and a magnetic stir rod, as described by Pate and Condon (1982). Pyrex bottles and contents were placed in a 35ºC water bath and left to gently mix for 10 mins. The culture medium was then decanted from the tissue, and 20-30 mL of fresh media containing type 1 collagenase (2000 U/g tissue; Worthington LS004197) was added. The tissue-collagenase mixture was placed back into the 35ºC water bath and left to stir for 45 mins. Every 15 mins the tissue was slowly passed through with a plastic 25 mL serological pipette 15 – 20 times to facilitate tissue disruption. The supernatant was then decanted from the residual tissue chunks and filtered with a 100 µm cell strainer into a 50 mL conical on ice for later processing. The residual tissue chunks were resuspended in another 20 mL of collagenase-containing medium for further digestion. Once all tissue was fully digested and filtered, cells were centrifuged at 300 × g for 7 mins at 4^{O}C. Cells were then resuspended and rinsed in 1× dPBS, followed by subsequent centrifugation for 3 rounds, or until the supernatant was clear of debris. Cells were then filtered a final time using a 100 µm cell strainer, resuspended in Ham's F-12 Nutrient Mix supplemented with 1× antibiotic-antimycotic, 1× Insulin-Transferrin-Selenium (ITS-G; ThermoFisher 41400045) and 10% fetal bovine essence (FBE; VWR 3100-500GH); complete culture medium. Cell concentration and viability was determined using a hemacytometer and trypan blue exclusion. Cells were either plated immediately for select assays or were plated in T-225 flasks (2×10^7 cells/ flask) for future experiments and incubated at 37 °C in 5% CO2 and 95% humidified air. The incubation parameters were consistent across all *in vitro* experiments.

Estimation of Steroidogenic Cell Population in Bovine Luteal Cell Culture

A nitro tetrazolium blue chloride assay was performed as per Hryciuk et al. (2019) to estimate the percentage of cells containing 3β-HSD (steroidogenic cells) in culture at 1, 7, and 11 days following the CL enzymatic digestion. Cells were first removed from respective flasks and $1x10^6$ cells were used in each assay. Cells were rinsed three times (300 x g for 5 mins) in 1× dPBS, and subsequently fixed in 1% paraformaldehyde for 15 mins at 37ºC. Cells were rinsed three more times in 1× dPBS and incubated for 24 hrs at 37ºC in 200 µL of dPBS containing 0.25 mM nitro tetrazolium blue chloride (Sigma-Aldrich N6876), 1.5 mM β-nicotinamide adenine dinucleotide hydrate (NAD^+; Sigma-Aldrich N1636), 0.2 mM 5-pregnen-3B-ol-20-one (Sigma-Aldrich P9129), 0.2 mM ethylenediaminetetraacetic acid disodium salt (EDTA; Sigma-Aldrich E7889) and 0.1% BSA. Pregnenolone was withheld from negative staining controls.

Data Analysis

The percentage of steroidogenic cells (purple cells) was quantified using a hemocytometer ($\frac{\#\ purple\ cells}{\#\ \text{total cells}}$) after 24 hrs of incubation.

Statistical Analysis

All data were first assessed for normality using the Shapiro-Wilk test ($p>0.05$= normal distribution) with Python (version 3.10.6) prior to statistical analysis. A PROC MIXED model was used to determine statistical differences between variables using the Statistical Analysis Software (SAS). Variables included in the model were stage of CL and day of culture post-enzymatic digestion, with the CL number as a random variable.

Omega-3 Fatty Acid Pre-Treatment

Cells that were pre-treated with omega-3 fatty acids prior to cytokine exposure were first trypsinized with 0.5% Trypsin-EDTA (ThermoFisher 15400054) from T-225 flasks one day following CL enzymatic digest. Cells were pre-treated with 10 µM eicosapentaenoic acid (EPA; Sigma-Aldrich 73167-03-0) and 10 µM docosahexaenoic acid (DHA; Sigma-Aldrich D8768) in complete medium. The concentration of EPA and DHA (ω-3) were previously established by Plewes et al. (2017) in a dose-response experiment on bovine luteal cell cultures. To ensure the complete solubilization of the ω-3, fatty acids were pre-bound to 33 mg/mL bovine serum albumin (BSA; Sigma-Aldrich 7030) in complete medium prior to exposure to cells, as previously described by Mattos et al. (2003). Cells were then left to incubate for 72 hrs in the ω-3-integrated culture medium.

Cytokine Exposure to Mixed Bovine Luteal Cells

When investigating the influence of ω-3 or luteal stage on the cytotoxicity of tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ), all cytokine concentrations and plating numbers were the same.

Cells were first plated in triplicate in a 96-well plate (3000 cells/well) in complete medium. Cells were serum starved with serum-starve culture media (complete culture medium with only 1% FBE) for 12 hrs to force all cells into the G0 phase of the cell cycle. Next, cells were exposed to 2.5 nM bovine recombinant TNFα (R&D Systems 210-TA-100) and 2.3 nM bovine recombinant IFNγ (R&D Systems 2300-BG-025) in serum-starve culture media and cultured for 0, 12, 24, 36, or 48 hrs. Concentrations of TNFα and IFNγ were previously established by Hojo et al. (2010). Depending on the relevant experiment, treatment groups

included BSA control (BSA), TNFα & IFNγ treated (BSA TI), EPA & DHA pre-treated control (ω-3), and ω-3 pre-treated and TNFα & IFNγ treated (ω-3 TI).

Adenosine 5-Triphosphate
Luminescence Cell
Viability Assay

Following exposure to TNFα and IFNγ, cellular viability was assessed using the luminescent CellTiter-Glo^{R} 2.0 Cell Viability Assay (Promega G9241), which quantifies ATP levels. The protocol provided by Promega (TM403; Revised 10/18) was followed. Briefly, before the addition of the CellTiter-Glo^{R} reagent (100 uL) to the 96 wells, 100 uL of the culture media was removed and discarded to ensure the final volume was compatible with the 96 well format. Following the addition of CellTiter-Glo^{R} reagent and complete cellular lysis, the contents of each well were transferred to identical wells in a white opaque-walled 96-well plate to reduce luminescent cross talk. Before the final analysis, any bubbles were removed using a propane torch briefly held over the top of the 96-well plate. Luminescence was assessed using the Perkin Elmer Victor X5.

Data Analysis

An average value per treatment (BSA Cont, BSA TI, ω-3 Cont, ω-3 TI), per CL was normalized to the respective non-cytokine treated BSA controls

$$\left(\frac{\text{treatment average luminescence value}}{\text{BSA control average luminescence value}}\right)$$

, providing relative viability.

Statistical Analysis

All data were assessed for normality using the Shapiro-Wilk test, as previously outlined. If data were normally distributed following transformation, a PROC MIXED model was used to determine statistical differences between variables with SAS. When analyzing the influence of luteal stage on the cytotoxicity of TNFα and IFNγ, variables included in the model were isolated

CL, stage of CL, time of cytokine exposure, and all possible interactions. If main effects or interactions were significant ($p<0.05$), a preplanned pairwise *t*-test comparisons analysis was used with the PDIFF function to determine statistical differences between groups. When assessing the influence of EPA & DHA pre-treatment on the cytotoxicity of TNFα and IFNγ, variables included stage of CL, time of cytokine exposure, treatment group, and all possible interactions. The number of isolated CL was set as a random variable. If main effects of interactions were significant ($p<0.05$), a preplanned pairwise *t*-test comparisons analysis was used with the PDIFF function to determine statistical differences between groups.

Ribonucleic Acid Extraction and Complementary Deoxyribonucleic Acid Synthesis

Total RNA was isolated to investigate the influence of ω-3 fatty acid treatment and luteal stage on apoptotic, necroptotic, programmed cell death associated, and steroidogenic genes. Cells were first plated in duplicate in a 12 well dish (1×10^5 cells/well). All EPA & DHA and TNFα & IFNγ pre-treatment methodology and concentrations were as previously mentioned. Cells were exposed to cytokines for 24 h, prior to RNA isolation.

RNA was isolated from cell culture experiments using the Direct-zol™ RNA Kit (Zymogen) protocol supplied by the manufacturer. Of note, following the addition of TRIzol to cells, samples were stored at -80ºC until further processing. Following isolation, RNA concentrations were quantified using a Thermo Scientific NanoDrop 2000C Spectrophotometer. The integrity of the RNA was assessed using a 1% agarose gel electrophoresis.

For the subsequent synthesis of cDNA, the QuantiTect Rev. Transcription Kit (Qiagen 205313) was utilized per the protocol supplied by the manufacturer. Briefly, 1 µg of RNA was utilized in the genomic DNA wipeout reaction, which was then utilized for cDNA synthesis, described by Plewes et al. in detail (2018). In brief, the genomic wipeout consisted of a 2-minute

incubation period at 42°C, following which tubes were directly transported back on ice. Reactions for cDNA synthesis were incubated at 42°C for 30 mins, followed by a 95°C incubation for 3 mins. Both the genomic wipeout and cDNA synthesis reactions were performed using a Bio-Rad T100 PCR Thermal Cycler. cDNA samples were stored at -20°C until time of qPCR.

Quantitative Polymerase Chain Reaction and Analysis of Steady State Messenger Ribonucleic Acid

All primers used for qPCR (table 1) were first validated (in duplicate) for binding efficiency using a 10-fold dilution of cDNA commencing with 5 ng, 300 nM of forward and reverse primers, and 1X QuantiTect SYBR Green PCR Kit (Qiagen 204145) in a 10 uL total reaction. Thereafter, qPCR reactions (run in duplicate) were run with 5 ng of cDNA, 300 nM of forward and reverse primers, and 1X QuantiTect SYBR Green PCR Kit in a 10 uL total reaction. All binding efficiency and qPCR reactions were run for a total of 40 cycles: denaturing at 94°C for 15 s, annealing at 60°C for 30 s, and amplification at 72°C for 30 s. Analysis of melt curves following each experiment were performed to ensure single product amplification. All qPCR reactions were run on a Bio-Rad CFX384 real-time PCR Thermal Cycler. All qPCR experiments contained a negative template control per gene analyzed.

Table 1

List of Primer Sequences, Subsequent Product Length (BP) and Sources of Retrieval

Gene	gene name	Forward sequence	Reverse sequence	BP	Source
ACTB	Beta-actin	TTCCAGCAGATGTGGATCAG	AGCCATGCCAATCTCATCTC	154	Plewes et al. (2018)
CASP3	caspase 3	TCAGTCAGTTGGGCACTCTG	CACACCCGTAGCTGTGAAGA	144	Cedillo (2019)
BCL2	B-cell lymphoma 2	TTTGCTTCAGGGTTTCATCC	ATCCTCTGCAGCTCCATGTT	147	Cedillo (2019)
BAX	Bcl-2 Associated X	TCTGACGGCAACTTCAACTG	TCGAAGGAAGTCCAATGTCC	135	Cedillo (2019)
STARD1	Steroidogenic acute regulatory protein	CAGCAGAAGGGTGTCATCAGA	GAGAGGACCTGGTTGATGATG	152	Plewes et al. (2018)
CYP11A1	Cholesterol sidechain cleavage	AGGCAGAGGGAGACATAAGCA	GTGTCTTGGCAGGAATCAGGT	156	Plewes et al. (2018)
RIPK1	Receptor interacting protein kinase 1	GCAATAGCTCCAAGCAGGTC	TGTGCAGCAGGAAGTCATTC	148	Hojo et al. (2019)
RIPK3	Receptor interacting protein kinase 3	CCAGAGAGAGCAGGTTCCAC	AATCAGGCGGTTGTTGTTTC	219	Hojo et al. (2019)
MLKL	Mixed lineage kinase domain-like protein	ACTTCCATCAGCCGACAAAC	CTCCCAGAGGACAATTCCAA	144	Hojo et al. (2019)
NFKB2	Nuclear factor kappa B subunit 2	CCTGCTGAATGCTCTGTCTG	TCCTCCTTCACCTCTGTGCT	102	Talukder et al. (2017)
TNFα	Tumor necrosis factor alpha	CAAAAGCATGATCCGGGATG	TTCTCGGAGAGCACCTCCTC	51	Talukder et al. (2017)
TNFR1	Tumor necrosis factor alpha receptor 1	CACCACCACCATCTGCTT	TCTGAACTGGGGTGCAGA	257	Sakumoto et al. (2000)

Data Analysis

When analyzing quantity of steady state mRNA for target genes, *beta-actin* (ACTB) was used as a reference gene. The fold change of gene expression was quantified using the delta delta C_t ($2^{-\Delta\Delta Ct}$) method.

Statistical Analysis

All data were assessed for normality in the same manner previously mentioned. If raw data did not show normal distribution, data were transformed and reassessed. Optimal transformation exponents were determined using the Box-Cox Normality Plot using Python. If data were normally distributed, a PROC MIXED statistical model was used to determine statistical differences between variables, as previously outlined. When comparing gene expression across treatments (BSA, BSA TI, ω-3, ω-3 TI), the tested variables included stage of CL and treatment, with the number of CL used set as a random variable. If data were not normally distributed, a Kruskal-Wallis one-way analysis of variance was run on SAS to determine overall effects. If effects were significant ($p<0.05$), a post hoc Conover-Iman Test was utilized to determine differences of gene expression across treatments. Values were reported as significant if $p<0.05$.

The Effects Fish Meal Supplementation on Luteal Function, Serum Progesterone, Luteal Blood Flow, and Luteal Mass Following Two Low Dose Intra-Uterine Infusions of Prostaglandin F2 Alpha

Animal Husbandry

Mixed breed beef cows were purchased from the Centennial Livestock Auction house in Larimer County, CO. Cattle were transported to, and subsequently maintained for the extent of the study at the Animal Reproduction Biotechnology Laboratory at Colorado State University in Fort Collins, CO. All animal care and experimental protocols were pre-approved by the Colorado State University Institutional Animal Care and Use Committee (Approval #1366). Inclusion criteria for cows admitted to the study included lack of gross reproductive abnormalities, lack of reproductive adhesions, lack of cystic follicles, non-pregnant, ovaries present, and normal ovarian cyclicity. All examinations were performed per rectum palpation and with trans-rectal ultrasonography in B-mode using a linear rectal probe and ExaPad Mini (IMV Imaging).

Supplementation and Synchronization Period

Upon admittance to the study, cows were fed a diet of 95% mixed hay and 5% supplement (table 2). Dry matter intake was at 2.0% total body weight, which averaged 13.6 kg mixed hay and 0.68 kg supplement per cow. Cows were matched by body weight and randomly assigned to a fish meal supplementation group or a corn gluten meal supplementation group (5% total diet)—both of which were isocalorically and isonitrogenously matched by addition of 25 mL of vegetable oil to corn gluten meal groups (table 3). All nutritional parameters met or exceeded guidelines set by the National Research Council for non-lactating beef cows. Cows were individually penned twice daily from 06:00-09:00 and 18:00-21:00 (or until complete dietary consumption) and received respective hay and supplement. When animals were not

feeding, water and shelter were accessed *ad libitum.* Animal body weights were assessed every three weeks, and respective dry matter intake masses were altered accordingly. The total supplementation period consisted of approximately 63 days (Figure 2.1).

Cows' estrous cycles were synchronized with 25 mg Dinoprost (a prostaglandin F2 alpha synthetic analogue; Zoetis 708-901) on day 35 and 49 of the approximately 63-day supplementation and synchronization period in preparation for the experimental period. Cows were observed for signs of estrus behaviors 30 mins prior to and following morning and evening feedings. Estrus detector patches (Estrotect) were also applied to the tail-head region of the cow to aid in detection of estrus (figure 2.1).

Table 2

Mixed Hay Nutrient Dry Matter Basis

Nurtient	Dry Matter Basis
Crude protein	18.1 %
Fat	2.81 %
Acid detergent fiber	30.2 %
Neutral detergent fiber	49.0 %
Starch	1.1 %
Water soluble carbohydrates with fructans—starch	9.4 %
Ethanol soluble carbohydrates—startch	7.0 %

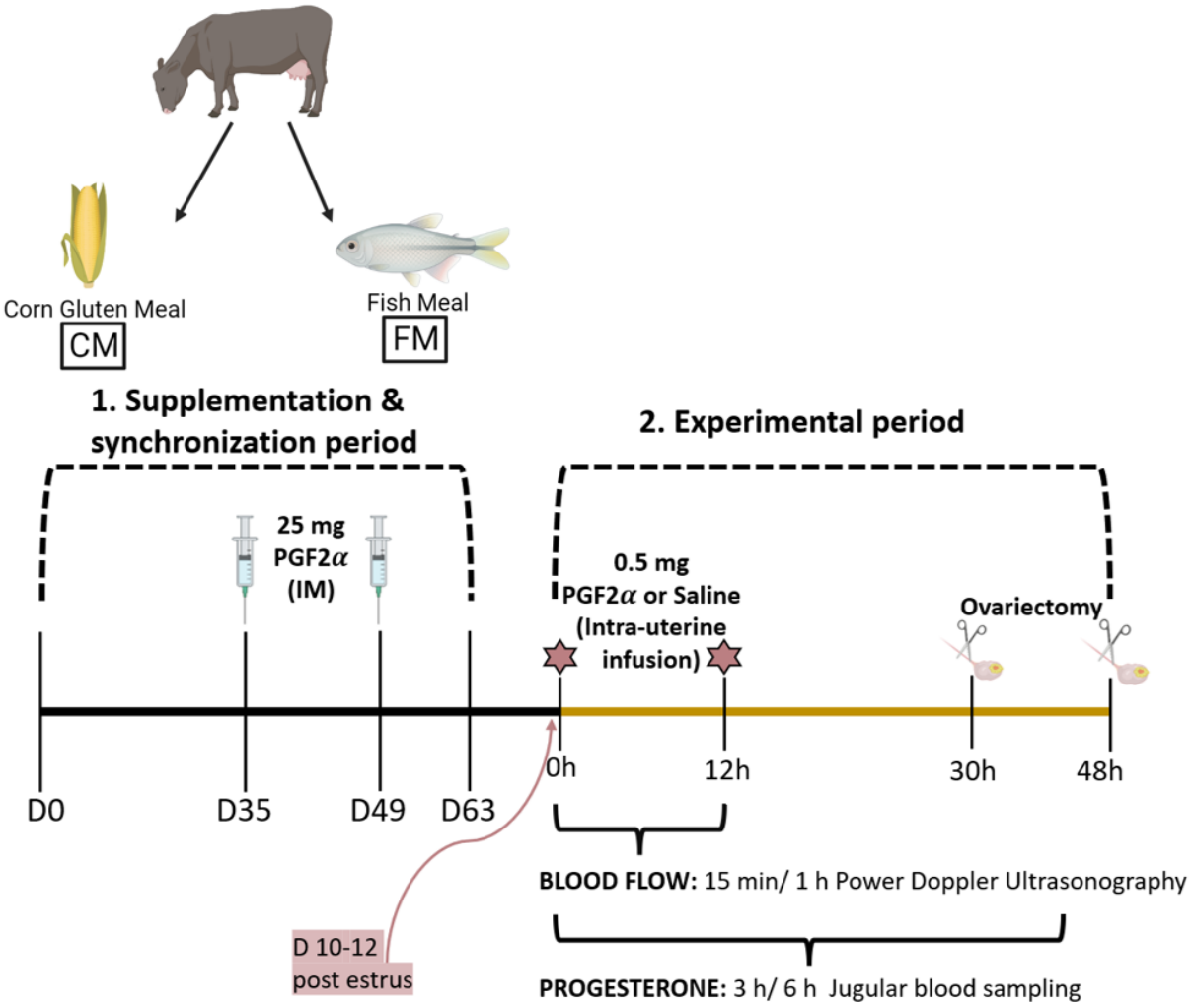
Table 3

Percent of Crude Fat and Protein in Corn Gluten Meal (CM) and Fish Meal (FM) Supplementations

Nutrient	CM Supplement	FM Supplement
Crude fat	3.0 %	9.0 %
Crude protein	60.0 %	60.0 %

Figure 2.1

Schematic Diagram of the Supplementation & Synchronization Period and the Experimental Period



Note. During the supplementation and syncornization period, cows were supplemeneted with fish meal (FM) or corn gluten meal (CM) for 63 days, in conjunction with mixed hay. On days 35 and 49 of the supplementation and syncronization period, estrous cycles were syncronized with 25 mg intramuscular (IM) injections of a prostaglandin F2 alpha (PGF2α) synthetic analouge, Dinoprost. At the start of the experimental period (day 63, 0 h), cows' were of day 10-12 post estrus, bearing a middle cycle corpus luteum. At 0 h and 12 h of the experimental period, cows were infused in the uterine horn ipsolateral to the corpus luteum-bearing ovary with low dose infusions of either Dinaprost (0.5 mg in 0.25 mL saline) or saline (0.25 mL). Following each infusion, luteal blood flow was analyzed with power Doppler ultrasongraphy (frequency 6 MHz, PFR 2000 Hz, wall filter 2, gain 18, and depth 60 mm) every 15 mins for the first hour, and every 60 mins for the following 6 h. Following each infusion, jugular blood was drawn for future analysis of serum progesterone concentrations. Ovariectomies were performed 30 or 48h following the first infusion.

Plasma Fatty Acid Composition and Abundance

Previous studies have demonstrated that fish meal supplementation at an identical dry matter intake rate is sufficient for fatty acid incorporation into the blood (Plewes et al., 2018). To ensure cows from the study were undergoing the same rate of incorporation, weekly plasma samples were acquired for future analysis with liquid gas chromatography (GC). All blood samples were collected from the jugular vein of cows and inverted in BD vacutainer EDTA blood collection tubes to prevent clotting. Samples were transported back to the laboratory at the University of Northern Colorado, wherein samples were spun down at 1500 x g for 15 mins at 4^{o}C. The plasma was then isolated and stored at -20^{o}C until further analysis.

Gas Liquid Chromatography

Prior to analysis of plasma fatty acid composition and abundance, samples first underwent saponification and methylation reactions, as described by O'Fallon et al. (2007). Concisely, following a room temperature thaw, 1 mL of plasma sample was subsequently transferred to a 13 X 100 mm Borosilicate glass tubes (VWR 47729-572). Samples were then dried with a freeze drier overnight. Thereafter, 5.3 mL of methanol and 0.7mL of 10 M potassium hydroxide were added to samples. Teflon tape was used on the glass tube and cap threads to ensure formation of a complete seal. Samples were then incubated at 55^{o}C for 1.5 h, thereby allowing for optimal saponification conditions. Samples were vortexed every 20 mins for 5 s to dissolve, hydrolyze, and permeate the sample. Next, samples were placed in a cold-water bath and left to cool until room temperature. Then, 0.58 mL of 12.5 M sulfuric acid was added to each sample and tubes were subsequently inverted until formation of white potassium sulfate precipitate. Tubes and contents were then incubated at 55^{o}C for 1.5 hrs, effectively methylating all samples. Throughout the incubation period, tubes were vortexed every 20 mins for 5 s. Tubes

were once again placed in a cold-water bath and left to cool until room temperature. Once cooled, 3 mL of hexane was added to each tube. Tubes were then vortexed for 5 mins. Samples were centrifuged for 5 mins at 450 x g The hexane layer was then removed from the glass tubes and placed into 15x 28 mm glass GC vials (Kimble 60810 1528). Samples were either dried down with nitrogen gas for immediate analysis on the Agilent 7890A Series GLC with spectrometry detector or stored at -20^{o}C for later analysis.

Data Analysis. GLC analysis was performed as previously described by Plewes et al. (2017).

Statistical Analysis. Prior to statistical analysis of data, normality was assessed using methods previously mentioned. A PROC MIXED with repeated measures was used as previously outline. An autoregressive covariance structure (type=AR (1)) was used in the repeated measures model. Variables included in the model were cow, day, supplementation, and supplementation x day. If main effects of interactions were significant ($p<0.05$), a preplanned pairwise *t*-test comparisons analysis was used with the PDIFF function to determine statistical differences between groups.

Experimental Period

Baseline Luteal Blood Flow and Serum Blood Draws

Cows in the experimental period were all of day 10-12 post estrus, therefore all CL were of the middle luteal stage. Prior to commencement of the experimental period, all cows' ovaries were scanned to ensure singular ovulation (polyovulation would exclude cows from the study) and presence of a CL. A baseline ultrasound scan (-15 mins) of the luteal blood flow was taken trans-rectally in power mode. All ultrasound videos performed during the experimental period

were as follows; frequency 6 MHz, PFR 2000 Hz, wall filter 2, gain 18, and depth 60 mm. Data from vessels with a flow range of 0-0.3 cm/s were captured.

A baseline jugular vein blood draw (-15 mins), for future serum progesterone analysis, was also performed. All blood samples for the experimental period were collected using glass vacutainer blood collection tubes with no additives. Samples were left to clot at 4^{o}C for 2 hrs. Thereafter, using wooden applicators, platelets from blood samples were shear activated against the wall of the blood tubes. Samples were then centrifuged at 1500 X g for 15 mins at 4^{o}C. The serum fraction was isolated and stored at -80^{o}C for future serum progesterone analysis.

Intrauterine Infusions

Following baseline assessments, cows were infused with an artificial insemination stylet preloaded with either 0.25 mL saline or 0.5 mg Dinoprost (prostaglandin F2 alpha analogue) in 0.25 mL saline. All artificial insemination straws (0.25 cc polyvinyl AI straws) were directly pre-loaded before infusions. Infusions were given in the uterine horn ipsilateral to the CL-bearing ovary at 0 hrs and 12 hrs of the experimental period. Cows were randomly assigned to an infusion group prior to the commencement of the experimental period.

Luteal Blood Flow and Serum Progesterone

Following infusions, luteal blood flow was analyzed using transrectal ultrasonography in power mode with the same parameters as previously listed in the baseline assessment. Luteal ultrasound scans occurred every 15 mins for the first hour, and every 60 mins thereafter for a total of 6 hrs—12 hrs total when considering both infusions. A final ultrasound scan was taken immediately prior to ovariectomies (30 hrs or 48 hrs following first infusion). Jugular vein blood draws for further serum progesterone analysis were also taken every 3 hrs for the first 24 hrs, and every 6 hrs until time of ovariectomy, using the same methodology as in the baseline assessment.

Quantification of Luteal Blood Flow

To quantify the influence of fish meal supplementation on luteal blood flow following low dose intra-uterine infusions of Dinaprost, we measured the changes in luteal blood coverage from the ultrasound scans. All luteal scans were stored in CINELOOP files and were converted to AVI files on the ExaPad mini. Files were then transported to a computer, wherein ImageJ (Fiji: version 2.6.0) was used for further analysis. The analyzer was blinded to all file names (including cow number) to reduce bias. The AVI files were selectively reduced to frames that contained minimal artifact and a full luteal scan (from apex to base of the CL). Artifact was defined as red spots appearing and disappearing within a single or double frame. Any uncertainty in artifact exclusions were secondarily assessed by an additional trained professional. Thereafter, pixel length was converted to cm (analyze>set measurement) using the provided scale on each ultrasound image. The maximal diameter of the gland was determined using the measure function (analyze>measure) on the identical and adjacent B-mode image to ensure accuracy of the measurement, as red coloration tended to conceal the outer limit of the CL. The entire gland was then circled using the "polygon selections" tool (figure 2.2A). The circle drawn around the outline of the CL (region of interest; ROI) was then manually moved over to the power mode image, identical to how it was placed on B mode. In doing so, we ensured consistency when determining the ROI, as we only accounted for intra-luteal blood flow. The circled area was then duplicated (figure 2.2B) and converted to grey scale (Image>adjust>color threshold), wherein all red pixels were converted to black, and all non-red pixels were converted to white (threshold color B&W; figure 2.2C-D). The hue of the color threshold was set to 1, saturation set to 1 and thresholding method set to default (figure 2.2D). With the gland still outlined, the percentage of

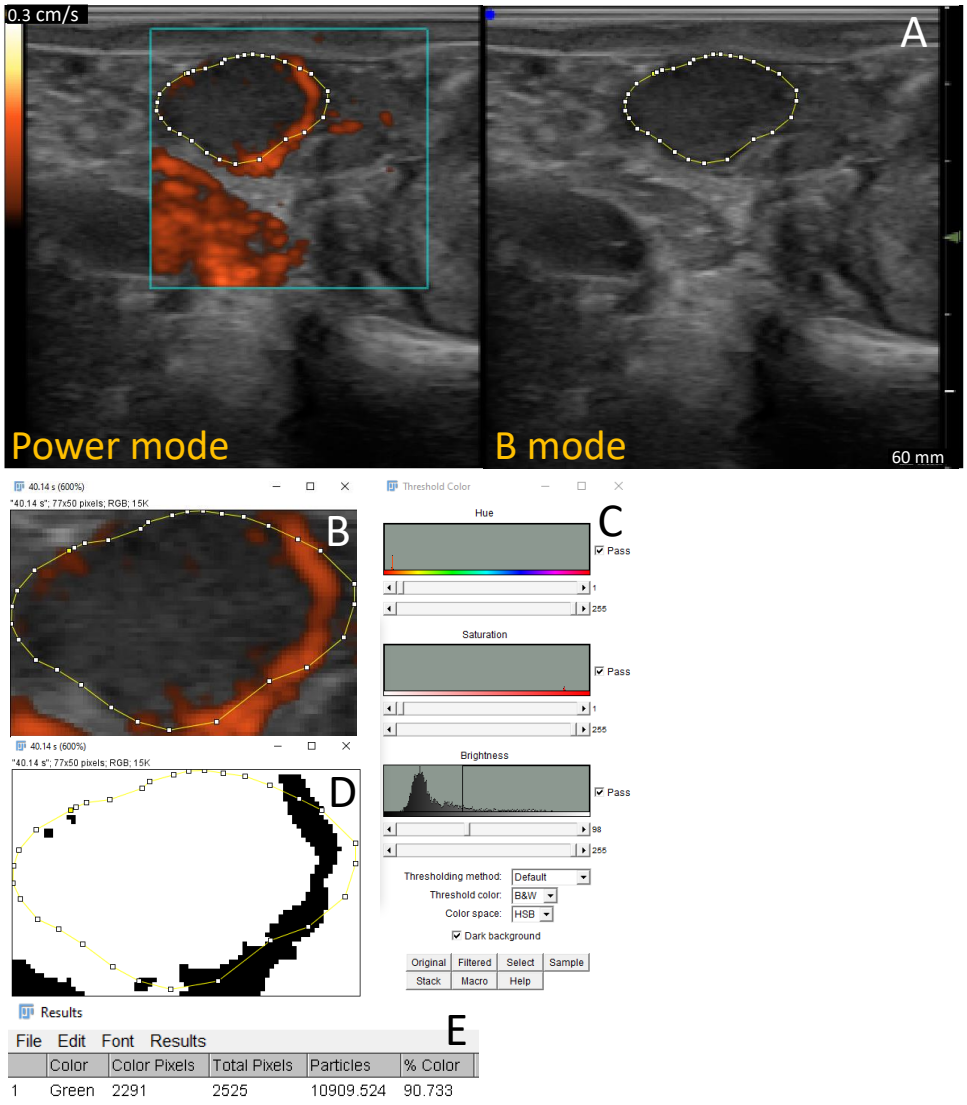
total black pixels was quantified using the color pixel counter plugin (plugins>color pixel counter; plugin:color:color_pixel_counter:start; figure 2.2E).

Data Analysis. The "% colored pixels" (percent non-colored pixels) was then subtracted from 1 to determine percent of black pixilation (100-% colored pixels). The same processes were repeated from frame to frame, until the glandular diameter was 10% of the maximal diameter. The maximal percentage of blood coverage falling within the 10% window was reported as the CL blood flow for the animal at said timepoint.

Statistical Analysis. With a sample size of 30, assessment of normality was not required nor performed. A PROC MIXED model with repeated measures was used to determine overall effects, as previously outlined. An autoregressive covariance structure was used in the repeated measures model, consistent with the previously mentioned. Variables assessed included cow, time of scan, supplementation, infusion, CL functionality and all possible interactions. If main effects of interactions were significant ($p<0.05$), a preplanned pairwise *t*-test comparisons analysis was used with the PDIFF function to determine statistical differences between groups.

Figure 2.2

Quantifying Luteal Blood Flow From Ultrasonography Scans Using ImageJ Software



Note. A) Representative power Doppler ultrasound image of the corpus luteum (CL) and associated blood flow on the left, and B mode of same scan to demonstrate CL boundaries. Region of interest (ROI) was outlined in yellow. Settings used for all ultrasound scans were as follows; frequency 6 MHz, PFR 2000 Hz, wall filter 2, gain 18, and depth 60 mm. Blood flow was only captured in vessels with flow slower than 0.3 cm/s. Range of speeds per pixel is indicated by color of pixel; black=0 cm/s, white= 0.3 cm/s (left color bar). B) The ROI was then duplicated. C-D) The duplicated RIO was color threshold adjusted to black and white (B&W), hue set to 1, saturation set to 1, and thresholding method set at default. E) Using the color pixel counter plugin, the percentage of colored pixels (white) compared to black pixels was compared. The percentage of blood flow was quantified by subtracting the % color value from 100 (100-% color).

Serum Progesterone Extraction and Quantification

Prior to the quantification of serum progesterone, to remove any progesterone bound to carrier molecules, a double organic phase extraction was performed. Stored serum samples were first thawed at room temperature. A total of 100 uL of serum was added to a 13 X 100 disposable Borosilicate glass tube, followed by the addition of 1 mL of petroleum ether, performed in a fume hood. The tubes and contents were vortexed for 1 min to allow for complete extraction of the upper organic phase (containing progesterone) from the lower inorganic phase (containing aqueous binding proteins). Thereafter, tubes were placed in a -80^{o}C freezer for 15-20 mins, or until solidification of the aqueous phase. The organic phase was then decanted into a new set of clean glass tubes. Tubes were then placed in a 42^{o}C heat block, and the petroleum ether was further evaporated with nitrogen gas. The process was repeated one more time with the original (thawed) aqueous phase. The dried, double extracted samples were reconstituted in 1.5 mL of Elisa buffer, which was provided in the Cayman Chemical Progesterone ELISA Kit (582601). Samples were vortexed, covered with parafilm, and left to incubate at 4^{o}C overnight to ensure complete reconstitution of progesterone into the buffer. The following day, the Progesterone ELISA Kit was used to quantify the concentration of progesterone in serum, as per the manual provided by Cayman Chemical. Briefly, all samples were run in duplicate, and an additional control was run with all samples to quantify the assay coefficient of variation ($\frac{Standard\ deviation}{mean}\ X\ 100$). All samples were measured at 412 nm on a SpectraMax® 190 Absorbance Microplate Reader (Molecular Devices).

Data Analysis. A CL was defined as functional if the cows' blood progesterone was maintained above 1 ng/ mL at time of ovariectomy (30 or 40 hrs following first infusion). The ratio of functional CL was defined as $(\frac{\#functional\ CL}{\#total\ CL} X\ 100)$.

Statistical Analysis. With a sample size greater than 30, assessment of normality was not required nor performed. A PROC MIXED with repeated measures was used to determine main effects, as previously outlined. An autoregressive covariance structure was used in the repeated measures model, as previously defined. Variables included in the model were cow, hour of blood draw, supplementation, infusion group and all possible interactions. If main effects of interactions were significant ($p<0.05$), a preplanned pairwise *t*-test comparisons analysis was used with the PDIFF function to determine statistical differences between groups. When determining statistical differences of functional CL between supplementation groups, a chi-squared test was utilized.

Ovariectomies and Luteal Tissue Preparation

Cows were then prepared for standing flank single ovariectomies (surgery) 48 hrs following the first infusion. Prior to surgery, cows were held from feed for 24 hrs and water for 12 hrs. Immediately before surgery, cows were administered 650 mg of Flunixin Meglumine (Mereck NADA 101-479) I.V. and 9900 mg of Noromycin 300 LA Oxytetracycline (Norbrooks Labs NADA 141-143) I.M. Next, a 60 cm X 60 cm square was shaved in and around the paralumbar fossa with shears, and thoroughly cleaned with 70% ethanol and betadine. Cows were then locally anesthetized in an inverted-L infiltration pattern (targeting the T13-L5 vertebra nerve plexus) with 4% subdermal Lidocaine (VetOne NDC 13985-222-04) injections. Following the confirmation of a successful nerve block, a sterile surgical drape was placed across the cow to maintain a clean incision site, and a 30 cm X 30 cm hole was incised into the drape. Next, a

lateral 20 cm incision was made through the integument of the cow with a surgical blade. All abdominal muscles were bluntly dissected in the direction of muscle striation to avoid excessive bleeding, and a small incision was made in the peritoneum. The ovary was then located, and blood supply was clamped off at the ovarian peduncle with an umbilical cord clamp. The ovary was removed from the mesovarium using surgical scissors. Upon removal of the ovary from the cow, the CL was dissected from the ovarian cortex, weighed, measured, and photographed. Following removal of the ovary, the peritoneum and abdominal muscles were sutured closed with #3 Catgut (Jorgensen Laboratories J0091C20), using a continuous suture pattern. The integument was further sutured closed with #2 Braunamid Suture (Jorgensen Laboratories JOR0091030) using a continuous cross stitch pattern.

Every 24 hrs, and for three days post-surgery, cows were administered 650 mg of Flunixin Meglumine IV, rectal temperatures were recorder for monitoring of infection, and sutures were examined for inflammation or infection. Sutures were removed two weeks following surgery.

CHAPTER III

RESULTS

The Influence of Docosahexaenoic Acid and Eicosapentaenoic Acid on Tumor Necrosis Factor Alpha and Interferon Gamma-Induced Cytotoxicity on Bovine Luteal Cells

Steroidogenic Cell Population in Culture

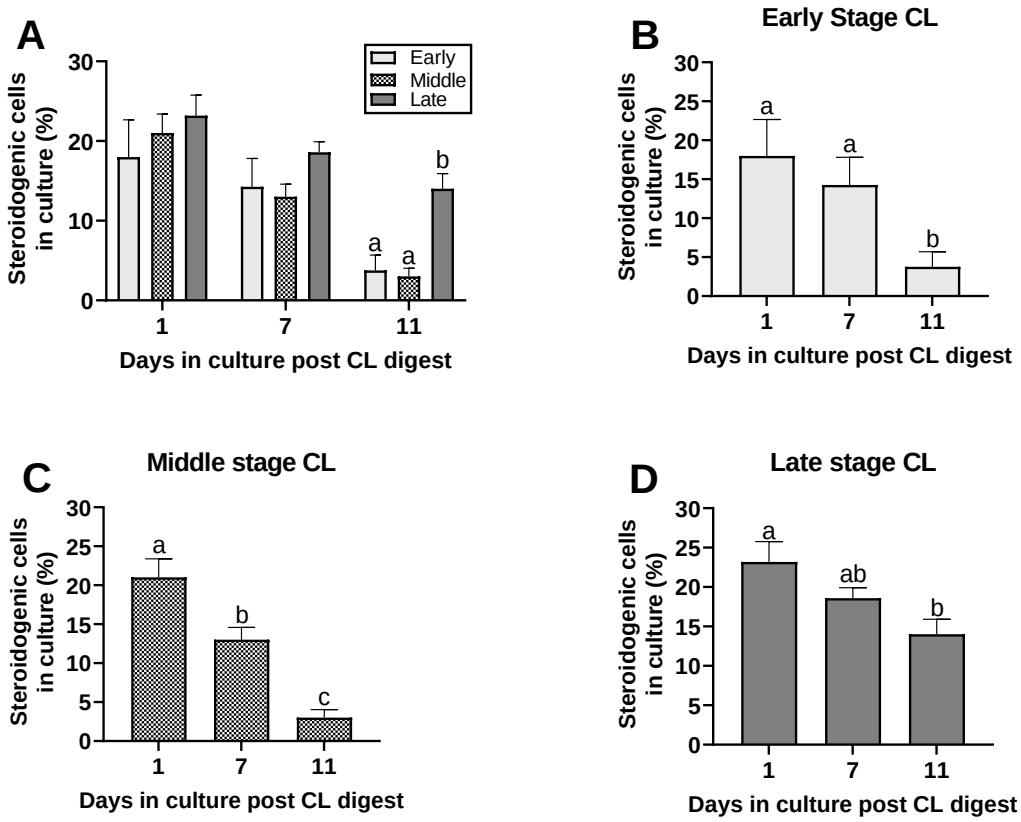
A nitro tetrazolium blue assay was used to estimate the percentage of steroidogenic cells at 1, 7 and 11 days of culture. There was a main effect of stage of CL on percentage of steroidogenic cells. The percentage of steroidogenic cells isolated from early (day 1-5 of the estrous cycle [EC]; n=4), middle (day 6-12 of the EC; n=6) or late (day 13-17 of the EC; n=5) did not differ at 1 or 7 days of culture ($p>0.05$; figure 3.1A). However, there was a greater decrease in percentage of steroidogenic cells at 11 days of culture for tissue obtained from early and mid CL as compared to cells obtained from late CL.

There was a main effect of time on percentage of steroidogenic cells. When comparing the percentage of steroidogenic cells over time in culture within early staged cells, populations did not significantly differ ($p<0.05$) until day 11 in culture (figure 3.1B), indicating experiments should not proceed longer than 7 days. The percentage of steroidogenic cells in middle staged cells significantly decreased ($p<0.05$) from day 1, 7, and 11 in culture (figure 3.1C). These results indicate that the population of phenotypically conserved steroidogenic cells was not consistent by the end of experiments in middle staged cells. In late staged cells, the steroidogenic population was maintained until day 7 in culture, followed by a significant decreased ($p<0.05$) at

day 11 compared to day 1 (figure 3.1D), meaning steroidogenic cells were most likely preserved in experiments. Although late-stage cells maintain a greater steroidogenic phenotype by day 11 in culture compared to the early and middle staged cells, the relative percentage (within stage) significantly declines over time, like the other two stages.

Figure 3.1

3 Beta-Hydroxysteroid Dehydrogenases (3βHSD) Positive Steroidogenic Cell Populations Declined 11 Days in Culture Following Enzymatic Digest



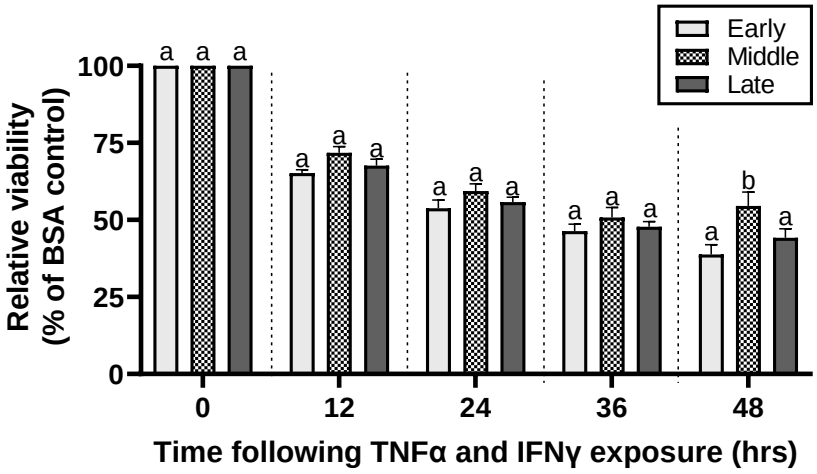
Note. Corpora lutea (CL) were first staged as early: days 1-5 of the estrous cycle (EC; n=4), middle: days 6-12 of the EC (n=6), or late: days 13-17 of the EC (n=5). Following CL staging and enzymatic digest, cells were plated and cultured for 1, 7, or 11 days. The percentage of steroidogenic luteal cells was determined using a nitro tetrazolium blue (NTT) assay, wherein cells containing active 3βHSD convert NTT (and so cells) to a dark blue color. Letters (a, b, c) indicate statistically significant differences ($P<0.05$) within day and between stage (A) and within stage between days (B-D). Data are presented as mean ± SEM.

The Cytotoxicity of Tumor Necrosis Factor Alpha and Interferon Gamma on Differentially Staged Bovine Luteal Cells

Cells isolated from early (n=5), middle (n=5), or late (n=10) CL were exposed to tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ) for 0 to 48 hrs to investigate the influence of CL stage on cytokine cytotoxicity. Cells obtained from middle stage CL were more viable ($p<0.05$) 48 hrs following exposure to cytokines when compared to cells obtained from early and late staged CL (figure 3.2).

Figure 3.2

Mixed Bovine Luteal Cells of a Middle-Staged Corpus Luteum (CL) Were Less Sensitive to the Cytotoxic Effects of Tumor Necrosis Factor Alpha (TNFα) and Interferon Gamma (IFNγ) Exposure



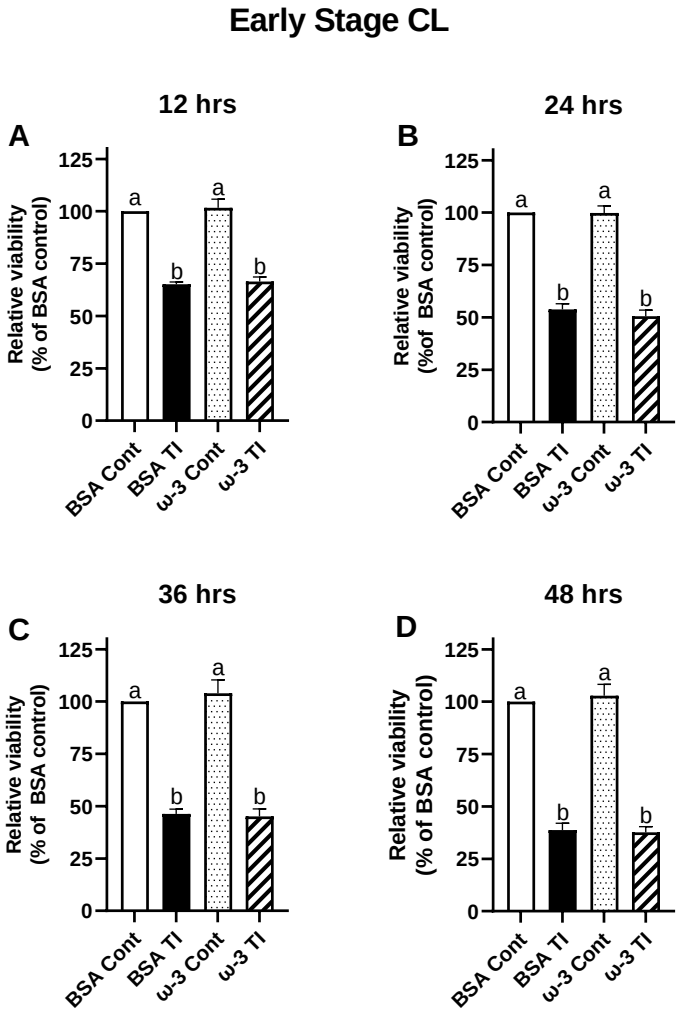
Note. Cells were exposed to 2.3 nM TNFα and 2.5 nM IFNγ for 0, 12, 24, 36, or 48 hrs. An adenosine 5-triphosphate luminescence assay was utilized to assess cellular viability, and all values were normalized to untreated controls. Early= cells from CL isolated between days 1-5 of the estrous cycle (EC; n=5). Middle= days 6-12 of the EC (n=5). Late=days 13-17 of the EC (n=10). Differences in letters (a, b) indicate significant differences ($p<0.05$) between stages within time following TNFα and IFNγ exposure. Data are presented as mean ± SEM.

Eicosapentaenoic Acid and Docosahexaenoic Acid Pre-Treatment on Tumor Necrosis Factor Alpha and Interferon Gamma Induced Cytotoxicity

Cells isolated from early (n=5), middle (n=5), and late CL (n=10) were pre-treated with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) to determine the influence of omega-3 fatty acids on the cytotoxic effects (relative viability) of tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ). Cellular viability in early staged cultures did not differ ($p>0.05$) between bovine serum albumin controls (BSA Cont) and EPA and DHA pre-treated controls (ω-3 Cont) 12, 24, 36, or 48 hrs of culture (figure 3.3A-D). Cells exposed to TNFα and IFNγ (BSA TI and ω -3 TI) underwent a significant reduction ($p<0.05$) in relative viability (compared to controls) at all time points (Figure 3.3A-D). BSA TI and ω -3 TI groups did not significantly differ ($p>0.05$) in relative viability (Figure 3.3A-D). Cellular viability was identical for middle staged cells 12-, 24-, and 36-hrs following cytokine exposure (figure 3.4A-C). However, cellular viability was significantly increased ($p<0.05$) in the ω -3 Cont when compared to the BSA Cont, indicating that in middle stage cells, DHA and EPA may increase relative viability 48 hrs following pre-treatment (figure 3.4D). Viability in late staged cells were like that of early staged cells, in that no significant difference ($p>0.05$) occurred between controls (BSA Cont and ω -3 Cont), nor between cells exposed to TNFα and IFNγ with different pre-treatment groups (BSA TI and ω -3 TI: figure 3.5A-D). Likewise, cellular viability between unexposed controls (BSA Cont and ω -3 Cont) did not significantly differ ($p>0.05$) between TNFα and IFNγ exposed cells (BSA TI and ω -3 TI).

Figure 3.3

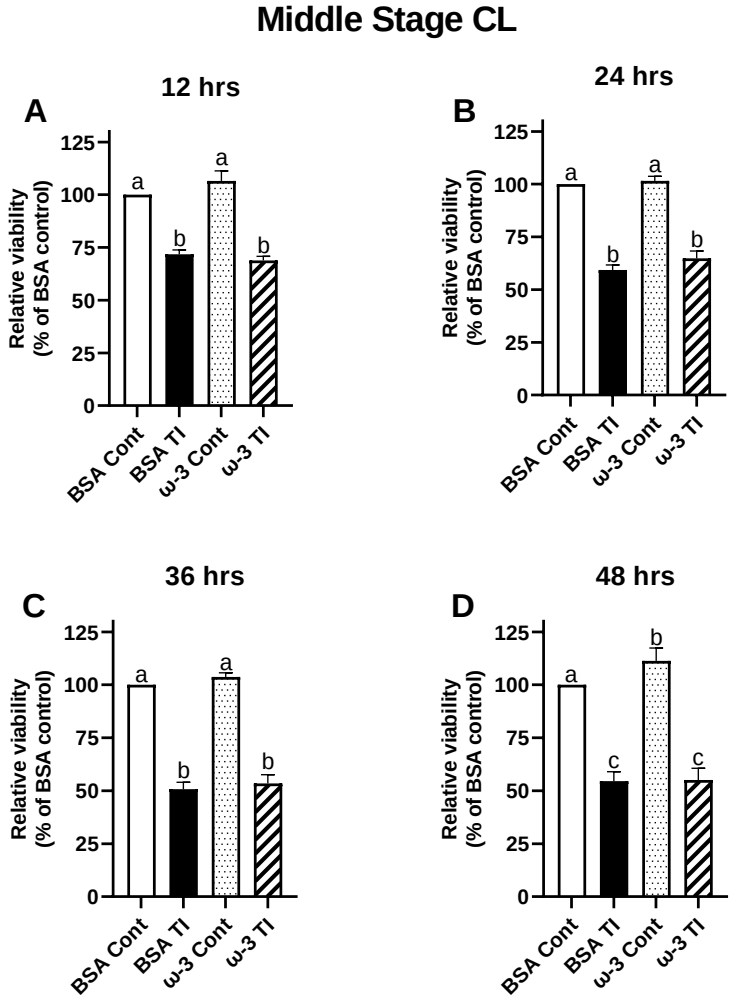
Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) Pre-Treatment Did Not Protect Luteal Cells Isolated From an Early-Staged Corpus Luteum (CL) From the Cytotoxic Effects of Tumor Necrosis Factor Alpha (TNFα) and Interferon Gamma (IFNγ)



Note. Cells were pre-treated with 10 uM DHA and 10 uM EPA for 72 hrs. Cells were then exposed to 2.3 nM TNFα & 2.5 nM IFNγ for 12 (A), 24 (B), 36 (C), or 48 hrs (D). An adenosine 5-triphosphate luminescence assay was utilized to assess cellular viability, and all values were normalized to BSA controls. Early-stage CL= isolated between days 1-5 of the estrous cycle (n=5 CL). BSA Cont= bovine serum albumin control; BSA TI= BSA and TNFα & IFNγ exposure; ω-3 Cont= DHA & EPA control; ω-3 TI= DHA & EPA and TNFα & IFNγ. A-D) Differences in letters (a, b) indicate statistical differences ($P<0.05$) between treatment groups. Data are presented as mean ± SEM.

Figure 3.4

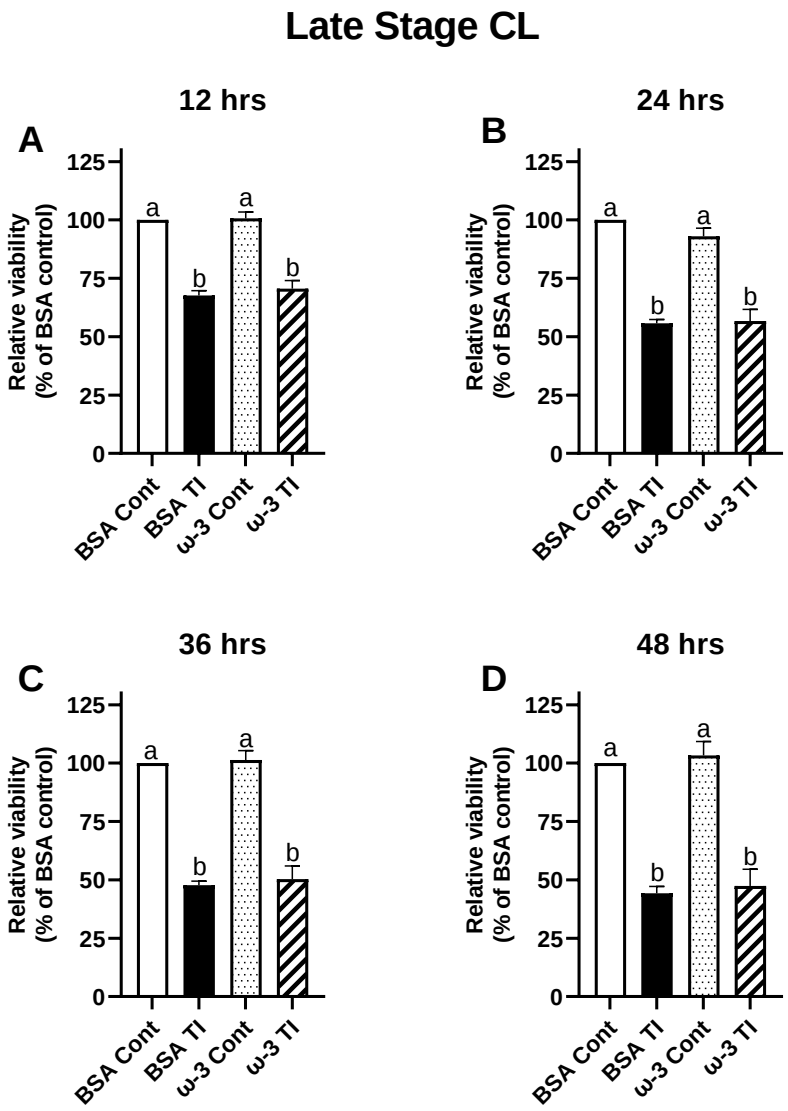
Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) Pre-Treatment Did Not Protect Luteal Cells Isolated From a Middle-Staged Corpus Luteum (CL) From the Cytotoxic Effects of Tumor Necrosis Factor Alpha (TNFα) and Interferon Gamma (IFNγ)



Note. Cells were pre-treated with 10 uM DHA and 10 uM EPA for 72 hrs. Cells were then exposed to 2.3 nM TNFα & 2.5 nM IFNγ for 12 (A), 24 (B), 36 (C), or 48 hrs (D). An adenosine 5-triphosphate luminescence assay was utilized to assess cellular viability, and all values were normalized to BSA controls. Middle-stage CL= isolated between days 6-12 of the estrous cycle (n=5 CL). BSA cont= bovine serum albumin control; BSA Cont= bovine serum albumin control; BSA TI= BSA and TNFα & IFNγ exposure; ω-3 Cont= DHA & EPA control; ω-3 TI= DHA & EPA and TNFα & IFNγ. A-D) Differences in letters (a, b, c) indicate statistical differences ($P<0.05$) between treatment groups. Data are presented as mean ± SEM.

Figure 3.5

Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) Pre-Treatment Did Not Protect Luteal Cells Isolated From a Late-Staged Corpus Luteum (CL) From the Cytotoxic Effects of Tumor Necrosis Factor Alpha (TNFα) and Interferon Gamma (IFNγ)



Note. Cells were pre-treated with 10 uM DHA and 10 uM EPA for 72 hrs. Cells were then exposed to 2.3 nM TNFα & 2.5 nM IFNγ for 12 (A), 24 (B), 36 (C), or 48 hrs (D). An adenosine 5-triphosphate luminescence assay was utilized to assess cellular viability, and all values were normalized to BSA controls. Late-stage CL= isolated between days 13-17 of the estrous cycle (n=10). BSA cont= bovine serum albumin control; BSA Cont= bovine serum albumin control; BSA TI= BSA and TNFα & IFNγ exposure; ω-3 Cont= DHA & EPA control; ω-3 TI= DHA & EPA and TNFα & IFNγ. Differences in letters (a, b) indicate statistical differences ($P<0.05$) between treatment groups. Data are presented as mean ± SEM.

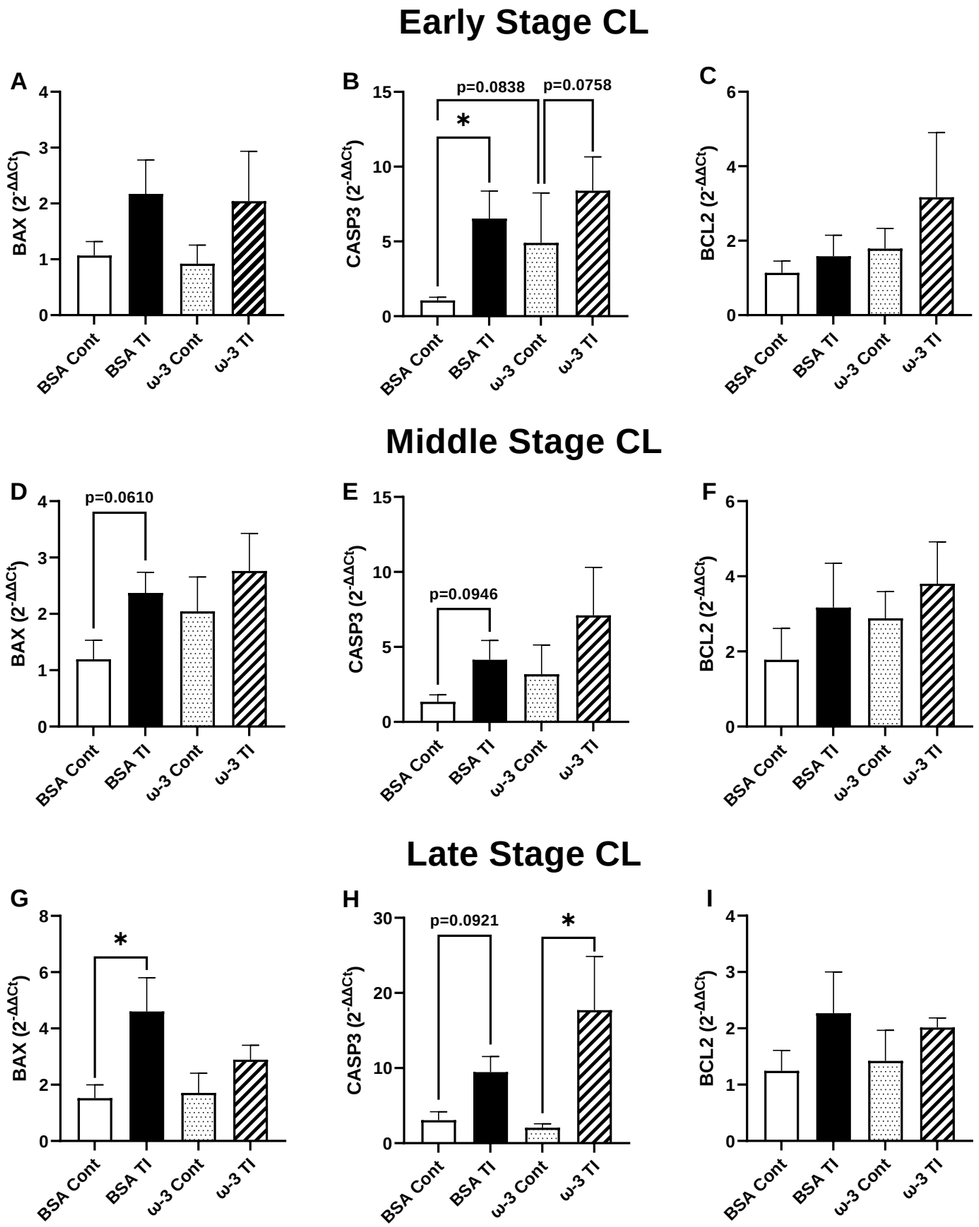
Apoptotic Gene Expression

Mixed bovine luteal cells isolated from early (n=4), middle (n=5), or late (n=5) CL were pre-treated with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) to determine the influence of omega-3 fatty acids on apoptotic gene expression following tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ) exposure. No significant differences ($p>0.05$) or trends ($p>0.1$) between *BAX* or *BCL2* were observed in early staged cultures, regardless of treatment (figure 3.6A, figure 3.6C). There was, however, a statistical increase in CASP3 expression between the BSA Cont and BSA TI groups, indicating TNFα and IFNγ exposure increased expression of a pro-apoptotic gene (figure 3.6B). Furthermore, cells within the ω -3 Cont had a higher expression (trend; $p<0.1$) of *CASP3* when compared to the BSA Cont, indicating EPA and DHA regulate transcriptional control of the apoptotic pathway, independent of external stimuli in the early CL. However, the effect of EPA and DHA pre-treatment did not appear to predispose cells for elevated *CASP3* expression following TNFα and IFNγ exposure, as BSA TI and ω -3 TI groups did not significantly differ ($p>0.05$). Within middle staged cultures, the only trends ($p<0.1$) observed were increased expression of *BAX* and *CASP3* in the BSA TI group when compared to the BSA control, indicating exposure to TNFα and IFNɣ, but not EPA and DHA pre-treatment, increase apoptotic gene expression (figure 3.6D, figure 3.6E). Gene expression for *BCL2* did not significantly differ ($p>0.05$) and no trends were observed ($p>0.1$; Figure 3.6F), indicating neither EPA and DHA pre-treatment or TNFα and IFNɣ exposure influenced *BCL2* expression in middle staged cultures. Late staged cultures exposed to TNFα and IFNɣ had a significant increase ($p<0.5$; BSA TI) in *BAX* expression compared to the BSA Cont, independent of EPA and DHA pre-treatment ($p>0.5$; figure 3.6G). *CASP3* expression was significantly elevated ($p<0.05$; ω -3 Cont) and trended towards elevation ($p<0.1$; BSA TI)

following TNFα and IFNɣ exposure, independent of EPA and DHA pre-treatment as neither BSA Cont vs. ω -3 Cont nor BSA TI vs. ω -3 TI significantly differed ($p>0.05$; figure 3.6H). Consistent with early and middle staged cultures, late cultures had no significant changes ($p>0.5$) or trends ($p>0.1$) in *BCL2* expression between groups (figure 3.6I).

Figure 3.6

Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) Pre-Treatment Altered Apoptotic Gene Expression in Cells From Early-Staged Corpora Lutea (CL), but Not Those of a Middle or Late-Staged CL Following Exposure to Tumor Necrosis Factor Alpha (TNFα) and Interferon Gamma (IFNɣ)



Note. Cells were pre-treated with 10 uM EPA and 10 uM DHA for 72 hrs, and subsequently exposed to 2.3 nM TNFα and 2.5 nM IFNɣ for 24 hrs. The deltadelta C_t method ($2^{-\Delta\Delta Ct}$) was used when analyzing fold change of steady state mRNA. A-C) Cells isolated from early staged CL= day 1-5 of the estrous cycle (EC; n=4), D-F) Cells isolated from middle staged CL= day 6-12 of the EC (n=5). G-I) Cells isolated from late staged CL= day 13-17 of the EC (n=5). BSA Cont= bovine serum albumin control; BSA TI= BSA and TNFα & IFNγ exposure; ω-3 Cont= DHA & EPA control; ω-3 TI= DHA & EPA and TNFα & IFNγ. *Significant difference ($p<0.05$) between treatments. p values were reported if $p<0.1$, indicating statistical trend. Data are presented as mean ± SEM.

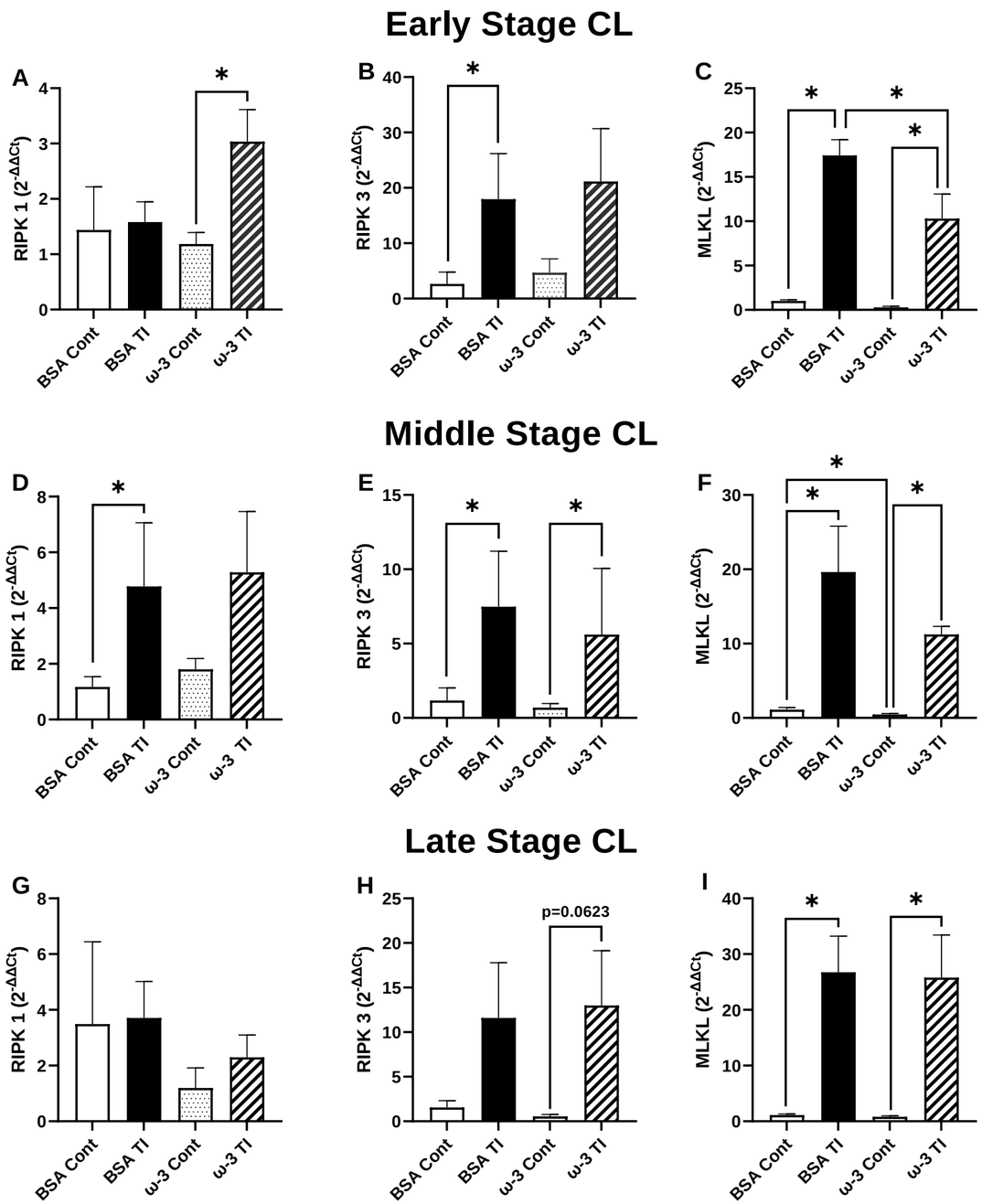
Necroptotic Gene Expression

Mixed bovine luteal cells isolated from early (n=4), middle (n=5), or late (n=5) CL were pre-treated with EPA and DHA to determine the influence of omega-3 fatty acids on necroptotic gene expression following TNFα and IFNɣ exposure. Within early staged cultures, only cells from the ω -3 TI group had significantly elevated expression of *RIPK1* ($p<0.05$; figure 3.7A). However, because the BSA TI and the ω-3 TI did not statistically differ, it cannot be concluded that EPA and DHA pre-treatment differentially influenced the expression of *RIPK1*. *RIPK3* gene expression was significantly increased ($p<0.05$) following TNFα and IFNɣ exposure and was not differentially influenced by EPA and DHA pre-treatment (figure 3.7B). *MLKL* gene expression was elevated in both BSA TI, and ω-3 TI groups compared to controls, however, ω-3 TI was significantly lower ($p<0.05$) when compared to BSA TI (figure 3.7C). These results partially support the hypothesis that EPA and DHA pre-treatment reduce necroptotic gene expression following exposure to TNFα and IFNɣ in the early phase CL. Middle staged cultures exposed to TNFα and IFNɣ had increased expression ($p<0.05$) of *RIPK1* (ω-3 TI; figure 3.7D) and *RIPK3* (ω-3 TI and BSA TI; figure 3.7E). However, elevation in gene expression within both genes was independent of EPA and DHA pre-treatment due to an insignificant difference ($p>0.05$) between BSA TI and ω-3 TI groups. *MLKL* was significantly lower ($p<0.05$) in the ω-3 Cont cultures compared to the BSA Cont, indicating that EPA and DHA pre-treatment may reduce basal transcriptional activation of *MLKL* (figure 3.7F). However, the reduction in gene expression was not observed following TNFα and IFNɣ exposure; *MLKL* expression was significantly elevated ($p<0.05$) following TNFα and IFNɣ exposure in both BSA TI and ω-3 TI groups, and expression between groups did not significantly differ ($p>0.05$). Cells from late staged cultures had no significant difference ($p>0.05$) in expression of *RIPK1* (figure 3.7G). *RIPK3* expression trended

towards significant elevation ($p<0.1$; ω-3 TI) following TNFα and IFNɣ but did not significantly differ ($p>0.05$) nor trend different ($p>0.1$) between BSA TI and ω-3 TI groups, indicating changes in gene expression were independent of EPA and DHA pre-treatment (figure 3.7H). *MLKL* expression was significantly elevated ($p<0.05$) in both BSA TI and ω-3 TI groups but did not significantly differ ($p>0.05$) between groups, indicating *MLKL* expression was independent of EPA and DHA pre-treatment (figure 3.7I).

Figure 3.7

Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) Pre-Treatment Altered Necroptotic Gene Expression in Cells From Early and Middle Staged Corpora Lutea (CL), but Not Those of a Late-Staged CL Following Exposure to Tumor Necrosis Factor Alpha (TNFα) and Interferon Gamma (IFNγ)



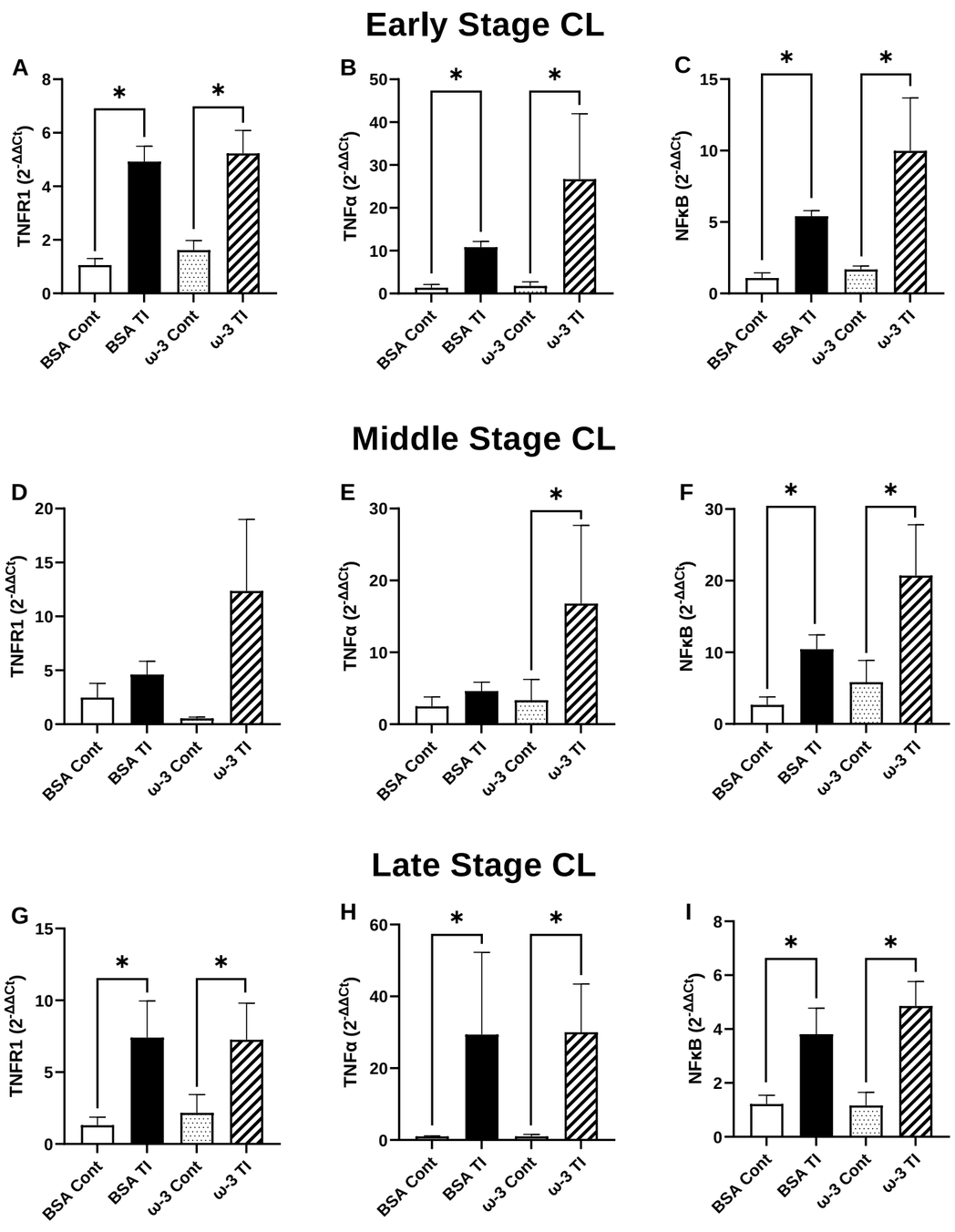
Note. Cells were pre-treated with 10 uM EPA and 10 uM DHA for 72 hrs, and subsequently exposed to 2.3 nM TNFα and 2.5 nM IFNγ for 24 hrs. The delta delta C_t method ($2^{-\Delta\Delta Ct}$) was used when analyzing fold change of steady state mRNA. A-C) Cells isolated from early staged CL= day 1-5 of the estrous cycle (EC; n=4). D-F) Cells isolated from middle staged CL= day 6-12 of the EC (n=5). G-I) Cells isolated from late staged CL= day 13-17 of the EC (n=5). BSA Cont= bovine serum albumin control; BSA TI= BSA and TNFα & IFNγ exposure; ω-3 Cont= DHA & EPA control; ω-3 TI= DHA & EPA and TNFα & IFNγ. *Significant difference ($p<0.05$) between treatments. p values were reported if $p<0.1$, indicating statistical trend. Data are presented as mean ± SEM.

Programed Cell Death Related Gene Expression

Mixed bovine luteal cells isolated from early (n=4), middle (n=5), or late (n=5) CL were pre-treated with EPA and DHA to determine the influence of omega-3 fatty acids on cell death related genes following TNFα and IFNɣ exposure. Cells from early staged cultures exposed to *TNFα* and *IFNγ* had a significant increase ($p<0.05$) in *TNFR1* (figure 3.8A), *TNFα* (figure 3.8B), and *NFκB* (figure 3.8C) expression. However, as both BSA TI and ω-3 TI groups did not significantly differ ($p>0.05$), expression pattern of all genes was independent of EPA and DHA pre-treatment. No significant changes ($p>0.05$) nor trended towards differences ($p>0.1$) were detected in TNFR1 gene expression between groups of a middle CL (figure 3.8D). This indicates *TNFR1* expression may be differentially regulated in a middle CL. However, it is important to note the variation as a potentially confounding source. *TNFα* expression was significantly elevated ($p<0.05$) following TNFα and IFNγ exposure in the ω-3 TI group (figure 3.8E). Although the BSA TI groups did not significantly differ from controls ($p<0.05$), results also did not differ from the ω-3 TI group, suggesting the elevated expression is not a result of the EPA and DHA pre-treatment. *NFκB* gene expression was significantly elevated ($p<0.05$) in both the BSA TI and the ω-3 TI groups, but because expression did not significantly differ ($p>0.05$) between groups, results are not due to EPA and DHA pre-treatment (figure 3.8F). Within late staged cultures, *TNFR1* (figure 3.8G), *TNFα* (figure 3.8H), and *NFκB* (figure 3.8I) were all significantly elevated ($p<0.05$) in BSA TI and ω-3 TI groups, but not between groups ($p>0.05$), indicating changes were independent of EPA and DHA pre-treatment.

Figure 3.8

Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) Pre-Treatment Did Not Differentially Alter Programmed Cell-Death Associated Gene Expression in Cells From Early, Middle, or Late-Staged Corpora Lutea (CL) Following Expos ure to Tumor Necrosis Factor Alpha (TNFα) and Interferon Gamma (IFNγ)



Note. Cells were pre-treated with 10 uM EPA and 10 uM DHA for 72 hrs, and subsequently exposed to 2.3 nM TNFα and 2.5n M IFNγ for 24 hrs. The delta delta C_t method ($2^{-\Delta\Delta Ct}$) was used when analyzing fold change of steady state mRNA. A-C) Cells isolated from early staged CL= day 1-5 of the estrous cycle (EC; n=4). D-F) Cells isolated from middle staged CL= day 6-12 of the EC (n=5). G-I) Cells isolated from late staged CL= day 13-17 of the EC (n=5). BSA Cont= bovine serum albumin control; BSA TI= BSA and TNFα & IFNγ exposure; ω-3 Cont= DHA & EPA control; ω-3 TI= DHA & EPA and TNFα & IFNγ. *Significant difference ($p<0.05$) between treatments. p values were reported if $p<0.1$, indicating statistical trend. Data are presented as mean ± SEM.

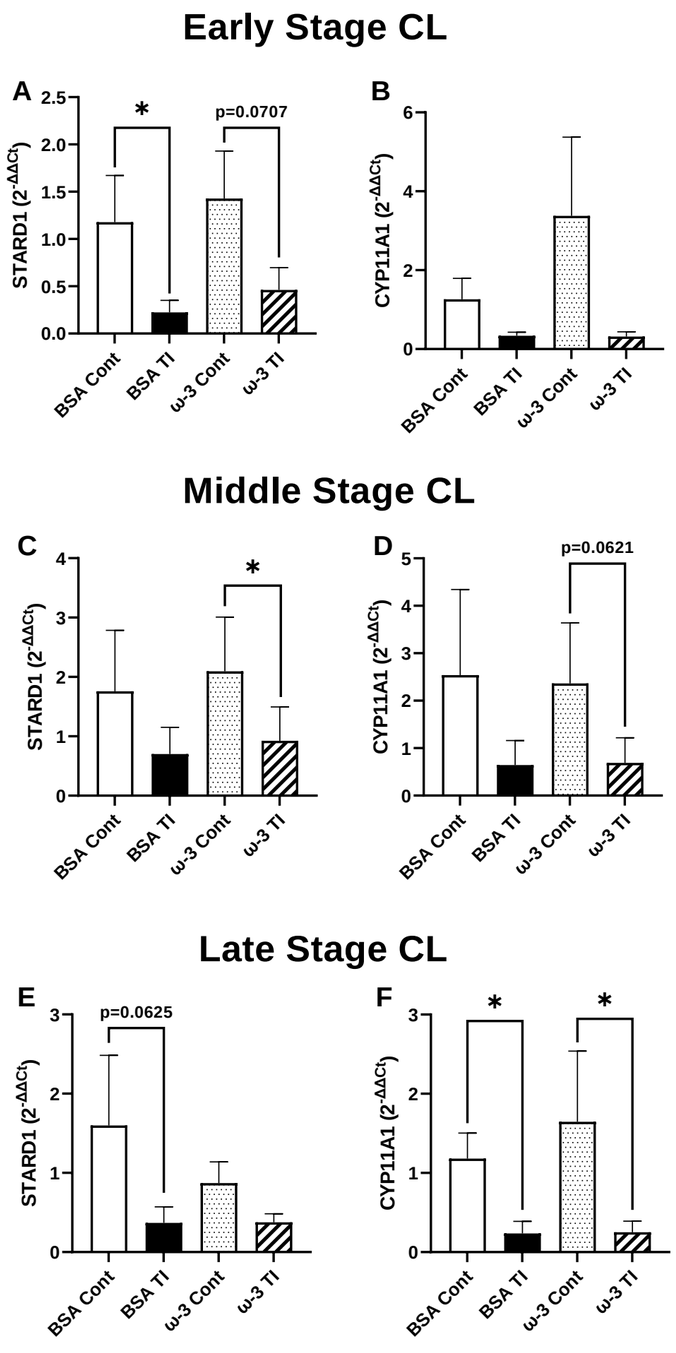
Steroidogenic Gene Expression

Mixed bovine luteal cells isolated from early (n=4), middle (n=5), or late (n=5) CL were pre-treated with EPA and DHA to determine the influence of omega-3 fatty acids on steroidogenic genes following TNFα and IFNɣ exposure. Early staged cultures exposed to TNFα and IFNɣ underwent a significant decrease ($p<0.05$; BSA TI) and trended towards a decrease ($p<0.1$; ω-3 TI) in *STARD1* gene expression (figure 3.9A). Since no significant difference ($p>0.05$) was detected between BSA TI and ω-3 TI groups, the reduction in *STARD1* expression is not equated to EPA and DHA pre-treatment. No significant differences ($p>0.05$) or trends ($p>0.1$) existed with regards to *CYP11A1* gene expression (figure 3.9B). Following exposure to TNFα and IFNɣ, middle staged cultures had a significant reduction ($p<0.05$; ω-3 TI) in *STARD1* gene expression (figure 3.9C). *CYP11A1* trended toward decreasing gene expression ($p<0.1$) in both BSA TI and ω-3 TI groups (figure 3.9D). Within both *STARD1* and *CYP11A1*, no statistical difference ($p>0.05$) occurred between BSA TI, and ω-3 TI treated groups, indicating reduction in steroidogenic gene expression is solely influenced by TNFα and IFNɣ exposure. In late staged cultures, cells exposed to TNFα and IFNɣ had a trending reduction ($p<0.1$; BSA TI) in *STARD1* gene expression (figure 3.9E). Since no statistical difference was found between BSA TI and ω-3 TI, the reduced gene expression was independent of EPA and DHA pre-treatment. *CYP11A1* significantly decreased in expression ($p<0.05$) in both BSA TI and ω-3 TI groups (figure 3.9F).

The overall reduction in steroidogenic gene expression following TNFα and IFNɣ supports the hypothesis that the cytokines reduce steroidogenic mRNA transcripts. Moreover, the hypothesis that EPA and DHA pre-treatment would maintain steroidogenic gene expression following TNFα and IFNɣ exposure was rejected.

Figure 3.9

Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) Pre-Treatment Did Not Differentially Alter Steroidogenic Gene Expression in Cells from Early, Middle, or Late-Staged Corpora Lutea (CL) Following Exposure to Tumor Necrosis Factor Alpha (TNFα) and Interferon Gamma (IFNɣ)



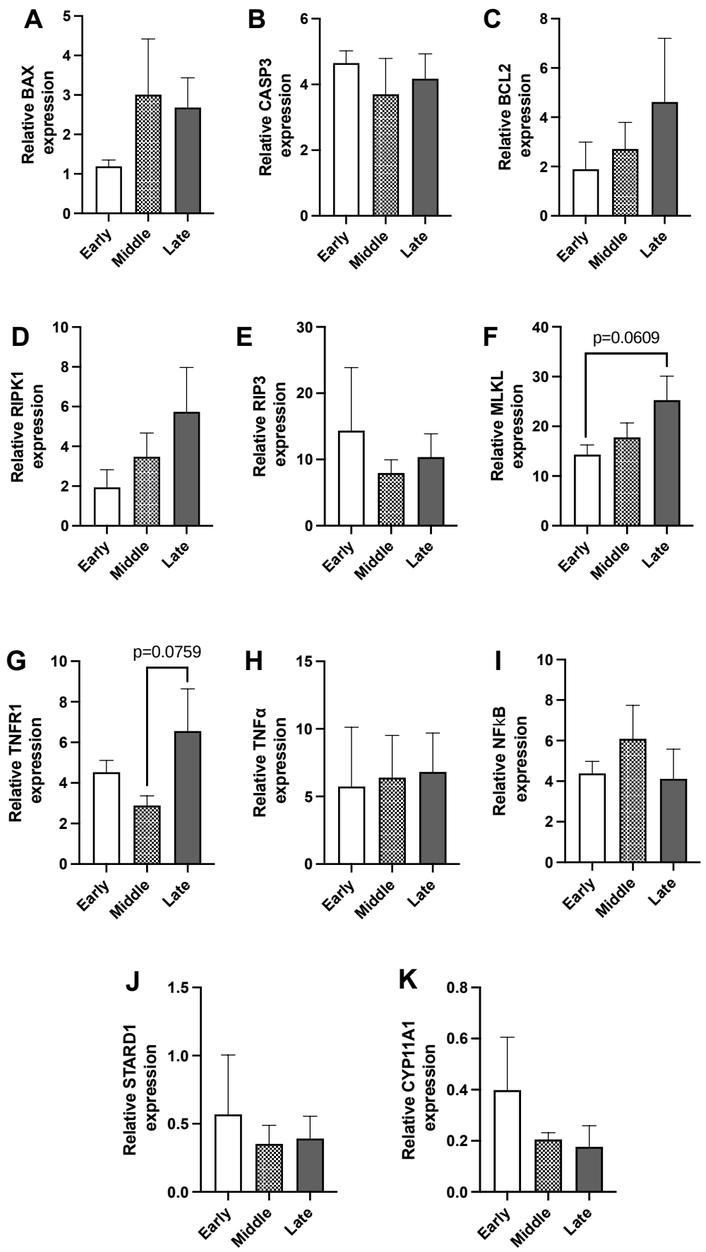
Note. Cells were pre-treated with 10 uM EPA and 10 uM DHA for 72 hrs, and subsequently exposed to 2.3 nM TNFα and 2.5 nM IFNγ for 24 hrs. The delta delta C_t method $3(2^{-\Delta\Delta Ct})$ was used when analyzing fold change of steady state mRNA. A-B) Cells isolated from early staged CL= day 1-5 of the estrous cycle (EC; n=4). C-D) Cells isolated from middle staged CL= day 6-12 of the EC (n=5). E-F) Cells isolated from late staged CL= day 13-17 of the EC (n=5). BSA Cont= bovine serum albumin control; BSA TI= BSA and TNFα & IFNγ exposure; ω-3 Cont= DHA & EPA control; ω-3 TI= DHA & EPA and TNFα & IFNγ. *Significant difference ($p<0.05$) between treatments. P values were reported if $p<0.1$, indicating statistical trend. Data are presented as mean ± SEM.

Gene Expression Across Stages Following Cytokine Exposure

We also investigated the influence of luteal stage (early [n=4], middle [n=5], and late [n=5]) on gene expression following exposure of mixed bovine luteal cells to TNFα and IFNγ. Changes in gene expression across luteal stages were quantified by generating a ratio of the delta C_t (2^{-DCt}) values in the BSA TI groups to the 2^{-DCt} in BSA Cont groups (relative gene expression). No differences ($p>0.05$) or trends ($p>0.1$) were observed between luteal stages for apoptosis associated genes, *BAX*, *CASP3* and *BCL2* (Figure 3.10A-C) nor in the steroidogenesis associate genes, *STARD1* and *CYP11A1* (Figure 3.10J-K). Interestingly, MLKL in cells isolated from a late CL had a near significant increase in expression following TNFα and IFNγ when compared to cells from early staged CL, indicating late CL may be more adept for necroptosis associated signaling. Moreover, cells isolated from late staged CL trended towards increased expression of *TNFR1* gene expression (figure 3.10G). The two aforementioned results are of particular interest as necroptotic signaling occurs via activation of the TNFRI.

Figure 3.10

Genes Associated With Necroptotic Cell Death Are More Abundant in Cells Isolated From Late-Stage Corpora Lutea (CL) Following Exposure to Tumor Necrosis Factor Alpha (TNFα) and Interferon Gamma (IFNγ)



Note. Cells were exposed to 2.3 nM TNFα and 2.5 nM IFNγ for 24 hrs, following which RNA was isolated. The relative expression of apoptotic (A-C), necroptotic (D-F), programmed cell death (G-I), and steroidogenic related genes was reported as ratio of the delta C_t ($2^{-\Delta Ct}$) of cells exposed to TNFα and IFNγ (BSA TI) to the $2^{-\Delta Ct}$ of cells exposed to BSA alone (BSA Cont). Cells isolated from early staged CL= day 1-5 of the estrous cycle (EC; n=4), middle staged CL= day 6-12 of the EC (n=5), and late staged CL= day 13-17 of the EC (n=5). P <0.1 indicates strong statistical trend. Data are presented as mean ± SEM.

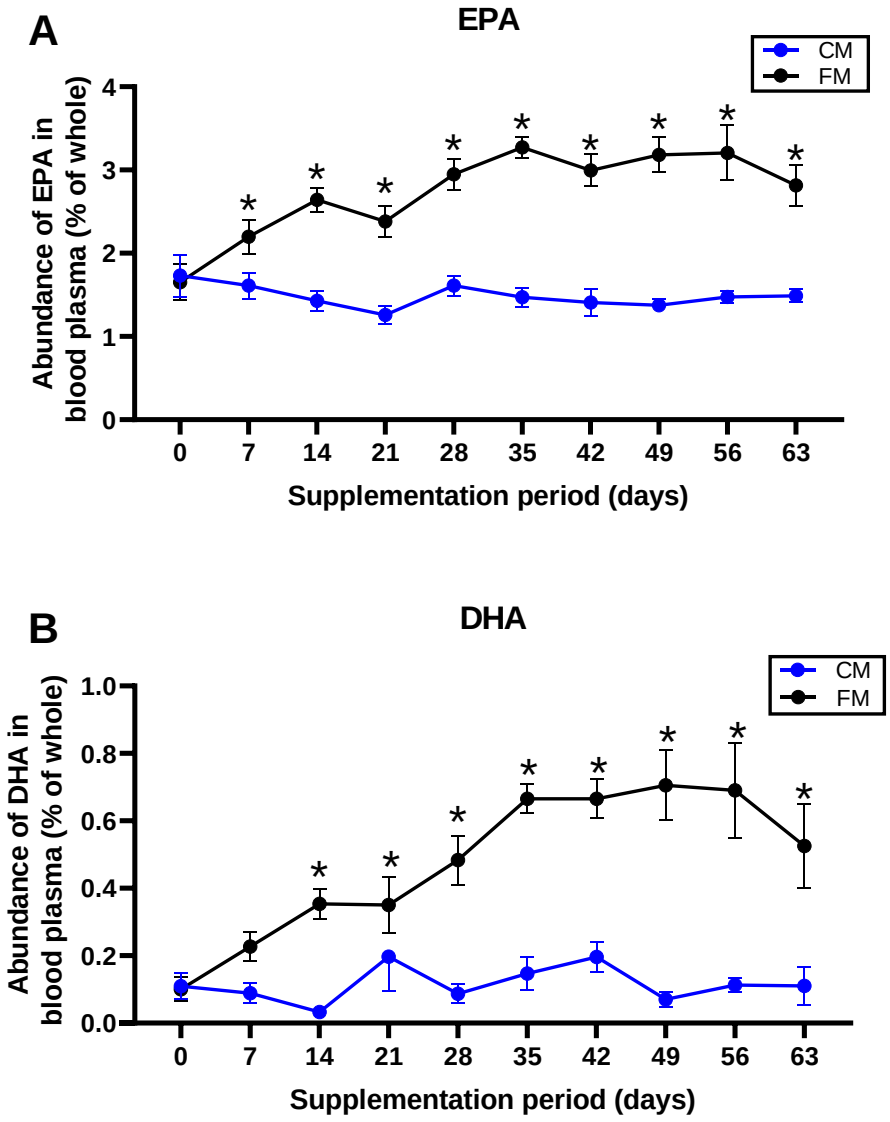
Fish Meal Supplementation on Luteal Blood Flow Following Two Low Dose Intra-Uterine Infusions of Prostaglandin F2 Alpha

Abundance of Plasma Eicosapentaenoic Acid and Docosahexaenoic Acid in Plasma

Cows were given either corn gluten meal (CM; n=9) or fish meal (FM; n=10) supplement for 63 days and weekly blood samples were drawn to quantify abundance of EPA and DHA in the plasma. Blood plasma EPA in FM supplemented cows was significantly more abundant ($p<0.05$) by day 7, and until day 63, when compared to CM supplemented cows (figure 3.11A), indicating EPA from the FM supplementation was successfully incorporated into the blood of animals. Blood plasma DHA in FM supplemented cows was significantly elevated ($p<0.05$) by day 14, and remained elevated by day 63, when compared to CM supplemented cows (figure 3.11B), therein signifying successful incorporation of DHA into blood. Both omega-3 fatty acids were effectively, and differentially incorporated into blood plasma within two weeks of supplementation. By the time of the experimental period (directly following day 63 of supplementation), cows had a significant difference in both EPA and DHA concentrations, indicating any results of the experimental period could be due to supplementation effects.

Figure 3.11

Fish Meal (FM) Supplementation Increases the Abundance of Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) in Blood Plasma When Compared to Corn Gluten Meal (CM) Supplemented Cows



Note. Cows were supplemented with either CM (n=9) or FM (n=10) for 63 days, during which weekly jugular vein blood draws were performed to quantify abundance of EPA (A) and DHA (B) in the blood plasma. Following plasma separation and subsequent methylation, fatty acid

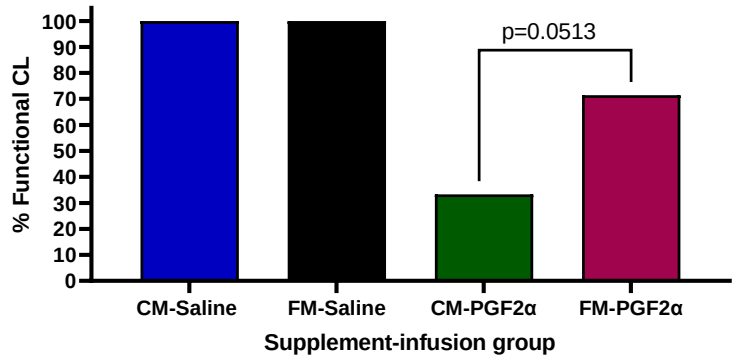
abundance was assessed using liquid gas chromatography. *Statistical difference ($p<0.05$) between supplementation groups within day. Data are presented as mean ± SEM.

Percentage of Functional Corpus Luteum at Time of Ovariectomy

Following the 63-day supplementation period, cows received two low dose infusions of either prostaglandin F2 alpha (PGF2α) or saline, 12 hrs apart. Luteal functionality was defined as serum progesterone >1 ng/mL at time of ovariectomy (30 or 48 hrs following first infusion). As was expected, cows supplemented with CM or FM and infused with saline (CM-Saline [n=5]; FM-Saline[n=4]) all maintained a functional CL (figure 3.12). Cows supplemented with CM and that received two PGF2α infusions (CM-PGF2α; n=11) maintained a functional CL 33.3% of the time, whereas FM supplemented and PGF2α infused (FM-PGF2α n=14) maintained a functional CL 71.4% of the time, equating to a 114.4% increase in functionality in the FM- PGF2α group relative to CM-PGF2α. There was a tendency for increased functional CL in FM supplemented cows ($p<0.1$). From these results it can be extrapolated that FM supplementation reduces rates of regression following PGF2α exposure.

Figure 3.12

Fish Meal (FM) Supplementation Maintains a Greater Proportion of Functional Corpus Luteum (CL) Following Two Low Dose Intra-Uterine Infusions of Prostaglandin F2 Alpha (PGF2α), When Compared to Corn Gluten Meal (CM) Supplemented Cows



Note. Cows were supplemented with either CM or FM for 63 days, during which estrus were synchronized. On day 10-12 following onset of estrus, cows were infused with either 0.25mL saline or 0.5mg of PGF2α (in 0.25 mL saline) in the uterine horn ipsilateral to the CL-bearing ovary. The cows were infused twice, each infusion 12 hrs apart. Jugular vein blood draws were performed, and serum progesterone concentrations later quantified using a progesterone ELISA. CL were defined as functional if serum progesterone > 1 ng/mL or regressed if <1 ng/mL at time of ovariectomy (30 or 48 hrs following first infusion). CM-Saline (n=5); FM- Saline (n=4); CM-PGF2α (n=11); FM-PGF2α (n=14). Statistical significance $p<0.05$.

Serum Progesterone Following Low Dose Infusions of Prostaglandin F2 Alpha

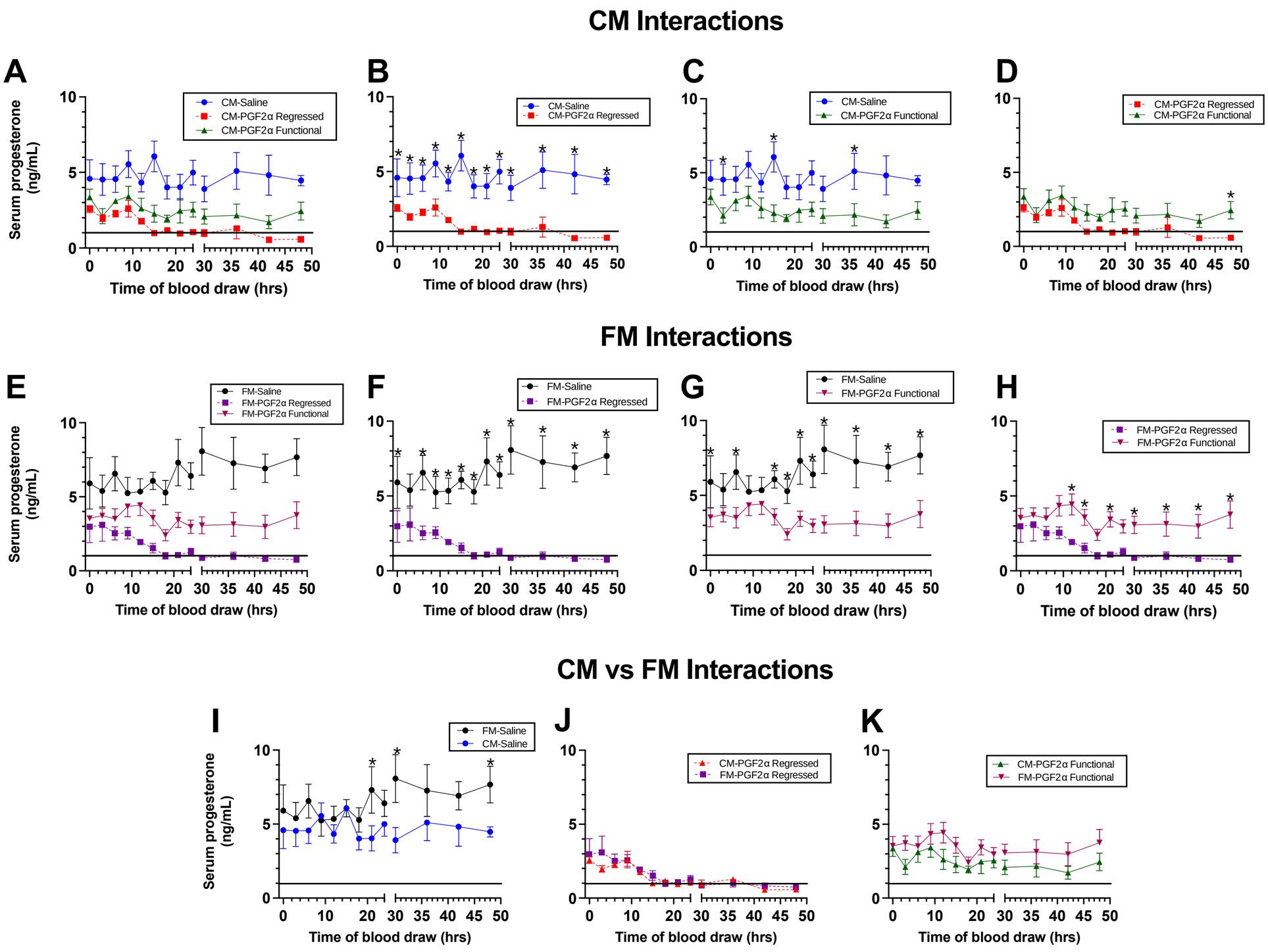
Single infusions of either PGF2α or saline occurred at hour 0 and 12. As previously stated, if serum progesterone was >1 ng/mL the CL was defined as functional and if < 1 ng/mL, regressed. All groups within CM supplemented cows (infusion groups and functional state of CL) are shown in figure 3.13A, and all groups within FM supplementation groups in figure 3.13E. When comparing means of CM-Saline (n=5) and CM-PGF2α-Regressed cows (n=7; figure 3.13B), serum progesterone was significantly lower ($p<0.05$) at all time points. CM-PGF2α Regressed cows declined below the functional threshold (serum progesterone <1ng/mL)

42 hrs following the first infusion. When comparing means of CM-Saline and CM-PGF2α Functional groups (n=4; figure 3.13C), only 3 time points (3 hrs, 15 hrs, and 36 hrs) were significantly lower ($p<0.05$) in the CM- PGF2α Functional group, but at all time points, progesterone concentrations were lower than the saline control, indicating PGF2α exposure reduced progesterone output. Interestingly, CM Functional cows only had a significant difference in serum progesterone 48 hrs following infusion (figure 3.13D). When comparing the means of FM-Saline (n=4) and FM-PGF2α Regressed cows (n=4; figure 3.13F), serum progesterone was significantly lower ($p<0.05$) at all time points, except for at 3 hrs post infusion. Moreover, the concentration of progesterone in FM-PGF2α Regressed cows declined below 1 ng/mL at 42 hrs post infusion, like the CM-PGF2α Regressed group. When comparing concentration means of FM-Saline and FM-PGF2α Functional cows (n=10; figure 3.13G), the latter had a significantly lower ($p<0.05$) concentration of progesterone, except at 3-, 9-, and 12-hrs following the first infusion, indicating glandular exposure to PGF2α reduced progesterone output. Serum progesterone in the FM-PGF2α Regressed cows began to significantly decline by 12 hrs post infusion when compared to FM-PGF2α Functional cows, and only FM-PGF2α Functional cows underwent a rebound in serum progesterone by 21 hrs post infusion (figure 3.13H). Between the CM-Saline and FM-group, progesterone concentrations were significantly higher ($p<0.05$) in FM-Saline cows at 21-, 30-, and 48-hrs following the first infusion, indicating the overall basal progesterone concentrations were not influenced by supplementation group (figure 3.13 I). Between CM- and FM- PGF2α regressed cows, no significant differences ($p>0.05$) were detected at any time point (figure 3.13J), indicating supplementation does not influence progesterone output in regressing CL. Within both supplementation groups, blood progesterone steadily declined over the 48-hour experimental period. When CM- and FM-

PGF2α Functional cows were compared, no significant differences ($p>0.05$) were detected at any time point (figure 3.13K). However, both groups had a rebound in progesterone concentrations 3- to 6- hrs following the first infusion, a decrease after the second infusion (at 12 hrs), and eventually a plateau in progesterone concentrations. The ability of a CL to rebound its output of serum progesterone appears to be a distinguishing factor between a regressing and functional CL.

Figure 3.13

Supplementation Group (Corn Gluten Meal [CM] or Fish Meal [FM]) Did Not Differentially Alter Progesterone Concentrations Following Two Low Dose Intra-Uterine Infusions of Prostaglandin F2 Alpha (PGF2α)



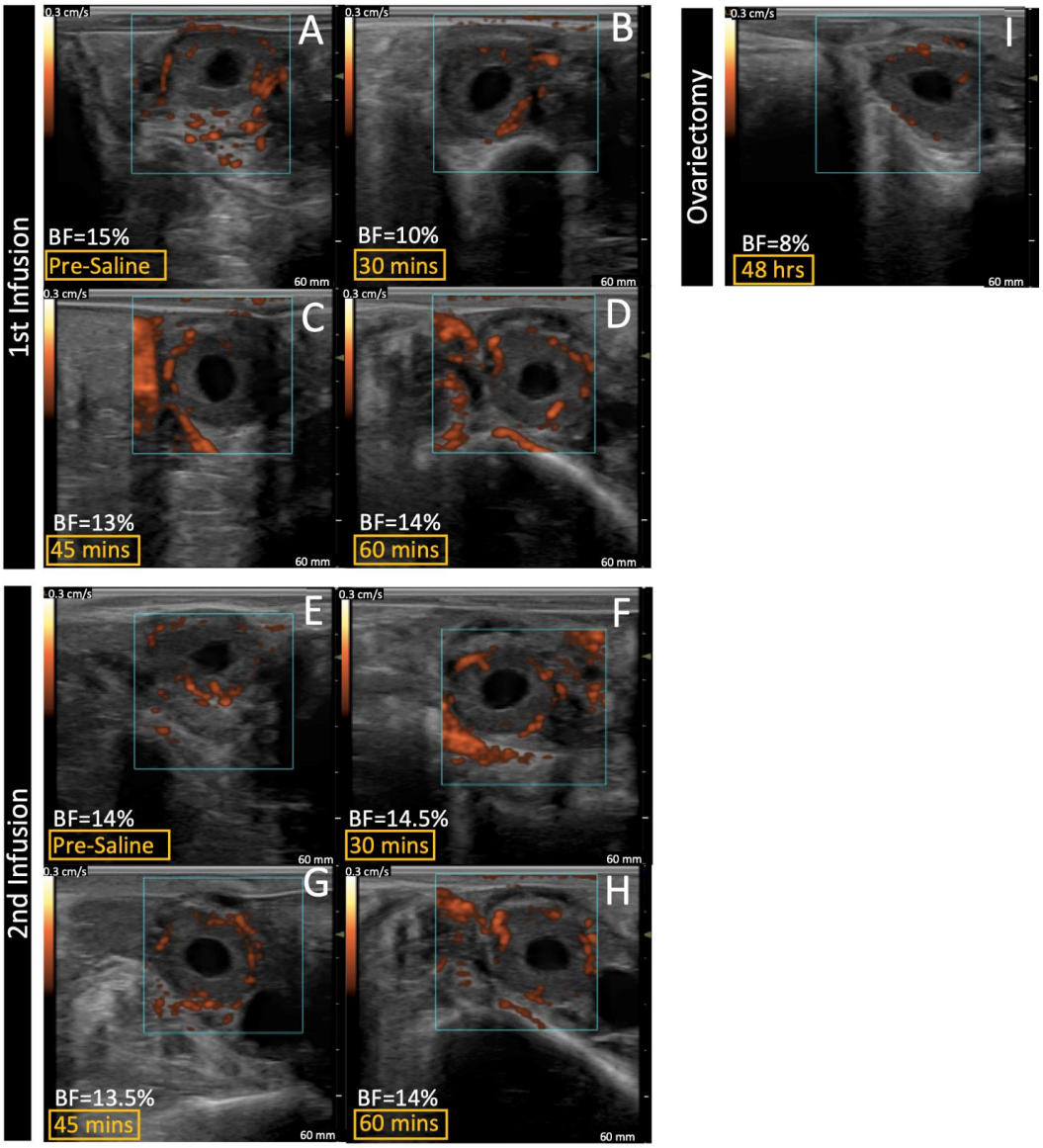
Note. Cows were supplemented with either CM or FM for 63 days, during which estrus was synchronized. On day 10-12 following onset of estrus, cows were infused with either 0.25 mL saline or 0.5 mg of PGF2α (in 0.25 mL saline) in the uterine horn ipsilateral to the CL-bearing ovary. The cows were infused twice, each infusion 12 hrs apart. Jugular vein blood draws were collected every 3 hrs for the first 24 hrs, and every 6 hrs, until time of ovariectomy (48h following first infusion). Serum progesterone concentrations were then quantified using a progesterone ELISA. Black line on graphs was set at 1 ng/mL to indicate functional CL threshold. CL were deemed regressed if serum progesterone <1 ng/mL. All CM interactions (A-D), FM interactions (E-H), and CM versus FM interactions (I-K) between saline or PGF2α infused groups. CM-Saline (n=5); CM-PGF2α Regressed (n=7); CM-PGF2α Functional (n=4); FM-Saline (n=5); FM-PGF2α Regressed (n=4); FM-PGF2α Functional (n=10). *Significant differences between supplementation or infusion group within time of blood draw. Data are presented as mean ± SEM. Note: overlapping error bars were removed for effective visualization purposes.

Representative Ultrasound Images of Luteal Blood Flow Following Infusions

To assess the influence of CM and FM supplementation on luteal blood flow following the two low dose intra-uterine infusions of PGF2α, power Doppler ultrasound scans were taken before and after time of infusion every 15 mins for an hour, and every hour thereafter for 6 hrs. A final scan was taken at 48 hrs post infusion. Representative images from each group (CM-Saline [Figure 3.14]; CM-PGF2α-Regressed [Figure 3.15]; CM=PGF2α-Functional [Figure 3.16]; FM-Saline [figure 3.17]; FM-PGF2α Regressed [figure 3.18]; FM-PGF2α Functional [figure 3.19]) and repeated scan times for the first infusion (pre infusion/-15 mins [A]; 30 mins post infusion [B]; 45 mins post infusion [C], 1 hr post infusion [D]) and second infusion (pre infusion/-15 mins [E]; 30 mins post infusion [F]; 45 mins post infusion [G], 1 hr post infusion [H], 48 hrs post infusion [F])

Figure 3.14

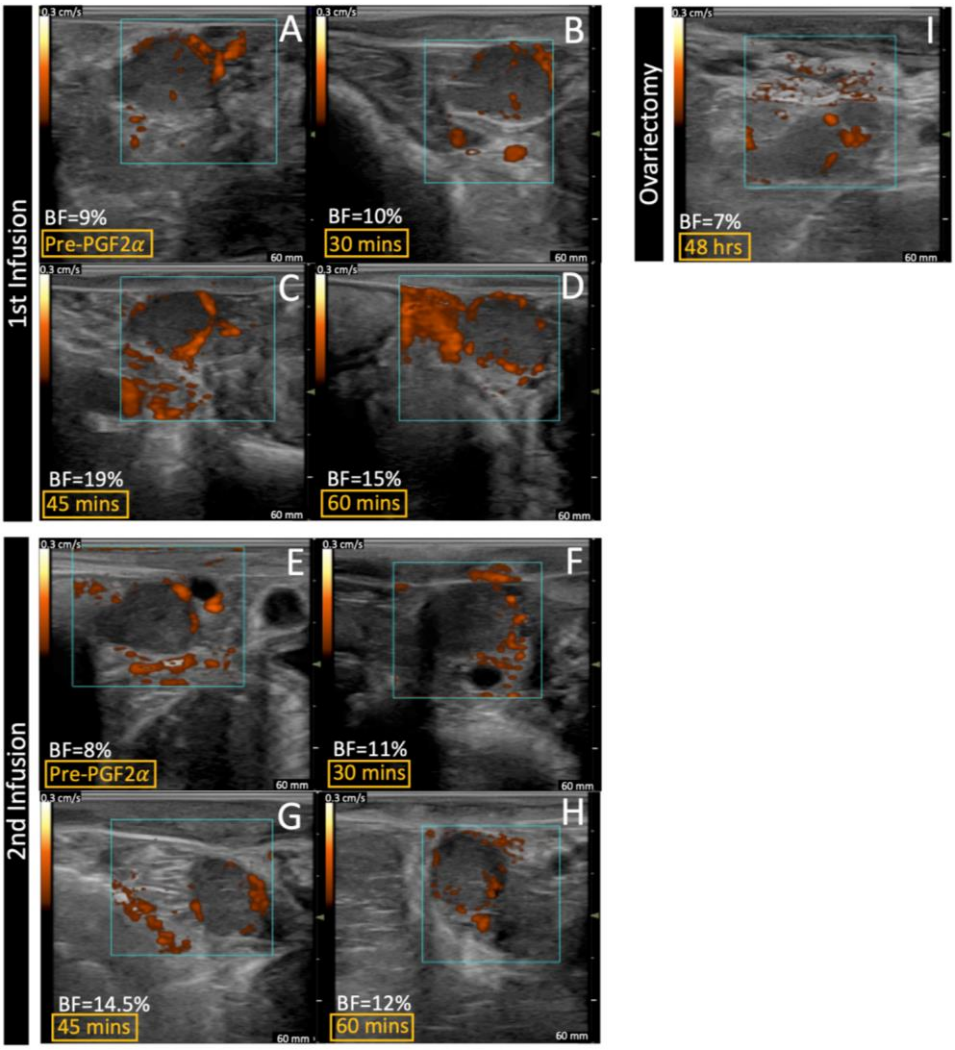
Representative Power Doppler Ultrasound Images of Luteal Blood Flow in Corn Gluten Meal (CM) Supplemented, and Saline Infused Cows



Note. Cows were given CM supplement for 63 days, during which estrus was synchronized. On day 10-12 following onset of estrus, cows were infused with 0.25mL saline in the uterine horn ipsilateral to the CL-bearing ovary. Luteal blood flow was assessed using trans-rectal power Doppler ultrasonography (Frequency 6 MHz, PFR 2000 Hz, and depth 60 mm) at multiple time points pre- and post-infusion. Colored pixels within the blue box demonstrate differing speeds of detected flow (upper left color bar; black=0 cm/s, white= 0.3 cm/s). BF= quantified luteal blood flow of said image. Representative images of blood flow prior to the first saline infusion (A), 30 mins post (B), 45 mins post (C), and 60 mins post (D). Representative images of blood flow prior to the second saline infusion (E), 30 mins post (F), 45 mins post (G), and 60 mins post (H). I) Blood flow 48 hrs following the first infusion.

Figure 3.15.

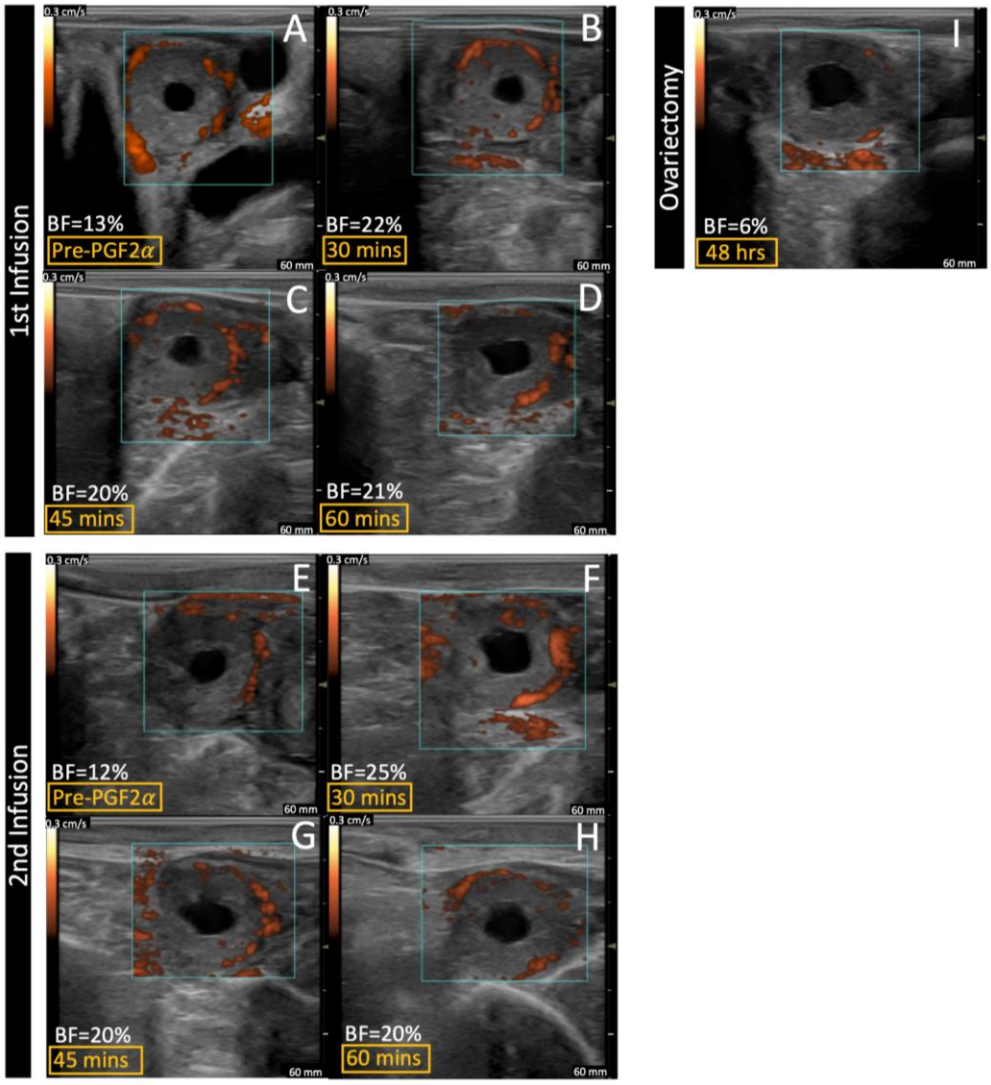
Representative Power Doppler Ultrasound Images of Luteal Blood Flow in Corn Gluten Meal (CM) Supplemented, Prostaglandin F2 Alpha (PGF2α) Infused, and Regressed Corpus Luteum (CL) Cows



Note. Cows were given CM supplement for 63 days, during which estrus was synchronized. On day 10-12 following onset of estrus, cows were infused with 0.5mg of PGF2α (in 0.25 mL saline) in the uterine horn ipsilateral to the CL-bearing ovary. Luteal blood flow was assessed using trans-rectal power Doppler ultrasonography (Frequency 6 MHz, PFR 2000 Hz, and depth 60 mm) at multiple time points pre- and post-infusion. Colored pixels within the blue box demonstrate differing speeds of detected flow (upper left color bar; black=0 cm/s, white= 0.3 cm/s). CL were defined as regressed if serum progesterone <1 ng/mL at time of ovariectomy (48 hrs). BF= quantified luteal blood flow of said image. Representative images of blood flow prior to the first PGF2α infusion (A), 30 mins post (B), 45 mins post (C), and 60 mins post (D). Representative images of blood flow prior to the second PGF2α infusion (E), 30 mins post (F), 45 mins post (G), and 60 mins post (H). I) Blood flow 48 hrs following the first infusion.

Figure 3.16

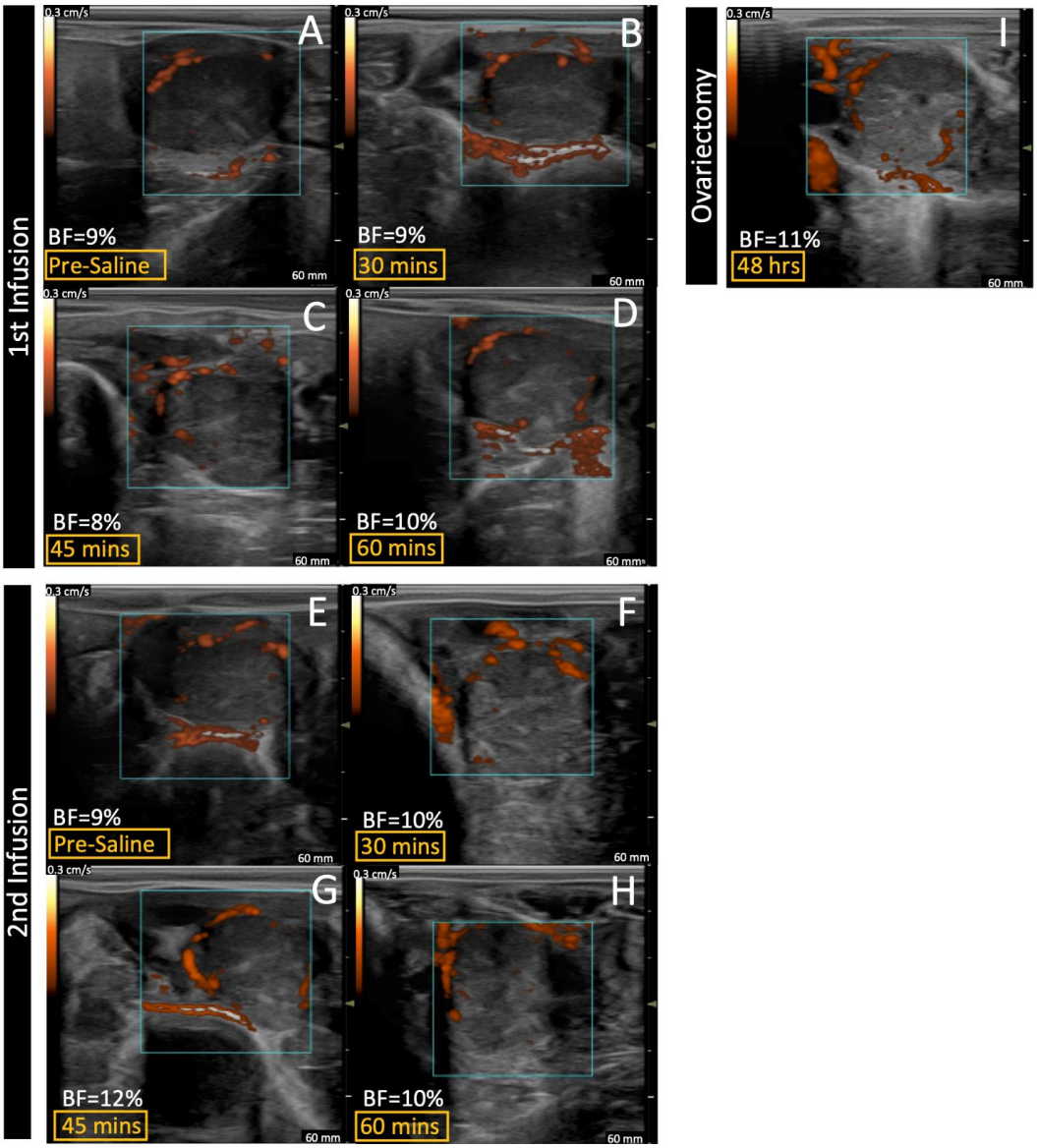
Representative Power Doppler Ultrasound Images of Luteal Blood Flow in Corn Gluten Meal (CM) Supplemented, Prostaglandin F2 alpha (PGF2α) Infused, and Functional Corpus Luteum (CL) Cows



Note. Cows were given CM supplement for 63 days, during which estrus was synchronized. On day 10-12 following onset of estrus, cows were infused with 0.5mg of PGF2α (in 0.25 mL saline) in the uterine horn ipsilateral to the CL-bearing ovary. Luteal blood flow was assessed using trans-rectal power Doppler ultrasonography (Frequency 6 MHz, PFR 2000 Hz, and depth 60 mm) at multiple time points pre- and post-infusion. Colored pixels within the blue box demonstrate differing speeds of detected flow (upper left color bar; black=0 cm/s, white= 0.3 cm/s). CL were defined as functional if serum progesterone >1 ng/mL at time of ovariectomy (48 hrs). BF= quantified luteal blood flow of said image. Representative images of blood flow prior to the first PGF2α infusion (A), 30 mins post (B), 45 mins post (C), and 60 mins post (D). Representative images of blood flow prior to the second PGF2α infusion (E), 30 mins post (F), 45 mins post (G), and 60 mins post (H). I) Blood flow 48 hrs following the first infusion.

Figure 3.17

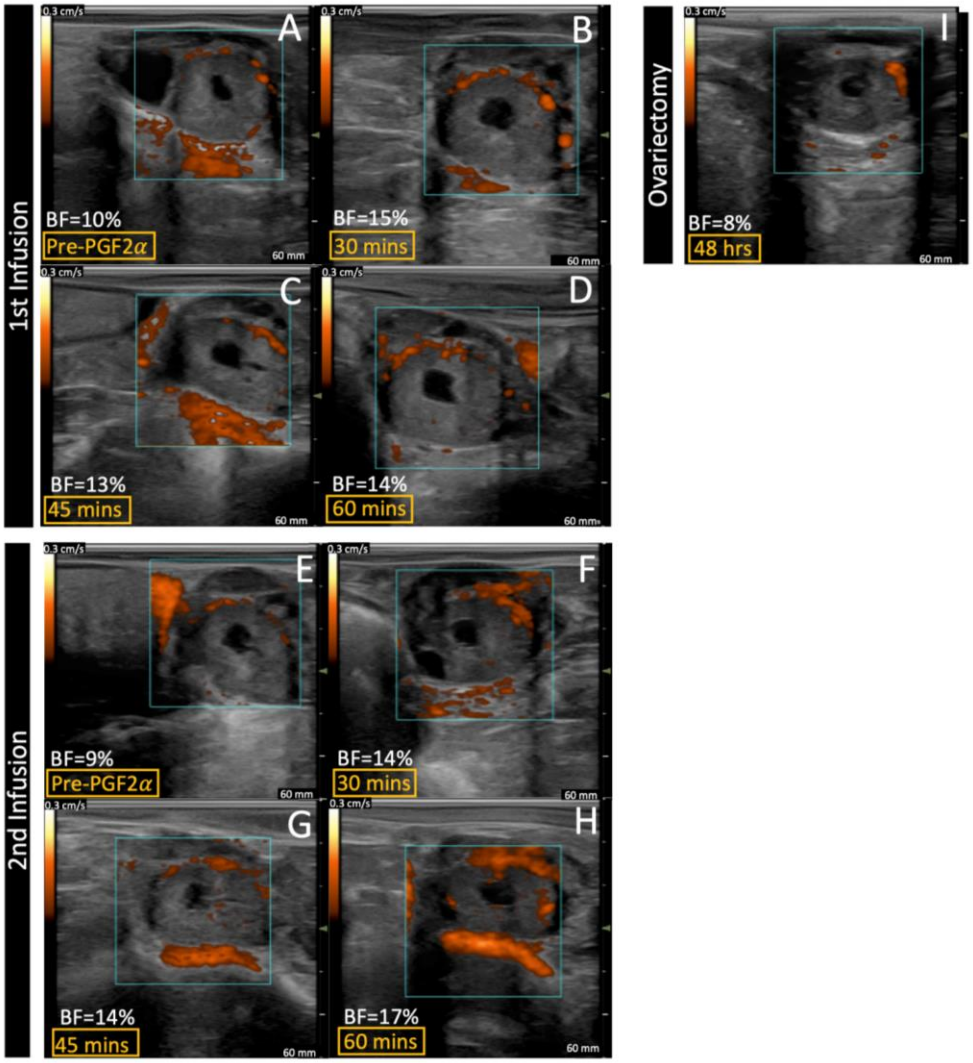
Representative Power Doppler Ultrasound Images of Luteal Blood Flow in Fish Meal (FM) Supplemented, and Saline Infused Cows



Note. Cows were given FM supplement for 63 days, during which estrus was synchronized. On day 10-12 following onset of estrus, cows were infused with 0.25mL saline in the uterine horn ipsilateral to the CL-bearing ovary. Luteal blood flow was assessed using trans-rectal power Doppler ultrasonography (Frequency 6 MHz, PFR 2000 Hz, and depth 60 mm) at multiple time points pre- and post-infusion. Colored pixels within the blue box demonstrate differing speeds of detected flow (upper left color bar; black=0 cm/s, white= 0.3 cm/s). BF= quantified luteal blood flow of said image. Representative images of blood flow prior to the first saline infusion (A), 30 mins post (B), 45 mins post (C), and 60 mins post (D). Representative images of blood flow prior to the second saline infusion (E), 30 mins post (F), 45 mins post (G), and 60 mins post (H). I) blood flow 48 hrs following the first infusion.

Figure 3.18

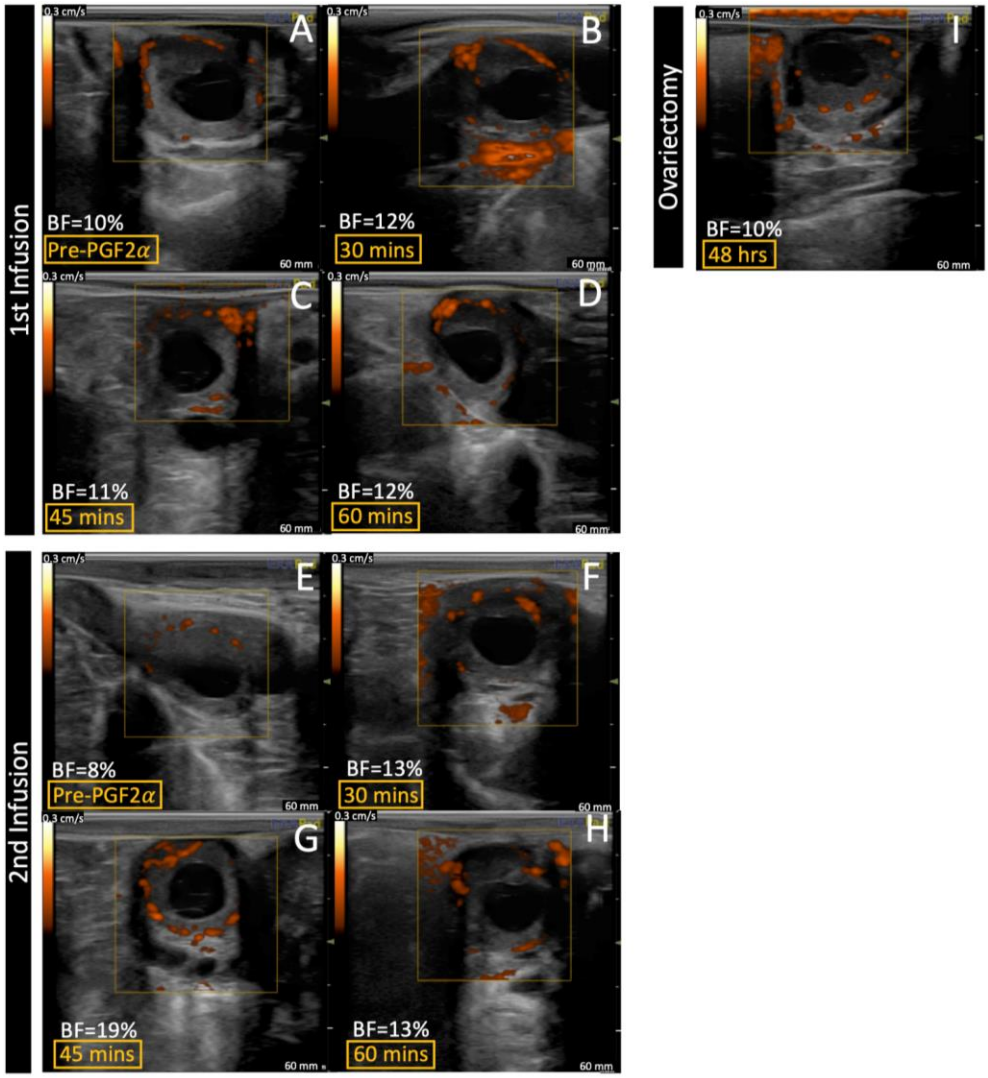
Representative Power Doppler Ultrasound Images of Luteal Blood Flow in Fish Meal (FM) Supplemented, Prostaglandin F2 Alpha (PGF2α) Infused, and Regressed Corpus Luteum (CL) Cows



Note. Cows were given FM supplement for 63 days, during which estrus was synchronized. On day 10-12 following onset of estrus, cows were infused with 0.5mg of PGF2α (in 0.25 mL saline) in the uterine horn ipsilateral to the CL-bearing ovary. Luteal blood flow was assessed using trans-rectal power Doppler ultrasonography (Frequency 6 MHz, PFR 2000 Hz, and depth 60 mm) at multiple time points pre- and post-infusion. Colored pixels within the blue box demonstrate differing speeds of detected flow (upper left color bar; black=0 cm/s, white= 0.3 cm/s). CL were defined as regressed if serum progesterone <1 ng/mL at time of ovariectomy (48 hrs). BF= quantified luteal blood flow of said image. Representative images of blood flow prior to the first PGF2α infusion (A), 30 mins post (B), 45 mins post (C), and 60 mins post (D). Representative images of blood flow prior to the second PGF2α infusion (E), 30 mins post (F), 45 mins post (G), and 60 mins post (H). I) Blood flow 48 hrs following the first infusion.

Figure 3.19

Representative Power Doppler Ultrasound Images of Luteal Blood Flow in Fish Meal Supplemented, Prostaglandin F2 Alpha (PGF2α) Infused, and Functional Corpus Luteum (CL) Cows



Notes. Cows were given FM supplement for 63 days, during which estrus was synchronized. On day 10-12 following onset of estrus, cows were infused with 0.5mg of PGF2α (in 0.25 mL saline) in the uterine horn ipsilateral to the CL-bearing ovary. Luteal blood flow was assessed using trans-rectal power Doppler ultrasonography (Frequency 6 MHz, PFR 2000 Hz, and depth 60 mm) at multiple time points pre- and post-infusion. Colored pixels within the orange box demonstrate differing speeds of detected flow (upper left color bar; black=0 cm/s, white= 0.3 cm/s). CL were defined as functional if serum progesterone >1 ng/mL at time of ovariectomy (48 hrs). BF= quantified luteal blood flow of said image. Representative images of blood flow prior to the first PGF2α infusion (A), 30 mins post (B), 45 mins post (C), and 60 mins post (D). Representative images of blood flow prior to the second PGF2α infusion (E), 30 mins post (F), 45 mins post (G), and 60 mins post (H). I) Blood flow 48 hrs following the first infusion.

Luteal Blood Flow Following Prostaglandin F2 Alpha Infusions

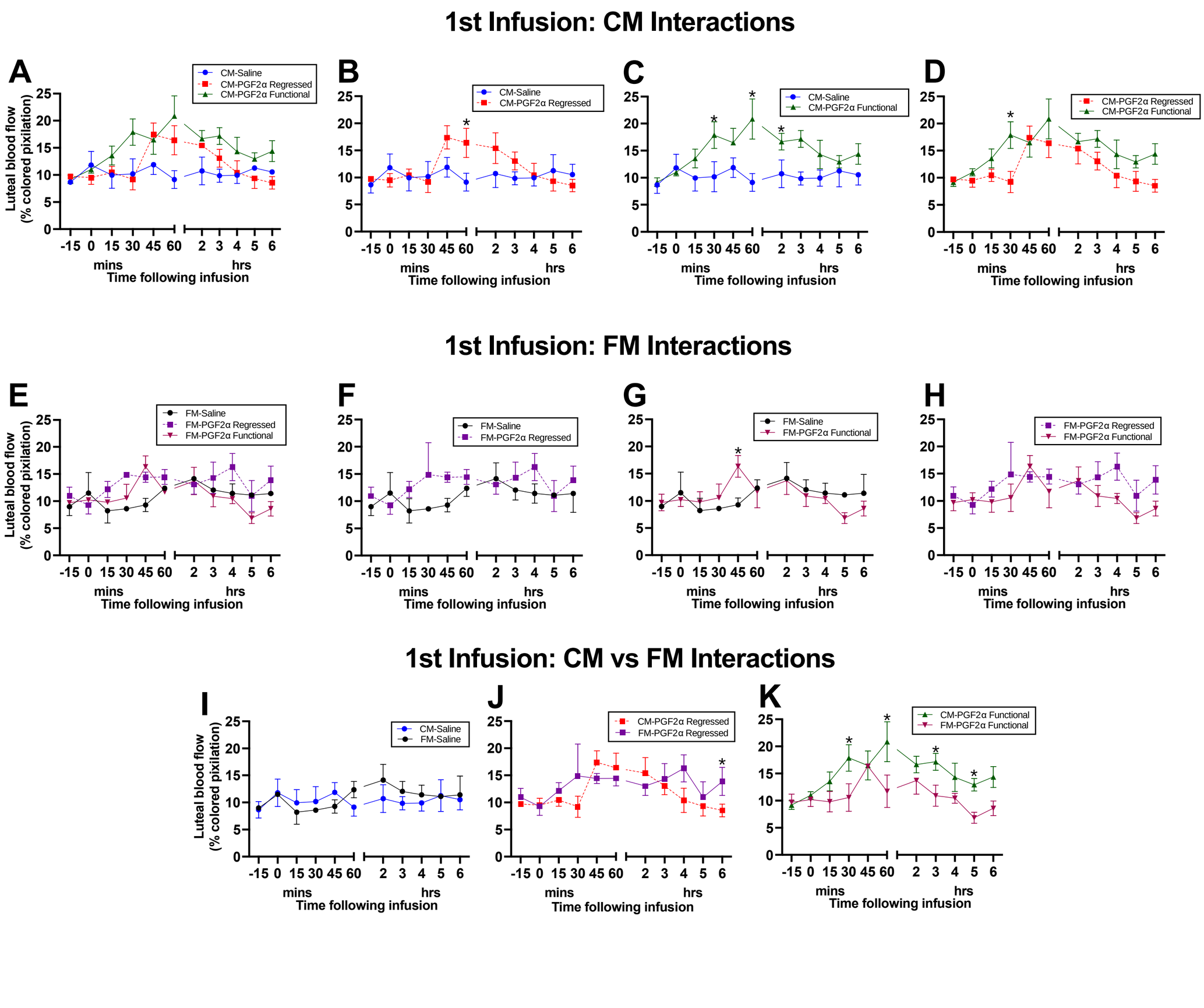
First Infusion

Images from the power Doppler ultrasonography CL scans were quantified to generate numerical values for statistical comparisons. All CM associated groups can be visualized in figure 3.19A, and all FM associated groups in figure 19E. When comparing CM interactions, both CM-PGF2α Regressed (figure 3.20B; n=6) and CM-PGF2α Functional (figure 3.20C; n=4) cows had a significant spike ($p<0.05$) in luteal blood flow within 60 (CM-PGF2α Regressed) or 30 mins (CM-PGF2α Functional) following infusions compared to CM-Saline (n=4), indicating PGF2α influences luteal blood flow in an acute time span. As early as 2 hrs (CM-PGF2α Regressed) and 4 hrs (CM-PGF2α Functional) post infusion, luteal blood flow re-stabilized to match control means. Moreover, while both Regressed and Functional groups underwent an increase in luteal blood flow post infusion, Functional cows underwent this effect 15 mins earlier ($p<0.05$), though more conservatively is due to animal variation and small sample size than to biological effect. When comparing FM interactions, luteal blood flow did not significantly differentiate ($p>0.05$) at any point between the FM- saline (n=3) and PGF2α Regressed groups (figure 3.20F; n=4), indicating detectable blood flow was not influenced by PGF2α, unlike in the CM-PGF2α Regressed group (relative to respective supplement control). However, the lack of effect may be due to extensive variability at that time point. Furthermore, FM-PGF2α Functional cows (figure 3.20G; n=9) only significantly differed from the FM-Saline control 45 mins following infusion ($p<0.05$), and immediately re-stabilized within 15 mins (by 60 mins post infusion); a much faster recovery when compared to the CM-PGF2α Functional group. Interestingly, Regressed and Functional cows had no significant difference ($p>0.05$; figure

3.20H) at any time point. Additionally, it can be inferred that supplementation did not affect basal luteal blood flow, as both CM- and FM-Saline groups had no significant differences ($p>0.05$; figure 3.20I). Despite the presence and lack of significant differences between CM-PGF2α Regressed vs CM-Saline (figure 3.20B) and FM-PGF2α Regressed vs FM-Saline groups (figure 3.20F), respectively, no significant differences ($p>0.05$) were detected between CM- and FM-PGF2α Regressed groups (figure 3.20J). Within PGF2α Functional groups, FM supplementation appeared to have blunted the spike in luteal blood flow that occurred in the CM supplemented cows, made evident by the significant elevation ($p<0.05$) in blood flow 30 mins, 60 mins, and 2 hrs following infusion in CM-PGF2α Functional cows (figure 3.20K).

Figure 3.20

Fish Meal (FM) Supplemented Cows Had a Blunted Response in Luteal Blood (Percentage of Luteal Blood Flow) When Compared to Corn Gluten Meal (CM) Supplemented Cows Following the First Low Dose Intra-Uterine Infusion of Prostaglandin F2 Alpha (PGF2α)



Note. Cows were supplemented with either CM or FM for 63 days, during which estrus was synchronized. On day 10-12 following onset of estrus, cows were infused with either 0.25 mL saline or 0.5 mg of PGF2α (in 0.25 mL saline) in the uterine horn ipsilateral to the corpus luteum (CL)-bearing ovary. Luteal blood flow was assessed using trans-rectal power Doppler ultrasonography (Frequency 6 MHz, PFR 2000 Hz, and depth 60 mm) 15 mins prior to the first infusion (-15 mins), at time of the first infusion (0 mins), every 15 mins for the following hour, and every hour thereafter (for 6 hrs). The percentage of luteal blood flow was later quantified using ImageJ software. CL were deemed functional if serum progesterone levels were >1 ng/mL or regressed if < 1 ng/mL at time of ovariectomy (48 hrs following first infusion). All CM (A-D), FM (E-H), and CM versus FM interactions (I-K) between saline or PGF2α infused groups, and PGF2α-functional and non-functional CL groups. CM-Saline (n=4); CM-PGF2α Regressed (n=6); CM-PGF2α Functional (n=4); FM-Saline (n=3); FM-PGF2α Regressed (n=4); FM-PGF2α Functional (n=9). *Significant differences between supplementation or infusion group within time of scan. Data are presented as mean ± SEM. Note: overlapping error bars were removed for effective visualization purposes.

Second Infusion

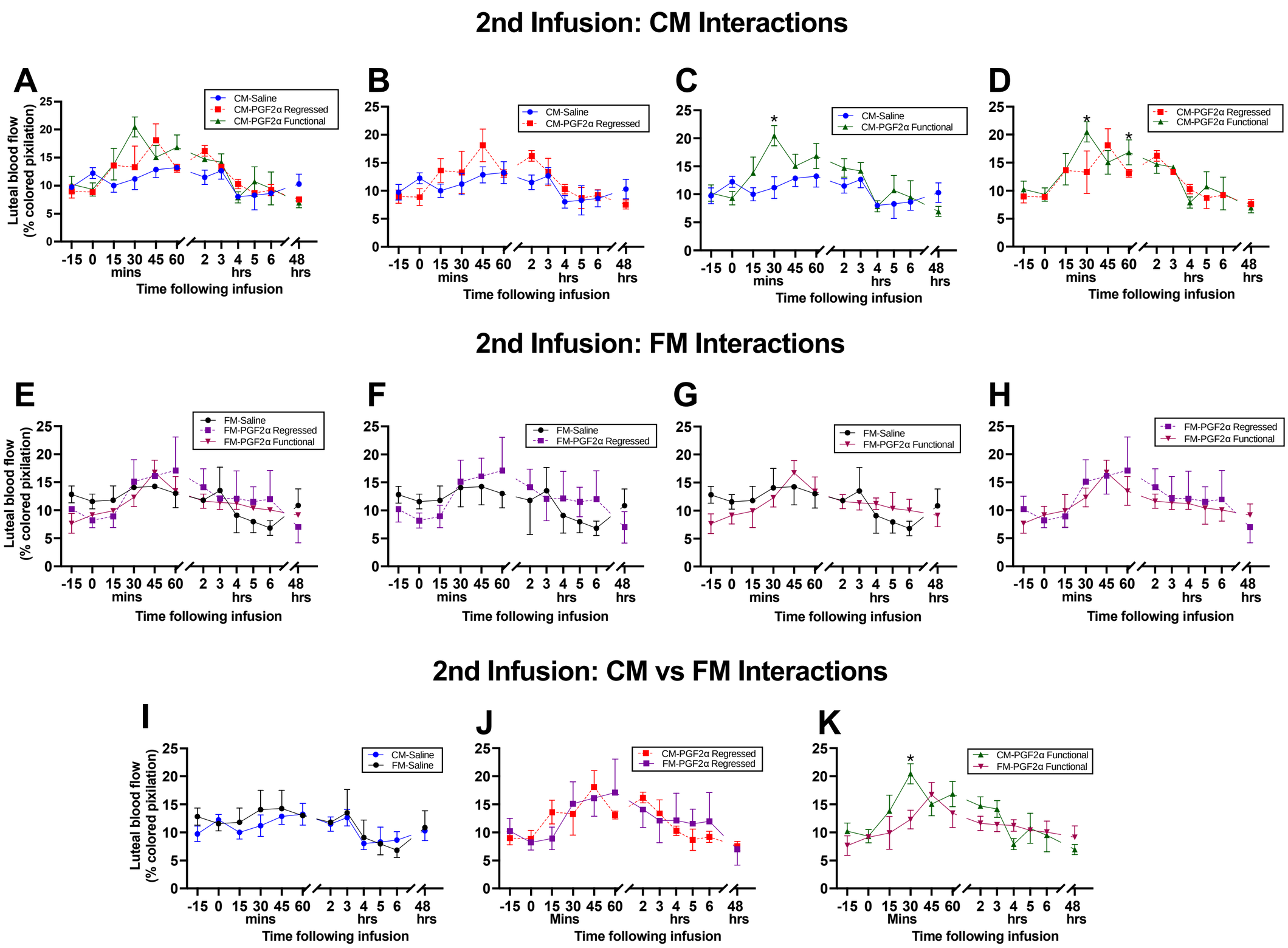
All CM associated groups can be visualized in figure 3.21A, and all FM associated groups can be visualized in figure 3.21E. We found that blood flow did not significantly differentiate ($p<0.05$) between CM-Saline (n=4) and CM-PGF2α Regressed (n=6; figure 3.21B) groups, despite an apparent increase in the CM-PGF2α Regressed group. These results indicate the second infusion of PGF2α did not influence blood flow as drastically in CM supplemented cows with a regressing CL. Luteal blood flow in CM-PGF2α Functional cows (n=4) was differentially influenced however, as blood flow was significantly elevated ($p<0.05$) 30 mins post infusion compared to both CM-Saline (figure 3.21C) and CM-PGF2α Functional groups (figure 3.21D). Interestingly, no FM supplemented groups significantly differed from the FM-Saline control (n=3), however, both the FM-PGF2α Regressed (n=4; figure 3.21F) and the FM-PGF2α Functional (n=9) had a lower starting blood flow, denoting the importance of assessing the change in percent luteal blood flow. Furthermore, we found that supplementation group (CM vs FM-Saline; figure 3.21I) did not differentially influence basal luteal blood flow. Why both control groups underwent a reduction in blood flow from 3- to 6-hrs post infusion is unclear. Similarly, when comparing the patterns in blood flow between CM- and FM-PGF2α Regressed groups, no significant difference ($p>0.05$) was observed, showing that supplement group had no effect on luteal blood flow in regressing CL, as was to be expected. Finally, following analysis of CM and FM-PGF2α Functional groups, CM supplemented animals had an earlier, and more robust increase in luteal blood flow, as evident by the significant increase in flow 30 mins post infusion.

While supplementation does not appear to maintain luteal blood flow as we had hypothesized, nor is luteal blood flow different in functional vs regressing CL, it would appear as

if FM supplementation reduced the intensity of the luteal blood flow response within the first hour following infusion.

Figure 3.21

Fish Meal (FM) Supplemented Cows Had a Blunted Response in Luteal Blood (Percentage of Luteal Blood Flow) When Compared to Corn Gluten Meal (CM) Supplemented Cows Following the Second Low Dose Intra-Uterine Infusion of Prostaglandin F2 Alpha (PGF2α)



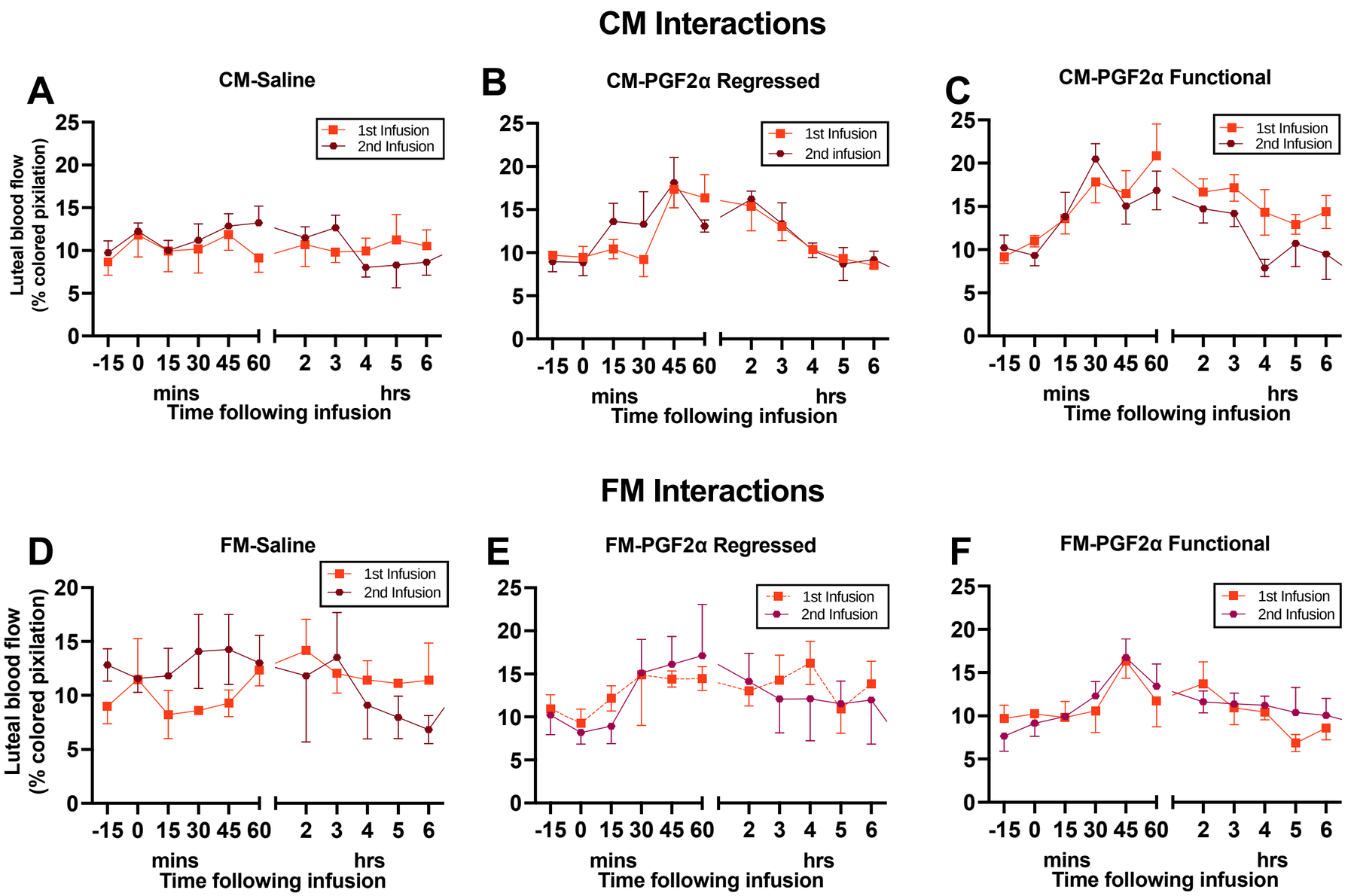
Note. Cows were supplemented with either CM or FM for 63 days, during which estrus was synchronized. On day 10-12 following onset of estrus, cows were infused with either 0.25mL saline or 0.5mg of PGF2α (in 0.25 mL saline) in the uterine horn ipsilateral to the CL-bearing ovary. The first infusion occurred 12 hrs prior to the second infusion. Luteal blood flow was assessed using trans-rectal power Doppler ultrasonography (Frequency 6 MHz, PFR 2000 Hz, and depth 60 mm) 15 mins prior to the second infusion (-15 mins), at time of the second infusion (0 mins), every 15 mins for the following hour, and every hour thereafter (for 6 hrs). Blood flow was also assessed directly prior to the time of ovariectomy (48 hrs following first infusion). The percentage of luteal blood flow was later quantified using ImageJ software. CL were deemed functional if serum progesterone levels were >1 ng/mL or regressed if < 1 ng/mL at time of ovariectomy. All CM (A-D), FM (E-H), and CM versus FM interactions (I-K) between saline or PGF2α infused groups, and PGF2α-functional and non-functional CL groups. CM-Saline (n=4); CM-PGF2α Regressed (n=6); CM-PGF2α Functional (n=4); FM-Saline (n=3); FM-PGF2α Regressed (n=4); FM-PGF2α Functional (n=9). *Significant differences between supplementation or infusion group within time of scan. Data are presented as mean ± SEM. Note: overlapping error bars were removed for effective visualization purposes.

First and Second Infusions

We found no significant differences ($p>0.05$) in luteal blood flow between the first and second infusions, independent of supplementation or infusion group, indicating the sequence of infusions did not differentially influence luteal blood flow (figure 3.22A-F). This was of particular interest specifically with regards to the CM-PGF2α Regressed group, considering blood flow was significantly elevated following the first infusion when compared to the control (figure 3.20B), but not following the second (figure 3.21B). Similarly, with regards to FM-PGF2α Functional cows, a significant difference in blood flow was observed in the first infusion (figure 3.20G), but not the second (figure 3.21G). In fact, both CM and FM respective groups closely matched each other in flow fluctuation trends between the first and second infusion. This could be due to the variability in controls associated with a low sample size and early phase data collection.

Figure 3.22

Infusion Number (1st vs 2nd) of Prostaglandin F2 Alpha (PGF2α) Did Not Influence the Response in Luteal Blood Flow (Percentage of Luteal Blood Flow) in Corn Gluten Meal (CM) or Fish Meal (FM) Supplemented Cows



Note. Cows were supplemented with either CM or FM for 63 days, during which estrus was synchronized. On day 10-12 following onset of estrus, cows were infused with either 0.25mL saline or 0.5mg of PGF2α (in 0.25 mL saline) in the uterine horn ipsilateral to the corpus luteum (CL)-bearing ovary. The first infusion occurred 12 hrs prior to the second infusion, both infusions occurring at 0 mins. Luteal blood flow was assessed using trans-rectal power Doppler ultrasonography (Frequency 6 MHz, PFR 2000 Hz, and depth 60 mm) Percentage of luteal blood flow was later quantified using ImageJ software. CL were deemed functional if serum progesterone levels were >1 ng/mL or regressed if < 1 ng/mL at time of ovariectomy. All CM (A-C) or FM (D-F) interactions between saline or PGF2α infused groups, and PGF2α-functional and non-functional CL groups. CM-Saline (n=4); CM-PGF2α Regressed (n=6); CM-PGF2α Functional (n=4); FM-Saline (n=3); FM-PGF2α Regressed (n=4); FM-PGF2α Functional (n=9). Data are presented as mean ± SEM. Note: overlapping error bars were removed for effective visualization purposes.

Percent Difference of Luteal Blood Flow Following Infusions

First Infusion

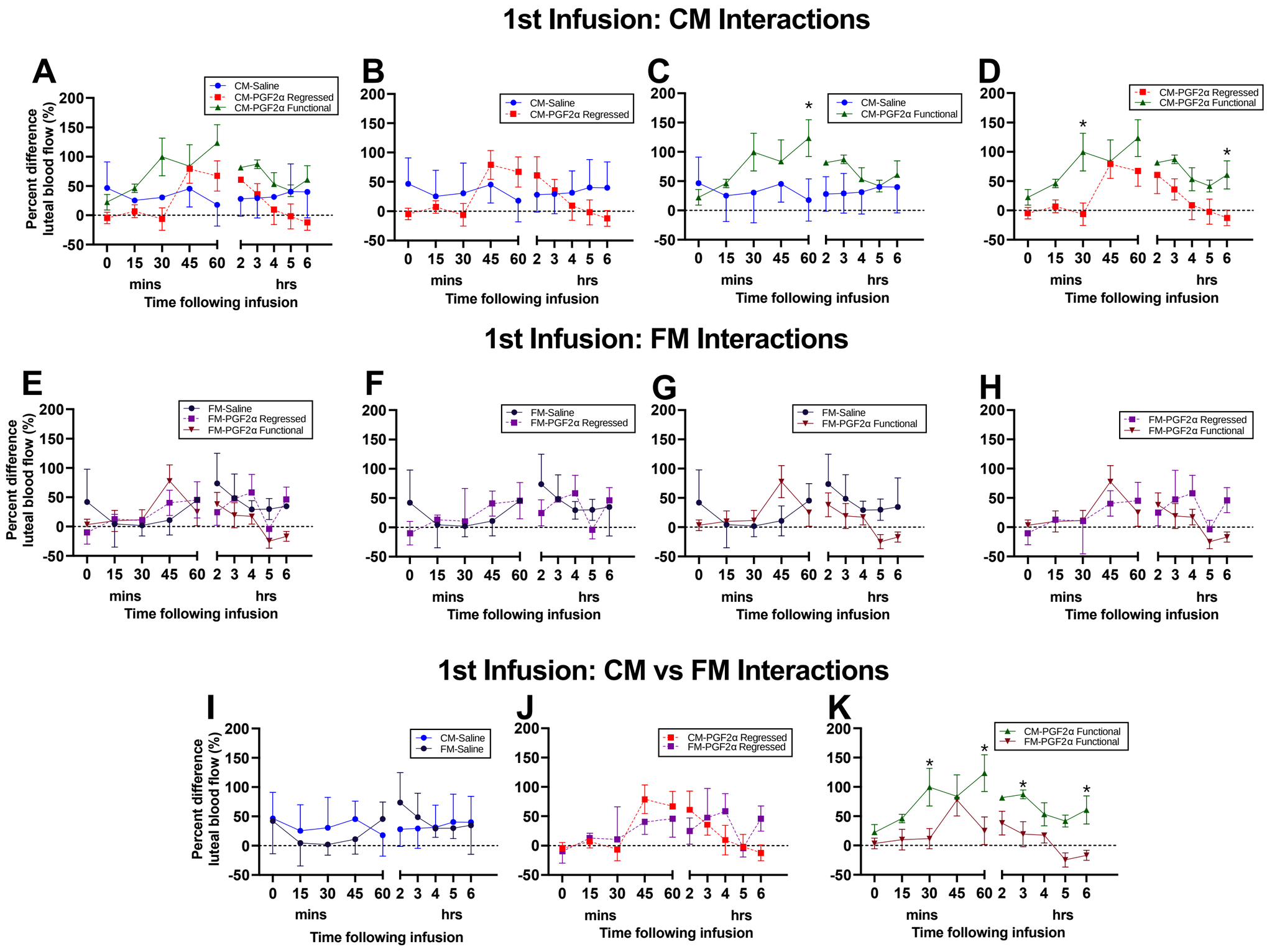
The percent difference in luteal blood flow was also quantified to assess relationships with respect to the pre-infusion control (-15 mins of first infusion), however, variability within controls was greatly increased as a result, and so potential relationships may be lost. All CM interactions are summarized in figure 3.23A and all FM in figure 3.23E. By assessing percent difference, the previously significant increase ($p<0.05$) in luteal blood flow between CM-Saline and CM-PGF2α Regressed was lost, and no significant difference was detected (figure 3.23B). However, the significant increase ($p<0.05$) between CM-Saline and CM-PGF2α Functional groups was retained (figure 3.23C). Furthermore, the relationship between CM-PGF2α Regressed and CM-PGF2α Functional groups was also maintained in that the CM-PGF2α Functional group had an earlier, more robust increase in blood flow ($p<0.05$; figure 3.23D). Interestingly, by comparing the percent differences in blood flow, CM-PGF2α Functional cows had a significantly greater ($p<0.05$) blood flow 6 hrs post infusion, indicating prolonged blood flow.

In assessing the data as percent difference, the variability and fluctuations occurring in the FM-saline controls are made apparent, as means shift from ~0 to ~75% at the 2 hrs mark. The lack of significant difference ($p>0.05$) between FM-Saline and FM-PGF2α Regressed groups was replicated even after accounting for percent difference, as such the lack of a blood flow response was made more apparent (figure 3.23F). The significant response in the FM-PGF2α Functional group compared to the FM-Saline group, was however lost ($p>0.05$) despite a promising spike 45 mins post infusion in the Functional group (Figure 3.23G). No differences were detected between FM-PGF2α Regressed and FM-PGF2α groups (figure 3.23H), as would

be expected considering a lack of independent effects relative to the control. When comparing supplementation groups (figure 3.23I-K), only the PGF2α-Functional groups (figure 3.23K) had significant differences in blood flow, as was observed prior to percent difference conversion. The results demonstrate that conversion to percentage difference presents a more honest assessment of the data and is more conservative with respect to generation of differential relations between data as far as first infusion comparisons were concerned. Furthermore, other than the FM-PGF2α Regressed group, all PGF2α infused groups underwent an increase in luteal blood flow within the first 1 hr following exposure.

Figure 3.23

Fish Meal (FM) Supplemented Cows Had a Blunted Response in Luteal Blood Flow (Percentage Difference of Luteal Blood Flow) When Compared to Corn Gluten Meal (CM) Supplemented Cows Following the First Low Dose Intra-Uterine Infusion of Prostaglandin F2 Alpha (PGF2α)



Note. Cows were supplemented with either CM or FM for 63 days, during which estrus was synchronized. On day 10-12 following onset of estrus, cows were infused with either 0.25mL saline or 0.5mg of PGF2α (in 0.25 mL saline) in the uterine horn ipsilateral to the corpus luteum (CL)-bearing ovary. Luteal blood flow was assessed using trans-rectal power Doppler ultrasonography (Frequency 6 MHz, PFR 2000 Hz, and depth 60 mm) 15 mins prior to the first infusion (-15 mins), at time of the first infusion (0 mins), every 15 mins for the following hour, and every hour thereafter (for 6 hrs). The percentage difference of luteal blood flow relative to the pre-infusions scan (-15 min) was later quantified using ImageJ software. CL were deemed functional if serum progesterone levels were >1 ng/mL or regressed if < 1 ng/mL at time of ovariectomy (48 hrs following first infusion). All CM (A-D), FM (E-H), and CM versus FM interactions (I-K) between saline or PGF2α infused groups, and PGF2α-functional and non-functional CL groups. CM-Saline (n=4); CM-PGF2α Regressed (n=6); CM-PGF2α Functional (n=4); FM-Saline (n=3); FM-PGF2α Regressed (n=4); FM-PGF2α Functional (n=9). *Significant differences between supplementation or infusion group within time of scan. Data are presented as mean ± SEM. Note: overlapping error bars were removed for effective visualization purposes.

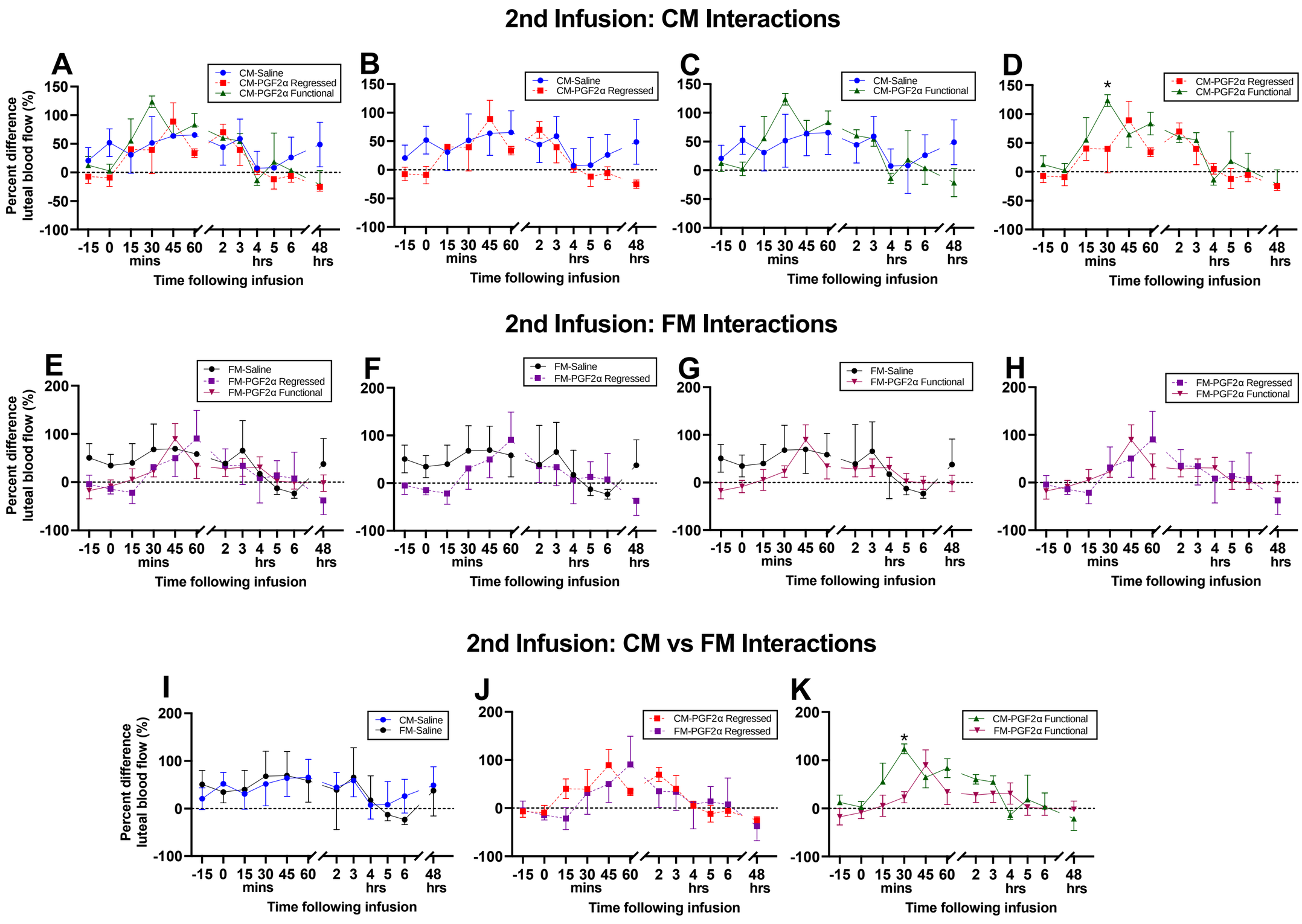
Second Infusion

All CM interactions are summarized in figure 3.24A and all FM in figure 3.24E. When assessing CM-Saline and CM-PGF2α Regressed groups, blood flow did not significantly differ ($p>0.05$) at any time (figure 3.24B). However, CM-PGF2α Regressed cows underwent a clear increase in blood flow up to 45 mins post infusion, and subsequently declined back to starting concentrations. A similar trend was observed in the CM-Saline and CM-PGF2α Function comparison (figure 3.24C). The insignificance may be due, in part, to the unexpected variability within saline control cows. While both CM-PGF2α Regressed and CM-PGF2α Functional cows followed a similar trend of increased percent blood flow following PGF2α exposure, Functional cows had an earlier, more robust response (figure 3.24D), a comparative trend to the first infusion (figure 3.23D). Across all FM-FM interactions, no significant differences ($p>0.05$) were observed (Figure 3.24F-H). However, despite a lack of significance, blood flow values in PGF2α infused groups (both FM Regressed and FM functional) followed a similar trend to that of CM-PGF2α groups, wherein blood flow spiked within the first hour following infusion and returned to base level within 6 hrs post infusion. When assessing relationships between CM and FM groups, we observed once again that supplement did not significantly alter ($p>0.05$) base luteal blood flow (figure 3.24I). Also, no significant differences ($p>0.05$) were detected between CM-PGF2α Regressed and FM-PGF2α Regressed groups (figure 3.24J). While both CM-PGF2α Functional and FM-PGF2α Functional groups underwent a similar spike in luteal blood flow within the first 1 hr following infusion, the spike in CM cows pre-ceded those of FM supplemented cows ($p<0.05$; figure 3.24K), although this did not appear to differentiate long term luteal blood flow, as flow was comparable from 45 mins post infusion until time of ovariectomy. Akin to findings in figure 20, no significant differences ($p>0.05$) were found by the

48 hrs time point between any groups, indicating that blood flow was conserved independent of the CL functional state. Moreover, while converting data to percent difference reduced the number of significant relationships, a more consistent trend was observed in luteal blood flow following exposure to PGF2α; luteal blood flow increases within 1 hr following exposure.

Figure 3.24

Fish Meal (FM) Supplemented Cows Had a Blunted Response in Luteal Blood Flow (Percentage Difference of Luteal Blood Flow) When Compared to Corn Gluten Meal (CM) Supplemented Cows Following the Second Low Dose Intra-Uterine Infusion of Prostaglandin F2 Alpha (PGF2α)



Note. Cows were supplemented with either CM or FM for 63 days, during which estrus was synchronized. On day 10-12 following onset of estrus, cows were infused with either 0.25mL saline or 0.5mg of PGF2α (in 0.25 mL saline) in the uterine horn ipsilateral to the corpus luteum (CL)-bearing ovary. The first infusion occurred 12 hrs prior to the second infusion. Luteal blood flow was assessed using trans-rectal power Doppler ultrasonography (Frequency 6 MHz, PFR 2000 Hz, and depth 60 mm) 15 mins prior to the second infusion (-15 mins), at time of the second infusion (0 mins), every 15 mins for the following hour, and every hour thereafter (for 6 hrs). Blood flow was also assessed directly prior to the time of ovariectomy (48 hrs following first

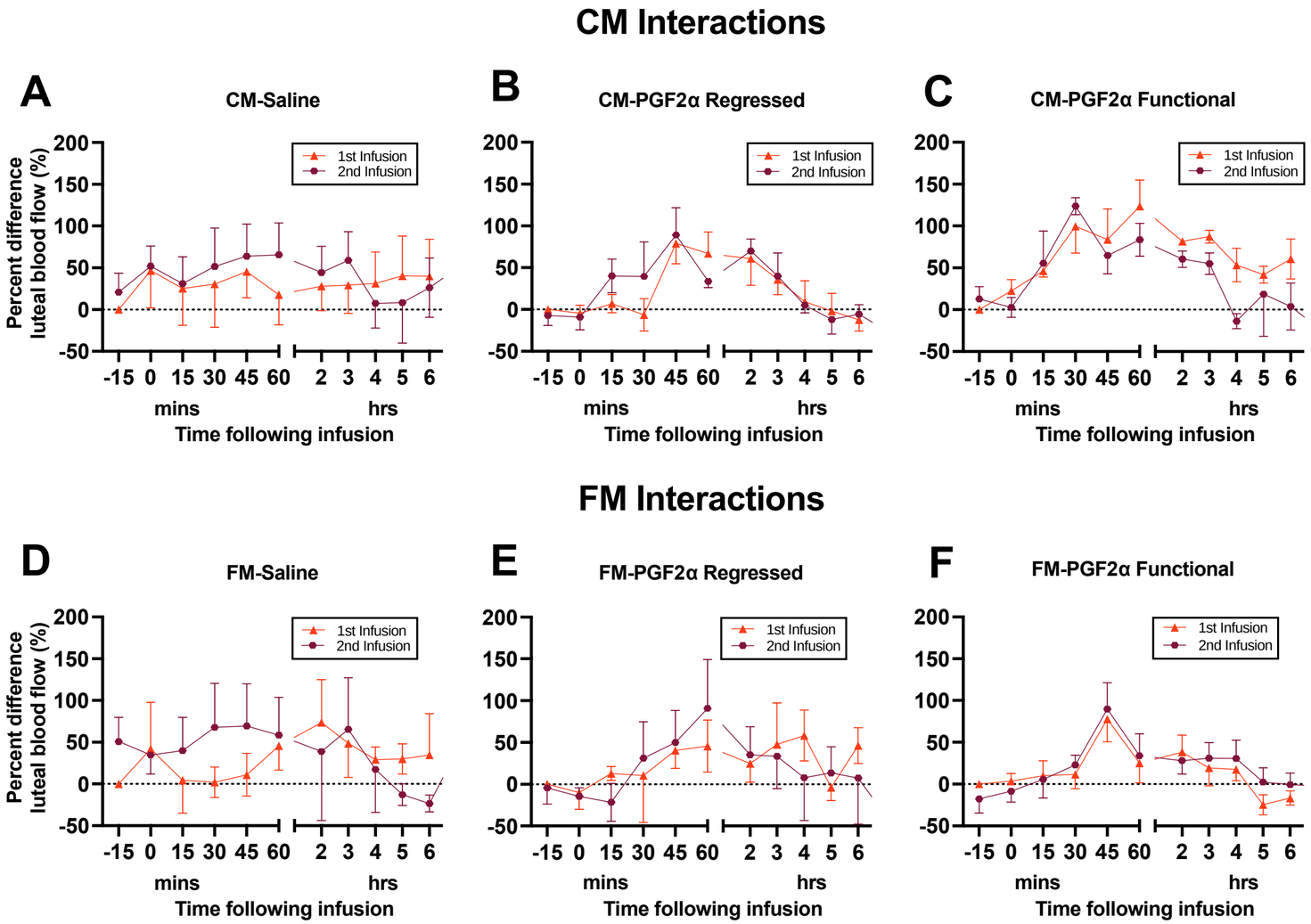
infusion). Percentage difference of luteal blood flow relative to the pre-infusions scan (-15 min of first infusion) was later quantified using ImageJ software. CL were deemed functional if serum progesterone levels were >1 ng/mL or regressed if < 1 ng/mL at time of ovariectomy. All CM (A-D), FM (E-H), and CM versus FM interactions (I-K) between saline or PGF2α infused groups, and PGF2α-functional and non-functional CL groups. CM-Saline (n=4); CM-PGF2α Regressed (n=6); CM-PGF2α Functional (n=4); FM-Saline (n=3); FM-PGF2α Regressed (n=4); FM-PGF2α Functional (n=9). *Significant differences between supplementation or infusion group within time of scan. Data are presented as mean ± SEM. Note: overlapping error bars were removed for effective visualization purposes.

First and Second Infusions

Comparable to results from the raw luteal blood flow (figure 3.22), data converted to percentage difference of luteal blood flow (all time points relative to the first scan prior to any infusions; -15 min of first infusion) yielded no significant differences ($p>0.05$) between the first or second infusion of PGF2α (figure 3.25). These results indicate that the succession of PGF2α infusions did not influence percentage difference of luteal blood flow. Furthermore, transforming data to percentage difference generated no new information with respect to relationships between infusions.

Figure 3.25

Infusion Number (1st vs 2nd) of Prostaglandin F2 Alpha (PGF2α) Did Not Influence the Response in Luteal Blood Flow (Percentage Difference of Luteal Blood Flow) in Corn Gluten Meal (CM) or Fish Meal (FM) Supplemented Cows



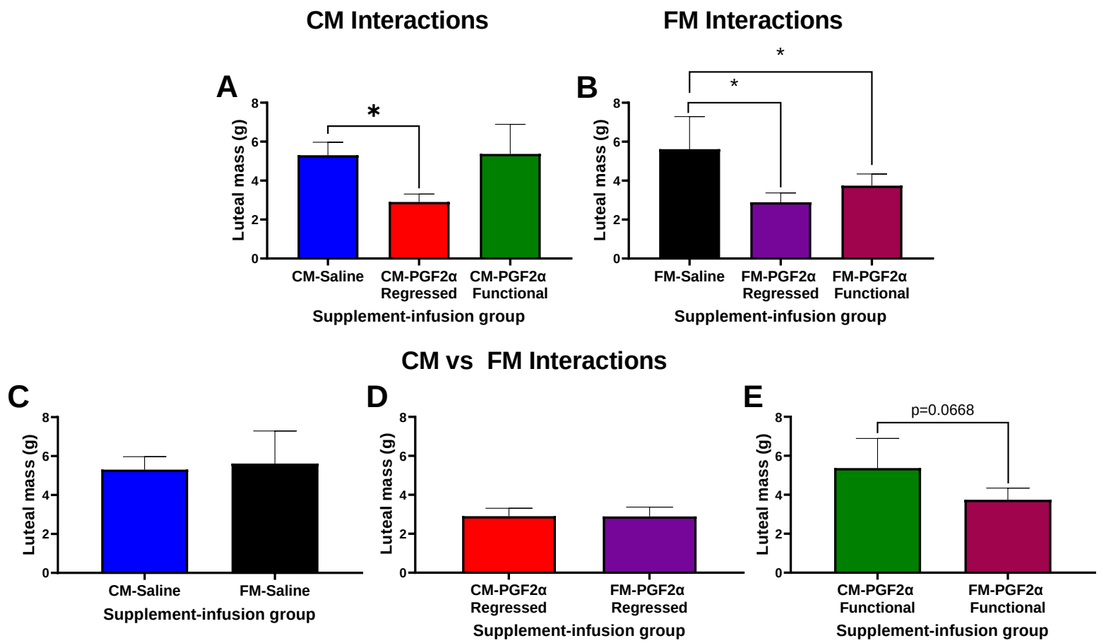
Note. Cows were supplemented with either CM or FM for 63 days, during which estrus was synchronized. On day 10-12 following onset of estrus, cows were infused with either 0.25mL saline or 0.5mg of PGF2α (in 0.25 mL saline) in the uterine horn ipsilateral to the CL-bearing ovary. The first infusion occurred 12 hrs prior to the second infusion, both infusions occurring at 0 mins. Luteal blood flow was assessed using trans-rectal power Doppler ultrasonography (Frequency 6 MHz, PFR 2000 Hz, and depth 60 mm) Percentage difference of luteal blood flow relative to the pre-infusions scan (-15 min of first infusion) was later quantified using ImageJ software. CL were deemed functional if serum progesterone levels were >1 ng/mL or regressed if < 1 ng/mL at time of ovariectomy. All CM (A-C) or FM (D-F) interactions between saline or PGF2α infused groups, and PGF2α-functional and non-functional CL groups. CM-Saline (n=4); CM-PGF2α Regressed (n=6); CM-PGF2α Functional (n=4); FM-Saline (n=3); FM-PGF2α Regressed (n=4); FM-PGF2α Functional (n=9). Data are presented as mean ± SEM. Note: overlapping error bars were removed for effective visualization purposes.

Luteal Mass at Time of Ovariectomy

Mass of the CL at time of ovariectomy was assesed to determine the influence of CM or FM supplementation on luteal involution following PGF2α exposure. Here, reported masses were from 48 hrs ovariectomies only. Luteal mass was not influenced by PGF2α infusions in the CM-PGF2α Functional group (n=3), as the mass did not signficantly differ ($p>0.05$) from CM-Saline controls (n=4) but did ($p<0.05$) from the CM-PGF2α Regressed group (n=6; figure 3.26A). Luteal mass was, however, influenced by PGF2α infusions in the FM-PGF2α Functional group (n=8), as CL mass signficiantly differed ($p<0.05$) from the FM-Saline control (n=4) but not ($p>0.05$) the FM-PGF2α Regressed group (n=4; figure 3.26B). The influence of CM supplementation on luteal mass following PGF2α exposure is furtherd by a trending increase ($p<0.1$) in mass in the CM- vs FM-PGF2α Functional group (figure 3.26E). Neither supplementaiton group (CM or FM) influenced basal mass of the CL ($p>0.05$; figure 3.26C), nor the mass of the regressed CL ($p>0.05$; figure 3.26D).

Figure 3.26

Structural Regression Occurred in Both Corn Gluten Meal (CM) and Fish Meal (FM) Supplemented Cows Following Two Low Dose Intra-Uterine Infusion of Prostaglandin F2 Alpha (PGF2α)



Note. Cows were supplemented with either CM or FM for 63 days, during which estrus was synchronized. On day 10-12 following onset of estrus, cows were infused with either 0.25 mL saline or 0.5 mg of PGF2α (in 0.25 mL saline) in the uterine horn ipsilateral to the corpus luteum (CL)-bearing ovary. The cows were infused twice, each infusion 12 hrs apart. 48 hrs following the first infusion, CL were removed via trans-rectal ovariectomy. CL were dissected from the ovary and massed (g). Any CL containing a fluid filled cavity were massed following removal of fluid. CL were deemed functional if serum progesterone levels were >1 ng/mL or regressed if < 1 ng/mL at time of ovariectomy. All CM (A), FM (B), and CM versus FM interactions (C-E) between saline or PGF2α infused groups, and PGF2α-functional and non-functional CL groups. CM-Saline (n=4); CM-PGF2α Regressed (n=6); CM-PGF2α Functional (n=3); FM-Saline (n=4); FM-PGF2α Regressed (n=4); FM-PGF2α Functional (n=8). *Significant difference ($p<0.05$) between treatments. p values were reported if $p<0.1$, indicating statistical trend. Data are presented as mean ± SEM.

CHAPTER IV

DISCUSSION

Cytokine Exposure and Omega-3 Fatty Acid Pre-Treatment

Middle Stage Corpora Lutea are Less Sensitive to Cytokines

We first sought to explore if the cytotoxic effects of tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ) on bovine luteal cells was dependent on the luteal stage from which cells were derived. From our results, we found a reduced, though marginal, reduction in sensitivity to TNFα and IFNγ by cells from middle staged CL when compared to those of early or late staged CL. The difference in viability between the mixed luteal cell population isolated form middle and early or late staged CL may be because each luteal stage presents a unique microenvironment with differing external stimuli, and as such, may pre-dispose cells to differential transcriptional regulation and subsequent differing responses to stimuli. For example, cells in the early CL undergo extensive programmed cell death during development of the tissue, have an abundance of neutrophils and macrophages, and luteal cells express MHC II. The abundance of M2 and N2 macrophages and neutrophils, respectively, would insinuate that while an inflammatory process, the early CL may be more regulatory and akin to a wound healing environment (Jiemtaweeboon et al., 2011; Penny et al., 1999; Petroff et al., 1999; Reibiger & Spanel-Borowski, 2000; Sakumoto et al., 2000; Terranova & Rice, 1997). However, the unique environment may condition cells to be more receptive to cell death stimuli. Late-stage CL begin to transition towards a pro-cell death and inflammatory environment (regardless of the presence

of prostaglandin F2 alpha [PGF2α]) as evident by increased expression of the pro-inflammatory TNFα and expression of MHC II, the latter of which is supressed only by pregnancy (Benyo et al., 1991; Miyamoto et al., 2005; Pate, 1995; Penny et al., 1999). Finally, middle stage CL generally represent a regulatory environment focused on stabilization and establishment of progesterone output, as evident by a significant presence of gamma delta regulatory T cells and interlukin-10 expression (Shirasuna & Miyamoto, 2016; Walusimbi & Pate, 2014).

Moreover, it is plausible that the difference in cellular viability between stages is related to the expression of the pro-death *TNFRI.* Interestingly, we found that following TNFα and IFNγ exposure, cells from a middle stage CL had a near significant reduction in *TNFR1* steady state mRNA expression when compared to the late staged CL. Since cellular exposure to TNFα and IFNγ synergistically increases gene expression for the TNFR1 receptor (Hojo et al., 2010; Tsujimoto et al., 1986), a reduced capacity for the cytokines to increase expression of *TNFRI* may protect cells from unwarranted cellular death to optimize luteal function. However, why early-stage CL were significantly more sensitive to TNFα and IFNγ, despite insignificant differences in *TNFR1* expression, remains unclear and assessment of protein expression is necessary to further understand the relationship between TNFRI abundance and TNFα and IFNγ sensitivity. It is also plausible that the differential expression of *TNFR1* could be due to the differential abundance of cell types. While the ratio of 3β-HSD positive cells (presumably steroidogenic cells) was relatively consistent across stages in our co-cultures, endothelial and fibroblast cells may not have been equally represented due to differential ratios across the luteal cycle (Lei et al., 1991).

Additionally, since cellular viability was different between CL staged groups, we explored potential differences in biomarkers for programmed cell death (apoptosis and

necroptosis). Interestingly, we found that *MLKL* gene expression following TNFα and IFNγ was elevated in late luteal stages when compared to early staged CL. Necroptosis is a more aggressive form of cellular death, wherein cells are lysed open and contents expelled (Newton & Manning, 2016; Pasparakis & Vandenabeele, 2015). As such, necroptosis may stimulate a larger inflammatory response when compared to the more controlled apoptosis. A CL transitioning to regression may be more inclined to undergo cellular death by way of necroptosis, as it may expedite the establishment of the pro-inflammatory environment contributing to regression. It is also plausible that the middle stage resistance to cell death is accomplished through posttranslational regulation of apoptotic and necroptotic biomarkers (Murphy & Vince., 2015; Zamaraev et al., 2017), and as such, further experimentation is required. Regardless, our findings consistent with previous works, wherein TNFα and IFNγ increased biomarker expression of both apoptotic and necroptotic programmed cell death signaling pathways (Hojo et al., 2016, 2019). These findings potentiate the notion that the cytokines TNFα and IFNγ induce differing degrees and mechanisms of action of cellular death in bovine luteal cells depending on the luteal stage.

Omega-3 Fatty Acid Pre-Treatment Does Not Reduce Cytokine Cytotoxicity

It has been demonstrated on multiple accounts that in cellular culture, TNFα and IFNγ reduce cellular viability by way of reactive oxygen species (ROS) production, as treatment with reactive oxygen scavenger molecules such as glutathione and SOD reduced a complete or partial proportion of the cytokine-induced cellular death (Petroff et al., 2001; Pru et al., 2003). Omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been shown to reduce ROS activity (Garrel et al., 2012; Giordano & Visioli, 2014; Jones et al., 2013). However, in this study, EPA and DHA pre-treatment did not influence (positively or negatively) the cytotoxicity of TNFα and IFNγ. Unsurprisingly, apoptotic gene expression at any luteal stage of culture was not influenced by omega-3

fatty acid pre-treatment, however, necroptotic signaling (*MLKL* gene expression, but not *RIPK1 or RIPK3*) was significantly reduced following pre-treatment in early staged cultures exposed to TNFα and IFNγ. Furthermore, in middle staged cultures, EPA and DHA pre-treatment reduced the basal levels of *MLKL* expression, but not following cytokine exposure. While EPA and DHA pre-treatment did not influence the degree of cell viability, the omega-3 fatty acids may none the less regulate the type of cellular death (apoptotic vs necroptotic) in early luteal staged cultures exposed to TNFα and IFNγ. Similar anti-necroptotic effects of EPA and DHA have been observed in the porcine intestine (Xiao et al., 2020), though potential mechanisms by which the omega-3 fatty acids are influencing necroptosis have scarcely been explored in the literature. Independent of luteal stage, these findings support the rejection of our initial hypothesis, as EPA and DHA did not differentially influence cellular viability following exposure to TNFα and IFNɣ, nor did it influence apoptotic gene expression. However, EPA and DHA did appear to reduce TNFα and IFNɣ induced necroptotic gene expression.

Moreover, it is plausible that while omega-3 fatty acids are not reducing the cytotoxic effects of TNFα and IFNɣ in vitro, omega-3 fatty acids may be reducing the release of TNFα and IFNɣ following PGF2*α* exposure *in vivo*, there in reducing the amount of cell death and preserving luteal function. In fact, in a review by McDaniel et al. (2008), omega-3 fatty acids reduce basal levels of pro-inflammatory cytokines, suggesting a potential influential role in pro-inflammatory cytokine management in the CL.

Cytokines Induced Expression of Pro-Inflammatory Pathways

We demonstrated that *TNFα* expression, independent of luteal stage, is elevated following TNFα and IFNγ exposure, indicating intracellular readiness for a feed forward loop. The notion of a feed forward loop is of relevance when considering the event of luteal regression, wherein a positive feedback loop accelerating cell death and local inflammation may

expedite luteal regression and thus reduce the time for a subsequent estrous cycle (chance at fertilization).

Additionally, we demonstrated that following cytokine exposure, *NFκB* gene expression was also elevated. NFκB regulates an array of cellular processes, including regulation of immunoregulatory proteins, cytokines (such as TNFα), growth factors, and cell survival (Oeckinghaus & Ghosh, 2009). The activation of NFκB by way of TNFα is a well-supported mechanism and may be an additional source of immunological regulation and propagation by luteal cells during luteal regression. In fact, a study demonstrated that TNFα and IFNγ synergistically reduced protein abundance of the NFκB inhibitor, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα), in bovine luteal cell cultures (Suter et al., 2001), indicating subsequent activation of the NFκB transcription factor. It is also plausible that elevated expression of NFκB —when assuming equal translation of protein expression and activity—further propagates synthesis of TNFα therein contributing to further cellular death during luteal regression; all of which requiring further experimentation to conclude. As such, how the changes in gene expression that we observed translate to abundance and activity of NFκB protein must be further explored.

While we found no effects of EPA and DHA pre-treatment on the modulation of *TNFR1, TNFα* or *NFκB* expression, it remains plausible that because we did not replicate the complexity of tissue in a two-dimensional environment, nor did we accommodate interactions between immune cells, steroidogenic cells and endothelial cell, physiological effects may have been missed.

Cytokines Regulate Steroidogenic Gene Expression

In a purified small and large steroidogenic cell culture, PGF2α does not simply or directly reduce steroidogenic capacity or cellular viability (Alila et al., 1988; Pate, 1996; Pate & Condon, 1989). Instead, it has been demonstrated that the environment of the culture, including the concoction of molecules and cellular interactions (i.e., co-cultures), determines the effects of PGF2α (Korzekwa et al., 2008). In fact, Pate and Keyes (2001) considered the potential role that immune cells and their secretions play during luteal regression and inhibition of steroidogenesis, a theory that has, and continues to be supported by the evidence that pro-inflammatory cytokines such as TNFα and IFNγ are 1) present during luteal regression (Kliem et al., 2009; Petroff et al., 1999) 2) are cytotoxic to isolated bovine luteal endothelial cells and isolated luteal steroidogenic cells (Hojo et al., 2016) 3) reduce LH-stimulated progesterone output (Benyo & Pate, 1992; Fairchild & Pate, 1991) and 4) *in vivo* studies have demonstrated that exposure to low doses of TNFα reduces progesterone output comparable to PGF2α exposure (Korzekwa et al., 2008; Skarzynski et al., 2003a). Here, we furthered this theory by demonstrating that TNFα and IFNγ reduced abundance of steroidogenic genes *STARD1* and *CYP11A1*. However, it is unclear whether the reduction in steroidogenic gene expression is a result of transcriptional repression or programmed cell death in steroidogenic populations, therefore the subject requires further experimentation to clarify. Of note, *CYP11A1* gene expression appeared to be insignificantly influenced by the cytokine exposure in early staged cultures, though staged cultures that underwent a significant reduction in *CYP11A1* (i.e., middle, and late) had a comparably low abundance of gene expression to that of the early staged cultures following cytokine exposure. In relation to our findings, studies have demonstrated that ceramide, which is elevated following TNFRI activation in bovine luteal derived endothelial cells (Pru et al., 2003), reduces activity of

a steroidogenic enzymes (CYP19A1) in non-luteinized granulosa cells (Santana et al., 1995)—but whether ceramide has similar effects on progesterone synthesizing enzymes in luteinized cells has yet to be gleaned.

Taken together, the inhibitory influence of TNFα and IFNγ on steroidogenic gene expression occurred independent of luteal stage, indicating that steroidogenic luteal cells may be susceptible to the cytotoxic or inhibitory effects of TNFα and IFNγ at all luteal stages. However, evidence by some research groups demonstrates that certain endothelial and fibroblast cells express STARD1, and cells of the skin express the CYP11A1 gene (Razin et al., 2021; Slominski et al., 2004). As such, it is plausible that cytokine-induced cell death in the endothelial and fibroblast cells in our co-cultures may be responsible for the decline in steroidogenic gene expression. Therefore, it is imperative we further elucidate if the decline in steroidogenic gene expression is occurring in large and small luteal cells. Regardless of the cell types targeted, results here support the notion that TNFα and IFNγ are one of many players aiding in the demise of the corpus luteum.

Fish Meal Supplementation on Luteal Blood Flow

A Pulse in the Percentage of Luteal Blood Flow During Regression

The CL is an extremely vascularized gland, a necessity for the adequate delivery of cholesterol and nutrients to sustain the high demands of steroidogenesis. As such, ameliorating our understanding of luteal blood flow throughout the estrous cycle is imperative and may provide key insight into prolonging the lifespan of the gland, as reduced CL vascularization is an indicator of late luteal regression. It has been demonstrated on multiple accounts that 30 mins, and up until 2 hrs following exposure to PGF2α, luteal blood flow increases (Acosta et al., 2002; Ginther et al., 2007), a result we repeated here as all PGF2α infused groups had an increase in

the percentage difference in luteal blood flow. It is important to note that, in our results, not all groups exposed to PGF2α underwent a significant increase in blood such that it differed from saline controls. However, all PGF2α -infused groups did undergo an apparent increase within 1 hr following infusion time— made apparent when assessing percent difference (percent change) in luteal blood flow. The lack of statistical significance between PGF2α and saline infused groups may well be due to the variation in our control cows associated with a small sample size, as we expected blood flow to be unchanged following saline infusions, as per findings in the literature (Ginther et al., 2007). It is therefore imperative we augment numbers to distinguish between biology and inter-animal variation.

It is also plausible that our use of power Doppler ultrasonography (with an upper blood flow detection limit of 0.3 cm/s) compared to Acosta et al. (2002) and Ginther et al.'s (2007) use of color Doppler ultrasonography (lower detection limit of 6-10 cm/s) undervalued the true spike in percent of luteal blood flow that occurs because of our inability to detect and transmit signal for faster moving blood. Regardless, an irrefutable increase in the percent of luteal blood flow occurs following PGF2α exposure. A frequently hypothesized mechanism by which luteal blood flow increases is by way of nitric oxide (NO) release in response to PGF2α, as NO is a potent vasodilator present following PGF2α administration and following the onset of natural luteolysis (Shirasuna et al., 2004). A study by Shirasuna et al., (2008) demonstrated that administration of a nitric oxide analogue replicated the spike in luteal blood flow 2 hrs following exposure. Furthermore, a research group showed that inhibition of nitric oxide synthase (NOS) reduced PGF2α-induced luteal regression (both structural and functional), indicating it is an imperative molecule downstream of the initial PGF2α pulses (Jaroszewski & Hansel, 2000). While NO clearly follows PGF2α exposure, how PGF2α induces activation of NOS and subsequent NO

release has yet to be demonstrated in the cow CL. A plausible mechanism includes PGF2α binding to the G-protein coupled PGF2α receptor (FP) and activating NOS activity; PGF2α binding to the FP on luteal endothelial cells of smooth muscle-encircled arterioles (Lee et al., 2009; Shirasuna et al., 2008) could transduce activation of NOS via $Ca2^{+}$ influx and phosphorylation events which would increase NO synthesis (Rafikov et al., 2011; Skarzynski et al., 2000). The elevated synthesis of NO would lead to vasodilation in the adjacent smooth muscle cells of arterioles, and the hallmark surge in blood flow would occur. In support of this theory, both endothelial NOS (eNOS) and inducible NOS (iNOS) protein and mRNA expression are elevated in the late corpus luteum, which may be in preparation for the onset of luteal regression allowing for a rapid, but non-immediate increase in luteal blood flow (Miyamoto et al., 2005; Skarzynski et al., 2003b). Shirasuna et al., (2008) also demonstrated that expression of eNOS was specific to the luteal periphery, and FPs were more abundant on peripheral luteal cells proximal to larger smooth muscle arterioles, therein supporting the notion that PGF2α would be primarily activating endothelial cell NOS. An additional mechanism potentially denoting the changes in blood flow during luteal regression includes an ischemia reperfusion model. Ischemia occurs when occlusion of blood vessels—most commonly due to a physical blockade such as atherosclerosis, thromboses, embolism, or torsion—interfere with normal flow. As a result, oxygen distribution to cells is diminished such that metabolic processes slow, ROS accumulate, and intracellular damage occurs (Collard & Gelman, 2001; Kalogeris et al., 2012). While prolonged ischemia can cause extensive damage to tissue, a rapid, reintroduction of blood (reperfusion) to provide oxygen to the deprived cells can expedite, if not worsen, the damage done to cells (Collard & Gelman, 2001). The flux in blood flow that we see following PGF2α exposure may in fact be a reperfusion event following moderate ischemia, as we observed

similar increases in luteal blood flow as other induced ischemia-reperfusion models (Miao et al., 2015). We propose moderate, rather than severe ischemia, as we detect no decrease in luteal blood flow 15 mins following either infusion. It is possible that arteriolar occlusion occurs shortly after PGF2α administration, due to the vasoconstrictive potential of the prostaglandin, but has been missed as a single group has yet to document real time changes in luteal blood flow between 0- and 15-mins post PGF2α administration. Classical signs of ischemia include production of ROS, elevation in cytokine and chemokines, increased neutrophil recruitment and diapedesis, and endothelial cell dysfunction; all of which occur at differential time points in the CL following PGF2α exposure (Dorweiler et al., 2007; Kalogeris et al., 2012; Kato et al., 1997; Sawyer et al., 1990; Shirasuna et al., 2012;). A brief reduction in metabolic demands due to the hypoxic environment would diminish production of progesterone in the CL (Nishimura et al., 2008), and as such, an ischemic event may be responsible for the functional regression preceding that of structural regression. An ischemic event may also explain why we see a brief rebound in serum progesterone around time of recovery in blood flow.

Understanding the true cause and effect of the spike in percentage of luteal blood flow during luteal regression is essential, as to truly generate strategies for interventions in luteal regression, we must uncover the physiological processes at play. Moreover, understanding the physiology regulating blood flow during luteal regression augments possibilities for using the CL as a model system for understanding vascular physiology and pathology interventions.

Luteal Blood Flow is Maintained Forty-Eight Hours Following Infusions Regardless of Luteal Functional State

Following the spike in luteal blood flow, it has been reported that a steady decline occurs until 48 hrs post PGF2α exposure, wherein flow is significantly reduced compared to pre-

exposure ultrasound scans (Acosta et al., 2002; Ginther et al., 2007). However, these results were not repeated in our study. Instead, we found that following the spike in percent of luteal blood flow 30 mins to 1 hr following the first infusion of PGF2α, blood flow recovered and matched that of the control within 4 hrs following time of infusion and did not significantly differ thereafter. One potential reason for our differing results may be due to the differential power in detection of slower moving blood. As previously mentioned, both Acosta et al. (2002) and Ginther et al. (2007) used color Doppler when gathering data, and as such were limited to assessing flows faster than 6-10 cm/s. The apparent reduction in blood flow may therefore be due to an inability to detect and transmit the signal from slower flowing blood that is in fact maintained. In using power Doppler, we were able to detect and transmit signals from blood flow ranging 0-0.3 cm/s and demonstrated that while blood flow did drop back to a basal level, it did not significantly differ from pre-PGF2α exposure scans. Thus, it is worth considering that following PGF2α exposure, whole blood flow does slow, it may not be to the degree implied by Acosta et al. (2002) and Ginther et al. (2007). Also, a group tracking luteal blood flow following onset of natural luteolysis found that blood flow underwent a much slower decline, to such a degree that the percentage of luteal blood flow did not significantly differ from pre-infusion values 48 hrs following pulsatile secretions of PGF2α (Shirasuna et al., 2008). In naturally occurring luteolysis, luteal derived vasoconstrictive factors ET-1, ANG II, and PGF2α reach uppermost means 3 days following onset of luteolysis. If we then compare trends for luteal blood flow and expression of vasoconstrictive factors, the reduction in luteal blood flow (below the original flow threshold) occurs roughly 2-3 days following onset (Miyamoto et al., 2005; Shirasuna et al., 2004, 2008). Maintenance of some degree of blood supply throughout the tissue would be of particular importance to sustain the metabolic demands and metabolite clearance

required for tissue remodeling and glandular involution (Pober & Sessa, 2015). In fact, following a luteolytic dose of PGF2α, the population of endothelial cells does not significantly differ 12 hrs post exposure nor in naturally regressing CL (Hojo et al., 2009). Additionally, smooth muscle encapsulated arteriole density increases during both natural and induced luteal regression (Hojo et al., 2009), and smooth muscle cell actin is maintained (Vonnahme et al., 2006). Therefore, evidence would suggest the vasculature supplying the gland does not recede directly following exposure, and in fact is maintained relative to other cells, if not at a greater proportion when considering smooth muscle arterioles. Visualization and quantification of the vasculature in our collected tissue at time of ovariectomy may elucidate why we see no reduction in luteal blood flow. Additionally, the speed by which luteal blood flow recovered in our study follows the temporal secretion pattern of vasoconstrictive factors in a naturally regressing CL, indicating our results may be representative of a more physiological response; 2 hrs following the start of natural luteolysis, luteal secretions of ET-1, ANG II, and PGF2α (potent vasoconstrictors) all significantly rise (Shirasuna et al., 2004). Miyamoto et al. proposed that the ET-1 and ANGII are released because of the shear stress from the acute spike in luteal blood flow to reestablish flow to homeostatic levels (2005). While vasculature to the gland does eventually dwindle days following PGF2α exposure (exogenous or endogenous), the timing of the decline requires further elucidation, as differing results were found here with use of a more sensitive ultrasound technique.

Moreover, it is also important to consider we may be missing potential differences in the percentage of luteal blood between supplementation groups as all data collection stopped 48 hrs following the first infusion, and so key differences may be occurring past our window of

analysis. We therefore may miss an effect such as maintenance of the percentage of luteal blood flow that could explain the luteoprotective effects of FM supplementation.

These preliminary findings do not support the initial hypothesis that luteal blood flow is prolonged in CL that did not regress 48 hrs following PGF2α exposure, however, a more extensive sample size is required to truly reject the hypotheses.

Fish Meal Supplementation Creates the Optimal Response in Luteal Blood Flow Following Infusions

As previously discussed, NO plays a critical role in luteal regression. As suggested by Shirasuna et al. (2004), the increase in luteal blood flow stimulated in part by the vasodilator NO may be a crucial step in the instigation of luteal regression. In fact, despite prevalence of PGF2α receptors on cells of the early CL, prior to day 4 of the estrous cycle, luteal exposure to PGF2α has no effect on luteal regression nor on luteal blood flow (Acosta et al., 2002; Tsai et al., 1996). Likewise, administration of an NO analogue does not elicit the increased percentage of luteal blood flow observed in the middle-staged CL, and protein expression for eNOS was significantly decreased in the early-stage CL relative to the middle and late luteal stages (Skarzynski et al., 2003b). The culmination of reported findings insinuates that the changes in luteal blood flow play a crucial role in luteal regression, and as such, it is plausible that a reduced response in luteal blood flow following PGF2α may prolong the lifespan of the gland. Interestingly, we observed changes in responsiveness between CM and FM supplemented groups, wherein FM supplemented cows comparatively had a less robust and shorter duration of elevated percent luteal blood flow following PGF2α exposure. This was denoted by a quicker recovery time of 1 hr following the first PGF2α exposure in FM supplemented cows, versus a lack of complete recovery 6 hrs post infusion (percent luteal blood flow did not return to starting blood flow values) in the CM supplemented cows. While the differences between supplementation groups

were less pronounced following the second dose, a similar trend was none-the less observed. Since FM supplementation led to a 114.4% increase in the rate of functional CL following PGF2α infusions when compared to CM supplemented cows (a near significant difference), a blunted increase in luteal blood flow following PGF2α administration may favor CL functionality. However, we would then expect regressing CL to have a significantly pronounced response to luteal blood flow, yet this is not the case for FM-PGF2α Regressed CL. From our results, it is plausible that components of FM supplementation (presumably the omega-3 fatty acids) may dampen the flux in blood flow following PGF2α exposure, such that only minor structural and regressive trends occur that none the less allow for maintenance of a functional gland, as was evident by reduced CL mass and a slight decline in, but ultimate maintenance of, serum progesterone above 1 ng/mL at time of ovariectomy in PGF2α-Functional glands. In other words, the degree of the response in blood flow dictates the fate of the gland. Earlier, we proposed that an ischemia-reperfusion event may occur during luteal regression. In fact, differing lengths of ischemia, as well as the tissue response following reperfusion greatly impacts the outcome of a tissue (Kalogeris et al., 2012). Omega-3 fatty acids have been shown to reduce cellular injury caused by reperfusion injuries (Jing et al., 2014; Kim et al., 2013; Qi et al., 2017), and so it remains plausible that FM supplementation reduces the degree of reperfusion and as such, more effectively prolongs glandular viability. It is also plausible that omega-3 fatty acids reduced cellular damage following a reperfusion event, and to confirm such, assessment of cellular stress biomarkers and programmed cell death between supplementation functional groups is required. While FM supplementation does not appear to maintain luteal blood flow, it does appear to alter the luteal flow response, which requires further exploration.

Progesterone Decline During Luteal Regression is Not Linearly Associated with Luteal Blood Flow

In our current study, we found no correlation (data not shown) between serum progesterone or luteal blood flow between any group 48 hrs following infusion, despite serum progesterone being significantly different between groups. These findings are consistent with those of Kaya et al. (2017) and Lüttgenau et al. (2011), who found that cows with differential luteal blood flow had no statistical difference in progesterone output. However, other groups found contradictory results, wherein blood flow was correlated with serum progesterone (Miyazaki et al., 1998). Interestingly, the reduction in progesterone output during luteal regression far precedes the eventual decrease in luteal blood flow, and as such, provides evidence that blood flow may not be initially correlated with the progesterone decline soon after PGF2α exposure. However, as previously discussed, it is plausible that early reduction in progesterone synthesis may be because of moderate ischemia occurring following exposure to PGF2α.

CHAPTER V

CONCLUSION

Early pregnancy loss continues to be a problematic in the cattle industry. Fish meal dietary supplementation has demonstrated potential in improving overall pregnancy rates. Moreover, fish meal supplementation reduces rates of luteal regression following low dose infusions of PGF2α, and as such, may reduce rates of pregnancy loss on days 16-32 of gestation in cattle. Prior to running large scale breeding trials, understanding the mechanisms by which fish meal supplementation is luteoprotective will bolster our understanding of luteal regression, as well as the role of omega-3 fatty acids in reproductive health and management. Here, we hypothesized two mechanisms by which fish meal supplementation may reduce rates of luteal regression. In the first mechanism, we postulated that EPA and DHA would reduce the cytotoxic effects of two cytokines prevalent during luteal regression (TNFα and IFNγ) in bovine luteal cell culture, as reduced cell death during luteal regression would, in theory, prolong glandular function. The second mechanism we postulated suggested that fish meal supplementation would retain the percentage of luteal blood flow to the corpus luteum in cows that did not regress 48 hrs following exposure to low doses of PGF2α. Loss in luteal blood flow is hallmark of luteal regression, and as such, maintenance of flow may prolong glandular function.

Cytokines and Omega-3 Fatty Acids

Findings here suggest that the cytokines TNFα and IFNγ have differing mechanisms of action on cellular death in bovine luteal cells depending on the luteal stage. EPA and DHA pre-

treatment did not influence the degree of cytotoxicity invoked by cytokines TNFα and IFNγ, therein refuting our initial hypotheses and mechanism postulated. However, EPA and DHA pre-treatment did reduce expression of the necroptotic biomarker *MLKL* either following TNFα and IFNγ exposure in the case of early staged CL or reduced basal MLKL expression in the case of middle staged CL. Taken together, EPA and DHA may have a potential regulatory role of the mode of cellular death in bovine luteal cells. We also found that both *TNFα* and *NFκB* gene expression were elevated following exposure to TNFα and IFNγ, which is the first study to our knowledge demonstrating the cytokine specific activation of genes involved in a pro-inflammatory feed forward loop in bovine luteal cells. Additionally, we contribute to the notion that cytokines TNFα and IFNγ contribute to luteal regression by way of reducing steroidogenic gene expression; whether this is in relation to functional or structural regression, however, requires further exploration.

Findings here collectively contribute to our understanding of EPA and DHA and their role in disrupting translational regulation of necroptotic signaling, as well as the transcriptional influence of the pro-inflammatory molecules TNFα and IFNγ on pro-inflammatory markers in bovine luteal cells.

Fish Meal Supplementation and Luteal Blood Flow

In this study, we replicated findings in the literature wherein the percentage of luteal blood flow drastically increases following PGF2α administration in mid-cycle CL. However, contrary to other groups, we demonstrated no change in the percentage difference in luteal blood flow 48 hrs following PGF2α exposure. Instead, all PGF2α groups (independent of supplementation) recovered back to pre-infusion blood flow values despite evidence of structural and functional regression. We contribute these differences in findings to the type of Doppler

ultrasonography used, as we were able to detect slower moving blood, and so were able to document sustained acute flow. Moreover, we compared the spike in percentage of luteal blood flow following PGF2α administration to an ischemic-reperfusion event, as a portion of the blood flow pattern and the entirety of cellular characteristics during luteal regression coincide with reported features of ischemia and reperfusion related damage. Induction of ischemia reperfusion would provide an effective mechanism for enhancement of luteal regression. Markedly, extensive research is required to elucidate the entirety of physiological events culminating to luteal regression, specifically with regards to the blood flow response. Our findings also demonstrate that FM supplementation may be altering the degree of the blood flow reperfusion in such a capacity that tissue health and maintenance is favored, as demonstrated by a greater percent of functional CL in FM supplemented cows compared to CM supplemented cows following two intrauterine PGF2α infusions, and a blunted increase in luteal blood flow. However, a larger sample size is required to clarify the degree of error in saline infused cows. Additionally, we demonstrated that serum progesterone declined independent of luteal blood flow 48 hrs following infusion, though the relationship between blood flow and progesterone may not be so simplistic as a linear relationship. While findings here do not support our initial hypothesis that blood flow would be maintained in FM supplemented cows 48 hrs following low dose, intra-uterine infusion of PGF2α, FM supplementation does appear to be altering luteal blood flow; the implications of which require further exploration.

Future Directions

Omega-3 Fatty Acids in the Inhibitory Regulation of Necroptosis

In this study, we demonstrated that omega-3 fatty acids reduced TNFα and IFNγ-induced necroptotic gene expression. However, whether inhibition of mRNA abundance corelates to protein translation and subsequent activity, remains unclear. It is therefore imperative we follow-up with protein analysis to properly conclude that omega-3 fatty acids are influencing necroptotic signaling in a mixed bovine luteal cell population.

Ischemia-Reperfusion Model

Here, we proposed that the pattern of luteal blood flow (rapid hyperemia followed by a slow decline) in response to PGF2α exposure resembles that of an ischemic-reperfusion event. If PGF2α exposure indeed causes vasoconstriction and subsequent ischemia, we would expect the effect to occur within the first 15 mins. Therefore, a study assessing luteal blood flow for the entire 15 mins following PGF2α administration is imperative. If an ischemic event were to succeed PGF2α exposure, then the percentage difference in luteal blood flow would decline relative to the pre-infusion scan values.

Immune Cell Infiltration

While the purpose of the hyperemic event following PGF2α exposure remains unclear, it appears to be a conserved portion of luteal regression. We demonstrated here that FM supplementation appears to blunt and delay the PGF2α -induced hyperemic event. As such, we are particularly interested in the biological processes occurring during the first hour post PGF2α exposure, and how FM supplementation may be influencing these events. As was previously discussed, immune cells play an apparent role during luteal regression (Pate et al., 2012; Pate &

Keyes, 2001). An acute neutrophil and macrophage infiltration has been theorized to play an important role in luteolysis (Penny et al., 1999; Shirasuna et al., 2012; Townson et al., 2002). Omega-3 fatty acids have been shown to reduce adhesion molecule expression on endothelial cells, and subsequently reduce immune cell infiltration, as reviewed by Calder (2012). Moreover, during a pro-inflammatory event, immune cells release factors that aid in vasodilation to enhance the immune response. Therefore, assessing changes in immune cell infiltration, as well as phenotyping the immune cell population to distinguish between a net pro-inflammatory versus an anti-inflammatory phenotype may provide insight as to how FM supplementation is luteal protective, and why it is dampening the spike in luteal blood flow.

REFERENCES

Acosta, T. J., Yoshizawa, N., Ohtani, M., & Miyamoto, A. (2002). Local changes in blood flow within the early and midcycle corpus luteum after prostaglandin F2α injection in the cow. *Biology of Reproduction, 66*(3), 651-658.

Aggarwal, B. B. (2003). Signalling pathways of the TNF superfamily: A double-edged sword. Nature Reviews. *Immunology, 3*(9), 745-756.

Al-Gubory, K. H. (2012). Mitochondria: omega-3 in the route of mitochondrial reactive oxygen species. *The International Journal of Biochemistry & Cell Biology, 44*(9), 1569-1573.

Alila, H. W., Dowd, J. P., Corradino, R. A., Harris, W. V., & Hansel, W. (1988). Control of progesterone production in small and large bovine luteal cells separated by flow cytometry. *Reproduction, 82*(2), 645-655.

Arosh, J. A., Banu, S. K., Kimmins, S., Chapdelaine, P., Maclaren, L. A., & Fortier, M. A. (2004). Effect of interferon-τ on prostaglandin biosynthesis, transport, and signaling at the time of maternal recognition of pregnancy in cattle: evidence of polycrine actions of prostaglandin E2. *Endocrinology, 145*(11), 5280-5293.

Aten, R. F., Duarte, K. M., & Behrman, H. R. (1992). Regulation of ovarian antioxidant vitamins, reduced glutathione, and lipid peroxidation by luteinizing hormone and prostaglandin *F2α. Biology of Reproduction, 46*(3), 401-407.

Avramis, C. A., Wang, H., McBride, B. W., Wright, T. C., & Hill, A. R. (2003). Physical and processing properties of milk, butter, and Cheddar cheese from cows fed supplemental fish meal. *Journal of Dairy Science, 86*(8), 2568-2576.

Bach, E. A., Aguet, M., & Schreiber, R. D. (1997). The IFNγ receptor: a paradigm for cytokine receptor signaling. *Annual Review of Immunology, 15*(1), 563-591.

Baddela, V. S., Koczan, D., Viergutz, T., Vernunft, A., & Vanselow, J. (2018). Global gene expression analysis indicates that small luteal cells are involved in extracellular matrix modulation and immune cell recruitment in the bovine corpus luteum. *Molecular and Cellular Endocrinology, 474*, 201-213.

Bane, A., & Rajakoski, E. (1961). The bovine estrous cycle. *The Cornell Veterinarian, 51*, 77 95.

Bartol, F. F., Thatcher, W. W., Lewis, G. S., Bliss, E. L., Drost, M., & Bazer, F. W. (1981). Effect of estradiol-17β on PGF and total protein content in bovine uterine flushings and peripheral plasma concentration of 13, 14-dihydro-15-keto PGF2α. *Theriogenology, 15*(4), 345-358.

Bauer, M., Reibiger, I., & Spanel-Borowski, K. (2001). Leucocyte proliferation in the bovine corpus luteum. *Reproduction (Cambridge, England), 121*(2), 297-305.

Bellows, R. A., & Short, R. E. (2021). *Reproductive losses in the beef industry.* In Factors Affecting Calf Crop (pp. 109-133). CRC Press.

Benyo, D. F., Haibel, G. K., Laufman, H. B., & Pate, J. L. (1991). Expression of major histocompatibility complex antigens on the bovine corpus luteum during the estrous cycle, luteolysis, and early pregnancy. *Biology of Reproduction, 45*(2), 229–234.

Benyo, D. F., & Pate, J. L. (1992). Tumor necrosis factor-alpha alters bovine luteal cell synthetic capacity and viability. *Endocrinology, 130*(2), 854-860.

Berisha, B., Schams, D., Kosmann, M., Amselgruber, W., & Einspanier, R. (2000). Expression and tissue concentration of vascular endothelial growth factor, its receptors, and localization in the bovine corpus luteum during estrous cycle and pregnancy. *Biology of Reproduction, 63*(4), 1106-1114.

Berisha, B., Steffl, M., Amselgruber, W., & Schams, D. (2006). Changes in fibroblast growth factor 2 and its receptors in bovine follicles before and after GnRH application and after ovulation. *Reproduction (Cambridge, England), 131*(2), 319–329.

Berisha, B., Steffl, M., Welter, H., Kliem, H., Meyer, H. H., Schams, D., & Amselgruber, W. (2008). Effect of the luteinising hormone surge on regulation of vascular endothelial growth factor and extracellular matrix-degrading proteinases and their inhibitors in bovine follicles. *Reproduction, Fertility and Development, 20*(2), 258-268.

Besbaci, M., Abdelli, A., Belabdi, I., & Raboisson, D. (2021). Non-steroidal anti-inflammatory drugs at embryo transfer on pregnancy rates in cows: A meta-analysis. *Theriogenology, 171*, 64-71.

Bimbo, A. P., & Crowther, J. B. (1992). Fish meal and oil: Current uses. *Journal of the American Oil Chemists Society, 69*(3), 221-227.

Bishop, C. V., Selvaraj, V., Townson, D. H., Pate, J. L., & Wiltbank, M. C. (2022). History, insights, and future perspectives on studies into luteal function in cattle. *Journal of Animal Science, 100*(7), skac143.

Burke, J. M., Staples, C. R., Risco, C. A., De La Sota, R. L., & Thatcher, W. W. (1997). Effect of ruminant grade menhaden fish meal on reproductive and productive performance of lactating dairy cows. *Journal of Dairy Science, 80*(12), 3386-3398.

Burns, P. D., Bonnette, T. R., Engle, T. E., & Whittier, J. C. (2002). Effects of fishmeal supplementation on fertility and plasma Ω-3 fatty acid profiles in primiparous, lactating beef cows. *The Professional Animal Scientist, 18*(4), 373-379.

Calder, P. C. (2012). The role of marine omega-3 (n-3) fatty acids in inflammatory processes, atherosclerosis and plaque stability. *Molecular Nutrition & Food Research, 56*(7), 1073 1080.

Cannon, M. J., & Pate, J. L. (2003). The role of major histocompatibility complex molecules in luteal function. *Reproductive Biology and Endocrinology, 1*(1), 1-7.

Cannon, M. J., & Pate, J. L. (2006). Indoleamine 2, 3-dioxygenase participates in the interferon gamma-induced cell death process in cultured bovine luteal cells. *Biology of Reproduction, 74*(3), 552-559.

Carswell, E., Old, L. J., Kassel, R., Green, S., Fiore, N., & Williamson, B. (1975). An endotoxin induced serum factor that causes necrosis of tumors. *Proceedings of the National Academy of Sciences, 72*(9), 3666-3670.

Cedillo, J. C. (2019). *The Effects of Fish Meal Supplementation on Gene Expression in the Bovine Corpus Luteum Following Low Dose Administration of Prostaglandin F2A* (Unpublished master's thesis). The University of Northern Colorado, Greeley.

Childs, S., Hennessy, A. A., Sreenan, J. M., Wathes, D. C., Cheng, Z., Stanton, C., Diskin, M. G., & Kenny, D. A. (2008). Effect of level of dietary n-3 polyunsaturated fatty acid supplementation on systemic and tissue fatty acid concentrations and on selected reproductive variables in cattle. *Theriogenology, 70*(4), 595–611.

Clemente, M., de La Fuente, J., Fair, T., Al Naib, A., Gutierrez-Adan, A., Roche, J. F., Rizos, D., & Lonergan, P. (2009). Progesterone and conceptus elongation in cattle: a direct effect on the embryo or an indirect effect via the endometrium?. *Reproduction (Cambridge, England), 138*(3), 507–517.

Coelho, S., Ambrose, J. D., Binelli, M., Burke, J., Staples, C. R., Thatcher, M. J., & Thatcher, W. W. (1997). Menhaden fish meal attenuates estradiol-and oxytocin-induced uterine secretion of PGF 2 α in lactating dairy cattle. *Theriogenology, 1*(47), 144.

Cohen, G. M. (1997). Caspases: the executioners of apoptosis. *Biochemical Journal, 326*(1), 1 16.

Collard, C. D., & Gelman, S. (2001). Pathophysiology, clinical manifestations, and prevention of ischemia-reperfusion injury. *The Journal of the American Society of Anesthesiologists, 94*(6), 1133-1138.

Davis, T. C., & White, R. R. (2020). Breeding animals to feed people: The many roles of animal reproduction in ensuring global food security. *Theriogenology, 150*, 27–33.

Davis, T. L., & Pate, J. L. (2007). Bovine luteal cells stimulate proliferation of major histocompatibility nonrestricted gamma delta T cells. *Biology of Reproduction, 77*(6), 914-922.

Dirandeh, E., Towhidi, A., Zeinoaldini, S., Ganjkhanlou, M., Ansari Pirsaraei, Z., & Fouladi Nashta, A. (2013). Effects of different polyunsaturated fatty acid supplementations during the postpartum periods of early lactating dairy cows on milk yield, metabolic responses, and reproductive performances. *Journal of Animal Science, 91*(2), 713–721.

Donaldson, L. E., & Hansel, W. (1965). Prolongation of life span of the bovine corpus luteum by single injections of bovine luteinizing hormone. *Journal of dairy science, 48(*7), 903-904.

Dorweiler, B., Pruefer, D., Andrasi, T. B., Maksan, S. M., Schmiedt, W., Neufang, A., & Vahl, C. F. (2007). Ischemia-reperfusion injury. *European Journal of Trauma and Emergency Surgery*, *33(*6), 600-612.

Endo, T. I., Aten, R. F., Wang, F. R., & Behrman, H. R. (1993). Coordinate induction and activation of metalloproteinase and ascorbate depletion in structural luteolysis. *Endocrinology*, *133*(2), 690-698.

Fairchild, D. L., & Pate, J. L. (1989). Interferon-γ induction of major histocompatibility complex antigens on cultured bovine luteal cells. *Biology of Reproduction*, *40*(3), 453-457.

Fairchild, D. L., & Pate, J. L. (1991). Modulation of bovine luteal cell synthetic capacity by interferon-γ. *Biology of Reproduction*, *44*(2), 357-363.

Fallarino, F., Grohmann, U., Vacca, C., Bianchi, R., Orabona, C., Spreca, A., Fioretti, M., & Puccetti, P. (2002). T cell apoptosis by tryptophan catabolism. *Cell Death and Differentiation*, *9*(10), 1069-1077.

Farin, P. W., Piedrahita, J. A., & Farin, C. E. (2006). Errors in development of fetuses and placentas from in vitro-produced bovine embryos. *Theriogenology, 65*(1), 178-191.

Faustman, D. L., & Davis, M. (2013). TNF receptor 2 and disease: autoimmunity and regenerative medicine. *Frontiers in Immunology*, *4*, 478.

Ford, S. P., & Chenault, J. R. (1981). Blood flow to the corpus luteum-bearing ovary and ipsilateral uterine horn of cows during the oestrous cycle and early pregnancy. *Reproduction, 62*(2), 555-562.

Fortier, M. A., Guilbault, L. A., & Grasso, F. (1988). Specific properties of epithelial and stromal cells from the endometrium of cows. *Reproduction, 83*(1), 239-248.

Fotin-Mleczek, M., Henkler, F., Samel, D., Reichwein, M., Hausser, A., Parmryd, I., Scheurich, P., Schmid, J., & Wajant, H. (2002). Apoptotic crosstalk of TNF receptors: TNF-R2 induces depletion of TRAF2 and IAP proteins and accelerates TNF-R1-dependent activation of caspase-8. *Journal of Cell Science, 115*(13), 2757-2770.

Fraenkel, L., & Cohn, F. (1901). Experimentelle Untersuchungen über den Einfluss des Corpus luteum auf die Insertion des Eies. *Anat. Anz, 20*, 294-300.

Fraser, H. M., Wilson, H., Morris, K. D., Swanston, I., & Wiegand, S. J. (2005). Vascular endothelial growth factor Trap suppresses ovarian function at all stages of the luteal phase in the macaque. *The Journal of Clinical Endocrinology & Metabolism, 90*(10), 5811-5818.

Friedman, A., Weiss, S., Levy, N., & Meidan, R. (2000). Role of tumor necrosis factor α and its type I receptor in luteal regression: induction of programmed cell death in bovine corpus luteum-derived endothelial cells. *Biology of Reproduction, 63*(6), 1905-1912.

Funston, R. N. (2004). Fat supplementation and reproduction in beef females. *Journal of Animal Science, 82*(suppl_13), E154-E161.

Galluzzi, L., Kepp, O., Chan, F. K. M., & Kroemer, G. (2017). Necroptosis: mechanisms and relevance to disease. *Annual Review of Pathology, 12*, 103.

Garrel, C., Alessandri, J. M., Guesnet, P., & Al-Gubory, K. H. (2012). Omega-3 fatty acids enhance mitochondrial superoxide dismutase activity in rat organs during post-natal development. *The International Journal of Biochemistry & Cell Biology, 44*(1), 123-131.

Geary, T. W., Ansotegui, R. P., MacNeil, M. D., Roberts, A. J., & Waterman, R. C. (2010). Effects of flunixin meglumine on pregnancy establishment in beef cattle. *Journal of Animal Science, 88*(3), 943-949.

Ginther, O. J., Silva, L. A., Araujo, R. R., & Beg, M. A. (2007). Temporal associations among pulses of 13, 14-dihydro-15-keto-PGF2alpha, luteal blood flow, and luteolysis in cattle. *Biology of Reproduction, 76*(3), 506-513.

Giordano, E., & Visioli, F. (2014). Long-chain omega 3 fatty acids: molecular bases of potential antioxidant actions. *Prostaglandins, Leukotrienes and Essential Fatty Acids, 90*(1), 1-4.

Girsh, E., Greber, Y., & Meidan, R. (1995). Luteotrophic and luteolytic interactions between bovine small and large luteal-like cells and endothelial cells. *Biology of Reproduction, 52*(4), 954-962.

Godkin, J. D., Smith, S. E., Johnson, R. D., & Doré, J. J. (1997). The role of trophoblast interferons in the maintenance of early pregnancy in ruminants. *American Journal of Reproductive Immunology, 37*(1), 137-143.

Gong, Y. N., Guy, C., Crawford, J. C., & Green, D. R. (2017). Biological events and molecular signaling following MLKL activation during necroptosis. *Cell Cycle, 16*(19), 1748-1760.

Gulliver, C. E., Friend, M. A., King, B. J., & Clayton, E. H. (2012). The role of omega-3 polyunsaturated fatty acids in reproduction of sheep and cattle. *Animal Reproduction Science, 131*(1-2), 9–22.

Hammond, J. (1927). *The physiology of reproduction in the cow*. Cambridge University Press, Cambridge.

Hansen, T. R., Henkes, L. K., Ashley, R. L., Bott, R. C., Antoniazzi, A. Q., & Han, H. (2010). Endocrine actions of interferon-tau in ruminants. *Society of Reproduction and Fertility Supplement, 67*, 325.

Hedges, J. F., Cockrell, D., Jackiw, L., Meissner, N., & Jutila, M. A. (2003). Differential mRNA expression in circulating γδ T lymphocyte subsets defines unique tissue-specific functions. *Journal of Leukocyte Biology, 73*(2), 306-314.

Helmer, S. D., Hansen, P. J., Thatcher, W. W., Johnson, J. W., & Bazer, F. W. (1989). Intrauterine infusion of highly enriched bovine trophoblast protein-1 complex exerts an antiluteolytic effect to extend corpus luteum lifespan in cyclic cattle. *Reproduction, 87*(1), 89-101.

Hess, B. W., Moss, G. E., & Rule, D. C. (2008). A decade of developments in the area of fat supplementation research with beef cattle and sheep 1. *Journal of Animal Science, 86*, E188-E204.

Hojo, T., Al-Zi'Abi, M. O., Skarzynski, D. J., Acosta, T. J., & Okuda, K. (2009). Changes in the vasculature of bovine corpus luteum during the estrous cycle and prostaglandin F2α induced luteolysis. *Journal of Reproduction and Development*, 0906090178-0906090178.

Hojo, T., Oda, A., Lee, S. H., Acosta, T. J., & Okuda, K. (2010). effects of tumor necrosis factor α and interferon γ on the viability and MRNA expression of TNF receptor type I in endothelial cells from the bovine corpus luteum. *Journal of Reproduction and Development*, 1006110282-1006110282.

Hojo, T., Piotrowska-Tomala, K. K., Jonczyk, A. W., Lukasik, K., Jankowska, K., Okuda, K., Witek, K. J., & Skarzynski, D. J. (2019). Receptor interacting protein kinases-dependent necroptosis as a new, potent mechanism for elimination of the endothelial cells during luteolysis in cow. *Theriogenology, 128*, 193-200.

Hojo, T., Siemieniuch, M. J., Lukasik, K., Piotrowska-Tomala, K. K., Jonczyk, A. W., Okuda, K., & Skarzynski, D. J. (2016). Programmed necrosis-a new mechanism of steroidogenic luteal cell death and elimination during luteolysis in cows. *Scientific reports, 6*(1), 1-14.

Howe, P. R., Evans, H. M., Kuszewski, J. C., & Wong, R. H. (2018). Effects of long chain omega-3 polyunsaturated fatty acids on brain function in mildly hypertensive older adults. *Nutrients, 10*(10), 1413.

Hryciuk, M. M., Braun, B. C., Bailey, L. D., & Jewgenow, K. (2019). Functional and morphological characterization of small and large steroidogenic luteal cells from domestic cats before and during culture. *Frontiers in endocrinology, 10*, 724.

Hsu, H. C., Chen, C. Y., & Chen, M. F. (2014). N-3 polyunsaturated fatty acids decrease levels of doxorubicin-induced reactive oxygen species in cardiomyocytes--involvement of uncoupling protein UCP2. *Journal of Biomedical Science, 21*(1), 1-11.

Hünigen, H., Bisplinghoff, P., Plendl, J., & Bahramsoltani, M. (2008). Vascular dynamics in relation to immunolocalisation of VEGF-A, VEGFR-2 and Ang-2 in the bovine corpus luteum. *Acta Histochemica, 110*(6), 462-472.

Inskeep, E. K. (1973). Potential uses of prostaglandins in control of reproductive cycles of domestic animals. *Journal of Animal Science, 36*(6), 1149-1157.

Ireland, J. J., Murphee, R. L., & Coulson, P. B. (1980). Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum. *Journal of Dairy Science, 63*(1), 155-160.

Jaroszewski, J. J., & Hansel, W. (2000). Intraluteal Administration of a Nitric Oxide Synthase Blocker Stimulates Progesterone and Oxytocin Secretion and Prolongs the Life Span of the Bovine Corpus Luteum (44514). *Proceedings of the Society for Experimental Biology and Medicine, 224*(1), 50-55.

Jaśkowski, B. M., Opałka, A., Gehrke, M., Herudzińska, M., Czeladko, J., Baumgartner, W., & Jaśkowski, J. M. (2021). A Critical Overview on Prostaglandin Inhibitors and Their Influence on Pregnancy Results after Insemination and Embryo Transfer in Cows. *Animals, 11*(12), 3368.

Jiemtaweeboon, S., Shirasuna, K., Nitta, A., Kobayashi, A., Schuberth, H. J., Shimizu, T., & Miyamoto, A. (2011). Evidence that polymorphonuclear neutrophils infiltrate into the developing corpus luteum and promote angiogenesis withinterleukin-8 in the cow. *Reproductive Biology and Endocrinology: RB&E, 9*, 79.

Jing, H., Yao, J., Liu, X., Fan, H., Zhang, F., Li, Z., Tian, X., & Zhou, Y. (2014). Fish-oil emulsion (omega-3 polyunsaturated fatty acids) attenuates acute lung injury induced by intestinal ischemia reperfusion through adenosine 5′-monophosphate-activated protein kinase–sirtuin1 pathway. *The Journal of Surgical Research, 187*(1), 252-261.

Jonczyk, A. W., Piotrowska-Tomala, K. K., & Skarzynski, D. J. (2019). Effects of prostaglandin F2α (PGF2α) on cell-death pathways in the bovine corpus luteum (CL). *BMC Veterinary Research, 15*(1), 1-16.

Jones, M. L., Mark, P. J., Mori, T. A., Keelan, J. A., & Waddell, B. J. (2013). Maternal dietary omega-3 fatty acid supplementation reduces placental oxidative stress and increases fetal and placental growth in the rat. *Biology of Reproduction, 88*(2), 37.

Juengel, J. L., Meberg, B. M., McIntush, E. W., Smith, M. F., & Niswender, G. D. (1998). Concentration of mRNA encoding 3β-hydroxysteroid dehydrogenase/Δ5, Δ4 isomerase (3β-HSD) and 3β-HSD enzyme activity following treatment of ewes with prostaglandin F2α. *Endocrine*, *8*(1), 45-50.

Kairenius, P., Ärölä, A., Leskinen, H., Toivonen, V., Ahvenjärvi, S., Vanhatalo, A., Huhtanen, P., Hurme, T., Griinari, J. M., & Shingfield, K. J. (2015). Dietary fish oil supplements depress milk fat yield and alter milk fatty acid composition in lactating cows fed grass silage-based diets. *Journal of Dairy Science*, *98*(8), 5653–5671.

Kalogeris, T., Baines, C. P., Krenz, M., & Korthuis, R. J. (2012). Cell biology of ischemia/reperfusion injury. *International review of cell and molecular biology*, *298*, 229-317.

Karasahin, T., Alkan, H., Satilmis, F., Dursun, S., & Erdem, H. (2021). Effect of flunixin meglumine treatment during and after embryo transfer on the pregnancy rate in cattle. *Reproduction in Domestic Animals, 56*(12), 1555-1561.

Kato, H., Sugino, N., Takiguchi, S., Kashida, S., & Nakamura, Y. (1997). Roles of reactive oxygen species in the regulation of luteal function. *Reviews of Reproduction, 2*, 81-83.

Kaya, S., Kaçar, C., Polat, B., Çolak, A., Kaya, D., Gürcan, I. S., Bollwein, H., & Aslan, S. (2017). Association of luteal blood flow with follicular size, serum estrogen and progesterone concentrations, and the inducibility of luteolysis by $PGF_{2\alpha}$ in dairy cows. *Theriogenology*, *87*, 167–172.

Kim, K., Jung, N., Lee, K., Choi, J., Kim, S., Jun, J., Kim, E., & Kim, D. (2013). Dietary omega 3 polyunsaturated fatty acids attenuate hepatic ischemia/reperfusion injury in rats by modulating toll-like receptor recruitment into lipid rafts. *Clinical Nutrition (Edinburgh, Scotland), 32*(5), 855-862.

Kim, M., Lee, S. H., Lee, S., & Kim, G. Y. (2019). Expression of fas and TNFR1 in the luteal cell types isolated from the ovarian corpus luteum. *Biomedical Science Letters, 25*(1), 107-112.

King, S. R., & LaVoie, H. A. (2012). Gonadal transactivation of STARD1, CYP11A1 and HSD3B. *Frontiers in Bioscience-Landmark, 17*(3), 824-846.

Kizuka, F., Tokuda, N., Takagi, K., Adachi, Y., Lee, L., Tamura, I., Maekawa, R., Taketani, T., Tamura, H., Suzuki, T., Owada, Y., & Sugino, N. (2012). Involvement of bone marrow derived vascular progenitor cells in neovascularization during formation of the corpus luteum in mice. *Biology of Reproduction, 87*(3), 55-55.

Kliem, H., Berisha, B., Meyer, H. H. D., & Schams, D. (2009). Regulatory changes of apoptotic factors in the bovine corpus luteum after induced luteolysis. *Molecular Reproduction and Development, 76*(3), 220-230.

Kliem, H., Rodler, D., Ulbrich, S. E., Sinowatz, F., Berisha, B., Meyer, H. H., & Schams, D. (2013). Dexamethasone-induced eosinopenia is associated with lower progesterone production in cattle. *Reproduction in Domestic Animals, 48*(1), 137-148.

Korzekwa, A. J., Jaroszewski, J. J., Woclawek-Potocka, I., Bah, M. M., & Skarzynski, D. J. (2008). Luteolytic effect of prostaglandin F2α on bovine corpus luteum depends on cell composition and contact. *Reproduction in Domestic Animals, 43*(4), 464-472.

Lafrance, M., Goff, A. K., Guay, P., & Harvey, D. (1989). Failure to maintain luteal function: a possible cause of early embryonic loss in a cow. *Canadian Journal of Veterinary Research=Revue Canadienne de Recherche Veterinaire, 53*(3), 279–284.

Langbeen, A., Bartholomeus, E., Leroy, J. L., & Bols, P. E. (2015). Bovine in vitro reproduction models can contribute to the development of (female) fertility preservation strategies. *Theriogenology*, *84*(4), 477-489.

LaVoie, H. A. (2016). Luteal steroidogenesis. *The life cycle of the corpus luteum* (pp. 37-55). Springer International Publishing.

Lee, H. J., Han, J., Jang, Y., Kim, S. J., Park, J. H., Seo, K. S., Jeong, S., Shin, S., Lim, K., Heo, J. Y., & Kweon, G. R. (2015). Docosahexaenoic acid prevents paraquat-induced reactive oxygen species production in dopaminergic neurons via enhancement of glutathione homeostasis. *Biochemical and Biophysical Research Communications*, *457*(1), 95-100.

Lee, S. H., Acosta, T. J., Yoshioka, S., & Okuda, K. (2009). Prostaglandin F2α regulates the nitric oxide generating system in bovine luteal endothelial cells. *Journal of Reproduction and Development*, 0904240164-0904240164.

Lei, Z. M., Chegini, N., & Rao, C. V. (1991). Quantitative cell composition of human and bovine corpora lutea from various reproductive states. *Biology of Reproduction, 44*(6), 1148 1156.

Lescano de Souza Junior, A., Mancini Filho, J., Pavan Torres, R., Irigoyen, M. C., & Curi, R. (2017). Pretreatment with fish oil attenuates heart ischaemia consequences in rats. *Experimental physiology*, *102*(11), 1459–1473.

Linkermann, A., & Green, D. R. (2014). Necroptosis. *New England Journal of Medicine, 370*(5), 455-465.

Liszewska, E., Rekawiecki, R., & Kotwica, J. (2005). Effect of progesterone on the expression of bax and bcl-2 and on caspase activity in bovine luteal cells. *Prostaglandins & Other Lipid Mediators, 78*(1-4), 67–81.

Liu, T., Zhang, L., Joo, D., & Sun, S. C. (2017). NF-κB signaling in inflammation. *Signal Transduction and Targeted Therapy*, *2*(1), 1-9.

Louis, T. M., Parry, D. M., Robinson, J. S., Thorburn, G. D., & Challis, J. R. (1977). Effects of exogenous progesterone and oestradiol on prostaglandin F and 13,14-dihydro-15-oxo prostaglandin F2alpha concentrations in uteri and plasma of ovariectomized ewes. *The Journal of Endocrinology, 73*(3), 427–439.

Lüttgenau, J., Ulbrich, S. E., Beindorff, N., Honnens, A., Herzog, K., & Bollwein, H. (2011). Plasma progesterone concentrations in the mid-luteal phase are dependent on luteal size, but independent of luteal blood flow and gene expression in lactating dairy cows. *Animal Reproduction Science*, *125*(1-4), 20-29.

Magnus, V. (1901). Ovariets betydning for svangerskabet med saerligt hensyn til corpus luteum. *Nor. Mag. Laegevidensk*, *62*, 1138-1145.

Malhi, P. S., Adams, G. P., & Singh, J. (2005). Bovine model for the study of reproductive aging in women: follicular, luteal, and endocrine characteristics. *Biology of reproduction*, *73*(1), 45-53.

Mamluk, R., Chen, D. B., Greber, Y., Davis, J. S., & Meidan, R. (1998). Characterization of messenger ribonucleic acid expression for prostaglandin F2α and luteinizing hormone receptors in various bovine luteal cell types. *Biology of Reproduction, 58*(3), 849-856.

Mann, G. E., & Lamming, G. E. (2001). Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. *Reproduction (Cambridge, England), 121*(1), 175–180.

Mattos, R., Guzeloglu, A., Badinga, L., Staples, C. R., & Thatcher, W. W. (2003). Polyunsaturated fatty acids and bovine interferon-τ modify phorbol ester-induced secretion of prostaglandin F2α and expression of prostaglandin endoperoxide synthase-2 and phospholipase-A2 in bovine endometrial cells. *Biology of Reproduction, 69*(3), 780 787.

Mattos, R., Staples, C. R., Arteche, A., Wiltbank, M. C., Diaz, F. J., Jenkins, T. C., & Thatcher, W. W. (2004). The effects of feeding fish oil on uterine secretion of PGF2α lpha, milk composition, and metabolic status of periparturient Holstein cows. *Journal of Dairy Science, 87*(4), 921–932.

McCracken, J. A., Custer, E. E., & Lamsa, J. C. (1999). Luteolysis: a neuroendocrine-mediated event. *Physiological Reviews, 79*(2), 263-323.

McDaniel, J. C., Belury, M., Ahijevych, K., & Blakely, W. (2008). Omega-3 fatty acids effect on wound healing. *Wound Repair and Regeneration, 16*(3), 337-345.

Meidan, R., Girsh, E., Mamluk, R., Levy, N., & Farberov, S. (2016). Luteolysis in ruminants: Past concepts, new insights, and persisting challenges. *The life cycle of the corpus luteum* (pp. 159-182). Springer International Publishing.

Meissner, N., Radke, J., Hedges, J., White, M., Behnke, M., Bertolino, S., Abrahamsen, M., & Jutila, M. (2003). Serial analysis of gene expression in circulating gamma delta T cell subsets defines distinct immunoregulatory phenotypes and unexpected gene expression profiles. *The Journal of Immunology, 170*(1), 356-364.

Melmed, S., Polonsky, K. S., Larsen, P. R., & Kronenberg, H. M. (2015). *Williams textbook of endocrinology E-Book*. Elsevier Health Sciences.

Merrill, M. L., Ansotegui, R. P., Burns, P. D., MacNeil, M. D., & Geary, T. W. (2007). Effects of flunixin meglumine and transportation on establishment of pregnancy in beef cows. *Journal of Animal Science, 85*(6), 1547-1554.

Meyer, H. H., Mittermeier, T., & Schams, D. (1988). Dynamics of oxytocin, estrogen and progestin receptors in the bovine endometrium during the estrous cycle. *Acta Endocrinologica, 118(*1), 96–104.

Meyer, M. D., Desnoyers, G. D., Oldick, B., Thatcher, W. W., Drost, M., Schalue, T. K., & Roberts, R. M. (1996). Treatment with recombinant bovine interferon-τ in utero attenuates secretion of prostaglandin F from cultured endometrial epithelial cells. *Journal of Dairy Science, 79*(8), 1375-1384.

Miao, Y. F., Wu, H., Yang, S. F., Dai, J., Qiu, Y. M., Tao, Z. Y., & Zhang, X. H. (2015). 5′ adenosine monophosphate-induced hypothermia attenuates brain ischemia/reperfusion injury in a rat model by inhibiting the inflammatory response. *Mediators of Inflammation, 2015*.

Miller, W. L., & Bose, H. S. (2011). Early steps in steroidogenesis: intracellular cholesterol trafficking: thematic review series: genetics of human lipid diseases. *Journal of Lipid Research, 52(*12), 2111-2135.

Mirando, M. A., Harney, J. P., Zhou, Y., Ogle, T. F., Ott, T. L., Moffatt, R. J., & Bazer, F. W. (1993). Changes in progesterone and oestrogen receptor mRNA and protein and oxytocin receptors in endometrium of ewes after intrauterine injection of ovine trophoblast interferon. *Journal of Molecular Endocrinology, 10*(2), 185-192.

Miyamoto, A., Shirasuna, K., Wijayagunawardane, M. P. B., Watanabe, S., Hayashi, M., Yamamoto, D., Matsui, M., & Acosta, T. J. (2005). Blood flow: a key regulatory component of corpus luteum function in the cow. *Domestic Animal Endocrinology, 29*(2), 329-339.

Miyazaki, T., Tanaka, M., Miyakoshi, K., Minegishi, K., Kasai, K., & Yoshimura, Y. (1998). Power and colour Doppler ultrasonography for the evaluation of the vasculature of the human corpus luteum. *Human Reproduction (Oxford, England), 13*(10), 2836-2841.

Moore, D. P., Cantón, G. J., & Louge Uriarte, E. L. (2021). Infectious diseases affecting reproduction and the neonatal period in cattle. *Frontiers in Veterinary Science, 8*, 679007.

Morgan, D. R., Dixon, L. J., Hanratty, C. G., El-Sherbeeny, N., Hamilton, P. B., McGrath, L. T., Leahey, W. J., Johnston, G. D., & McVeigh, G. E. (2006). Effects of dietary omega-3 fatty acid supplementation on endothelium-dependent vasodilation in patients with chronic heart failure. *The American Journal of Cardiology, 97*(4), 547-551.

Moussavi, A. H., Gilbert, R. O., Overton, T. R., Bauman, D. E., & Butler, W. R. (2007). Effects of feeding fish meal and n-3 fatty acids on ovarian and uterine responses in early lactating dairy cows. *Journal of Dairy Science, 90*(1), 145-154.

Murphy, J. M., & Vince, J. E. (2015). Post-translational control of RIPK3 and MLKL mediated necroptotic cell death. *F1000Research, 4*.

Musicki, B., Kodaman, P. H., Aten, R. F., & Behrman, H. R. (1996). Endocrine regulation of ascorbic acid transport and secretion in luteal cells. *Biology of Reproduction, 54*(2), 399 406.

Ndiaye, K., Poole, D. H., Walusimbi, S., Cannon, M. J., Toyokawa, K., Maalouf, S. W., Dong, J., Thomas, P., & Pate, J. L. (2012). Progesterone effects on lymphocytes may be mediated by membrane progesterone receptors. *Journal of Reproductive Immunology, 95*(1-2), 15-26.

Nett, T. M., McClellan, M. C., & Niswender, G. D. (1976). Effects of prostaglandins on the ovine corpus luteum: blood flow, secretion of progesterone and morphology. *Biology of Reproduction, 15*(1), 66-78.

Neuvians, T. P., Schams, D., Berisha, B., & Pfaffl, M. W. (2004). Involvement of pro-inflammatory cytokines, mediators of inflammation, and basic fibroblast growth factor in prostaglandin F2α-induced luteolysis in bovine corpus luteum. *Biology of Reproduction, 70*(2), 473-480.

Newton, K., & Manning, G. (2016). Necroptosis and inflammation. *Annual Review of Biochemistry, 85*, 743-763.

Nishimura, R., Komiyama, J., Tasaki, Y., Acosta, T. J., & Okuda, K. (2008). Hypoxia promotes luteal cell death in bovine corpus luteum. *Biology of Reproduction, 78*(3), 529-536.

Nishimura, R., & Okuda, K. (2010). Hypoxia is important for establishing vascularization during corpus luteum formation in cattle. *Journal of Reproduction and Development, 56*(1), 110 116.

Niswender, G. D., Juengel, J. L., Silva, P. J., Rollyson, M. K., & McIntush, E. W. (2000). Mechanisms controlling the function and life span of the corpus luteum. *Physiological Reviews, 80*(1), 1-29.

Niswender, G. D., Reimers, T. J., Diekman, M. A., & Nett, T. M. (1976). Blood flow: a mediator of ovarian function. *Biology of Reproduction, 14*(1), 64-81.

Oakley, O. R., Kim, H., El-Amouri, I., Lin, P. C., Cho, J., Bani-Ahmad, M., & Ko, C. (2010). Periovulatory leukocyte infiltration in the rat ovary. *Endocrinology, 151*(9), 4551–4559.

Odde, K. G. (1990). A review of synchronization of estrus in postpartum cattle. *Journal of Animal Science, 68(*3), 817-830.

OECD/FAO (2021). *OECD-FAO Agricultural Outlook 2021-2030*. OECD Publishing, Paris. https://doi.org/10.1787/19428846-en

Oeckinghaus, A., & Ghosh, S. (2009). The NF-κB family of transcription factors and its regulation. *Cold Spring Harbor Perspectives in Biology*, *1*(4), a000034.

O'Fallon, J. V., Busboom, J. R., Nelson, M. L., & Gaskins, C. T. (2007). A direct method for fatty acid methyl ester synthesis: application to wet meat tissues, oils, and feedstuffs. *Journal of Animal Science*, *85*(6), 1511-1521.

Oono, K., Ohtake, K., Watanabe, C., Shiba, S., Sekiya, T., & Kasono, K. (2020). Contribution of Pyk2 pathway and reactive oxygen species (ROS) to the anti-cancer effects of eicosapentaenoic acid (EPA) in PC3 prostate cancer cells. *Lipids in Health and Disease, 19*(1), 1-12.

O'shea, J. D., Rodgers, R. J., & D'occhio, M. J. (1989). Cellular composition of the cyclic corpus luteum of the cow. *Reproduction, 85*(2), 483-487.

Pasparakis, M., & Vandenabeele, P. (2015). Necroptosis and its role in inflammation. *Nature, 517*(7534), 311-320.

Pate, J., Poole, D., Walusimbi, S., & Brzezicka, E. (2012). Immune cells in the ruminant corpus luteum: Friends or foes? *Biology of Reproduction, 87*(Suppl_1), 99-99.

Pate, J. L. (1995). Involvement of immune cells in regulation of ovarian function. *Journal of Reproduction and Fertility-Supplements only, 49*, 365-378.

Pate, J. L. (1996). Intercellular communication in the bovine corpus luteum. *Theriogenology, 45*(7), 1381-1397.

Pate, J. L., & Condon, W. A. (1982). Effects of serum and lipoproteins on steroidogenesis in cultured bovine luteal cells. *Molecular and Cellular Endocrinology, 28*(3), 551-562.

Pate, J. L., & Condon, W. A. (1989). Regulation of steroidogenesis and cholesterol synthesis by prostaglandin F-2α and lipoproteins in bovine luteal cells. *Reproduction, 87*(2), 439-446.

Pate, J. L., & Keyes, P. L. (2001). Immune cells in the corpus luteum: friends or foes?. *Reproduction-Cambridge-, 122*(5), 665-676.

Pate, J. L., Toyokawa, K., Walusimbi, S., & Brzezicka, E. (2010). The interface of the immune and reproductive systems in the ovary: lessons learned from the corpus luteum of domestic animal models. *American Journal of Reproductive Immunology, 64*(4), 275 286.

Penny, L. A., Armstrong, D., Bramley, T. A., Webb, R., Collins, R. A., & Watson, E. D. (1999). Immune cells and cytokine production in the bovine corpus luteum throughout the oestrous cycle and after induced luteolysis. *Reproduction, 115*(1), 87-96.

Pescador, N., Soumano, K., Stocco, D. M., Price, C. A., & Murphy, B. D. (1996). Steroidogenic acute regulatory protein in bovine corpora lutea. *Biology of Reproduction, 55*(2), 485 491.

Petit, H. V., Dewhurst, R. J., Scollan, N. D., Proulx, J. G., Khalid, M., Haresign, W., Twagiramungu, H., & Mann, G. E. (2002). Milk production and composition, ovarian function, and prostaglandin secretion of dairy cows fed omega-3 fats. *Journal of Dairy Science, 85(*4), 889–899.

Petroff, B. K., Ciereszko, R. E., Dabrowski, K., Ottobre, A. C., Pope, W. F., & Ottobre, J. S. (1998). Depletion of vitamin C from pig corpora lutea by prostaglandin F2α-induced secretion of the vitamin. *Reproduction, 112*(2), 243-247.

Petroff, M. G., Mark Coggeshall, K., Jones, L. S., & Pate, J. L. (1997). Bovine luteal cells elicit major histocompatibility complex class II-dependent T-cell proliferation. *Biology of Reproduction, 57*(4), 887-893.

Petroff, M. G., Petroff, B. K., & Pate, J. L. (1999). Expression of cytokine messenger ribonucleic acids in the bovine corpus luteum. *Endocrinology, 140*(2), 1018-1021.

Petroff, M. G., Petroff, B. K., & Pate, J. L. (2001). Mechanisms of cytokine-induced death of cultured bovine luteal cells. *Reproduction-Cambridge, 121*(5), 753-760.

Plewes, M. R., Burns, P. D., Graham, P. E., Bruemmer, J. E., Engle, T. E., & Barisas, B. G. (2017). Effect of fish meal supplementation on spatial distribution of lipid microdomains and on the lateral mobility of membrane-bound prostaglandin F2α receptors in bovine corpora lutea. *Domestic Animal Endocrinology, 60,* 9-18.

Plewes, M. R., Cedillo, J. C., Burns, P. D., Graham, P. E., Bruemmer, J. E., & Engle, T. E. (2018). Effect of fish meal supplementation on luteal sensitivity to intrauterine infusions of prostaglandin F2α in the bovine. *Biology of Reproduction, 98*(4), 543-557.

Pober, J. S., & Sessa, W. C. (2015). Inflammation and the blood microvascular system. *Cold Spring Harbor Perspectives in Biology, 7*(1), a016345.

Poole, D. H., & Pate, J. L. (2009). Loss of regulatory T lymphocytes in the regressing bovine corpus luteum. *Biology of Reproduction, 81*(Suppl_1), 87-87.

Pope, W. F. (1988). Uterine asynchrony: a cause of embryonic loss. *Biology of Reproduction, 39*(5), 999-1003.

Porter, F. D., & Herman, G. E. (2011). Malformation syndromes caused by disorders of cholesterol synthesis. *Journal of Lipid Research, 52*(1), 6-34.

Pru, J. K., Lynch, M. P., Davis, J. S., & Rueda, B. R. (2003). Signaling mechanisms in tumor necrosis factor alpha-induced death of microvascular endothelial cells of the corpus luteum. *Reproductive Biology and Endocrinology, 1*(1), 1-11.

Purcell, S. H., Beal, W. E., & Gray, K. R. (2005). Effect of a CIDR insert and flunixin meglumine, administered at the time of embryo transfer, on pregnancy rate and resynchronization of estrus in beef cattle. *Theriogenology, 64*(4), 867-878.

Qi, X., Qin, Z., Tang, J., Han, P., Xing, Q., Wang, K., Yu, J., Zhou, G., Tang, M., Wang, W., & Zhang, W. (2017). Omega-3 polyunsaturated fatty acids ameliorates testicular ischemia reperfusion injury through the induction of Nrf2 and inhibition of NF-κB in rats. *Experimental and Molecular Pathology, 103*(1), 44-50.

Rafikov, R., Fonseca, F. V., Kumar, S., Pardo, D., Darragh, C., Elms, S., Fulton, D., & Black, S. M. (2011). eNOS activation and NO function: structural motifs responsible for the posttranslational control of endothelial nitric oxide synthase activity. *The Journal of Endocrinology, 210*(3), 271.

Razin, T., Melamed-Book, N., Argaman, J., Galin, I., Lowy, Y., Anuka, E., Naftali-Shani, N., Kandel-Kfir, M., Garfinkel, B. P., Brielle, S., Granot, Z., Apte, R. N., Conway, S. J., Molkentin, J. D., Kamari, Y., Leor, J., & Orly, J. (2021). Interleukin-1α dependent survival of cardiac fibroblasts is associated with StAR/STARD1 expression and improved cardiac remodeling and function after myocardial infarction. *Journal of Molecular and Cellular Cardiology, 155*, 125-137.

Reese, S. T., Franco, G. A., Poole, R. K., Hood, R., Fernadez Montero, L., Oliveira Filho, R. V., Cooke, R. F., & Pohler, K. G. (2020). Pregnancy loss in beef cattle: A meta-analysis. *Animal Reproduction Science, 212,* 106251.

Reibiger, I., & Spanel-Borowski, K. (2000). Difference in localization of eosinophils and mast cells in the bovine ovary. *Journal of Reproduction and Fertility, 118*(2), 243-250.

Rekawiecki, R., & Kotwica, J. (2007). Molecular regulation of progesterone synthesis in the bovine corpus luteum. *Veterinari Medicina-Praha-, 52*(9), 405.

Rekawiecki, R., Nowik, M., & Kotwica, J. (2005). Stimulatory effect of LH, PGE2 and progesterone on StAR protein, cytochrome P450 cholesterol side chain cleavage and 3β hydroxysteroid dehydrogenase gene expression in bovine luteal cells. *Prostaglandins & Other Lipid Mediators, 78*(1-4), 169-184.

Reynolds, L. P., Grazul-Bilska, A. T., Killilea, S. D., & Redmer, D. A. (1994). Mitogenic factors of corpora lutea. *Progress in Growth Factor Research, 5*(2), 159-175.

Reynolds, L. P., & Redmer, D. A. (1999). Growth and development of the corpus luteum. *Journal of Reproduction and Fertility. Supplement, 54*, 181–191.

Roberts, J. S., McCracken, J. A., Gavagan, J. E., & Soloff, M. S. (1976). Oxytocin-stimulated release of prostaglandin F2alpha from ovine endometrium in vitro: correlation with estrous cycle and oxytocin-receptor binding. *Endocrinology, 99*(4), 1107–1114.

Roberts, R. M., Cross, J. C., & Leaman, D. W. (1992). Interferons as hormones of pregnancy. *Endocrine reviews, 13*(3), 432–452.

Robinson, R. S., Hammond, A. J., Hunter, M. G., & Mann, G. E. (2005). The induction of a delayed post-ovulatory progesterone rise in dairy cows: a novel model. *Domestic Animal Endocrinology, 28*(3), 285-295.

Robinson, R. S., Hammond, A. J., Nicklin, L. T., Schams, D., Mann, G. E., & Hunter, M. G. (2006). Endocrine and cellular characteristics of corpora lutea from cows with a delayed post-ovulatory progesterone rise. *Domestic Animal Endocrinology, 31*(2), 154-172.

Robinson, R. S., Nicklin, L. T., Hammond, A. J., Schams, D., Hunter, M. G., & Mann, G. E. (2007). Fibroblast growth factor 2 is more dynamic than vascular endothelial growth factor A during the follicle-luteal transition in the cow. *Biology of Reproduction, 77*(1), 28-36.

Rohm, F., Spanel-Borowski, K., Eichler, W., & Aust, G. (2002). Correlation between expression of selectins and migration of eosinophils into the bovine ovary during the periovulatory period. *Cell and Tissue Research, 309*(2), 313-322.

Sakai, C., Ishida, M., Ohba, H., Yamashita, H., Uchida, H., Yoshizumi, M., & Ishida, T. (2017). Fish oil omega-3 polyunsaturated fatty acids attenuate oxidative stress-induced DNA damage in vascular endothelial cells. *PloS one*, *12*(11), e0187934.

Sakumoto, R., Berisha, B., Kawate, N., Schams, D., & Okuda, K. (2000). Tumor necrosis factor α and its receptor in bovine corpus luteum throughout the estrous cycle. *Biology of Reproduction, 62*(1), 192-199.

Sakumoto, R., Vermehren, M., Kenngott, R. A., Okuda, K., & Sinowatz, F. (2011). Localization of gene and protein expressions of tumor necrosis factor-α and tumor necrosis factor receptor types I and II in the bovine corpus luteum during the estrous cycle. *Journal of Animal Science, 89*(10), 3040-3047.

Santana, P., Llanes, L., Hernandez, I., Gallardo, G., Quintana, J., Gonzalez, J., Estevez, F., Ruiz de Galarreta, C., & Fanjul, L. F. (1995). Ceramide mediates tumor necrosis factor effects on P450-aromatase activity in cultured granulosa cells. *Endocrinology, 136*(5), 2345–2348.

Sawyer, H. R., Niswender, K. D., Braden, T. D., & Niswender, G. D. (1990). Nuclear changes in ovine luteal cells in response to PGF2α. *Domestic Animal Endocrinology, 7*(2), 229-237.

Schoenemann, H. M., Humphrey, W. D., Crowder, M. E., Nett, T. M., & Reeves, J. J. (1985). Pituitary luteinizing hormone-releasing hormone receptors in ovariectomized cows after challenge with ovarian steroids. *Biology of Reproduction, 32*(3), 574-583.

Schroder, K., Hertzog, P. J., Ravasi, T., & Hume, D. A. (2004). Interferon-gamma: an overview of signals, mechanisms and functions. *Journal of Leukocyte Biology, 75*(2), 163–189.

Schwarz, C., Wirth, M., Gerischer, L., Grittner, U., Witte, A. V., Köbe, T., & Flöel, A. (2018). Effects of omega-3 fatty acids on resting cerebral perfusion in patients with mild cognitive impairment: a randomized controlled trial. *The Journal of Prevention of Alzheimer's Disease, 5*(1), 26-30.

Serhan, C. N., Chiang, N., & Van Dyke, T. E. (2008). Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nature Reviews Immunology, 8*(5), 349-361.

Shaw, D. W., & Britt, J. H. (1995). Concentrations of tumor necrosis factor α and progesterone within the bovine corpus luteum sampled by continuous-flow microdialysis during luteolysis in vivo. *Biology of Reproduction, 53*(4), 847-854.

Shimizu, T., Kaji, A., Murayama, C., Magata, F., Shirasuna, K., Wakamiya, K., Okuda, K., & Miyamoto, A. (2012). Effects of interleukin-8 on estradiol and progesterone production by bovine granulosa cells from large follicles and progesterone production by luteinizing granulosa cells in culture. *Cytokine, 57*(1), 175-181.

Shirasuna, K., Asaoka, H., Acosta, T., Wijayagunawardane, M., Ohtani, M., Hayashi, M., Matsui, M., & Miyamoto, A. (2004). Real-time relationships in intraluteal release among prostaglandin F2α, endothelin-1, and angiotensin II during spontaneous luteolysis in the cow. *Biology of Reproduction, 71*(5), 1706-1711.

Shirasuna, K., Jiemtaweeboon, S., Raddatz, S., Nitta, A., Schuberth, H., Bollwein, H., Shimizu, T., & Miyamoto, A. (2012). Rapid accumulation of polymorphonuclear neutrophils in the corpus luteum during prostaglandin F2α-induced luteolysis in the cow. *PLoS One, 7*(1), e29054.

Shirasuna, K., & Miyamoto, A. (2016). Immune cells and their effects on the bovine corpus luteum. *The life cycle of the corpus luteum* (pp. 99-116). Springer International Publishing.

Shirasuna, K., Watanabe, S., Asahi, T., Wijayagunawardane, M. P. B., Sasahara, K., Jiang, C., Matsui, M., Sasaki, M., Shimizu, T., Davis, J. S., & Miyamoto, A. (2008). Prostaglandin F2 increases endothelial nitric oxide synthase in the periphery of the bovine corpus luteum: The possible regulation of blood flow at an early stage of luteolysis. *Reproduction (Cambridge, England), 135*(4), 527-539.

Sinn, N., & Howe, P. R. C. (2008). Mental health benefits of omega-3 fatty acids may be mediated by improvements in cerebral vascular function. *Bioscience Hypotheses, 1*(2), 103-108.

Skarzynski, D. J., Bah, M. M., Deptula, K. M., Woclawek-Potocka, I., Korzekwa, A., Shibaya, M., Pilawski, W., & Okuda, K. (2003a). Roles of tumor necrosis factor-α of the estrous cycle in cattle: an in vivo study. *Biology of Reproduction, 69*(6), 1907-1913.

Skarzynski, D. J., Jaroszewski, J. J., Bah, M. M., Deptula, K. M., Barszczewska, B., Gawronska, B., & Hansel, W. (2003b). Administration of a nitric oxide synthase inhibitor counteracts prostaglandin F2-induced luteolysis in cattle. *Biology of Reproduction, 68*(5), 1674 1681.

Skarzynski, D. J., Kobayashi, S., & Okuda, K. (2000). Influence of nitric oxide and noradrenaline on prostaglandin F2α-induced oxytocin secretion and intracellular calcium mobilization in cultured bovine luteal cells. *Biology of Reproduction, 63*(4), 1000-1005.

Slominski, A., Zjawiony, J., Wortsman, J., Semak, I., Stewart, J., Pisarchik, A., Sweatman, T., Marcos, J., Dunbar, C., & C. Tuckey, R. (2004). A novel pathway for sequential transformation of 7-dehydrocholesterol and expression of the P450scc system in mammalian skin. *European Journal of Biochemistry, 271*(21), 4178-4188.

Smith, G. W., & Meidan, R. (2014). Ever-changing cell interactions during the life span of the corpus luteum: relevance to luteal regression. *Reproductive biology, 14*(2), 75–82.

Spencer, T. E., Becker, W. C., George, P. H. I. L. I. P., Mirando, M. A., Ogle, T. F., & Bazer, F. W. (1995). Ovine interferon-tau inhibits estrogen receptor up-regulation and estrogen induced luteolysis in cyclic ewes. *Endocrinology, 136*(11), 4932-4944.

Spencer, T. E., Johnson, G. A., Bazer, F. W., Burghardt, R. C., & Palmarini, M. (2006). Pregnancy recognition and conceptus implantation in domestic ruminants: roles of progesterone, interferons and endogenous retroviruses. *Reproduction, Fertility and Development, 19*(1), 65-78.

Steck, T. L., & Lange, Y. (2010). Cell cholesterol homeostasis: mediation by active cholesterol. *Trends in Cell Biology, 20*(11), 680-687.

Still, J. G., & Greiss, F. C., Jr. (1978). The effect of prostaglandins and other vasoactive substances on uterine blood flow and myometrial activity. *American Journal of Obstetrics and Gynecology, 130*(1), 1–8.

Summons, K. R., & Hansel, W. (1964). Nature of the luteotropic hormone in the bovine. *Journal of Animal Science, 23*(1), 136-141.

Suter, J., Hendry, I. R., Ndjountche, L., Obholz, K., Pru, J. K., Davis, J. S., & Rueda, B. R. (2001). Mediators of interferon γ-initiated signaling in bovine luteal cells. *Biology of Reproduction, 64(*5), 1481-1486.

Talbott, H., Hou, X., Qiu, F., Zhang, P., Guda, C., Yu, F., Cushman, R. A., Wood, J. R., Wang, C., Cupp, A. S., & Davis, J. S. (2017). Early transcriptome responses of the bovine midcycle corpus luteum to prostaglandin F2α includes cytokine signaling. *Molecular and Cellular Endocrinology, 452*, 93-109.

Talukder, A. K., Yousef, M. S., Rashid, M. B., Awai, K., Acosta, T. J., Shimizu, T., Okuda, K., Shimada, M., Imakawa, K., & Miyamoto, A. (2017). Bovine embryo induces an anti-inflammatory response in uterine epithelial cells and immune cells in vitro: Possible involvement of interferon tau as an intermediator. *The Journal of Reproduction and Development, 63*(4), 425-434.

Terranova, P. F., & Rice, V. M. (1997). Cytokine involvement in ovarian processes. *American Journal of Reproductive Immunology, 37*(1), 50-63.

Townson, D. H., O'Connor, C. L., & Pru, J. K. (2002). Expression of monocyte chemoattractant protein-1 and distribution of immune cell populations in the bovine corpus luteum throughout the estrous cycle. *Biology of Reproduction, 66*(2), 361-366.

Tsai, S. J., & Wiltbank, M. C. (1998). Prostaglandin F2α regulates distinct physiological changes in early and mid-cycle bovine corpora lutea. *Biology of Reproduction, 58*(2), 346-352.

Tsai, S. J., Wiltbank, M. C., & Bodensteiner, K. J. (1996). Distinct mechanisms regulate induction of messenger ribonucleic acid for prostaglandin (PG) G/H synthase-2, PGE (EP3) receptor, and PGF2 alpha receptor in bovine preovulatory follicles. *Endocrinology, 137*(8), 3348-3355.

Tsujimoto, M., Yip, Y. K., & Vilcek, J. (1986). Interferon-gamma enhances expression of cellular receptors for tumor necrosis factor. *The Journal of Immunology, 136*(7), 2441 2444.

Turzillo, A. M., & Nett, T. M. (1999). Regulation of GnRH receptor gene expression in sheep and cattle. Journal of reproduction and fertility. *Supplement, 54*, 75–86.

Vonnahme, K. A., Redmer, D. A., Borowczyk, E., Bilski, J. J., Luther, J. S., Johnson, M. L., Reynolds, L. P., & Grazul-Bilska, A. T. (2006). Vascular composition, apoptosis, and expression of angiogenic factors in the corpus luteum during prostaglandin F2α-induced regression in sheep. *Reproduction, 131*(6), 1115-1126.

Walser, B., Giordano, R. M., & Stebbins, C. L. (2006). Supplementation with omega-3 polyunsaturated fatty acids augments brachial artery dilation and blood flow during forearm contraction. *European Journal of Applied Physiology, 97*(3), 347-354.

Walusimbi, S. S., & Pate, J. L. (2013). Physiology and Endocrinology Symposium: role of immune cells in the corpus luteum. *Journal of Animal Science, 91*(4), 1650-1659.

Walusimbi, S. S., & Pate, J. L. (2014). Luteal cells from functional and regressing bovine corpora lutea differentially alter the function of gamma delta T cells. *Biology of Reproduction, 90*(6), 140-1.

Weber, A. F., Morgan, B. B., & McNutt, S. N. (1948). A histological study of metrorrhagia in the virgin heifer. *The American Journal of Anatomy, 83*(2), 309–327.

Wendorf, G. L., Lawyer, M. S., & First, N. L. (1983). Role of the adrenals in the maintenance of pregnancy in cows. *Journal of reproduction and fertility, 68*(2), 281–287.

Wheelock, E. F. (1965). Interferon-like virus-inhibitor induced in human leukocytes by phytohemagglutinin. Science, 149(3681), 310-311.

Wise, T. H., Caton, D., Thatcher, W. W., Barron, D. H., & Fields, M. J. (1982). Ovarian function during the estrous cycle of the cow: Ovarian blood flow and progesterone release rate. *Journal of Animal Science, 55*(3), 627.

Wistuba, T. J., Kegley, E. B., & Apple, J. K. (2006). Influence of fish oil in finishing diets on growth performance, carcass characteristics, and sensory evaluation of cattle. *Journal of Animal Science, 84*(4), 902-909.

Woad, K. J., & Robinson, R. S. (2016). Luteal angiogenesis and its control. *Theriogenology, 86* (1), 221–228.

Wu, H., Ichikawa, S., Tani, C., Zhu, B., Tada, M., Shimoishi, Y., Murata, Y., & Nakamura, Y. (2009). Docosahexaenoic acid induces ERK1/2 activation and neuritogenesis via intracellular reactive oxygen species production in human neuroblastoma SH-SY5Y cells. B*iochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 1791*(1), 8 16.

Xiao, K., Liu, C., Qin, Q., Zhang, Y., Wang, X., Zhang, J., Odle, J., Lin, X., Hu, C. A., & Liu, Y. (2020). EPA and DHA attenuate deoxynivalenol-induced intestinal porcine epithelial cell injury and protect barrier function integrity by inhibiting necroptosis signaling pathway. *The FASEB Journal, 34*(2), 2483-2496.

Yamamoto, Y. L., Feindel, W., Wolfe, L. S., Katoh, H., & Hodge, C. P. (1972). Experimental vasoconstriction of cerebral arteries by prostaglandins. *Journal of neurosurgery, 37*(4), 385-397.

Yan, L., Zheng, D., & Xu, R. H. (2018). Critical role of tumor necrosis factor signaling in mesenchymal stem cell-based therapy for autoimmune and inflammatory diseases. *Frontiers in immunology, 9*, 1658.

Yancopoulos, G. D., Davis, S., Gale, N. W., Rudge, J. S., Wiegand, S. J., & Holash, J. (2000). Vascular-specific growth factors and blood vessel formation. *Nature (London), 407*(6801), 242-248.

Zamaraev, A. V., Kopeina, G. S., Prokhorova, E. A., Zhivotovsky, B., & Lavrik, I. N. (2017). Post-translational modification of caspases: the other side of apoptosis regulation. *Trends in Cell Biology, 27*(5), 322-339.

APPENDIX A

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE PROTOCOL

From: no-reply=kuali.co@mx3.kuali.co <no-reply=kuali.co@mx3.kuali.co> **On Behalf Of** Kuali Notifications
Sent: Monday, November 16, 2020 7:15 AM
To: Engle,Terry <Terry.Engle@ColoState.EDU>
Subject: KP IACUC Initial 1366 Approved Monday, November 16th 2020

The protocol listed below has been approved on Monday, November 16th 2020 by CSU IACUC (DMR and FCR).

A Continuing Review by Monday, November 15th 2021 is required to keep this protocol active.

- Principal Investigator: Engle,Terry
- Submission Type and ID: Initial 1366
- Title: Influence of fish oil on corpus luteum function
- Approval Date: Monday, November 16th 2020
- Committee: CSU IACUC (DMR and FCR)
- Link to this form: colostate.kuali.co/protocols/protocols/5fb28903834e4800337be84f

Please Note:

- If this IACUC protocol needs VTH CRB review, an amendment will be created for you that will go to the CRB for review/approval of your consent form. No client owned animal activities may be initiated until VTH CRB approval is secured.
- Your Research Continuity Application must be approved prior to starting work. For more details, see the CSU return to work website.

If you have additional questions about this please contact RICRO IACUC Staff.

AMENDMENT TO 1366

From: no-reply=kuali.co@mx3.kuali.co <no-reply=kuali.co@mx3.kuali.co> **On Behalf Of** Kuali Notifications
Sent: Monday, May 3, 2021 11:10 AM
To: Engle,Terry <Terry.Engle@ColoState.EDU>
Subject: KP IACUC Amendment 1366 Approved Monday, May 3rd 2021

The protocol listed below has been approved on Monday, May 3rd 2021 by CSU IACUC (DMR and FCR).

A Continuing Review by Monday, November 15th 2021 is required to keep this protocol active.

- Principal Investigator: Engle,Terry
- Submission Type and ID: Amendment 1366
- Title: Influence of fish oil on corpus luteum function
- Approval Date: Monday, May 3rd 2021
- Committee: CSU IACUC (DMR and FCR)
- Link to this form: colostate.kuali.co/protocols/protocols/6086c377060baa0038bc17ee

Please Note:

- If this IACUC protocol needs VTH CRB review, an amendment will be created for you that will go to the CRB for review/approval of your consent form. No client owned animal activities may be initiated until VTH CRB approval is secured.
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