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THE EFFECTS OF FORM OF SELENIUM ON THE BOVINE CORPUS LUTEUM, UTERINE ENDOMETRIUM, AND DEVELOPMENT OF THE CONCEPTUS

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THE EFFECTS OF FORM OF SELENIUM ON THE BOVINE CORPUS LUTEUM, UTERINE ENDOMETRIUM, AND DEVELOPMENT OF THE CONCEPTUS

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture, Food and Environment
at the University of Kentucky

By
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Lexington, Kentucky
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2021

ABSTRACT OF DISSERTATION

THE EFFECTS OF FORM OF SELENIUM ON THE BOVINE CORPUS LUTEUM, UTERINE ENDOMETRIUM, AND DEVELOPMENT OF THE CONCEPTUS

Widespread regions of the southeast United States have soils deficient in selenium (Se), necessitating Se supplementation to cattle grazing in these areas. Adequate dietary Se is required for optimal immune function, growth, and fertility. In forages, Se is predominantly found in the organic form (OSe), which is known to increase bioavailability. However, the inorganic form (ISe) is typically found in commercial mineral mixes. We previously reported that supplementation with an isomolar 1:1 mix (MIX) of ISe (sodium selenite, Prince Se Concentrate; Prince Agri Products, Inc., Quincy, IL) and OSe (SEL-PLEX, Alltech Inc., Nicholasville, KY) increases early luteal phase concentrations of progesterone (P4) above that in cows on ISe or OSe alone. Research has demonstrated that increased early luteal phase P4 advances embryonic development. A series of experiments were performed to investigate the effect of form of supplemental Se on 1) the early bovine corpus luteum and 2) the uterine endometrium and conceptus at maternal recognition of pregnancy. The objective of Experiment 1 was to investigate the effect of form of supplemental Se on the transcriptome of the bovine corpus luteum (CL) with the goal of elucidating form of Se-regulated luteal processes affecting fertility. Results indicated that MIX-supplemented cows had increased mRNA abundance of transcripts regulating cholesterol biosynthesis and increased CL content of several immune-response transcripts compared to cows supplemented with ISe alone. These results suggest that the MIX-induced increase in early luteal phase P4 is due to an increase in cholesterol availability and that the form of dietary Se affects immune function of the CL. Experiment 2 examined the effect of form of supplemental Se on the uterine endometrium and conceptus development at maternal recognition of pregnancy. The objective was to determine changes induced by the form of supplemental Se on the bovine endometrium and developing conceptus on day 17 of pregnancy. Form of supplemental Se differentially affected the transcriptome of the uterine endometrium at maternal recognition of pregnancy, resulting in form-induced effects on embryonic length. Combining the results from both experiments, supplemental Se in the MIX versus ISe form alters the transcriptome of the bovine CL on day 7 of the estrous cycle, the transcriptome of the uterine endometrium at maternal recognition of pregnancy and advances embryonic development. In conclusion, incorporating Se into a mineral supplementation strategy as MIX is an easy transition for producers that can ultimately increase the fertility levels in their herds. An increase in fertility can cause a shift in the calving distribution, leading to more calves born earlier in the season and subsequently calves are older and heavier at weaning, ultimately leading to increased profit potential.

KEYWORDS: Selenium, Corpus luteum, Progesterone, Endometrium, Conceptus development

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CHAPTER 1. Introduction

The selenium (Se) content of soils varies with type, texture, organic matter content and precipitation (Mehdi and Dufrasne, 2016). Likewise, the Se content of forages varies with the type of feed, the soil type, and the region (Mehdi and Dufrasne, 2016). In the United States, the distribution of Se content of grains and forages varies greatly in different geographical regions (Ammerman and Miller, 1975). The majority of producers in the southeast, including Kentucky, have forages and grains that are low (<0.05 ppm) to variable (~50% contain >0.1 ppm) in Se (Ammerman and Miller, 1975). Therefore, it is not unexpected that producers in the southeast have the highest proportion of cattle classified as Se-deficient compared to other geographical regions (Dargatz and Ross, 1996). In cattle, deficiencies in Se have been demonstrated to decrease immunity (Erskine et al., 1989), growth (Gleed et al., 1983), and fertility (McClure et al., 1986).

Cattle grazing Se-deficient forages necessitates the need to provide supplemental Se to these animals. The suggested dietary requirement of Se for beef cattle is at 0.1 ppm (National Academies of Sciences and Medicine, 2016). The FDA has approved both inorganic (ISe) and organic (OSe) forms of Se for beef cattle production (FDA, 2020). In the feed industry Se is commonly supplemented as ISe. However, the bioavailability of Se is significantly higher for OSe than ISe (Khanam and Platel, 2016).

Another challenge faced by many cattle producers is a high percentage of early embryonic loss which can have a detrimental impact on the profit potential for both beef and dairy operations. In cattle, fertilization rates are between 90 and 100%, when semen of known high fertility is used in artificial insemination (Diskin et al., 2006; Diskin and Morris, 2008). However, it has been reported that only 50 to 60% of beef cows

inseminated remain pregnant by day 30 (Bridges et al., 2013). More specifically, it is estimated that 70-80% of the embryonic loss occurs between day 8 and 16 after insemination (Sreenan and Diskin, 1986).

Lower concentrations of progesterone (P4) during the early luteal phase after artificial insemination have been associated with lower conception rates (Mann and Lamming, 1999). In contrast, increased concentrations of P4 immediately following conception have been associated with advanced conceptus elongation (Carter et al., 2008) and increased interferon tau (IFNT) production (Mann and Lamming, 2001). IFNT inhibits development of the endometrial luteolytic mechanism required for the pulsatile release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), thereby ensuring continued production of P4 by the corpus luteum (CL) (Spencer et al., 2007).

Our lab has previously reported increased early concentrations of P4 in cows supplemented with a 1:1 combination of ISe:OSe (MIX) compared to cows supplemented with OSe or ISe alone on days 6 (Cerny et al., 2016b) and 7 (Carr et al., 2020) of the estrous cycle. However, to our knowledge, studies regarding the mechanism of form of Se-induced increased concentrations of early luteal phase P4 or effects on the uterine endometrium and conceptus have not been reported. The specific goals and objectives of this dissertation are stated in Chapter 4.

CHAPTER 2. Literature Review

2.1. Selenium Supplementation of Cattle

The basic element selenium (Se) was discovered by Swedish chemist, Berzelius in 1817 (Johansson et al., 2005) and named, Selene, after the Greek goddess of the moon (Shini et al., 2015). Selenium was initially considered a toxin because it was responsible for a disorder in livestock that grazed the plains of the Nebraska and Dakota territories (Hatfield et al., 2014). In the 1930's, it was reported that foot and hair disorders occurring in animals grazing in these areas resulted from the animal's consumption of seleniferous plants with high levels of Se accumulated from the soil (Franke, 1934). Up to 1957 Se was regarded as a toxin, until it was found to prevent liver necrosis in rats (Sohwarz and Foltz, 1958). It has become clear that Se is toxic at high levels, but considered an essential dietary micronutrient at low levels (Hatfield et al., 2014). However, there is a narrow range between dietary adequacy and toxicity (Shini et al., 2015).

2.1.1. Selenium Deficiency

In soils, the Se content varies with type, texture, organic matter content and precipitation (Mehdi and Dufrasne, 2016). Likewise, the Se content of forages varies with the type of feed, the soil type, and the region (Mehdi and Dufrasne, 2016). Similarly, additional research has shown a wide range (0.23 to 2.663 ppm) of Se in a variety of feedstuffs (Perry et al., 1976). Moreover, within a type of feedstuff, Se content ranged

from 0.017 to 0.219 ppm in 12 samples of shelled corn (Perry et al., 1976). The variation of Se content within shelled corn alone presents many challenges with respect to supplementation with Se, as it is a common feed ingredient used in livestock production.

In the United States, the distribution of Se content of grains and forages varies greatly in different geographical regions (Ammerman and Miller, 1975). The majority of producers in the southeast, including Kentucky, have forages and grains that are low (<0.05 ppm) to variable (~50% contain >0.1 ppm) in Se (Ammerman and Miller, 1975). Therefore, it is not unexpected that producers in the southeast have the highest proportion of cattle classified as Se-deficient compared to other geographical regions (Dargatz and Ross, 1996). Interestingly, survey results from the same study indicated that more producers located in the southeast U.S. were providing supplemental Se to their herds compared to other regions (Dargatz and Ross, 1996).

Research has demonstrated that dietary Se is essential for an optimum immune response (Arthur et al., 2003). In cattle, deficiencies in Se have been demonstrated to decrease immunity (Erskine et al., 1989), growth (Gleed et al., 1983), and fertility (McClure et al., 1986), with supplementation of a Se-enriched yeast reported to improve growth performance, immune function, and antioxidant status in newly received cattle (Sgoifo Rossi et al., 2017).

2.2. Supplemental Selenium

Cattle grazing Se-deficient forages necessitates the need to provide supplemental Se to these animals. The suggested dietary requirement of Se for beef cattle is at 0.1 ppm

(National Academies of Sciences and Medicine, 2016). Additionally, the FDA has determined a maximum Se supplementation rate of 0.3 ppm in feed and a daily maximum intake not to exceed 3 mg Se per head (FDA, 2020). Common mineral supplementation methods include *ad-libitum* access to loose minerals, pressed blocks, liquid supplements, and molasses-based tubs (Greene, 2000). However, it appears that Se supplementation is not consistent in maintaining or achieving adequate concentrations of Se in whole blood (Dargatz and Ross, 1996). The study by Dargatz and Ross (1996), found that over 16% of cattle sampled were considered severely deficient (whole blood Se content <0.05 ppm) even though supplemental Se was provided (Dargatz and Ross, 1996). Patterson et al. (2013) also reported a wide variation in *ad libitum* mineral intake, ranging from 18.8 to 106.5 g/day. The authors reported a mean mineral consumption of 54.00 ± 7.43 g/day, which is 36% less than the typical formulation intake expectations of 85 g/day (Patterson et al., 2013). Perhaps the variation in *ad libitum* mineral intake reported by Patterson et al. (2013) could explain the proportion of cattle considered Se deficient by Dargatz and Ross (1996) even though supplemental Se was provided.

The FDA has approved both inorganic and organic forms of Se for beef cattle production (FDA, 2020). In the inorganic form, Se can be present as sodium selenite and sodium selenate (Suzuki, 2005). In the feed industry, sodium selenite is the most common supplemental form (Podoll et al., 1992). However, sodium selenate is absorbed ten-fold better by plants than sodium selenite (Terry et al., 2000). Organic forms of Se include selenomethionine (SeMet) and selenocysteine (SeCys), and are the two most common ones in forages (Mehdi and Dufrasne, 2016). The bioavailability of Se is significantly higher for organic forms than inorganic forms (Khanam and Platel, 2016). As reviewed

by Daniels (1996), organic Se is more effective at raising blood Se than sodium selenite or sodium selenate. Sulfur present in methionine and cysteine is substituted by Se to form SeMet and SeCys, respectively (Mehdi and Dufrasne, 2016). Commercially available sources of organic Se include specially cultivated strains of yeast (*Saccharomyces cerevisiae*) and are available from several manufacturers (e.g., SEL-PLEX, Alltech, KY, USA). In these Se enriched yeasts, SeMet is the predominant form of Se (Korhola et al., 1986).

2.2.1. Inorganic Se

Sodium selenite and sodium selenate are well known inorganic sources of Se (Suzuki, 2005). The absorption rates of sodium selenite and sodium selenate differ when added to a milk-based infant formula, 73.4% and 97.1% respectively, and the mean urinary excretion also differs between sodium selenite (9.7%) and sodium selenate (36.4%) (Van Dael et al., 2002). Therefore, the mean apparent retention of Se from sodium selenite (63.7%) or sodium selenate (60.7%) is comparable (Van Dael et al., 2002). Sodium selenite and selenate can be reduced simply to selenide as demonstrated in Figure 2.1 (Suzuki, 2005) and the amount not immediately converted into SeCys is methylated and excreted (Sgoifo Rossi et al., 2017). However, selenite is readily reduced to selenide by glutathione, while selenate requires more reducing conditions (Suzuki, 2005). Selenide of selenite and selenate origin are taken up differently by the liver and utilized for synthesizing selenoprotein P and cellular glutathione peroxidase (Suzuki, 2005). Supplemental Se as sodium selenate or sodium selenite supports normal serum

concentrations of Se and glutathione peroxidase activities in lambs, horses, and lactating dairy cows (Podoll et al., 1992). However, the efficiency of absorption of Se varies greatly between ruminants and nonruminants (Romero-Pérez et al., 2010). In sheep, absorption of Se was 34% compared to 85% in swine when administered orally (Mahan et al., 1999). This low absorption rate in ruminants is believed to occur from the reduction of dietary Se to insoluble forms in the rumen environment (Spears, 2003). Additionally, inorganic Se may not be readily stored, but remains in a separate pool and is utilized for the immediate synthesis of functional selenoproteins (Daniels, 1996).

2.2.2. Organic Se I: Selenomethionine

Organic Se, specifically SeMet, is more bioavailable than inorganic Se (Daniels, 1996; Khanam and Platel, 2016). Selenomethionine can be nonspecifically substituted for methionine (Met) in a large number of proteins, especially skeletal muscle protein, and skeletal muscle SeMet incorporated in this manner represents 40-50% of the total body Se pool (Daniels, 1996). In plants, the predominant form of Se exists as SeMet (Mangiapane et al., 2014). Since the chemical and physical properties of Se and sulfur are similar, plants tend to synthesize SeMet when Se is available, as they cannot distinguish between Se and sulfur (Lyons et al., 2007). Additionally, SeMet is the predominant form of Se in organic Se-enriched yeasts, as previously mentioned (Korhola et al., 1986). Interestingly, animals and humans are unable to synthesize SeMet as they have no efficient mechanism for Met synthesis (Schrauzer, 2000). However, exogenous sources of SeMet can be incorporated in its intact form into proteins by the same AUG codon as

that to the Met codon without distinguishing between SeMet and Met (Suzuki, 2005). Proteins containing Se in the form of SeMet are called Se-containing proteins, but not selenoproteins (Suzuki, 2005). Interestingly, only SeMet is incorporated into body proteins, which allows Se to be stored in the organism and reversibly released by normal metabolic processes (Schrauzer, 2000). Ingested SeMet can be absorbed in the small intestine via the Na^+-dependent neutral amino acid transport system (Vendeland et al., 1994). However, several transporter systems have been proposed to be involved in Se uptake (Cousins and Liuzzi, 2018). As illustrated in Figure 2.1, after absorption, SeMet can be incorporated into proteins to replace the methionine, or catabolized and the Se can be utilized to synthesize SeCys (Sgoifo Rossi et al., 2017). SeMet is transformed to SeCys through the trans-selenation pathway (Suzuki, 2005). The SeCys formed is then degraded further into the liver to serine and selenide (Schrauzer, 2000). Selenide is either used for selenoprotein synthesis or methylated and exhaled or excreted as shown in Figure 2.1 (Schrauzer, 2000).

2.2.3. Organic Se II: Selenocysteine

Selenocysteine is a major form of Se in the cell and is the 21st naturally occurring amino acid (Labunskyy et al., 2014). SeCys is a cysteine (Cys) residue analogue with a Se-containing selenol group in place of the sulfur-containing thiol group in Cys (Johansson et al., 2005). Proteins containing Se in the form of SeCys are called selenoproteins (Suzuki, 2005). SeCys is present as an amino acid residue in selenoproteins in plants and animals and is incorporated into amino acid sequences of

selenoproteins by the specific codon to SeCys residue, UGA (Suzuki, 2005). In animal tissues, the principal chemical form of Se is SeCyst (Mangiapane et al., 2014). Unlike SeMet, animals are able to endogenously synthesize SeCys from inorganic Se (Daniels, 1996). The pK_a for SeCys is lower than for Cys, 5.2 versus 8.3, respectively (Huber and Criddle, 1967). Consequently, at physiological pH, the selenol of SeCys is mainly in its anionic selenolate form, making SeCys significantly more reactive than Cys (Johansson et al., 2005). SeCys is required for Se-dependent enzyme functions (Khanam and Platel, 2016). The majority of characterized selenoproteins are enzymes and their SeCys residue is essential for the catalytic activity (Johansson et al., 2005). As reviewed in 1996 and shown in Figure 2.1, SeCys does not accumulate and the Se is released by SeCys β lyase to be reduced to selenide and becomes available for selenoprotein synthesis in the liver (Daniels, 1996). Biosynthesis of SeCys represents the main regulatory point for selenoprotein biosynthesis and not absorption as occurs with many nutrients (Shini et al., 2015).

2.3. Selenoprotein Synthesis

The biological effects of Se are largely mediated by selenoproteins (Labunskyy et al., 2014) and are present in all three domains of life: eubacteria, archaebacteria and eukaryotes (Böck et al., 1991; Hatfield et al., 2014; Labunskyy et al., 2014). Approximately 100 selenoprotein families have been discovered (Hatfield et al., 2014). However, there are 25 genes encoding selenoproteins in humans (Hatfield et al., 2014; Labunskyy et al., 2014) and approximately half of these genes code for proteins with

known functions (Kryukov et al., 2003; Hatfield et al., 2014). Although the specific functions of selenoproteins are diverse, the principal function is their participation in redox homeostasis (Hatfield et al., 2014).

Selenoproteins contain Se in the form of SeCys being a Cys-analogue with a Se atom replacing the sulfur atom in Cys (Johansson et al., 2005). There are no known human or animal functionally active selenoproteins that contain SeMet (Shini et al., 2015) and all selenoproteins contain at least one SeCys (Labunskyy et al., 2014). The codon for incorporating SeCys residues is UGA, which is the stop codon in general (Suzuki, 2005). A unique feature of SeCys is that is has its own tRNA (Labunskyy et al., 2014) and is the only known tRNA that controls the expression of an entire class of proteins (Hatfield et al., 2014). Since the biosynthesis of SeCys occurs on its tRNA and the SeCys moiety is synthesized from serine, the SeCys tRNA molecule is designated as SeCys-tRNA$^{[Ser]SeCys}$ (Hatfield et al., 1994; Hatfield et al., 2006; Hatfield et al., 2014).

There are five components required for the translation of UGA codon to SeCys residue in selenoproteins (Suzuki, 2005). This includes two cis-sequences, a SeCys-insertion sequence (SECIS) element, a SeCys codon (UGA) in the coding region and three *trans*-acting factors, a SeCys-specific translation elongation factor (eEFSeCys), the SeCys ^{SeCys}tRNA, and a SECIS-binding protein (SBP2) (Suzuki, 2005; Hatfield et al., 2006; Labunskyy et al., 2014) as shown in Figure 2.2. The SECIS elements in the 3'-untranslated region of selenoprotein mRNAs are responsible for recoding the UGA codeword as SeCys and bypassing stop (Hatfield et al., 2006). Additionally, essential functions of SBP2 include binding to the SECIS core, binding to the ribosome, and

insertion of SeCys into selenoprotein (Hatfield et al., 2006). Finally, expression of selenoproteins is differentially regulated by Se availability (Labunskyy et al., 2014).

2.4. Effects of Supplemental Se on Cattle Reproduction

Supplementing dairy cows during the pre- and postpartum periods with a Se-enriched yeast increased postpartum plasma concentrations of progesterone (P4) compared to providing no supplemental Se (Kamada, 2017). Importantly, it has been reported that postpartum concentrations of P4 are positively correlated with conception rate (Inskeep, 2004). Similarly, our lab has previously reported increased early concentrations of P4 in cows supplemented with a 1:1 combination (MIX) of inorganic Se and organic Se (SEL-PLEX; Alltech, Inc., Nicholasville, KY, USA) compared to cows supplemented with inorganic Se alone on days 6 (Cerny et al., 2016b) and 7 (Carr et al., 2020) of the estrous cycle. Lower concentrations of P4 during the early luteal phase after artificial insemination have been associated with lower conception rates (Mann and Lamming, 1999).

Additionally, providing supplemental Se has demonstrated to reduce the incidence of metritis and ovarian cysts (Wilde, 2006) and to increase first service pregnancy rates (McClure et al., 1986) in dairy cattle. Moreover, supplementing dairy cattle with organic Se increased second-service pregnancy rates compared to inorganic Se (Thatcher et al., 2010). This increase in fertility could be attributed to the reduction in early embryonic death (Mehdi and Dufrasne, 2016).

2.5. Corpus Luteum

Corpora lutea were first identified in rabbits in 1573 by Volcherus Coiter (Asdell, 1928). Latin for "yellow body," corpora lutea were named by Marcello Malpighi in 1689 and first accurately described by Regnier de Graff in 1672 (Asdell, 1928; Smith et al., 1994; Niswender et al., 2000). De Graaf described them as globular bodies that formed only after coitus (Asdell, 1928) and appeared on the ovary and remained there until after parturition (Niswender et al., 2000). Moreover, de Graaf observed that the number of corpora lutea was related to the number of offspring (Asdell, 1928; Short, 1977; Di Renzo et al., 2020) and that removal of the ovaries during pregnancy caused parturition in cows (Di Renzo et al., 2020). Similarly, Frankel found that removing ovaries or corpora lutea from pregnant rabbits resulted in abortion or resorption of the embryos, as reviewed by Niswender et al. (2000).

For nearly 150 years, it was believed that corpora lutea formed only after coitus. However, Home in 1817 found that corpora lutea are present on the ovaries of virgins (Asdell, 1928). Interestingly, Prenant and Born in 1898 suggested that the CL is responsible for secretions that support the early embryo and facilitates the implantation process in the uterus (Corner, 1974). Nearly 30 years later, the term "progestin" was proposed to describe the substance produced by the CL that exerts "pro-gestation" activity (Allen and Corner, 1929; Corner and Allen, 1929; Allen, 1930). Virtually simultaneously, four research groups, including Butenandt and Westphal, Slotta, Hartmann and Wettstein, and Corner and Allen, purified and crystallized the luteal factor produced by the CL (Smith et al., 1994; Niswender et al., 2000; Di Renzo et al., 2020).

Finally, in 1935 the luteal factor being referred to generically as “corpus luteum hormone,” was agreed to be named “progesterone” (for ***proge****stational* ***ster****oidal ket****one***) (Allen et al., 1935; Allen, 1970).

2.5.1. Luteal Development

The CL in ruminants and other mammals contains specific hormone-producing luteal cells along with several other cell types (O'Shea, 1987). Steroidogenic luteal cells include both small and large luteal cells (Hansel et al., 1987). Other cell types identified in the CL include fibroblasts, endothelial cells and pericytes (Farin et al., 1986; Hansel et al., 1991). Non-steroidogenic luteal cells including macrophages and endothelial cells, along with the capillary system account for approximately 14% of the volume and just over half (53%) of the cells in the mature bovine CL (Parry et al., 1980; O'Shea et al., 1989). Additionally, fibroblasts are classically associated with the structural component of a tissue (Fields and Fields, 1996) and are approximately 6% of the total volume of the bovine CL (O'Shea et al., 1989).

Corpora lutea are a continuation of follicular maturations and form after ovulation from the remaining follicular cells (Smith et al., 1994). The theca and granulosa cells of the follicle differentiate into the small and large luteal cells, respectively (Donaldson and Hansel, 1965; Alila and Hansel, 1984; Niswender et al., 1986). The preovulatory surge of luteinizing hormone (LH) causes differentiation of follicular cells into luteal cells, a process known as luteinization (Schams and Berisha, 2004; Stocco et al., 2007). Luteinization is characterized by increased steroid production, a switch from producing

estradiol (E2) to progesterone (P4) and of enzymes responsible for these changes (Juengel and Niswender, 1999).

2.5.2. Luteinization

In follicular cells, the activation of the LH receptor (LH-R) by the LH surge causes ovulation and rapidly initiates a program of terminal differentiation of the ovulated follicle into a CL through a process known as luteinization (Stocco et al., 2007). Luteinization involves the transition of a periovulatory follicle into a highly vascular CL capable of secreting large quantities of P4 (Smith et al., 1994). The granulosa layer is thrown into folds about the follicular antrum and theca cells are borne into the developing CL by invasion of connective tissue and vascular tissue at these folds (Murphy, 2000). The reprogramming of follicular cells into luteal cells is irreversible and requires exit from the cell cycle (Murphy, 2000; Stocco et al., 2007). Activation of the LH-R is coupled to the stimulatory guanine nucleotide binding protein Gs and signals to adenylyl cyclase to increase cyclic AMP (cAMP) and activate cAMP-dependent protein kinase A (PKA) (Richards, 2001). Once PKA is activated, its catalytic unit moves to the nucleus where it phosphorylates numerous transcription factors (Stocco et al., 2007).

Altering cellular responsiveness to external signals, allowing luteal cells to respond to a new set of hormones, is one of the more important changes during luteinization (Stocco et al., 2007). The LH surge causes silencing of the follicle stimulating hormone receptor (FSH-R), a transient decline in the LH-R, and sustained stimulation of the prolactin (PRL) receptor (PRL-R) in some species, such as mice and

rats (Stocco et al., 2007). However, prolactin does not appear to be essential for normal luteal function during the estrous cycle in cows and ewes (Niswender et al., 2000). Additionally, within 6 h following the LH surge, there is a transient increase in P4 receptor (PR) mRNA in the granulosal layer of the bovine periovulatory follicle (Cassar et al., 2002; Jo et al., 2002). Although the LH induction of PR in granulosa cells is a central event in ovulation, its role in luteinization is not clear (Stocco et al., 2007). Interestingly, PR-null mice treated with gonadotropin are able to form CL, which contain trapped oocytes (Lydon et al., 1996). Furthermore, the LH surge causes a shift in the expression of the estrogen receptor (ER) from predominately ERβ to Erα, with levels of ERα found at levels 10-fold higher in the CL (Telleria et al., 1998).

2.5.3. Steroidogenic Cells

2.5.3.1. Steroidogenic Cells: Small Luteal Cells

As mentioned earlier, small luteal cells originate from thecal cells (Donaldson and Hansel, 1965). Small luteal cells are less than 23 μm in diameter, comprise 26% of the luteal cells and 28% of the CL volume (Fields and Fields, 1996). In addition, small luteal cells are more numerous than large luteal cells and can be identified by their elongated shape using light microscopy (O'Shea et al., 1979; Farin et al., 1986). These cells are known for low basal production of P4 that when stimulated with LH responds with increased secretion of P4 (Hansel and Dowd, 1986). The magnitude of LH-stimulated secretion of P4 is greater in small versus large luteal cells (Smith et al., 1994).

Interestingly, most of the LH receptors are located on the small luteal cells (Schams and Berisha, 2004). The LH receptors are coupled to the PKA second messenger pathway which stimulates many components of the synthetic pathway for P4 as demonstrated in Fig. 2.3 (Niswender et al., 2007). Simulating small luteal cells stimulated with LH or analogs of cyclic AMP can increase the secretion of P4 by as much as 20-fold (Fitz et al., 1982). However, as highlighted in Fig. 2.3, activation of the protein kinase C (PKC) pathway in small luteal cells inhibits LH-stimulated secretion of P4 (Wiltbank et al., 1991).

2.5.3.2. Steroidogenic Cells: Large Luteal Cells

As one would expect, large luteal cells have a diameter (24-45 μm) that is larger compared to small luteal cells (Fields and Fields, 1996). Using light microscopy, large luteal cells appear spherical or polyhedral and the nucleus appears rounded (Niswender et al., 2007). Although large luteal cells comprise only 3% of all luteal cells, they account for approximately 40% of the volume of the CL (O'Shea et al., 1989). In contrast to small luteal cells, large luteal cells do not respond to LH (Alila et al., 1988). However, they are the steroidogenic cells that secrete oxytocin (Fields et al., 1992). This production of oxytocin from large luteal cells occurs during the estrous cycle, but not during the post-implantation period of pregnancy, and indicates the role of this oxytocin is for luteal regression (Fields et al., 1992).

The basal secretion rates of P4 by large luteal cells are 10- to 20-fold higher than small luteal cells on a per cell basis (Fitz et al., 1982). Importantly, large luteal cells are

responsible for 80% of the total production of P4 by the CL (Niswender et al., 1985). Interestingly, it has been suggested there are at least two populations of secretory granules: one, containing oxytocin, of the estrous cycle, and one of the post-implantation period, with unknown contents (Fields et al., 1992). It has been suggested that PKC does not play an essential role in large luteal cells as made evident by the failure of phospholipase C to stimulate the synthesis of P4, whereas the synthesis of P4 was stimulated after phospholipase C was added to small luteal cells (Alila et al., 1988). However, stimulating large luteal cells with prostaglandin $F_{2}\alpha$ ($PGF_{2\alpha}$) activates the PKC second messenger pathway which inhibits the synthesis of P4 (Wiltbank et al., 1991; Juengel and Niswender, 1999). In contrast, prostaglandin E_2 (PGE_2) has been shown to increase production of P4 from luteal cells in cows and sheep (Fitz et al., 1984a; Fitz et al., 1984b; Alila et al., 1988; Shelton et al., 1990; Bennegård-Edén et al., 1995). There are multiple types of receptors for PGE_2 that are linked to different second messenger systems (Narumiya, 1997), that ultimately lead to increased synthesis of P4 (Niswender et al., 2000).

Receptors for growth hormone (GH) are located mainly on large luteal cells (Lucy et al., 1993). As reviewed by Schams and Berisha (2004), research has demonstrated that GH stimulates the secretion of P4 and oxytocin by bovine CL *in vitro* and supports the development of the CL *in vivo*. Moreover, GH may influence luteal function indirectly by increasing expression of IGF-I, which may stimulate secretion of P4 through modification of the cytoskeleton (Niswender et al., 2000). In addition, studies have shown that GH is a more powerful stimulator of the production of $PGF_{2\alpha}$ and P4 in the early bovine CL than LH (Kobayashi et al., 2001). Furthermore, in contrast to small

luteal cells, large luteal cells of ewes, pigs, cows, and humans, increased synthesis of P4 in response to luteotropins such as PGI_2, PGE_2, GH, and IGF-I is not mediated through increased activation of PKA

2.5.4. Angiogenesis

As discussed earlier, changes that occur during CL formation include the differentiation of follicular cells to luteal cells, tissue remodeling and growth, a switch in steroidogenesis, and increasing production of P4 (Robinson et al., 2007). However, in order to meet these demands, the growth of blood vessels and establishment of a blood supply (angiogenesis) is essential (Niswender et al., 2000). The development of capillaries from preexisting blood vessels is essential for the formation and function of the CL (Fraser et al., 2000; Reynolds et al., 2000). The extent of angiogenesis within the CL reaches a maximum within 2-3 days after ovulation (Reynolds et al., 2000). Each luteal cell is in direct contact with several capillaries (Stocco et al., 2007), giving the CL one of the highest rates of blood flow, per unit of tissue, of any adult organ as reviewed by (Reynolds et al., 2000).

Angiogenesis is a complex process, a delicate balance between promoters and inhibitors, and precise control in the ovary is critical for normal luteal function (Schams and Berisha, 2004). Several important promoters of angiogenesis include vascular endothelial growth factor A (VEGFA), acidic and basic fibroblast growth factor (FGF-1 and FGF-2), insulin like growth factors (IGF-1 and IGF-2) and angiopoietins (ANPT-1 and ANPT-2) (Schams and Berisha, 2004). The mRNA expression of VEGF and its

receptor are highest in the early luteal phase of angiogenesis (Neuvians et al., 2004). However, data suggest that FGF2 plays a more important role in the initiation of angiogenesis post-ovulation, while VEGFA plays a more constitutive role in maintaining the development of developing capillaries/blood vessels (Robinson et al., 2007). Interestingly, LH increased the production of FGF2 both *in vivo* and *in vitro* (Robinson et al., 2007). Additionally, secreted protein, acidic, cysteine-rich (SPARC) was present at constant levels throughout the development of the CL and works with VEGFA in the maintenance of the vasculature (Robinson et al., 2007). As reviewed by Townson and Liptak (2003), monocyte chemoattractant protein-1, a chemokine specific for monocytes and T-lymphocytes, is expressed during ovulation and CL formation (Townson and Liptak, 2003). It appears that the combined actions of chemokines and leukocytes may encourage vascularization during luteal development (Townson and Liptak, 2003). The establishment of an inadequate vascular supply to the CL is postulated to have significant ramifications on steroid secretion later in the luteal phase (Fraser and Wulff, 2001).

2.5.5. Immune Cell Function in the Corpus Luteum

Immune cells play an active role in controlling the lifespan and function of the CL (Penny et al., 1999). During the early luteal phase, production of P4 requires rapid growth of the CL which is dependent on angiogenesis (Robinson et al., 2007). Angiogenic factors, including VEGF and FGF along with their receptors, are strongly regulated during the development of the bovine CL (Neuvians et al., 2004). Additionally, a high concentration of interleukin-8 (IL8) is present in the bovine CL during the early luteal

phase (Jiemtaweeboon et al., 2011), which effectively stimulates production of P4 in bovine luteinizing granulosa cells (Shimizu et al., 2012). Receptors for IL8 include CXCR1 and CXCR2 (Shirasuna and Miyamoto, 2017).

On the contrary, production of P4 is inhibited by tumor necrosis factor α (TNFα), gamma-interferon (IFNG) and interleukin 1β (IL-1β) (Pate, 1995). These three cytokines are also potent stimulators of prostaglandin production (Pate, 1995). However, research indicates that mRNA encoding TNFα is present in the bovine CL before luteolysis (Pate, 1995). It has also been proposed that TNFα promotes formation of the CL by increasing proliferation and steroidogenesis of luteinizing granulosa cells (Yan et al., 1993). Luteolytic effects of TNFα and IFNG on human and non-human primate luteal cells also involve Fas cell surface death receptor and Fas ligand (Pate et al., 2010).

2.5.6. Luteal Steroidogenesis of Progesterone

The first challenge for a steroid producing cell, including luteal cells, is obtaining the precursor, cholesterol (Christenson and Devoto, 2003). A constant supply of cholesterol is needed for the synthesis of steroid hormones in the CL (Stocco et al., 2007). Cholesterol used for steroid synthesis in the ovary may come from *de novo* synthesis or through cellular uptake of lipoprotein cholesterol transported by low (LDL) or high (HDL) lipoproteins (Grummer and Carroll, 1988). Additionally, mobilization of cholesterol esters contributes to the supply of free cholesterol for steroid synthesis (Gwynne and Strauss III, 1982). Producing cholesterol from *de novo* synthesis typically plays a minor role in the normal functioning tissue, as evidenced by low levels of 3-

hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthetic pathway (Gwynne and Strauss, 1982; Grummer and Carroll, 1988). However, cholesterol from circulating lipoproteins appears to provide the major source of substrate used for steroid synthesis (Pate and Condon, 1989).

The biosynthesis of P4 requires two enzymatic steps; 1) the conversion of cholesterol to pregnenalone, catalyzed by P450 side chain cleavage (P450scc) located on the inner mitochondrial membrane, and 2) the subsequent conversion of pregnenalone to P4, catalyzed by 3β-hydroxysteroid dehydrogenase (3β-HSD) present in the smooth endoplasmic reticulum as shown in Fig. 2.3 (Christenson and Devoto, 2003). Cholesterol cannot freely diffuse in the cytosol and reach the mitochondria without first binding to carrier proteins due to its hydrophobic nature (Stocco et al., 2007). Sterol carrier protein-2 is considered to play a role in the intracellular movement of cholesterol in steroidogenic cells (Seedorf et al., 2000). Once cholesterol has reached the outer mitochondrial membrane, it is transported to the inner mitochondrial membrane (Stocco et al., 2007). In 1994, it was discovered that an LH-induced protein, named steroidogenic acute regulatory protein (StAR), is localized in the mitochondria, and allows for the transfer of cholesterol to the inner mitochondrial membrane (Clark et al., 1994). There is a very tight, positive correlation with StAR protein and steroidogenesis (Manna et al., 2009). Additionally, the transport of cholesterol to the inner mitochondrial membrane by StAR protein is the rate-limiting step in steroidogenesis (Clark et al., 1994; Manna et al., 2016). The expression, activation, and extinction of StAR protein is mediated by PKA, PKC, and a host of other signaling pathways (Fig. 2.3) (Stocco and Clark, 1996; Manna et al., 2009; Manna et al., 2016). Steroidogenesis is mediated by mechanisms that enhance

transcription, translation, or the activity of StAR (Manna et al., 2009). Several transcription factors including SF-1, C/EBPβ, SREBP-1a, cFOS, GATA-4, Sp-1 and CREB family members have been implicated in the transcriptional stimulation of the StAR gene (Manna et al., 2003). In contrast, dosage-sensitive sex reversal-adrenal hypoplasia congenital region on the X chromosome gene 1 (DAX-1) and forkhead box protein L2 (FOXL2) were demonstrated to play key roles in the repression of StAR, by binding to a recognition motif found in the promoter region of the StAR gene, and serving as a marker for granulosa cell differentiation (Pisarska et al., 2004). The preovulatory LH surge results in acquisition of the 3βHSD enzyme by granulosa cells and an overall increase in the enzyme activity within corpora lutea, which facilitates high rates of P4 biosynthesis (Smith et al., 1994). Progesterone is then thought to diffuse from the cell and there is no evidence that P4 can be stored in high quantities in luteal tissue (Niswender et al., 2000). After P4 enters the circulation, it is subject to metabolism in the blood (Gomes and Erb, 1965). The half-life of P4 has been estimated to be approximately 30 minutes in cows, 7-8 minutes in the ewe, and only 3-5 minutes in humans as reviewed by Gomes and Erb (1965). Additional studies indicate that P4 is converted to androgenic substances in the liver and excreted via the bile into the feces (Miller and Turner, 1961, 1963).

Hormones that support the growth and/or function of the CL, known as luteotropic hormones, include LH, GH, PRL, IGF-1, oxytocin, prostaglandin E_2 (PGE_2), and prostaglandin I_2 (PGI_2) (Niswender et al., 2000). It seems clear that LH is required to maintain normal expression of mRNA, and presumably proteins, encoding StAR, P450scc, and 3βHSD (Niswender et al., 2000). Although the pulsatile release of LH is

required for luteal development in cattle, pulses of LH are not required for maintenance of P4 secretion in cattle (Peters et al., 1994). However, the CL is dependent on LH, as research has demonstrated that the removal of the pituitary results in regression of the CL (Denamur et al., 1966, 1973; Haworth, 1997). Concentrations of P4 in serum are dependent on the amount of steroidogenic tissue, blood flow, and capacity of the steroidogenic tissue to synthesize P4 (Niswender et al., 2000). Moreover, the steroidogenic capacity of individual luteal cells, and their ability to respond to LH, increases during luteal development (Niswender et al., 2000).

As previously discussed, it is generally accepted that steroidogenic tissues can derive cholesterol from circulating lipoproteins that may provide the major source of substrate for the synthesis of steroids (Pate and Condon, 1989). However, $PGF_{2\alpha}$ can inhibit the utilization of lipoproteins for the synthesis of P4 (Pate and Nephew, 1988). Limiting the supply of substrate for steroidogenesis could be one mechanism by which $PGF_{2\alpha}$ exerts is luteolytic effect (Pate and Condon, 1989). It has been demonstrated that the synthesis of cholesterol and production of P4 is regulated by the availability of lipoprotein, which can be inhibited by $PGF_{2\alpha}$ (Pate and Condon, 1989). Additionally, $PGF_{2\alpha}$ can suppress *de novo* sterol synthesis, further limiting the pool of cholesterol that would be available for the production of P4 (Pate and Condon, 1989). In fact $PGF_{2\alpha}$ was identified as the uterine-derived luteolytic hormone responsible for the regression of the CL and cessation of the secretion of progesterone by luteal cells (Schramm et al., 1983).

2.5.7. Luteal Regression

Luteolysis is defined as lysis or structural demise of the CL (Niswender et al., 2000). In the absence of pregnancy, the CL undergoes structural and functional luteolysis on days 15-16 and the cycle begins again (Bazer et al., 1998). During normal luteolysis there is a loss of the capacity to synthesize and secrete P4 (Miyashita et al., 1994) followed by the loss of the cells that comprise the CL (Knickerbocker et al., 1988; Pate, 1994). As mentioned in the section 2.5.6., $PGF_{2\alpha}$ is the factor from the uterus that initiates luteolysis (Schramm et al., 1983). Interestingly, P4 exposure during the early to mid-luteal phase of the estrous cycle is essential for initiation of endometrial $PGF_{2\alpha}$ production and luteolysis (Bazer et al., 1998). $PGF_{2\alpha}$ enters the ovarian artery from the utero-ovarian vein, via a counter current exchange mechanism (Staples and Whylie, 1984).

Uterine release of luteolytic $PGF_{2\alpha}$ is regulated primarily by estrogen, P4, and oxytocin as reviewed in Bazer et al. (1998). To modulate uterine responses to oxytocin, estrogen and P4 regulate oxytocin receptor (OTR) gene expression in the endometrial epithelium (McCracken et al., 1984). Endometrial OTR synthesis is blocked for 10 to 12 days by P4, a phenomenon termed the "progesterone block" to endometrial OTR formation (Bazer et al., 1998). However, continuous exposure of the endometrium to P4 negatively regulates PR expression in the luminal and glandular epithelium (Spencer et al., 2004; Spencer et al., 2008b). Without sufficient PR, the endometrial epithelium expresses ER, responds to estrogen and up-regulates expression of OTR (Spencer and Bazer, 1995; Spencer et al., 1995). Estrogen up-regulates OTR gene expression, while oxytocin, acting through OTR, induces pulsatile release of $PGF_{2\alpha}$ (Hixon and Flint, 1987). $PGF_{2\alpha}$ then initiates a positive-feedback loop involving release of additional luteal

oxytocin and $PGF_{2\alpha}$ of both luteal and uterine origin as reviewed by Niswender et al. (2000).

2.5.8. Luteal Function

2.5.8.1. Maternal Recognition of Pregnancy

The major role of the CL is the biosynthesis of P4 (Behrman et al., 1971), which is required for the maintenance of normal pregnancy in mammals (Niswender et al., 2000). As discussed earlier, the functional lifespan of the CL is controlled by the release of $PGF_{2\alpha}$ from the uterus (Bazer et al., 1998). Maintenance of luteal function in most pregnant mammals is dependent upon the interrelationship between the embryo, the ovaries, and hypophysis (Moor, 1968). In 1969, Roger Short coined the term "maternal recognition of pregnancy" (MRP) and is defined as the process where a chemical signal from the conceptus prevents luteolysis caused by the release of $PGF_{2\alpha}$ from the endometrium, therefore sustaining secretion of P4 beyond the length of a normal estrous cycle (Short, 1969). Later, researchers at the University of Florida determined that the MRP signal was interferon tau (IFNT) in ovine (Wilson et al., 1979) and bovine (Lewis et al., 1979). The pregnancy recognition signal occurs on day 12 in pigs, days 13-14 in sheep, and days 16-17 in cattle (Spencer, 2013). The antiluteolytic effect of IFNT produced by the conceptus is the primary cause of the maintenance of the CL in sheep and cattle (Thatcher et al., 1995). The oxytocin receptors are blocked by IFNT and as a result, the uterus does not produce the pulsatile release of $PGF_{2\alpha}$ initiating luteal

regression (Spencer, 2013). Additionally, IFNT may inhibit the action of TNFα and oxytocin and therefore stop the pulsatile release of $PGF_{2\alpha}$ from continuing (Okuda et al., 2002). The effects of IFNT on the CL and endometrium are further discussed in Section 1.6. *Uterine Function at Maternal Recognition of Pregnancy*.

2.5.8.2. Implications of Concentrations of Progesterone

Lower concentrations of P4 during the early luteal phase after artificial insemination have been associated with lower conception rates (Mann and Lamming, 1999). Moreover, low circulating concentrations of P4 in the first week after ovulation are associated with under-developed conceptuses (Forde and Lonergan, 2012), transcriptomic alterations (Barnwell et al., 2016), and a low likelihood of establishing pregnancy (Wiltbank et al., 2016). In contrast, increased concentrations of P4 immediately following conception has been associated with advanced conceptus elongation (Carter et al., 2008) and increased IFNT production (Mann and Lamming, 2001). Furthermore, embryo quality on day 16 was greater in cows with an earlier increase in concentrations of P4 after ovulation (Mann and Lamming, 2001). Artificially increasing early luteal-phase concentrations of P4 has been demonstrated to increase development of the conceptus (Garrett et al., 1988). Likewise, providing exogenous P4 between days 3 to 7 post-insemination resulted in increased pregnancy rates (Yan et al., 2016).

In the uterus, P4 acts on the endometrium as a differentiation factor (Cummings and Yochim, 1984). Progesterone stimulates secretions in the glandular epithelium

(Maslar et al., 1986) and changes the patterns of proteins secreted by endometrial cells (Maslar et al., 1986). Together, these proteins provide an environment that supports early embryonic development (Niswender et al., 2000). The effects of P4 on the uterus will be discussed in greater detail in the next section, *Uterine Function at Maternal Recognition of Pregnancy*.

2.6. Uterine Function at Maternal Recognition of Pregnancy

2.6.1. Overview

In cattle, fertilization rates are high and between 90 and 100%, when semen of known high fertility is used in artificial insemination (Diskin et al., 2006; Diskin and Morris, 2008). However, some studies reported that fertilization rates in lactating beef cows averaged 75%, with a range of 60 to 100% (Breuel et al., 1993; Santos et al., 2004). In comparison, beef heifers showed higher fertilization rates, averaging 88%, with a range between 75 and 100% (Maurer and Chenault, 1983; Dunne et al., 2000). However, it has been reported that only 50 to 60% of beef cows inseminated remain pregnant by day 30 (Bridges et al., 2013). Embryonic losses are defined as those that occur from fertilization until day 42 of pregnancy when differentiation and implantation has occurred (Santos et al., 2004). Embryonic losses are further divided into two categories and classified as early embryonic loss (EEL; fertilization to day 27) and late embryonic loss (LEL; day 28 to 42) (Santos et al., 2004).

Under the heightened physiological demands observed in high producing, lactating dairy cows, 20-50% have experienced pregnancy loss during the first week of gestation (Wiltbank et al., 2016). It is estimated that 70-80% of the embryonic loss occurs between day 8 and 16 after insemination (Sreenan and Diskin, 1986). Similarly, from days 8 to 27 of gestation, a period of time encompassing embryo elongation and MRP, pregnancy losses average approximately 30% (Wiltbank et al., 2016). Late embryonic loss occurs in a smaller percentage of females, ranging from 3 to 14% of beef cows and

heifers (Humblot, 2001; Santos et al., 2004; Perry et al., 2005). The loss of pregnancy at any stage of gestation has a clear negative impact for the reproductive performance and profit potential in beef and dairy operations.

After hatching from the zona pellucida, blastocysts develop into a tubular form, and then elongate on day 15 in cattle, to filamentous conceptuses that occupy much of the length of the uterine horn (Spencer et al., 2008b). Progesterone acts on the uterus to indirectly stimulate pre-implantation blastocyst growth and elongation (Garrett et al., 1988; Mann and Lamming, 2001). During this critical window of MRP, the elongating conceptus must secrete a chemical signal to signal pregnancy and to prevent uterine release of luteolytic pulses of $PGF_{2\alpha}$ and therefore luteal regression (Short, 1969). As discussed earlier, IFNT has been identified as the MRP signal in cattle (Lewis et al., 1979) and occurs around days 16-17 in cattle (Spencer, 2013). This type I interferon is exclusively produced by the mononuclear trophectoderm cells of the elongating conceptus during the peri-implantation period (Farin et al., 1990). During MRP, the conceptus trophectoderm secretes IFNT between days 10 and 21, with maximal production on days 14 to 16 (Roberts et al., 1999). IFNT acts in a paracrine manner on the endometrium to inhibit development of the endometrial luteolytic mechanism required for the pulsatile release of $PGF_{2\alpha}$, thereby ensuring continued production of P4 by the CL (Spencer et al., 2007). Specifically, IFNT acts on luminal epithelia and superficial glandular epithelia to suppress transcription of ER and OTR (Spencer and Bazer, 1996; Fleming et al., 2001), thereby abrogating development of the endometrial luteolytic mechanism (Spencer and Bazer, 1995; Spencer et al., 1995; Choi et al., 2001). Moreover, the antiestrogenic actions of IFNT prevent estrogen-induced increases in ER

and thus PR expression, OTR synthesis, and hence, production of luteolytic pulses of $PGF_{2\alpha}$ as detailed in Fig. 2.4 (Spencer and Bazer, 2002).

Additionally, IFNT increases PGE_2 synthase (PGES) in the CL and increases the PGE receptor (EP), EP2, in endometrial stroma (Arosh et al., 2004). However, in the endometrium, IFNT decreases expression of $PGF_{2\alpha}$ synthase (PGFS) (Arosh et al., 2004) and high concentrations of IFNT stimulate PGE_2 production or increase its production relative to $PGF_{2\alpha}$ (Parent et al., 2003). This key alteration leads to an increase in the PGES to PGFS ratio in the endometrium, suggesting that although levels of expression of PGES and PGFS are the same in a given tissue, prostaglandin biosynthesis would be favorably directed toward PGE_2, rather than $PGF_{2\alpha}$ (Arosh et al., 2004). PGE_2 is considered a luteoprotective or luteotrophic mediator at the time of MRP (Pratt et al., 1977; Magness et al., 1981) and it is well known that PGE_2 stimulates luteal secretion of P4 in small luteal cells by increasing cAMP (Hansel and Blair, 1996). Additionally, IFNT stimulates a number of genes in a cell-specific manner within the endometrium that are implicated in uterine receptivity and conceptus development (Spencer et al., 2007), and will be discussed in more detail in Section 2.6.3

Successful pregnancy in mammals requires both a viable embryo and a receptive endometrium (Walker et al., 2010). Synchronous signaling between the endometrium and embryo during the pre-implantation period is critical for normal embryo development, implantation of the embryo, and placentation (Wolf et al., 2003). Uterine receptivity has been shown to be dependent on P4 (Mansouri-Attia et al., 2009). Additionally, uterine factors include enzymes, cytokines, growth factors, ions, hormones, glucose, transport proteins, and adhesion molecules, collectively termed “histotroph,” have been shown to

be mainly synthesized by the endometrial glands (Martal et al., 1997). Research has indicated that P4-induced changes in endometrial gene expression leads to changes in the composition of histotroph that are required for post-hatching conceptus survival and growth (Spencer et al., 2008a). The importance of histotroph for conceptus development was demonstrated in the uterine gland knockout (UGKO) model in sheep in which embryos fail to develop beyond the blastocyst stage in adult UGKO ewes (Spencer and Gray, 2006).

The early embryo is nourished by histotroph from the uterine glands which are located in the intercaruncular endometrium (ICAR) (Atkinson et al., 1984). While, small aglandular caruncular (CAR) areas of stromal origin are scattered over the endometrium surface (Mansouri-Attia et al., 2009). The CAR and ICAR areas encompass two distinct endometrial zones, with apparent differences in structure and biological functions (Mansouri-Attia et al., 2009). The endometrial glands of the ICAR areas have been shown to be crucial for the development of the conceptus (Gray et al., 2001). Whereas the CAR areas are present in the cyclic endometrium and they fuse with the fetal cotyledons to form placentomes in the pregnant animals (Atkinson et al., 1984). During this pivotal time period encompassing MRP, failure or delays in trophoblast elongation and/or embryonic development result in loss of pregnancy possibly due to suboptimal histotroph (Wiltbank et al., 2016).

Pregnancy also represents an immunological contradiction, in that the immunologically foreign embryo is able to form a close physical relationship with the maternal endometrium that lasts throughout pregnancy (Walker et al., 2010). Interestingly, apposition, adhesion, and invasion processes are thought to be controlled by

the endometrium (von Rango, 2008). The fact that an embryo can survive in the presence of the maternal immune system has led to the hypothesis that the uterus is an immunologically privileged site (Bainbridge, 2000). The immune response to pregnancy may be one of the key regulators of pregnancy maintenance, and deregulation of the immune response may be responsible, at least in part, for the large number of pregnancy losses that occur near the time of MRP (Walker et al., 2010).

2.6.2. Progesterone Induced Changes

Progesterone is the hormone of pregnancy and unequivocally required in all mammals for maternal support of conceptus survival and development (Spencer and Bazer, 2002). The actions of P4 are mediated by PR (Spencer and Bazer, 2002). Progesterone stimulates and maintains endometrial functions necessary for conceptus growth, implantation, placentation, and development to term (Bazer, 1975; Bazer et al., 1979; Spencer and Bazer, 2002; Spencer et al., 2004). Heifers and ewes with lower concentrations of P4 in the early luteal phase had smaller conceptuses that secreted less IFNT (Nephew et al., 1991; Mann and Lamming, 2001). Conversely, increased concentrations of P4 immediately following conception has been associated with advanced conceptus elongation (Carter et al., 2008) and increased IFNT production (Mann and Lamming, 2001).

From the perspective of the maternal system, a key regulator of uterine function and histotroph involves the circulating concentrations of P4 (Wiltbank et al., 2016). Progesterone both positively and negatively regulates expression of genes in the

endometrium, and P4 and IFNT stimulate a number of genes, particularly in the endometrial epithelia (Spencer et al., 2008b). Progesterone receptors are expressed in endometrial epithelia and stroma during the early to mid-luteal phase, allowing direct regulation of genes by P4 (Spencer et al., 2008b). However, continuous exposure of the endometrium to P4 negatively regulates PR expression in the luminal and glandular epithelium, and the down regulation of PR is temporally associated with the induction of many P4-stimulated genes (Spencer et al., 2004; Spencer et al., 2008b). As reviewed by Spencer et al. (2004), the paradigm of loss of PR in uterine epithelia immediately before implantation is common to sheep, cattle, pigs, western spotted skunks, baboons, rhesus monkeys, humans, and mice. Thus, regulation of endometrial epithelial function during the peri-implantation period must be directed by specific factors produced by PR-positive stromal cells in response to P4 (Cunha et al., 1985).

Regulation of diacylglycerol O-acyltransferase homolog 2 (DGAT2) and myostatin (MSTN) have been associated with P4-dominant environments (Forde et al., 2009). DGAT2 catalyzes the final step in the formation of triglyceride to acylcoenzyme A and that triglyceride is a potential energy source up to the blastocyst stage in cattle (Forde et al., 2009). Interestingly, a P4-induced earlier increase in DGAT2 expression may increase availability of triglyceride as an energy source for the developing conceptus (Forde et al., 2009). Similarly, P4 induction of MSTN may increase glucose secretion into histotroph, contributing to the advanced development of the conceptus after hatching (Forde et al., 2009).

One of the first PR targets identified and known to be central to uterine function is the growth factor, Indian hedgehog (IHH), which is induced in the epithelium and exerts

paracrine effects on the stroma (Matsumoto et al., 2002; Takamoto et al., 2002). Importantly, epithelial IGG induces stromal chicken ovalbumin upstream promoter-transcription factor II (COUPTFII) expression (Takamoto et al., 2002; Lee et al., 2006), which both inhibits estrogen-induced epithelial proliferation to allow implantation and induces bone morphogenetic protein 2 (BMP2) in the stroma to effect the decidualization response (Kurihara et al., 2007; Lee et al., 2010). Another PR target in the endometrium is homeobox protein-A10 (HOXA10), and HOXA10 knockout mice are infertile due to uterine defects that appear to be a result of lost stromal P4 responsiveness (Benson et al., 1996; Lim et al., 1999). Interestingly, PR in decidualized stromal cells activates the insulin-like growth factor binding protein-1 (IGFBP-1) promoter (Gao et al., 1999) that modulate insulin-like growth factor activity and bioavailability (Satterfield et al., 2008). In situ hybridization analyses revealed that IGFBP-1 mRNAs were expressed specifically in luminal epithelia and superficial glandular epithelia of ICAR and in luminal epithelia of CAR tissue (Satterfield et al., 2008). Importantly, IGFBP1 is upregulated in the endometrium during early pregnancy and is implicated as a regulator of blastocyst implantation and placental growth and development (Giudice and Saleh, 1995). Additionally, P4 regulates forkhead Box L2 (FOXL2) expression in the endometrium of ruminants and stimulates FOXL2 promoter activity through PR nuclear receptors (Eozenou et al., 2020). In bovine endometrium, a negative correlation between circulating concentrations of P4 and FOXL2 gene expression exists (Eozenou et al., 2012). FOXL2 appears to be important in the endometrium as well as a key gene involved in ovarian differentiation and maintenance of ovarian function (Eozenou et al., 2012; Georges et al., 2014; Elzaiat et al., 2017). Interestingly, it has been revealed that

FOXL2 is a transcriptional repressor of the StAR protein gene (Pisarska et al., 2004). As discussed in section 2.5.6, StAR protein transports cholesterol to the inner mitochondrial membrane and is the rate-limiting step in steroidogenesis (Clark et al., 1994; Manna et al., 2016).

Uterine receptivity to implantation is P4-dependent; however, implantation events are preceded by loss of PR and estrogen receptors by uterine epithelia (Spencer and Bazer, 2002; Spencer et al., 2008b). It is likely that P4 stimulates PR-positive stromal cells to express one or more progestamedins (eg., fibroblast growth factors-7 and -10, and/or hepatocyte growth factor), that act via their respective receptors on uterine epithelia and trophectoderm to regulate expression of interferon stimulated genes (ISGs) (Bazer et al., 2008). Moreover, for most, if not all, actions of type I/II interferons on the uterus, P4 is permissive to ISG expression, with genes being induced by interferons or induced by P4 and stimulated by interferon (Bazer et al., 2008).

2.6.3. Interferon Stimulated Genes

In addition to its antiluteolytic actions, IFNT acts on endometrial genes, ISGs, in a specific spatial and temporal manner (Bazer et al., 2008; Bazer et al., 2009). Interestingly, in a comparison of pregnant and cyclic heifers, differentially expressed genes identified on day 16 of pregnancy were found to be due to the presence of the conceptus and the majority were expressed in response to IFNT produced by the conceptus (Forde et al., 2011). Similarly, ISGs were among the most up-regulated group of genes in pregnant animals; this is consistent with maximal production of the MRP

signal, IFNT, by the embryo during this time (Walker et al., 2010). Following binding of IFNT to its receptors (IFNAR1 and IFNAR2), it initiates cell signaling via Janus activation kinases (JAKs) and tyrosine kinase 2 (TYK2) as demonstrated in Fig. 2.5 (Bazer et al., 2008; Walker et al., 2010). IFNT then induces the expression of classical ISGs in the stromal cells that express interferon sensitive response elements (ISREs) (Forde et al., 2011). In contrast, expression of interferon regulatory factor 2 (IRF2) in the luminal and glandular epithelium of sheep inhibits classical ISG expression (Choi et al., 2001; Spencer et al., 2008b). However, IFNT does stimulate a number of genes in the endometrial epithelia that transport nutrients or enhance genes for proteins important for conceptus elongation and uterine receptivity to implantation (Forde et al., 2011). These include wingless-type mouse mammary tumor virus integration site family member 7A (WNT7A), as well as galectin, proteases, transporters for glucose and amino acids, and IGFBP1 (Bazer et al., 2008). WNT7A, a luminal epithelia-specific gene in all mammals studied, stimulates ovine trophectoderm cell proliferation by activating the canonical WNT signaling pathway which is proposed to coordinate conceptus-endometrial interactions required for implantation in mice and humans (Hayashi et al., 2007). The WNT family of genes encode highly conserved secreted glycoproteins that regulate cell and tissue growth and differentiation during embryonic development (Polakis, 2000).

The energy substrate for mammalian conceptuses switches from pyruvate to glucose at the blastocyst stage, which coordinates with increases in expression of uterine glucose transport proteins during early pregnancy (Das et al., 1998; Zhao et al., 2005; Riley and Moley, 2006). An increase in the uterine glucose transport proteins is especially important, as neither conceptuses nor uterine endometrium can carry-out

gluconeogenesis (Bazer et al., 2008). Specifically, two glucose transporters, SLC2A1 and SLC5A11, were identified as P4-induced and IFNT-stimulated during the peri-implantation period (Bazer et al., 2008).

Several ISGs have been identified as being differentially expressed between pregnant and cyclic animals; many of which may function to provide localized immune system suppression to allow the embryo to survive within the uterus (Walker et al., 2010). Specifically, IFITM, TAP, and OAS proteins were upregulated in pregnant animals and are involved in local immune suppression (Walker et al., 2010). In particular, silencing major histocompatibility complex (MHC) class I alpha chain and beta2 microglobulin (B2M) genes in endometrial luminal and superficial glandular epithelia during pregnancy may be critical in preventing immune rejection of the conceptus allograft (Bazer et al., 2008).

OAS upregulation during early pregnancy is also involved in regulating the production of osteopontin (SPP1) (Spencer et al., 1999; McAveney et al., 2000), which is also up-regulated in pregnant animals (Walker et al., 2010). Additionally, upregulation of SPP1 in pregnant animals promotes adhesion of the trophoblast to the endometrium, stimulates morphological changes in the trophoblast (Johnson et al., 2003) and regulates the immune response (Walker et al., 2010). Upregulation of these genes may be an important mechanism to enhance the response to potential viral pathogens during the time of local immune suppression that occurs in response to the embryo (Walker et al., 2010). The upregulation of MX1 and MX2, both ISGs, supports this hypothesis and are upregulated in response to viral infection (Hicks et al., 2003; Bauersachs et al., 2009). Interestingly, MX2 is also upregulated in peripheral blood leukocytes during pregnancy

(Gifford et al., 2007) and suggests that the innate immune system is active during early pregnancy (Walker et al., 2010). Additionally, some of these genes are also thought to be important regulators of luteal regression (Hicks et al., 2003). For example, OAS inhibits $PGF_{2\alpha}$ synthesis, possibly through the alteration of arachidonic acid metabolism (Schmitt et al., 1993).

Intriguingly, several reports indicate induction or increases in ISGs in peripheral blood lymphocytes and the CL during pregnancy or in ewes receiving intrauterine injections of IFNT (Spencer et al., 2008b). Thus, IFNT or IFNT-stimulated immune cells may traffic out of the uterus to exert systemic effects that alter maternal physiology, particularly in the CL of pregnancy (Spencer et al., 2008b). The immune response to pregnancy may be one of the key regulators of pregnancy maintenance, and deregulation of the immune response may be responsible, at least in part, for the large percentage of pregnancies lost during the time around MRP (Walker et al., 2010).

2.7. Working Mechanism of Selenium-Induced Increased Conception Rate

Research has demonstrated that organic forms of Se have increased levels of bioavailability compared to inorganic Se. Our lab has previously demonstrated increased concentrations of early luteal phase P4 on days 6 and 7 of the estrous cycle in cows supplemented with MIX compared to OSe or ISe alone. However, the mechanism responsible for the Se-induced increased P4 remain unknown. Increased concentrations of P4 could be due in part to 1) increased availability of the steroid precursor, cholesterol, 2) increased expression of enzymatic transcripts involved in steroidogenesis, or 3) decreased catabolism of P4 in the liver. Perhaps cows supplemented with MIX have increased expression of StAR, the rate-limiting enzyme in steroidogenesis, increased enzymatic activity involved in cholesterol biosynthesis, or decreased expression of liver enzymes involved in catabolism of P4. It is plausible that some combination of the thoughts listed above are responsible for the MIX-induced increased concentrations of P4 observed in our lab.

The benefits of increased early luteal phase concentrations of P4 are well known. Additionally, inducing increased concentrations of P4 earlier in the cycle has demonstrated to stimulate secretory activity of the uterine endometrium, which stimulates advance conceptus development. Perhaps the MIX-induced increased concentrations of P4 earlier in gestation leads to conceptuses further along in development, that produce large quantities of IFNT earlier after insemination, thus advancing the timing of maternal recognition of pregnancy, and ultimately leading to increased conception rates. Increased concentrations of IFNT earlier would result in earlier suppression of ER and OTR,

therefore suppressing the luteolytic effects of oxytocin induced pulsatile release of $PGF_{2\alpha}$.

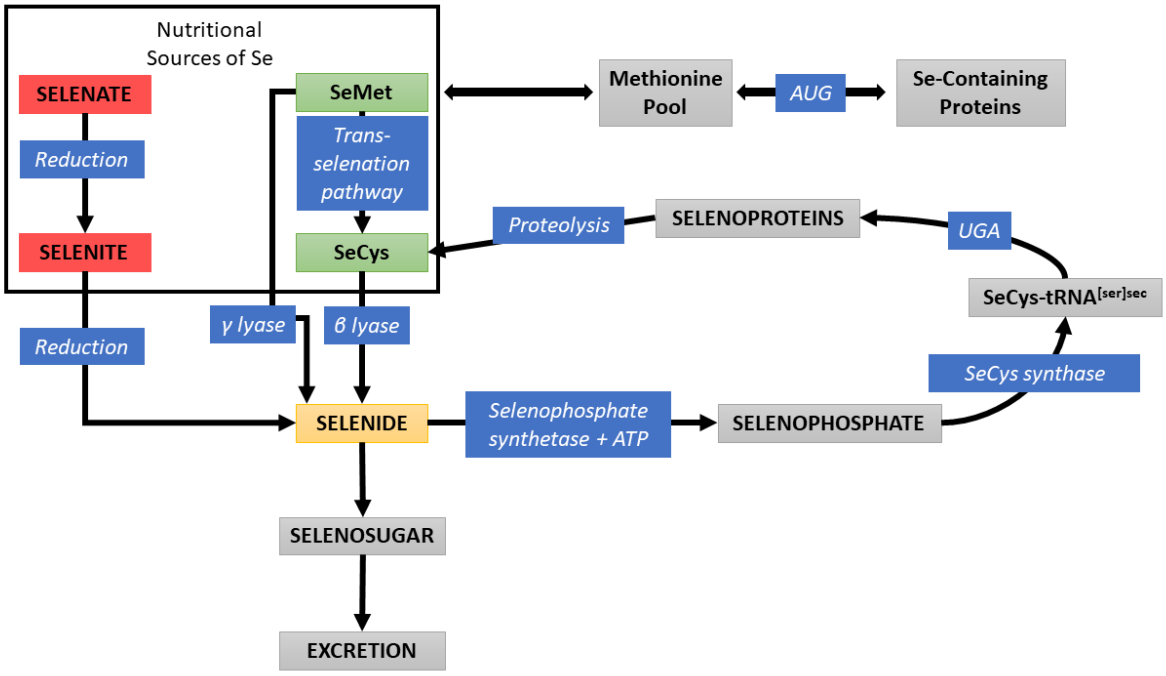
Throughout the estrous cycle and gestation, follicles grow and regress in patterns of two or three follicular waves. Research has demonstrated that cows with three follicular waves are more fertile, the occurrence of 2- or 3-waves is random, and the number of follicular waves can be controlled with exogenous hormones. Cows exhibiting two follicular waves have a dominant follicle present for a longer period, thus prolonging the exposure of E2. It is possible there is simply a shift in the population of females exhibiting three follicular waves rather than two in those supplemented with MIX instead of ISe.

Furthermore, successful pregnancy outcome involves a receptive uterine environment and regulation of the immune response. Histotroph secretion from the uterine endometrium nourishes the developing embryo. Genes involved in histotroph secretions have been well documented. Perhaps cows or heifers supplemented with MIX have increased abundance of transcripts associated with these secretions necessary for the developing embryo/conceptus. The process of pregnancy involves the invasion of a foreign embryo and is quite astonishing as it's an immunological contradiction. Research has shown that Se is necessary for an optimum immune response. It is possible that MIX supplemented animals have an increased ability to deregulate the immune response necessary for pregnancy establishment and maintenance compared to animals supplemented with ISe alone.

A successful pregnancy outcome is dependent upon a multitude of factors beginning before insemination and throughout gestation. Taking everything into

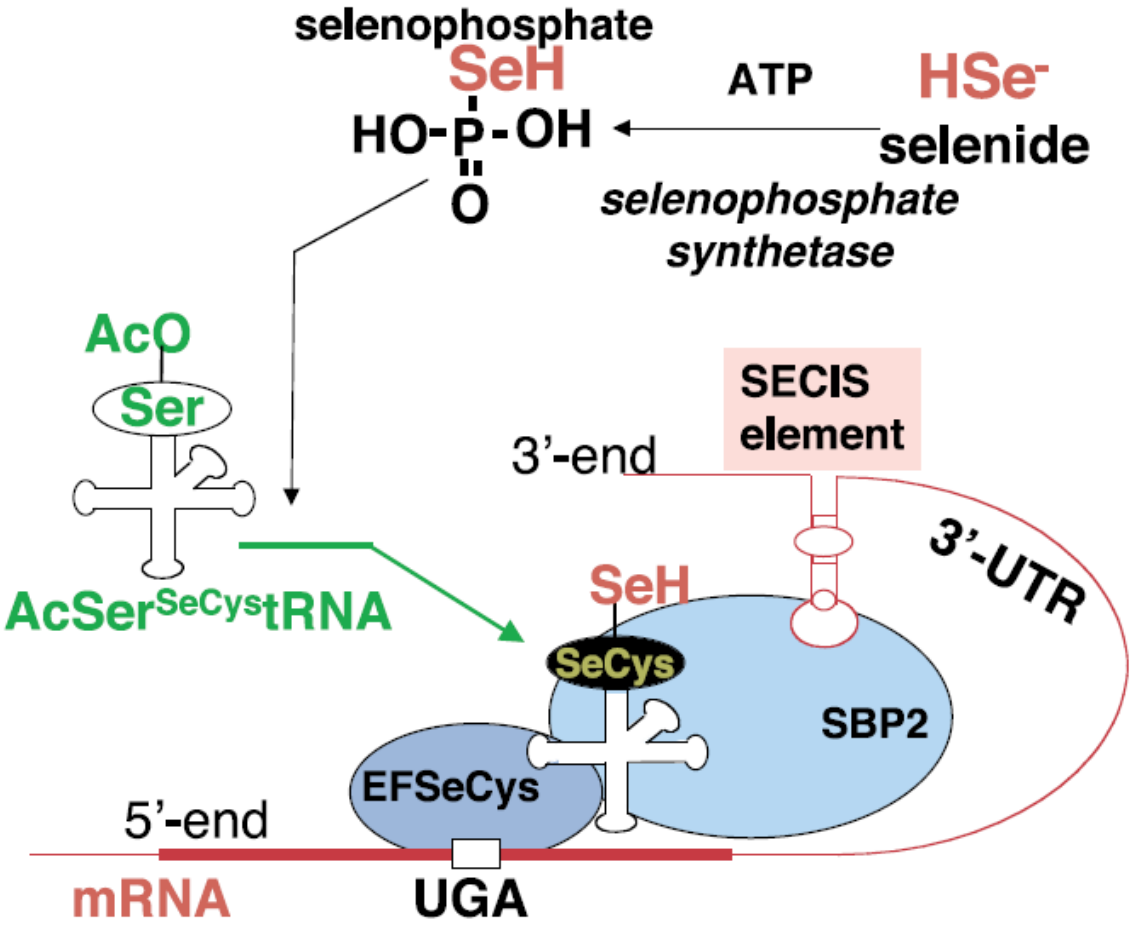
consideration, the MIX-induced increase in early concentrations of P4 could be explained, at least in-part, by increased expression of transcripts involved in histotroph secretion from the endometrium, which would enhance the growth and development of the conceptus, stimulating an earlier release of IFNT, and thus signaling MRP earlier after insemination. Collectively, these speculations could lead to increased conception rates in MIX-supplemented animals compared to ISe-supplemented counterparts. Increasing conception rates is economically relevant to beef and dairy operations alike and can have a tremendous impact on their profit potential.

Figure 2.1 Illustration of metabolic pathway for selenium.[1]



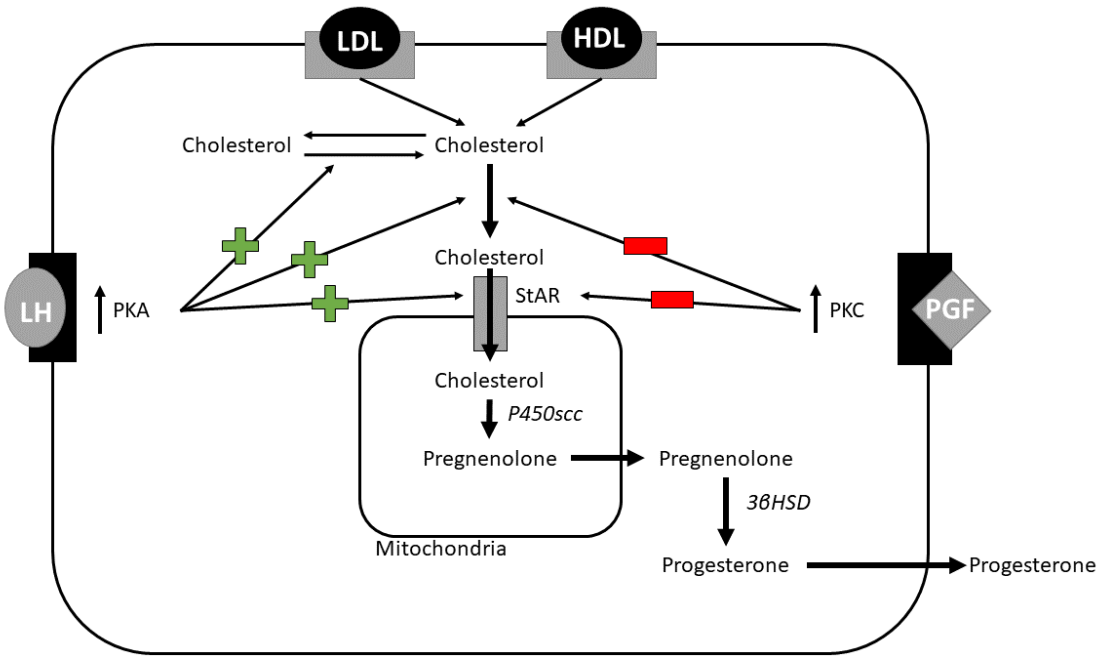
[1]Abbreviations: SeMet, selenomethionine; SeCys, selenocysteine.
Adapted from Suzuki (2005).

Figure 2.2 Illustration of translation mechanism for the synthesis of selenoprteins.[1]



[1] Two cis-sequences, a SECIS element in the 3'-untranslated region and a SeCys codon (UGA) in the coding region, and three trans-acting factors, a SeCys-specific translation elongation factor (EFSeCys), the SeCys ^{SeCys}tRNA, and a SECIS-binding protein (SBP2) are proposed for the translation of the UGA codon to the SeCys sequence. Adapted from Suzuki (2005).

Figure 2.3 Model showing the acutely regulated steps in the steroidogenic pathway in a generic luteal cell.[1]



[1]The increased activity of PKA activation indicated by green plus signs, while decreased activity from PKC activation indicated by red minus sign.

Figure 2.4 Schematic illustrating current working hypothesis of IFN tau action to regulate OTR gene expression during maternal recognition of pregnancy in sheep.[1]

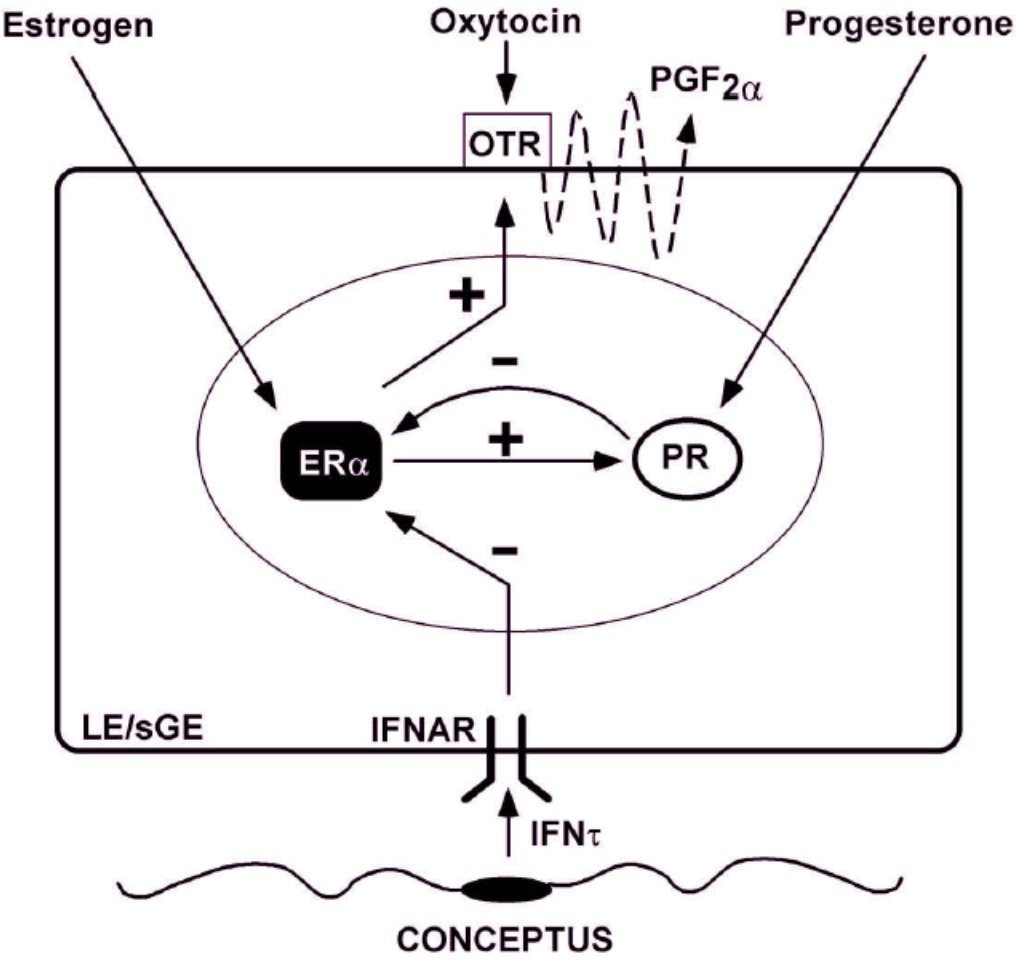
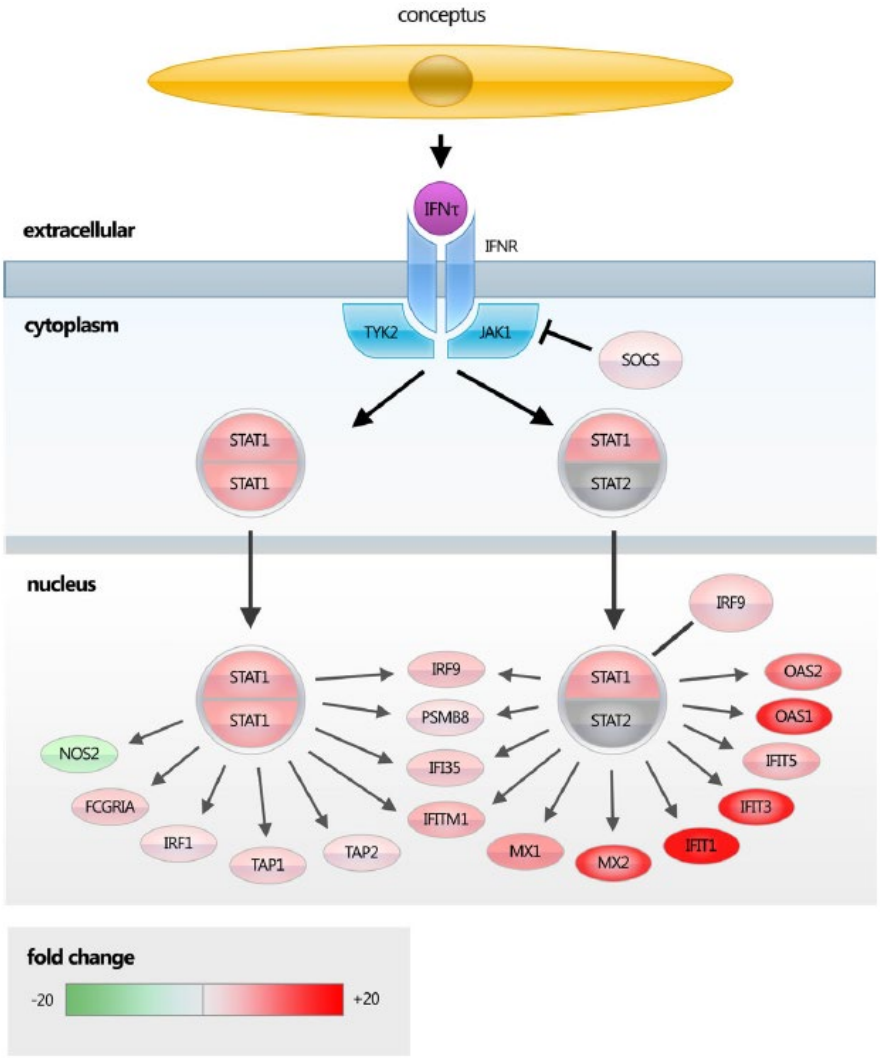


Figure 2.5 Genes differentially expressed between pregnant and cyclic dairy cows are shaded red (up-regulated in pregnant) and green (down-regulated in pregnant).[1]



[1]The bovine embryo produces IFNt which binds to type-1 interferon receptors, leading to the activation of the JAK-STAT (Janus kinase-signal transducer and activator of transcription) pathway and the synthesis of a range of interferon stimulated gene products. Adapted from (Walker et al., 2010).

CHAPTER 3. Statement of the Problem

In the United States, the distribution of Se content of grains and forages varies greatly in different geographical regions (Ammerman and Miller, 1975). The majority of producers in the southeast, including Kentucky, have forages and grains that are low (<0.05 ppm) to variable (~50% contain >0.1 ppm) in Se (Ammerman and Miller, 1975). The suggested dietary requirement of Se for beef cattle is at 0.1 ppm (National Academies of Sciences and Medicine, 2016). Cattle grazing Se-deficient forages necessitates the need to provide supplemental Se to these animals.

In cattle, deficiencies in Se have been demonstrated to decrease immunity (Erskine et al., 1989), growth (Gleed et al., 1983), and fertility (McClure et al., 1986). Supplementing dairy cows during the pre- and postpartum periods with a Se-enriched yeast increased postpartum plasma concentrations of progesterone (P4) compared to providing no supplemental Se (Kamada, 2017). Importantly, it has been reported that postpartum concentrations of P4 are positively correlated with conception rate (Inskeep, 2004).

Lower concentrations of P4 during the early luteal phase after artificial insemination have been associated with lower conception rates (Mann and Lamming, 1999). Moreover, low circulating concentrations of P4 in the first week after ovulation are associated with under-developed conceptuses (Forde and Lonergan, 2012), transcriptomic alterations (Barnwell et al., 2016), and a low likelihood of establishing pregnancy (Wiltbank et al., 2016). In contrast, increased concentrations of P4 immediately following conception has been associated with advanced conceptus

elongation (Carter et al., 2008) and increased IFNT production (Mann and Lamming, 2001). The pregnancy recognition signal, IFNT, occurs on days 16-17 in cattle (Spencer, 2013). The antiluteolytic effect of IFNT produced by the conceptus is the primary cause of the maintenance of the CL in sheep and cattle (Thatcher et al., 1995). IFNT acts in a paracrine manner on the endometrium to inhibit development of the endometrial luteolytic mechanism required for the pulsatile release of $PGF_{2\alpha}$, thereby ensuring continued production of P4 by the CL (Spencer et al., 2007)

In cattle, fertilization rates are high and between 90 and 100%, when semen of known high fertility is used in artificial insemination (Diskin et al., 2006; Diskin and Morris, 2008). However, it has been reported that only 50 to 60% of beef cows inseminated remain pregnant by day 30 (Bridges et al., 2013). It is estimated that 70-80% of the embryonic loss occurs between day 8 and 16 after insemination (Sreenan and Diskin, 1986).

Therefore, the overall goals of this dissertation were to investigate whether the form of supplemental Se (ISe and MIX) in vitamin-mineral mixes would affect the early cycle bovine CL and the uterine endometrium and conceptus at maternal recognition of pregnancy. More specifically, the objectives were 1) to investigate the effect of form of supplemental Se on the transcriptome of the bovine CL with the goal of elucidating form of Se-regulated luteal processes affecting fertility (Experiment 1, Chapter 4) and 2) to determine changes induced by the form of supplemental Se on the bovine endometrium and developing conceptus on day 17 of pregnancy (Experiment 2, Chapter 5).

CHAPTER 4. Form of dietary selenium affects mRNA encoding cholesterol biosynthesis and immune response elements in the early luteal phase bovine corpus luteum.

4.1. Abstract

Widespread regions of the southeast United States have soils, and hence forages, deficient in selenium (Se), necessitating Se supplementation to grazing cattle for optimal immune function, growth, and fertility. We have reported that supplementation with an isomolar 1:1 mix (MIX) of inorganic (ISe) and organic forms of Se increases early luteal phase (LP) progesterone (P4) above that in cows on ISe alone. Increased early LP P4 advances embryonic development. Our objective was to determine the effect of form of Se on the transcriptome of the early LP corpus luteum (CL) with the goal of elucidating form of Se-regulated processes affecting steroidogenesis and fertility. Non-lactating, three-year-old Angus-cross cows underwent 45-day Se-depletion then repletion periods, then at least 90 days of supplementation (TRT) with 35 ppm Se/day as either ISe (n=5) or MIX (n=5). CL were then recovered on Day 7 of the estrous cycle, total RNA isolated and the effect of TRT on the luteal transcriptome evaluated using bovine gene 1.0 ST arrays (Affymetrix, Inc., Santa Clara, CA). The level of expression of transcripts in each CL was subjected to one-way ANOVA using Partek Genomic Suite software to determine TRT effects. Microarray analysis indicated a total of 887 transcripts that were differentially expressed and functionally annotated, with 423 and 464 up- and down-regulated ($P<0.05$) in MIX vs. ISe CL, respectively. Bioinformatic analysis (Ingenuity Pathway Analysis) revealed the top TRT-affected canonical pathways to include seven

specific to cholesterol biosynthesis and two to inflammatory responses. Results from the microarray analysis were corroborated by targeted real-time PCR. MIX CL had increased (P<0.05) abundance of transcripts regulating cholesterol biosynthesis including *DHCR7*, *DHCR24*, and *CYP51A1* (fold changes of 1.65, 1.48, and 1.40, respectively), suggesting MIX-induced increases in P4 to be due, in part, to increased availability of substrate to luteal cells. In addition, MIX CL had increased (P<0.05) abundance of immune-response transcripts including *C1QC*, *FAS*, *ILR8B*, and *IL1R1* (fold changes of 2.30, 1.74, 1.66, and 1.63, respectively). *SREBF1* mRNA was also increased (1.32-fold, P<0.05) in the MIX CL, which increases cholesterol synthesis and stimulates IL1B, linking effects of form of supplemental Se (TRT) on cholesterol biosynthesis and immune function in the CL.

Keywords: Corpus luteum, progesterone, selenium

4.2. Introduction

In the United States, the distribution of selenium (Se) in soils, and hence grains and forages, varies across the country (Ammerman and Miller, 1975). Particularly in the southeast, including Kentucky, producers are faced with grazing forages deficient in this mineral (Ammerman and Miller, 1975). In turn, this leads to deficiencies in whole blood Se in animals grazing in these regions (Dargatz and Ross, 1996). In cattle, deficiencies in Se have been demonstrated to decrease immunity (Erskine et al., 1989), growth (Gleed et al., 1983), and fertility (McClure et al., 1986). Ultimately, this necessitates the inclusion of Se in a mineral supplementation strategy. Mineral supplements are commonly provided *ad libitum* in the form of loose minerals, pressed blocks, liquid supplements, and molasses-based tubs (Greene, 2000). Most often, Se is supplemented in the inorganic (ISe) dietary form of sodium selenite or sodium selenate (Podoll et al., 1992). However, research has demonstrated lower concentrations of Se in whole blood in cows supplemented with ISe compared to cows supplemented with organic (OSe**)** Se (Sel-Plex; Alltech, Inc., Nicholasville, KY, USA) (Patterson et al., 2013; Cerny et al., 2016b; Jia et al., 2018).

In dairy cattle, supplementing with Se has been demonstrated to reduce the incidence of metritis and ovarian cysts (Wilde, 2006) and to increase first service pregnancy rates (McClure et al., 1986). Additionally, supplementing with OSe increased second-service pregnancy rates in dairy cattle when compared to ISe (Thatcher et al., 2010). This increase in fertility could be attributed to the reduction in early embryonic death (Mehdi and Dufrasne, 2016). Supplementing dairy cows during the pre- and

postpartum periods with a Se-enriched yeast increased postpartum plasma concentrations of progesterone (P4) compared to providing no supplemental Se (Kamada, 2017). Importantly, it has been reported that postpartum concentrations of P4 are positively correlated with conception rate (Inskeep, 2004).

Similarly, our lab has previously reported increased early concentrations of P4 in cows supplemented with a 1:1 combination (MIX) of ISe (sodium selenite; Prince Agri Products, Inc. Quincy, IL) and OSe (SEL-PLEX; Alltech, Inc., Nicholasville, KY, USA) compared to cows supplemented with ISe or OSe on day 6 (Cerny et al., 2016b), or ISe on day 7 (Carr et al., 2020) of the estrous cycle. Lower concentrations of P4 during the early luteal phase after artificial insemination have been associated with lower conception rates (Mann and Lamming, 1999). It is known that P4 positively impacts the oocyte, endometrium, and embryo (Lonergan, 2011). Artificially increasing early luteal-phase concentrations of P4 has been demonstrated to increase development of the conceptus (Garrett et al., 1988). Furthermore, embryo quality on day 16 was greater in cows with an earlier increase in concentrations of P4 after ovulation (Mann and Lamming, 2001). Results from a meta-analysis indicated that providing exogenous P4 between days 3 to 7 post-insemination resulted in increased pregnancy rates (Yan et al., 2016).

The objective of this study was to investigate the effect of form of supplemental Se on the transcriptome of the bovine corpus luteum (CL) with the goal of elucidating form of Se-regulated luteal processes affecting steroidogenesis and fertility. We hypothesized that the form of Se supplemented to cows would alter the transcriptome of the bovine CL that favored increased concentrations of early luteal phase P4 as previously reported in our lab (Cerny et al., 2016b; Carr et al., 2020). Mineral

supplementation is a production practice that is easily implemented by producers and incorporating Se as MIX would be a simple transition with the potential to increase fertility in their herds.

4.3. Material and Methods

The experimental procedures in this project were approved by the Institutional Animal Care and Use Committee at the University of Kentucky, protocol number 2017-2828.

4.3.1. Animals and Experimental Procedure

Non-lactating, three-year-old Angus-cross cows (N=10) were randomly selected from pre-existing, Se form-specific cow herds as previously described (Patterson et al., 2013; Matthews et al., 2014; Cerny et al., 2016a; Cerny et al., 2016b). At the beginning of this experiment, animals received *ad libitum* access to a basal mineral mix with no Se for a 45-day Se-depletion period. This was followed by a 45-day period with *ad libitum* access to the mineral mix formulated to contain 35-ppm Se as ISe for 45 days to return systemic blood Se in all cows to adequate concentrations (National Academies of Sciences and Medicine, 2016). Following the period of Se repletion, cows were randomly assigned to have at least 90 days of individual access to a basal mineral mix containing 35-ppm Se as inorganic (n=5; ISe; sodium selenite; Prince Agri Products, Inc. Quincy, IL) or as 1:1 combination of ISe and OSe (n=5; MIX; SEL-PLEX; Alltech, Inc.,

Nicholasville, KY) as described by (Carr et al., *In Review*). Cows were individually fed their respective vitamin-mineral mixes using in-pasture Calan gates (Patterson et al., 2013).

To ensure cows maintained adequate status of total blood Se for the duration of the study, whole blood was collected from each cow at the start and endpoint of the depletion and repletion periods and bimonthly until the end of the experiment for the determination of total whole blood concentrations of Se (Patterson et al., 2013; Cerny et al., 2016a).

4.3.2. Diet

All cows grazed a common, novel, nontoxic endophyte-infected tall fescue (Lacefield MaxQ II) pasture, with the addition of a common corn silage diet during the winter months. Forage and silage samples were collected and analyzed (Dairy One Forage Testing Laboratory, Ithaca, NY) for Se and trace mineral content. Concentration of Se in the silage was 0.03 mg/kg as fed and 0.08 mg/kg on a dry matter basis and in the forage was 0.01 mg/kg as fed and 0.04 mg/kg on a dry matter basis. These values are consistent with being classified as Se deficient for cattle (National Academies of Sciences and Medicine, 2016).

4.3.3. Experimental Regimen and Tissue Collection

Cows were supplemented with their respective mineral treatment for at least 90 days before being randomly injected with dinoprost tromethamine (25 mg, Lutalyse, Zoetis, Parisppany, NJ) to induce regression of the CL and then monitored for behavioral estrus (Day 0) using visual appraisal and electronic cow monitoring technology (CowManager, Gerverscop 9, The Netherlands). Presence of a preovulatory follicle and subsequent ovulation were confirmed via transrectal ultrasonography using a 5-8 MHz linear transducer (Ibex Pro, E.I. Medical Imaging, Loveland, CO). On days 5, 6, and 7, the diameter of the CL was determined by transrectal ultrasonography and approximately 8 ml of blood was collected into additive-free tubes (Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ) by jugular venipuncture for retrieval and quantification of serum P4 via radioimmunoassay. On day 7, CL were removed by supra-vaginal lutectomy and immediately placed in ice-cold culture media (24 mM HEPES-buffered Ham's F-12 (1x) culture medium plus L-glutamine and sodium bicarbonate containing 0.5% bovine serum albumin and 20 µg/mL gentamicin), weighed, and then cut into two halves. One half was transported to the laboratory on ice in culture media for dissociation and culture of the fully differentiated luteal cells as reported in (Carr et al., *In Review*). The second half was divided into eight pieces and snap frozen in liquid nitrogen to be used for RNA extraction and the determination of transcript expression after hybridization to microarray chips and by real-time polymerase chain reaction (qPCR).

4.3.4. RNA Extraction

Total RNA was extracted from 400-600 mg of frozen luteal tissue using TRIzol Reagent (Invitrogen Corporation, Carlsbad, CA) following the manufacturer's instructions. The purity and concentration of total RNA samples were analyzed using a NanoDrop ND-100 Spectophotometer (NanoDrop Technologies, Wilmington, DE, USA). All samples had high purity, with 260/280 absorbance ratios of 1.97 or greater. The integrity of total RNA was examined by gel electrophoresis using an Aligent 2100 Bioanalyzer System (Agilent Technologies, Santa Clara, CA) at the University of Kentucky Microarray Core Facility. Visualization of gel images and electropherograms verified that all RNA samples were of high integrity with 28S/18S rRNA absorbance ratios ranging from 1.9-2.3.

4.3.5. Microarray Analysis

The effect of form of Se on the transcriptome of the bovine CL was evaluated using Bovine gene 1.0 ST Arrays (GeneChip; Affymetrix, Inc., Santa Clara, CA, USA) and conducted according to the manufacturer's standard protocol at the University of Kentucky Microarray Core Facility as described (Bridges et al., 2011; Bridges et al., 2012; Li et al., 2019). Briefly, RNA (3 μg/sample) was reverse-transcribed to double-stranded cDNA and then to single stranded complementary RNA (cRNA). The cRNA was labeled with biotin and then further fragmented to be used as probes to hybridize the gene chips in the GeneChip Hybridization Oven 640 (Affymetrix), using 1 chip per RNA sample. Following hybridization, the chips were washed and stained on a GeneChip Fluidics Station 450 (Affymetrix.) The reaction image and signals were read with a

GeneChip Scanner (GCS 3000, 7G; Affymetrix) and the data collected using the GeneChip Operating Software (GCOS, version 1.2; Affymetrix).

For each sample, the raw expression intensity values from the GCOS (i.e., 10 *.cel files from the raw methylation measurements) were imported into Partek Genomics Suite software (PGS, version 6.6; Partek Inc., St. Louis, MO). Robust Multichip Averaging algorithm adjusted with probe length and GC Oligo contents was implemented for GeneChip background correction (Irizarry et al., 2003). The background corrected data were further converted into expression values using quantile normalization across all chips and median polish summarization of multiple probes for each probe set.

All the GeneChip transcripts were annotated using the NetAffx annotation database for Gene Expression on Bovine GeneChip Array ST 1.0, provided by the manufacturer (http://affymetrix.com/analysis/index.affx, last accessed in August 2019, annotation file last updated in July 2016). Quality of data was assessed using light intensity expression values on a per chip and per gene basis and visualized as box plots. Pearson (linear) Correlation generated the similarity matrix (last accessed in August 2019, Partek Genomics Suite 6.6 6.15.0422). The average correlation between any of the 10 GeneChips was 0.98 (Fig. 4.1) and all GeneChips were further analyzed. Principal component analysis (Fig. 4.2) was performed to elucidate the quality of the microarray hybridization and visualize the general data variation among the chips.

To assess treatment effects on the relative expression of the CL gene transcripts, qualified microarray data were subjected to one-way ANOVA using the same PGS software. Gene chip transcripts were annotated using the NetAffx annotation database for Gene Expression on Bovine GeneChip Array ST 1.0, provided by the manufacturer

(http://affymetrix.com/analysis/index.affx, last accessed in August 2019, annotation file last updated in July 2016). A total of 887 transcripts showed treatment effects at the significance level of $P<0.05$ with false discovery rate (FDR) of <56%. The high FDR in this experiment appears to be due to the relatively small sample size and/or the increased variation that was observed by principal component analysis (described in Section 4.4.3) in the CL retrieved from MIX versus ISe supplemented cows. However, corroborating microarray data with a high FDR by qPCR on multiple gene transcripts allows confirmation of the observed changes (Rockett and Hellmann, 2004).

These differentially expressed genes/gene transcripts (DEGs) were subjected to hierarchical clustering analysis using PGS software and to canonical, functional, and network pathway analyses using QIAGEN'S Ingenuity Pathway Analysis (IPA, QIAGEN, Redwood City, CA, USA, www.qiagen.com/ingenuity). The raw data (10 *.cel files) collected by GCOS plus the GC Robust Multichip Averaging corrected data processed by PGS software of this manuscript have been deposited into the Gene Expression Omnibus (National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo/) as accession number GSE190092.

4.3.6. Real-time PCR Analysis

The relative quantification of mRNA for genes of interest was performed using qPCR using standard procedures in our laboratory, as described previously (Cerny et al., 2016a). Briefly, 1 µg of each cow's CL RNA was reverse transcribed to cDNA using the SuperScript™ IV VILO™ Master Mix with ezDNAse™ Enzyme (Invitrogen by Thermo

Fisher Scientific, Vilnius, Lithuania). Additionally, a no reverse-transcription control sample was transcribed and analyzed via qPCR to ensure that products from the targeted transcripts were not obtained from genomic DNA.

The relative abundance of mRNA encoding cholesterol-associated transcripts: *ACAT1, CYP51A1, DHCR7, DHCR24, FGF2, LSS, NSHDL, POR, SCD, SF1, SREBF, SQLE and TM7SF2* and the immune related transcripts: *C1QC, FAS, FDFT1, HMGCS1, IL18, IL1R1, ILR8A, ILR8B, IFNG, ITGAM, TNFα, VEGFA* and *VEGFC* were quantified. Primer sequences used and GenBank accession numbers for the cholesterol- and immune-related transcripts are listed in Table 4.1 and 4.2, respectively. The qPCR procedures were performed using the Bio-Rad CFX Maestro™ thermal cycler (Bio-Rad, Hercules, CA, USA) with iTaq Universal SYBR® Green Supermix (Bio-RAD, Hercules, CA, USA). A total volume of 25 µl was used in each qPCR reaction containing 5 µl of cDNA, 1 µl of a 10 µM stock of each primer (forward and reverse), 12.5 µl of 2 x SYBR Green PCR Master Mix, and 5.5 µl of nuclease-free water. The relative amount of each transcript was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Primer sets for genes of interest were designed and obtained from NCBI Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primerblast/) against RefSeq.

All cDNA products were validated via DNA sequencing for verification of target at Eurofins MWG Operon LLC (Louisville, KY, USA), as previously described (Cerny et al., 2016a; Jia et al., 2018). The resulting sequences were then compared to the NCBI RefSeq mRNA sequences used for primer templates. The primer pair design, amplicon length of product, and product identity for each targeted transcript are shown in Tables 4.1 and 4.2. Three constitutively expressed genes (*β-ACTIN*, *HPRT1* and *SDHA*) with CT

values not affected (P > 0.05) by Se-form treatment were used to normalize the relative mRNA expression to the geometric mean of the three. For qPCR analysis, n = 5 for ISe and MIX treatments. All reactions were performed in triplicate.

4.3.7. Se and P4 Analysis

As indicated in Carr et al. (Unpublished), total blood Se was determined by the University of Kentucky's Veterinary Diagnostics Laboratory (Lexington, KY) and Se was quantified using an Agilent 7900 inductively coupled plasma-mass spectrometer, as described previously (Wahlen et al., 2005).

Concentrations of P4 were quantified in samples of serum by a commercially available competitive RIA without extraction (*ImmuChem*™ Coated Tube Progesterone 125-I RIA Kit, MP Biomedicals, Costa Mesa, CA) according to manufacturer's instructions. One assay performed for analysis of the serum with an intra assay CV of 4.99%.

4.3.8. Statistical Analysis

Data are presented as least square means (±SEM) with individual cow as the experimental unit. Data were analyzed for normal distribution and homogeneity. When appropriate, data were transformed for normality by natural log transformation. Microarray hybridization data (relative expression of CL gene transcripts) were subjected to one-way ANOVA using the PGS software as described above.

To determine the effect of form of Se on concentrations of systemic Se and P4, data were analyzed using the PROC MIXED procedure of SAS statistical software package (version 9.4; SAS Institute, Inc. Cary, NC) as an ANOVA with repeated measures. The form of dietary Se was considered the fixed effect for both and the P4 data were natural log transformed due to being not normally distributed.

Additionally, the effect of form of Se supplementation on the relative abundance of CL mRNA transcripts were analyzed as an ANOVA using the PROC GLM procedure of SAS statistical software package (version 9.4; SAS Institute, Inc. Cary, NC). For all analyses, n=5 for ISe and MIX, and significance was declared at $P < 0.05$ and a tendency to differ was declared when $0.05 \leq P \leq 0.10$.

4.4. Results

4.4.1. Concentrations of Whole Blood Se

Cows were maintained on form of Se-specific treatments that provided adequate concentrations of whole blood Se (Gerloff, 1992; Dargatz and Ross, 1996) for the duration of this experiment (Fig. 4.3, adapted from (Carr et al., *In Review*)). Although the concentration of total blood Se remained numerically higher in the ISe- than MIX-treated cows, differences were not significant (*P>0.05*) at any time point during the experiment (Carr et al., *In Review*).

4.4.2. Progesterone

Serum collected from cows on days 5 through 7 of the estrous cycle was used to determine concentrations of P4. Previous studies from our lab indicate an increase of ~1 ng/mL at these same time points (Cerny et al., 2016b; Carr et al., 2020). In the current study, concentrations of P4 and CL diameter and weight were not affected (P>0.05) by form of Se-treatment (Table 4.3, adapted from Carr et al. (*In Review*). However, the difference of ~1 ng/mL and no differences in CL size are consistent with our previous studies (Cerny et al., 2016b).

4.4.3. Differentially Expressed Genes

Principle component analysis (PCA) of all microarray data was performed to evaluate the correlation and variation among gene chips. This allows for visualization of patterns through the distribution of samples to highlight similarities and differences (Partek, 2009). The score plot (Fig. 4.2) showed the first principal component (PC #1, x-axis) explained 16.3% of the total variance, whereas PC #2 (y-axis) and PC #3 (z-axis) explained 13% and 11.6% of the variance, respectively. Total variance (40.9%) is the cumulative percent of variance accounted for in our datasets based upon eigenvector multivariate analysis. Overall, the score plot indicated that the chips in the ISe treatment group were closely clustered together, whereas the chips in the MIX treatment group displayed greater variation among each cow.

Individual ANOVA were conducted to identify altered expression of mRNA transcripts between the CL of MIX and ISe supplemented cows. With $P < 0.05$ a total of

887 gene transcripts were identified as functionally annotated, with 423 and 464 transcripts up- and down-regulated, respectively. Hierarchical clustering analysis of the 887 DEGs indicated a complete separation among Se treatment groups (Fig. 4.4). The 10 most highly up- and down-regulated genes in the CL of MIX versus ISe treated cows identified are provided in Table 4.4.

4.4.4. Pathways and Gene Network Analysis

To determine the physiological significance of Se treatment induced DEGs bioinformatics analysis of canonical, functional, and network pathway analysis were performed using QIAGEN'S Ingenuity Pathway Analysis (IPA, QIAGEN, Redwood City, CA, www.qiagen.com/ingenuity). Canonical pathway analysis indicated that of the top 10 pathways affected, seven were specific to cholesterol biosynthesis and 2 involved in inflammatory responses (Table 4.5). Additionally, overlaying of canonical pathways revealed cross talk among cholesterol biosynthesis and inflammatory response pathways (Fig. 4.5).

4.4.5. Real-time PCR Analysis of Selected mRNA Transcripts

Real-time PCR analysis was used to corroborate the microarray analysis-identified altered expression of transcripts involved in cholesterol biosynthesis and immune response elements in MIX versus ISe-treated cows (Table 4.6 and 4.7, respectively). The results of these two analyses were consistent in terms of increased

expression and statistical significance (P<0.05) in MIX versus ISe treated cows for the following transcripts in cholesterol biosynthesis: *CYP51A1, DHRC24, DHRC7* and *SREBF1* along with transcripts related to immune response: *C1QC, FAS, IL1R1* and *ITGAM.* The cholesterol-related transcripts *ACAT1, FDFT1, FGF2, HMGC1, LSS, NSDHL, POR, SF1, SQLE* and *TM7SF2* as well as the immune-related transcripts *IL18, TNFα, VEGFA* and *VEGFC* were not detected to be different (*P>0.05*) by qPCR. Although the microarray analysis did not identify them as DEGs, the expression of *ILR8A, ILR8B* and *IFNG* was assessed by qPCR. Real-time PCR indicated that *ILR8A* did not differ (*P=0.19*), however *ILR8B* and *IFNG* was increased (*P<0.05*) in MIX versus ISe treated cows.

4.5. Discussion

The aim of this study was to investigate the effect of form of supplemental Se on the transcriptome of the bovine CL. Moreover, we sought to determine form of Se-regulated luteal processes affecting fertility that could, in-part, explain the increased early luteal phase concentrations of P4 previously reported by our lab (Cerny et al., 2016b; Carr et al., 2020). In the present study, MIX supplemented cows had numerically greater concentrations of P4 compared to ISe treated cows. Although not statistically significant, the difference of 1.1 ng/mL is similar to the 1.7 ng/mL and 1.0 ng/mL MIX-induced differences in concentrations of P4 reported by our lab in previous studies that used a larger number of animals (Cerny et al., 2016b; Carr et al., 2020). The inability to detect statistical significance of a MIX-induced increased concentration of P4 is likely limited

by the number of animals used in the present study; however, cows in the present study were also managed on novel, non-toxic endophyte-infected tall fescue pastures and received a common corn silage diet during the winter months. Cows used in previous studies from our lab were grazing toxic endophyte-infected tall fescue pastures (Cerny et al., 2016b; Carr et al., 2020). It may be that there is a relationship between endophyte toxicity and form of supplementary Se that accounts for the differences between these studies. Interestingly, research has also demonstrated that peripheral concentrations of P4 are decreased by an increased plane of feed intake due to increased metabolic clearance rate of the steroids (Sangsritavong et al., 2002).

Previous research has indicated increased concentrations of P4 at different times of the estrous cycle and throughout gestation when exogenous Se is supplemented to the diet of dairy cows and heifers (Kamada and Hodate, 1998; Kamada et al., 2014; Kamada, 2017). Supplementing with inorganic Se, specifically sodium selenite, increased plasma concentrations of P4 during the estrous cycle in non-lactating dairy cows compared to cows lacking supplemental Se (Kamada and Hodate, 1998). Similarly, providing dairy cows with supplemental Se-yeast increased early luteal phase concentrations of P4 when estrus occurred between 60 and 80 days postpartum (Kamada, 2017). Additionally, supplementation with sodium selenite increased concentrations of P4 during the last 10 weeks of gestation in dairy heifers compared to cows receiving a basal diet without supplemental Se (Kamada et al., 2014). However, it is important to note that these studies examined the effects of supplemental Se compared to control diets versus examining a Se-form effect on concentrations of P4.

Concentrations of P4 during early pregnancy have a large effect on the outcome of insemination success (Mann and Lamming, 1999). As reviewed by Mann and Lamming (1999), delayed increases in the postovulatory rise of P4 and low early luteal phase concentrations of P4 lead to impaired embryo development and ultimately reduced pregnancy rates. Increased concentrations of P4 during the first five days of pregnancy stimulate changes in uterine secretions that advance conceptus growth and development (Garrett et al., 1988). Further, there appears to be an optimal range of concentrations of P4 on day 7 of gestation that is conducive to pregnancy (Barnwell et al., 2015). Pregnancy rates were higher when serum concentrations of P4 were between 2-5 ng/mL at the time of embryo transfer on day 7 of the estrous cycle (Niemann et al., 1985).

The precursor for steroid synthesis is cholesterol (Grummer and Carroll, 1988; Rekawiecki et al., 2008). Cholesterol used for steroid synthesis in the ovary may come from *de novo* synthesis or through cellular uptake of lipoprotein cholesterol transported by low (LDL) or high (HDL) lipoproteins (Grummer and Carroll, 1988). Additionally, mobilization of cholesterol esters contributes to the supply of free cholesterol for steroid synthesis (Gwynne and Strauss III, 1982). However, cholesterol from circulating lipoproteins appears to provide the major source of substrate used for steroid synthesis (Pate and Condon, 1989). The proportion of cholesterol produced from *de novo* synthesis varies across species (Grummer and Carroll, 1988). When bovine granulosa cells were cultured with lipoproteins, *de novo* synthesis accounted for 25% to 36% of production of P4 (Savion et al., 1982).

Cholesterol biosynthesis from acetyl-CoA is accomplished in a process involving more than 30 different enzymes and numerous cofactors (Vance and Van den Bosch,

2000). An important metabolic pathway involved in cholesterol biosynthesis is the mevalonate pathway (Pool et al., 2018). The mevalonate pathway begins with the reversible condensation of two molecules of acetyl-CoA to acetoacetyl-CoA using the enzyme acetyl-CoA acetyltransferase (ACAT1; ACAT2) (Mazein et al., 2013). Hydroxymethylglutaryl-CoA synthase (HMGCS1) then forms 3-hyrdoxy-3-methylglutaryl-CoA (HMG-CoA) from acetoacetyl-CoA (Mazein et al., 2013). Next, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) catalyzes the conversion of HMG-CoA into mevalonic acid (Mazein et al., 2013). Importantly, HMGCR is the rate limiting enzyme in *de novo* synthesis of cholesterol (Grummer and Carroll, 1988).Sterol regulatory element binding protein (SREBP) also known as sterol regulatory element binding factor (SREBF), regulates the transcription of HMGCR and HMGCS (Brown and Goldstein, 1997). Insufficient levels of cholesterol cause the SREBP precursor to be cleaved, SREBP migrates to the nucleus, and promotes expression of the genes involved in cholesterol biosynthesis (Vance and Van den Bosch, 2000). After forming mevalonic acid, a series of phosphorylations and decarboxylations forms isopentenyl pyrophosphate (IPP), that is polymerized to form geranyl pyrophosphate (GPP) and farnesyl pyrophosphate (FPP), and FPP may condense to form squalene and subsequently be converted to the sterols (Rudney and Sexton, 1986). Squalene synthetase (FDFT1) is the first committed enzyme involved in sterol synthesis (Goldstein and Brown, 1990).

Results from the microarray analysis indicate a MIX-induced increase in the expression of key transcripts involved in cholesterol biosynthesis. Transcripts encoding the following proteins (CYP51A1, DHCR24, DHCR7, FDFT1, HMGCS1, HMGCR, LSS, NSDHL, SCD, SREBF1, and SQLE) were found to be upregulated in MIX

supplemented cows compared to those supplemented with ISe alone. As depicted in Fig. 4.6, the involvement of these key transcripts involved in cholesterol biosynthesis should contribute to increased early luteal phase concentrations of P4. Importantly, these findings occur in the presence of similar whole blood concentrations of Se in ISe and MIX supplemented cows.

Immune cells play an active role in controlling the lifespan and function of the CL (Penny et al., 1999). During the early luteal phase, production of P4 requires rapid growth of the CL, which is dependent on angiogenesis (Robinson et al., 2007). Angiogenic factors, including vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) along with their receptors, are strongly regulated during the development of the bovine CL (Neuvians et al., 2004). Additionally, a high concentration of interleukin-8 (IL8) is present in the bovine CL during the early luteal phase (Jiemtaweeboon et al., 2011), which effectively stimulates production of P4 in bovine luteinizing granulosa cells (Shimizu et al., 2012). Receptors for IL8 include CXCR1 and CXCR2 (Shirasuna and Miyamoto, 2017). Microarray analysis indicated increased mRNA expression of *FAS*, *VEGFA*, and *VEGFC*, while qPCR identified increased expression of *CXCR2* in MIX versus ISe supplemented cows.

On the contrary, production of P4 is inhibited by tumor necrosis factor α (TNFα), gamma-interferon (IFNG) and interleukin 1β (IL-1β) (Pate, 1995). These three cytokines are also potent stimulators of prostaglandin production (Pate, 1995). However, research indicates that mRNA encoding *TNFα* is present in the bovine CL before luteolysis (Pate, 1995). It has also been proposed that TNFα promotes formation of the CL by increasing proliferation and steroidogenesis of luteinizing granulosa cells (Yan et al., 1993).

Luteolytic effects of TNFα and IFNG on human and non-human primate luteal cells also involve Fas cell surface death receptor and Fas ligand (Pate et al., 2010). Interestingly, mRNA encoding *FAS*, *TNFα*, *IFNG*, and interleukin 1 receptor type 1 (receptor for IL-1β) were upregulated in MIX supplemented cows. Perhaps indicative of MIX supplemented cows being further along in their preparation for luteolysis. Similarly, lactating dairy cows supplemented with OSe had greater serum concentrations of IL-1 and slightly increased serum concentrations of TNFα compared to cows supplemented with ISe (Gong et al., 2014). In contrast, Se deficiency in broilers has shown to increase levels of IL-1β and IFNG (Liu et al., 2016).

Bioinformatic analysis revealed that the LXR/RXR Activation pathway connected pathways associated with cholesterol biosynthesis and immune response elements. Intriguingly, the LXR pathways maintains cholesterol homeostasis and regulates immune and inflammatory responses (Wang and Tontonoz, 2018). The LXR family consists of two isotopes, LXRα and LXRβ (Noelia and Castrillo, 2011). Mice lacking LXRα accumulate large amounts of cholesterol in the liver when fed a high cholesterol diet (Peet et al., 1998). Importantly, LXRs work with SREBP2 to maintain cellular and systemic sterol levels (Wang and Tontonoz, 2018). Low levels of cholesterol cause SREBP2 to drive the transcription of genes encoding proteins involved in cholesterol biosynthesis and uptake of LDL cholesterol (Hua et al., 1993). Additionally, LXRs inhibit inflammatory genes, such as COX-2 and IL-1β, after TNFα or IL-1β stimulation as reviewed by Noelia and Castrillo (2011). Research has demonstrated that IL-1β inhibits LH-stimulated steroidogenesis (Pate, 1995) and COX-2 is involved in the production of prostaglandins (Simon, 1999).

4.5.1. Conclusion

Evidence from this study supports our hypothesis that form of supplemental Se positively influences the expression of key transcripts that favor increased concentrations of early luteal phase P4. Cows supplemented with MIX had increased expression of several key transcripts involved in cholesterol biosynthesis and immune response elements compared to those supplemented with ISe alone. The results from the microarray analysis on the CL confirmed the top canonical pathways were those associated with cholesterol biosynthesis and inflammatory responses. Importantly, MIX-induced upregulation of cholesterol biosynthesis pathways and associated transcripts play a pivotal role in increasing the early luteal phase concentration of P4, which is a salient finding of this area of research.

Figure 4.1 Microarray array-array intensity correlation plot.

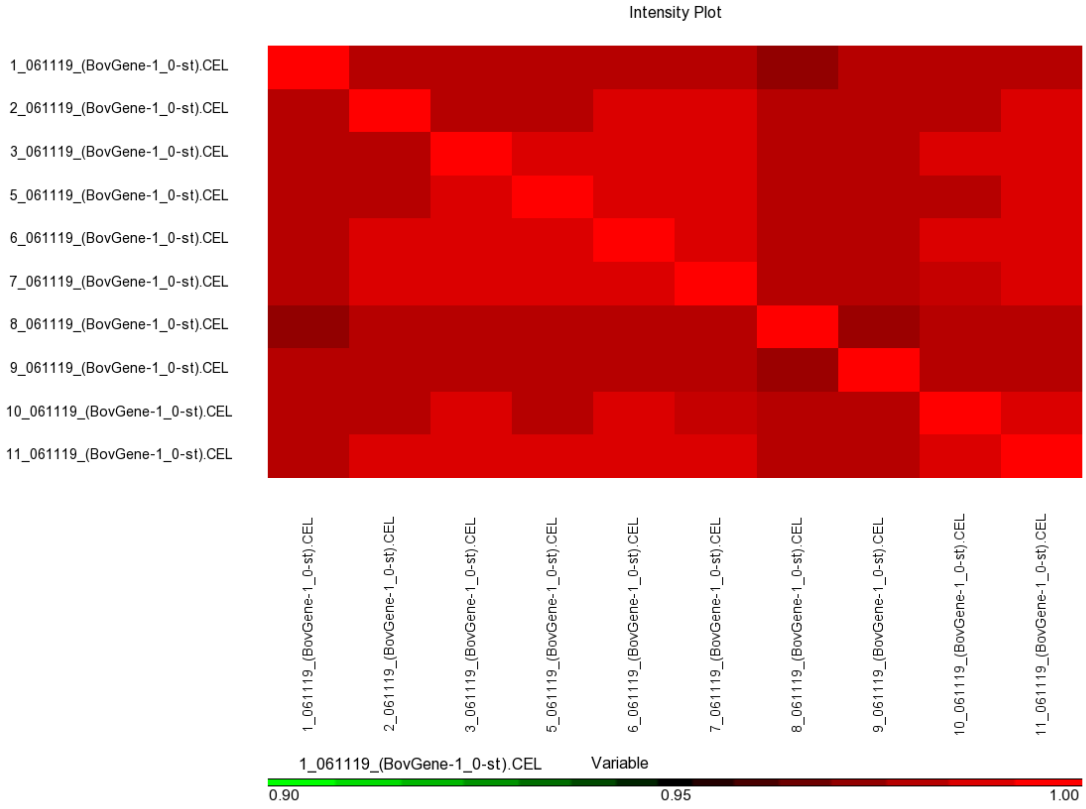


Figure 4.2 Score plot of principal component analysis of microarray transcriptome analysis of 10 CL samples from cows supplemented with 3 mg Se/d in vitamin-mineral mixes as sodium selenite (ISe, n=5, red sphere) or a 1:1 blend of ISe and OSe (MIX, n=5)

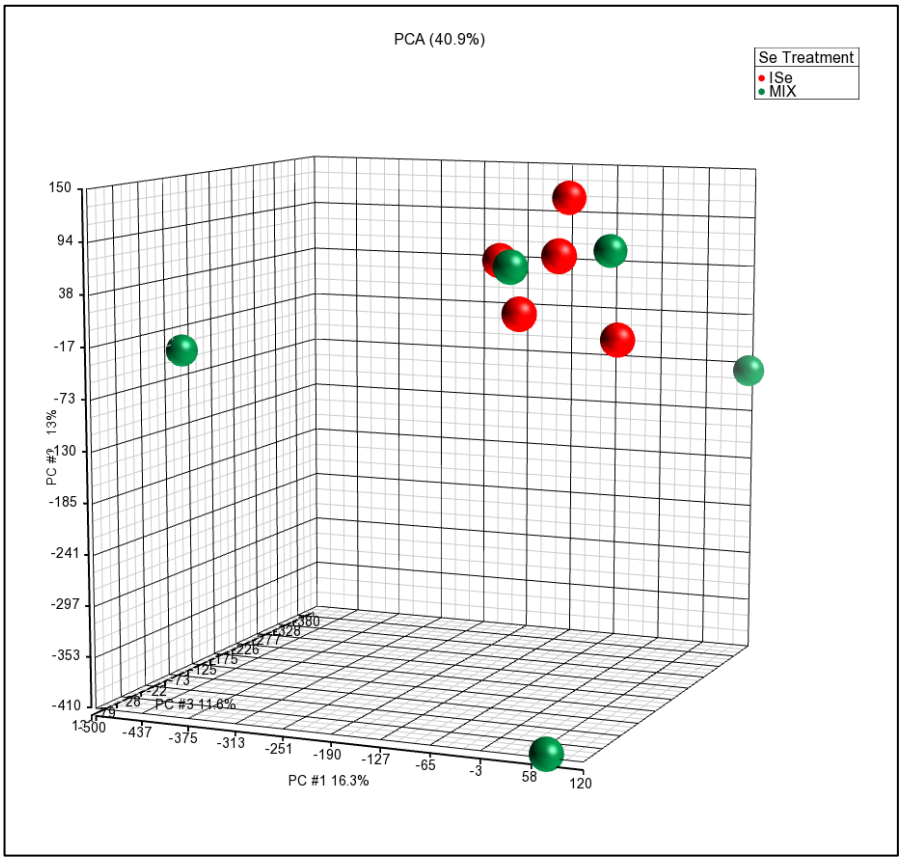
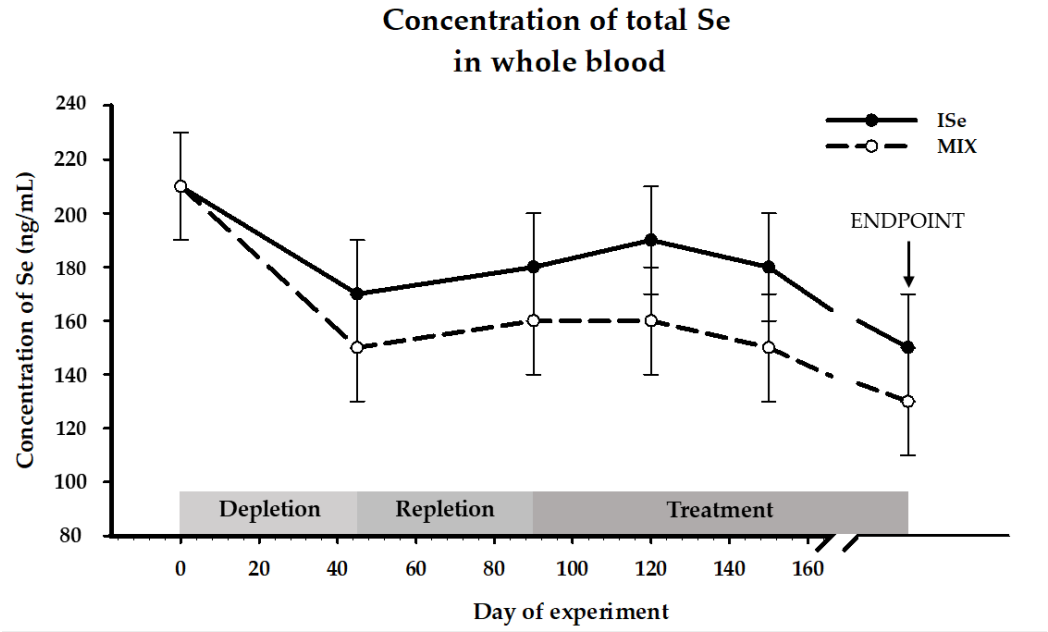


Figure 4.3 Effect of form of Se on whole blood concentrations (ppm; LS Mean ± SEM) of Se in cows supplemented with either ISe (Sodium selenite; n = 5) or a 1:1 combination (MIX) of ISe and OSe (Sel-Plex; n = 5).[1]



[1]Data were analyzed as an ANOVA with repeated measures. Whole blood Se was not affected by treatment ($P = 0.2393$) but was affected by time ($P < 0.0001$). Adapted from Carr et al. (Unpublished).

Figure 4.4 Hierarchical cluster analysis of the 887 genes identified as differentially expressed (ANOVA P-values of <0.05) by the CL of cows supplemented with 3 mg Se/d in vitamin-mineral mixes as sodium selenite (ISe, n=5) or a 1:1 blend of ISe and OSe (MIX, n=5).

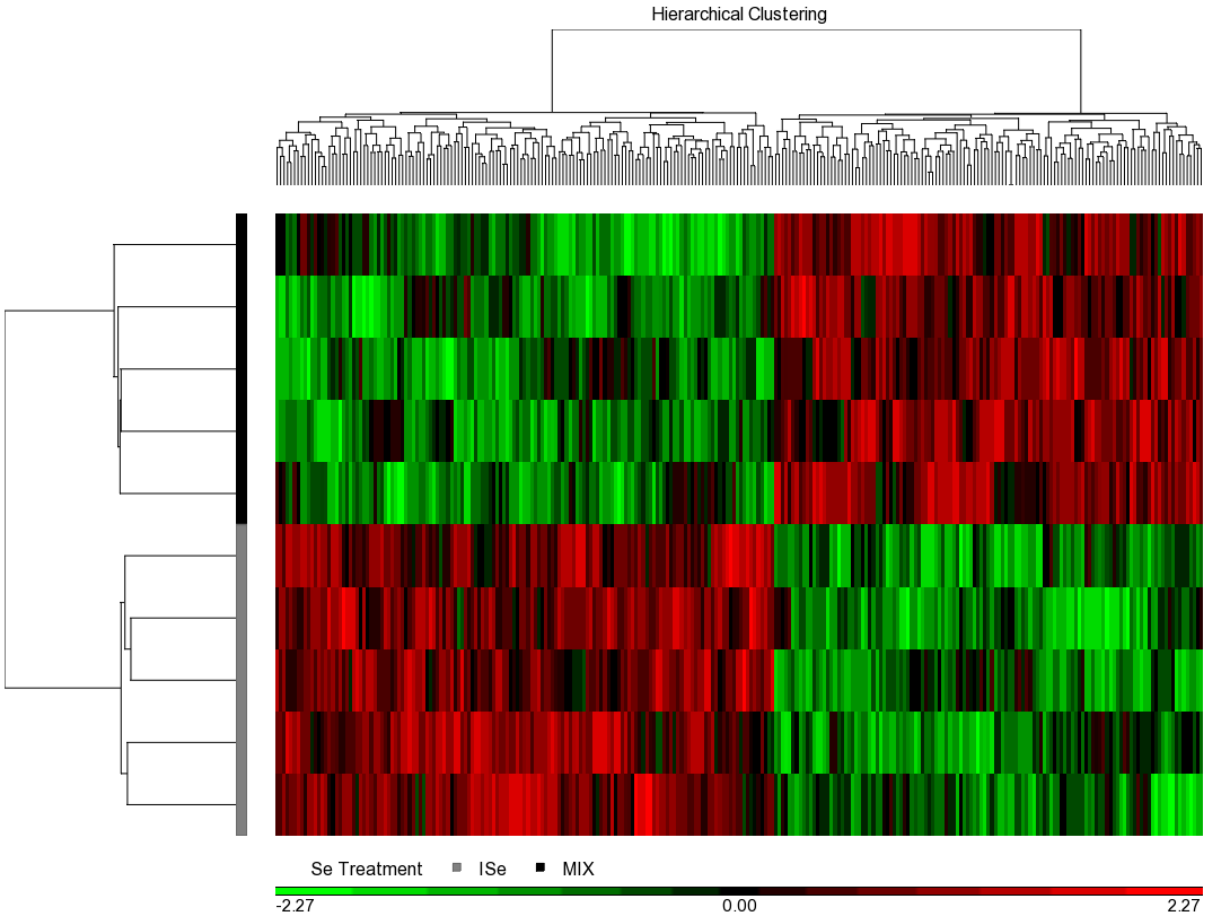


Figure 4.5 Overlapping canonical pathways associated with cholesterol biosynthesis and inflammatory responses from the bioinformatic analysis (Ingenuity Pathway Analysis).

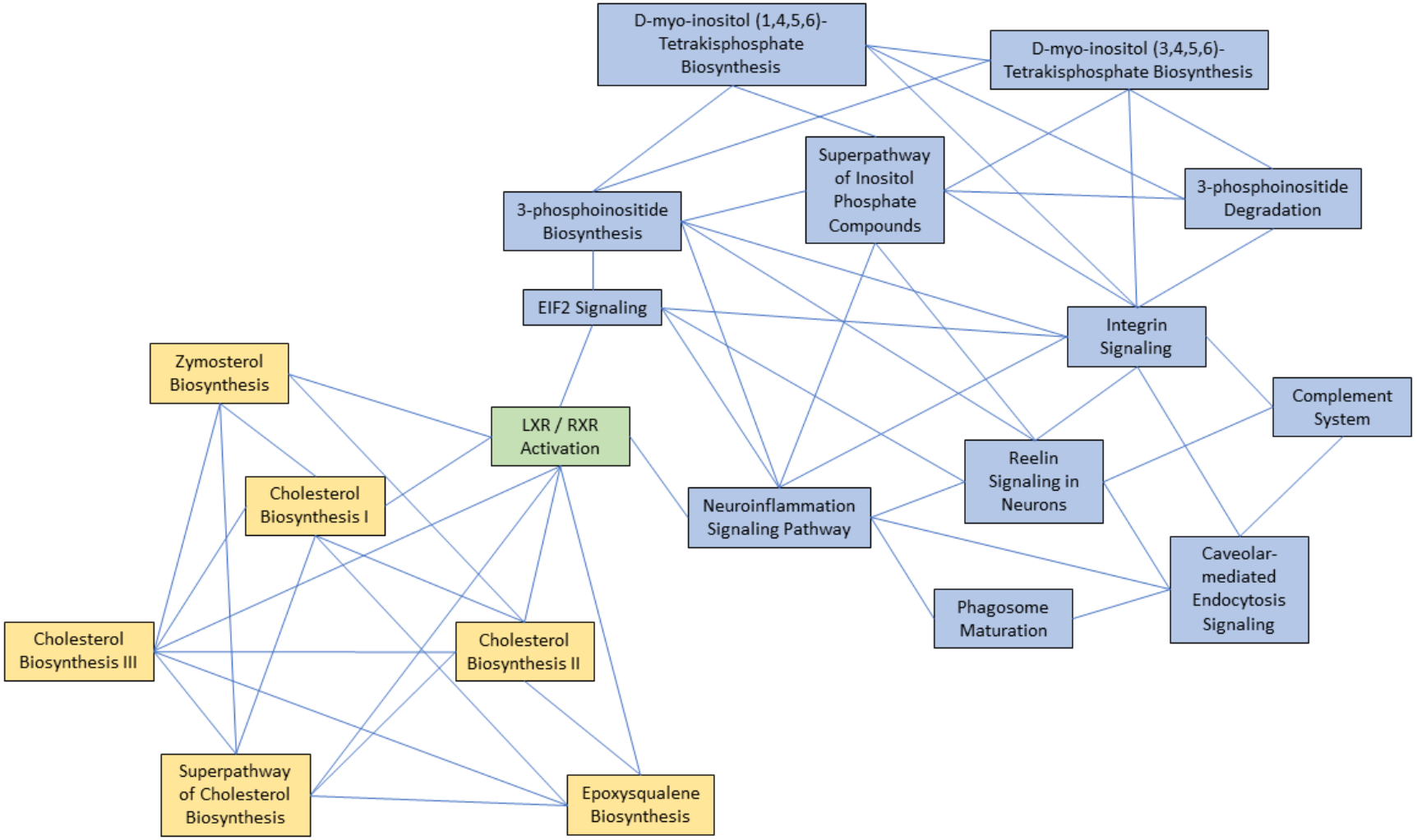


Figure 4.6 Mechanism of cholesterol availability and steroidogenesis with an emphasis on cholesterol biosynthesis. Green arrows indicate increased mRNA expression of transcripts identified from the microarray analysis.

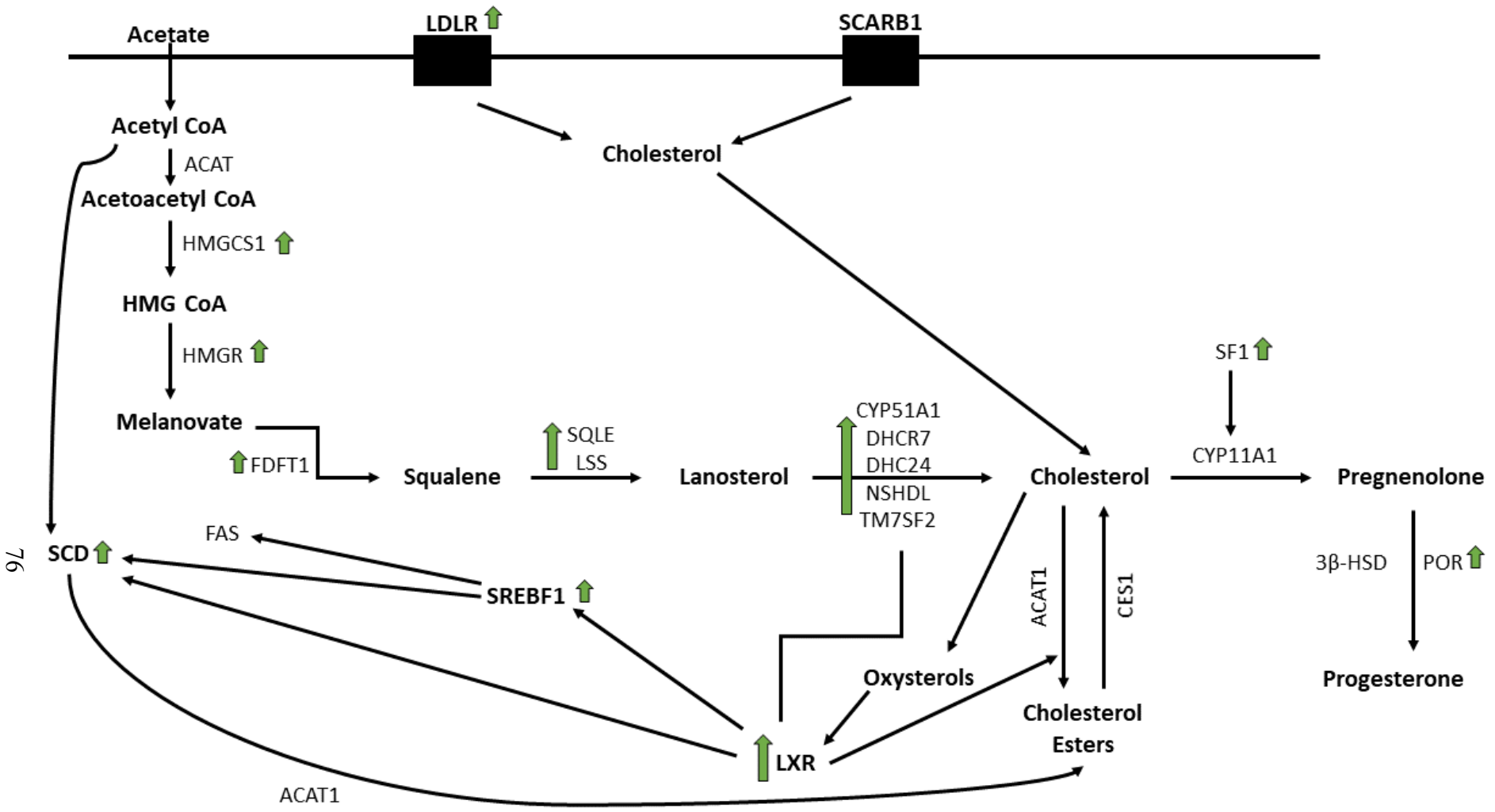


Table 4.1 Primer sets and product identities of qPCR analysis of cholesterol-associated genes.

Gene	Gene Name	Accession Number[1]	Oligonucleotide Primer Design (5' to 3') direction	Amplicon length (bp)	Product identity [2]
ACAT1	Acetyl-CoA acetyltransferase 1	NM_001046075.1	F: GAACAGAGGAGCAACACCA R: CCATCATTCAGTGTGCTGGC	379	99%
CYP51A1	cytochrome P450, family 51, subfamily A, polypeptide 1	NM_001025319.2	F: CTCGCATCCATGCTGCTCAT R: CTCCCCAGCAGGTAGGTAA	261	100%
DHCR7	7-dehydrocholesterol reductase	NM_001014927.1	F: AGAGGTTGGAGGGCCCAAT R: CGTGACCCATGCTGTGTAGA	332	100%
DHCR24	24-dehydrocholesterol reductase	NM_001103276.1	F: GGGCTGGAGTTCGTTCTCAT R: ACCCGCAGTGAAACAGTGA	249	99%
FGF2	Fibroblast growth factor 2	NM_174056.4	F: AAGCGGCTGTACTGCAAGAA R: ACACTCGTCTGTAACACATTTAGAA	216	100%
FDFT1	Farnesyl-diphosphate farnesyltransferase 1	NM_001013004.1	F: TGGAGTTCGTGAAGTGCTTGG R: ATACTGCATGGCGCATTTCC	207	100%
HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1	NM_001206578.1	F: ACCTCAGTGCATTAGACCGC R: ATCCCCAAAGGCTTCTAGGC	236	98%
LSS	Lanosterol synthase	NM_001046564.1	F: CCCAGTCCCAGATCCACAAC R: AGGGTGCAGACGGGAGAA	239	100%

Table 4.1 (continued)

NSDHL	NAD(P) Dependent Steroid Dehydrogenase-Like	NM_001035482.2	F: CGAATCCTGACAGGCCTCAA R: CTCCTCACGGTCTTGTCCAC	254	100%
POR	Cytochrome p450 oxidoreductase	NM_001035390.1	F: GCCCAGGACTTCTACGACTG R: TACTGGCGAATGCTGGACTC	290	99%
SCD	Stearoyl-CoA desaturase	NM_173959.4	F: TCCGACCTAAGAGCCGAGAA R: TGGGCAGCACTATTCACCAG	200	100%
SF1	Splicing factor 1	NM_001081614.1	F: CAGAGAGTCGGCCCTACCAT R: CTGATCCATTGGAGGAGGG	228	99%
SREBF1	Sterol regulatory element binding transcription factor 1	NM_001113302.1	F: ACATCTCTTGGAGCGAGCAC R: CACCACAGCTGTCAGAGAGG	229	99%
SQLE	Squalene epoxidase	NM_001098061.1	F: AGCTTCCTCCCTTCTTCACCA R: TGCAACACATTTTCCACCAAGT	339	99%
TM7SF2	Transmembrane 7 superfamily member 2	NM_174622.3	F: CTTTGGTACGAGGAGGCAGTC R: TCTCAAGGTCAGCCACTCTG	271	100%
Housekeeping Transcripts					
ACTB	Actin beta	NM_173979.3	F: AGCGGGAAATCGTCCGTGAC R: TGTTGGCGTAGAGGTCCTTGC	278	99%

Table 4.1 (continued)

HPRT1	Hypoxanthine phosphoribosyltransferase 1	NM_001034035.2	F: GCCAGCCGGCTACGTTAT R: ATCCAACAGGTCGGCAAAGA	256	100%
SDHA	Succinate dehydrogenase complex flavoprotein subunit A	NM_174178.2	F: GCAGAACCTGATGCTTTGTG R: CGTAGGAGAGCGTGTGCTT	185	99%

[1] These contents are associated with each gene symbol and are the accession numbers of the sequences retrieved from the NCBI RefSeq database for designing primers and probes.
[2] All qPCR products were validated by sequencing. The identity values (%) presented are the base pair ratios between the total amplicon length and the number of identical base pairs.

Table 4.2 Primer sets and product identities of qPCR analysis of immune-related genes.

Gene	Gene Name	Accession Number[1]	Oligonucleotide Primer Design (5' to 3') direction	Amplicon length (bp)	Product identity [2]
C1QC	Complement C1q C chain	NM_001206396.1	F: TGGCCCCTCCTGGTACTAAA R: TTCCCAGGATAGCCAGGTGT	236	100%
FAS	Fas cell surface death receptor	NM_174662.2	F: TTATGGGCCCTCCTGATCCT R: TCCTCCATACCGTTCTTCCG	230	100%
IFNG	Interferon gamma	NM_174086.1	F: TAGCTAAGGGTGGGCCTCTC R: TCTCCGGCCTCGAAAGAGAT	356	99%
IL1R1	Interleukin 1 receptor type 1	NM_001206735.1	F: GAGTTTGTGCAGCATGAGCC R: CACTGTGTGCTGTGTTCACG	241	100%
IL18	Interleukin 18	NM_174091.2	F: TGGCTGCAGAACAAGTAGAAGA R: TGGTTACGGCCAGACCTCTA	297	100%
IL8RA	Chemokine (C-X-C motif) receptor 1 (CXCR1)	NM_174360.3	F: TGGTTGGTGACTCAGTCTTTC R: CCCAGGAGGCTTAGCAAGAA	229	100%
IL8RB	Chemokine (C-X-C motif) receptor 2 (CXCR2)	NM_001101285.1	F: ACACTGACCTGCCCTCTATTC R: CCCAACCCTTTGCCTTGGA	261	100%

Table 4.2 (continued)

ITGAM	Integrin subunit alpha M	NM_001039957.1	F: CAGATCTCCCCCAAGCTTCA R: TGGAGGCAGACTTTCACCTG	245	99%
TNFα	Tumor necrosis factor	NM_173966.3	F: TGCCTTGGCTCAGATGTGTT R: GGTTACAGGCATGACTCCCC	205	100%
VEGFA	Vascular endothelial growth factor A	NM_174216.2	F: AAGAAAATCCCTGTGGGCCTT R: TCTGGTTCCCGAAACCCT	206	100%
VEGFC	Vascular endothelial growth factor C	NM_174488.2	F: CTCCTGCCGATGCATGTCTA R: GAAGCCTGAAGTCCCCCTTT	285	100%

[1] These contents are associated with each gene symbol and are the accession numbers of the sequences retrieved from the NCBI RefSeq database for designing primers and probes.
[2] All qPCR products were validated by sequencing. The identity values (%) presented are the base pair ratios between the total amplicon length and the number of identical base pairs.

Table 4.3 Effect of form of Se on concentrations of P4 in the peripheral plasma of cows during the early luteal phase of the estrous cycle.[1]

Variable	Treatment		P-value[2]
	ISe	MIX	
	LS Mean ±SEM	LS Mean ±SEM	
Progesterone (ng/mL)			
Year 1*			
(n=)	9	9	
Day 6†	3.44 ± 0.18	5.14 ± 0.60	0.035
CL diameter (mm)	18.2 ± 0.5	18.8 ± 0.6	
Year 2**			
(n=)	12	12	
Day 7†	2.92 ± 0.27	3.91 ± 0.16	0.006
Year 3			
(n=)	5	5	
Day 5	0.59 ± 0.58	1.20 ± 0.55	0.456
Day 6	0.86 ± 0.55	1.19 ± 0.55	0.678
Day 7	1.87 ± 0.55	2.92 ± 0.55	0.198
CL weight (g)	6.07 ± 0.82	6.77 ± 0.82	0.563
CL diameter (mm)	22.3 ± 1.09	23.2 ± 1.09	0.576

[1] Selenium was supplemented at 35 ppm as either inorganic (ISe; sodium selenite) or a 1:1 combination (MIX) of ISe and OSe (SEL-PLEX). Selenium was supplemented individually using in-pasture Calan gates(Patterson et al., 2013).
[2] P-values associated with one-way ANOVA (year1, OSe treatment not shown), one-way ANOVA with repeated measures (year 2 and 3), and t-test (CL weight and CL diameter).
† Means with different superscripts differ, $P < 0.05$.
*Reported in (Cerny et al., 2016b)
**Reported in (Carr et al., 2020)

Table 4.4 Top 10 most highly up- and down-regulated DEGs in the CL of MIX versus ISe treated cows.[1]

Gene Symbol	Gene Description	Ratio	Fold change	*P-value*[2]
LOC505037	similar to egf-like module containing, mucin-like, h	1.89	0.89	0.014
FGG	fibrinogen gamma chain	1.81	0.81	0.020
LPL	lipoprotein lipase	1.80	0.80	0.014
DRAM1	DNA-damage regulated autophagy modulator 1	1.69	0.69	0.002
SPP1	secreted phosphoprotein 1	1.63	0.63	0.013
ITIH5	inter-alpha (globulin) inhibitor H5	1.62	0.62	0.019
VSIG4	V-set and immunoglobulin domain containing	1.60	0.60	0.007
SLC5A6	solute carrier family 5 (sodium-dependent vitamin transporter	1.59	0.59	0.022
KLRD1	KLRD1 // natural killer cells antigen CD94-like	1.57	0.57	0.017
GABRB1	gamma-aminobutyric acid (GABA) A receptor, beta 1	1.56	0.56	0.019

Gene Symbol	Gene Description	Ratio	Fold change	*P-value*[2]
DFNA5	deafness, autosomal dominant 5	0.45	-0.55	0.020
LOC786728	similar to ribosomal protein L27a-like	0.46	-0.54	0.002
FAM127A	family with sequence similarity 127, member A	0.50	-0.50	0.044
KCNIP4	Kv channel interacting protein 4	0.51	-0.49	0.009
NEFH	neurofilament, heavy polypeptide	0.55	-0.45	0.018
VCAM1	vascular cell adhesion molecule 1	0.59	-0.41	0.041
PTN	pleiotrophin	0.62	-0.38	0.007
LOC785533	UL16 binding protein 1-like	0.62	-0.38	0.001
CLEC12B	C-type lectin domain family 12, member B	0.66	-0.34	0.045
ANKRD1	ankyrin repeat domain 1 (cardiac muscle)	0.66	-0.34	0.026

[1] Selenium was supplemented at 35 ppm as either inorganic (ISe; sodium selenite) or a 1:1 combination (MIX) of ISe and OSe (SEL-PLEX). Selenium was supplemented individually using in-pasture Calan gates (Patterson et al., 2013).

[2] For statistical analysis, one-way ANOVA using the Partek Genomic Suite program was used to determine significance of each transcript in each comparison.

Table 4.5 Top 10 IPA-identified canonical pathways of genes differentially expressed from CL of cows supplemented with 3 mg Se/d in vitamin-mineral mixes as sodium selenite (ISe, n=5) or a 1:1 belnd of ISe and OSe (MIX, n=5)..

Canonical Pathway	Gene Symbol(s)	Ratio[1]	-log(p-value)
Cholesterol Biosynthesis I	CYP51A1, DHCR24, DHCR7, FDFT1, LSS, NSDHL, SQLE, TM7SF2	0.615	8.85
Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)	CYP51A1, DHCR24, DHCR7, FDFT1, LSS, NSDHL, SQLE, TM7SF2	0.615	8.85
Cholesterol Biosynthesis III (via Desmosterol)	CYP51A1, DHCR24, DHCR7, FDFT1, LSS, NSDHL, SQLE, TM7SF2	0.615	8.85
Superpathway of Cholesterol Biosynthesis	CYP51A1, DHCR24, DHCR7, FDFT1, HMGCS1, LSS, NSDHL, SQLE, TM7SF2	0.321	6.79
Assembly of RNA Polymerase II Complex	GTF2E1, GTF2F1, GTF2H5, POLR2B, POLR2F, POLR2G, POLR2H, TAF1	0.160	3.69
LXR/RXR Activation	ACACA, CD14, CYP51A1, FASN, FDFT1, IL18, IL1R1, LPL, NCOR2, SAA1, SCD, SREBF1	0.099	3.2
Zymosterol Biosynthesis	CYP51A1, NSDHL, TM7SF2	0.500	3.19
Nucleotide Excision Repair Pathway	GTF2H5, POLR2B, POLR2F, POLR2G, POLR2H, RAD23B	0.171	3.06
Epoxysqualene Biosynthesis	FDFT1, SQLE	1.000	2.97
Caveolar-mediated Endocytosis Signaling	ARCN1, B2M, COPB1, COPE, ITGA6, ITGAM, ITGB2, ITGB3	0.110	2.58

[1]The ratio calculated as the number of differentially expressed genes (P<0.05) in a given pathway divided by the total number of genes that make up that pathway.

Table 4.6 Microarray and real-time RT-PCR identification of selected cholesterol-related genes from CL of cows supplemented with 3 mg Se/d in vitamin-mineral mixes as sodium selenite (ISe, n=5) or a 1:1 blend (MIX, n=5) of ISe and OSe (SEL-PLEX).[1]

Gene	Gene Name	Microarray[2]			RT-PCR[2]			
		Ise	MIX	P-value	ISe	Mix	SEM	P-value
ACAT1	Acetyl-CoA acetyltransferase 1	1.0	0.93	0.34	1.03	0.96	0.09	0.5891
CYP51A1	Cytochrome P450, family 51, subfamily A, member 1	1.00[a]	1.19[b]	0.0159	1.008[a]	1.416[b]	0.064	0.002
DHCR24	24-dehydrocholesterol reductase	1.00[a]	1.12[b]	0.0039	1.017[a]	1.507[b]	0.122	0.0217
DHCR7*	7-dehydrocholesterol reductase	1.00[a]	1.09[b]	0.0103	1.011[a]	1.670[b]	0.206	0.0457
FDFT1	Farnesyl-diphosphate farnesyltransferase 1	1.00[a]	1.21[b]	0.0419	1.040	1.180	0.112	0.4028
FGF2	Fibroblast growth factor 2	.	.	.	1.39	1.66	0.56	0.6396
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase	1.00[x]	1.22[y]	0.0538	.	.	.	.
HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1	1.00[a]	1.10[b]	0.0207	1.030	1.061	0.164	0.8962
LSS	Lanosterol synthase	1.00[a]	1.13[b]	0.0011	1.020	1.250	0.110	0.1771
NSDHL	NAD(P) dependent steroid dehydrogenase-like	1.00[a]	1.08[b]	0.0083	1.007	1.024	0.052	0.8238
POR	P450 (cytochrome) oxidoreductase	1.00[a]	1.10[b]	0.009	1.06	1.36	0.16	0.2092
SCD	Stearoyl-CoA desaturase (delta-9-desaturase)	1.00[a]	1.07[b]	0.009	1.02	0.89	0.11	0.4347

Table 4.6 (continued)

SF1	Splicing factor 1	1.00	1.02	0.404	1.05	0.83	0.11	0.2142
SREBF1	Sterol regulatory element-1	1.00[a]	1.10[b]	0.0124	1.023[a]	1.346[b]	0.088	0.0314
SQLE	Squalene epoxidase	1.00[a]	1.14[b]	0.0236	1.036	1.310	0.244	0.3747
TM7SF2	Transmembrane 7, superfamily, member 2	1.00[a]	1.34[b]	0.0232	1.053	1.129	0.220	0.8139

[1] Selenium was supplemented at 35 ppm as either inorganic (ISe; sodium selenite) or a 1:1 combination (MIX) of ISe and OSe (SEL-PLEX). Selenium was supplemented individually using in-pasture Calan gates(Patterson et al., 2013).
[2] Data are expressed as a ratio of MIX relative to ISe expression.
[a,b] Means within a row that lack a common superscript differ ($P<0.05$)
[x,y] Means within a row that lack a common superscript tend to differ ($0.05 < P < 0.10$)
*Natural log transformed due to lack of normality

Table 4.7 Microarray and real-time RT-PCR identification of selected immune-related genes from CL of cows supplemented with 3 mg Se/d in vitamin-mineral mixes as sodium selenite (ISe, n=5) or a 1:1 blend (MIX, n=5) of ISe and OSe (SEL-PLEX).[1]

Gene	Gene Name	Microarray[2]			RT-PCR[2]			
		Ise	MIX	P-value	ISe	Mix	SEM	P-value
C1QC	Complement C1q C chain	1.00[a]	1.32[b]	0.0149	1.023[a]	2.349[b]	0.216	0.0025
FAS	Fas cell surface death receptor	1.00[a]	1.33[b]	0.0247	1.021[a]	1.776[b]	0.245	0.0357
IFNG	Interferon gamma		.	.	1.18[a]	3.05[b]	0.51	0.0319
IL18	Interleukin 18, interferon-gamma-inducing factor	1.00[a]	1.24[b]	0.0471	1.027	1.246	0.115	0.2146
IL1R1	Interleukin 1 receptor type 1	1.00[a]	1.34[b]	0.0428	1.040[a]	1.693[b]	0.196	0.0468
ILR8 A*	Chemokine (C-X-C motif) receptor 1 (CXCR1)	.	.	.	1.051	2.881	0.818	0.1948
ILR8 B	Chemokine (C-X-C motif) receptor 2 (CXCR2)	.	.	.	1.007[a]	1.671[b]	0.107	0.0023
ITGAM	Integrin subunit alpha M	1.00[a]	1.23[b]	0.0291	1.042[a]	1.766[b]	0.132	0.0046
TNFα	Tumor necrosis factor	1.00	1.07	0.3	1.23	2.28	0.59	0.2425
VEGFA	Vascular endothelial growth factor A	1.00	1.09	0.24	1.03	1.41	0.21	0.2295
VEGFC	Vascular endothelial growth factor C	1.00	1.04	0.417	1.01	1.08	0.06	0.4521

[1] Selenium was supplemented at 35 ppm as either inorganic (ISe; sodium selenite) or a 1:1 combination (MIX) of ISe and OSe (SEL-PLEX). Selenium was supplemented individually using in-pasture Calan gates(Patterson et al., 2013).
Table 4.7 (continued)
[2] Data are expressed as a ratio of MIX relative to ISe expression.
[3] Means within a row that lack a common superscript differ ($P<0.05$)
*Natural log transformed due to lack of normality

CHAPTER 5. Form of dietary selenium affects mRNA encoding interferon stimulated and progesterone induced genes in the bovine endometrium and conceptus length at maternal recognition of pregnancy.

5.1. Abstract

Widespread regions of the southeast United States have soils, and hence forages, deficient in selenium (Se), necessitating Se supplementation to grazing cattle for optimal immune function, growth, and fertility. We have reported that supplementation with an isomolar 1:1 mix (MIX) of inorganic (ISe) and organic forms of Se increases early luteal phase (LP) progesterone (P4) above that in cows on ISe alone. Increased early LP P4 advances embryonic development. Our objective was to determine effects of form of Se on the development of the bovine conceptus and the endometrium using targeted qPCR on day 17 of gestation, the time of maternal recognition of pregnancy (MRP). Angus-cross yearling heifers underwent 45-day Se-depletion then repletion periods, then at least 90 days of supplementation (TRT) with 35 ppm Se/day as either ISe (n=10) or MIX (n=10). Heifers were inseminated to a single sire after a detected estrus (Day 0). On Day 17 of gestation, caruncular (CAR) and intercaruncular (ICAR) endometrium samples and the developing conceptus were recovered. Real-time PCR (qPCR) was performed to determine the relative abundance of targeted transcripts in CAR and ICAR samples and were subjected to one-way ANOVA to determine TRT effects. Effects of TRT on conceptus development were analyzed using a one-tailed student T-Test. MIX heifers had decreased ($P<0.05$) abundance of several P4-induced and interferon-stimulated mRNA

transcripts, including *IFIT3, ISG15, MX1, OAS2, RSAD2, DGAT2, FGF2* in CAR and *DKK1* in ICAR. Additionally, MIX heifers tended (*0.05 ≤ P ≤ 0.10*) to have decreased mRNA abundance of *IRF1, IRF2, FOXL2,* and *PGR* in CAR, and *HOXA10* and *PAQR7* in ICAR. In contrast, MIX-supplemented heifers had increased (*P<0.05*) mRNA abundance of *MSTN* in ICAR. Importantly, MSTN increases glucose secretion into histotroph and contributes to advanced conceptus development. Interestingly, and a salient finding of this experiment, a MIX-induced increase (25.96 ± 3.95 cm vs. 17.45 ± 3.08 cm*; P=0.0533)* in conceptus length was observed when compared to heifers supplemented with ISe alone. Intriguingly, this advancement in conceptus development occurs in the presence of similar concentrations of serum P4 (*P=0.88*) and whole blood Se (*P=0.07*) at MRP. Collectively, results from this experiment suggests that the onset of MRP may be shifted and occurs earlier in MIX vs. ISe supplemented heifers.

Keywords: Selenium, progesterone, endometrium, conceptus

5.2. Introduction

The majority of producers in the southeast United States, including Kentucky, have forages and grains that are low (<0.05 ppm) to variable (~50% contain >0.1 ppm) in selenium (Se) (Ammerman and Miller, 1975). Therefore, it is not unexpected that producers in the southeast have the highest proportion of cattle classified as Se-deficient compared to other geographical regions (Dargatz and Ross, 1996). In cattle, deficiencies in Se have been demonstrated to decrease immunity (Erskine et al., 1989), growth (Gleed et al., 1983), and fertility (McClure et al., 1986). In the feed industry, Se is commonly supplemented in the inorganic (ISe) dietary form of sodium selenite or sodium selenate (Podoll et al., 1992). However, research has demonstrated lower concentrations of Se in whole blood in cattle supplemented with ISe compared to cattle supplemented with organic (OSe**)** Se (Sel-Plex; Alltech, Inc., Nicholasville, KY, USA) (Patterson et al., 2013; Cerny et al., 2016b; Jia et al., 2018).

Supplementing dairy cows during the pre- and postpartum periods with a Se-enriched yeast increased postpartum plasma concentrations of progesterone (P4) compared to providing no supplemental Se (Kamada, 2017). Importantly, it has been reported that postpartum concentrations of P4 are positively correlated with conception rate (Inskeep, 2004). Similarly, our lab has previously reported increased early luteal phase concentrations of P4 in cows supplemented with a 1:1 combination (MIX) of ISe (sodium selenite; Prince Agri Products, Inc. Quincy, IL) and OSe (SEL-PLEX; Alltech, Inc., Nicholasville, KY, USA) compared to cows supplemented with ISe or OSe on day 6 (Cerny et al., 2016b), or ISe on day 7 (Carr et al., 2020) of the estrous cycle. Moreover,

we have recently reported that this increase in P4 could be explained, in-part, by increased cholesterol biosynthesis (Crites et al., Unpublished) and increased cholesterol uptake (Carr et al., Unpublished) in corpora lutea recovered from MIX versus ISe supplemented cows.

Progesterone plays a crucial role in creating an optimal uterine environment that favors advanced conceptus elongation (Lonergan, 2011). Progesterone acts on the uterus to indirectly stimulate pre-implantation blastocyst growth and elongation (Garrett et al., 1988; Mann and Lamming, 2001). The elongating conceptus must secrete a chemical signal to signal pregnancy and to prevent uterine release of luteolytic pulses of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and therefore luteal regression, a process referred to as "maternal recognition of pregnancy" (MRP) (Short, 1969). In cattle that signal is interferon tau (IFNT) (Lewis et al., 1979) and MRP occurs around days 16-17 (Spencer, 2013).

In the uterus, P4 acts on the endometrium as a differentiation factor (Cummings and Yochim, 1984) and stimulates secretions in the glandular epithelium. Increased concentrations of P4 immediately following conception have been associated with advanced conceptus elongation (Garrett et al., 1988; Carter et al., 2008) and increased IFNT production (Mann and Lamming, 2001).

Additionally, IFNT stimulates a number of genes in a cell-specific manner within the endometrium that are implicated in uterine receptivity and conceptus development (Spencer et al., 2007). Together, these proteins provide an environment that supports early embryonic development (Niswender et al., 2000). The early embryo is nourished by histotroph from the uterine glands which are located in the intercaruncular endometrium

(ICAR) (Atkinson et al., 1984), and have shown to be crucial for the development of the conceptus (Gray et al., 2001). Additionally, small aglandular caruncular (CAR) areas of stromal origin are scattered over the endometrium surface (Mansouri-Attia et al., 2009). The CAR areas are present in the cyclic endometrium and fuse with the fetal cotyledons to form placentomes in pregnant animals (Atkinson et al., 1984). During this pivotal time period encompassing MRP, failure or delays in trophoblast elongation and/or embryonic development result in loss of pregnancy possibly due to suboptimal histotroph (Wiltbank et al., 2016). Although fertilization rates are high in cattle (Diskin and Morris, 2008), pregnancy losses average approximately 30% from days 8 to 27 of gestation, a period of time encompassing embryo elongation and MRP (Wiltbank et al., 2016). The loss of pregnancy at any stage of gestation has a clear negative impact on the reproductive performance and profit potential in beef and dairy operations.

The objectives of this study were to investigate the effects of form of supplemental Se on gene expression in bovine endometrium and the developing conceptus on day 17 of pregnancy, further contributing to our long-term goal of elucidating form of Se-regulated processes affecting fertility. We hypothesized that the form of Se supplemented to cows would alter gene expression in both CAR and ICAR bovine endometrium, with MIX creating an environment that favors conceptus development. More specifically, we hypothesized that conceptus development would be more advanced in heifers supplemented with MIX versus ISe. Providing supplemental mineral is a production practice that is easily implemented by producers and incorporating Se as MIX would be a simple transition with the potential to increase fertility in their herds.

5.3. Material and Methods

The experimental procedures in this project were approved by the Institutional Animal Care and Use Committee at the University of Kentucky, protocol number 2017-2828.

5.3.1. Animals and Experimental Procedure

Fall-born, Angus-cross yearling heifers (N=20) were randomly selected from pre-existing, Se form-specific cow herds as previously described (Patterson et al., 2013; Matthews et al., 2014; Cerny et al., 2016a; Cerny et al., 2016b) and were housed at the C. Oran Little Research Center at the University of Kentucky. At the beginning of this experiment, animals received *ad libitum* access to a basal mineral mix with no exogenous source of Se for a 45-day Se-depletion period. This was followed by a 45-day period with *ad libitum* access to the mineral mix with 35-ppm Se as ISe for 45 days to return systemic blood Se in all cows to adequate concentrations (National Academies of Sciences and Medicine, 2016). Following the period of Se repletion, cows were randomly assigned to have at least 90 days of *ad libitum* access to a basal mineral mix containing 35-ppm Se as inorganic (n=10; ISe; sodium selenite; Prince Agri Products, Inc. Quincy, IL) or as 1:1 combination of ISe and OSe (n=10; MIX; SEL-PLEX; Alltech, Inc., Nicholasville, KY) as described by (Carr et al., *In Review*). Throughout the depletion and repletion periods, all heifers grazed a common, novel, nontoxic endophyte-infected tall fescue (Lacefield

MaxQ II) pasture and during the Se-specific treatment periods, heifers grazed toxic endophyte-infected tall fescue pastures with the addition of a common corn silage diet.

To ensure heifers maintained adequate status of total blood Se for the duration of the study, whole blood was collected from each heifer at the start and endpoint of the depletion and repletion periods and bimonthly until the end of the experiment for the determination of total whole blood concentrations of Se (Patterson et al., 2013; Cerny et al., 2016a).

5.3.2. Experimental Regimen and Tissue Collection

Heifers were supplemented with their respective mineral treatment for at least 90 days before being randomly injected with one or two doses of dinoprost tromethamine (25 mg, Lutalyse, Zoetis, Parisppany, NJ) to induce regression of the corpus luteum (CL) and then monitored for behavioral estrus (Day 0), twice daily, using visual appraisal and electronic cow monitoring technology (CowManager, Gerverscop 9, The Netherlands). At detected estrus (0 h) presence of a preovulatory follicle was confirmed via transrectal ultrasonography using a 5-8 MHz linear transducer (Ibex Pro, E.I. Medical Imaging, Loveland, CO) prior to insemination. Heifers were inseminated at 0 h, 12 h, and 24 h using frozen semen from a single bull with known high fertility. On days 0, 5, 6, 7, 8, 11, 14 and 17 approximately 8 mL of blood was collected into additive-free tubes (Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ) by jugular venipuncture for retrieval and quantification of serum P4 via radioimmunoassay. Additionally, on days 0 and 17, 8 mL of whole blood was collected in EDTA-containing

(2.7 mg/mL) blood collection tubes (Becton Dickinson, Franklin Lakes, NJ) by jugular venipuncture for concentration of whole blood Se. The presence and diameter of the CL was determined by transrectal ultrasonography on the morning of day 17 prior to transport to the University of Kentucky Meat Laboratory.

On day 17 after insemination, the ovaries and uterus were collected from each heifer after euthanasia by captive bolt stunning and exsanguination at the USDA inspected University of Kentucky Meat Laboratory. Reproductive tracts were immediately removed, placed on ice, and processed for the collection of the conceptus and tissue samples from the CL and endometrium. Specifically, the uterine horn contralateral to the CL was ligated approximately 4 cm from the uterine bifurcation and the ovary bearing the CL was removed. Subsequently, an artificial insemination sheath (Alpha sheath, IMV Technologies USA, Maple Grove, MN) was inserted transcervical to the internal os and 20 mL of ice-cold physiologically buffered saline (PBS) was infused into the uterine horn, massaged gently, and exited through an incision at the tip of the ipsilateral uterine horn. Uterine luminal flushing media and the conceptus were recovered in a sterile 100 by 15-mm Petri dish. If no conceptus was recovered in the first flush, then an additional flush of 20 mL of PBS was performed. If no conceptus was recovered after flushing four consecutive times, heifers were determined to be nonpregnant and were removed from the experiment. A complete, intact conceptus was recovered from MIX (n=6) and ISe (n=6) heifers. Only heifers with a recovered conceptus were included in all analyses. Digital images of the conceptuses were captured over 6.35 mm grid paper and conceptus lengths were measured using the software program, Digimizer (version 5.6.0),

by two independent technicians. Conceptus lengths were averaged to determine a composite conceptus length to be used in statistical analyses.

Endometrial samples were only collected from the ipsilateral horn to the CL for gene expression analysis. The ipsilateral uterine horn was longitudinally opened by scissors and carcuncular (CAR) and intercaruncular (ICAR) endometrium samples were collected by the same individual for all animals using an 8mm biopsy punch (Integra LifeSciences Production Corporation, Mansfield, MA). After collection, the conceptus, CAR, ICAR and luteal samples were snap-frozen in liquid nitrogen and stored at -80°C to be used for RNA extraction and the determination of transcript expression by real-time polymerase chain reaction (qPCR).

5.3.3. RNA Extraction

Total RNA was extracted from ~200 mg of frozen CAR and ICAR endometrial tissue using TRIzol Reagent (Invitrogen Corporation, Carlsbad, CA) following the manufacturer's instructions. The purity and concentration of total RNA samples were analyzed using a NanoDrop ND-100 Spectophotometer (NanoDrop Technologies, Wilmington, DE, USA). All samples had high purity, with 260/280 absorbance ratios of 1.88 or greater.

5.3.4. Real-time PCR Analysis

The relative quantification of mRNA for genes of interest was performed using qPCR using standard procedures in our laboratory, as described previously (Cerny et al., 2016a; Carr et al., *In Review*). Briefly, 1 µg of each cow's CL RNA was reverse transcribed to cDNA using the SuperScript™ IV VILO™ Master Mix with ezDNAse™ Enzyme (Invitrogen by Thermo Fisher Scientific, Vilnius, Lithuania). Additionally, a no reverse-transcription control sample was transcribed and analyzed via qPCR to ensure that products from the targeted transcripts were not obtained from genomic DNA.

The relative abundance of mRNA encoding interferon-stimulated associated transcripts: *ACKR3, IFIT3, IRF1, IRF2, ISG15, MSXI, MX1, MX2, OAS1, OAS2* and *RSAD2* and the P4-induced transcripts: *DGAT2, DKK1, FABP3, FGF2, MSTN, SLC1A5, SLC1A3, SLC46A3, FOXL2, IHH, HOXA10,* and *IGFBP1* were quantified. Next, the relative abundance of P4-associated enzymatic transcripts: *PTGS2* and *PGES* and the receptor transcripts: *OXTR, ESR1, PGR, PGRMC1, PGRMC2, PAQR5, PAQR7, PAQR8, EP1, EP2, EP3, EP4, PGFR, IFNAR1* and *IFNAR2* were quantified. Primer sequences used and GenBank accession numbers are listed in Tables 5.1 and 5.2, respectively. The qPCR procedures were performed using the Bio-Rad CFX Maestro™ thermal cycler (Bio-Rad, Hercules, CA, USA) with iTaq Universal SYBR® Green Supermix (Bio-RAD, Hercules, CA, USA). A total volume of 25 µl was used in each qPCR reaction containing 5 µl of cDNA, 1 µl of a 10 µM stock of each primer (forward and reverse), 12.5 µl of 2 x SYBR Green PCR Master Mix, and 5.5 µl of nuclease-free water. The relative amount of each transcript was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Primer sets for genes of interest were designed and obtained from NCBI Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primerblast/) against RefSeq.

All cDNA products were validated via DNA sequencing for verification of target at ACGT, Inc. (Wheeling, IL, USA). The resulting sequences were then compared to the NCBI RefSeq mRNA sequences used for primer templates. The primer pair design, amplicon length of product, and product identity for each targeted transcript are shown in Tables 5.1 and 5.2. Three constitutively expressed genes (*β-ACTIN*, *GAPDH* and *SDHA*) with CT values not affected ($P > 0.05$) by Se-form treatment were used to normalize the relative mRNA expression to the geometric mean of the three. For qPCR analysis, $n = 6$ and 6 for ISe and MIX treatments, respectively. Data were normalized to ISe expression and all reactions were performed in duplicate.

5.3.5. Se and P4 Analysis

Total blood Se was determined by the University of Kentucky's Veterinary Diagnostics Laboratory (Lexington, KY) and Se was quantified using an Agilent 7900 inductively coupled plasma-mass spectrometer, as described previously (Wahlen et al., 2005).

Concentrations of P4 were quantified in samples of serum by a commercially available competitive RIA without extraction (*ImmuChem*™ Coated Tube Progesterone 125-I RIA Kit, MP Biomedicals, Costa Mesa, CA) according to manufacturer's instructions. There was one assay performed for analysis of the serum with an intra assay CV of 10.16%.

5.3.6. Statistical Analysis

Data are presented as least square means (±SEM) with individual cow as the experimental unit. Data were analyzed for normal distribution and homogeneity. When appropriate, data were transformed for normality by natural log transformation.

To determine the effect of form of Se on concentrations of systemic Se and P4, data were analyzed using the PROC MIXED procedure of SAS statistical software package (version 9.4; SAS Institute, Inc. Cary, NC) as an ANOVA with repeated measures. The form of dietary Se was considered the fixed effect for both and the P4 data were natural log transformed due to being not normally distributed. The effect of form of Se supplementation on conceptus length was analyzed as a one-tailed student's T-test, using the PROC TTEST procedure of SAS statistical software package (version 9.4; SAS Institute, Inc. Cary, NC).

Additionally, the effect of form of Se supplementation on the relative abundance of CAR and ICAR mRNA transcripts were analyzed as an ANOVA using the PROC GLM procedure of SAS statistical software package (version 9.4; SAS Institute, Inc. Cary, NC). For all analyses n=6 for ISe and MIX treatments and significance was declared at $P < 0.05$ and a tendency to differ was declared when $0.05 \leq P \leq 0.10$.

5.4. Results

5.4.1. Concentrations of Whole Blood Se

Cows were maintained on form of Se-specific treatments (ISe vs. MIX) that provided adequate concentrations of whole blood Se (Gerloff, 1992; Dargatz and Ross, 1996) for the duration of this experiment (Fig. 5.1). There was an effect of time ($P<0.0001$) and tended to be a main effect of form of Se ($P=0.0704$), but there was no significant treatment by time interaction ($P=0.5066$).

5.4.2. Progesterone

Serum collected from heifers on days 0, 5, 6, 7, 8, 11, 14 and 17 of gestation was used to determine concentrations of P4. Previous studies from our lab indicate an increase of ~1 ng/mL on days 6 and 7 of the estrous cycle (Cerny et al., 2016b; Carr et al., 2020). In the current study, concentrations of P4 were not affected ($P=0.88$) by form of Se-treatment (Fig. 5.2). However, there was an effect of day ($P<0.0001$), but there was no treatment by day interaction ($P=0.77$). Although not significant, there is a form of Se-induced numerical increase in concentrations of P4 on days 6 and 7 in the study herein, a time consistent with our previous studies (Cerny et al., 2016b).

5.4.3. Conceptus Length

Form of Se supplementation affected conceptus length on day 17 of gestation. Conceptus length was increased (*$P=0.0533$*) in heifers supplemented with MIX (25.96 ± 3.95 cm) compared to ISe (17.45 ± 3.08 cm) (Fig. 5.3). A representative image of a collected conceptus is shown in Fig. 5.4.

5.4.4. Real-time PCR Analysis of Selected mRNA Transcripts

The relative abundance of 40 mRNA encoding interferon-stimulated associated transcripts, P4-induced transcripts, P4-associated enzymatic transcripts and receptor transcripts were analyzed in both CAR and ICAR samples via qPCR.

Of the 11 interferon-stimulated associated transcripts (Table 5.3), the level of expression of mRNA encoding *IFIT3, ISG15, MX1, OAS2* and *RSAD2* was lower (*P<0.05)* in CAR retrieved from MIX versus ISe supplemented heifers (Fig. 5.5). Similarly, the relative abundance of *IRF1* and *IRF2* tended ($0.05 \leq P \leq 0.1$) to be lower in CAR retrieved from MIX versus ISe supplemented heifers (Fig. 5.5).

Of the 12 targeted P4-induced transcripts (Table 5.4), the level of expression of mRNA encoding *DGAT2* and *FGF2* was lower (*P<0.05*) in CAR retrieved from MIX versus ISe supplemented heifers (Fig. 5.6). Additionally, the relative abundance of *FOXL2* tended ($0.05 \leq P \leq 0.1$) to be lower in CAR retrieved from MIX versus ISe supplemented heifers. Similarly, MIX supplemented heifers had lower (*P<0.05*) levels of expression of mRNA encoding *DKK1* and tended ($0.05 \leq P \leq 0.1$) to have increased expression of mRNA encoding *HOXA10* in ICAR samples compared to heifers supplemented with ISe. However, MIX supplemented heifers had greater (*P<0.05*) levels of expression of mRNA encoding *MSTN* in ICAR compared to ISe supplemented heifers (Fig. 5.6). In addition, of the 17 targeted receptor transcripts (Table 5.5), the expression of mRNA encoding the nuclear P4 receptor (*PGR*) in CAR samples and the P4 membrane

receptor (*PAQR7*) in ICAR samples tended ($0.05 \leq P \leq 0.1$) to be decreased in MIX versus ISe treated cows (Fig. 5.7).

5.5. Discussion

The aim of this study was to investigate the effect of form of supplemental Se on the uterine endometrium and developing conceptus at the time of MRP in cattle. More specifically, we sought to determine alterations in conceptus development and targeted gene expression in the bovine endometrium at MRP on day 17 of gestation. Recently we have observed a MIX-induced increase in CL mRNA transcripts associated with cholesterol biosynthesis (Crites et al., Unpublished) and cholesterol uptake (Carr et al., *In Review*), which could partly explain the increased concentrations of P4 in the early luteal phase observed previously in our lab (Cerny et al., 2016b; Carr et al., 2020).

In the present study, MIX supplemented cows had numerically greater concentrations of P4 versus ISe supplemented cows on days 6 and 7 of gestation. Although not statistically significant, the increased concentrations of P4 occur at similar times reported in our previous studies that used a larger number of animals (Cerny et al., 2016b; Carr et al., 2020). The inability to detect statistical significance of a MIX-induced increased concentration of P4 is likely limited by the number of animals used in the present study; however, cows in the present study were also managed on both non-toxic and toxic endophyte-infected tall fescue pastures and received a common corn silage diet during the winter months. Cows used in previous studies from our lab were grazing toxic endophyte-infected tall fescue pastures (Cerny et al., 2016b; Carr et al., 2020). Previous research has demonstrated that heifers fed endophyte-infected fescue had lower luteal

phase concentrations of P4 than heifers consuming a diet containing endophyte-free fescue (Jones et al., 2003). Perhaps there is a relationship between endophyte toxicity and form of supplementary Se that accounts for the differences between these studies.

Successful pregnancy in mammals requires both a viable embryo and a receptive endometrium (Walker et al., 2010). Synchronous signaling between the endometrium and embryo during the pre-implantation period is critical for normal embryo development, implantation of the embryo, and placentation (Wolf et al., 2003). Uterine receptivity has been demonstrated to be dependent on P4 (Mansouri-Attia et al., 2009). Additionally, uterine factors including enzymes, cytokines, growth factors, ions, hormones, glucose, transport proteins, and adhesion molecules, collectively termed "histotroph," have been shown to be mainly synthesized by the endometrial glands (Martal et al., 1997). Research has indicated that P4-induced changes in endometrial gene expression leads to changes in the composition of histotroph that are required for post-hatching conceptus survival and growth (Spencer et al., 2008a).

The actions of P4 are mediated by P4 receptors (PR) (Spencer and Bazer, 2002). Progesterone receptors are expressed in endometrial epithelia and stroma during the early to mid-luteal phase, allowing direct regulation of genes by P4 (Spencer et al., 2008b). However, continuous exposure of the endometrium to P4 negatively regulates PR expression in the luminal and glandular epithelium, and the down regulation of PR is temporally associated with the induction of many P4-stimulated genes (Spencer et al., 2004; Spencer et al., 2008b). As reviewed by Spencer et al. (2004), the paradigm of loss of PR in uterine epithelia immediately before implantation is common to sheep, cattle, pigs, and mice, amongst other species. Furthermore, the loss of PR is associated with the

induction of numerous gene associated with cell adhesion (Spencer et al., 2008b). Results from qPCR performed in the study herein indicated a tendency for the relative abundance of mRNA encoding the nuclear P4 receptor (*PGR*) and progestin and adipoQ receptor family member 7 (*PAQR7*) to be decreased in ICAR tissue recovered from MIX compared to ISe supplemented heifers. Since PR are downregulated from continuous exposure to P4, this MIX-induced reduction in PGR and PAQR7 fits in with the increased concentration of P4 previously reported in our lab (Cerny et al., 2016b; Carr et al., *In Review*) and the numerical increase on days 6 and 7 in the present study.

Progesterone regulates forkhead Box L2 (FOXL2) expression in the endometrium of ruminants and stimulates FOXL2 promoter activity through nuclear P4 receptors (PGR) (Eozenou et al., 2020). Results from qPCR indicated that heifers supplemented with MIX had decreased mRNA abundance of *FOXL2* and *PGR.* In bovine endometrium, a negative correlation between circulating concentrations of P4 and FOXL2 gene expression exists (Eozenou et al., 2012). FOXL2 appears to be important in the endometrium as well as a key gene involved in ovarian differentiation and maintenance of ovarian function (Eozenou et al., 2012; Georges et al., 2014; Elzaiat et al., 2017). Interestingly, it has been revealed that FOXL2 is a transcriptional repressor of the StAR protein gene (Pisarska et al., 2004). Importantly, StAR protein transports cholesterol to the inner mitochondrial membrane and is the rate-limiting step in steroidogenesis (Clark et al., 1994; Manna et al., 2016). Therefore, repressing the StAR protein gene would lead to reduced concentrations of progesterone.

Histotroph represents maternal contributions to uterine luminal fluid and is composed of glucose, fatty acids, and amino acids (Forde et al., 2014). Although

gluconeogenesis does not occur in the uterus (Zimmer and Magnuson, 1990; Yánez et al., 2003), glucose is stored as glycogen (Demers et al., 1972; Greenstreet and Fotherby, 1973). It is likely that uterine glycogen reserves are an important source of energy for pre-embryonic growth and development (Dean et al., 2014). Diacylglycerol O-acyltransferase homolog 2 (DGAT2) catalyzes the final step in the formation of triglyceride to acylcoenzyme A and that triglyceride is a potential energy source up to the blastocyst stage in cattle (Forde et al., 2009). In the present study, qPCR results revealed that MIX supplemented heifers had decreased relative abundance of DGAT2 in CAR tissue compared to heifers supplemented with ISe. Moreover, a P4-induced earlier increase in DGAT2 expression may increase availability of triglyceride as an energy source for the developing conceptus (Forde et al., 2009). Additionally, P4 induction of myostatin (MSTN) may increase glucose secretion into histotroph, contributing to the advanced development of the conceptus after hatching (Forde et al., 2009). Intriguingly, MIX supplemented heifers had increased relative abundance of MSTN compared to ISe heifers in ICAR tissue. These results suggest that conceptus recovered from MIX heifers were more advanced in their development due to an earlier increase P4, leading to an earlier increase in DGAT2 and increased MSTN on day 17 of gestation.

Similarly, in spermatogenesis, the preferred energy substrate of round spermatids by sertoli cells is lactate (Goddard et al., 2003; Boussouar and Benahmed, 2004). Previous research from our lab indicated increased expression of mRNA encoding lactate dehydrogenase A (LDHA) in testis of calves born to MIX-dams (Cerny et al., 2016a) and importantly, LDHA is required for the production of lactate. Additionally, MIX steers had 99% more hepatic glutamine synthetase (GS) activity than ISe steers (Jia et al., 2018)

and GS is involved in scavenging ammonia that escapes periportal hepatocyte detoxification by synthesizing glutamine from sinusoidal ammonia and glutamate (Wagenaar et al., 1994).

Progesterone stimulates and maintains endometrial functions necessary for conceptus growth, implantation, placentation, and development to term (Bazer, 1975; Bazer et al., 1979; Spencer and Bazer, 2002; Spencer et al., 2004). Heifers and ewes with lower concentrations of P4 in the early luteal phase had smaller conceptuses that secreted less IFNT (Nephew et al., 1991; Mann and Lamming, 2001). Conversely, increased concentrations of P4 immediately following conception has been associated with advanced conceptus elongation (Carter et al., 2008) and increased IFNT production (Mann and Lamming, 2001). Although concentrations of P4 were similar between treatments at MRP in the present study, conceptus development was advanced in MIX versus ISe supplemented heifers. Importantly, the MIX-induced increase in conceptus development occurred in the presence of similar concentrations of whole blood Se. This MIX-induced increase in conceptus development is a salient finding of this research and could be, in-part, due to the increased relative abundance of MSTN discussed earlier. It has been suggested that heifers with artificially increased concentrations of P4 soon after insemination had protein products required to advance conceptus development transported to the uterine lumen at an earlier stage than in normal P4 environments (Forde et al., 2009). Perhaps the increased concentrations of progesterone previously reported by our lab on days 6 (Cerny et al., 2016b) and 7 (Carr et al., 2020; Carr et al., *In Review*) and the numerical increase in concentrations of P4 on days 6, 7, and 11 in MIX versus ISe heifers in the present study allowed for an earlier induction of MSTN,

ultimately contributing to the advanced conceptus development of MIX embryos recovered herein.

In addition to its antiluteolytic actions, IFNT produced by the conceptus acts on endometrial genes, known as interferon stimulated genes (ISGs), in a specific spatial and temporal manner (Bazer et al., 2008; Bazer et al., 2009). Interestingly, in a comparison of pregnant and cyclic heifers, differentially expressed genes identified on day 16 of pregnancy were due to the presence of the conceptus and the majority were expressed in response to IFNT produced by the conceptus (Forde et al., 2011).

Following binding of IFNT to its receptors (IFNAR1 and IFNAR2), it initiates cell signaling via Janus activation kinases (JAKs) and tyrosine kinase 2 (TYK2) (Bazer et al., 2008; Walker et al., 2010) and can induce ISG expression (Michalska et al., 2018). Similarly, IRF1 can regulate expression of ISGs in response to type I and II interferons (Michalska et al., 2018). In the present study, qPCR results indicated MIX supplemented heifers tended to have decreased abundance of IRF1 and IRF2 compared to ISe heifers. Treating ovine luminal epithelia cells with IFNT induced IRF1 expression within 1 h, maximal expression at 3 h, and steadily declined through 48 h (Stewart et al., 2001). This suggests that the maximal expression of IFNT, and thus the timing of MRP, is shifted and occurs earlier in the MIX supplemented heifers.

In sheep, classical ISGs, such as interferon stimulated gene 15 (ISG15), mouse myxovirus resistance 1 (MX1), and 2',5' oligoadenylate synthase (OAS), induced by IFNT are limited to uterine glandular epithelia and stromal cells because uterine luminal epithelia and superficial glandular epithelia express interferon regulatory factor 2 (IRF2) which is a potent inhibitor of gene transcription that silences expression of genes such as

estrogen receptor 1 (ESR1) and signal transducer and activator of transcription factor 1 (STAT1) (Bazer et al., 2008).

OAS upregulation during early pregnancy is involved in regulating the production of osteopontin (SPP1) (Spencer et al., 1999; McAveney et al., 2000), which is also up-regulated during pregnancy (Walker et al., 2010). In the present study, MIX heifers had decreased abundance of mRNA encoding *OAS2* in CAR tissue compared to ISe heifers. Additionally, upregulation of SPP1 in pregnant animals promotes adhesion of the trophoblast to the endometrium, stimulates morphological changes in the trophoblast (Johnson et al., 2003) and regulates the immune response (Walker et al., 2010). Upregulation of these genes may be an important mechanism to enhance the response to potential viral pathogens during the time of local immune suppression that occurs in response to the embryo (Walker et al., 2010). The upregulation of MX1 and MX2, both ISGs, supports this hypothesis and are upregulated in response to viral infection (Hicks et al., 2003; Bauersachs et al., 2009). Heifers supplemented with MIX had decreased relative abundance of MX1, but similar abundance of MX2 compared to ISe heifers in the present study. Additionally, MX genes induced by IFNT at pre-implantation may play a role in pregnancy recognition, uterine reception, and/or conceptus attachment to the endometrium (Shirozu et al., 2015).

Additionally, supplementing heifers with MIX reduced the relative abundance of mRNA encoding radical S-adenosyl methionine domain containing 2 (RSAD2) in CAR. RSAD2 is produced during viral infection in response to interferons to limit viral replication and modulate adaptive immunity (Helbig et al., 2005). Moreover, RSAD2 could act as an immunomodulatory factor preventing viral infection of the uterus during

the critical stage of implantation (Mansouri-Attia et al., 2009). Interestingly, expression of RSAD2 and MX1 was not limited to the stroma or the glandular epithelium but was also detectable in the luminal epithelium at implantation (Mansouri-Attia et al., 2009).

5.5.1. Conclusion

Evidence from this study supports our hypothesis that form of supplemental Se influences the expression of transcripts in the bovine endometrium at MRP and that MIX advances conceptus development compared to ISe. Results from qPCR indicated that heifers supplemented with MIX had decreased expression of several transcripts known to be induced by P4 and/or stimulated by interferons. Additionally, compared to ISe supplemented heifers, MIX supplemented heifers tended to have decreased abundance of mRNA encoding PGR and PAQR7, and the paradigm of loss of PR in uterine epithelia immediately before implantation occurs in both sheep and cattle, amongst other species. Similarly, MIX heifers tended to have decreased abundance of mRNA encoding IRF1, which is stimulated by IFNT and has reduced responsiveness to IFNT overtime. Moreover, MIX supplemented heifers had increased abundance of mRNA encoding MSTN, which increases glucose secretion into histotroph, thus advancing conceptus development. Interestingly, a MIX-induced increase in conceptus length was observed compared to heifers supplemented with ISe and is a salient finding of this experiment. Collectively, these results suggest that the onset of MRP may be shifted and occurs earlier in MIX supplemented heifers compared to those supplemented with ISe alone.

Table 5.1 Primer sets and product identities of qPCR analysis of progesterone-induced and interferon-stimulated genes.

Gene	Gene Name	Accession Number[1]	Oligonucleotide Primer Design (5' to 3') direction	Amplicon length (bp)	Product identity [2]
Progesterone-induced transcripts					
DGAT2	Bos taurus diacylglycerol O-acyltransferase 2	NM_205793.2	F: AACACACCCAAGAAAGGTGGC R: GCTTACTTCTGTGGCCTCTGT	204	100%
DKK1	Bos taurus dickkopf WNT signaling pathway inhibitor 1	NM_001205544.1	F: GGCAGCAAGTACCAGACCAT R: AGAAGGCATGCATATCCCGTT	207	100%
FABP3	Bos taurus fatty acid binding protein 3	NM_174313.2	F: TGAAGTCACTCGGTGTCGGT R: TCAACCATCTCCCGCACAAG	271	100%
FGF2	Bos taurus fibroblast growth factor 2	NM_174056.4	F: AAGCGGCTGTACTGCAAGAA R: ACACTCGTCTGTAACACATTTAGAA	216	100%
FOXL2	Bos taurus forkhead box L2	NM_001031750.1	F: GCAGAAGCCCCCATACTCTT R: GGTCCAGCGTCCAGTAGTTG	239	100%
HOXA10	Bos taurus homeobox A10	NM_001105017.1	F: TTTCGGAAATGTGTCAAGGCAA R: CGGATCCGGTTTTCTCGGTT	262	100%
IGFBP1	Bos taurus insulin like growth factor binding protein 1	NM_174554.3	F: CAGCGATGAGGCTACAGATAC R: GCTGCTCCCTGGCTAATCTG	257	99%
IHH	Bos taurus Indian hedgehog signaling molecule	NM_001076870.2	F: GCCAACAATCACACTGAGCC R: CCAAGCTGTGAAACAGTCGC	274	100%
MSTN	Bos taurus myostatin	NM_001001525.3	F: TGCCCACGGAGTCTGATCTT R: TGCCTGGGTTCATGTCAAGT	237	100%
SLC1A3	Bos taurus solute carrier family 1 member 3	NM_174600.2	F: GGGCGCCGTGATAAACAATG R: GAGGGGCGTACCACATGAT	242	100%
SLC1A5	Bos taurus solute carrier family 1 member 5	NM_174601.2	F: CAAGGAGGTGCTCGATTCGT R: ACAGGGGCGTACCACATGAT	306	100%
SLC46A3	Bos taurus solute carrier family 46 member 3	NM_001103303.2	F: TCTACTGAGCAAGGGACCAT R: CCCGTATTCCTGCTGACGTA	200	100%
Interferon-stimulated transcripts					

Table 5.1 (continued)

ACKR3	Bos taurus atypical chemokine receptor 3	NM_001098381.2	F: TACTCAGAGCCGGGGAACTT R: TGTAGCAGTGCGTGTCGTAG	226	99%
IFIT3	Bos taurus interferon induced protein with tetratricopeptide repeats 3	NM_001075414.1	F: ATTCTGAAGCAGGCCGTTGA R: TCCAGTGCCCTTAGCAACAG	224	100%
ISG15	Bos taurus ISG15 ubiquitin like modifier	NM_174366.1	F: CCATCCTGGTGAGGAACGAC R: GAACACGGTGCACCCCTTCA	200	99%
MSXI	Bos taurus msh homeobox 1	NM_174798.2	F: CCATTTCTCGGTGGGAGGAC R: GTACTGCTTCTGGCGGAACT	241	100%
MX1	Bos taurus MX dynamin like GTPase 1	NM_173940.2	F: ACATGATCGTCAAGTGCCGT R: ACAGGGGCAGAGTTTTACAAATG	201	100%
MX2	Bos taurus MX dynamin like GTPase 2	NM_173941.2	F: GCTCCAGAAGGCCATGGAAAT R: AACCACGCCGTAAATCTGGT	208	100%
OAS1	Bos taurus 2',5'-oligoadenylate synthetase 1	NM_001029846.2	F: GGAGACGTGCTTCCAAGAGT R: TCTTCAGTCACCTGAGCTTGTG	381	99%
OAS2	Bos taurus 2'-5'-oligoadenylate synthetase 2	NM_001024557.1	F: ACTGGTTTCAAAAGTGCCAGG R: CAGCCAGCAGGTGTTATCCA	314	98%
RSAD2	Bos taurus radical S-adenosyl methionine domain containing 2	NM_001045941.1	F: GTGGTTCCAGAAGTACGGTGA R: AACCGTTCCGCTTCTCTCAG	315	100%

Table 5.2 Primer sets and product identities of qPCR analysis of steroidogenesis-associated and reference genes.

Gene	Gene Name	Accession Number[1]	Oligonucleotide Primer Design (5' to 3') direction	Amplicon length (bp)	Product identity [2]
Enzymatic transcripts					
PTGES	Prostaglandin E synthase	NM_174443.2	F: CGCTGCTGGTCATCAAAATGT R: GGTCTCCATGTCATTCCGGT	173	97%
PTGS2	Prostaglandin-endoperoxide synthase 2	NM_174445.2	F: CCCATGGGTGTGAAAGGGAG R: TCCACCCCATGGTTCTTTCC	203	100%
Receptor transcripts					
IRF1	Bos taurus interferon regulatory factor 1	NM_001191261.2	F: ACAGCCCCGATACCTTCTCT R: CTTCCCATCCACGCTTGTCT	338	100%
IRF2	Bos taurus interferon regulatory factor 2	NM_001205793.2	F: TGGGCCATCCATACAGGAAA R: CCGTCCAGATGTGACTGTCC	383	99%
OXTR	Bos taurus oxytocin receptor	NM_174134.2	F: GCAGCTTCTGTGGGACATCA R: TCCACGTGATGTAGGCCTTG	326	99%
ESR1	Estrogen receptor 1	NM_001001443.1	F: ATGGCCATGGAATCTGCCAA R: GGTCTTTCCGTATTCCGCCT	256	99%
PGR	Nuclear progesterone receptor	NM_001205356.1	F: CCCACAGGAGTTTGTGAAGC R: AGTGCCCGGGACTGGATAAA	291	99%
PGRMC1	Progesterone receptor membrane component 1	NM_001075133.1	F: GGCCGTATGGAGTCTTTGCT R: TTGTCTGAGTACACGGTGGG	217	100%
PGRMC2	Progesterone receptor membrane component 2	NM_001099060.1	F: GCTTGCGGTCAATGGGAAAG R: GACGGTTCTTCCCCTGGTTT	264	99%
EP1 [3]	Prostaglandin E receptor 1	NM_001192148.1	F: GGCCGCTGTTTTTGGCCGTG R: CCTCCATGGCTGCCCTTGGC	142	100%
EP2	Prostaglandin E receptor 2	NM_174588.2	F: GCTTCATCGGACACAAGCAG R: CTCCGCCATGGATACCCTTT	197	100%
EP3	Prostaglandin E receptor 3	NM_181032.1	F: CGCCGTTGCTGATAATGATGT R: GTCCTTTCAAAAGCTGGCAA	204	100%
EP4	Prostaglandin E receptor 4	NM_174589.2	F: CGGGACCAATGCATCATCCT R: TTGGCCCTTCAAGTAGGTGG	241	100%

Table 5.2 (continued)

PAQR5 [4]	Progestin and adipoQ receptor family member 5 (mPR_{γ})	XM_024997926.1	F: GGTTCTTCTCGTGGAGGTTTGT R: GTTCCTGGACATGGAGCTGAA	151	96%
PAQR7 [4]	Progestin and adipoQ receptor family member 7 (mPR_{α})	NM_001038553.1	F: CCGGCGGTCCATCTATGA R: CCACCCCCTTCACTGAGTCTT	159	99%
PAQR8	Progestin and adipoQ receptor family member 8 (mPR_{β})	NM_001101135.2	F: TGTAGCCTTGCGAGACACAG R: CAGCATCGCAGAAGAATGCC	214	100%
PTGFR	Prostaglandin F receptor	NM_181025.3	F: TGGTGTTCTCTGGTCTGTGC R: GGCTAGGAGCCCCAGAAAAG	293	100%
IFNAR1	Bos taurus interferon alpha and beta receptor subunit 1	NM_174552.2	F: ACAGGCGGAATAAAGGGAGC R: GGCTGATCGGAGAAATACTCGT	220	99%
IFNAR2	Bos taurus interferon alpha and beta receptor subunit 2	NM_174553.2	F: CCCAGACGAGAATCAGAGTCAT R: TGGGGAGCTGCCTCATTTTC	299	100%
Housekeeping Transcripts					
ACTB	Actin beta	NM_173979.3	F: GAGCGGGAAATCGTCCGTGAC R: GTGTTGGCGTAGAGGTCCTTGC	278	99%
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	NM_001034034.2	F: ACATCAAGTGGGGTGATGCT R: GGCATTGCTGACAATCTTGA	201	97%
SDHA	Succinate dehydrogenase complex flavoprotein subunit A	NM_174178.2	F: GCAGAACCTGATGCTTTGTG R: CGTAGGAGAGCGTGTGCTT	185	99%

Table 5.3 Real-time PCR[1] identification of selected interferon-stimulated genes from CAR and ICAR endometrium of heifers supplemented with 3 mg Se/d in vitamin-mineral mixes as sodium selenite (ISe, n=6) or a 1:1 blend (MIX, n=6) of ISe and OSe (SEL-PLEX).[2]

		CAR[3]				ICAR[3]			
Gene	Gene Name	Ise	MIX	SEM	P-value	ISe	Mix	SEM	P-value
ACKR3**	Bos taurus atypical chemokine receptor 3	1.05	0.82	0.128	0.2175	1.03	0.86	0.159	0.2866
IFIT3**	Bos taurus interferon induced protein with tetratricopeptide repeats 3	1.08[a]	0.65[b]	0.13	0.0415	1.07	0.85	0.148	0.370
IRF1*	Bos taurus interferon regulatory factor 1	1.05[x]	0.75[y]	0.111	0.0815	1.03	0.94	0.119	0.5845
IRF2	Bos taurus interferon regulatory factor 2	1.02[x]	0.83[y]	0.059	0.0564	1.02	0.87	0.076	0.1884
ISG15**	Bos taurus ISG15 ubiquitin like modifier	1.04[a]	0.76[b]	0.088	0.0476	1.05	0.94	0.104	0.650
MSXI	Bos taurus msh homeobox 1	1.05	1.08	0.121	0.8452	1.08	0.85	0.146	0.2896
MX1**	Bos taurus MX dynamin like GTPase 1	1.00[a]	0.79[b]	0.059	0.0282	1.02	0.94	0.076	0.5332
MX2	Bos taurus MX dynamin like GTPase 2	1.03	0.79	0.100	0.1064	1.04	0.82	0.123	0.2401
OAS1	Bos taurus 2',5'-oligoadenylate synthetase 1	1.02	0.82	0.154	0.3843	1.04	0.94	0.229	0.7507
OAS2*	Bos taurus 2'-5'-oligoadenylate synthetase 2	1.03[a]	0.67[b]	0.097	0.0133	1.05	0.88	0.116	0.3358
RSAD2*	Bos taurus radical S-adenosyl methionine domain containing 2	1.05[a]	0.57[b]	0.137	0.0109	1.02	0.82	0.080	0.1005

[1] Data are expressed as a ratio of MIX relative to ISe expression.

Table 5.3 (continued)
[2] Selenium was supplemented at 35 ppm as either inorganic (ISe; sodium selenite) or a 1:1 combination (MIX) of ISe and OSe (SEL-PLEX). Selenium was supplemented to treatment groups *ad libitum*.
[3]Values are LS means and SEM.
[a,b] Means within a row that lack a common superscript differ ($P<0.05$)
[x,y] Means within a row that lack a common superscript tend to differ ($0.05 < P < 0.10$)
*CAR natural log transformed due to lack of normality
**ICAR natural log transformed due to lack of normality
***CAR and ICAR natural log transformed due to lack of normality

Table 5.4 Real-time PCR[1] identification of selected progesterone-induced genes from CAR and ICAR endometrium of heifers supplemented with 3 mg Se/d in vitamin-mineral mixes as sodium selenite (ISe, n=6) or a 1:1 blend (MIX, n=6) of ISe and OSe (SEL-PLEX).[2]

		CAR[3]				ICAR[3]			
Gene	Gene Name	Ise	MIX	SEM	P-value	ISe	Mix	SEM	P-value
DGAT2**	Bos taurus diacylglycerol O-acyltransferase 2	1.05[a]	0.64[b]	0.109	0.0254	1.03	0.88	0.130	0.3518
DKK1	Bos taurus dickkopf WNT signaling pathway inhibitor 1	1.07	0.93	0.166	0.5515	1.10[a]	0.64[b]	0.147	0.0496
FABP3*	Bos taurus fatty acid binding protein 3	1.38	1.38	0.426	0.9208	1.77	1.82	0.464	0.9375
FGF2	Bos taurus fibroblast growth factor 2	1.04[a]	0.71[b]	0.097	0.0407	1.02	0.95	0.145	0.7203
FOXL2	Bos taurus forkhead box L2	1.05[x]	0.70[y]	0.119	0.0657	1.02	0.79	0.120	0.2088
HOXA10	Bos taurus homeobox A10	1.04	0.91	0.096	0.3343	1.07[x]	0.71[y]	0.143	0.0974
IGFBP1***	Bos taurus insulin like growth factor binding protein 1	1.09	0.78	0.201	0.2356	1.21	0.98	0.239	0.6958
IHH	Bos taurus Indian hedgehog signaling molecule	1.16	1.23	0.241	0.8348	1.08	0.89	0.163	0.4307
MSTN	Bos taurus myostatin	1.05	1.45	0.177	0.1370	1.03[a]	1.71[b]	0.140	0.0070
SLC1A3**	Bos taurus solute carrier family 1 member 3	1.04	1.09	0.129	0.7999	1.04	0.90	0.122	0.4045
SLC1A5	Bos taurus solute carrier family 1 member 5	1.06	0.91	0.124	0.4239	1.04	1.01	0.128	0.8698

Table 5.4 (continued)

SLC46A3	Bos taurus solute carrier family 46 member 3	1.05	1.25	0.149	0.3831	1.09	1.19	0.189	0.7140

[1] Data are expressed as a ratio of MIX relative to ISe expression.
[2] Selenium was supplemented at 35 ppm as either inorganic (ISe; sodium selenite) or a 1:1 combination (MIX) of ISe and OSe (SEL-PLEX). Selenium was supplemented to treatment groups *ad libitum*.
[3]Values are LS means and SEM.
[a,b] Means within a row that lack a common superscript differ ($P \leq 0.05$)
[x,y] Means within a row that lack a common superscript tend to differ ($0.05 < P < 0.10$)
*CAR natural log transformed due to lack of normality
**ICAR natural log transformed due to lack of normality
***CAR and ICAR natural log transformed due to lack of normality

Table 5.5 Real-time PCR[1] identification of selected steroidogenic enzymes and receptor genes from CAR and ICAR endometrium of heifers supplemented with 3 mg Se/d in vitamin-mineral mixes as sodium selenite (ISe, n=6) or a 1:1 blend (MIX, n=6) of ISe and OSe (SEL-PLEX)[2].

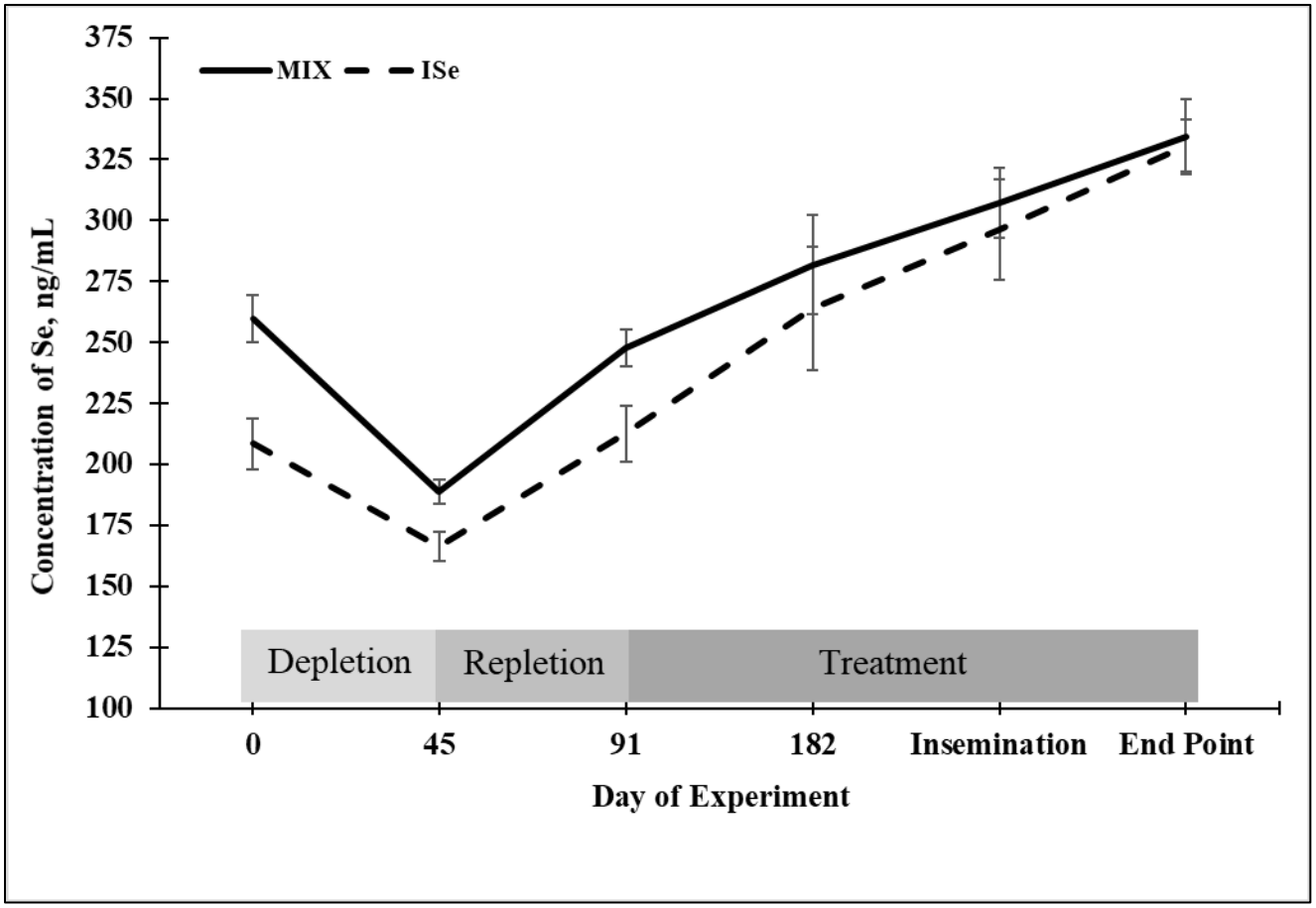
		CAR[3]				ICAR[3]			
Gene	Gene Name	Ise	MIX	SEM	P-value	ISe	Mix	SEM	P-value
OXTR**	Bos taurus oxytocin receptor	1.54	1.40	0.489	0.8533	2.2	2.03	0.563	0.9095
ESR1	Estrogen receptor 1	1.07	0.79	0.130	0.1620	1.04	0.86	0.107	0.2603
PGR	Nuclear progesterone receptor	1.03^{x}	0.77^{y}	0.094	0.0738	1.06	1.01	0.175	0.8259
PGRMC1**	Progesterone receptor membrane component 1	1.02	1.10	0.078	0.4961	1.05	0.98	0.108	0.8517
PGRMC2	Progesterone receptor membrane component 2	1.05	1.02	0.119	0.8486	1.03	1.03	0.094	0.9656
EP1[4]**	Prostaglandin E receptor 1	1.04	0.89	0.100	0.3295	1.02	1.11	0.223	0.5907
EP2	Prostaglandin E receptor 2	1.18	1.09	0.207	0.7714	1.09	1.39	0.167	0.2335
EP3**	Prostaglandin E receptor 3	1.20	0.75	0.212	0.1687	1.34	1.07	0.39	0.4176
EP4*	Prostaglandin E receptor 4	1.01	0.88	0.107	0.2024	1.04	1.12	0.142	0.6971
PAQR5[5]*	Progestin and adipoQ receptor family member 5 (mPR_{γ})	1.31	1.24	0.246	0.6879	1.07	1.15	0.167	0.7570
PAQR7	Progestin and adipoQ receptor family member 7 (mPR_{α})	1.05	0.78	0.132	0.1772	1.06^{x}	0.73^{y}	0.128	0.0934

Table 5.5 (continued)

PAQR8	Progestin and adipoQ receptor family member 8 (mPRβ)	1.07	0.89	0.141	0.3700	1.06	1.03	0.151	0.9067
PTGFR***	Prostaglandin F receptor	2.28	1.22	0.716	0.8113	4.19	5.08	2.513	0.2158
IFNAR1	Bos taurus interferon alpha and beta receptor subunit 1	1.05	0.86	0.103	0.2297	1.04	0.94	0.130	0.6095
IFNAR2*	Bos taurus interferon alpha and beta receptor subunit 2	1.02	0.89	0.094	0.3071	1.02	0.91	0.114	0.4811
PTGES	Prostaglandin E synthase	1.11	0.92	0.154	0.3875	1.36	0.83	0.276	0.2095
PTGS2***	Prostaglandin-endoperoxide synthase 2	1.05	0.91	0.194	0.4047	1.20	1.56	0.228	0.2484

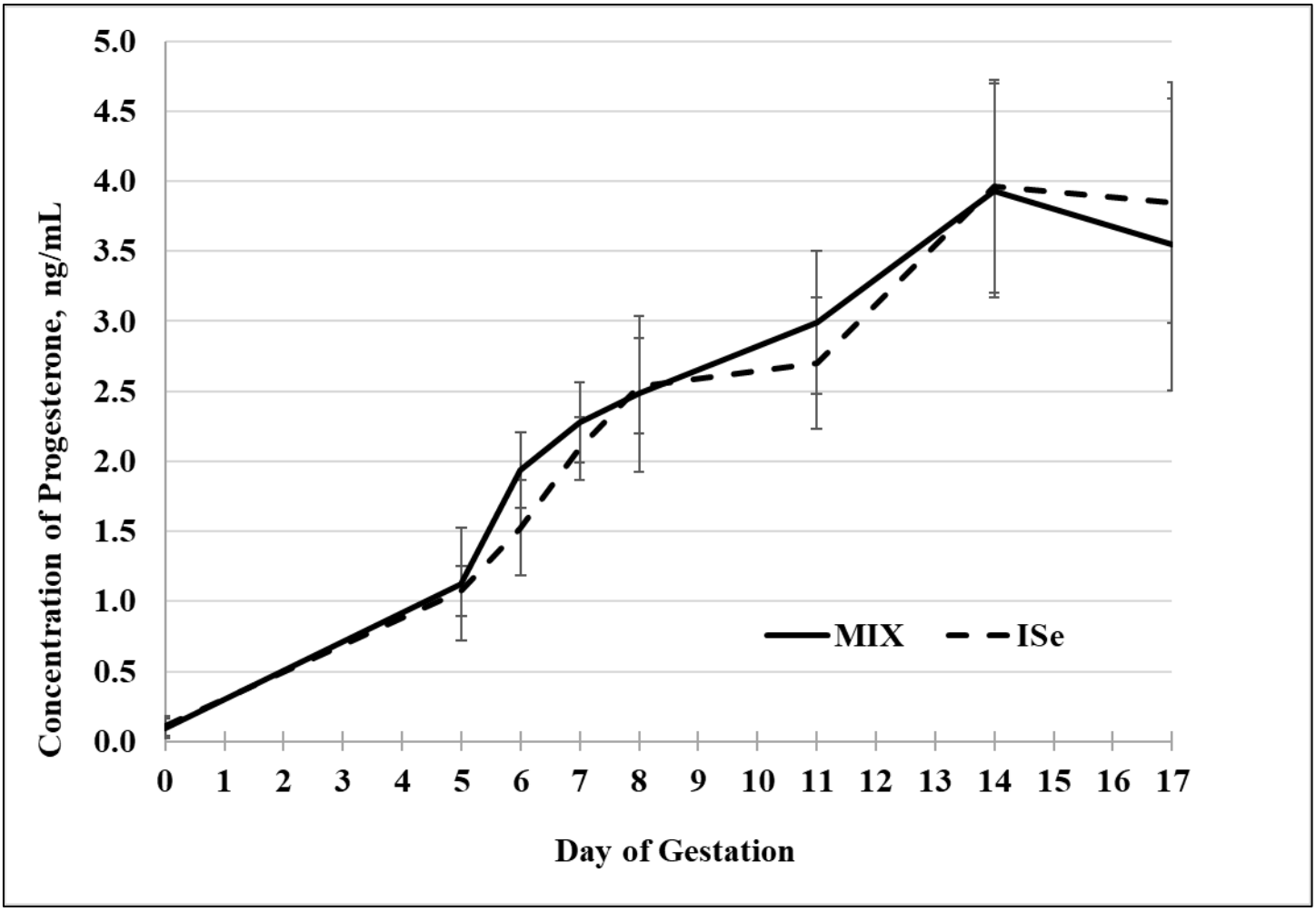
[1] Data are expressed as a ratio of MIX relative to ISe expression.
[2] Selenium was supplemented at 35 ppm as either inorganic (ISe; sodium selenite) or a 1:1 combination (MIX) of ISe and OSe (SEL-PLEX). Selenium was supplemented to treatment groups *ad libitum*.
[3]Values are LS means and SEM.
[4] From (Weems et al., 2012).
[5] From (Kowalik et al., 2018).
[a,b] Means within a row that lack a common superscript differ ($P \leq 0.05$)
[x,y] Means within a row that lack a common superscript tend to differ ($0.05 < P < 0.10$)
*CAR natural log transformed due to lack of normality
**ICAR natural log transformed due to lack of normality
***CAR and ICAR natural log transformed due to lack of normality

Figure 5.1 Concentration of Total Se in Whole Blood.



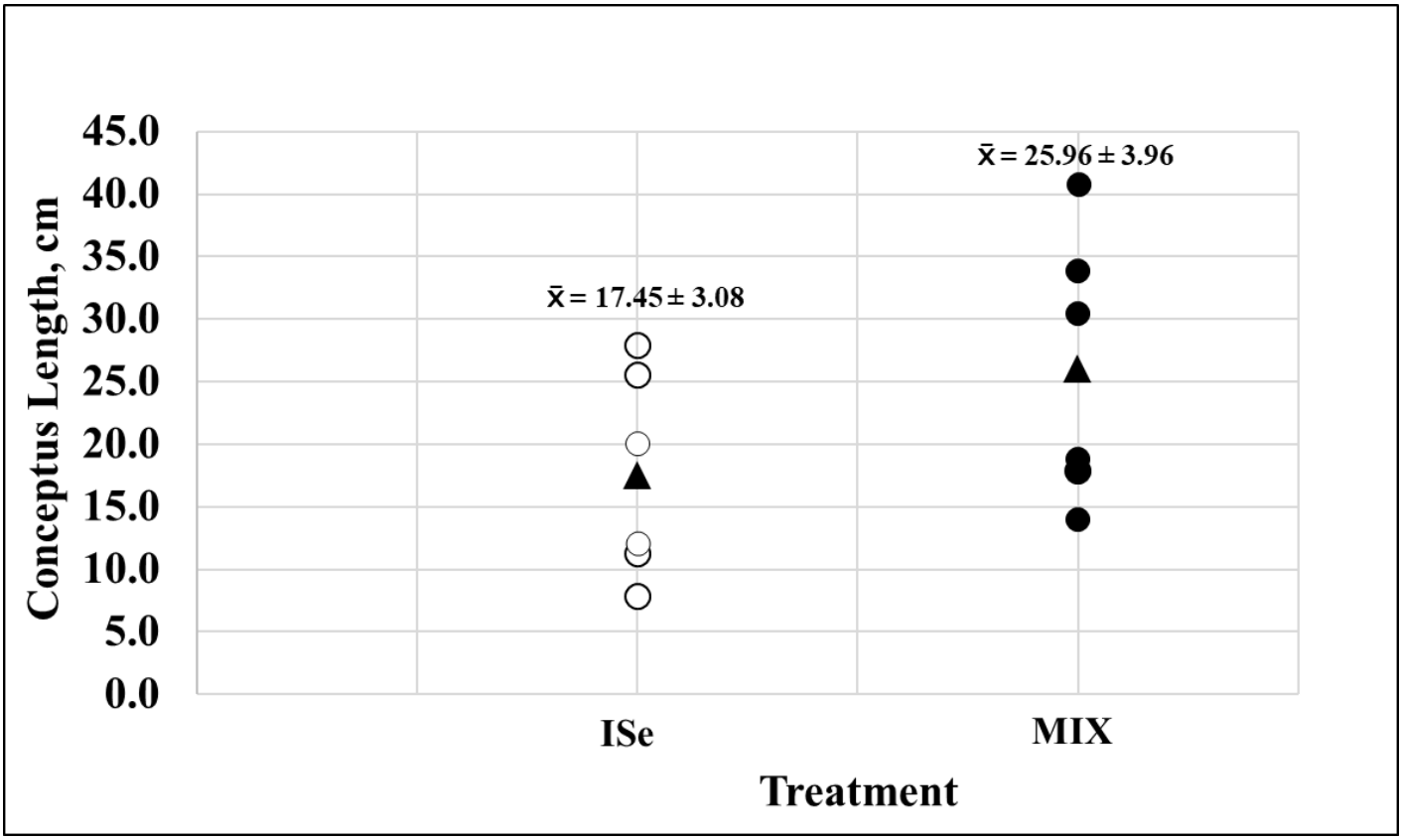
Effect of form of Se on whole blood concentrations (ppm; LS Mean ± SEM) of Se in cows supplemented with either ISe (Sodium selenite; n = 6) or a 1:1 combination (MIX) of ISe and OSe (Sel-Plex; n = 6). Data were analyzed as an ANOVA with repeated measures. Whole blood Se tended to be affected by treatment ($P = 0.0704$) and was affected by time ($P < 0.0001$), but there was no significant treatment by time interaction ($P=0.5066$).

Figure 5.2 Concentration of Progesterone in Serum.



Effect of form of Se on serum concentrations (ng/mL; LS Mean ± SEM) of Se in cows supplemented with either ISe (Sodium selenite; n = 6) or a 1:1 combination (MIX) of ISe and OSe (Sel-Plex; n = 6). Data were analyzed as an ANOVA with repeated measures. Serum progesterone was not affected by treatment (*P = 0.88*) but was affected by day (*P <0.0001*), however there was no treatment by day interaction (*P=0.77*).

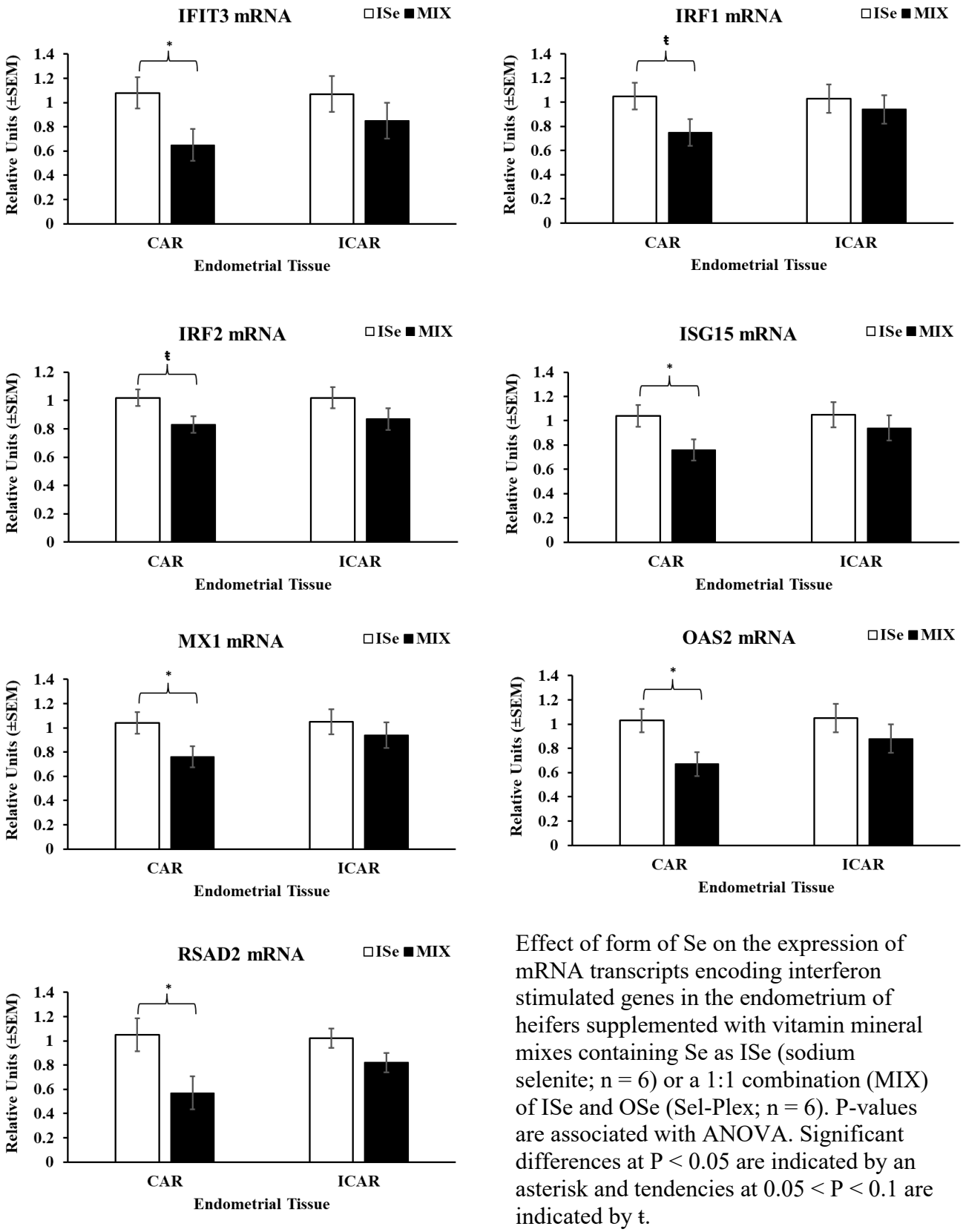
Figure 5.3 Effect of Treatment on Conceptus Length.



Effect of form of Se on average conceptus length (cm; LS Mean ± SEM) in heifers supplemented with either ISe (Sodium selenite; n = 6) or a 1:1 combination (MIX) of ISe and OSe (Sel-Plex; n = 6). Recovered conceptuses are represented by individual dot in their respective treatments and within treatment the mean is represented by a black triangle. Data were analyzed as a one-tailed student's T-TEST.
[a,b]Means with different superscripts differ *p=0.0533.*

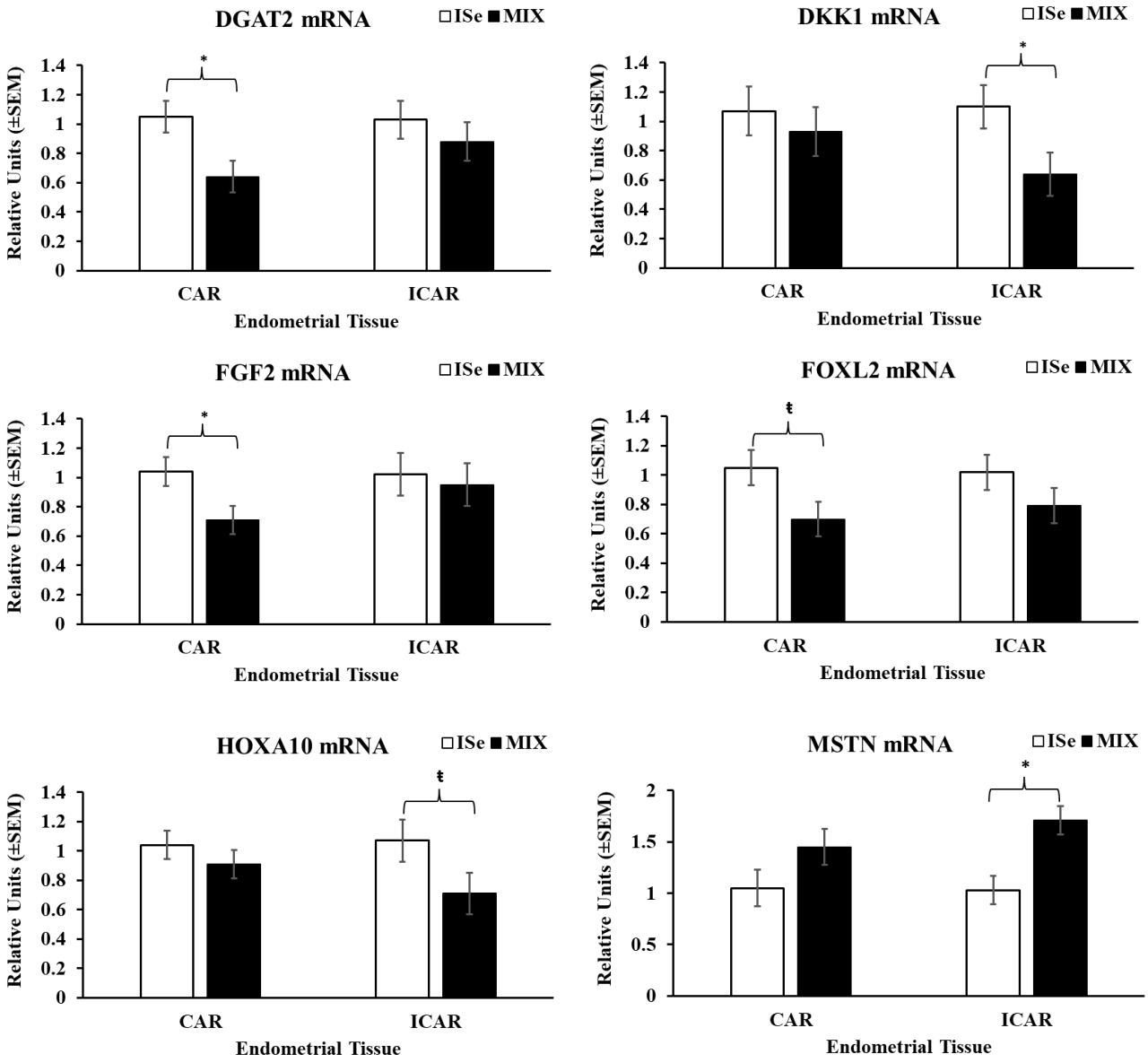
Figure 5.4 Representative image of conceptus collected on day 17 of gestation.

Figure 5.5 Relative expression of mRNA transcripts encoding interferon stimulated genes.



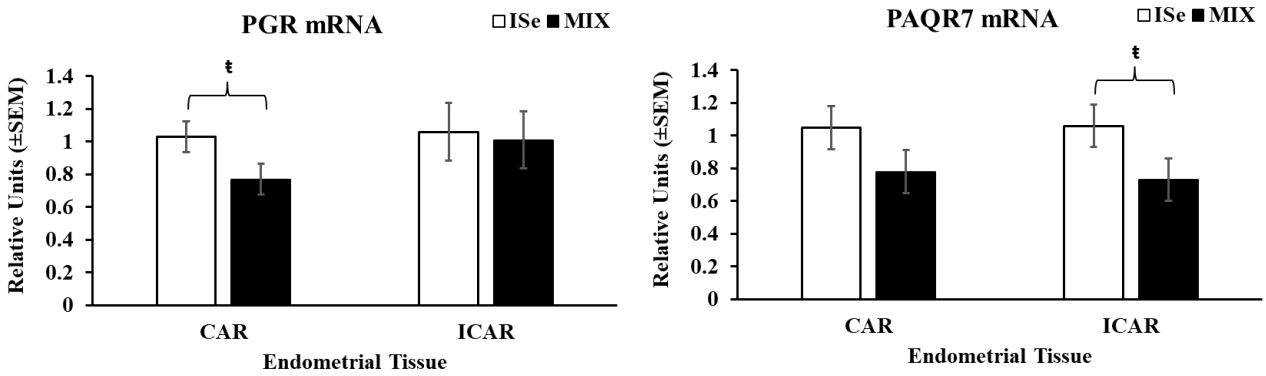
Effect of form of Se on the expression of mRNA transcripts encoding interferon stimulated genes in the endometrium of heifers supplemented with vitamin mineral mixes containing Se as ISe (sodium selenite; n = 6) or a 1:1 combination (MIX) of ISe and OSe (Sel-Plex; n = 6). P-values are associated with ANOVA. Significant differences at $P < 0.05$ are indicated by an asterisk and tendencies at $0.05 < P < 0.1$ are indicated by ŧ.

Figure 5.6 Relative expression of mRNA transcripts encoding progesterone-induced genes.



Effect of form of Se on the expression of mRNA transcripts encoding interferon stimulated genes in the endometrium of heifers supplemented with vitamin mineral mixes containing Se as ISe (sodium selenite; n = 6) or a 1:1 combination (MIX) of ISe and OSe (Sel-Plex; n = 6). P-values are associated with ANOVA. Significant differences at $P < 0.05$ are indicated by an asterisk and tendencies at $0.05 < P < 0.1$ are indicated by ŧ.

Figure 5.7 Relative expression of mRNA transcripts encoding recepto r genes.



Effect of form of Se on the expression of mRNA transcripts encoding interferon stimulated genes in the endometrium of heifers supplemented with vitamin mineral mixes containing Se as ISe (sodium selenite; n = 6) or a 1:1 combination (MIX) of ISe and OSe (Sel-Plex; n = 6). P-values are associated with ANOVA. Tendencies at $0.05 < P < 0.1$ are indicated by ŧ.

CHAPTER 6. Summary and Conclusions

Cattle operations in the southeast United States are challenged by grazing Se-inadequate forages due to Se-deficient soils. The effects of Se-deficiencies have been evaluated in many studies and have been implicated with immune, growth, and reproductive challenges. Our lab has previously reported increased early concentrations of P4 in cows supplemented with a 1:1 combination of ISe:OSe (MIX) compared to cows supplemented with OSe or ISe alone on days 6 (Cerny et al., 2016b) and 7 (Carr et al., 2020) of the estrous cycle. The benefits of increased early luteal phase P4 on endometrium secretions, conceptus development, IFNT production, and conception rates are well known. However, to our knowledge, studies regarding the mechanism of form of Se-induced increased concentrations of early luteal phase P4 or effects on the uterine endometrium and conceptus have not been reported. Therefore, the overall goals of this dissertation were to investigate whether the form of supplemental Se (ISe and MIX) in vitamin-mineral mixes would affect the early cycle bovine CL and the uterine endometrium and conceptus at maternal recognition of pregnancy. More specifically, the objectives were 1) to investigate the effect of form of supplemental Se on the transcriptome of the bovine CL with the goal of elucidating form of Se-regulated luteal processes affecting fertility (Experiment 1, Chapter 4) and 2) to determine changes induced by the form of supplemental Se on the bovine endometrium and developing conceptus on day 17 of pregnancy (Experiment 2, Chapter 5).

In the first experiment (Chapter 4), Angus-cross cows were supplemented (3 mg/d) with MIX or ISe forms of Se to evaluate the transcriptome profiles (microarray) of

CL on day 7 of the estrous cycle. The concentrations of Se in whole blood was not statistically different between treatments, however we found that cows supplemented with MIX had increased expression of several key transcripts involved in cholesterol biosynthesis and immune response elements compared to those supplemented with ISe alone. The results from the microarray analysis on the CL confirmed the top canonical pathways were those associated with cholesterol biosynthesis and inflammatory responses. Importantly, MIX-induced upregulation of cholesterol biosynthesis pathways and associated transcripts play a pivotal role in increasing the early luteal phase concentration of P4, which is a salient finding of this research.

The timing and process of MRP are essential for the maintenance of both the CL and developing conceptus. In a two-week period surrounding MRP, pregnancy losses average ~30%. This reduction in pregnancies has a negative impact on the reproductive performance and profit potential within a herd. Therefore, the objectives of Experiment 2 (Chapter 5), were to investigate the effect of form of supplemental Se on gene expression in bovine endometrium and the developing conceptus on day 17 of pregnancy. Angus-cross, fall yearling heifers were supplemented (3 mg/d) with MIX or ISe forms of Se to evaluate the gene expression in the endometrium as well as alterations to the developing conceptus on day 17 of gestation. Results from this experiment indicated that ISe-supplemented heifers had increased expression of several P4-induced and interferon-stimulated mRNA transcripts, including the mRNA encoding the nuclear P4 receptor. Additionally, MIX-supplemented heifers had increased mRNA abundance of *MSTN*, which increases glucose secretion into histotroph, thus advancing conceptus development. Interestingly, and a salient finding of this experiment was a MIX-induced

increase in conceptus length was observed compared to heifers supplemented with ISe. It appears that the onset of MRP may be shifted and occurs earlier in MIX supplemented heifers compared to those supplemented with ISe alone.

In conclusion, this dissertational research describes novel effects of different forms of dietary Se (ISe or MIX) on the CL and endometrium gene expression profiles and conceptus development. MIX treated cows and heifers had increased expression of transcripts involved in cholesterol biosynthesis and advanced conceptus development compared to those supplemented with ISe. The translational impact of these studies has the potential to improve fertility and profit potential in beef and dairy operations. By understanding how the CL and endometrium responds to supplemental form of Se, targeted vitamin-mineral mix supplementation strategies can be explored in the future. Further research is warranted to determine form of Se-induced changes after MRP and implantation have occurred, which would add to the overall impact on fertility.

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Publications

Refereed Journal Articles

B.R. Crites, R. Vishwanath, A. Arnett, P.J. Bridges, W.R. Burris, and L.H. Anderson. 2021. Conception risk of beef heifers inseminated with either SexedULTRA 4M™ or conventional semen after ovulation is synchronized using a modified 14-Day CIDR-PG protocol. *In preparation.*

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