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SELENIUM FORM-INDUCED CHANGES IN THE EARLY LUTEAL PHASE CORPUS LUTEUM, THE BLOOD, AND THE ENDOMETRIUM DURING EARLY GESTATION IN BEEF COWS

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SELENIUM FORM-INDUCED CHANGES IN THE EARLY LUTEAL PHASE CORPUS LUTEUM, THE BLOOD, AND THE ENDOMETRIUM DURING EARLY GESTATION IN BEEF COWS

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:
Sarah Nancy Carr

Lexington, Kentucky

Director: Dr. Phillip J. Bridges, Associate Professor of Animal and Food Sciences

Lexington, Kentucky

2023

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ABSTRACT OF DISSERTATION

SELENIUM FORM-INDUCED CHANGES IN THE EARLY LUTEAL PHASE CORPUS LUTEUM, THE BLOOD, AND THE ENDOMETRIUM DURING EARLY GESTATION IN BEEF COWS

Selenium (Se) deficient soils and thus forages are widespread throughout the United States, necessitating supplementation of this trace mineral in the diet of grass-fed cattle to negate detrimental effects of a deficiency on immune function, growth, and fertility. Conventionally, Se is supplemented to cattle in a vitamin-mineral mix as an inorganic form (ISe) although the organic forms (OSe) are available when cattle consumer forage, and OSe has been shown to increase the bioavailability of Se in cattle. Previous research has demonstrated supplementation with 1:1 mixture (MIX) of ISe and OSe compared to ISe alone increases systemic concentrations of progesterone (P4) in the early luteal phase of cows at a time in which the concentration of P4 can critically influence the endometrium and developing conceptus. Herein, a series of experiments were conducted to 1) determine the mechanism responsible for the MIX-induced increase in the early luteal phase concentration of systemic P4, 2) identify form of Se effects on systemic blood metabolites during early gestation, and 3) investigate interferon-induced changes in the caruncular (CAR) tissue of the endometrium as a response of different supplemental forms of Se (ISe vs. MIX) which may affect the ensuing process of implantation. The objectives of Experiment 1 were to confirm and expand upon the initial report of the MIX-induced increase in early luteal phase P4, determining systemic changes in P4 throughout gestation, and prolactin (PRL) throughout lactation. We observed a MIX-induced increase in systemic concentrations of P4 on day 7 of the estrous cycle, consistent with our initial report, with circulating P4 then remaining higher throughout gestation. In contrast, prolactin was lower in the MIX supplemented heifers at months 5 and 6 of lactation; supplemental form of Se can be used to manipulate early luteal phase and gestational concentrations of P4, and postpartum concentration of PRL. The objective of Experiment 2 was to investigate the mechanistic changes in the CL contributing to the increase in systemic P4 by analyzing changes in mRNA transcripts in the CL and the responses of steroidogenic luteal cells to select agonists *in vitro*. Initially, we observed human chorionic gonadotropin (hCG)-induced increases in P4 in culture media of luteal cells obtained from ISe-supplemented cows. Interestingly, qPCR revealed

no significant differences in the steroidogenic transcripts in the CL, however the abundance of mRNA encoding 2 key transcripts regulating cholesterol uptake (Ldlr and Hsl) was increased in MIX-supplemented cows, collectively suggesting that the form of Se-induced effects on luteal production of P4 are the result of changes in cholesterol availability, rather than a direct effect on the expression of steroidogenic enzymes within the CL. Following Experiment 2, we investigated changes in systemic blood metabolites during early gestation in response to the form of Se. Therefore, the objective of Experiment 3 was to quantify relative changes in serum metabolic parameters at estrus, during the early luteal phase, and at maternal recognition of pregnancy (MRP). MIX-supplemented heifers compared to ISe had significantly lower concentration of total serum cholesterol and low/very low-density lipoproteins, as well as changes in serum glucose, aspartate aminotransferase (AST), and beta hydroxybutyrate (BHBA). The objective of Experiment 4 was to investigate transcriptomic changes in the CAR regions of the endometrium at MRP. Results revealed significant changes in interferon signaling and endometrial restructuring that would be expected just prior to implantation. Given the advanced conceptus development observed in MIX vs. ISe-supplemented heifers, coincident with the concurrent decrease in the level of expression of several known MRP-associated transcripts, it appears that the MIX supplement is shifting the timing of MRP, resulting in hastened conceptus development. Encompassing all results, manipulating the form of Se in the diet of grazing beef cattle significantly alters systemic levels of P4, blood metabolites, and transcriptomic changes in the CL and endometrium that can advance conceptus development during early gestation. MIX Se is a novel producer-friendly supplement that can ultimately improve whole animal physiology and fertility.

KEYWORDS: selenium; corpus luteum; progesterone; endometrium; gestation; maternal recognition of pregnancy

Sarah Nancy Carr

04/20/2023

Date

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Chapter 1. Introduction

Selenium (Se) is a trace mineral naturally occurring in the soils and the content varies according to the type, texture, organic matter, and amount of precipitation in the geographic region (Mehdi and Dufrasne, 2016). Selenium is an essential trace mineral in the diet of cattle, and this is problematic in regions where the soils are deficient because it translates into forages that are deficient (Ammerman and Miller, 1975). There is a widespread deficiency in Se across the United States, particularly in the Southeast region; therefore, it is unsurprising that producers in this region have the highest proportion of cattle classified as Se-deficient (Dargatz and Ross, 1996). This necessitates supplemental dietary Se to alleviate the negative effects of a Se deficiency on immunity (Erskine et al., 1989), growth (Gleed et al., 1983), and fertility (McClure et al., 1986). Sodium selenate and sodium selenite, the inorganic forms of Se (ISe), as well as selenomethionine (SeMet) and selenocysteine (SeCys), the organic forms of Se (OSe), have been approved for supplementation by the Food and Drug Administration (FDA (FDA, 2020)). Producers conventionally supplement Se as ISe although OSe-forms are available when cattle consume forage (Ammerman and Miller, 1975) and are more bioavailable than ISe (Khanam and Platel, 2016) presenting a conundrum that warrants further investigation.

Additionally, cattle producers are challenged with a high percentage of early embryonic loss directly impacting the profitability of beef and dairy operations. Rate of fertilization to artificial insemination (AI) ranges between 90-100% given high fertility semen is used (Diskin et al., 2006; Diskin and Morris, 2008); however, it is estimated that only 50-60% of inseminated cows remain pregnant by day 30 of gestation (Bridges et al., 2013). This large proportion of early embryonic mortality primarily occurs between days

8 and 16 of gestation with 70-80% of loss occurring during this time (Sreenan and Diskin, 1986).

Successful establishment and maintenance of pregnancy is dependent on progesterone (P4), which is commonly referred to as the hormone of pregnancy. During the early luteal phase, a relatively lower concentration of P4 can lead to lower conception rates (Mann and Lamming, 1999) whereas elevated concentrations of P4 have resulted in advanced development of the conceptus (Carter et al., 2008) and increased production of interferon tau (IFNτ (Mann and Lamming, 2001)). IFNτ is the signal for maternal recognition of pregnancy (MRP) in cattle and is produced by the mononuclear trophoblastic cells of the trophectoderm to inhibit increasing pulses of luteolytic prostaglandin F2α (PGF2α) and thus maintain the secretion of P4 by the corpus luteum (CL). This, in turn, will stimulate the production of histotroph by uterine glands in the endometrium to nourish the developing conceptus, as well as signal changes in the endometrium allowing embryonic implantation (Spencer et al., 2007).

Concerning the aforementioned challenges in the beef industry, our lab determined that feeding a MIX form of Se containing a 1:1 ratio of ISe:OSe compared to ISe or OSe alone resulted in a significant increase in the peripheral concentration of P4 on day 6 of the estrous cycle, with no changes to the diameter of the CL (Cerny et al., 2016b). This piloted the current research which has investigated the Se-form induced mechanistic changes in the early luteal phase, as well as changes in the blood and endometrium during early gestation in cows. The specific goals of this dissertation are identified in section 3.1. Objectives and hypotheses.

Chapter 2. Review of literature

2.1. Selenium in cattle nutrition

2.1.1. Supplementation of selenium

Selenium (Se) is a trace mineral that was first discovered by Jöns Jacob Berzelius in 1817 (Johansson et al., 2005; Boyd, 2011). It was primarily recognized as having toxic effects on both humans and livestock (Franke, 1934); however, it was then appreciated as essential in the diet of animals in 1957 and the role of this mineral was defined within the subsequent years (Schwarz and M., 1957; McCoy and Weswig, 1969; Thompson and Scott, 1969). Selenium is an essential micronutrient at low levels, but is toxic at high levels (Hatfield et al., 2014), providing a narrow aperture between dietary adequacy and toxicity (Shini et al., 2015).

The content of Se in the soil varies depending on the composition of the soil, including the content of organic matter, type, texture, and levels of precipitation, and similarly, the content of Se in forages varies depending on the composition of species of the forage available, the type of soil in which the forage is grown, and the region of production (Mehdi and Dufrasne, 2016). Generally, quantification of Se in different feedstuffs has significant variation ranging from 0.23 to 2.663 ppm Se, and particularly in shelled corn, the concentration of Se ranges from 0.017 to 0.219 ppm (Perry et al., 1976) which undoubtedly provides challenges for cattle producers particularly in Se-deficient regions.

Across the United States, the concentration of Se in the soils and subsequently the forages available varies by geographic region (Ammerman and Miller, 1975) and the concentration of Se in the forages and grains in the southeastern United States including

Kentucky are low, at less than 0.05 ppm, to variable with only about 50% of the feedstuffs produced containing greater than 0.1 ppm. In beef cattle, the minimal recommended amount of dietary Se is 0.1 ppm (NASEM, 2016), and the maximum recommendation is 0.3 ppm in feed with the maximum Se intake being no greater than 3 mg Se per animal per day (FDA, 2020). Unsurprisingly, producers in the southeast region have the greatest amount of cattle considered Se-deficient, and a higher proportion of these producers compared to those in other geographical regions supplement Se to their cattle to maintain Se-adequate status (Dargatz and Ross, 1996). Supplementation conventionally occurs by providing *ad-libitum* access to a vitamin-mineral block, loose mixture, liquid supplement, or molasses-based tub (Greene, 2000). Providing this auxiliary Se to overcome a deficiency in this trace mineral is essential for various physiological processes including immune function (Erskine et al., 1989; Arthur et al., 2003), growth (Gleed et al., 1983), antioxidant status (Sgoifo Rossi et al., 2017), and fertility (McClure et al., 1986).

From a panoptic perspective, Se exists in either inorganic or organic forms. Organic forms of Se (OSe) are the primary forms in forages; however, the inorganic forms (ISe) are more commonly used in commercial vitamin-mineral mixes supplemented to livestock in Se deficient regions (Ammerman and Miller, 1975; Podoll et al., 1992). The inorganic forms of Se are sodium selenate (Na_2SeO_4) and sodium selenite (Na_2SeO_3) which are produced synthetically and are classified as selenols (R-Se-H, (Reddy and Massaro, 1983; Suzuki, 2005)). First, elemental Se is oxidized to form selenous acid and then subsequently neutralized to form selenium selenite. This product

can be further oxidized with hydrogen peroxide (H_2O_2) to form sodium selenate (Björnberg et al., 1984) and both Se salts can be incorporated into the diet of ruminants.

The organic forms of Se are selenocysteine (SeCys) and selenomethionine (SeMet, (Suzuki, 2005)). Structurally, SeMet is an analogue of methionine where Se has replaced the sulfur, and SeCys is an analogue of cysteine where sulfur is replaced by Se (Reddy and Massaro, 1983). Due to the physical and chemical similarities between Se and sulfur, plants cannot determine the differences between the two minerals and will synthesize SeMet rather than methionine when Se is available (Lyons et al., 2007) resulting in SeMet existing in the greatest abundance in plants (Mangiapane et al., 2014). Commercially, OSe is made available through cultivated strains of yeast (*Saccharomyces cerevisiae*), and similar to forages, the Se enriched yeasts contain SeMet as the dominant form (Korhola et al., 1986). SeCys is a cysteine residue with a selenol group in place of the thiol group in this amino acid (Johansson et al., 2005), and SeCys is incorporated into selenoproteins via translation with a UGA codon specific to the SeCys residue (Suzuki, 2005).

2.1.2 Selenium metabolism

Upon consumption, different forms of Se have different fates in the gastrointestinal tract of the ruminants due to the metabolic activity of the microbial population. In the rumen, selenate may be reduced to selenite (Paulson et al., 1968) and selenite can be further reduced to insoluble, lowly digestible Se compounds. Alternatively, SeMet appears less susceptible to chemical changes in the rumen (Paulson et al., 1968).

Regardless of the source, Se is absorbed through the epithelium of the duodenum, jejunum, and ileum of the small intestine through various mechanisms dependent on the form available (Burk and Hill, 2015). The absorption of SeMet is rapid through all portions of the small intestine (Vendeland et al., 1992). The inorganic forms, selenate and selenite are absorbed primarily through the distal regions of the small intestine in the rat (Ardüser et al., 1985; Wolffram et al., 1985). However, in ruminants, the absorption of sodium selenite appears to occur primarily in the duodenum, and there is little evidence to support absorption through the wall of the rumen, abomasum, jejunum, or ileum (Wright and Bell, 1966). Additionally, it appears that Se is not absorbed as efficiently in ruminants as it is in monogastric species where the absorption of selenite was about 34% in sheep compared to 85% in swine as determined by an orally dosed Se^{75} disappearance study (Wright and Bell, 1966; Mahan et al., 1999). The efficiency of absorption of selenite in ruminants has been reported to be as low as 29% (Mehdi et al., 2013; Mehdi and Dufrasne, 2016). This lower absorption efficiency of Se in ruminants is thought to be attributed to activity of the ruminal microbial environment reducing dietary Se to insoluble forms of Se such as elemental Se or selenides (Cousins and Cairney, 1961; Peterson and Spedding, 1963; Spears, 2003).

Generally, the uptake of Se occurs via diffusion (McConnell and Cho, 1965; Wolffram et al., 1985), though some research suggests a role for various transport systems (Cousins and Liuzzi, 2018) including the X_c^- cysteine/glutamate antiporter (Olm et al., 2009), the amino acid transporter 1 (SLC3A1) and the neutral amino acid transporter (SLC1A4) similarly to amino acid transport (Cousins and Liuzzi, 2018).

Once absorbed, Se is directly transported to the liver through the portal drainage system (Kato et al., 1992) where the liver critically regulates whole body concentrations of Se via selenoprotein synthesis or catabolism for excretion (Burk and Hill, 2015). The inorganic forms of Se, sodium selenite and sodium selenate, can be reduced to selenide in the liver (Schrauzer, 2000; Suzuki, 2005) and if not readily converted to SeCys for incorporation into a selenoprotein, it is methylated and excreted (Schrauzer, 2000; Sgoifo Rossi et al., 2017). Excretion of Se occurs predominantly through feces as a result of failed intestinal absorption, and the concentration of Se secreted in urine is relatively lower (Mehdi et al., 2013). Selenosugar is the only identified Se compound excreted in feces (Suzuki et al., 2010); whereas, both the trimethylselenonium ion and selenosugar compounds have been identified in urine (Kobayashi et al., 2002).

Alternatively, synthesized selenoproteins can be secreted into systemic circulation to be transported to other tissues (Hill et al., 2012). Notably, the main physiological function of the idiosyncratic Selenoprotein P (SELENOP) is to maintain ubiquitous concentrations of Se (Hill et al., 2012; Burk and Hill, 2015). This protein is synthesized in the liver and transported to target tissues to be internalized via receptor-mediated endocytosis (Hill et al., 2012; Burk and Hill, 2015).

The organic forms of Se are more bioavailable than the inorganic forms (Khanam and Platel, 2016), and organic selenium can raise whole blood levels of Se more effectively compared sodium selenite or sodium selenate (Daniels, 1996). Additionally, transcriptomic analysis in the liver of growing beef heifers supplemented Se as either ISe, OSe, or a 50%:50% mixture of ISe or OSe (MIX) demonstrated that the form of Se creates specific phenotypes respective to chemical form with unique physiological

capabilities including changes in capacity or redox potential. Additionally, these transcriptomic changes were distinct and not indicative of a gradient effect respective to the different forms of Se (Matthews et al., 2014).

2.1.3. Selenoprotein synthesis

In the liver and other target tissues, physiological incorporation of Se is dependent on its available form, whether SeMet, SeCys, selenite, or selenate, (Suzuki, 2005). Selenomethionine enters the methionine metabolic pool and is nonspecifically incorporated into proteins in positions that could be occupied by methionine using the same AUG codon without bias (Figure 2.1, (Suzuki, 2005)). The amount of incorporation of SeMet compared to methionine is dependent on the relative concentration of Se available (Burk and Hill, 2015), and incorporation into these proteins allows for an effective mechanism for the body to store this mineral for subsequent utilization as needed (Schrauzer, 2000). Importantly, proteins that contain SeMet are not selenoproteins rather they are referred to as Se-containing proteins (Suzuki, 2005).

Alternatively, SeMet can be converted to SeCys via the intermediate selenocystathionine with the enzymatic activity of cystathionine β-synthase and cystathionine, and this process is known as the trans-selenation pathway (Suzuki, 2005; Mattmiller et al., 2013). The synthesized SeCys or dietary SeCys is then transformed into selenide by β-lyase activity (Suzuki, 2005).

The formation of selenide is where the metabolic pathways for OSe and ISe converge. Contrasted to the organic forms of Se, once consumed, selenate is easily

converted to selenite and then further converted to selenide (Mattmiller et al., 2013). Selenide formed from either source of Se can be converted into selenophosphate and then to the SEC tRNA$^{[SER]SEC}$ which incorporates the SeCys into the peptide sequence of selenoproteins during synthesis (Figure 2.2, (Veres et al., 1992; Glass et al., 1993)). Proteins containing SeCys residues are classified as selenoproteins (Suzuki, 2005), and many of these have enzymatic function as the SeCys residue exerts catalytic activity (Kryukov et al., 2003; Johansson et al., 2005).

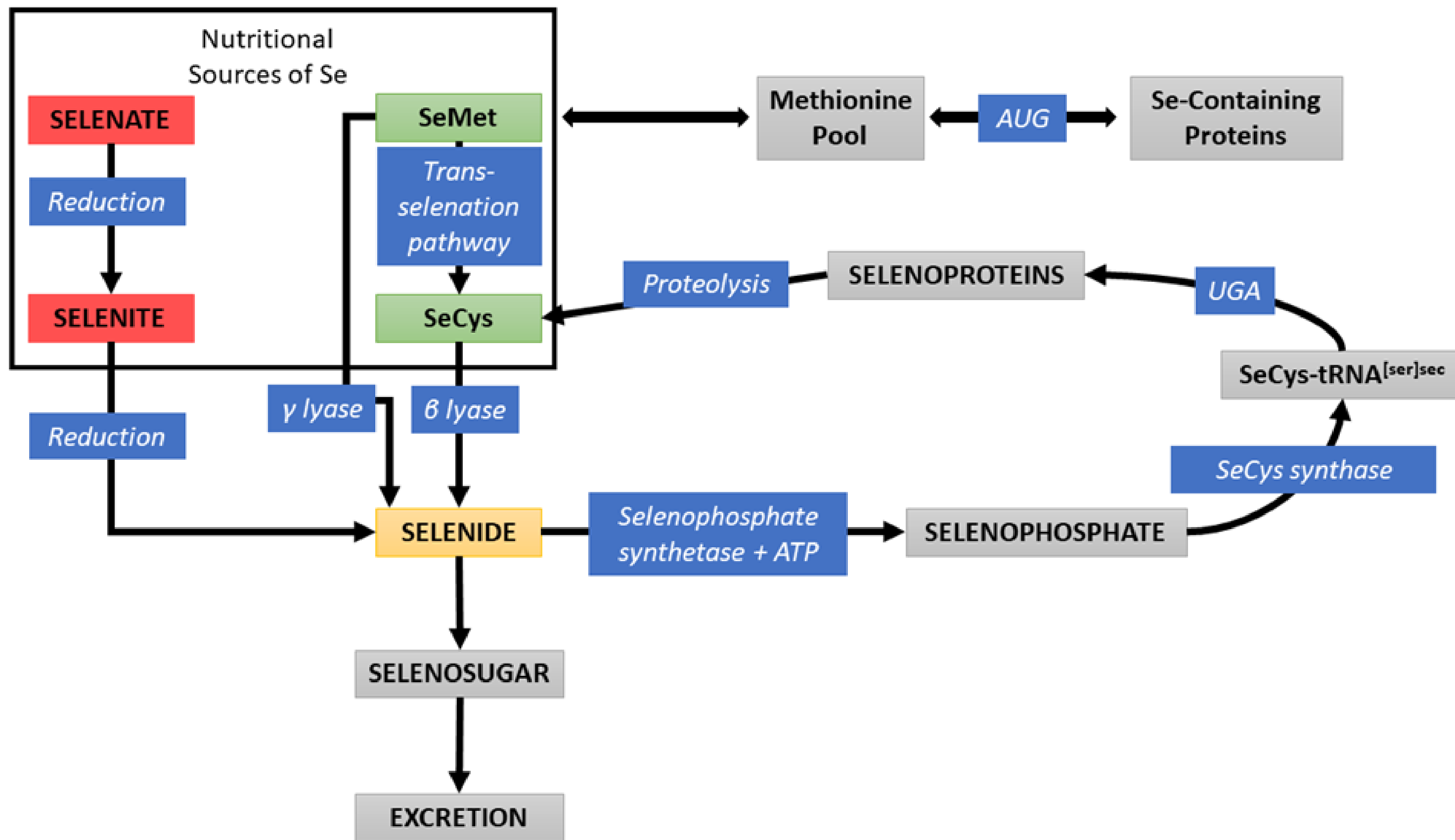

Figure 2.1 Schematic of selenium metabolism. Figure is adapted from (Suzuki, 2005) and available in (Crites, 2021). Abbreviations: selenomethionine (SeMet), selenocysteine (SeCys).

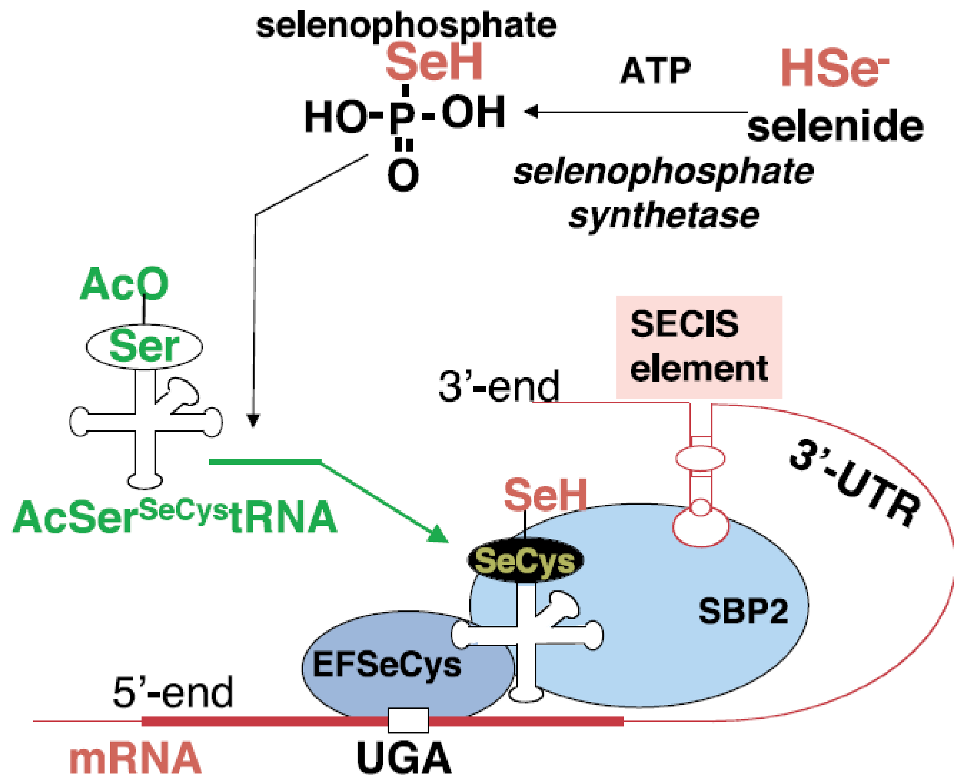

Figure 2.2 Translation mechanism for selenoprotein synthesis. The translation of the UGA codon to the selenocyetine (SeCys) sequence requires two cis-sequences, a selenocysteine insertion sequence (SECIS) element in the 3' untranslated region and a SeCys codon (UGA) in the coding region. It also requires three trans-activating factors, a SeCys-specific translation elongation factor (EFSeCys), the SeCys ^{SeCys}tRNA and a SECIS-binding protein (SBP2). The figure is available in (Suzuki, 2005).

2.1.4. Selenoproteins

The physiological relevance of Se was initially identified as a structural component of glutathione peroxidase (GPX; (Flohe et al., 1973)), and presently, there are 25 selenoprotein genes identified in mammals (Labunskyy et al., 2014; Chen et al., 2018), and many are thought to have antioxidant activities due to the presence of at least one SeCys residue (Kryukov et al., 2003).

2.1.4.1. Glutathione peroxidases

The first group of selenoproteins identified in mammals was glutathione peroxidases (GPX, (Flohe et al., 1973)), and of the eight known homologs, five are selenoproteins (GPX1, GPX2, GPX3, GPX4, GPX6). The other three homologs are not considered selenoenzymes because the active site contain cysteine (Cys) rather than a SeCys residue (Kryukov et al., 2003). As demonstrated in Figure 2.3, the GPX family of oxidoreductases exert antioxidant activity by catalyzing the reduction of hydrogen peroxide (H_2O_2) in conjugation with the glutathione cofactor to effectively reduce oxidative damage and maintains cellular redox homeostasis (Margis et al., 2008). Mechanistically, glutathione is a tripeptide and in its reduced state, it can interact with an organic peroxide (H_2O_2) resulting in a disulfide bridge between two glutathione molecules rendering these molecules oxidized. Simultaneously, GPX is necessary to catalyze the conversion of glutathione to glutathione disulfide and H_2O_2 to H_2O during this process. The glutathione disulfide can be recycled into 2 glutathione molecules via the enzymatic activity of glutathione reductase (Margis et al., 2008).

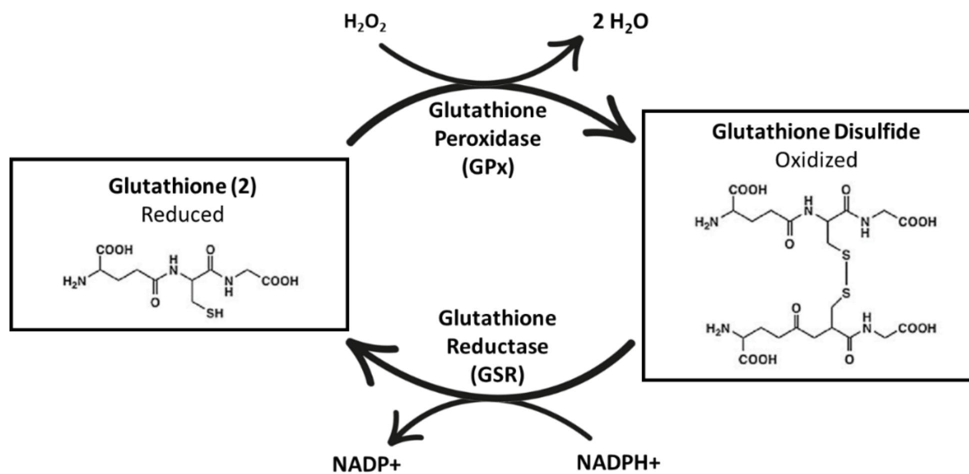

Figure 2.3. Mechanism demonstrating the catalytic reduction of H_2O_2.

The cytosolic and ubiquitously expressed GPX1 is the most abundant and characterized selenoprotein. It has also been localized within mitochondria (Brigelius-Flohé and Maiorino, 2013). Structurally this enzyme forms a homotetramer (Flohe et al., 1973; Awasthi et al., 1975) and catalyzes the reduction of H_2O_2 in a glutathione-dependent manner as discussed above (Rotruck et al., 1973; Gladyshev and Hatfield, 1999). This GPX homolog is considered one of the most significant antioxidant enzymes that protects cells from toxic reactive oxygen species (ROS, (Lubos et al., 2011)); however, it is sensitive to a deficiency in Se (Sunde, 1994).

The gastrointestinal specific GPX2 is structurally and functionally similar to GPX1. It is a tetramer that functions to reduce H_2O_2 in the cytosol coupled with glutathione (Brigelius-Flohé and Maiorino, 2013).

Next, blood specific GPX3 is a glycosylated protein that is primarily secreted from the kidneys. This enzyme is a tetramer with broad specificity that can reduce H_2O_2 in the presence of glutathione but it can additionally utilize thioredoxin or glutaredoxin as reductants due to plasma concentration of glutathione being relatively lower than intracellular glutathione (Björnstedt et al., 1994). Over 20% of the total plasma concentration of Se is present in GPX3 proteins which provides a reliable marker of systemic Se status (Gandin et al., 2009).

The unique GPX4 can reduce phospholipid hydroperoxides to protect membrane integrity (Herbette et al., 2007) because it lacks an integral loop in its monomeric region allowing it to interact with these bulky structures (Sherrer et al., 2011). Electron donors for GPX4 are glutathione as discussed above and protein thiols (Ursini et al., 1999; Imai and Nakagawa, 2003). It is one of the most abundant selenoproteins and is ubiquitously

expressed in the cytosol. It has also been localized to the mitochondria and nucleus of the testes (Brigelius-Flohé et al., 1994; Labunskyy et al., 2014). Unlike GPX1, GPX4 is not sensitive to a deficiency in Se and is referenced as a housekeeping gene in a Se deficient status (Bermano et al., 1995; Hara et al., 2001).

Lastly, GPX6 has conventionally been localized to embryos and the olfactory epithelium (Kryukov et al., 2003), and although little is known about this enzyme compared to the other GPX homologs, it is thought to possess capabilities to reduce H_2O_2 due to the presence of the SeCys residue (Labunskyy et al., 2014).

2.1.4.2. Thioredoxin reductases

Thioredoxins are a group of small ubiquitous proteins that protect against oxidative stress as redox proteins and can catalyze dithiol-disulfide exchange reactions in various tissues. Although thioredoxin is not a selenoprotein, once thioredoxin is oxidized, it must be reduced by the selenoproteins thioredoxin reductases (TRXR) (Arnér, 2009). There are three known TRXR (TRXR1, TRXR2, and TRXR3 (Labunskyy et al., 2014)) that are the only identified enzymes to catalyze the reduction of oxidized thioredoxin and this occurs in a NAPDH-dependent manner (Mustacich and Powis, 2000). Uniquely, the SeCys residue of TRXR is in the C-terminal, diverging from the normal structure of selenoproteins that have the SeCys residue in the N-terminal (Gladyshev et al., 1996).

The cytosolic TRXR1 is the major protein disulfide reductase in mammalian cells and the primary substrate for this reaction is thioredoxin (Arnér and Holmgren, 2000). Additionally, TRXR2 can reduce other enzymes include ribonucleotide reductase and methionine sulfoxide reductases (Stubbe and Riggs-Gelasco, 1998; Stadtman et al.,

2002), as well as the non-disulfide compounds, hydroperoxides, and selenite. Relatedly, selenite can be further reduced to hydrogen selenide by TRXR1 to prepare the Se donor for incorporation into selenoproteins (Ganther, 1999).

The widely expressed mitochondrial TRXR2 (Rundlöf et al., 2001; Lillig and Holmgren, 2007) catalyzes the reduction of thioredoxin to regulate the homeostasis of intra-mitochondrial ROS (Nalvarte et al., 2004). This selenoprotein may also be involved in regulating cell signaling (Prasad et al., 2014) and cell proliferation (Kim et al., 2003).

Finally, TRXR3 exists primarily in the testes (Arnér, 2009) and contains a glutaredoxin domain in the N-terminal in addition to the FAD-binding, NADPH, and interface domains of TRXR1 and TRXR2 (Biterova et al., 2005) which allows for glutathione and glutaredoxin reductase activity (Sun et al., 1999; Sun et al., 2001). Interestingly, due to its specificity, it is highly expressed after puberty and speculatively plays a role in sperm maturation (Su et al., 2005).

2.1.4.3. Iodothyronine deiodinases

Another family of selenoproteins are known as iodothyronine deiodinases (DIOs) that regulate the activation and deactivation of thyroid hormones, and subsequently growth, development, thermogenesis, and energy metabolism (Mullur et al., 2014). There are three thyroid hormone structures. Thyroxine (T4) is secreted from the thyroid gland, and can be converted to the bioactive 3,3',5-triiodothyronine (T3) by reductive deiodination of T4 (Labunskyy et al., 2014). Additionally, T3 can be further converted to the bioactive diiodothyronine (T2). Alternatively, T4 can be converted into the inactive reverse-triiodothyronine (rT3) particularly during periods of stress, and then rT3 be

further converted into T2. All conversions are facilitated by two of the three isoforms of DIOs (Lu and Holmgren, 2009).

DIO1 is bound in the plasma membrane and has been primarily localized to the liver, kidney, and thyroid (Labunskyy et al., 2014). This selenoprotein converts T4 to T3 by deiodination of the outer ring, and it can convert T3 and T4 into the inactive T2 or rT3 respectively via inner ring deiodination (Bianco et al., 2002). Therefore, DIO1 may be responsible for regulating the circulating levels of the bioactive T3 (Gladyshev and Hatfield, 1999). Whereas, DIO2 and DIO3 may regulate the concentration of intracellular T3 (Gereben et al., 2008a).

Intracellular DIO2 is located in the endoplasmic reticulum, and is primarily expressed in the nervous system, pituitary, thyroid, and brown adipose tissue (Labunskyy et al., 2014). Low levels of serum T4 can result in an elevation in the activity of DIO2 (Gereben et al., 2008b), and the intracellularly derived T3 is an effective regulator of gene transcription (Sagar et al., 2007).

DIO3 is another membrane-bound selenoprotein that is primarily expressed in vascular tissue, skin, and the placenta, as well as being highly expressed in fetal and neonatal tissues (Mullur et al., 2014). Its high level of expression in the placenta is to protect the growing fetus from the activity of maternal thyroid hormone (Forhead and Fowden, 2014).

2.1.4.4. Other selenoproteins

The other known selenoproteins are selenophosphate synthetase 2 (SEPHS2), selenoprotein P (SELENOP), selenoprotein W (SELENOW), selenoprotein F

(SELENOF), selenoprotein H (SELENOH), selenoprotein I (SELENOI), selenoprotein K (SELENOK), selenoprotein M (SELENOM), selenoprotein N (SELENON), selenoprotein O (SELENOO), selenoprotein R (SELENOR, also known as methionine sulfoxide reductase B1, MSRB1), selenoprotein S (SELENOS), selenoprotein T (SELENOT), and selenoprotein V (SELENOV). Of these, SELENOP (Takebe et al., 2002), SELENOW (Beilstein et al., 1996), SELENOH (Novoselov et al., 2007), SELENOK (Lu et al., 2006), SELENOM (Reeves et al., 2010), and SELENOR (Kryukov et al., 2002) have documented or proposed antioxidant properties. Below is an introduction of a select group of these selenoproteins.

SEPHS2 is a unique selenoprotein in that it constitutively catalyzes the conversion of selenide to selenophosphate, the Se donor in selenoprotein synthesis (Xu et al., 2007), and may regulate its own biosynthesis (Guimarães et al., 1996) as well as selenoprotein synthesis as illustrated in Figure 2.2 (Kim et al., 1997).

Also called 15-kDa selenoprotein (SEP15), SELENOF is one of the earliest identified selenoproteins and was named for its molecular mass (Behne et al., 1997). It is widely expressed in many tissues with the highest concentrations being in the liver, kidney, brain, testes, and prostate (Kumaraswamy et al., 2000). The expression of this selenoprotein is dependent on the availability of dietary Se (Ferguson et al., 2006).

Intriguingly, SELENOP has redox capabilities; however, its main physiological function is to maintain homeostasis of the concentration of Se (Burk and Hill, 2009), due to the presence of 9-12 SeCys residues per molecule compared to one SeCys residue in the other selenoproteins in mammals (Read et al., 1990). Selenoprotein P is primarily synthesized in the liver and transported through the plasma to target tissues to be

internalized in the cell by receptor-mediated endocytosis (Burk and Hill, 2009). SELENOP accounts for approximately 50% of the plasma concentration of Se (Burk and Hill, 2015) rendering it a reliable marker of Se status (Xia et al., 2005). It is delivered to target tissues by binding to endocytic receptors from the low-density lipoprotein (LDL) receptor family. The first receptor is the apolipoprotein E receptor-2 (apoER2) which is also known as the low-density lipoprotein receptor-related protein 2 (LRP2) (Olson et al., 2007) is highly expressed in the testes, placenta, bone marrow, muscle and brain and lowly to moderately expressed in numerous other tissues (Burk and Hill, 2015). The second receptor that binds SELENOP is megalin, also known as low-density lipoprotein receptor-related protein 8 (LRP8, (Olson et al., 2008)), and it is primarily responsible for the uptake of plasma SELENOP into the kidneys (Avissar et al., 1994), but it is also involved in Se metabolism in the brain (Chiu-Ugalde et al., 2010). Regardless of the target tissue, endocytosed and degraded SELENOP provides SeCys residues to be subsequently incorporated into other selenoproteins (Richardson, 2005).

Finally, SELNOW is one of the earliest identified and most abundant selenoproteins in mammals (Labunskyy et al., 2014). This protein is ubiquitously expressed and most highly concentrated in the muscles and the brain (Gu et al., 2000). It has been localized to the cytosol and is bound to the cell membrane in small fractions (Yeh et al., 1995). The expression of SELENOW is regulated by the availability of Se in the diet (Howard et al., 2013), and in response to a deficiency in Se, the transcription of SELENOW in the muscle, heart, intestines, skin, and prostate is downregulated whereas the transcription of this protein remains stable in the brain (Whanger, 2000). It appears

that SELENOW is involved in redox-related processes because it binds glutathione (GSH) with high affinity (Beilstein et al., 1996).

2.2. Metabolic physiology

2.2.1. Glutamine and glutamate metabolism

Metabolism of glutamine (Gln) and glutamate (Glu) occurs primarily in the liver and is critical for ureagenesis, the production of glutathione, *de novo* protein synthesis, and gluconeogenesis (Meijer et al., 1990; Watford, 2000; Matthews, 2005). Form of Se supplemented to growing beef steers affects hepatic glutamine synthetase (GS/ GLUL) activity. When compared to ISe alone, 1:1 mixture of ISe:OSe increased protein content of GS by 94% and activity of GS by 99% in the liver (Jia et al., 2018). Further research indicated that this effect was at least partially mediated by an upregulation of mRNA encoding *Glul*, glutamate dehydrogenase (*Glud1*), and the glutamine transporter (*Slc38a2*) in MIX supplemented steers, occurring concurrently with relatively lower levels of mRNAs encoding glutamine I and II (*Gls1, Gls2*, (Jia, 2019)).

Extrahepatically, Glu and Gln are dispersed systemically. In the CL and the uterus, these amino acids are necessary for processes including conversion into glutathione, the production of energy upon entry into the TCA cycle, or to disseminate these amino acids to the developing conceptus and fetus (Xu et al., 2022).

Glutamine (Gln) is imported into target cells through the neutral amino acid transporter (solute carrier family 1 member 5, SLC1A5) where intracellular Gln can be converted into Glu in the mitochondria via a deamination reaction with the glutaminases

(GLS1 and GLS2). Glutamate can be converted into α-ketoglutarate via the activity of glutamate dehydrogenase (GLUD1 or GLUD2), and alanine transaminase (ALT1/ GPT). Glutamate can then be converted to α-ketoglutarate via the activity of GLUD1 or GLUD2, and alanine transaminase (ALT1/GPT) to supply the tricarboxylic acid (TCA) cycle ultimately contribution to the production of intracellular ATP (Xu et al., 2022). Alternatively, Glu in conjugation with Cys can be converted to glutathione via the enzymatic activity of glutamate-cysteine ligase catalytic subunit (GCLC), to exert antioxidant capabilities by neutralizing free radicals as discussed above. Glutamate can serve as a bioactive precursor in regulating amino acid pools and can act as a nitrogen donor in the synthesis of nitrogen-containing amino acids (Xu et al., 2022).

2.2.2. Metabolic serological parameters (relevant to the studies described herein)

Analyses of metabolic profiles are routinely used to deduce nutritional status, incidence of disease, and assess fertility status in cattle (Ingraham and Kappel, 1988). Glucose, triglycerides, non-esterified fatty acids (NEFA), and beta hydroxybutyrate (BHBA) are energy analytes (Van Saun, 2023). In addition to the prominent short chain fatty acids (SCFA) acetate, butyrate, and propionate, glucose is a ubiquitous precursor for intracellular energy production. Glucose directly provides energy via glycolysis, but relatively, it is a major precursor for milk lactose, and to a lesser extent, milk citrate, non-essential amino acids, and glycerol (Ortega Cerrilla, 2003). Additionally, it is the primary energy source for the post-hatched conceptus prior to implantation and formation of the placenta during early gestation (Atkinson et al., 1984; Martal et al., 1997). Circulating BHBA is increased in the blood when energy demands are greater than the amount of

available glucose (Benedet et al., 2019); thus, rendering it an appropriate marker of energy availability. BHBA is primarily synthesized from fatty acids in the liver and is commonly associated with ketosis in dairy cows (Lei and Simões, 2021).

Next, hemoglobin, urea nitrogen, total protein, and albumin are used to identify an animal's protein status, liver function, and kidney function. They are quantified to ensure that there are adequate substrates for growth, lactation, reproduction, and constitutive protein synthesis from amino acid precursors (Van Saun, 2023).

Albumin, the most abundant serum protein accounts for ~50% of the total circulating protein (Rothschild et al., 1972) and is synthesized and secreted from hepatocytes. Albumin is primarily a circulating depot and transport protein for metabolites and molecules including fatty acids and cholesterol (Kaneko et al., 2008). It is synthesized and secreted from hepatocytes in the liver (Peters, 1977), and in response to demanding circumstances, the production of albumin in the liver can be upregulated by 200-300% (Evans, 2002). This serum protein is routinely quantified as an indicator of blood protein concentrations and an increase in the circulating albumin can indicate dehydration (Doumas and Peters, 1997). A relatively low concentration of serum albumin may indicate a disease present in the liver, kidney, or gastrointestinal tract, malnutrition, or a loss of blood (Kaneko et al., 2008).

Finally, it is common to quantify the serological abundance of the lipids cholesterol and non-esterified fatty acids (NEFA) and lipoproteins to analyze liver function, protein status, and energy availability (Van Saun, 2023). Cholesterol is a vital metabolic hormone but also the primary substrate for the synthesis of steroid hormones.

Serum cholesterol concentrations fluctuate proportionally with dietary intake and is also used to ascertain the liver's ability to export lipoproteins (Van Saun, 2023). Conversely, NEFA are fatty acid products of adipose tissue lipolysis, and NEFA are mobilized in the absence of sufficient glucose to meet metabolic demands. The systemic concentration of NEFA are increased by low insulin and elevated cholesterol (Van Saun, 2023). As deducible, the ratio of systemic NEFA:cholesterol is used to assess fatty liver status in cattle (Holtenius and Hjort, 1990).

2.3. Development and function of the corpus luteum

2.3.1. Luteal transition and development

The CL is one of the prominent functional structures on the ovary, and it is present following ovulation producing P4 during the luteal phase and pregnancy. The CL is comprised of both large and small steroidogenic luteal cells and non-luteal cell constituents (Hansel et al., 1987; O'Shea, 1987) including immune cells, fibroblasts, endothelial cells, and pericytes (Farin et al., 1986; Hansel et al., 1991). This transient structure is a continuation of follicular maturation and forms via transition of follicular cells in response to the preovulatory surge of gonadotropins (Smith et al., 1994). During the process of luteinization, granulosa cells from the periovulatory follicle terminally differentiate into large luteal cells and theca cells terminally differentiate into small luteal cells (Donaldson and Hansel, 1965; Alila and Hansel, 1984; Niswender et al., 1986; Schams and Berisha, 2004; Stocco et al., 2007). This process is characterized by a diversion from the production of estradiol (E2) to the production of progesterone (P4)

(Juengel and Niswender, 1999) to prepare the endometrium in anticipation of a potential pregnancy.

2.3.2. Luteinization

Ovulation is a cataclysmic event that is luteinizing hormone (LH)-dependent, culminating in release of the oocyte and a follicular-luteal shift in steroidogenesis (Fortune et al., 2009). This transition includes several primary events: (1) meiotic maturation of the oocyte, (2) expansion of cumulus cells surrounding the periovulatory oocyte, (3) rupture of the follicle, and (4) a shift in steroidogenesis from the production of E2 to P4 (Bilodeau-Goeseels, 2007; Fortune et al., 2009; Turathum et al., 2021).

Prior to ovulation the dominant follicle with the enclosed oocyte is comprised of granulosa and theca cells populations separated by a basement membrane (Niswender et al., 2000). The process of ovulation and luteinization occurs when the LH receptor (LHCGR) in follicular cells is activated by the preovulatory surge of gonadotropins from the adenohypophysis and includes the rapid and terminal differentiation of follicular to steroidogenic luteal cells (Stocco et al., 2007). Structurally, the granulosa layer of the ovulated follicle collapses into the follicular antrum and the theca cells are unraveled into the developing structure via the invasion of vascular and connective tissue (Murphy, 2000). The CL is a highly vascularized structure that can efficiently secrete large quantities of P4 into the systemic circulation given sufficient vascularization during luteinization (Smith et al., 1994).

The terminal differentiation of steroidogenic luteal cells is characterized by an exit from the cell cycle (Murphy, 2000; Stocco et al., 2007), and alterations in cellular responses to external signals (Stocco et al., 2007). Specifically, the process results in activation of the guanine nucleotide binding protein Gs (G-protein) coupled-LHCGR in small luteal cells which activates adenyl cyclase (ADCY) to increase cAMP and activate cAMP-dependent protein kinase A (PKA) signal transduction (Richards, 2001) necessary for intracellular production of P4.

The preovulatory LH surge upregulates hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (3βHSD) in granulosa cells and subsequently an overall increase in enzymatic activity in the CL to facilitate increasing amounts of P4 biosynthesis (Smith et al., 1994). The steroidogenic capacity of luteal cells and responsiveness to LH increase during luteal development (Niswender et al., 2000). Signaling from LH is required to maintain expression of steroidogenic mRNA and presumably proteins (Niswender et al., 2000); however, past luteinization, LH secretion is not required for maintenance of P4 secretion from the CL in cattle (Peters et al., 1994). Although curiously, mid-luteal phase hypophysectomy resulted in subsequent regression of the CL thus indicating a role for LH in maintenance of this transient ovarian structure (Denamur et al., 1966, 1973).

As previously mentioned, vascularization of the CL is necessary for function of the CL and production of P4 (Niswender et al., 2000). Angiogenesis is the formation of new blood vessels by migration, growth, and differentiation of endothelial cells. In the CL, establishment of the blood supply occurs using the preexisting capillary network (Reynolds et al., 2000). Structurally, individual luteal cells are in direct contact of the

capillary system to provide with CL with a relatively high rate of blood flow per unit of tissue compared to other adult organs (Reynolds et al., 2000; Stocco et al., 2007).

2.3.3. Steroidogenic cells

2.3.3.1. Small luteal cells

The small luteal cells originate from the theca interna of the ovulating follicle (Donaldson and Hansel, 1965). They are less than 23 μm in diameter but are more abundant numerically than large luteal cells. In comparison to large luteal cells, small luteal cells are significantly smaller in size, elongated in shape and contain numerous lipid droplets in their cytoplasm (O'Shea et al., 1979; Farin et al., 1986). These cells comprise approximately 26% of the luteal cell population and approximately 28% of the total volume of the CL (Fields and Fields, 1996).

Comparatively, small luteal cells produce only basal levels of P4; however, they respond with increasing amounts of P4 in response to LH stimulation (Hansel and Dowd, 1986). In the cow, LH is considered the primary luteotropic hormone. The magnitude of responsiveness to LH stimulation is greater in small versus large luteal cells (Smith et al., 1994), and stimulation of small luteal cell with exogenous LH or an analog of cyclic AMP can increase P4 up to 20-fold in sheep (Fitz et al., 1982; Alila et al., 1988b). The small steroidogenic cells of the CL contain receptors for LH (luteinizing hormone G-protein coupled receptor, LHCGR) which can assert its effects by acting through the protein kinase A (PKA) second messenger system to stimulate production of P4 (Fitz et al., 1982; Alila et al., 1988b; Niswender et al., 2007). Even though large steroidogenic luteal cells contribute a 20-fold greater amount of the P4 per cell, they are devoid of the

LH receptor; thus, they do not respond to simulation by this gonadotropic hormone (Fitz et al., 1982; Hoyer and Niswender, 1986).

2.3.3.2. Large luteal cells

The large luteal cells originate from granulosa cells of the ovulating follicle. They range from 24-45 μm in diameter (Fields and Fields, 1996) and appear spherical or polyhedral with a rounded nucleus and visible lipid droplets (Niswender et al., 2007). Unlike small luteal cells, large luteal cells contain many dense secretory granules that are located closely to the plasma membrane (Fields et al., 1992). These granules contain oxytocin (OT) in the cyclic CL but not in the pregnant CL, and it has been postulated that the granules contain relaxin in the pregnant CL (Stoelk et al., 1991; Fields et al., 1992; Kohsaka et al., 2001). Numerically, large luteal cells comprise 3% of the population of luteal cells yet comprise 14% of the total volume of the CL due to significant hypertrophic growth during luteinization (O'Shea et al., 1989). Unlike small luteal cells, large luteal cells do not respond to LH (Alila et al., 1988b), but they are responsible for approximately 80% of the total production of P4 from the CL (Niswender et al., 1985). These large steroidogenic cells also synthesize and secrete OT from the cyclic CL but not the post-implantation CL (Fields et al., 1992) which indicates OT has a role in luteolysis. This will be further discussed in *2.3.4. Luteolysis.*

As mentioned above, LH signaling activates the PKA signaling pathway in the small luteal cell; however, it appears that this mechanism is not essential for P4 synthesis in large steroidogenic cells (Alila et al., 1988a). However, large luteal cells are responsive to agonistic and antagonistic ligands that bind to both membrane and nuclear

receptors. The luteotropic hormones PGE_2 and growth hormone (GH) will be discussed herein, and the luteolytic hormone $PGF_{2\alpha}$ will be discussed in *2.3.4. Luteolysis.*

Treatment with luteotropic and luteoprotective PGE_2 has been reported to increase the production of P4 from luteal cells in ruminants (Fitz et al., 1984a; Fitz et al., 1984b; Alila et al., 1988b; Shelton et al., 1990). *In vitro*, treating steroidogenic luteal cells with PGE_2 resulted in an increase in the production of P4 (Fitz et al., 1982; Fitz et al., 1984a; Alila et al., 1988a) and increased protection of the CL from luteolysis (Henderson et al., 1977; Pratt et al., 1977; Magness et al., 1981; Reynolds et al., 1981). PGE_2 stimulated the production of P4 by binding the membrane prostaglandin E receptors 1-4 (EP1, EP2, EP3, and EP4) and activating various signaling pathway (Weems et al., 2002; Kotwica et al., 2003).

Next, receptors for GH are located primarily on large luteal cells which directly influences luteal function (Lucy et al., 1993). Exogenous administration to GH to large steroidogenic cells *in vitro* stimulates the secretion of both P4 and OT, and treatment with GH *in vivo* supports development of the CL (Schams and Berisha, 2004). Moreover, it has been reported that GH may indirectly influence luteal function by increasing expression of insulin-like growth factor 1 (IGF1) which stimulates modification of the cytoskeleton and may stimulate secretion of P4 (Niswender et al., 2000). It has been speculated that compared to LH, GH may be a more potent stimulator of the production of P4 in the early CL (Kobayashi et al., 2001).

2.3.3.3. The production of progesterone

Mechanistically, the primary substrate for the production of P4 is cholesterol, which is made available to steroidogenic luteal cells via four mechanisms: conversion of low-density lipoproteins (LDL), uptake of high-density lipoproteins (HDL), availability of free cholesterol, or *de novo* synthesis (Cook et al., 1967; Cook and Nalbandov, 1968; Kaltenbach et al., 1968).

The conversion of LDL and HDL into free cholesterol is the prominent pathway to make this substrate available for the production of P4 by the CL (Ohashi et al., 1982; Pate and Condon, 1982; Hwang and Menon, 1983). The uptake of LDL occurs by binding to the LDL receptor (LDLR) and triggering receptor mediated endocytosis (Brown and Goldstein, 1986). The endosome containing the LDL combines with lysosomes and LDL is dissociated from the receptor to be broken down into free cholesterol (Grummer and Carroll, 1988). Transport out of the lysosome occurs by binding the cholesterol transporters: Niemann-Pick C1 protein (NPC1) and Niemann-Pick C2 protein (NPC2 (Infante et al., 2008; Kwon et al., 2009; Wang et al., 2010)). LDL-derived free cholesterol is rapidly exported out of the lysosomal compartments via the actions of NPC1 and NPC2, and without these transporters, cholesterol and other lipids would accrete within lysosomes (Sleat et al., 2004). In contrast, HDL is utilized as a substrate for cholesterol by first binding to the scavenger receptor class B type 1 (SCARB1) on the cell surface. The lipoprotein is not entirely internalized; rather, the cholesteryl esters are selectively delivered into the cell to be hydrolyzed into free cholesterol (Ferreri and Menon, 1992; Kraemer et al., 2004; Shen et al., 2016). Although, delivery to peripheral tissues occurs via the activity of LDL, HDL is primary lipoprotein

responsible for the recycling of cholesterol, phospholipids, and proteins back to the liver (Bruss, 2008).

Another mechanism associated with the availability of this substrate for conversion into P4 in steroidogenic cells is the hydrolysis of cholesteryl esters to free cholesterol from lipid droplets. This reaction is stimulated by hormone-sensitive lipase (HSL), also known as lipase E, hormone sensitive type (LIPE), which results in free cholesterol and fatty acids that can then act as precursors for various physiological functions, including the production of steroid hormones and cellular energy (Fredrikson et al., 1981; Cook et al., 1982; Cook et al., 1983; Fredrikson et al., 1986; Yeaman, 2004).

Additionally, cholesterol can be synthesized intracellularly via *de novo* cholesterol synthesis from acetyl-CoA as demonstrated in Figure 2.5 (Vance and Van den Bosch, 2000). The mevalonate pathway is a vital pathway of cholesterol synthesis (Pool et al., 2018) and this starts with the reversible condensation of two acetyl-CoA molecules to acetoacetyl-CoA using acetyl-CoA acetyltransferase (ACAT) which has two homologs (ACAT1 and ACAT2, (Mazein et al., 2013)), and acetyoacetyl-CoA in conjugation with the enzyme hydoxymethylglutaryl-CoA synthase (HMGCS1) forms 3-hyrdoxy-3-methylglutaryl-CoA (HMG-CoA) (Mazein et al., 2013). Next, the rate limiting enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) catalyzes the conversion of HMG-CoA into mevalonic acid (Grummer and Carroll, 1988; Mazein et al., 2013). The sterol regulatory element binding protein/ factor (SREBP or SREBF) regulates the transcription of HMGCR and HMGCS (Brown and Goldstein, 1997), and in a state of low cholesterol availability, SREBP is cleaved and translocates into the nucleus to upregulate the expression of aforementioned genes involved in cholesterol biosynthesis

(Vance and Van den Bosch, 2000). In continuing this pathway, after forming mevalonic acid, sequential phosphorylations and decarboxylations drive the conversion of mevalonic acid through the intermediate structures isopentenyl pyrophosphate (IPP), geranyl pyrophosphate (GPP), and farnesyl pyrophosphate (FPP) to form squalene for conversion into sterols (Rudney and Sexton, 1986). The synthesis of squalene via squalene synthetase (FDFT2) is the first committed step in the *de novo* production of cholesterol (Goldstein and Brown, 1990).

As further demonstrated in Figure 2.5, squalene can be converted into lanosterol via the enzymatic activity of squalene epoxidase (SQLE) and lanosterol synthase (LSS), which is then converted into cholesterol through a multistep pathway involving the enzymes cytochrome P450, family 51, subfamily A, polypeptide 1 (CYP51A1), 7-dehydrocholesterol reductase (DHCR7), 24-dehydrocholesterol reductase (DHCR24), NAD(P) dependent steroid dehydrogenase-like (NSDHL), and Transmembrane 7 superfamily member 2 (TM7SF2, (Mazein et al., 2013)).

Cholesterol derived from *de novo* synthesis has been reported to play a relatively minor role in contribution to the production of P4 due to the relatively low levels of the HMG-CoA formed as a result of the rate-limiting enzymatic activity of HMGCS1 (Gwynne and Strauss, 1982; Grummer and Carroll, 1988).

From any mechanism discussed above, once cholesterol is free, it is transported into the mitochondria by binding to the steroidogenic acute regulatory protein (STAR), which then interacts with membrane proteins on both the outer and inner mitochondrial membranes to facilitate transport into the inner mitochondrial matrix (Selvaraj et al., 2018). Cholesterol is hydrophobic, and therefore, it cannot freely diffuse across the

mitochondrial membrane without binding to a carrier protein (Stocco et al., 2007). This mechanism mediates the rate at which P4 can be produced (Stocco and Sodeman, 1991; Clark et al., 1994; Lin et al., 1995), and there is a strong correlation between the concentration of STAR protein and steroidogenesis (Manna et al., 2009). The transcription, activity, and degradation of STAR protein is under the control of the PKA and PKC second messenger signaling pathways (Manna et al., 2009; Manna et al., 2016) in conjugation with several transcription factors including SF-1, C/EBPβ, SREBP-1a, cFOS, GATA-4, Sp-1 and CREB family members (Manna et al., 2003). Alternatively, dosage-sensitive sex reversal-adrenal hypoplasia congenital region on the X chromosome gene 1 (DAX-1) and forkhead box protein L2 (FOXL2) may negatively regulate STAR by binding a specific recognition motif in the promoter region of this gene (Pisarska et al., 2004).

Within the inner mitochondrial matrix, cholesterol is then converted into pregnenolone via the actions of cytochrome P450 family 11 member 1 (CYP11A1) located on the inner mitochondrial membrane (Hanukoglu and Jefcoate, 1980; Hanukoglu et al., 1981; Mitani et al., 1982; Hanukoglu and Hanukoglu, 1986). Once transported out of the mitochondria, the enzymes hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta isomerase 1 (HSD3B1) located in the endoplasmic reticulum further converts pregnenolone into P4 (Labrie et al., 1992) allowing autocrine, paracrine, and endocrine activity of this hormone.

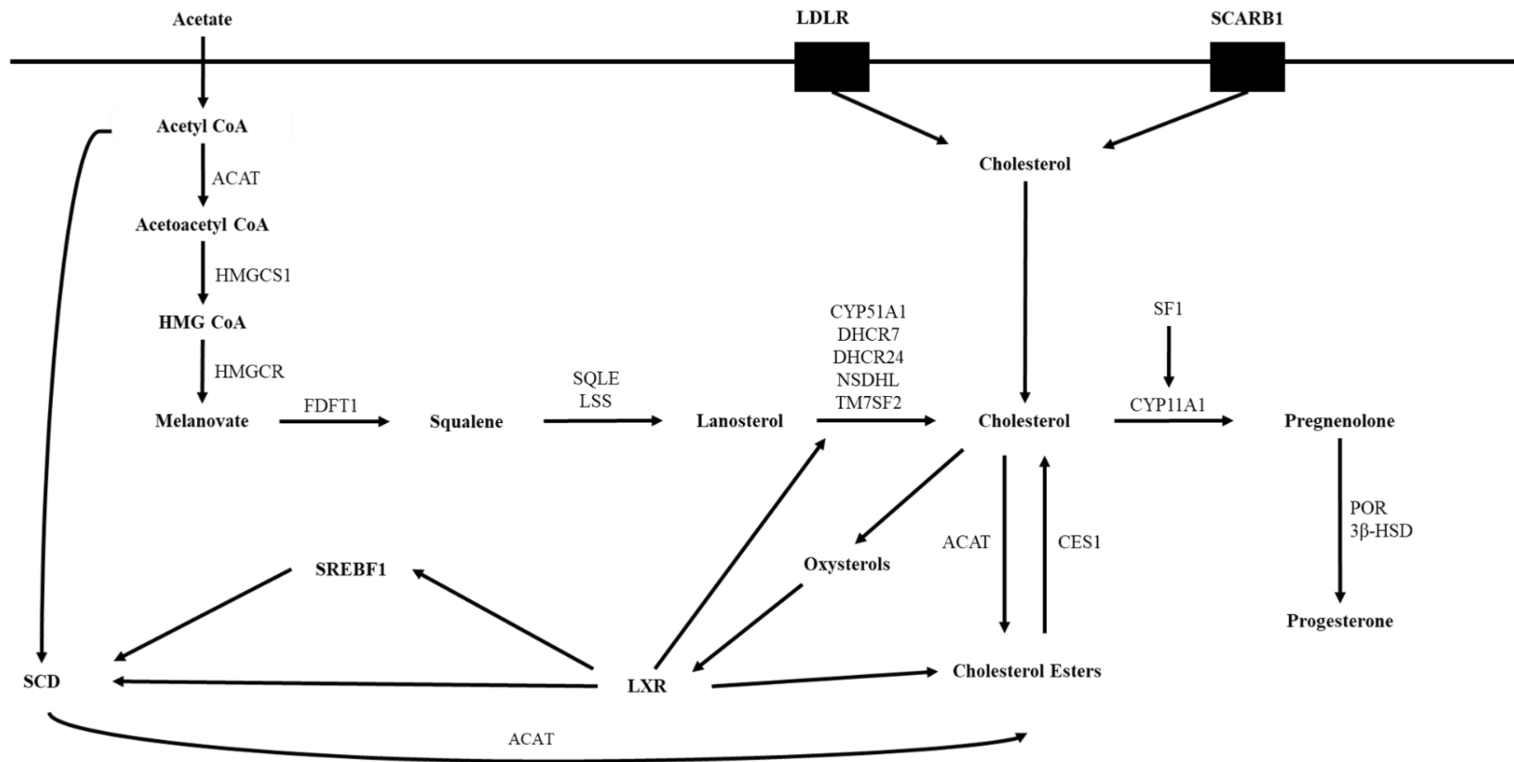

Figure 2.4. Mechanism for *de novo* cholesterol synthesis.

2.3.4. Luteolysis

Lysis of the CL, or luteolysis, is the process of luteal regression (Niswender et al., 2000) and is initiated by default on approximately day 17 of the estrous cycle in the absence of pregnancy (Sirois and Fortune, 1988). During normal luteal regression, the CL first loses the ability to synthesize and secrete P4, a process referred to as functional luteolysis (Miyashita et al., 1994). Subsequently there is a loss of the cellular constituents, referred to as structural luteolysis (Knickerbocker et al., 1988; Meidan et al., 2017). The luteolytic cascade includes changes in the steroidogenic capacity, vascularization, immune cell function, composition of the extracellular matrix, and cell viability. Additionally, luteolysis is characterized as undergoing programmed cell death (apoptosis, (Meidan et al., 2017)).

The luteolytic $PGF_{2\alpha}$ is a potent hormone with vasoconstricting effects that is synthesized and released from the uterine endometrium to effectively regress the CL of a nongravid cow, rapidly reducing the synthesis and secretion of P4 (Meidan et al., 2017). To effectively induce luteolysis, the uterus must secrete repeated pulses of PGF2α, and these effects are dependent on the stage of CL development (Levy et al., 2000; Mondal et al., 2011). The bovine CL is refractory to regression signaled by PGF2α prior to day 5 of the estrous cycle (Braun et al., 1988; Pursley et al., 1995; Tsai and Wiltbank, 1998; Levy et al., 2000) even though it contains receptors for PGF2α and demonstrates transcriptomic changes in response to exogenous administration of this hormone (Tsai and Wiltbank, 1998; Levy et al., 2000; Mondal et al., 2011; Zalman et al., 2012). However, it is responsive during the mid-luteal phase (Mondal et al., 2011) and therefore,

during this time, $PGF_{2\alpha}$ can effective reduce intracellular and systemic concentrations of P4.

The exact mechanism of the timing and sequential nature of signaling in luteal regression is still being investigated. Regarding the initiation of this process, P4 exposure to the endometrium during the mid-luteal phase of the estrous cycle is necessary for endometrial production of $PGF_{2\alpha}$, the preeminent luteolytic hormone. Uterine synthesis and release of $PGF_{2\alpha}$ is primarily regulated by P4, E2, and oxytocin (OT) (Bazer et al., 1998).

It is evident that OT from the corpus luteum as well as from the neurohypophysis promotes the release of $PGF_{2\alpha}$ from the endometrial luminal epithelial cells to act on the CL effectively removing the source of the P4 (Demmers et al., 2001). Large luteal cells produce and secrete OT during the estrous cycle, but this does not translate to the post-implantation period (Fields et al., 1992), thus indicating a roll for CL derived OT in luteolysis.

For the first 10-12 days of the estrous cycle, synthesis of the oxytocin receptor (OTR) in the endometrium is blocked by the production of P4, a phenomenon generally referred to as the "progesterone block" of OTR formation (Bazer et al., 1998). After P4 has continuously primed the endometrium, P4 begins negatively regulating the expression of the nuclear P4 receptor (PR) in the luminal and glandular epithelium (Spencer et al., 2004; Spencer et al., 2008). Repression of PR allows for increased expression of the estrogen receptor (ER) which responds to circulating E2 and upregulates the expression of OTR (Spencer and Bazer, 1995; Spencer et al., 1995). Follicular estrogen (E2) upregulates OTR in the endometrium facilitating OT signaling

induction of pulsatile $PGF_{2\alpha}$ (Hixon and Flint, 1987), thus promoting a positive feedback loop among luteal OT, luteal $PGF_{2\alpha}$, and endometrial $PGF_{2\alpha}$ (Niswender et al., 2000).

Once secreted, $PGF_{2\alpha}$ crosses from the utero-ovarian vein to the ovarian artery through a counter current exchange mechanism (Staples and Diskin, 1984). The pulses of $PGF_{2\alpha}$ bind to prostaglandin $PGF_{2\alpha}$ receptor (PTGFR) on the membrane of steroidogenic luteal cells to activate the protein kinase C (PKC) secondary messenger system. Resultantly, phosphorylation of STAR protein by PKC appears to reduce binding and transport of cholesterol across the mitochondrial cell membrane (Niswender et al., 2000) thus reducing the production of P4 from luteal cells. Additionally, activation of PTGFR promotes an influx of Ca^{2+} leading to cellular degeneration and apoptosis (Niswender et al., 2000).

In addition to the direct activity of $PGF_{2\alpha}$ on the production of P4 during luteolysis, $PGF_{2\alpha}$ indirectly affects function and structure of the CL by effecting changes in vascularization, immune cell function, composition of the extracellular matrix, and cell viability (Meidan et al., 2017). The CL is a highly vascularized tissue which is critical for the delivery of substrates to the CL and P4 to the endometrium, and endothelial cells that line capillaries contain PTGFR (Shirasuna et al., 2012). Of relevance, endothelial cells are the primary source for the transcription and translation of abundant and hormonally regulated endothelin-1 (EDN1) in the CL (Goede et al., 1998). The highest intracellular expression of EDN1 mRNA was reported on day 18 of the estrous cycle when compared to days 5 and 10 (Girsh et al., 1996b; Ohtani et al., 1998). Treatment with EDN1 *in vitro* inhibits both basal and LH-stimulated production of P4 in a dose-dependent manner (Girsh et al., 1996a; Hinckley and Milvae, 2001). It appears that PGF2α stimulates the

production of EDN1 in endothelial cells which then signals through the membrane endothelin receptor type A (EDNRA) on steroidogenic cells (Girsh et al., 1996a; Hinckley and Milvae, 2001), however the exact mechanism remains to be defined. Next, $PGF_{2\alpha}$ can further affect vascularization during luteolysis by transiently inducing the production of angiopoietin 2 (ANGPT2) which blocks endothelial expressed tyrosine kinase receptor thus disrupting vessel stabilization (Miyamoto et al., 2009).

Additionally, $PGF_{2\alpha}$ alters immune cell function in the CL during luteolysis by signaling changes in endothelial cells associated with both the innate and adaptive immune system (Mai et al., 2013). Transduction of $PGF_{2\alpha}$ signaling increased the production of inflammatory cytokines and chemokines to mediate extravasation and disbursement of immune cells (Mai et al., 2013). These endothelial cells also recruit adhesion molecules that facilitate leukocyte recruitment and endothelial transmigration (Bowen et al., 1999; Cheng et al., 2013).

2.3.5. Luteal function

2.3.5.1. Maternal recognition of pregnancy

The synthesis and secretion of P4 is the primary function of the CL (Behrman et al., 1971). One of the primary actions of P4 is to prepare the endometrium for a possible pregnancy. In the event of fertilization and the presence of a viable embryo, the continuous production of P4 is necessary for successful development of the conceptus, implantation, and maintenance of a normal pregnancy (Niswender et al., 2000). For this to occur, the CL must evade the luteolytic effects of $PGF_{2\alpha}$ from the endometrium. The developing conceptus must signal its presence at a time termed maternal recognition of

pregnancy (MRP). The trophectoderm derived protein IFNτ is the signal for MRP in cattle, and it blocks the production and release of $PGF_{2\alpha}$ preventing luteolysis and extending the functional lifespan of the CL past that of the normal estrous cycle (Short, 1969; Lewis et al., 1979). The role of the CL at MRP will be further discussed in in *2.4.2. Maternal recognition of pregnancy.*

2.3.5.2. Implications of concentrations of progesterone

The relative concentration of P4 during early pregnancy affects embryonic growth and development. Low systemic P4 during the early luteal phase after artificial insemination has been associated with lower conception rates (Mann and Lamming, 1999), and lower P4 in the first week after ovulation is associated with underdeveloped conceptuses (Forde and Lonergan, 2012), alterations in the conceptus transcriptome (Barnwell et al., 2016), and a lower likelihood of successful pregnancy (Wiltbank et al., 2016). Alternatively, an elevated concentration of P4 post-conception and during the early luteal phase has been reported to advance conceptus elongation and development (Garrett et al., 1988; Carter et al., 2008) and increase the production of IFNτ (Mann and Lamming, 2001). It has also been reported that an earlier increase in systemic P4 post-ovulation improves embryo quality on day 16 of pregnancy (Mann and Lamming, 2001) and can increase pregnancy rates (Yan et al., 2016).

More specifically, P4 is considered a differentiation factor in the uterus (Cummings and Yochim, 1984) because it stimulates the secretion of histotroph from the glandular epithelium (Maslar et al., 1986) and alters protein secretion patterns by endometrial cells (Maslar et al., 1986) to promote an environment to support

development of the post-hatched conceptus (Niswender et al., 2000). The effects of P4 on the uterus during early gestation will be further described in *2.4. Uterine function.*

2.4. Uterine function

2.4.1. Early pregnancy

Following ovulation and fertilization via a capacitated sperm, the zygote undergoes cleavage divisions, differentiates into a morula, and then a blastocyst consisting of an inner cell mass and the trophectoderm (Bo and Mapletoft, 2013). Arrival in the uterus is typically observed on approximately day 4 of pregnancy during the late stages of cleavage division or as an early morula. The embryo hatches from the protective zona pellucida of the expanded blastocyst on around day 8-9 after fertilization (Spencer, 2013) and begins a period of rapid growth into an ovoid shape and further into an elongated and filamentous conceptus that is dependent on a conducive uterine environment (Mathew et al., 2022).

Successful fertilization and embryonic development to the blastocyst stage occurs independent of maternal input; however, elongation cannot occur without interacting with a conducive uterine environment in the presence of histotroph, uterine secretions consisting of consists of a complex mixture of ions, amino acids, fatty acids, steroids, prostaglandins, proteins, retroviral components, and extracellular vesicles (Mathew et al., 2019; Mathew et al., 2022). Of consideration, the conceptus also does not elongate in the absence of uterine glands *in vivo* (Gray et al., 2001). The inability for the bovine embryo to elongate *in vitro* creates challenges studying of the conceptus of early pregnancy (Fléchon et al., 1986; Vajta et al., 2004; Clemente et al., 2009), although substantial work

is being conducted to create a co-culture protocol that allows for the interaction between the bovine embryo and endometrium (Mathew et al., 2019).

The hatched blastocyst begins secreting IFNτ, the signal of MRP, in increasing amounts until it peaks on approximately day 16 (Mansouri-Attia et al., 2009), and functionally it abrogates the luteolytic activity of the endometrial derived $PGF_{2\alpha}$, thus ensuring maintenance of the CL and continued production of P4 (Forde and Lonergan, 2017).

Histotroph is secreted from the uterine glands located in the intercaruncular endometrium (ICAR (Atkinson et al., 1984). In contrast, the small uniformly distributed aglandular regions of the endometrium of the uterine horn are called caruncular (CAR) regions (Mansouri-Attia et al., 2009). The ICAR and CAR are clearly distinct zones with differences in structure and biological functions (Mansouri-Attia et al., 2009). The ICAR primarily supports the growth and development of the post-hatched conceptus (Gray et al., 2001). Whereas, the CAR fuses with the extraembryonic membranes of the conceptus, called cotyledons, to form the collective maternal-fetal placentomes that are the placental attachments in the cow (Atkinson et al., 1984). The CAR tissue is the maternal contribution to this structure that provides nutrients to and removes waste from the fetus following implantation (Mathew et al., 2022).

2.4.2. Maternal recognition of pregnancy

Maternal recognition of pregnancy (MRP) is a critical series of events where the developing embryo signals its presence, blocks luteolytic pulses of PGF_{2a}, effectively extends

the functional lifespan of the CL and the production of P4 (Short, 1969). IFNτ is the signal in cattle (Spencer, 2013) and it is generally accepted that effects of this interferon on approximately day 16 are critical for MRP. Importantly, this signal must occur prior to natural regression of the CL that is initiated on approximately day 17 in a nongravid cow (Lewis et al., 1979; Sirois and Fortune, 1988; Forde and Lonergan, 2017). At MRP, a balance between the action of luteal P4 and embryonic IFNτ creates a consummate environment to support develop and prepare the endometrium for subsequent implantation and placentation (Garrett et al., 1988; Mann and Lamming, 2001).

IFNτ is a type I interferon that is exclusively synthesized and secreted by mononuclear cells of the trophectoderm (Farin et al., 1990) and it acts in a paracrine manner on the endometrium signaling the type I interferon receptors (IFNAR1 and IFNAR2) activating the janus kinase/ signal transducers and activators of transcription (JAK/STAT) signaling transduction pathway (Spencer et al., 2007).

IFNτ signaling results in various physiological changes in the endometrium including the paramount suppression of the transcription of the ER and OTR (Spencer and Bazer, 1996; Fleming et al., 2001), blocking the luteolytic activity of PGF2α (Spencer and Bazer, 1995; Spencer et al., 1995; Choi et al., 2001). Further IFNτ initiates transcriptomic changes that render the uterus to be luteoprotective rather than luteolytic by positively regulating the transcription of endometrial PGE_2 synthase (PGES) and PGE receptors (EP1-4), while decreasing the expression of $PGF_{2\alpha}$ synthase (PGFS) (Arosh et al., 2004) shifting the relative concentration in favor of the luteotrophic PGE_2 (Pratt et al., 1977; Magness et al., 1981; Parent et al., 2003; Arosh et al., 2004) and indirectly

stimulating the secretion of P4 from small luteal cells via activation of cAMP (Hansel and Blair, 1996).

Additionally in a cell specific manner in the endometrium, IFNτ stimulates multiple genes that result in stimulation of histotroph, development of the conceptus, and uterine receptivity (Spencer et al., 2007). Numerous endometrial genes with varied functionalities are expressed in response to IFNτ. Transcriptomic analysis of the bovine endometrium containing both CAR and ICAR recovered on day 16 of pregnancy revealed 764 DEGs in response to the presence or absence of the conceptus, and of these, 514 were greatest at MRP compared to earlier stages of pregnancy (Forde et al., 2011b) consistent with the time in which concentration of IFNτ is greatest (Walker et al., 2010). Effectively, IFNτ binds IFNAR1 and IFNAR2 which are associated with tyrosine kinase-2 (TYK2) and janus kinase 1 (JAK1), respectively. As demonstrated in Figure 2.6, following binding of IFNτ, activation of IFNAR1 and IFNAR2 result in dimerization following by autophosphorylation and activation of the downstream signal transducer and activator of transcription (STAT) 1 and 2 (Walker et al., 2010). Ligand binding cross-links the adjacent receptors and the JAKs cross-phosphorylate each other on tyrosines and phosphorylate receptors on tyrosines thus allowing STATs to dock on specific phosphotyrosines to be phosphorylated by JAKs. Once STATs dissociate from the receptor, they dimerize via their SH2 domains. Interestingly, STAT1 can form a homodimer that translocates into the nucleus to stimulate the transcription of specific genes. Alternatively, STAT1 and STAT2 form a heterodimer and interact with interferon regulatory factor 9 (IRF9) to form the transcriptionally active gene complex factor 3. This complex migrates into the nucleus to bind interferon stimulated response elements

(ISRE) that facilitate transcription of ISGs (Bazer et al., 2008; Walker et al., 2010; Forde et al., 2011b). In signaling via the STAT1 homodimer, the classic ISG are Fc fragment of IgG receptor 1a (FCGR1A), interferon regulatory factor 1 (IRF1), transporter 1, ATP binding cassette subfamily B member (TAP1), and transporter 2, ATP binding cassette subfamily B member (TAP2), while the STAT1/2 heterodimer stimulated ISG are MX dynamin like GTPase 1 (MX1), MX dynamin like GTPase 2 (MX2), interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), interferon induced protein with tetratricopeptide repeats 3 (IFIT3), interferon induced protein with tetratricopeptide repeats 5 (IFIT5), 2',5'-oligoadenylate synthetase 1 (OAS1), and 2',5'-oligoadenylate synthetase 2 (OAS2), with interferon regulatory factor 9 (IRF9), proteasome 20S subunit beta 8 (PSMB8), interferon induced protein 35 (IFI35), interferon induced transmembrane protein 1 (IFITM1) being activated by both transduction pathways (Walker et al., 2010).

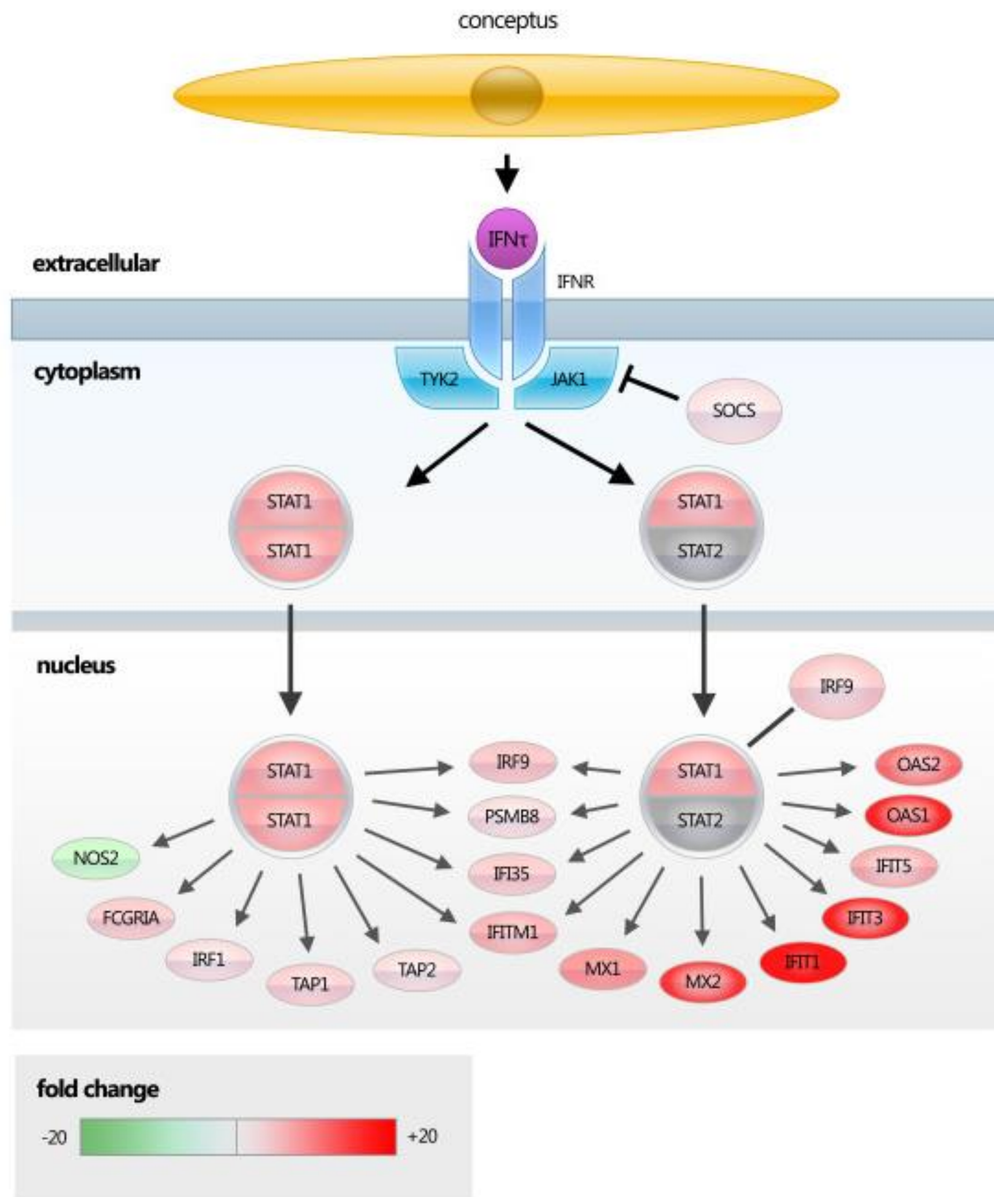

Figure 2.5. Interferon signaling via the Janus kinase-signaling transducer and activator of transcription (JAK-STAT) mechanism and resultant interferon stimulated genes (ISG). Comparisons for fold-change calculations are pregnant cows compared to cyclic cows on day 16 of gestation or the relative estrous cycle. Figure available in (Walker et al., 2010).

Chapter 3. Statement of the problem and dissertation objectives

A deficiency in Se and early embryonic loss are two challenges that can significantly affect profitability in beef cattle production. Soils across the United States, particularly in the southeast, are deficient in Se which problematically results in deficiencies of Se in the forages available (Ammerman and Miller, 1975), and producers in these regions have the highest proportion of grass-fed cattle classified as Se-deficient (Dargatz and Ross, 1996). This necessitates supplemental dietary Se to alleviate the negative effects of Se deficiency on immunity (Erskine et al., 1989), growth (Gleed et al., 1983), and fertility (McClure et al., 1986) including an increased rate of abortion and perinatal mortality (Enjalbert et al., 2006).

The relevance of Se as a trace mineral was identified due to its role as a structural component of glutathione peroxidases (GPX's; (Flohe et al., 1973)) that exert intracellular antioxidant effects. It then became evident that Se is incorporated into a wider range of selenoproteins such as thioredoxin reductases and iodothyronine deiodinases or can be stored as a selenium-containing proteins to serve as an unregulated pool of this mineral (Burk and Hill, 2015).

In supplementing Se in diets of cattle, commercial vitamin-mineral mixes are formulated with ISe, even though OSe-forms are available when cattle consume forages (Ammerman and Miller, 1975) and OSe is more bioavailable than ISe (Khanam and Platel, 2016) presenting a conundrum that warrants further investigation. Previously demonstrated, the form of Se consumed by cattle affects the bioavailability and bioactivity of Se in the blood and tissues (Slavik et al., 2008; Brennen et al., 2011; Liao et al., 2011). Additionally, OSe has been reported to increase the abundance of mRNA

transcripts promoting cellular growth, proliferation, and development in various tissues (Brennen et al., 2011; Liao et al., 2011). More recent research has investigated the effects of form of supplemental Se provided as ISe, OSe, or a novel 1:1 mixture (MIX) of ISe:OSe and have discovered that the form of supplemental Se results in specific transcriptomic phenotypes in liver of growing heifers and the physiological differences are not attributed to a gradient effect of each respective Se-form (Matthews et al., 2014). Additionally, Se-form effects on the transcriptome in the pituitary of growing steers (Li et al., 2019) and testis of neonatal calves (Cerny et al., 2016a) have been observed.

Interestingly, our lab determined that feeding this MIX form compared to ISe alone resulted in a 1.7 ng/mL increase in the peripheral concentration of P4 on day 6 of the estrous cycle with no changes in the diameter of the CL (Cerny et al., 2016b) which provides an opportunity to investigate a novel producer-friendly dietary supplement that may significantly affect fertility in beef cows. Relatedly, cattle producers are challenged with a high percentage of early embryonic loss during early pregnancy, with 70-80% loss occurring between day 8 and 16 of gestation (Sreenan and Diskin, 1986). Successful establishment and maintenance of pregnancy is dependent on P4, and an elevated concentrations of P4 during the early luteal phase results in advanced endometrial function (Forde et al., 2009), longer conceptuses at MRP (Carter et al., 2008), and increased production of IFNτ (Mann and Lamming, 2001).

Collectively, these challenges to beef producers and our previous findings where the novel MIX form of Se increased early luteal phase P4 presented an opportunity to investigate the physiological interrelationship among the form of Se, increased systemic P4, and fertility in beef cows.

3.1. Objectives and hypotheses

CHAPTER 4

TITLE: Form of supplemental selenium in vitamin-mineral premixes differentially affects early luteal and gestational concentrations of progesterone, and postpartum concentrations of prolactin in beef cows

OBJECTIVES: The objectives of this study were to (1) confirm and expand upon the timing of our previous report of a MIX-induced increase in early luteal phase P4, (2) to quantify the effects of the form of Se on concentrations of P4 throughout gestation, and (3) to determine the effects of the form of Se on concentrations of PRL during lactation.

HYPOTHESIS: We hypothesized that the MIX form of Se, versus ISe, would mimic previous findings of an early luteal phase increase in P4, as well as result in an increased concentration of P4 throughout gestation, and then of PRL during lactation.

CHAPTER 5

TITLE: Form of supplemental selenium affects the expression of mRNA transcripts encoding selenoproteins, and proteins regulating cholesterol uptake, in the corpus luteum of grazing beef cows

OBJECTIVES: The objectives of this study were to determine the effect of form of Se on (1) the relative abundance of mRNA transcripts that encode selenoproteins and targeted steroidogenic enzymes in the CL, and (2) the ability of dissociated luteal cells to synthesize P4 and respond to key agonists *in vitro*.

HYPOTHESIS: We hypothesized that there would be form of Se-induced changes in the expression of selenoprotein mRNAs, that CL from MIX-treated cows would express an increase in enzymatic transcripts that favor the production of P4 by luteal cells, and an increased ability for these luteal cells to respond to exogenous agonists *in vitro*.

CHAPTER 6

TITLE: Effect of form of selenium on serum metabolites during early gestation in beef heifers

OBJECTIVES: The objective of this study was to investigate Se-form-specific changes in serum metabolic parameters at estrus, during the early luteal phase, and at MRP, with special consideration given for effects on systemic concentrations of lipoproteins, glucose, cholesterol, and triglycerides.

HYPOTHESIS: We hypothesized that these proteins and metabolites would be elevated in MIX-Se form compared to ISe-supplemented heifers during the establishment of pregnancy.

CHAPTER 7

TITLE: Transcriptomic changes in response to form of selenium on the interferon-tau signaling mechanism in the caruncular tissue of beef heifers at maternal recognition of pregnancy

OBJECTIVES: The objective of this study was to determine form of Se-induced transcriptomic changes in the CAR tissue of the endometrium at MRP.

HYPOTHESIS: We hypothesized that there would be changes in the endometrium that advanced the development of CAR tissue in preparation for implantation of the previously observed advancing conceptus in MIX vs. ISe-supplemented heifers.

Chapter 4. Form of supplemental selenium in vitamin-mineral premixes differentially affects early luteal and gestational concentrations of progesterone, and postpartum concentrations of prolactin in beef cows

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4.1. Abstract

Soils with marginal to deficient levels of selenium (Se) are widespread in the northwest, northeast, and southeast US. Supplementation to the diet of forage-grazing beef cattle with a vitamin-mineral mix containing additional Se is recommended in these geographic regions. We have reported that the form of supplemental Se provided to Angus-cross beef cows can affect circulating levels of progesterone (P4) on day 6 of the estrous cycle, a time when increased P4 is known to promote fertility. The objectives of this study were to (1) confirm and expand upon our initial report that the form of Se provided to cows

affects early luteal-phase concentrations of systemic P4, (2) determine the effects of the form of Se on concentrations of P4 during gestation, and (3) determine the effects of the form of Se on concentrations of prolactin (PRL) during lactation. Throughout this study, Angus-cross beef cows had *ad libitum* access to a vitamin-mineral mix containing 35 ppm of Se in either an inorganic form (ISe) or a 1:1 mix of inorganic and organic forms (MIX). We observed a MIX-induced increase ($p = 0.006$) in systemic concentrations of P4 on day 7 but not on days 4 or 10 of the estrous cycle, consistent with our earlier report. We observed a MIX-induced increase ($p = 0.02$) in the systemic concentration of P4 at months 1, 3, 5, and 7 of gestation, and a MIX-induced decrease ($p < 0.05$) in systemic concentrations of PRL at months 5 and 6 of lactation. In summary, the form of Se provided to cows can be manipulated to affect the early luteal phase and gestational concentrations of P4, and postpartum concentrations of PRL.

Keywords: corpus luteum; progesterone; prolactin; gestation; lactation; selenium

4.2. Introduction

Dietary selenium (Se) is required for the synthesis of selenoproteins, including glutathione peroxidases and thioredoxin reductases, which catalyze the breakdown of hydrogen peroxide and lipid hydroperoxides, protecting extracellular and intracellular cell membranes (Combs et al., 1975; Paszkowski et al., 1995; Steinbrenner et al., 2006; Ghadi et al., 2012). In cattle, a deficiency in Se has been shown to reduce rates of growth (Gleed et al., 1983), alter immune responses (Boyne and Arthur, 1979; Erskine et al., 1989), and reduce indicators of fertility (McClure et al., 1986), including an increased rate of abortion and perinatal mortality (Enjalbert et al., 2006). Soils with marginal to

deficient levels of Se are widespread in the northwest, northeast, and southeast USA (Kubota and Allaway, 1972). Because the content of Se in forages is dependent upon the content of Se in the soil, supplementation to the diet of forage-grazing beef cattle with a vitamin-mineral mix containing Se is recommended in these areas.

Conventionally, free-choice vitamin-mineral mixes containing Se have been formulated with an inorganic form of Se, sodium selenate or sodium selenite. However, the available forms of Se that naturally occur in forages are the organic forms, selenomethionine and selenocysteine (Pereira et al., 2012). The form of Se available to an animal affects the bioavailability of Se in blood and tissues, and the bioactivity of Se by affecting blood glutathione peroxidases (Slavik et al., 2008; Brennen et al., 2011; Liao et al., 2011). Additionally, organic forms of supplemental Se have been shown to stimulate tissue assimilation as noted by the upregulation of mRNA associated with genes promoting cellular growth, proliferation, and development (Brennen et al., 2011; Liao et al., 2011).

We previously demonstrated that the consumption of equimolar amounts of Se in organic (OSe), inorganic (ISe), or mixed (MIX, 1:1 OSe:ISe) forms by beef cows throughout pregnancy resulted in distinct transcriptome profiles in the testes collected from their newborn bull calves, including the differential expression of mRNAs known to regulate gonadal steroidogenesis (Cerny et al., 2016a), and that cows consuming MIX versus ISe had an increased concentration of systemic progesterone (P4) on day 6 of the estrous cycle (Cerny et al., 2016b). Elevated early luteal phase concentrations of P4 have been reported to advance endometrial development (Forde et al., 2009), increase embryonic length (Garrett et al., 1988; Carter et al., 2008), and improve rates of

pregnancy (Wiltbank et al., 2012), suggesting that the form of Se supplied to cattle may be used to manipulate early luteal phase concentrations of P4 in a manner that promotes fertility.

Given these documented effects of the form of Se on steroidogenic gene expression in the neonatal testes, and on day 6 concentrations of P4 in the cycling cow, the first objectives of this study were to (1) confirm and expand upon the timing of our initial report of an MIX-induced increase in early luteal phase P4, and (2) to quantify the effects of the form of Se on concentrations of P4 throughout gestation itself. We also reported that the form of Se supplemented to steers affects the systemic concentration of prolactin (PRL, (Jia et al., 2018)). Although PRL is most widely recognized for its role in the development of the mammary gland and the induction of lactation (Stricker and Grueter, 1928), it has been further identified as a regulator of multiple physiological processes, including growth and development, metabolism, and immune function (Bole-Feysot et al., 1998; Goffin et al., 2002). Therefore, our third objective was to determine the effects of the form of Se on concentrations of PRL during lactation. Collectively, we hypothesized that the MIX form of Se, versus ISe, would increase concentrations of P4 throughout gestation, and then PRL during lactation. From this research, supplementation strategies using a defined form of Se may be adopted by beef producers to influence endocrine pathways that promote the establishment and maintenance of pregnancy in cows, followed by the growth and development of their offspring prior to weaning.

4.3. Methods

4.3.1. Animals and Experimental Procedure

All animal research protocols were approved by the University of Kentucky Institutional Animal Care and Use Committee (IACUC #2017-2828). Multiparous Angus-cross cows (4–11 years of age) were managed in a forage-based fall-calving production system and housed at the University of Kentucky Research and Education Center in Princeton, Kentucky. With the exception of months 5 to 7 of lactation, when all cows consumed a common silage ration, all animals continuously grazed endophyte-infected tall fescue pastures.

For the experiments described herein, cows were randomly selected from pre-existing Se form-specific cow herds. As described before (Patterson et al., 2013; Matthews et al., 2014; Cerny et al., 2016a; Cerny et al., 2016b), each cow had *ad libitum* access to a free-choice vitamin-mineral mix formulated to contain 35-ppm ISe (Sodium selenite; Prince Agri Products, Inc., Quincy, IL, USA) or a 1:1 combination (MIX) of ISe and OSe (SEL-PLEX; Alltech, Inc., Nicholasville, KY, USA) for the duration of this study. Details of individual *ad libitum* intake and the effects on blood Se have been previously reported (Goffin et al., 2002), as has the composition of the basal vitamin-mineral mix (Jia et al., 2018).

4.3.2. Experimental Regimen

4.3.2.1. Effect of Form of Se on Early Luteal Phase Concentrations of P4

To expand upon our earlier report that the MIX form of Se increased the concentration of systemic P4 on day 6 post-estrus, we determined the effect of supplementation with MIX versus ISe on concentrations of P4 on day 4, 7, and 10 post-estrus, spanning the interval where increased P4 is known to promote fertility (Stronge et al., 2005; Carter et al., 2008; Carter et al., 2010; Monteiro et al., 2014; Monteiro et al., 2015). Briefly, luteal function was confirmed in 24 cows (n = 12 (Carter et al., 2008) per treatment) by transrectal ultrasonography using a 5–8 MHz, 66-mm linear array transducer (Ibex Pro, E.I. Medical Imaging, Loveland, CO, USA). Cows were then administered i.m. with 25 mg prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Lutalyse, Pfizer Animal Health, New York, NY, USA) to induce regression of the corpus luteum (CL) and monitored for behavioral estrus (day 0). On days 4, 7, and 10 post-estrus, 8 mL of blood was collected via jugular venipuncture into sodium-heparin-containing tubes (Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for retrieval and quantification of plasma concentrations of P4 by radioimmunoassay (Hatler et al., 2008).

4.3.2.2. Effect of Form of Se on Concentrations of P4 during Gestation

To determine the effect of supplementation with MIX versus ISe on the concentration of systemic P4 during gestation, estrus was synchronized in cows using an intravaginal Controlled Internal Drug Releasing (CIDR) device (ZOETIS EAZI-BREED. CIDR ®1.38 g progesterone, Zoetis, Parsippany, NJ, USA) for 7 days, with each cow administered 25 mg of $PGF_{2\alpha}$ at CIDR removal. At observed estrus, cows were

artificially inseminated by an experienced technician. Pregnancy was confirmed via transrectal ultrasonography at 45 days after insemination, and only cows that conceived to artificial insemination (AI) were included in this study (ISe, n = 12; MIX, n = 14). At months 0, 1, 3, 5, and 7 of gestation, 8 mL of blood was collected via jugular venipuncture into sodium-heparin-containing tubes for retrieval and quantification of plasma concentrations of P4 by radioimmunoassay (RIA, (Hatler et al., 2008)).

4.3.2.3. Effect of Form of Se on Concentrations of PRL during Lactation

To determine the effect of supplementation with MIX versus ISe on the circulating concentration of PRL during the postpartum period, sampling of the same cows that were used to determine the effect of treatment on gestational concentrations of P4 was continued (n = 12 per treatment, as 2 cows were removed from this study due to management considerations). Beginning at 1 month postpartum, 8 mL of blood was collected via jugular venipuncture into additive-free tubes (Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) every 28 days for the following 6 months for the subsequent retrieval and quantification of serum concentrations of PRL by RIA (Schuenemann et al., 2005).

4.3.3. Se and Hormone Analyses

To verify the Se-adequate status of cows during this trial, whole blood was retrieved from cows throughout this study for subsequent analysis of total blood Se. We previously reported that liver and plasma levels of Se stabilize between 54 and 112 days

of supplementation (Brennen et al., 2011). Total blood Se was determined by Michigan State University Diagnostic Center for Population and Animal Health (DCPAH) using an Agilent 7900 inductively coupled plasma-mass spectrometer, as described previously (Wahlen et al., 2005). Concentrations of P4 were quantified in samples of plasma by a commercially available competitive RIA without extraction (ImmuChem. Coated Tube Progesterone 125-I RIA Kit, MP Biomedicals, Costa Mesa, CA, USA), as described previously (Hatler et al., 2008). Low (0.2 ng/mL), medium (1.6 ng/mL), and high (4 ng/mL) reference samples were included in the RIA. All samples were analyzed within a single assay, and the intra-assay coefficient of variation (CV) was 3.15%.

Concentrations of PRL were quantified by the laboratory of Dr. Lannett Edwards (University of Tennessee), using a double-antibody RIA as described previously (Bernard et al., 1993). Low (5 ng/mL) and high (10 ng/mL) reference samples were included in the RIA. The intra-assay CV was 4.49% and the inter-assay CV was 8.59%.

4.3.4. Statistical Analysis

The individual cow was the experimental unit. To determine the effect of the form of Se on concentrations of circulating P4 and PRL, data were subjected to ANOVA with repeated measures using the PROC Glimmix function of SAS statistical software package (version 9.4; SAS Institute, Inc. Cary, NC, USA). Results are presented as the least square means ± standard error of the mean (LS Means ± SEM). Significance was declared at $P < 0.05$, and a tendency to differ was declared when $0.05 < P < 0.10$.

4.4. Results

4.4.1. Concentrations of Se in Whole Blood

All cows were maintained on the form of Se-specific treatments that provided adequate concentrations of whole blood Se (0.14 to 0.17 ± 0.01 ng/mL) throughout the duration of this study (Gerloff, 1992; Dargatz and Ross, 1996).

4.4.2. Concentrations of P4 during the Early Luteal Phase

The concentrations of P4 were determined in plasma collected from cows on days 4, 7, and 10 of the estrous cycle. Cows maintained on the MIX treatment group versus ISe had a greater concentration of systemic P4 on day 7 ($P = 0.006$) but not on days 4 or 10 ($P > 0.05$) post-estrus (Table 4.1).

Table 4.1. Effect of form of supplemental Se on the concentration of progesterone (P4) in the peripheral plasma of cows during the early luteal phase of the estrous cycle[1].

Variable	**Treatment**		***P*-value [2]**
	ISe	**MIX**	
	Mean ± SEM	Mean ± SEM	
Progesterone (ng/mL)			
*Cerny et al., 2016 **			
No. of cows (n)	9	9	
Day 6 †	3.44 ± 0.18 [a]	5.14 ± 0.60 [b]	0.035
Experimental Study			
No. of cows (n)	12	12	
Day 4	1.02 ± 0.22	0.94 ± 0.12	0.740
Day 7 †	2.92 ± 0.27 [a]	3.91 ± 0.16 [b]	0.006
Day 10	7.17 ± 0.54	6.36 ± 0.55	0.308

[1] Selenium was supplemented at 35 ppm as either inorganic (ISe; sodium selenite), or a 1:1 combination of (MIX) of ISe and organic (OSe, SEL-PLEX). Selenium was provided ad libitum in free-choice vitamin-mineral premixes;
[2] P-values associated with one-way ANOVA; * Adapted from results reported in (Cerny et al., 2016b); Means ± SEM with different superscripted letters (a,b) differ ($P \leq 0.05$).

4.4.3. Concentrations of P4 during Gestation

The concentrations of P4 were determined in plasma collected from cows at months 0, 1, 3, 5, and 7 of confirmed pregnancy. Cows maintained on the MIX treatment group versus ISe had a greater (P = 0.02) concentration of systemic P4 at months 7 of gestation (Figure 4.1).

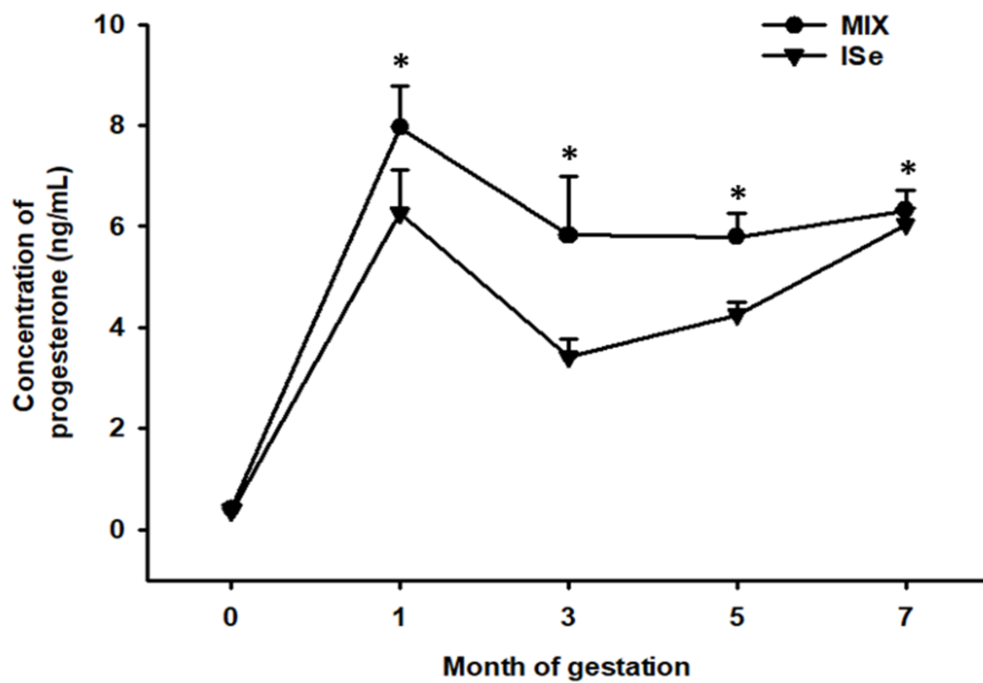

Figure 4.1. Effect of the form of Se on the systemic concentration of P4 during gestation. Cows had *ad libitum* access to vitamin-mineral premixes containing 35 ppm of either inorganic (ISe; sodium selenite; n = 12), or a 1:1 combination (MIX) of ISe and OSe (SEL-PLEX; n = 14) selenium. The concentrations of P4 was affected by treatment (P = 0.02) and time (P < 0.001) but not treatment by time (P = 0.2).

4.4.4. Concentrations of PRL during Lactation

Beginning at 1 month postpartum, the concentration of PRL was determined in serum retrieved from cows every 28 days for the following 6 months. Prolactin was affected by time ($P < 0.001$) and treatment by time ($P < 0.001$) and tended to be affected by treatment ($P = 0.08$). The form of Se did not affect ($P > 0.05$) the concentration of PRL in the systemic blood of cows during the first four (28 day) periods after calving. However, cows maintained on the MIX treatment group versus ISe had a lower ($P < 0.05$) concentration of systemic PRL during the fifth and sixth periods (February and March, Figure 4.2).

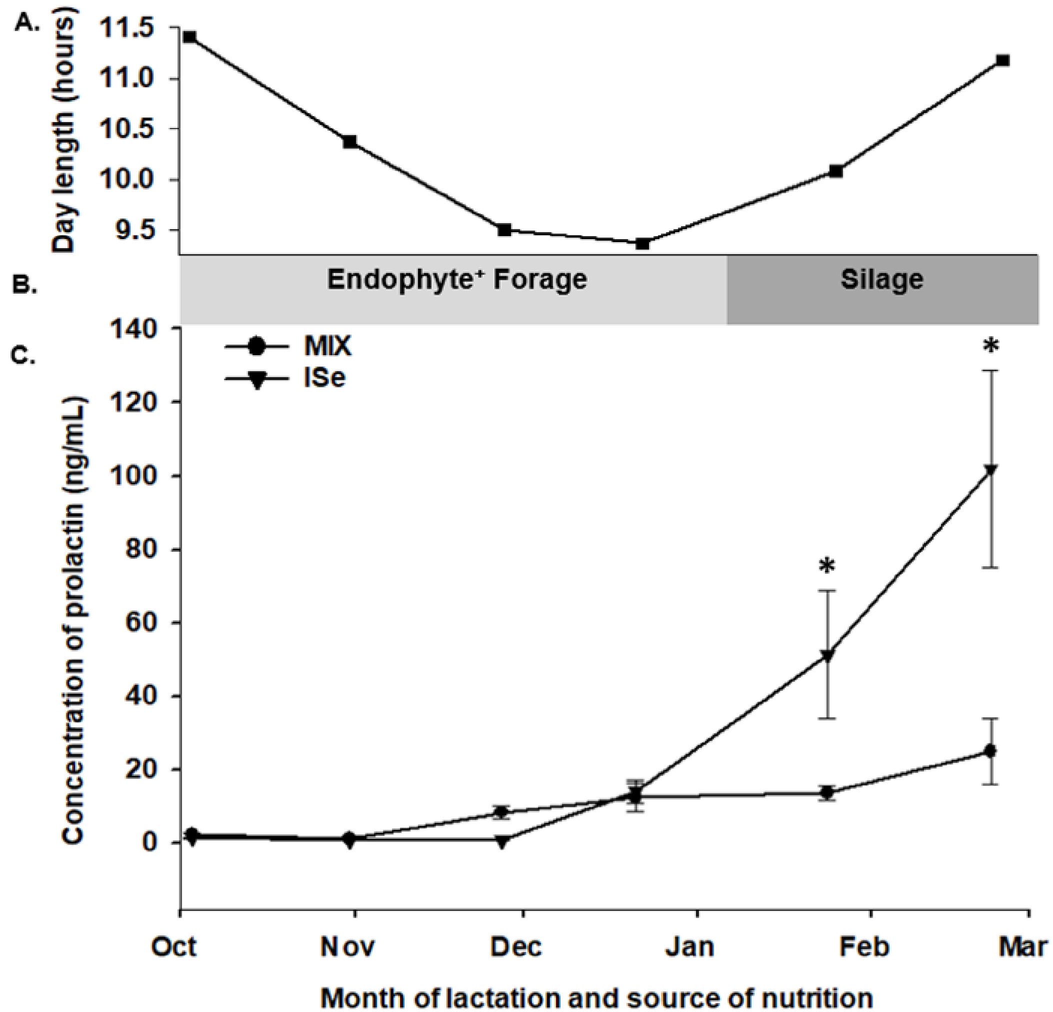

Figure 4.2. Effect of the form of Se on the systemic concentration of prolactin (PRL) during lactation. (A) Day length (hours), (B) basal diet (endophyte-infected tall fescue pasture versus silage), and (C) serum concentrations of PRL during lactation. Cows had *ad libitum* access to vitamin-mineral premixes containing 35 ppm of either inorganic (ISe; sodium selenite; n = 12), or a 1:1 combination (MIX) of ISe and OSe (SEL-PLEX; n = 12) selenium. The concentration of PRL was affected by time ($P < 0.001$) and treatment by time ($P < 0.001$) but not by treatment ($P = 0.8$).

4.5. Discussion

The objectives of this study were to determine, in Se-adequate cattle, the effect of the form of supplemental Se on concentrations of P4 during the early luteal phase of the estrous cycle and gestation, then on concentrations of PRL during lactation. Analysis of the concentrations of Se in the whole blood of cows during this study confirmed that these cows were maintained in a Se-adequate status throughout this study (Gerloff, 1992; Dargatz and Ross, 1996).

We previously reported that supplementing cows with the mixed blend of organic and inorganic Se (MIX), versus inorganic Se (ISe) alone, resulted in an increase in peripheral concentrations of P4 by 50% on day 6 of the estrous cycle (Cerny et al., 2016b). Herein, we confirm this stimulatory effect of the form of Se on early luteal phase P4, with MIX now reported to increase P4 on days 6 (Cerny et al., 2016b) and 7 (herein) but not days 4 or 10 of the estrous cycle. Multiple reports have indicated that increases in early luteal phase concentrations of P4 advance endometrial development, increase embryonic length, and improve rates of pregnancy. For example, Beltman et al. artificially increased P4 in heifers from day 3.5 to 6 or day 4.5 to 8 post-estrus. In both instances, they reported a significant relationship between the change in serum P4 and embryo survival rate (Beltman et al., 2009). Consistent with this, Mann et al. determined that P4 on days 4 and 5 differed among cows that had a large well-elongated embryo versus a less well-elongated embryo (Mann et al., 2003). Carter et al. artificially increased P4 in heifers from days 3 to 7 post-estrus and reported P4-dependent changes to the transcriptome of day 7-recovered blastocysts (Carter et al., 2010), and Forde et al. increased P4 in heifers from day 3 post-estrus and reported P4-dependent divergence of

the endometrial transcriptome by day 7, as well as a P4-dependent increase in conceptus development by day 14 (Forde et al., 2011a). Importantly, Yan et al. performed a comprehensive meta-analysis from the data reported from 53 publications and concluded that increased P4 (treatment with P4 initiated between days 3 and 7) post-estrus significantly increased the chance of pregnancy (Yan et al., 2016). With the MIX form of Se now shown to increase day 6 and 7 concentrations of systemic P4, we therefore confirm a novel producer-friendly management technique to increase the levels of circulating P4 at a time that is known to promote endometrial function, embryonic development, and conceptus survival.

In the current study, concentrations of P4 remained higher throughout gestation in the MIX- versus ISe-supplemented cows (Figure 4.1). For over a century, the requirement of P4 for the maintenance of pregnancy has been understood (Prenant, 1898), with removal of the CL (the primary source of P4 in the cow) prior to 200 days of gestation resulting in the termination of pregnancy (Fraenkel, 1901; Magnus, 1901; Fraenkel, 1903; Estergreen et al., 1967). However, the physiological importance of the MIX-induced increase in gestational levels of P4 observed herein is still hard to define. Although the level of gestational P4 has been reported to affect the incidence of retained placenta in dairy cows (McDonald et al., 1952; McDonald et al., 1954; Chew et al., 1977), with Erb et al. concluding that high levels of P4 in the blood are necessary for normal gestation and expulsion of fetal membranes (Erb et al., 1968), whether the MIX-induced increase in gestational levels of P4 observed herein provides a positive benefit to the maintenance of pregnancy in beef cows remains to be determined.

It is well established that the concentration of circulating PRL is affected by the photoperiod and temperature (Peters and Tucker, 1978; Tucker et al., 1984), as well as exposure to endophyte-infected tall fescue forages (Goetsch et al., 1987; Davenport et al., 1993; Li et al., 2019). Worthy to note, a decrease in circulating concentrations of PRL is considered a hallmark of endophyte-induced fescue toxicosis (Goetsch et al., 1987). Interestingly, the divergence in concentrations of PRL that we observed in cows on the MIX versus ISe treatment groups occurred during the winter months when all cattle were maintained on a common corn-based silage ration in lieu of grazing endophyte-infected tall fescue pastures. While the mechanism responsible for the more robust increase in PRL in ISe- versus MIX-supplemented cows in this study is unclear, we reported that in steers grazing endophyte-infected tall fescue pastures during the summer months and supplemented with either ISe or MIX, MIX-supplemented steers had increased concentrations of serum PRL (Jia et al., 2018). Whether the contrasting results in PRL between these two trials is reflective of differences in gender (steers versus cows) or diet (pasture versus silage) remains to be determined.

Although PRL is most recognized for its role in the development of the mammary gland and the induction of lactation (Stricker and Grueter, 1928), more recently defined are the pleiotrophic roles of PRL, including effects on immune function and osmoregulation (Bole-Feysot et al., 1998; Goffin et al., 2002). Extra-pituitary sources of PRL have been identified and include immune cells (macrophages, B cells, NK cells, T cells, thymocytes, and peripheral blood mononuclear cells (O'Neal et al., 1992; Pellegrini et al., 1992; Matera et al., 1997; Gingras and Margolin, 2000), with the PRL receptor also localized widely throughout the immune system (Orbach et al., 2007). Prolactin also

affects water transport across amniotic membranes, and PRL-induced solute transport during late pregnancy may be an important preparatory player for subsequent lactation (Freeman et al., 2000). We reported that the form of Se affects the expression of > 500 annotated genes in the pituitary gland of beef steers (Li et al., 2019), with a functional analysis of that microarray-based dataset revealing that the form of Se predominately affected a canonical pathway network between PRL and pro-opiomelanocortin (POMC), adrenocorticotropic hormone (ACTH), and α-melanocyte-stimulating hormone (α-MSH) synthesis-related hormones. Overall, it appears that there is an interplay between a direct effect of the form of Se on the bovine pituitary gland and its release of PRL, and the form of Se-mediated PRL-immune cell signaling.

4.5.1. Conclusion

In this report, we aimed to (1) confirm our initial report of an MIX-induced increase in early luteal phase concentrations of systemic P4, (2) define the effects of the form of Se on P4 throughout gestation, and (3) quantify the effects of the form of Se on concentrations of PRL during lactation. Even with the limitation of animal numbers, our hypothesis that the MIX treatment would increase circulating concentrations of P4 during gestation and PRL during lactation was confirmed for P4 but not for PRL in which the reverse was observed. With increased early luteal phase P4 known to promote fertility, and the absolute requirement for P4 on the maintenance of a pregnancy, it can be concluded that supplementation with the MIX form of Se can be considered a viable management tool to improve fertility in cows maintained in regions where the Se content of soils is inadequate and supplementation with this trace mineral is recommended.

Whether manipulation of the form of Se to affect postpartum concentrations of PRL can be used as a management tool to promote the growth, development, and immune function of suckling calves remains to be fully elucidated.

Chapter 5. Form of supplemental selenium affects the expression of mRNA transcripts encoding selenoproteins, and proteins regulating cholesterol uptake, in the corpus luteum of grazing beef cows

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5.1. Abstract

Selenium (Se)-deficient soils necessitate supplementation of this mineral to the diet of forage-grazing cattle. Functionally, Se is incorporated into selenoproteins, some of which function as important antioxidants. We have previously shown that the source of supplemental Se; inorganic (sodium selenite or sodium selenate; ISe), organic (selenomethionine or selenocysteine; OSe) or 1:1 mix of ISe and OSe (MIX), provided to Angus-cross cows affects concentrations of progesterone (P4) during the early luteal phase of the estrous cycle. In this study, we sought to investigate (1) the effect of form of Se on the expression of mRNA encoding selenoproteins in the corpus luteum (CL), and

(2) whether this previously reported MIX-induced increase in P4 is the result of increased luteal expression of key steroidogenic transcripts. Following a Se depletion and repletion regimen, 3-year-old, non-lactating, Angus- cross cows were supplemented with either ISe as the industry standard, or MIX for at least 90 days, with the CL then retrieved on Day 7 post-estrus. Half of each CL was used for analysis of targeted mRNA transcripts and the remainder was dissociated for culture with select agonists. The expression of three selenoprotein transcripts and one selenoprotein P receptor was increased ($P < 0.05$), with an additional five transcripts tending to be increased ($P < 0.10$), in cows supplemented with MIX versus ISe. In cultures of luteal cells, hCG-induced increases in P4 ($P < 0.05$) were observed in CL obtained from ISe-supplemented cows. The abundance of steroidogenic transcripts in the CL was not affected by the form of Se, however, the abundance of mRNA encoding 2 key transcripts regulating cholesterol uptake (*Ldlr* and *Hsl*) was increased ($P < 0.05$) in MIX-supplemented cows. Overall, the form of Se provided to cows is reported to affect the expression of mRNA encoding several selenoproteins in the CL, and that the form of Se-induced effects on luteal production of P4 appears to be the result of changes in cholesterol uptake, rather than a direct effect on the expression of steroidogenic enzymes within the CL.

Keywords: corpus luteum; selenium; selenoprotein; progesterone; cholesterol; steroidogenesis

5.2. Introduction

The physiological effects of a deficiency in selenium (Se) that occur as a result of cattle consuming forages from Se-deficient soils are well-documented (Boyne and

Arthur, 1979; Gleed et al., 1983; McClure et al., 1986; Erskine et al., 1989; Enjalbert et al., 2006). In Se-deficient regions, including the southeast United States, it is recommended that cattle producers supplement this trace mineral to ensure adequate rates of growth (Gleed et al., 1983), immune function (Boyne and Arthur, 1979), and reproductive health (Enjalbert et al., 2006). Naturally, Se can exist in various forms, including the organic forms, selenocysteine (SeCys) and selenomethionine (SeMet), and the inorganic forms, selenite and selenate (Suzuki, 2005). Organic forms of selenium are the most predominant form in forages; however, inorganic forms are more commonly used in commercial vitamin-mineral mixes supplemented to livestock in Se deficient regions such as the southeast United States (Ammerman and Miller, 1975).

The relevance of Se as a trace mineral was identified due to its role as a structural component of glutathione peroxidases (GPX's; (Flohe et al., 1973)), a family of enzymes with antioxidant capabilities. Presently, there have been > 20 genes for selenoproteins identified in humans (Labunskyy et al., 2014) and pigs (Chen et al., 2018). Many studies have demonstrated the antioxidant capabilities of selenoproteins to protect cells from harmful reactive oxygen species (ROS, (Beilstein et al., 1996; Rundlöf et al., 2001; Kryukov et al., 2002; Takebe et al., 2002; Lu et al., 2006; Lillig and Holmgren, 2007; Novoselov et al., 2007; Arnér, 2009; Reeves et al., 2010; Labunskyy et al., 2014)). GPX variants are some of the most common antioxidants that catalyze hydrogen peroxide into H_2O (Labunskyy et al., 2014), and thioredoxins protect against oxidative stress as redox proteins and can catalyze dithiol-disulfide exchange reactions in various tissues. Although thioredoxin is not a selenoprotein, once thioredoxin is oxidized, it must be reduced by the selenoproteins thioredoxin reductases (TRXR, (Arnér, 2009)).

Additionally, selenoprotein P (SELENOP (Takebe et al., 2002)), selenoprotein W (SELENOW (Beilstein et al., 1996)), selenoprotein H (SELENOH (Novoselov et al., 2007)), selenoprotein K (SELENOK (Lu et al., 2006)), selenoprotein M (SELENOM (Reeves et al., 2010), and selenoprotein R (SELENOR (Kryukov et al., 2002)) have documented or proposed antioxidant properties.

Physiological incorporation of Se is dependent on its respective form (Suzuki, 2005) and the form of Se available for uptake affects the transcriptome profile in tissues including the liver (Matthews et al., 2014), neonatal testis (Cerny et al., 2016a), and pituitary (Li et al., 2019) of cattle. The form of dietary Se affects the bioavailability and bioactivity of this trace mineral in blood and tissues, altering concentrations of selenoproteins including GPXs (Slavik et al., 2008; Brennen et al., 2011; Liao et al., 2011). Research has indicated differences in the assimilation of Se, dependent on the form being fed, with lower blood and milk concentrations of Se in cows supplemented with an inorganic (ISe) versus organic (OSe) form (Pehrson et al., 1999; Gunter et al., 2003; Givens et al., 2004; Muñiz-Naveiro et al., 2006; Slavik et al., 2008; Ceballos et al., 2009; Gunter et al., 2013; Patterson et al., 2013). Previously, our lab demonstrated that a 1:1 blend of ISe and OSe (MIX) provided to cows to achieve a Se-adequate status resulted in a 1.0 to 1.7 ng/mL increase in early (Days 6 and 7) luteal phase concentrations of P4 when compared to cows supplemented with ISe and/or OSe alone (Cerny et al., 2016b; Carr et al., 2020). This elevated P4 occurs without any effect on diameter of the CL (Cerny et al., 2016b). Importantly, multiple trials have reported that development of the endometrium (Forde et al., 2009) and consequently growth of the conceptus (Garrett

et al., 1988; Carter et al., 2008) are advanced with elevated early luteal phase concentrations of P4, increasing overall indicators of fertility (Wiltbank et al., 2012).

With no knowledge of how different forms of supplemental Se affect the expression of transcripts encoding selenoproteins in the CL, nor of the mechanism by which supplementation with MIX increases early luteal phase (Cerny et al., 2016b; Carr et al., 2020) or gestational (Carr et al., 2020) concentrations of systemic P4, the primary objectives of this study were to determine the effect of form of Se on (1) the relative abundance of mRNA transcripts that encode selenoproteins and targeted steroidogenic enzymes in the CL, and (2) the ability of dissociated luteal cells to synthesize P4 and respond to key agonists *in vitro*. To achieve this goal, we assigned cows to form of Se-treatment regimens (ISe versus MIX) that achieved a Se-adequate status in all cows, then collected CL from these cows on Day 7 of the estrous cycle for molecular and *in-vitro* analyses. We hypothesized that there would be form of Se-induced changes in the expression of selenoprotein mRNAs, that CL from MIX-treated cows would express an increase in enzymatic transcripts that favor the production of P4 by luteal cells, and an increased ability for these luteal cells to respond to exogenous agonists *in vitro*.

5.3. Methods

5.3.1. Animals and Experimental Procedure

All procedures involving animals were approved by the University of Kentucky's Institutional Animal Care and Use Committee (IACUC), protocol number 2017-2828. Fall-born, first-calf 3-year-old Angus-cross cows (N = 10) were selected from established, Se form-specific cowherds as previously described (Patterson et al., 2013;

Matthews et al., 2014; Cerny et al., 2016a; Cerny et al., 2016b). At the beginning of this trial, all cows were subject to a 45-day period where systemic levels of Se were depleted (supplementation of a vitamin-mineral mix containing no exogenous source of Se), followed by a 45-day period where all cows received supplement formulated with 35 ppm Se as ISe (repletion, Figure 5.1) to return total blood Se in all cows to a Se adequate status (Gerloff, 1992; Dargatz and Ross, 1996; Brennen et al., 2011). Following this, cows were assigned to at least 90 days of individual access to ISe (n = 5; Sodium selenite; Prince Agri Products, Inc. Quincy, IL, USA) or MIX (n = 5; 1:1 ISe:OSe; SEL-PLEX; Alltech, Inc., Nicholasville, KY, USA). Cows were individually supplemented with their respective treatments using in-pasture Calan gates (Patterson et al., 2013). Jugular blood was collected for the determination of total whole blood Se from each cow at the start, middle and endpoint of the depletion and repletion periods, then monthly until the end of the trial, similar to methods previously described (Patterson et al., 2013; Cerny et al., 2016b).

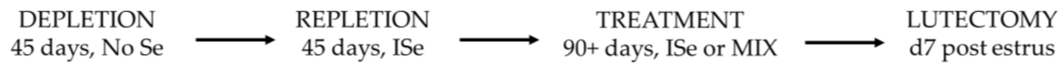

Figure 5.1. Experimental model of dietary supplementation. At the start of experimentation, cows were subject to a 45-day depletion during which they were supplemented with a Se-free vitamin-mineral mix, followed by a 45-day repletion period during which cows were supplemented with a vitamin-mineral mix formulated to contain 35 ppm ISe/cow/day. After repletion, cows were supplemented with a vitamin-mineral mix containing 35 ppm of their respective treatment for at least 90 days prior to estrous synchronization with an i.m. injection of 25 mg dinoprost tromethamine. On day 7 post-estrus, CL were collected via trans-vaginal lutectomy for analysis of key selenoprotein-encoding and steroidogenic transcripts by real-time PCR and *in vitro* culture of dissociated luteal cells with select agonists.

5.3.2. Diet

Cows were initially maintained on a common, nontoxic endophyte-infected tall fescue pasture (Lacefield MaxQ II), then transferred to a common silage diet during the winter months (January–April). Forage samples for analysis of Se and trace minerals were collected for the duration of experimentation. Pasture concentration of Se in the forage was approximately 0.01 mg Se/kg as fed and 0.04 mg Se/kg dry matter (Dairy One Forage Testing Laboratory, Ithaca, NY, USA). The concentration of Se in the dietary corn silage was approximately 0.03 mg Se/kg as fed and 0.08 mg Se/kg dry matter (Dairy One Forage Testing Laboratory, Ithaca, NY, USA). Both are consistent with being classified as Se deficient(Kubota and Allaway, 1972; NASEM, 2016).

5.3.3. Experimental Regimen

Following the 90 days of treatment with supplemental Se in either ISe or MIX forms, cows were randomly treated i.m. with 25 mg dinoprost tromethamine ($PGF_{2\alpha}$; Lutalyse, Pfizer Animal Health, New York, NY, USA) to induce luteal regression and monitored for behavioral estrus (Day 0). Development of the preovulatory follicle and ovulation was confirmed via trans-rectal ultrasonography using a 5–8 MHz, 66-mm linear array transducer (Ibex Pro, E.I. Medical Imaging, Loveland, CO, USA). On days 5, 6, and 7 post-estrus, the diameter of the CL was determined by ultrasonography and blood (8 mL) was collected into additive-free tubes (Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for retrieval of serum and quantification of P4 via radioimmunoassay (Hatler et al., 2008). On Day 7, each CL was collected by supra-vaginal lutectomy, and immediately placed in ice-cold culture medium (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) to be washed, weighed, and cut into two halves (Pate, 1993; Poole et al., 2013). One-half of the CL was transported in the culture medium on ice to the laboratory for dissociation and culture of the fully differentiated luteal cells. The second half was divided into 8 pieces, each of which was immediately snap frozen in liquid nitrogen to be used for RNA extraction and quantitative transcript expression by real-time polymerase chain reaction (qPCR).

5.3.4. Cell Culture

Luteal tissue was handled, dissociated, and incubated following established protocol (Pate, 1993; Poole et al., 2013). Briefly, luteal tissue was minced into ~1 mm^3 cubes and placed in 24 mM HEPES-buffered Ham's F-12 (1x) culture medium with L-glutamine and sodium bicarbonate (Gibco®, Life Technologies Corporation™, Grand Island, NY, USA), containing 0.5% BSA (Fisher BioReagents, Fair Lawn, NJ, USA), 20 µg/mL gentamicin (Gibco®, Life Technologies Corporation™, Grand Island, NY, USA), and 2000 U/g tissue collagenase type I (Worthington Biochemical Corporation, Lakewood, NJ, USA) to dissociate cells. Following dissociation, cells were re-suspended in Ham's F-12 solution with L-glutamine, sodium bicarbonate, HEPES buffer and gentamicin for the determination of cell numbers and viability.

Culture plates were prepared by coating each well in 250 µL culture medium with 10% newborn calf serum (Gibco®, Life Technologies Corporation™, Grand Island, NY, USA). Plates were incubated for 30 min and then washed with the serum-free medium. Luteal cells were plated at 0.6×10^6 cells/mL in 24-well plates in serum free medium

supplemented with L-glutamine and ITS (5 µg/mL insulin, 5 µg/mL transferrin, and 5 ng/mL selenium, Corning™ Premix Universal Culture Supplement, Corning, NY, USA); and 20 µg/mL gentamicin. Steroidogenic cells were cultured with or without PGE_2 (1 and 50 µM/mL, Cayman Chemical Company, Ann Arbor, MI, USA), LH (1 and 10 IU/mL, NIH LH-S26), or hCG (10 and 50 ng/mL, Sigma Aldrich, St. Louis, MO, USA). Treatment concentrations (low and high for each agonist) were based upon previous reports (Pate and Condon, 1984; Gu et al., 1990; Gregoraszczuk and Wojtusiak, 1992; Gregoraszczuk and Zieba, 1994; Cannon et al., 2007; Roy et al., 2009; Szóstek et al., 2011). All treatments and agonist-free control groups were performed in triplicate wells within each replicate. Cultures were incubated at 37 °C in a 5% CO2/95% air environment. Cultures were observed every 12 h during experimentation. All cultured wells were similarly confluent and did not display any loss of cells. Culture media were collected and replaced with fresh medium containing treatments every 24 h for a total of 96 h for subsequent determination of the concentration of P4 via RIA.

5.3.5. Real-Time PCR

Total RNA was extracted from 400–600 mg frozen luteal tissue using TRIzol Reagent (Invitrogen Corporation, Carlsbad, CA, USA) following the manufacturer's instructions. The purity and concentration of total RNA samples were analyzed using a NanoDrop ND-100 Spectrometer (Nanodrop Technologies, Wilmington, DE, USA). All samples had 260/280 absorbance ratios of 1.97 or greater.

The quantification of relative mRNA was performed using real-time polymerase chain reaction (qPCR) using cDNA. To do so, 1 µg of each cow's total RNA was reverse

transcribed into cDNA using the SuperScript™ IV VILO™ Master Mix with ezDNAse™ Enzyme (Invitrogen by Thermo Fisher Scientific, Vilnius, Lithuania). Additionally, a control reaction that did not contain reverse transcriptase was performed and analyzed via qPCR to ensure that products from the targeted transcripts were not obtained from genomic DNA contamination.

The relative abundance of the following mRNAs was quantified: the iodothyronine deiodinases *Dio1, Dio2,* and *Dio3,* the glutathione peroxidases *Gpx1, Gpx2, Gpx3, Gpx4,* and *Gpx6,* the thioredoxin reductases *Txnrd1, Txnrd2,* and *Txnrd3.* Additionally, we investigated the following selenoprotein mRNA's: *Selenof, Selenoh, Selenoi, Selenok, Selenom, Selenon, Selenoo, Selenop, Selenor, Selenos, Selenot, Selenov, Selenow, Sephs1,* and *Sephs2.* Finally, we analyzed the selenoprotein P receptor mRNA's *Lrp2, Lrp8,* and *Tfrc.*

Next, the relative abundance of P4-associated enzymatic transcripts: *Star, Cyp11a1, Hsd3b1, Ptgs2*, and *Ptges*, the receptor transcripts: *Lhcgr, Pgr, Pgrmc1, Pgrmc2, Paqr5, Paqr7, Paqr8, Ep1, Ep2, Ep3, Ep4*, and *Pgtfr*, and transcripts of proteins associated with cholesterol availability: *Ldl, Scarb1, Hsl, Npc1*, and *Npc2* were quantified. Primer sequences used in qPCR and GenBank accession numbers are listed in Table 5.1 and 5.2. Real-time PCR 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 2X 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/primer-blast/) against RefSeq sequence.

All cDNA products were validated via DNA sequencing for verification of targets 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 Table 5.1 and Table 5.2. 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 these three genes. For qPCR analysis, $n = 5$ and 5 for ISe and MIX treatments, respectively. All reactions were performed in triplicate.

Table 5.1. Ascession numbers, primer sets (forward and reverse), and product identities of qPCR analysis of selenoprotein-associated genes.

Gene	Gene Name	Accession Number[1]	Oligonucleotide Primer Design (5' to 3') direction	Amplicon length (bp)	Product identity [2]
Enzymatic transcripts					
DIO1	Iodothyronine deiodinase 1	NM_001122593.2	F: TCCTGTAGTCCGCCTGTCA R: TCCGGTGATTCTTGATGTCCA	242	99%
DIO2	Iodothyronine deiodinase 2	NM_001010992.4	F: GATGGGCATCCTCAGCGTAG R: TTCTCCTGGGCACCATTTCC	315	100%
DIO3	Iodothyronine deiodinase 3	NM_001010993.3	F: AAGTGGAGCTCAACAGCGAT R: AGTCGAGGATGTGCTGGTTC	213	100%
Glutathione peroxidases					
GPX1	Glutathione peroxidase 1	NM_174076.3	F: GCAACCAGTTTGGGCATCAG R: TAGGGTCGGTCATGAGAGCA	210	100%
GPX2	Glutathione peroxidase 2	NM_001163139.2	F: AACAGCCTCAAGTACGTCCG R: TCGGTCATGAGGGAAAACGG	158	100%
GPX3	Glutathione peroxidase 3	NM_174077.5	F: GCACCATCTATGAGTACGGGG R: CCCCATTCACATCGCCTTTC	315	100%
GPX4	Glutathione peroxidase 4	NM_174770.3	F: GATCAAAGAGTTCGCCGCTG R: CCATACCGCTTCACCACACA	198	100%

Table 5.1. (Continued)

GPX6	Glutathione peroxidase 6	NM_001163142.1	F: CACTGTTCCTGGTCGGCTTA R: CCCAGCACAACTACACCGAA	259	100%
Thioredoxin reductases					
TXNRD1	Thioredoxin reductase 1	NM_174625.5	F: AAGGCCGCGTTATTTGGGTA R: CCTGGTGTCCCTGCTTCAAT	306	100%
TXNRD2	Thioredoxin reductase 2	NM_174626.2	F: CAAATGGCTTCGCTGGTCAC R: TTCGTATGCACACCAGCCTT	230	100%
TXNRD3	Thioredoxin reductase 3	XM_015468824.1	F: CGGCGTATGACTACGACCTC R: GACTGTACTCCCAGCCGAAC	249	100%
Other selenoproteins					
SELENOF	Selenoprotein F	NM_001034759.2	F: GCAGCTCCTGTGATTTGCTT R: TTTAGCACAGGGTCTGAACCG	241	100%
SELENOH	Selenoprotein H	NM_001321327.1	F: CACGAGCTGACGAGTCTACG R: CTTCTTCAGCTCCTCCAGCA	235	100%
SELENOI	Selenoprotein I	NM_001075257.2	F: TCTGGCTTTCTGCTGGTTGT R: TGGTCAAAAAGCTCCCCCAG	212	100%
SELENOK	Selenoprotein K	NM_001037489.3	F: CCGTTTTGTCGATTCACGGC R: CAGATGAGCTTCCGTAGCCT	278	100%

Table 5.1. (Continued)

SELENOM	Selenoprotein M	NM_001163171.2	F: CCCACTCTACCACAACCTGG R: ACCTAAAGGTCTGCGTGGTC	249	100%
SELENON	Selenoprotein N	NM_001114976.2	F: GTGGCCATGTACCCCTTCAA R: GGGATGGGTTCTCCTGGTTG	265	100%
SELENOO	Selenoprotein O	NM_001163193.2	F: TGGACAGGTATGACCCCGAT R: ATCTTCTGCAGGTAGTGCCG	202	100%
SELENOP	Selenoprotein P	NM_174459.3	F: TCAGGTCTTCATCACCACCA R: GTGGCAACAGCAGCTACTCA	201	100%
SELENOR	Selenoprotein R, Methionine sulfoxide reductase B1	NM_001034810.2	F: GAACCACTTTGAGCCGGGTA R: GGCCATCGTTCAGGAACTCA	221	100%
SELENOS	Selenoprotein S	NM_001046114.3	F: CCCACCCTCGAGACCGA R: GCCCAGGACTGTCTTCTTCC	394	100%
SELENOT	Selenoprotein T	NM_001103103.2	F: TGGTCACCTTCCATCCATGC R: AAGAGGTACAACGAGCCTGC	240	100%
SELENOV	Selenoprotein V	NM_001163244.2	F: ACTCCATTGGCCACCGATTT R: AGGCCACAGTAAACCACTCG	224	100%
SELENOW	Selenoprotein W	NM_001163225.1	F: AGTGTTCGTAGCGGGAAAGC R: CGCGAGAACATCAGGGAAGG	233	98%

Table 5.1. (Continued)

SEPHS1	Selenophosphate synthetase 1	NM_001075316.1	F: CAAAGCGAACCGGTGGATCT R: GAGGTCACTGAGGACGTTGG	422	99%
SEPHS2	Selenophosphate synthetase 2	NM_001114732.2	F: GATCCCTACATGATGGGGCG R: GTTTACCACCGTTTGCCCAC	219	100%
Selenoprotein P receptors					
LRP2	LDL receptor related protein 2	XM_024983502.1	F: GTGGTTTGGGTTACCGTTGC R: GGCACCCTGTTAGCTGTGAT	304	99%
LRP8	LDL receptor related protein 8	NM_001097565.1	F: AGCCACCCTTTTGGGATAGC R: AAGGCACAGGTACTCACAGC	231	100%
TFRC	Transferrin receptor	NM_001206577.1	F: CCAGGTTTAGTCTGGCTCGG R: GGTCTGCCCAGAATATGCGA	339	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 5.2. Primer sets and product identities of qPCR analysis of reference and steroidogenesis-associated genes.

Gene	Gene Name	Accession Number[1]	Oligonucleotide Primer Design (5' to 3') direction	Amplicon length (bp)	Product identity [2]
Enzymatic transcripts					
STAR	Steroidogenic acute regulatory protein	NM_174189.3	F: CCCGAGACTTTGTGAGCGTA R: GCGCAGGTGATTGGCAAAAT	275	99%
CYP11A1	Cytochrome P450, family 11, subfamily A, polypeptide 1	NM_176644.2	F: TTCAACCTCATCCTGACGCC R: GTGCAAGAGGTGTGGACTGA	204	98%
HSD3B1	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	NM_174343.3	F: GGTTCCTGGTGAGCGTTTCT R: CAGCAGCTGGGTACCTTTCA	362	99%
PTGS2	Prostaglandin-endoperoxide synthase 2	NM_174445.2	F: CCCATGGGTGTGAAAGGGAG R: TCCACCCCATGGTTCTTTCC	203	100%
PTGES	Prostaglandin E synthase	NM_174443.2	F: CGCTGCTGGTCATCAAAATGT R: GGTCTCCATGTCATTCCGGT	173	97%

Receptor transcripts

Table 5.2. (Continued)

LHCGR	Luteinizing hormone (LH) G-protein coupled receptor	NM_174381.1	F: GCCTTTGACAACCTCCTCAAT R: TCCAGGGAAATCAGCGTTGT	332	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	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	Progestin and adipoQ receptor family member 5 (mPR_{γ})	XM_024997926.1	F: GGTTCTTCTCGTGGAGGTTTGT R: GTTCCTGGACATGGAGCTGAA	151	96%
PAQR7	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%
Cholesterol related transcripts					
LDLR	Low density lipoprotein receptor	NM_001166530.1	F: CCCTGACTGCAAGGACAAGT R: GGAGATGCACTCACCGCTTT	217	99%
SCARB1	Scavenger receptor class B member 1	NM_174597.2	F: CAGACATGGGCAACCTCTCT R: TGGATGATCCCCTCAGGGTT	244	99%
HSL	Lipase E, hormone sensitive type	NM_001080220.1	F: GGGATATCTGAAGAGGCCTGG R: GGCTGGTGCGAAAGAAGATG	362	99%

Table 5.2. (Continued)

NPC1	NPC intracellular cholesterol transporter 1	NM_174758.2	F: GGTCATGAGCTGTGGCATCT R: TAGTCCATGAGTGGCTCCCA	253	98%
NPC2	NPC intracellular cholesterol transporter 2	NM_173918.2	F: GGACTGCGGTTCTTGGGTC R: GGGGCATCTGATTCCAGACT	226	99%
Housekeeping Transcripts					
ACTB	Actin beta	NM_173979.3	[illegible]GGAAATCGTCCGTGAC R: GTGTTGGCGTAGAGGTCCTTGC	278	99%
HPRT1	Hypoxanthine phosphoribosyl-transferase 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.

5.3.6. Se and P4 Analysis

Total blood Se was assayed by the American Associates for Veterinary Laboratory Diagnosticians-approved University of Kentucky Veterinary Diagnostics Laboratory (Lexington, KY, USA), as previously reported (Wahlen et al., 2005).

In both samples of serum and *in vitro* media, concentrations of P4 were quantified by a commercially available competitive RIA without extraction (*ImmuChem*™ Coated Tube Progesterone 125-I RIA Kit, MP Biomedicals, Costa Mesa, CA, USA), as described previously (Givens et al., 2004). There was one assay performed for analysis of the serum with an intra-assay CV of 5%, and there were seven assays performed for analysis of culture media with inter-assay CV of 7.85% and intra-assay CVs ranging from 4.53–9.43%.

5.3.7. Statistical Analysis

In all statistical analyses, an individual cow was the experimental unit. All data were analyzed for a normal distribution and homogeneity of variance. When appropriate, data were natural log-transformed for normality. Results are presented as Least Square Means (LS Means) ± standard error of the mean (SEM). At $P < 0.05$ significance was declared, with a tendency to differ when $0.05 \leq P < 0.10$. The effect of treatment on concentrations of peripheral Se and P4 were analyzed as an ANOVA with repeated measures using the PROC MIXED function of SAS statistical software package (version 9.4; SAS Institute, Inc. Cary, NC, USA). The form of dietary Se was considered the fixed effect for both, and the P4 data were natural log-transformed due to not being normally distributed. Luteal weight, luteal diameter, and relative abundance of all mRNA

transcripts were analyzed using a Student's T-Test with the PROC TTest function of SAS statistical software package (version 9.4; SAS Institute, Inc. Cary, NC, USA). *In vitro* concentrations of P4 were analyzed using a split plot design for repeated measures (culture time) with Se treatment as the main-plot and LH, PGE_2, and hCG as the sub-plot factors. Data were blocked by CL (cow) and natural log transformed due to data not being normally distributed or homologous. Data were analyzed as a mixed ANOVA using the PROC GLIMMIX function of the SAS statistical software package (version 9.4; SAS Institute, Inc. Cary, NC, USA).

5.4. Results

5.4.1. Concentrations of Se in Whole Blood

All cows were maintained on their respective Se-form treatments (ISe vs. MIX) and concentrations of whole blood Se were adequate throughout the duration of this study (Figure 5.2, (Gerloff, 1992; Dargatz and Ross, 1996)). During the depletion period, whole blood concentrations of Se declined for both treatment groups, and subsequently increased during the period of repletion. On each treatment, the ISe supplemented cows had a numerically greater concentration of peripheral Se compared to the MIX treatment group. However, within each group, the levels remained relatively stable until the experimental endpoint. There was no effect ($P > 0.05$) of form of Se at any time point during the study. There was an effect of time ($P < 0.0001$), but there was no treatment X time interaction ($P > 0.1$).

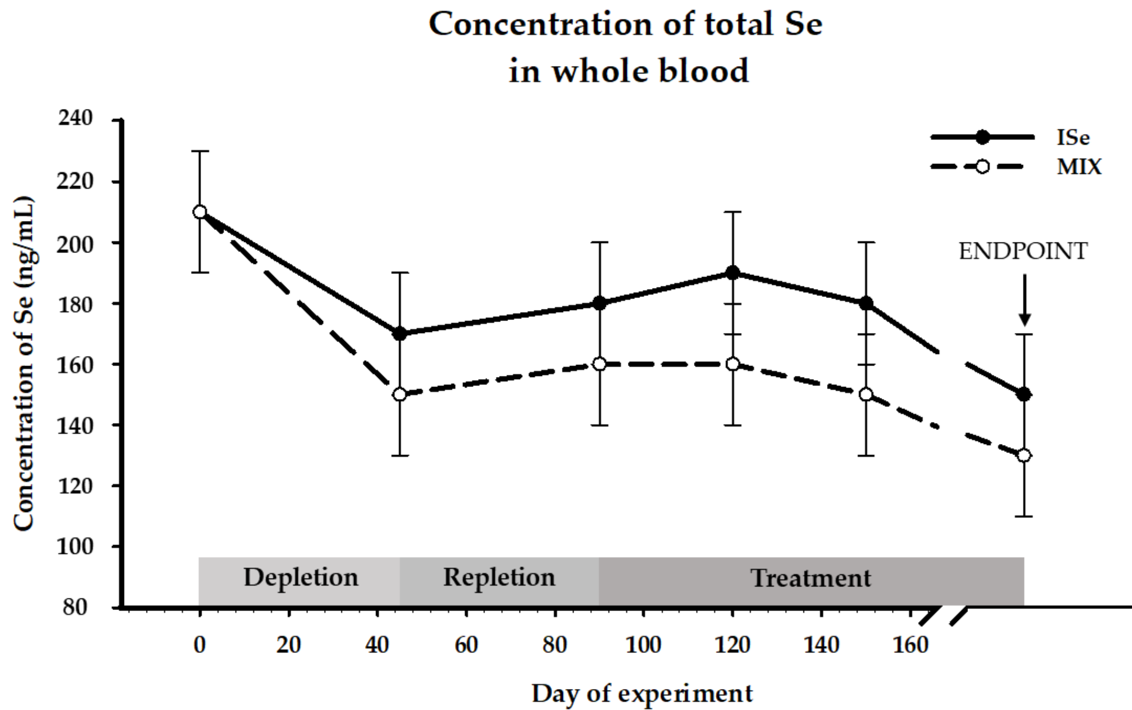

Figure 5.2. Effect of treatment (form of Se) on concentrations of Se in whole blood (ppm; LS Mean ± SEM) of Se in cows supplemented with either ISe (Sodium selenite; *n* = 5) or a 1:1 combination (MIX) of ISe and OSe (Sel-Plex; *n* = 5). Data were analyzed as an ANOVA with repeated measures. Whole blood Se was not affected by treatment (P = 0.2393) but was affected by time (P < 0.0001).

5.4.2. Real-Time RT-PCR Analysis of Selenoprotein and Receptor mRNA Transcripts

Twenty-six transcripts encoding selenoproteins and three transcripts encoding receptors for selenoprotein P were targeted via qPCR analysis. The MIX form of supplemental Se significantly ($P < 0.05$) increased the abundance of four key transcripts encoding *Gpx6, Selenor, Selenov,* and *Tfrc* in the CL (Figure 5.3 and Table 5.3). Additionally, the relative abundance of *Dio2, Gpx1, Gpx3, Selenoh,* and *Selenop* tended ($0.05 \le P < 0.1$) to be increased in the MIX treatment group. The relative level of expression of the other targeted selenoprotein transcripts or selenoprotein P receptors was not affected by treatment (Table 5.3).

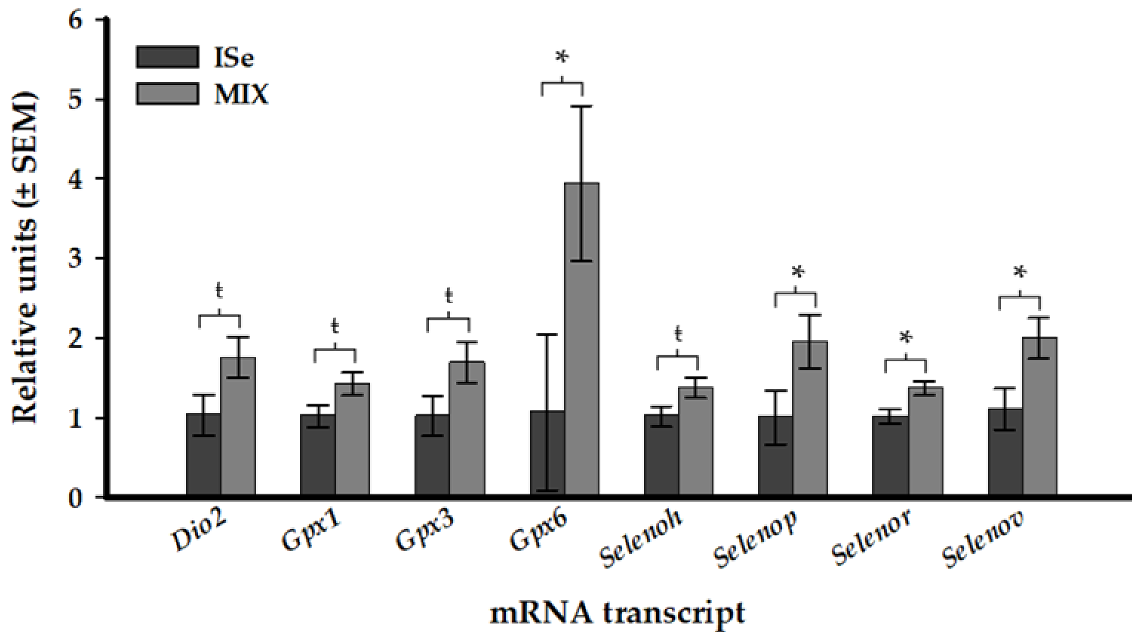

Figure 5.3. Effect of form of Se on the expression of mRNA transcripts encoding selenoproteins in the CL of cows supplemented with vitamin mineral mixes containing Se as ISe (sodium selenite; n = 5) or a 1:1 combination (MIX) of ISe and OSe (Sel-Plex; n = 5). P-values are associated with Student's T-Test. Significant differences at $P < 0.05$ are indicated by an asterisk and tendencies at $0.05 \leq P < 0.1$ are indicated by ŧ.

Table 5.3. Relative abundance of mRNA encoding selenoproteins and selenoprotein P receptors in the CL of cows supplemented with vitamin-mineral mixes containing Se as sodium selenite (ISe, n = 5) or a 1:1 equimolar mix of ISe and OSe (MIX, SEL-PLEX, n = 5) [1].

Gene	**Gene Name**	**qPCR**			
		ISe	**MIX**	**SEM**	***p*-Value** [2]
Iodothyronine deiodinases					
DIO1	Iodothyronine deiodinase 1	1.6668	1.3540	0.5915	0.89
DIO3	Iodothyronine deiodinase 3	1.8881	0.7281	0.5667	0.54
Glutathione peroxidases					
GPX2	Glutathione peroxidase 2	1.1133	1.3412	0.2026	0.45
GPX4	Glutathione peroxidase 4	1.0200	0.8963	0.0906	0.36
Thioredoxin reductases					
TXNRD1	Thioredoxin reductase 1	1.0338	1.2380	0.1528	0.37
TXNRD2	Thioredoxin reductase 2	1.0034	1.0816	0.0435	0.24
TXNRD3	Thioredoxin reductase 3	1.0357	1.2130	0.1613	0.50
Other selenoproteins					
SELENOF	Selenoprotein F	1.0223	0.8537	0.0983	0.26
SELENOI	Selenoprotein I	1.0089	1.2904	0.1215	0.14
SELNOK	Selenoprotein K	1.0143	1.2131	0.0810	0.12
SELNOM	Selenoprotein M	4.6687	7.5038	2.9960	0.52
SELENON	Selenoprotein N	1.0196	1.0072	0.0733	0.91
SELENOO	Selenoprotein O	1.0234	1.1227	0.0808	0.33
SELENOS	Selenoprotein S	1.0131	1.2860	0.1229	0.16
SELENOT	Selenoprotein T	1.0398	0.9553	0.1090	0.60
SELENOW	Selenoprotein W	1.0183	1.1465	0.1134	0.44
SEPHS1	Selenophosphate synthetase 1	1.0655	1.6289	0.2718	0.18
SEPHS2	Selenophosphate synthetase 2	1.0142	1.0127	0.0738	0.99
Selenoprotein P receptors					
LRP2	LDL receptor related protein 2	1.0335	1.2207	0.1478	0.40

LRP8	LDL receptor related protein 8	1.0647	1.2923	0.1858	0.41
TFRC †	Transferrin receptor	1.0104 [a]	1.5173 [b]	0.1116	0.01

[1] Se was supplemented at 35 ppm as either inorganic (ISe; sodium selenite), or a 1:1 combination (MIX) of ISe and OSe (SEL-PLEX). [2] P-values are associated with Student's T-Test. † Means with different superscripted letters differ at $P < 0.05$.

5.4.3. Concentrations of P4, Luteal Weight, and Luteal Diameter

The concentration of P4 was quantified in serum collected on Days 5, 6 and 7 of the estrous cycle. Although in the present study there was no observed effect of form of Se on systemic P4 (Table 2, Year 3), the difference of ~1 ng/mL between the treatment groups on Day 7 is consistent with previous studies from our lab (Cerny et al., 2016b; Carr et al., 2020) that used a larger number of animals (Table 5.4, Year 1 and Year 2). Additionally, there was no difference ($P > 0.05$) in the weight or the diameter of the CL, consistent with our previously reported results (Cerny et al., 2016b).

Table 5.4. Relative concentrations of treatment (form of supplemental Se) on the concentration of systemic P4 in cows between days 4 and 10 of the estrous cycle. Cows were supplemented with Se as sodium selenite (ISe, *n* = 5) or a 1:1 equimolar mix of ISe and OSe (MIX, SEL-PLEX, *n* = 5) [1,†].

	Treatment		
Variable	**ISe** LS Mean ± SEM	**MIX** LS Mean ± SEM	***p*-Value [2]**
Progesterone (ng/mL)			
Year 1 *			
No. of cows (*n*)	9	9	
Day 6 †	3.44 ± 0.18 [a]	5.14 ± 0.60 [b]	0.035
Year 2 **			
No. of cows (*n*)	12	12	
Day 4	1.02 ± 0.22	0.94 ± 0.12	0.740
Day 7 †	2.92 ± 0.27 [a]	3.91 ± 0.16 [b]	0.006
Day 10	7.17 ± 0.54	6.36 ± 0.55	0.308
Year 3			
No. of cows (*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 (year 1, OSe treatment not shown), ANOVA with repeated measures (year 2 and year 3), and Student's T-Test (CL weight and CL diameter). † Means with different superscripted letters differ at $P < 0.05$. * Reported in (Cerny et al., 2016b). ** Reported in (Carr et al., 2020).

5.4.4. Production of Progesterone *In Vitro*

Luteal production of P4 was determined *in vitro* by treating dissociated luteal cells with or without a low or high concentration of three agonists, PGE_2, LH, or hCG, for 96 h. Culture media was collected every 24 h for quantification of P4. Across all dietary and agonist treatments, there was a significant effect of time of culture ($P < 0.0001$) as concentrations of P4 in the culture media decreased with each subsequent 24-h period. There was a significant interaction ($P < 0.0001$) between time in culture and agonist treatment. There was no significant three-way interaction ($P > 0.05$) of dietary treatment, agonist treatment, and time in culture.

In basal, untreated cultures, there was no significant difference ($P > 0.1$) between dietary treatment groups in the production of P4; however, there tended (199.5 ± 13.9 vs. 163.0 ± 8.9, $P = 0.07$) to be a greater concentration of P4 in the media collected at 48 h from CL retrieved from MIX-treated cows compared to ISe-treated cows (Figure 5.4).

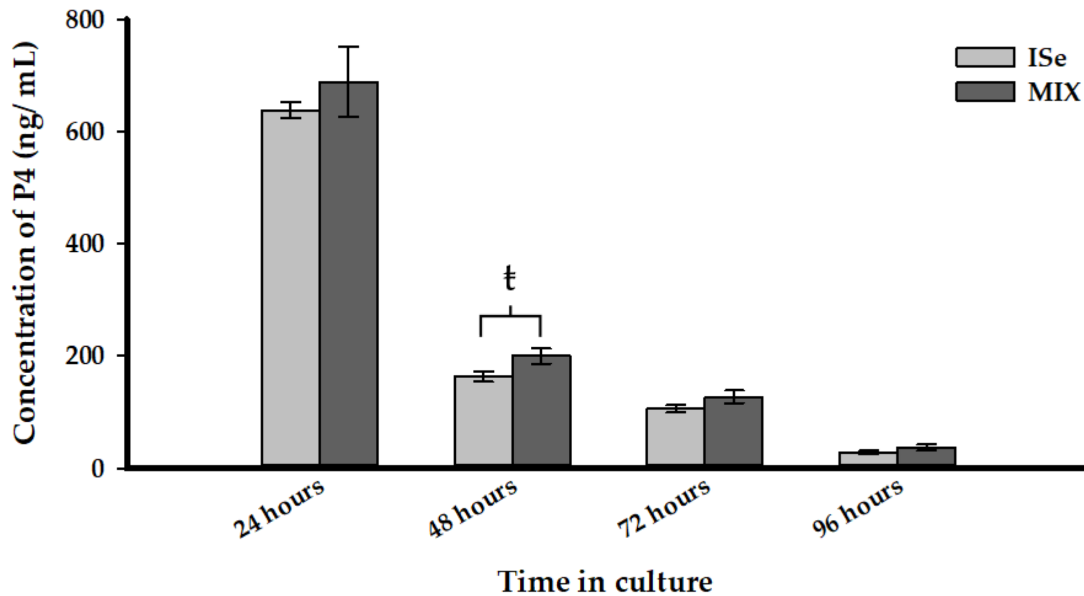

Figure 5.4. Effect of treatment (form of Se) on basal media concentrations of P4 (ng/mL; LS mean ± SEM) collected from *in vitro* culture every 24 h for 96 h from cows supplemented with either sodium selenite ISe (sodium selenite; $n = 5$) or a 1:1 combination (MIX) of ISe and OSe (Sel-Plex; $n = 5$) treatments. Data were analyzed using a split plot design for repeated measure. Significant differences were determined at $P < 0.05$ and tendencies were determined at $0.05 \leq P < 0.1$. Tendencies are indicated by ŧ.

After 24 h of culture, both the low and high doses of hCG increased the production of P4 in luteal cells collected from cows supplemented with ISe compared to MIX ($P < 0.05$, Figure 5.5 A,B), an unexpected result. There was no other significant difference at 24 h *in vitro* ($P > 0.1$). After 48 h of culture, the low dose of LH also increased the concentration of P4 in luteal cultures from ISe- compared to MIX-treated cows ($P < 0.05$). At 72 and 96 h of culture, there were no differences between dietary treatments in the production of P4 in response to LH or hCG. Furthermore, there was no effect of form of Se on PGE_2-treated luteal cells throughout the 96 h of culture ($P > 0.05$, data not shown).

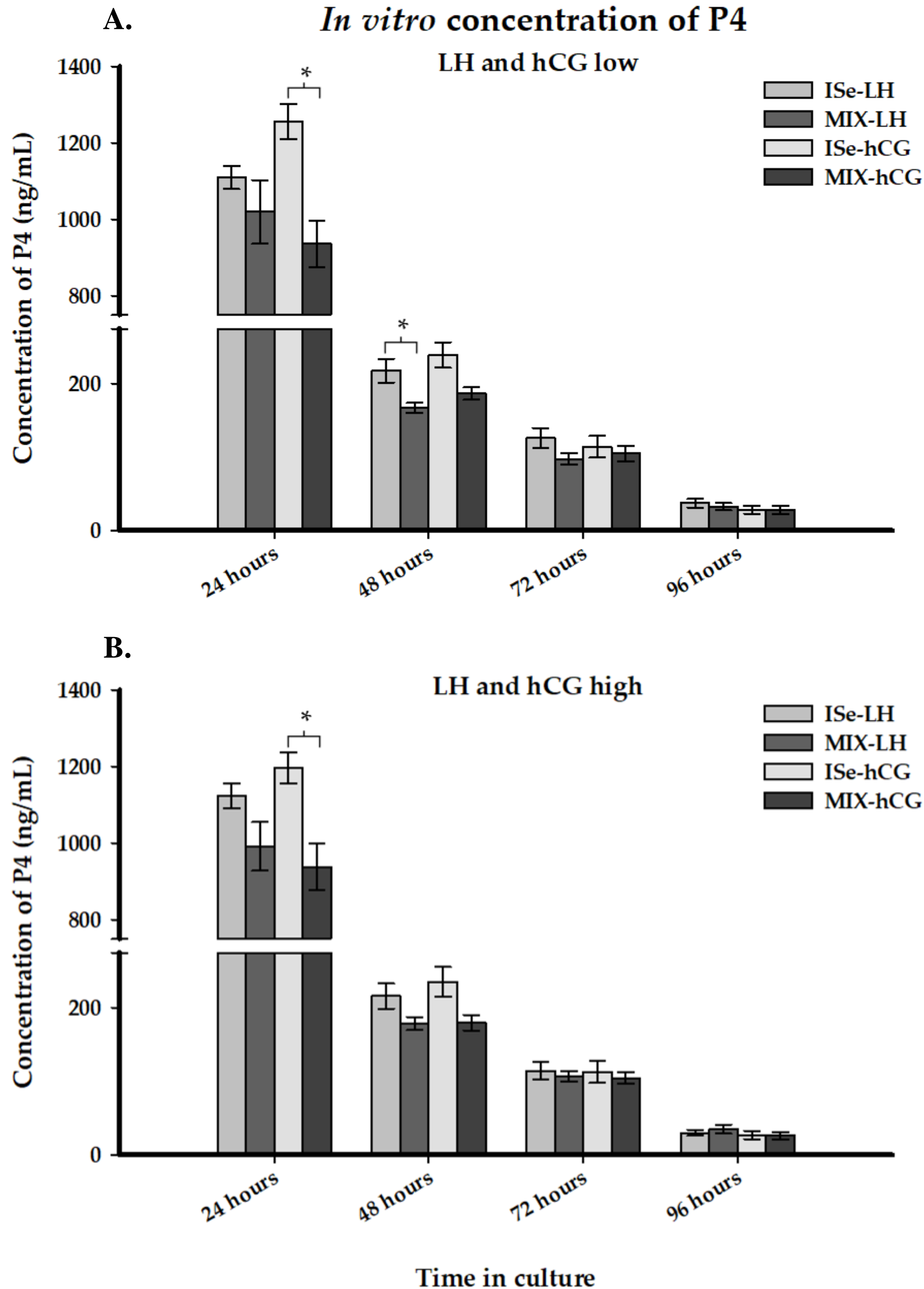

Figure 5.5. Effect of form of Se on concentrations of P4 in culture media (ng/mL; LS mean ± standard error of the mean) collected after *in vitro* culture of dissociated luteal cells with a low (A) or high (B) dose of LH (1 or 10 IU/mL) or hCG (10 or 50 ng/mL). Corpora lutea were recovered from cows supplemented with either ISe (Sodium selenite; $n = 5$) or a 1:1 combination (MIX) of ISe and OSe (Sel-Plex; $n = 5$) on Day 7 post-estrus. Data were analyzed using split plot design for repeated measure. Significant differences were determined at $P < 0.05$ and are designated by an asterisk.

5.4.5. Real-Time PCR Analysis of Steroidogenic and Cholesterol Related mRNA Transcripts

The relative abundance of 21 mRNA transcripts associated with P4 biosynthesis and regulation were analyzed via qPCR. Form of Se did not ($P > 0.05$) affect the abundance of the five targeted enzymatic transcripts (Table 5.5). Of the targeted receptor transcripts, the expression of mRNA encoding the nuclear P4 receptor (*Pgr*) was decreased in CL retrieved from MIX versus ISe treated cows ($P < 0.05$). Of the five targeted transcripts associated with cholesterol availability, the level of expression of mRNA encoding both *Ldlr,* and *Hsl* was increased in CL retrieved from MIX versus ISe supplemented cows (Figure 5.6, $P < 0.05$).

Table 5.5. Effect of form of Se on the relative expression of mRNA transcripts encoding steroidogenic enzymes and receptors in the d7 CL. Cows were supplemented with Se as ISe ($n = 5$) or MIX ISe:OSe ($n = 5$) [1].

Gene	Gene Name	qPCR			
		ISe	**MIX**	**SEM**	***p*-Value [2]**
Enzymatic transcripts					
STAR	Steroidogenic acute regulatory protein	1.0231	1.2259	0.1132	0.2410
CYP11A1	Cytochrome P450, family 11, subfamily A, polypeptide 1	1.0068	0.9822	0.0503	0.7371
HSD3B1	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	1.0014	0.9521	0.0398	0.4069
PTGS2	Prostaglandin-endoperoxide synthase 2 (COX2)	1.0079	0.9396	0.1574	0.7668
PTGES	Prostaglandin E synthase	1.4308	0.9376	0.3539	0.3533
Receptor transcripts					
LHCGR	Luteinizing hormone (LH) G-protein coupled receptor	1.0484	0.6859	0.1433	0.1112
PGR	Nuclear progesterone receptor	1.0076	0.8415	0.0508	0.0495
PGRMC1	Progesterone receptor membrane component 1	1.0355	0.9044	0.1060	0.4071
PGRMC2	Progesterone receptor membrane component 2	1.0248	1.0553	0.0970	0.8295
EP1	Prostaglandin E receptor 1	1.2310	1.4844	0.0721	0.5832
EP2	Prostaglandin E receptor 2	1.0385	1.0890	0.1392	0.8041
EP3	Prostaglandin E receptor 3	1.0286	0.7820	0.1045	0.1337
EP4	Prostaglandin E receptor 4	1.0313	1.2515	0.1524	0.3368
PAQR5	Progestin and adipoQ receptor family member 5 (mPR γ)	1.0342	0.9295	0.1187	0.5502
PAQR7	Progestin and adipoQ receptor family member 7 (mPR α)	1.0165	0.8922	0.0804	0.3058
PAQR8	Progestin and adipoQ receptor family member 8 (mPR β)	1.0042	1.0206	0.0678	0.8687
P*GTFR*	Prostaglandin F receptor	1.0493	0.8624	0.1735	0.4680

[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 Student's T-Test.

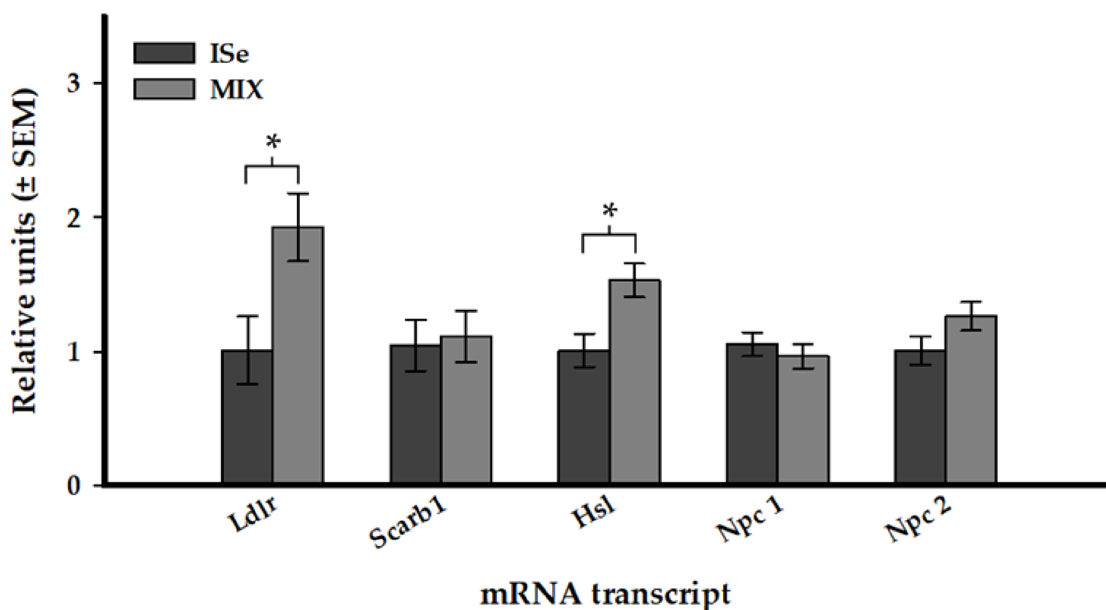

Figure 5.6. Effect of form of Se on the expression of mRNA transcripts associated with the availability of cholesterol in the CL of cows supplemented with Se in vitamin mineral mixes as ISe (sodium selenite; *n* = 5) or a 1:1 combination (MIX) of ISe and OSe (Sel-Plex; *n* = 5). P-values are associated with Student's T-test. Significant differences at $P < 0.05$ are indicated by an asterisk.

5.5. Discussion

In this study, we sought to investigate the mechanism responsible for increases in early luteal phase concentrations of peripheral P4 after supplementation with MIX versus the industry standard ISe (Cerny et al., 2016b; Carr et al., 2020). The specified objectives were to investigate the effect of supplementation with ISe or MIX-form Se on (1) the relative abundance of mRNA transcripts that encode selenoproteins and targeted steroidogenic enzymes in the CL, and (2) the ability of dissociated luteal cells to synthesize P4 in response to key agonists *in vitro.* We first verified our model by demonstrating the diets containing distinct Se-form specific vitamin-mineral mixes resulted in a selenium-adequate status in all animals during experimentation (Gerloff, 1992; Dargatz and Ross, 1996; Brennen et al., 2011). Further, we analyzed serum concentrations of P4 collected from cows on Days 5 to 7 of the early luteal phase to model the previously defined MIX-induced increase in P4. Our previous studies reported a 1.7 ng/mL increase in Day 6 (year one) concentrations of P4 (Cerny et al., 2016b), and a 1.0 ng/mL increase in Day 7 (year 2) P4 (Carr et al., 2020). In the present study, we mimicked this difference with a 1.1 ng/mL increase in P4 on day 7 of the estrous cycle in the MIX treatment group. Although this difference was not statistically significant, we suspect that the lack of an effect in the serum concentration of P4 may be due to (1) the smaller number of animals in this more intensive study, and/or (2) a possible dietary interaction because our previous studies were performed using cattle maintained on toxic endophyte-infected tall fescue pastures, versus the study herein where cattle grazed novel, nontoxic endophyte-infected tall fescue pastures, followed by a silage-based diet over the winter months of January to April. Importantly, in both (Cerny et al., 2016b) and

the current study, this increase in peripheral concentrations of P4 occurred in the absence of any treatment effect on weight or diameter of the CL, suggesting increased capacity of individual steroidogenic cells to synthesize P4.

Regarding selenoproteins, the physiological incorporation of Se into any tissue is dependent upon the form available (Suzuki, 2005). SeMet enters the methionine metabolic pool and readily competes with Met for incorporation into protein by tRNA. The amount of incorporation of SeMet compared to methionine is dependent on their relative concentrations (Burk and Hill, 2015). Alternatively, SeMet can be converted to SeCys via the intermediate selenocystathionine and the enzymatic activity of both cystathionine Β-synthase and cystathionine (Suzuki, 2005; Mattmiller et al., 2013). The synthesized SeCys or dietary SeCys is transformed into selenide by β-lyase activity (Suzuki, 2005). Contrary to the organic forms of Se, once consumed, selenate is easily converted to selenite and then further converted to selenide (Mattmiller et al., 2013). The formation of selenide is where the metabolic pathways for organic and inorganic forms of Se converge. Selenide is converted to the intermediate selenophosphate by the known selenoprotein enzymes selenophosphate synthetases (SEPHS1 and SEPHS2) (Veres et al., 1992; Glass et al., 1993). Selenophosphate then interacts with a charged serine present on the specific ^{SeCys}tRNA, followed by the conversion of Ser-^{SeCys}tRNA to SeCys-^{SeCys}tRNA for incorporation into selenoproteins (Suzuki, 2005; Xu et al., 2006).

In the CL, ROS including hydrogen peroxide and hydroxyl radicals are produced as a byproduct of aerobic metabolism when the P450/450 system catalyzes the reaction to produce P4 (Kato et al., 1997; Garrel et al., 2007). Similarly to what occurs in other cell types, ROS are also generated in the luteal cells during the production of ATP from

oxidative phosphorylation by the oxidases NADH and NADPH, and by the activity of xanthine oxidase (Chen et al., 2003; Agarwal et al., 2005). Therefore, antioxidants must be active to regulate the concentration of ROS and maintain cellular viability, as the concentration of ROS and antioxidants are closely related to luteal function (Riley and Behrman, 1991; Carlson et al., 1993; Hayashi et al., 2003; Al-Gubory et al., 2010; Kawaguchi et al., 2013). Intra-luteal concentrations of ROS can be inhibitory to the production of P4 during luteolysis and pregnancy in rats (Sawada and Carlson, 1991; Sugino et al., 1993). Hence, maintenance of cellular function and production of P4 requires the regulation of concentrations of ROS by antioxidants. Unregulated ROS or increases in intracellular concentrations of ROS have been associated with apoptosis of the steroidogenic cells and luteolysis of the CL (Nakamura et al., 2001; Vu et al., 2012; Vu et al., 2013; Vu and Acosta, 2014).

GPX variants are known to reduce hydrogen peroxide (Labunskyy et al., 2014) and relevant to the current study, we observed a significant increase in the abundance of mRNA encoding *Gpx6* and a tendency of increasing *Gpx1* and *Gpx3* mRNA content in the CL from MIX supplemented cows. The most abundant glutathione peroxidase is the cytosolic GPX1. Vu, et al. (Vu and Acosta, 2014) demonstrated that GPX1 protein abundance and activity is greatest in the early and mid-luteal stages aligning with the increases in the production of P4 at this time. In contrast, we did not observe any difference in the mitochondrial GPX4 which has the ability to alleviate apoptogenic protein release on the inner membrane of the mitochondria (Liang et al., 2009). The roles of the GPX3 and GPX6 are not clearly defined in the CL, although we speculate that they exert a similar function related to reducing ROS. GPX3 is most highly abundant in blood

(Brigelius-Flohé and Maiorino, 2013), and previously researchers have demonstrated that the mRNA encoding *Gpx6* has been located in limited tissues including embryos, olfactory epithelium, sperm, seminal plasma, and the ovaries in humans (Brigelius-Flohé and Maiorino, 2013; Kuchenbaecker et al., 2015; Chen et al., 2020; Souto et al., 2021) (Dear et al., 1991; Kryukov et al., 2003). The present study provides novel evidence of the presence and role for GPX6 in the CL of cows, and that the *Gpx6* mRNA abundance is significantly affected by the form of Se, with mRNA encoding *Gpx6* being increased in the CL from cows in the MIX treatment group. Other selenoproteins with antioxidant capabilities and in which we observed a significant effect or tendency to increase abundance in the MIX treatment group of the respective mRNA transcripts encoding *Selenop* (Takebe et al., 2002), *Selenoh* (Novoselov et al., 2007), and *Selenor* (Kryukov et al., 2002). Of note, SELENOP has redox capabilities; however, its main physiological function is to maintain homeostasis of the concentration of Se (Burk and Hill, 2009). It is primarily synthesized in the liver and transported through the plasma to target tissues to be internalized in the cell by receptor-mediated endocytosis (Burk and Hill, 2009). Hence, it is of interest that the mRNA encoding *Selenop* was significantly upregulated in MIX treated cows, although the relevance within the CL is unclear at this time.

Of equal importance to the current study, systemic P4 is affected by a plethora of factors, including the rate of P4 synthesis in the CL, the rate of catabolism in the liver, and various luteotropic and luteolytic hormones (Fitz et al., 1982; Harrison et al., 1987; Alila et al., 1988b; Weems et al., 2002; Kotwica et al., 2003; Hart et al., 2014). In the cow, LH is considered the primary luteotropic hormone. *In vitro* stimulation of luteal cells with LH can increase the production of P4 by up to 20-fold (Fitz et al., 1982; Alila

et al., 1988b). The small steroidogenic cells of the CL contain receptors for LH (luteinizing hormone G-protein coupled receptor, LHCGR) which can assert its effects by acting through the protein kinase A (PKA) second messenger system to stimulate production of P4 (Fitz et al., 1982; Alila et al., 1988b). However, large steroidogenic luteal cells that contribute a 20-fold greater amount of the P4 per cell are devoid of the LH receptor; thus, they do not respond to simulation by that gonadotropic hormone (Fitz et al., 1982; Hoyer and Niswender, 1986). In the present study, we unexpectedly observed minor ISe-induced increases in P4 after treatment of luteal cells with LH or hCG *in vitro*, with hCG stimulating the small luteal cell similarly to LH (De Rensis et al., 2010). Considering the relative level of expression of mRNA encoding the LH/hCG receptor (1.05 vs. 0.69 ± 0.14, ISe vs. MIX, relative units ± SEM, $p = 0.11$) that we observed in luteal tissue herein, a form of Se-induced effect on small luteal cell steroidogenesis may be apparent.

Large luteal cells contain receptors, including those for PGE_2 and $PGF_{2\alpha}$ (Fitz et al., 1982; Harrison et al., 1987). PGE_2 has a luteotropic and luteoprotective role in the CL and $PGF_{2\alpha}$ is luteolytic (Fitz et al., 1982; Harrison et al., 1987). In ruminants, treating luteal cells with PGE_2 has resulted in an increase in the production of P4 (Fitz et al., 1982; Fitz et al., 1984a; Alila et al., 1988a), increased protection of the CL from luteolysis (Henderson et al., 1977; Pratt et al., 1977; Magness et al., 1981; Reynolds et al., 1981), and stimulation of the secretion of P4 by binding to the prostaglandin E receptors 1-4 (EP1, EP2, EP3, and EP4) on the plasma membrane via activation of the cAMP-mediated signaling pathway (Weems et al., 2002; Kotwica et al., 2003). We did

not observe an effect of treatment with PGE_2 on luteal cell-production of P4 in the study, nor an effect of form of Se on the expression of mRNA encoding the *Ep1-4* receptors.

Intuitively, there is no clear connection between feeding a MIX Se-form supplement and the observed increases in systemic concentrations of P4. Therefore, to investigate this mechanism, we first analyzed for differences in the expression of transcripts for key steroidogenic enzymes and associated receptors. We hypothesized that there would be an increase in enzymatic transcripts and receptor transcripts that favor the production of P4. Our initial hypothesis was not substantiated. Interestingly, among the transcripts analyzed, the content of mRNA encoding the nuclear *Pgr* but not the membrane components *Pgrmc1* and *Pgrmc2* was decreased in CL from MIX-supplemented cows suggesting down-regulation of PGR mediated events in those animals. Both on and within steroidogenic luteal cells, P4 auto-regulates the further production of P4 by binding to either the nuclear membrane receptors (PGR-A and PGR-B), or the cytoplasmic and endoplasmic membrane bound P4 receptors, P4 receptor membrane components 1 and 2 (PGRMC1 and PGRMC2, respectively). P4 also binds the progestin and adipoQ receptor family members 5, 7, and 8 (PAQR5, PAQR7, and PAQR8, respectively, (Fernandes et al., 2005; Gellersen et al., 2009)).

After analyzing the results of transcripts related to the production of P4, concurrent with the results from our *in vitro* study, it was clear that the regulatory activity of the peripheral production of P4 had to be either upstream or downstream of the targeted steroidogenic pathway (Figure 5.7). Mechanistically, the primary substrate for the production of P4 is cholesterol, which is made available to steroidogenic luteal cells via four mechanisms: conversion of low-density lipoproteins (LDL), uptake of high-

density lipoproteins (HDL), the uptake of free cholesterol, or *de novo* synthesis (Cook et al., 1967; Cook and Nalbandov, 1968; Kaltenbach et al., 1968). The conversion of LDL and HDL into free cholesterol is the prominent pathway to make this substrate available for the production of P4 in the CL (Ohashi et al., 1982; Pate and Condon, 1982; Hwang and Menon, 1983). The uptake of LDL occurs by binding to the LDL receptor (LDLR) and triggering receptor mediated endocytosis (Brown and Goldstein, 1986). The endosome containing the LDL combines with lysosomes and LDL is dissociated from the receptor to be broken down into free cholesterol (Grummer and Carroll, 1988). Transport out of the lysosome occurs by binding the cholesterol transporters: Niemann-Pick C1 protein (NPC1) and Niemann-Pick C2 protein (NPC2 (Infante et al., 2008; Kwon et al., 2009; Wang et al., 2010)). LDL-derived free cholesterol is rapidly exported out of the lysosomal compartments via the actions of NPC1 and NPC2, and without these transporters, cholesterol and other lipids would accrete within the lysosomes (Sleat et al., 2004). In contrast, HDL is utilized as a substrate for cholesterol by first binding to the scavenger receptor class B type 1 (SCARB1) on the cell surface. The lipoprotein is not entirely internalized; rather, the cholesteryl esters are selectively delivered into the cell to be hydrolyzed into free cholesterol (Ferreri and Menon, 1992; Kraemer et al., 2004; Shen et al., 2016). Another mechanism relevant to the present study and associated with the availability of P4 in steroidogenic cells is the hydrolysis of cholesteryl esters to free cholesterol from lipid droplets. This reaction is stimulated by hormone-sensitive lipase (HSL) which results in free cholesterol and fatty acids that can then act as precursors for various physiological functions, including the production of steroid hormones and

cellular energy (Fredrikson et al., 1981; Cook et al., 1982; Cook et al., 1983; Fredrikson et al., 1986; Yeaman, 2004).

Once cholesterol is free, it is transported into the mitochondria by binding the steroidogenic acute regulatory protein (STAR), which then interactions with membrane proteins on both the outer and inner mitochondrial membranes to facilitate the transport into the inner mitochondrial matrix (Selvaraj et al., 2018). This mechanism mediates the rate at which P4 can be produced (Stocco and Sodeman, 1991; Clark et al., 1994; Lin et al., 1995). Cholesterol is then converted into pregnenolone via the actions of cytochrome P450 family 11 member1 (CYP11A1) located on the inner mitochondrial membrane (Hanukoglu and Jefcoate, 1980; Hanukoglu et al., 1981; Mitani et al., 1982; Hanukoglu and Hanukoglu, 1986). Once transported out of the mitochondria, the enzymes hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta isomerase 1 (HSD3B1) located in the endoplasmic reticulum further converts pregnenolone into P4 (Labrie et al., 1992) allowing autocrine, paracrine, and endocrine activity of the hormone.

We targeted five transcripts (*Ldl, Scarb1, Npc1, Npc2*, and *Hsl*) that are indicative of the availability of cholesterol to steroidogenic cells. Of these, transcripts encoding *Ldlr* and *Hsl* were significantly upregulated in the MIX Se treatment group suggesting that the MIX-induced increase in *in vivo* P4 is due, in part, to stimulation of cholesterol uptake. Since we observed increases in mRNA encoding the *Ldlr* transcript in the MIX-supplemented Day 7 CL, the failure to observe a MIX-induced increase in the production of P4 in vitro appears to be due to the fact that there is limited cholesterol available in the serum-free media for the cells to internalize as substrate. Thus, these cells cannot

recapitulate the *in vivo* increase in the production of P4 by the CL from cows supplemented with the MIX form of Se.

Given this, the mechanism between dietary form of Se and the MIX-induced increase in early luteal phase P4 requires further research to define. However, it appears that it could partially be due to differences in the physiological integration of both selenoproteins and cholesterol, with cholesterol being the primary substrate for the production of P4 by the CL. Selenium is an integral component of GPX enzymes, which have antioxidant activity by removing hydrogen peroxide and protecting against the aggregation of reactive oxygen species (ROS). We have previously demonstrated that feeding the MIX form or the organic form of Se significantly upregulates the *Gpx4* transcript in the pituitary of steers when compared to ISe (Li et al., 2019), which asserts antioxidant properties there. ROS are generated in the mitochondria of the CL and have been shown to reduce the production of P4 (Behrman and Aten, 1991; Gatzuli et al., 1991; Aten et al., 1992; Sugino et al., 1993; Carlson et al., 1995). Additionally, ROS damage LH receptors (Gatzuli et al., 1991; Vega et al., 1995), inhibit the transfer of cholesterol to the mitochondria for the synthesis of P4 (Behrman and Aten, 1991), and inhibit the enzymatic activity of P450scc (Carlson et al., 1995). In concert, the information discussed herein provides a plausible mechanism for the association between supplementing the MIX Se-form and increased production of P4. However, additional research that analyzes transcripts associated with cholesterol biosynthesis and the production of ROS within the CL, are warranted to further define the interrelationships between the form of dietary Se, systemic concentrations of cholesterol, and peripheral concentrations of P4.

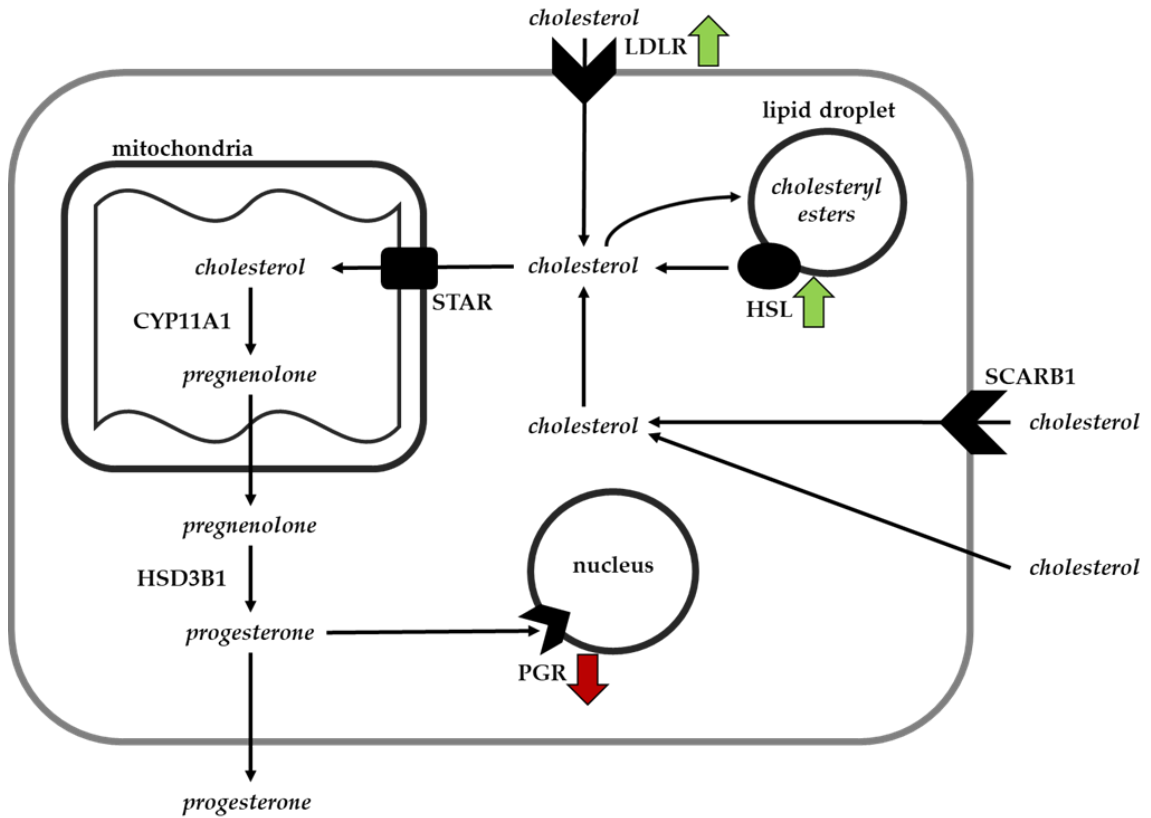

Figure 5.7. Mechanism of the availability of cholesterol and steroidogenesis in a steroidogenic luteal cell, emphasizing the four potential sources of cholesterol that are available as a substrate. Each arrow represents a significant difference in the abundance of mRNA associated with each respective protein. The arrow pointing upward or downward indicates that the mRNA is significantly upregulated (green) or downregulated (red) respectively in the MIX treatment group compared to the ISe treatment group.

5.5.1. Conclusions

In this study, we sought to quantify Se form-induced changes in the expression of mRNA encoding selenoproteins and to investigate the mechanism(s) responsible for the previously reported increase in early luteal phase concentrations of P4 in cows supplemented with the MIX form of Se. Though the mechanism is still not completely defined, we have demonstrated that the form of selenium does not affect steroidogenic enzyme mRNA expression but does alter mRNA transcripts associated with the availability of cholesterol to steroidogenic luteal cells. Further research is necessary to determine how the bioavailability of cholesterol is affected. Overall, it appears that the MIX-induced increase in early luteal P4 is not directly mediated by an increase in the key steroidogenic transcripts but by an increase in cholesterol uptake, through at least the LDLR. Understanding the mechanism of the MIX-induced increase in P4 is requisite, as this novel dietary approach offers a producer-friendly avenue to increase fertility outcomes in beef cows grazing forages in areas with soils deficient in Se.

Chapter 6. Effect of form of selenium on serum metabolites during early gestation in beef heifers

6.1. Abstract

It is necessary to supplement Se to the diet of beef cattle in regions such as the southeast United States in which the soils and forages are deficient in this trace mineral. This supplemental Se typically is provided using an inorganic form (ISe), although organic forms (OSe) are predominantly available when cattle graze forage. Previously we have investigated the physiological response of providing supplemental Se as ISe, or a 1:1 mixture of ISe to OSe (MIX) on grazing beef cattle and have observed significant effects in both corpus luteum (CL) and endometrial function. Compared to ISe, the MIX form of Se increased the concentration of progesterone (P4) during the early luteal phase of the estrous cycle at a time period that can significantly improve early embryonic development, and this appeared to be the result of an increase in the availability of cholesterol to the CL through uptake via the low-density lipoprotein receptor (LDLR), cleavage via hormone sensitive lipase (HSL), and *de novo* cholesterol synthesis. Further, we investigated the effects of MIX form Se versus ISe on the mRNA transcripts encoding IFNτ- and P4-induced proteins in the caruncular (CAR) and intercaruncular (ICAR) tissues of the uterine endometrium at maternal recognition of pregnancy (MRP). MIX-supplemented heifers had an increased abundance of mRNA encoding myostatin (MSTN) in ICAR, and a decrease in the relative abundance of diacylglycerol o-acyltransferase 2 (DGAT2) in CAR which may affect the availability of glucose and triglycerides to the developing conceptus, and this occurred concurrently with significantly longer

conceptuses at MRP in MIX-Se supplemented heifers. To further investigate potential metabolic changes affecting fertility, the objective of this study was to investigate Se-form specific changes in serum metabolic parameters at estrus, during the early luteal phase, and at MRP. Commercial Angus-cross heifers ($N = 20$) were subjected to a 45-day period of Se depletion following by a 45-day repletion period supplemented with only ISe. They were then randomly assigned to at least 90 days of treatment with a vitamin-mineral mix containing 35-ppm Se as either ISe ($n = 10$), or 1:1 ratio of ISe and OSe ($n = 10$, MIX). Following estrous synchronization and artificial insemination, heifers were killed at MRP (d 17) and reproductive tracts were collected. Only heifers in which a fully intact conceptus was recovered were utilized for statistical analyses. Whole blood and serum were collected during experimentation for quantification of lipoproteins and metabolites. All data were analyzed by an ANOVA with repeated measures. Heifers assigned to MIX versus ISe had a significantly lower concentration of total serum cholesterol ($P = 0.01$), and saliently, a decrease in both total and free low density lipoprotein/ very low density lipoprotein (LDL/VLDL) fractions on d 7 ($P < 0.05$) and d17 ($P < 0.05$) of gestation. Additionally, the form of Se significantly affected serum glucose ($P = 0.03$), aspartate aminotransferase (AST, $P < 0.01$), and beta hydroxybutyrate (BHBA, $P = 0.04$), with no effect observed on serum triglycerides ($P > 0.05$). Overall, it appears that the form of Se may be altering the serum metabolic and lipid profiles of the heifer, with changes to whole animal physiology affecting fertility.

Keywords: selenium; lipoproteins; cholesterol; glucose; maternal recognition of pregnancy; endometrium

6.2. Introduction

The necessity to supplement Se to the diet of beef cattle in regions such as the southeast United States where the soils and hence forages are deficient in this trace mineral is well documented (Boyne and Arthur, 1979; Gleed et al., 1983; McClure et al., 1986; Erskine et al., 1989; Enjalbert et al., 2006). This supplemental Se is conventionally provided using an inorganic form (ISe) even though organic forms (OSe) are predominant when cattle graze forage (Ammerman and Miller, 1975).

Previously our lab has investigated the physiological effects of supplementing different forms of Se as ISe, OSe, or a 1:1 mixture of ISe to OSe (MIX) on grazing beef cattle and have observed significant effects on the liver (Matthews et al., 2014), pituitary (Li et al., 2019), testis (Cerny et al., 2016a), corpus luteum (CL) (Carr et al., 2022; Crites et al., 2022b), and endometrium (Crites et al., 2022a). Importantly, we have reported that the MIX form of Se increases the concentration of progesterone (P4) by ~1 ng/ ml on d6 (Cerny et al., 2016b) and d7 (Carr et al., 2020) of the estrous cycle at a time period that can significantly improve early embryonic development (Garrett et al., 1988; Carter et al., 2008). Further results revealed that this increase in P4 was not a result of increased production of enzymes regulating steroidogenesis (Carr et al., 2022); rather, it appears to be supported by an increase in the availability of cholesterol to the CL through uptake via the low-density lipoprotein receptor (LDLR), cleavage via hormone sensitive lipase (HSL), and *de novo* cholesterol synthesis (Carr et al., 2022; Crites et al., 2022b) thus delivering cholesterol as a substrate for steroidogenesis and the production of P4.

Subsequently, we analyzed the effects of supplementing the MIX form of Se versus ISe alone on the expression of mRNA transcripts encoding proteins responsive to IFNτ- and P4 in the caruncular (CAR) and intercaruncular (ICAR) tissues of the endometrium of the uterus at maternal recognition of pregnancy (MRP). Germanely, the MIX-supplemented heifers had an increased abundance of mRNA encoding myostatin (MSTN) in ICAR tissue, and a decrease in the relative abundance of diacylglycerol o-acyltransferase 2 (DGAT2) in CAR tissue. The MSTN protein is thought to increase the availability of glucose (Forde et al., 2009), and DGAT2 may increase the availability of triglycerides to the histotroph for utilization of the developing conceptus (Forde et al., 2009). Ultimately, these uterine changes in mRNA transcripts presumably indicated an effect on the availability of energy sources for the developing conceptus, with significantly longer conceptuses recovered at MRP in MIX-Se supplemented heifers compared to those on ISe alone (Crites et al., 2022a). Glucose sequestered from maternal blood and secreted into histotroph is the primary energy source for the post-hatching conceptus prior to implantation (Atkinson et al., 1984; Martal et al., 1997), which provides a plausible mechanism for the observed advancement in conceptus development.

With the MIX-induced increase in availability of cholesterol to the CL, as well as changes in the mRNA transcripts including MSTN and DGAT2 in the endometrium (which may affect the contents of histotroph), the objective of this study was to investigate Se-form specific changes in serum metabolic parameters at estrus, during the early luteal phase, and at MRP, with special consideration given for effects on systemic concentrations of lipoproteins, glucose, cholesterol, and triglycerides. We hypothesized

that these proteins and metabolites would be elevated in MIX-Se form compared to ISe supplemented heifers during the establishment of pregnancy.

6.3. Methods

All procedures in this project were approved by the University of Kentucky's Institutional Animal Care and Use Committee referencing protocol number 2017-2828.

6.3.1. Animals and Experimental Procedure

Commercial non-lactating Angus-cross heifers (N = 20) were subjected to a 45-day period of Se depletion where they were not supplemented with any exogenous sources of this mineral. Subsequently, all heifers were fed with a vitamin-mineral mix containing 35-ppm Se as ISe for a 45-day period to return the blood concentrations of Se to adequate (Gerloff, 1992; Dargatz and Ross, 1996). After repletion, the heifers were randomly assigned to treatment for at least 90 days with a vitamin-mineral mix containing 35-ppm Se as either inorganic Se (n = 10, ISe, sodium selenite, Prince Agri Products, Inc. Quincy, IL, USA) or a 1:1 ratio of ISe and OSe (n = 10, MIX, SEL-PLEX; Alltech, Inc., Nicholasville, KY, USA). Whole blood was collected at the beginning of experimentation, start of repletion, start of treatment, and bi-monthly during treatment until the end of experimentation to ensure that heifers maintained a Se adequate status prior to breeding and tissue recovery.

6.3.2. Experimental Regimen and Blood Collection

Once heifers were on their respective Se treatment (ISe or MIX) for at least 90 days, they were randomly injected with one or two doses of dinoprost tromethamine (25 mg, Lutalyse, Zoetis, Parsippany, NJ) to induce regression of the CL. Heifers were then monitored daily for behavioral estrus (d 0) using visual observations and CowManager technology (Gerverscop 9, The Netherlands) which uses an electronic ear sensor to measure ear temperature and behavioral patterns to best predict when the heifer is displaying primary and secondary signs of estrus.

At detected estrus (0 h), transrectal ultrasonography using a 5-8 MHz linear transducer (Ibex Pro, E.I. Medical Imaging, Loveland, CO, USA) was used to verify the presence of a preovulatory follicle prior to artificial insemination at 0 h, 12 h, and 24 h with commercially available frozen semen from a bull with an established high level of fertility.

On d 0, d 7, and d 17 approximately 8 mL of blood was collected into additive-free tubes (Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) by jugular venipuncture for quantification of serum lipoproteins and the metabolites cholesterol, glucose, triglycerides, albumin, aspartate amino transferase (AST), beta-hydroxybutyrate (BHBA), non-esterified fatty acids (NEFA), and urea nitrogen. Additionally, on d 0 and d 17, 8 mL of whole blood was collected in EDTA-containing (2.7 mg/mL) blood collection tubes (Becton Dickinson, Franklin Lakes, NJ, USA) by jugular venipuncture for additional analysis of the effect of form of supplemental Se on whole blood Se content.

On d 17 after insemination, the ovaries, uterus, pituitary, and liver were collected from each heifer after euthanasia by captive bolt stunning and exsanguination at the USDA inspected University of Kentucky Meat Laboratory. These tissues were utilized in experimentation elsewhere and only blood and serum parameters are reported in the present study. Importantly, each uterus was flushed to collect the conceptus as described in (Crites et al., 2022a). Briefly, following ligation of the uterine horn contralateral to the CL, an artificial insemination sheath was inserted transcervically to flush out the uterine fluid and developing conceptus with ice-cold phosphate-buffered saline (PBS). If no conceptus was recovered after four consecutive flushing attempts, the animal was deemed non-pregnant and excluded from statistical analyses herein. An intact conceptus was recovered from six heifers in each treatment group (ISe, n = 6; MIX, n = 6).

6.3.3. Analysis of Se

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

6.3.4. Analysis of Metabolic Parameters

The serum metabolic parameters glucose, cholesterol, triglycerides, albumin, aspartate amino transferase (AST), beta-hydroxybutyrate (BHBA), non-esterified fatty acids (NEFA), and urea nitrogen were quantified at d 0, d 7, and d 17 of gestation at the Cornell University Animal Health Diagnostics Center (Ithaca, NY, USA). A brief

overview of methods employed by the lab are included below. All results are reported along with the provided species-specific reference ranges for each analysis.

Serum glucose was analyzed using the hexokinase method where hexokinase catalyzes the phosphorylation of glucose to form glucose-6-phosphate. This product is then oxidized by glucose-6-phosphate dehydrogenase to form 6-phosphogluconate. This reaction also results in the conversion of NAD+ to NADPH which is measured at $\lambda = 340$ nm and the product measured directly correlates to the concentration of glucose in the sample.

Total cholesterol was quantified by an end-point reaction using the CHOD-PAP method. Initially free fatty acids and cholesterol are released using cholesterol esterase, and then cholesterol is oxidized to cholest-4-en-3-one by cholesterol oxidase. During this second reaction, hydrogen peroxide is produced which then oxidizes a product that fluoresces at $\lambda ex = 500$/ $\lambda em = 550$ nm which is proportional to the concentration of cholesterol present in the sample.

Serum triglycerides were quantified by an end-point reaction using the GPO-PAP method. This method is based on the disruption of triglycerides by lipoprotein lipase resulting in NEFA and glycerol. The glycerol is measured by the assay. Briefly, lipoprotein lipase catalyzes the hydrolysis of the triglycerides to yield fatty acids and glycerol. Next, glycerol kinase catalyzes the phosphorylation of glycerol and then glycerophosphate oxidase catalyzes the oxidation of glycerol-3-phosphate to dihydroxyacetone phosphate and hydrogen peroxide (H_2O_2). This H_2O_2 product is measured at $\lambda = 500$ nm, which is directly proportional to the concentration of triglycerides in the sample.

The concentration of albumin in serum was quantified by a blanked end-point reaction using the bromocresol green method. First, albumin becomes charged in an acidic environment (pH = 4.1) and it binds to the bromocresol green dye forming a measurable fluorescent product proportional to the concentration of albumin in the sample.

AST, also called glutamate oxaloacetate transaminase (GOT), was quantified by a kinetic photometric reaction. Initially α-ketoglutarate and L-aspartate is catalyzed by AST to produce L-glutamate and oxaloacetate and it is carried out until equilibrium. The oxaloacetate is then reduced to L-malate by NADH catalyzed by malate dehydrogenase. The concentration of AST calculated by the rate of the production of oxaloacetate is proportional to the declining rate of NADH which is measured photometrically.

Serum BHBA was quantified using a kinetic reaction in a spectrophotometric assay where 3-hydroxybutyrate dehydrogenase catalyzes the oxidation of D-3-hydroxybutyrate to acetoacetate. During this process, NAD+ is reduced to NADH and this generated NADH is measured at $\lambda = 340$ nm which is directly proportional to the concentration of D-3-hydroxybutyrate in the sample.

NEFA were quantified using a blank endpoint reaction with a colorimetric enzymatic method. Briefly, acyl-CoA synthetase catalyzes the conversion of NEFAs to coenzyme A (CoA) producing acyl-CoA with H_2O_2 produced as a product of the oxidation of acyl-CoA. Then H_2O_2 along with peroxidase results in oxidative condensation of 4-aminoantipyrine with 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline (MEHA) which forms a colorimetric product $\lambda max = 550$ nm with the concentration of NEFA being proportional of the measured density of the dye.

Urea nitrogen was measured using a kinetic reaction. Initially, urease catalyzes the hydrolysis of urea resulting in the creation of ammonium and carbonate ions. In the presence of the enzyme glutamate dehydrogenase, the ammonium ion reacts with α-ketoglutarate and NADH to form L-glutamate. The concurrent oxidation of NADH to NAD+ results in an absorbance decrease that is measurable and proportional to the concentration of urea.

6.3.5. Analysis of lipoproteins

We investigated the systemic concentration of the lipoprotein fractions split into two groups: high-density lipoproteins (HDL), and low density lipoproteins (LDL)/ very low-density lipoproteins (VLDL) in heifers during early gestation (d 7) and at MRP (d 17). Protein fractions were quantified in serum samples by the commercially available HDL and LDL/VLDL Quantification Kit (Sigma-Aldrich®, St. Louis, MO, USA) according to manufacturer's instructions. Initially, the protein fractions were separated using a precipitation buffer and centrifugation. The concentration of each protein fraction was determined by a coupled enzyme assay resulting in fluorometric product (λex = 535/ λem = 587 nm) that is proportional to the concentration of HDL or LDL/VLDL target present. Fluorescence was measured and quantified in 96 well flat-bottom black plates using the SpectraMax® M2e Microplate Reader with the SoftMax® Pro 6.2.2 Software (Molecular Devices, San Jose, CA, USA).

In the present study, we analyzed the concentration of total serum HDL, free serum HDL, total serum LDL/VLDL, and free serum LDL/VLDL, and one assay was performed for quantification of each at d 7 and d 17 of experimentation. Across all plates,

the intra-assay CV ranged from 1.53 - 3.85 %. Each protein fraction was diluted to ensure reading within the linear range of the standard curve.

For quantification, each reaction consisted of 50 µl of sample and 50 µl of a master mix containing a cholesterol assay buffer, probe, and enzyme mix. Cholesterol esterase was added to the master mix used to quantify each standard and total HDL or LDL/VLDL fractions. No cholesterol esterase was used to measure free cholesterol.

6.3.6. Statistical Analysis

Data are presented as least square means (± SEM) with individual heifer as the experimental unit. Data were analyzed for normal distribution and homogeneity. When appropriate, data were natural log or inverse transformed given the samples were not distributed normally.

To determine the effect of form of Se on concentrations of systemic Se and each metabolite, 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 in all analyses and each treatment group contained 6 heifers (ISe, n = 6; MIX, n = 6). The concentrations of total LDL and BHBA were natural log transformed due to not being normally distributed. Serum glucose was inverse transformed because it was not normally distributed originally or following a natural log transformation. Results were deemed statistically significant at $P \leq 0.05$ or a tendency to differ at $0.05 < P \leq 0.10$.

6.4. Results

6.4.1. Concentrations of Whole Blood Se

Throughout experimentation, heifers in both ISe and MIX treatment groups maintained a Se adequate status (Figure 6.1, (Gerloff, 1992; Dargatz and Ross, 1996)) as expected for this experimental model. However, there was a significant effect of day of experiment ($P < 0.0001$) and there tended to be an effect of treatment ($P = 0.07$). There was no dietary treatment by day interaction ($P > 0.05$). These results are published in another study from our laboratory (Crites et al., 2022a).

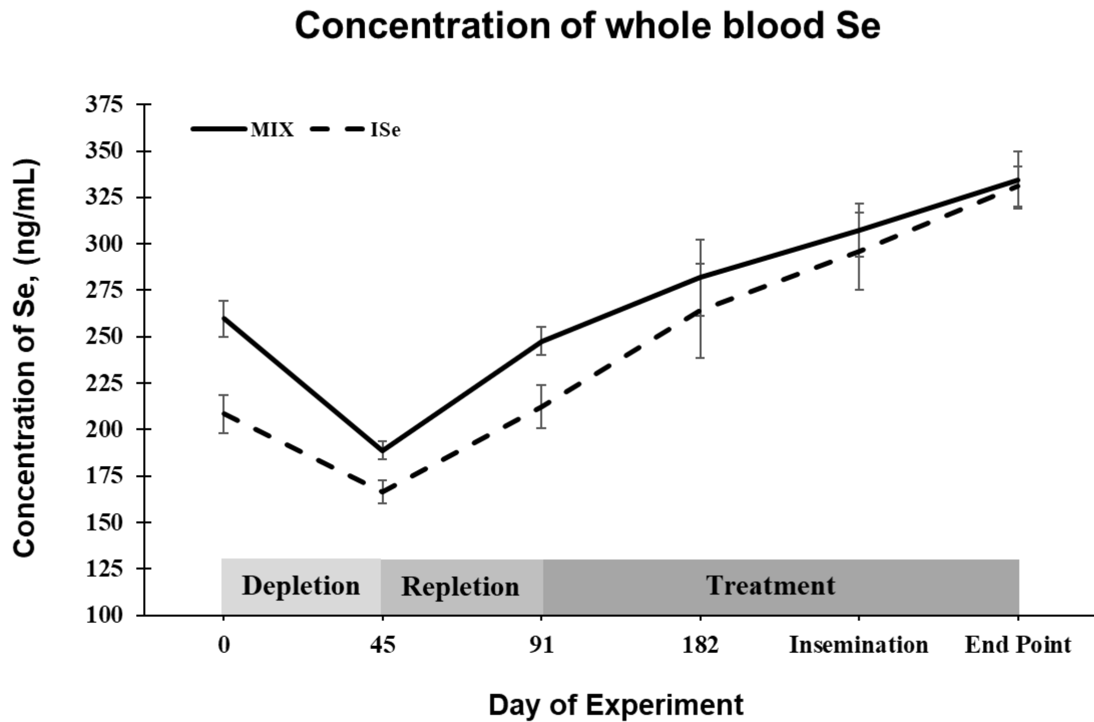

Figure 6.1. Concentration of whole blood Se throughout experimentation in heifers supplemented with ISe (sodium selenite; n = 6) or MIX (sodium selenite and Sel-Plex; n = 6). Data were analyzed as ANOVA with repeated measures and is presented as LS Means ± SEM. Whole blood Se tended to be affected by dietary treatment ($P = 0.07$) and day of experiment ($P < 0.0001$), but there was no interaction between dietary treatment and day of experiment ($P > 0.05$).

6.4.2. Total Cholesterol

Following our finding that the increase in early luteal phase P4 was likely the result of MIX-induced increases in cholesterol uptake and availability by luteal cells, total circulating cholesterol was quantified at d 0, d 7, and d 17 of gestation. Cholesterol is a vital membrane constituent and is the primary substrate for the production of sex steroids (Hu et al., 2010). With the exception of one time point, the serum concentration of cholesterol was below the established clinical reference range of 163-397 mg/dL for cattle. Circulating total cholesterol was significantly decreased in the MIX vs. ISe treatment group with the main effects of treatment ($P = 0.01$), and an effect of day of gestation ($P < 0.01$), but no treatment x day interaction ($P > 0.05$, Figure 6.2). We detected a tendency for MIX to have a lower concentration of serum cholesterol on d 0 (170.83 ± 15.88 v 134.33 ± 12.22 mg/dL, $P < 0.1$) but failed to detect a difference on d 7 or d 17 of gestation.

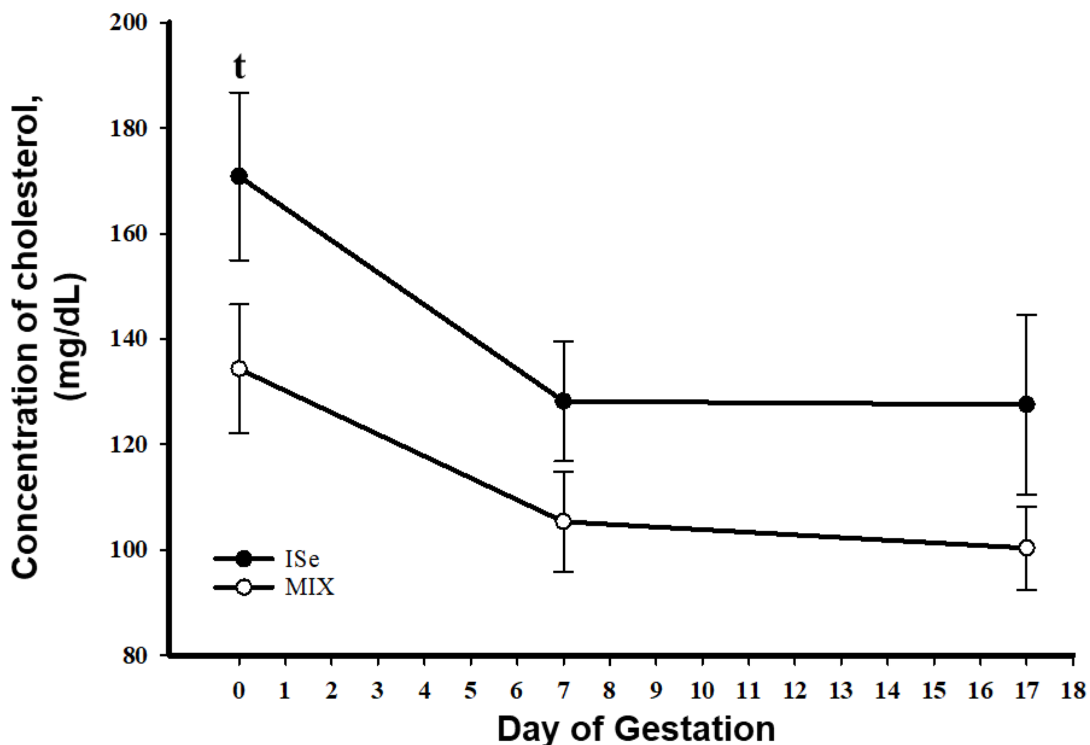

Figure 6.2. Concentration of total serum cholesterol at estrus (d 0) and gestational d 7 and d 17 in heifers supplemented with either ISe (n = 6) or MIX (n = 6) vitamin-mineral mix. Data were analyzed as an ANOVA with repeated measures. Serum cholesterol was affected by dietary treatment (P = 0.01) and day of gestation (P = 0.02). There was no treatment x day interaction (P > 0.05). An * indicates a significant difference at P ≤ 0.05 and a t indicates a tendency to differ at 0.05 < P ≤ 0.01. Comparisons represented are between ISe and MIX at each indicated time point.

6.4.3. Lipoproteins

Given our earlier finding that the expression of mRNA encoding the LDLR was increased in the corpus luteum on d 7 of the estrous cycle in MIX vs. ISe-supplemented heifers, total and free LDL/VLDL and HDL were quantified in serum on d 7 and d 17 of gestation. There was a significant main effect in the serum concentration of total ($P = 0.02$) and free LDL/VLDL ($P = 0.03$) protein fractions (Figure 6.3 a and b). There was a significant effect of form of Se on day of gestation ($P < 0.05$) in the free LDL/VLDL fraction, but this did not occur in the total LDL/VLDL protein fraction ($P > 0.05$). Additionally, there was no treatment by day interaction ($P > 0.05$) for either response variable.

On d 7 of gestation, the MIX form of Se compared to ISe alone resulted in a decrease in the total (55.08 v 78.79 ± 8.92 respectively, $P = 0.04$) and free (12.97 v 18.56 ± 1.82, $P < 0.05$) LDL/VLDL protein fractions. Similarly, on d17 of gestation, MIX form of Se treatment resulted in a decrease in the total (59.46 v 89.66 ± 8.92, $P = 0.03$) and free (15.57 v 21.17 ± 1.82, $P < 0.05$) LDL/VLDL protein fractions.

There was no difference in the blood concentrations of total or free HDL in response to form of Se ($P > 0.05$, Figure 6.3 c and d) or day of gestation ($P > 0.05$). Additionally, there was no significant treatment by day interaction ($P > 0.05$).

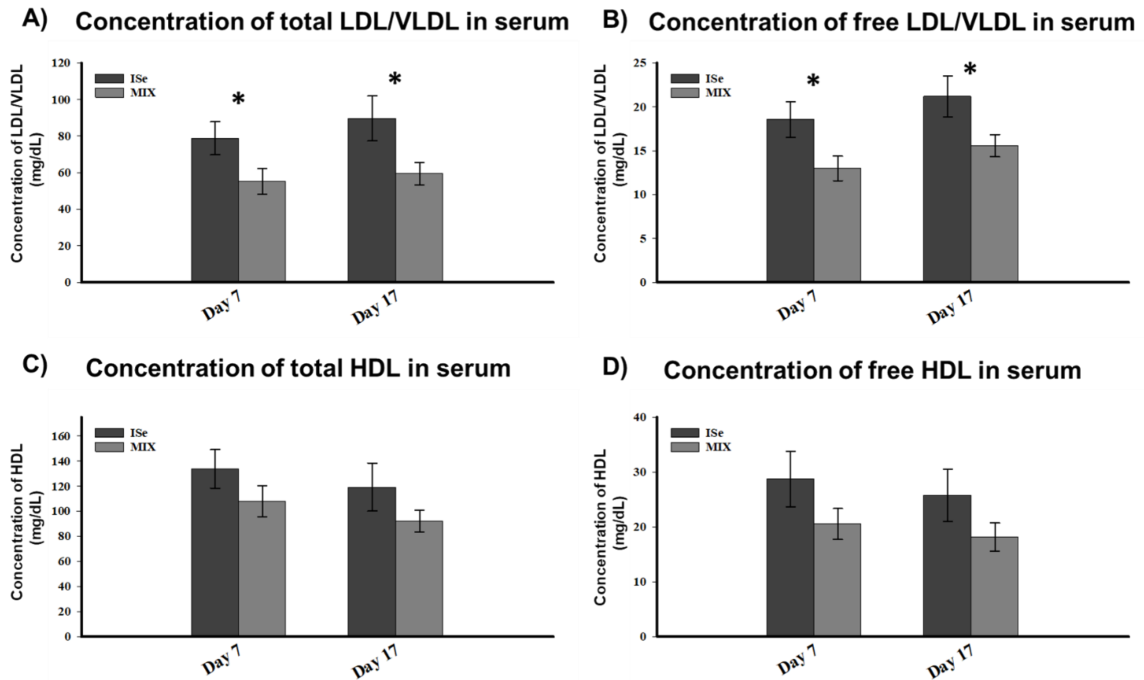

Figure 6.3. Concentration of serum lipoprotein fractions on gestational d 7 and d 17 in heifers supplemented with ISe (n = 6) or MIX (n = 6) vitamin-mineral mix. Data were analyzed as ANOVA with repeated measures. a) Total LDL/VLDL and b) free LDL/VLDL were significantly affected by diet ($P < 0.05$). Free LDL/VLDL was affected by time ($P < 0.05$), but total LDL/VLDL was not. Neither total nor free LDL/VLDL fractions had a treatment by day interaction ($P > 0.05$). c) Total HDL and d) free HDL were not affected by Se-treatment ($P > 0.05$), day of gestation ($P > 0.05$) or a treatment by day interaction ($P > 0.05$). In all figures, an * indicates statistical significance at $P \leq 0.05$ between the form of Se at each respective timepoint.

6.4.4. Glucose

Given the previously reported increase in the abundance of mRNA encoding myostatin in the ICAR tissue on d 17 of gestation and the potential to affect early development of the post-hatching, pre-implantation embryo, we quantified circulating concentrations of glucose at estrus and on d 7 and d 17 of gestation. Throughout experimentation the serum concentration of glucose was higher than the reported reference range (57-79 mg/dL, Figure 6.4). However relative to Se treatments, glucose was significantly lower in the MIX supplemented heifers compared to ISe across all three time points observed ($P = 0.03$), but there was no significant effect of day of gestation ($P > 0.05$) nor an interaction between dietary Se and day of gestation ($P > 0.05$). There tended to be a lower concentration of glucose at gestational d 7 (91.67 ± 7.45 v 77.17 ± 6.55 mg/dL, $P < 0.1$) but this was not detected at d 0 or d 17.

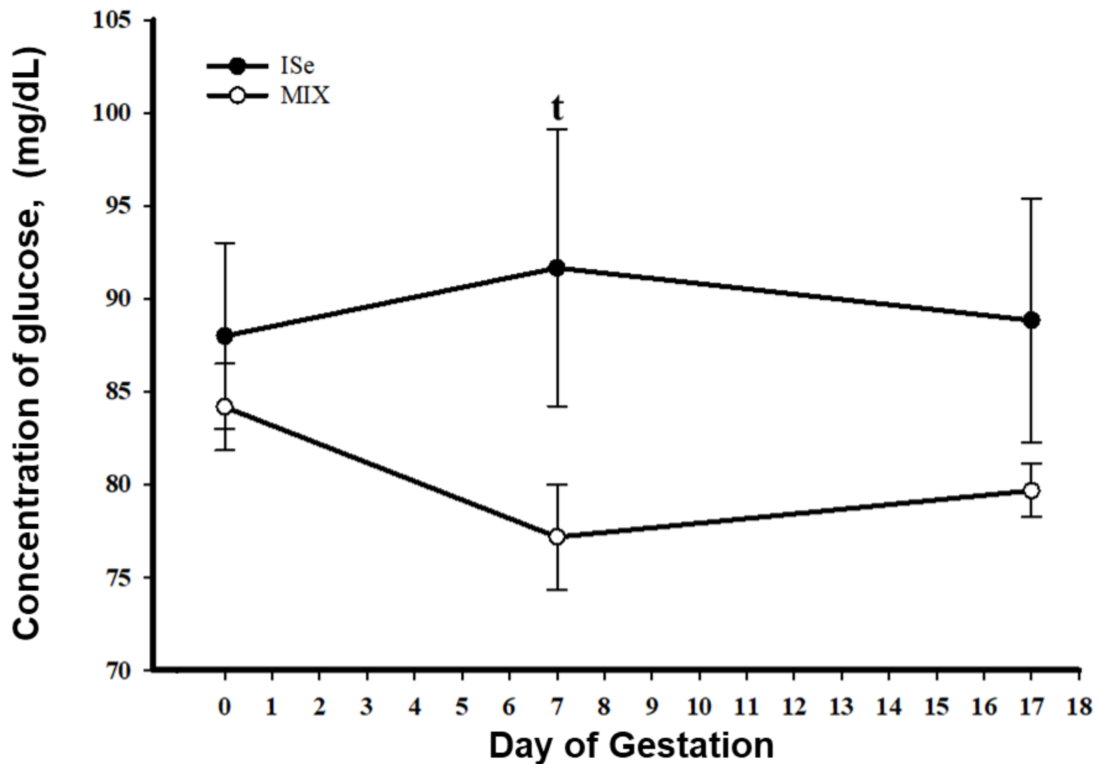

Figure 6.4. Concentration of serum glucose at estrus (d 0) and gestational d 7 and d 17 in heifers supplemented with either ISe (n = 6) or MIX (n = 6) vitamin-mineral mix. Data were analyzed as an ANOVA with repeated measures. Serum glucose was affected by dietary form of Se ($P = 0.03$), but not time ($P > 0.05$). Glucose was not affected by an interaction between form of Se and day of gestation ($P > 0.05$). An * indicates a significant difference at $P \leq 0.05$ and a t indicates a tendency to differ at $0.05 < P \leq 0.01$. Comparisons represented are between ISe and MIX at each respective time point.

6.4.5. Albumin

Albumin synthesized from hepatocytes is the most abundant serum protein (Rothschild et al., 1972), and it serves as a circulating reservoir and transport protein for metabolites and molecules including fatty acids and cholesterol (Bruss, 2008). In the present study, the serum concentration of albumin was within the reference range of 3.3-4.3 g/dL (Table 6.1). Throughout the experimental period, there was no significant effect ($P > 0.05$) of form of Se on albumin at d 0, d 7, and d 17 of gestation. Additionally, there was no significant interaction between dietary treatment and day of gestation ($P > 0.05$).

6.4.6. Aspartate amino transferase (AST)

The concentration of AST is regarded as a marker for liver damage (Sun et al., 2015) and fatty liver disease (Puppel and Kuczyńska, 2016). Moreover, it is directly responsible for regulating the concentration of cellular glutamate by catalyzing the conversion of aspartate and α-ketoglutarate to glutamate and oxaloacetate (Puppel and Kuczyńska, 2016). During the present experiment, the serum concentration of AST was within the normal reference range 54-135 U/L (Table 6.1). Overall, the MIX supplemental Se dietary treatment maintained a significantly higher concentration of serum AST compared to ISe at MRP (97.33 ± 3.73 v 72.50 ± 4.77 U/L respectively, $P < 0.01$). There was a significant main effect of form of dietary Se ($P = 0.02$) and day of gestation ($P = 0.03$) on the serum concentration of AST, and there was no significant interaction between dietary treatment and day ($P > 0.05$).

6.4.7. Beta-hydroxybutyrate (BHBA)

The serum concentration of BHBA is a primary indicator of metabolic status (Benedet et al., 2019), and presently, the concentrations of BHBA were within the normal reference range (2.3-10 mg/dL) for a healthy liver in cattle (Table 6.1). There was a significant main effect of dietary treatment ($P = 0.04$) and time ($P < 0.01$), with no interaction between treatment and time ($P > 0.05$). The concentration of BHBA tended to be higher in heifers supplemented with the MIX-Se form on d7 of gestation (5.43 ± 0.92 v 3.15 ± 0.21 mg/dL respectively, $P = 0.06$, Table 6.1) with no significant difference on at estrus and d17 ($P > 0.05$).

6.4.8. Non-esterified fatty acids (NEFA)

NEFA are a major component of triglycerides which contain a glycerol backbone with three fatty acids, and they are commonly measured as an indicator of negative energy balance in cows (Adewuyi et al., 2005). All heifers had serum concentrations of NEFA fall within the reference range 0.07-0.46 mE/L for cattle (Table 6.1). There was no significant effect of form of Se on the serum concentration of NEFA ($P > 0.05$) but there was a significant effect of time ($P = 0.04$). There was no significant interaction between dietary treatment and time ($P > 0.05$).

6.4.9. Triglycerides

Triglycerides are composed of a glycerol backbone with three fatty acids and function as a storage depot for excess lipids (Bobe et al., 2004). In cattle, triglycerides are

measured as a determination of metabolic status whereas a buildup of triglycerides in the liver decrease liver function and lead to a fatty liver disease (Bobe et al., 2004). All animals in the present study had higher concentrations of serum triglycerides than the reference concentrations of 10-19 mg/dL in cattle (Table 6.1). Further, the serum concentration of triglycerides was not affected by the form of supplemental Se ($P > 0.05$) but was affected by time ($P = 0.03$) with no significant interaction between the two ($P > 0.05$).

6.4.10. Urea nitrogen

The concentration of urea nitrogen in the serum is indicative of kidney function and mediated by a balance between the synthesis from the hepatic ornithine cycle and glomerular filtration in the kidneys (Archibeque et al., 2001). Throughout experimentation herein, the serum concentration of urea nitrogen was within the normal reference range of 7-19 mg/dL (Table 6.1). The serum concentration of urea nitrogen was significantly lower on d 0 of gestation ($P < 0.05$) but did not differ on d 7 and d 17 of gestation ($P > 0.05$). There was a significant main effect of dietary treatment ($P = 0.04$) and time ($P < 0.001$), with no significant interaction between dietary form of Se and day of gestation ($P > 0.05$).

Table 6.1. Concentration of metabolites in the serum of heifers supplemented with vitamin-mineral mixes containing Se as sodium selenite (ISe, n = 6) or a 1:1 mixture of ISe and OSe (MIX, SEL-PLEX, n = 6).[1]

Variable		**Treatment**		**P-Value[2]**
		ISe (*n = 6*)	**MIX** (*n = 6*)	
Albumin (g/dL)	d 0	3.783 ± 0.083	3.767 ± 0.080	ND
	d 7	3.767 ± 0.095	3.750 ± 0.112	ND
	d 17	3.767 ± 0.105	3.700 ± 0.089	ND
AST (U/L)	d 0	94.33 ± 7.260	105.83 ± 2.810	ND
	d 7	90.20 ± 13.97	106.33 ± 8.510	ND
	d 17	72.50 ± 4.770	97.33 ± 3.730	0.0056
BHBA (mg/dL)	d 0	3.683 ± 0.463	4.467 ± 0.327	ND
	d 7	3.150 ± 0.209	5.433 ± 0.923	0.0561
	d 17	2.447 ± 0.303	2.900 ± 0.543	ND
NEFA (mEq/L)	d 0	0.397 ± 0.116	0.290 ± 0.035	ND
	d 7	0.253 ± 0.061	0.257 ± 0.044	ND
	d 17	0.430 ± 0.054	0.332 ± 0.022	ND
Triglycerides (mg/dL)	d 0	36.83 ± 3.25	32.50 ± 5.35	ND
	d 7	28.00 ± 3.71	27.67 ± 1.96	ND
	d 17	35.17 ± 2.31	35.50 ± 5.29	ND
Urea nitrogen (mg/dL)	d 0	17.33 ± 1.944	12.00 ± 1.390	0.0490
	d 7	4.667 ± 0.494	4.833 ± 0.601	ND
	d 17	4.500 ± 0.719	4.167 ± 0.601	ND

[1] Se was supplemented with a vitamin-mineral mix formulated with 35 ppm of inorganic (ISe; sodium selenite), or a 1:1 combination (MIX) of ISe and OSe (SEL-PLEX).
[2] P-values are reported from ANOVA with repeated measures for the comparisons between dietary treatment at each time point. Significance is declared at $P \leq 0.05$ and a tendency to differ is considered $0.05 < P \leq 0.10$.

6.5. Discussion

This experiment expanded upon our previous findings that suggested changes in nutrient availability are occurring in the early luteal phase in cows (Carr et al., 2022; Crites et al., 2022b) and at MRP (Crites et al., 2022a) in heifers, and sought to investigate Se form-specific changes in serum metabolic parameters during estrus, the early luteal phase, and at MRP. Of particular importance was the concentration of systemic lipoproteins, glucose, cholesterol, and triglycerides, and we hypothesized that these would be elevated in the MIX-Se treatment group compared to ISe.

Previously we reported a 1.7 ng/mL increase in peripheral P4 on d 6 (Cerny et al., 2016b) and a 1.0 ng/mL increase on d 7 (Carr et al., 2022) of the estrous cycle in MIX versus ISe supplemented cows, which appears to be associated with the uptake and availability of cholesterol to the luteal cells as a substrate for this hormone. Particularly, the MIX diet increased the concentration of mRNA encoding the LDLR and HSL (Carr et al., 2022) and significantly upregulated the *de novo* cholesterol synthesis pathway in the early luteal phase CL (d7, (Crites et al., 2022b)). Unexpectedly in the present study, there was a significant decrease in the serum concentration of total and free LDL/VLDL lipoproteins on d 7, and d 17 in the MIX-Se supplemented heifers compared to ISe. There was no significant effect on the serum concentration of total and free HDL. Effectively, there are four lipoproteins that have different primary functions, VLDL, IDL, LDL, and HDL. The LDL and VLDL deliver lipids to peripheral tissues, and LDL is the primary lipoprotein that delivers cholesterol from the liver to target cells. Intermediate density lipoproteins (IDL) are transient proteins in lipid metabolism and HDL primarily carries excess cholesterol back the liver (Bruss, 2008). Therefore, it is perplexing that the MIX

supplemented heifers had lower serum concentrations of LDL/VLDL, given that LDL delivers cholesterol to the CL for conversion into P4.

These changes in the concentration of serum LDL/VLDL occurred concurrently with a significant decrease in the serum concentration of cholesterol throughout the present study in the MIX-supplemented heifers compared to ISe. Therefore, it is an unexpected discovery given our previous hypothesis that circulating cholesterol would be greater in MIX to correlate with the upregulated transcription of mRNA encoding LDLR in the CL on d7 of the estrous cycle (Carr et al., 2022). Speculatively, the opposite is plausible given that there is MIX-induced up-regulation of the LDLR in the CL at this time point to accommodate for the lower concentration of systemic cholesterol being transported by LDL.

Next, we investigated the effects of the dietary form of Se on systemic glucose elaborating on our previous finding that mRNA encoding MSTN was significantly upregulated in the ICAR of the MIX heifers on d 17 of gestation. We hypothesized that the concentration of glucose would be greater in the MIX-Se form supplemented heifers during the early luteal phase and at MRP. This metabolite is sequestered from the maternal blood and secreted into the histotroph as the primary source of energy for the post-hatched conceptus prior to implantation and formation of the placenta (Atkinson et al., 1984; Martal et al., 1997). Presently, glucose was significantly higher in ISe heifers at d 7 and d 17 of gestation. However, we were unable to determine significant differences at each time point presumably due to the high variance and limited number of experimental samples. Of relevance, Moraes, et al (2020) demonstrated that high fertility classified heifers had significantly less plasma glucose on d 17 of gestation compared to

infertile heifers, with sub-fertile classified heifers having an intermediate concentration of plasma glucose, but this trend did not translate to the concentration of glucose in the uterine lumen (Moraes et al., 2020). The previously observed increase in MSTN mRNA in MIX-Se form heifers could be a tissue specific metabolic change accounting for a relatively lower availability of this metabolic substrate to be sequestered from the blood.

We have also reported a decrease in levels of mRNA for DGAT2 in CAR tissue (Crites et al., 2022a), which may increase the availability of triglycerides to the histotroph for utilization by the early embryo (Forde et al., 2009). This protein catalyzes the final step in the formation of triglycerides to acylcoenzyme A (acyl-CoA), and triglycerides may provide energy to the developing conceptus in cattle (Forde et al., 2009; Ribeiro et al., 2016). We failed to detect a significant effect of form of Se on circulating triglycerides. The upregulation of DGAT2 mRNA in MIX-supplemented heifers could be contributing to a greater availability of triglycerides to the developing conceptus to compensate for the lack of differences in the availability of systemic triglycerides. However, this is yet to be investigated.

Next, albumin, the most abundant serum protein accounting for 50% of the total protein is synthesized and secreted from hepatocytes (Rothschild et al., 1972). Albumin is primarily a circulating depot and transport protein for metabolites and molecules including fatty acids and cholesterol (Bruss, 2008). Therefore, we quantified albumin in the serum of experimental heifers at estrus, d 7, and d 17 of gestation and were unable to detect a significant difference between MIX and ISe supplemented heifers which is consistent with previous studies measuring albumin in the serum of beef steers (Jia, 2019).

Circulating BHBA is a marker of energy availability in cattle and is increased in the blood when energy demands are greater than the amount of available glucose (Benedet et al., 2019). Serum BHBA was increased in MIX versus ISe supplemented heifers, at the time concurrent with previously observed form of Se-dependent increases in cholesterol uptake and production of progesterone by the early luteal phase CL (Carr et al., 2022; Crites et al., 2022b). BHBA is primarily synthesized from fatty acids in the liver and is commonly associated with ketosis in dairy cows (Lei and Simões, 2021), being mobilized when dietary nutrition does not meet energy demands and blood glucose concentrations are unable to meet the increased requirement for energy substrates (Grummer et al., 1995). Increased BHBA appears to be indicative of a MIX-induced increase in hepatic output at a time concurrent with the MIX-induced increases in cholesterol uptake, utilization, and luteal P4 secretion. However, further physiological significance remains to be investigated.

6.5.1. Conclusions

Overall, it appears that the form of Se is altering physiological processes that affect the serum concentrations of cholesterol, lipoproteins, glucose, AST, and BHBA, effectively altering the energy metabolism and the lipid profile of the cow and subsequently changing whole animal physiology and fertility. Further research is warranted to understand the metabolic changes in the liver and endometrium, and how these contribute to the MIX-Se form specific increase in the length of the conceptus at MRP in heifers.

Chapter 7. Transcriptomic changes in response to form of selenium on the interferon-tau signaling mechanism in the caruncular tissue of beef cows at maternal recognition of pregnancy

7.1. Abstract

It is necessary to supplement selenium (Se) to the diet of beef cattle in regions where the soils and therefore forages are deficient in this trace mineral. Supplementation of this mineral conventionally occurs using an inorganic form (ISe) although the organic forms (OSe) are predominantly available when cattle consume forage. Previously we have investigated the physiological effects of supplemental form of Se as ISe, or a 1:1 mixture of ISe to OSe (MIX) on grazing beef cows and heifers and have observed significant effects on the corpus luteum (CL) and endometrium. The MIX form of Se increased the concentration of progesterone (P4) during the early luteal phase of the estrous cycle at a time that can significantly improve early embryonic development. We subsequently investigated the effects of MIX form Se versus ISe on the mRNA transcripts encoding IFNτ- and P4-induced proteins in the caruncular (CAR) tissue of the uterine endometrium at maternal recognition of pregnancy (MRP). MIX-supplemented heifers had a decrease in the relative abundance of diacylglycerol o-acyltransferase 2 (DGAT2) in CAR which was coupled with significantly longer conceptuses at MRP in MIX-Se supplemented heifers. However, global changes in the CAR transcriptome in response to MIX- versus ISe supplementation are unclear, thus stimulating the objective of this study. Angus-cross heifers (N = 20) were exposed to a 45-day period of Se depletion with no exogenous supplementation of this mineral followed by a 45-day repletion period in which they were

provided with ISe alone. Heifers were then randomly assigned to 90 days of treatment with a vitamin-mineral mix containing 35-ppm Se as ISe (n = 10), or 1:1 mixture of ISe and OSe (n = 10, MIX). Following estrous synchronization and artificial insemination, heifers were killed at MRP (d 17) and the reproductive tracts were collected. Only heifers with a fully intact recovered conceptus were utilized for further analyses (n = 6 per treatment). Transcriptomic analysis using RNA-sequencing was conducted using total mRNA from caruncular samples. Differential gene expression (DEG) was determined using Integrated Differential Expression and Pathway Analysis (iDEP.96), and DEG were subjected to canonical, functional, and network analyses using QIAGEN's Ingenuity Pathway Analysis (IPA). RNA sequencing results were validated using qPCR. At $P < 0.05$, there was a total of 2029 DEGs with a total of 1038 transcripts upregulated, and 991 transcripts downregulated in MIX versus ISe heifers. Saliently, the interferon JAK/STAT pathway signaling through the STAT1/2 heterodimer was significantly down regulated in CAR samples from MIX heifers, as were the following interferon-responsive genes: IFIT1, IFIT2, IFIT3, IRF1, IRF9, ISG15, ISG20, OAS2, RSAD2, and STAT2. Considering the present results and our previous finding of significantly longer conceptus on d 17 of pregnancy in MIX versus ISe-treated heifers, it appears that MIX-supplemented heifers are experiencing an earlier timing of peak INFτ signaling and MRP.

Keywords: selenium; caruncle; endometrium; maternal recognition of pregnancy; interferon tau; interferon signaling

7.2. Introduction

It is necessary to supplement selenium (Se) in the diet for cattle grazing forages in regions in which the soils are deficient in this trace mineral (Boyne and Arthur, 1979; Gleed et al., 1983; McClure et al., 1986; Erskine et al., 1989; Enjalbert et al., 2006). Most commonly, supplemental Se is provided in a vitamin-mineral mix as an inorganic form (ISe) being sodium selenite or sodium selenate. However, organic forms of selenium (OSe), and particularly selenomethione, are available when cattle graze forage (Ammerman and Miller, 1975).

We have consistently studied the effects of form of supplemental Se as either ISe or a 50%:50% mixture (MIX) of ISe to OSe and have reported a MIX-induced increase in the systemic concentration of progesterone (P4) in the early luteal phase of the estrous cycle in cattle (Cerny et al., 2016b; Carr et al., 2020), with this increase occurring at a time that can significantly improve embryonic development by altering endometrial function that supports conceptus elongation (Garrett et al., 1988; Spencer and Bazer, 2002; Carter et al., 2008) and the production of interferon tau (IFNτ), a protein signal from the developing conceptus that signals maternal recognition of pregnancy (MRP (Mann and Lamming, 2001)).

Receptivity of the endometrium to allow implantation of the conceptus is dependent on P4 (Mansouri-Attia et al., 2009), uterine restructuring to promote placentation (Haeger et al., 2016), and the ability of the fetal allograft to evade the maternal immune response (Ott, 2019). Considerably, we analyzed changes in the relative abundance of P4 and IFNτ- regulated mRNA transcripts in the caruncular (CAR) tissues of the endometrium from ISe versus MIX-supplemented heifers at MRP (d 17 of

gestation) and observed a significant decrease in the expression of mRNAs encoding diacylglycerol o-acyltransferase 2 (DGAT2), fibroblast growth factor 2 (FGF2), interferon induced protein with tetratricopeptide repeats 3 (IFIT3), ISG15 ubiquitin like modifier (ISG15), MX dynamin like GTPase 1 (MX1), and 2'-5'- oligoadenylate synthetase 2 (OAS2, (Crites et al., 2022a)). Interferon stimulated genes affect uterine receptivity and the development of the conceptus (Spencer et al., 2007). In the same study, we saliently observed a significant increase in the length of the conceptus in MIX-supplemented heifers, which has clear implications on fertility outcomes as it may be better prepared for attachment and implantation (Crites et al., 2022a).

Finally, the ability of the developing conceptus to implant is dependent on successful evasion of the maternal immune system to allow the fetal allograft to closely interact with the maternal interface (Walker et al., 2010; Ott, 2019). This immunotolerant environment is the result of a careful balance between compositional changes in the innate immune system, which appears to remain active to protect against pathogens (Walker et al., 2010), and the adaptive immune system which must be repressed (Walker et al., 2010; Ott, 2019). During this time period, ligands and prostaglandins from the conceptus signal the endometrium to effect necessary changes, and in the presence of P4, there is an increase in macrophages, dendritic cells, and NK cells that migrate into uterine epithelial, endothelial, and stomal cells (Ott, 2019), as well as changes in the abundance of molecules that promote immune tolerance such as indoleamine 2,3-dioxygenase 1 (IDO1), transforming growth factor beta (TGFB), and interferon stimulated genes (ISG) (Ott, 2019), thus allowing for the impending processes of implantation and placentation.

All encompassing, we hypothesized that the maximal expression of IFNτ may be occurring earlier in MIX supplemented heifers, shifting the initial timing of MRP to an earlier timepoint, which is occurring concomitantly with the development of longer conceptuses from MIX versus ISe-treated heifers on d 17 of gestation (Crites et al., 2022a). We further surmise that there are changes occurring in the endometrium allowing for a more receptive environment for the implanting conceptus.

Se-form induced changes in CAR gene expression had not been defined at the global level. Therefore, with the absence of a global perspective of mRNA transcripts in the CAR in response to Se-form, the objective of this study was to examine transcriptomic changes in the CAR tissue of the endometrium at MRP, testing the hypothesis that there would be changes in the endometrium indicating a shift in the timing of MRP and an advancement in CAR preparation for attachment and implantation in MIX versus ISe-treated heifers.

7.3. Methods

This project (protocol number 2017-2828) was approved by the University of Kentucky's Institutional Animal Care and Use Committee.

7.3.1. Animals and Experimental Procedure

Angus-cross heifers (N = 20) received 45-days of Se depletion with no supplemental Se, followed by 45-days of Se repletion with a vitamin-mineral mix containing 35-ppm Se as only ISe to reestablish systemic Se adequacy status (Gerloff,

1992; Dargatz and Ross, 1996). The heifers were then randomly assigned to supplemental Se treatment formulated with a vitamin-mineral mix containing 35-ppm Se as either inorganic Se (n = 10, ISe, sodium selenite, Prince Agri Products, Inc. Quincy, IL, USA) or a 1:1 ratio of ISe and OSe (n = 10, MIX, SEL-PLEX; Alltech, Inc., Nicholasville, KY, USA). Heifers received dietary treatments for at least 90 days prior to estrous synchronization.

Whole blood was collected via jugular venipuncture prior to depletion, repletion, and the start of treatment. It was also recovered bi-monthly during treatment until the end of experimentation to confirm Se adequate status throughout. Whole blood was also collected from each heifer at estrus and prior to slaughter (d 17). Total blood Se was quantified by the University of Kentucky's Veterinary Diagnostics Laboratory (Lexington, KY) using an Agilent 7900 inductively coupled plasma-mass spectrometer (Wahlen et al., 2005). Throughout experimentation, all animals-maintained Se-adequate status (Gerloff, 1992; Dargatz and Ross, 1996), and there tended to be an effect of Se-treatment ($P = 0.07$) as discussed in Chapter 6 and previously published (Crites et al., 2022a).

7.3.2. Experimental Regimen and Serum Collection

After at least 90 days of Se supplementation with each respective treatment (ISe vs. MIX) heifers were randomly injected with one or two doses of dinoprost tromethamine (25 mg, Lutalyse, Zoetis, Parsippany, NJ) to induce regression of the CL and then monitored daily for behavioral estrus (d 0) by visual observation as well as using CowManager technology (Gerverscop 9, The Netherlands) to predict the timing of

estrus. The presence of a preovulatory follicle (estrus, d 0) was confirmed via transrectal ultrasonography using a 5-8 MHz linear transducer (Ibex Pro, E.I. Medical Imaging, Loveland, CO, USA) prior to artificial insemination at 0 h, 12 h, and 24 h using commercially available frozen semen from an established high fertility bull.

The ovaries, uterus, pituitary, and liver were collected from each heifer on d 17 following euthanasia via captive bolt stunning and exsanguination at the USDA inspected University of Kentucky Meat Laboratory. Of these tissues, only the uterus was utilized for experimentation in the present study. The ovaries, pituitary, and liver were used for experimentation elsewhere. Initially, the uterus was flushed to collect the conceptus as described in (Crites et al., 2022a). An intact conceptus was recovered from six heifers in each treatment group (ISe, n = 6; MIX, n = 6), and only these heifers were used for analyses in the present study.

Subsequently, caruncular endometrial samples were collected from the uterine horn ipsilateral to the ovary bearing the CL. This respective uterine horn was cut longitudinally to expose the uterine lumen, and an 8 mm biopsy punch (Integra LifeSciences Production Corporation, Mansfield, MA, USA) was used to collect CAR endometrial samples from all animals. These samples were flash frozen in liquid N_2 stored at −80°C for RNA extraction, RNA sequencing and for real-time PCR (qPCR) analysis.

7.3.3. RNA Extraction

Total RNA was extracted from approximately 200 mg CAR samples of the endometrium using TRIzol Reagent (Invitrogen Corporation, Carlsbad, CA). All samples had high purity, with 260/280 absorbance ratios of 1.88 or greater as quantified using a NanoDrop ND-100 Spectophotometer (NanoDrop Technologies, Wilmington, DE, USA).

7.3.4. RNA-Sequencing

Transcriptomic analysis using RNA-sequencing was conducted in mRNA extracted from CAR tissue. Library preparation was performed by Zymo Research Corporation (Irvine, CA, USA). Initially, 500 ng of total RNA was used to construct the total RNA-Seq libraries. The method described in (Bogdanova et al., 2011), was used to remove ribosomal RNA (rRNA) with some modifications, and libraries were prepared using the Zymo-Seq RiboFree Total RNA Library Prep Kit (Zymo Research Corporation, Irvine, CA, USA). The RNA-Seq libraries were then sequenced using an Illumina NovaSeq with a sequencing depth of at least 30 million read pairs per sample.

The RNA-Seq pipeline utilized by the Zymo Research Corporation (Irvine, CA, USA) was adapted from nf-core/rnaseq pipeline v1.4.2 (Ewels et al., 2020), and built using Nextflow (Di Tommaso et al., 2017). The quality of raw reads was analyzed using FastQC v0.11.9, and adaptor and low-quality reads were trimmed using Trim Galore! v0.6.6. The resultant trimmed reads were aligned to the reference genome using STAR v2.6.1d (Dobin et al., 2013). SAMtools v1.9 was used for BAM file filtering and indexing (Danecek et al., 2021), and library quality control was executed using QualiMap v2.2.2-dev (García-Alcalde et al., 2012) and RSeQC v4.0.0 (Wang et al., 2012).

Duplicated reads were marked using Picard tools v2.23.9 (Broad Institute, Cambridge, MA, USA), and quality control of the duplication rate was analyzed using dupRadar v1.18.0 (Sayols et al., 2016). The complexity of the library was estimated using Preseq v2.0.3 (Daley and Smith, 2013), and gene assignments were applied to reads that overlapped with exons using featureCounts v2.0.1 (Liao et al., 2014).

Count data was uploaded to Integrated Differential Expression and Pathway Analysis (iDEP.96, (Ge et al., 2018)), and lowly expressed transcripts were removed at < 1.0 count per million (CPM). Data were log transformed and then subjected to principal component analysis (PCA) and hierarchical clustering of the top 1000 expressed genes to visualize sample variation. For all samples, the average mapping percentage of the identified transcripts was 92.33%. The total counts for all samples are displayed in Figure 7.1A, and data for each sample were normally distributed (Figure 7.1B). The average correlation among all samples was 0.97 as indicated in Figure 7.2. Differentially expressed gene/transcript (DEG) expression analysis was completed using DESeq2 v1.28.0 (Love et al., 2014), which uses the Wald test for hypothesis testing by calculating the log fold change and dividing it by the standard error resulting in a z-statistic that can be used to ascertain the p-value. At the significance level $P < 0.05$, a total of 2029 gene transcripts demonstrated an effect of treatment with a False Discovery Rate (FDR) of < 40%. Presently, the high FDR appears to be due to the small sample size, sample variation, and relatively low proportion of DEGs in the MIX compared to ISe treatment groups. However, it is generally accepted that corroborating multiple gene transcripts after high throughput analysis by qPCR provides validation of the observed changes (Rockett and Hellmann, 2004).

To assess global effects of Se-treatment on the abundance of CAR transcripts at MRP, differentially expressed DEGs identified in DESeq2 analysis were analyzed for canonical, functional, and network analyses using QIAGEN's Ingenuity Pathway Analysis (IPA, QIAGEN, Redwood City, CA, USA, http://www.ingenuity.com). The raw data (FASTQ files) of this manuscript will be uploaded to the NCBI Sequence Read Archive (SRA) upon submission of this chapter for publication.

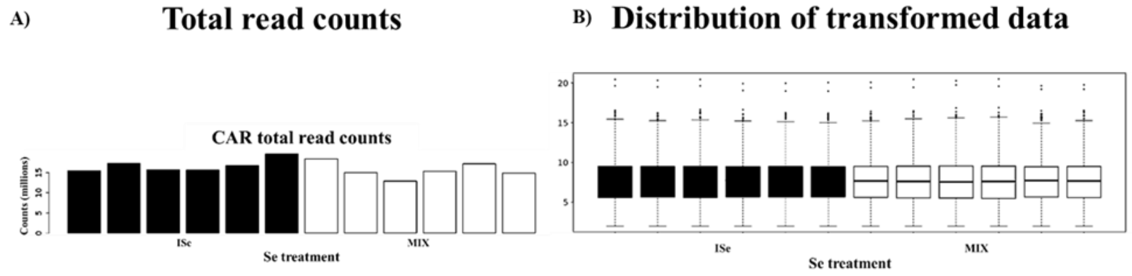

Figure 7.1. The A) total counts and B) distribution of log transformed data derived from RNA-Seq analysis of each sample of caruncular (CAR) tissue from heifers supplemented with 3 mg Se/d in vitamin-mineral mixes as ISe (n = 6) or MIX (n = 6).

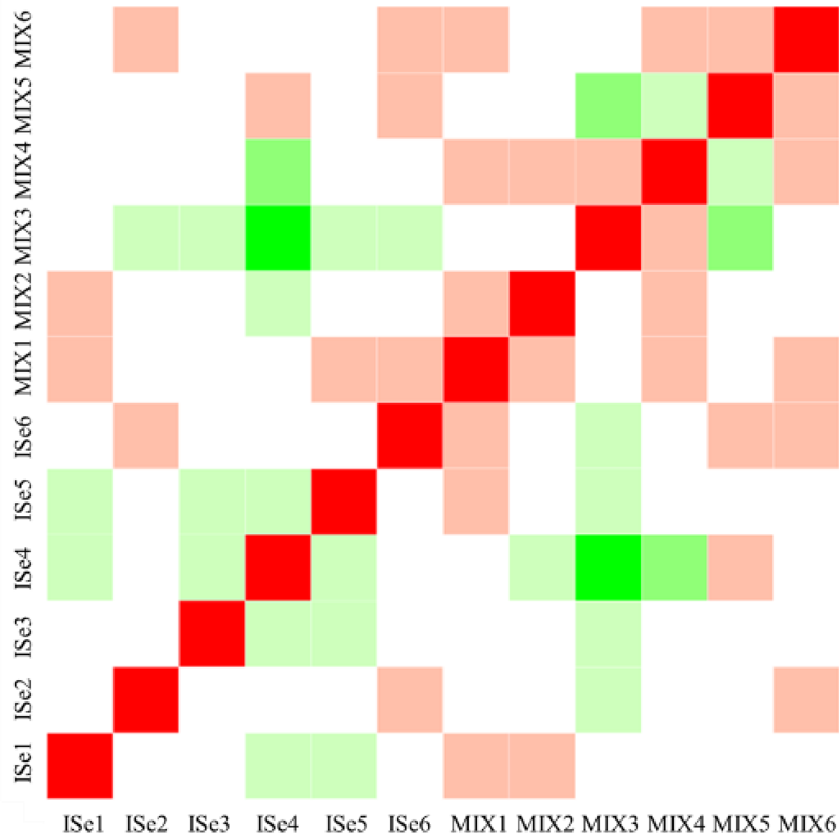

Figure 7.2. Correlation matrix of data derived from RNA-Seq analysis of each sample of caruncular (CAR) tissue from heifers supplemented with 3 mg Se/d in vitamin-mineral mixes as ISe (n = 6) or MIX (n = 6). The average correlation among all samples was 0.97.

7.3.5. qPCR Analysis

Real-time PCR (qPCR) was used to quantify the relative abundance of mRNA in CAR samples for identified genes that differed according to the RNA-Seq analysis using a technique that has been routinely used in our laboratory (Cerny et al., 2016a; Carr et al., 2022; Crites et al., 2022a; Crites et al., 2022b). Approximately 1 μg of RNA from each sample was reverse transcribed into cDNA using SuperScript IV VILO Master Mix with ezDNAse Enzyme (Invitrogen by Thermo Fisher Scientific, Vilnius, Lithuania). To ensure subsequent qPCR results are not a result of genomic DNA contamination, each sample was compared to a no-reverse transcription control.

The relative concentration of *Ifit2, Ifit3, Irf1, Irf9, Isg15, Isg20, Mmp19, Oas2, Rsad1, Rsad2, Scara5, Timp2, Timp3,* and *Trim56* was quantified to corroborate RNA-Seq results. Primers were designed against each respective RefSeq using the NCBI Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Target products in cDNA were verified by DNA sequencing at ACGT, Inc. (Wheeling, IL, USA), and sequencing results were compared to each respective primer template using the NCBI Nucleotide-BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_SPEC=GeoBlast&PAGE_TYPE=BlastSearch). The GenBank accession numbers, forward and reverse primer sequences, amplicon length of each product and product identify for each transcript of interest are listed in Table 7.1. To perform qPCR analysis, a total volume of 25 μL containing 5 μL of cDNA, 1 μL of a 10 μM stock of each primer (forward and reverse), 12.5 μL of 2 × SYBR Green PCR Master Mix (iTaq Universal SYBR Green Supermix, BIO-RAD, Hercules, CA, USA), and 5.5 μL of nuclease-free water. Reactions

were conducted using a Bio-Rad CFX Maestro thermal cycler (Bio-Rad, Hercules, CA, USA).

The relative abundance of each transcript was quantified using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) using transcripts for three consecutively expressed normally distributed genes that were not affected by Se-treatment to normalize the data: *Gapdh, Hprt1,* and *Ywhaz.* Additionally, data were normalized to the expression level of ISe. A total of 6 heifers per treatment were analyzed via qPCR and all reactions were performed in triplicate.

Table 7.1. Identified genes, GenBank accession numbers, primer sets (forward and reverse) and amplicon length (base pairs) for qPCR analysis of target transcripts.

Gene	Gene Name	Accession Number[1]	Primer Design (5' to 3') direction	Amplicon length (bp)	Citation
IFIT2	Interferon induced protein with tetratricopeptide repeats 2	XM_002698356.5	F: TGCAGAGGCCAGCATCAGGC R: GCCTGGTCAGCGTGCTCTCG	80	(Forde et al., 2011b)
IFIT3*	Interferon induced protein with tetratricopeptide repeats 3	NM_001075414.1	F: ATTCTGAAGCAGGCCGTTGA R: TCCAGTGCCCTTAGCAACAG	224	.
IRF1*	Interferon regulatory factor 1	NM_001191261.2	F: ACAGCCCCGATACCTTCTCT R: CTTCCCATCCACGCTTGTCT	338	.
IRF9	Interferon regulatory factor 2	NM_001024506.1	F: CAGTTCCCAGGAGTGTGCTG R: TATATCGCCCAGGCCTTGAA	125	(Shirozu et al., 2016)
ISG15*	ISG15 ubiquitin like modifier	NM_174366.1	F: CCATCCTGGTGAGGAACGAC R: GAACACGGTGCACCCCTTCA	200	.
ISG20	Interferon stimulated exonuclease, transcript variant 1	XM_002696514.5	F: GCAGGCAGCACACCTGAGGG R: TGCAGCGAGCCAAGCCACTC	117	(Forde et al., 2011b)
MMP19	Matrix metallopeptidase 19	NM_001075983.1	F: TGCTGGGCCACTGGAGAA R: AGGTCAAGGGAGCCACATTG	132	(Sponchiado et al., 2017)
OAS2*	2'-5'-oligoadenylate synthetase 2	NM_001024557.1	F: ACTGGTTTCAAAAGTGCCAGG R: CAGCCAGCAGGTGTTATCCA	314	.
RSAD2*	Radical S-adenosyl methionine domain containing 2	NM_001045941.1	F: GTGGTTCCAGAAGTACGGTGA R: AACCGTTCCGCTTCTCTCAG	315	.

Table 7.1. (Continued)

SCARA5	Scavenger receptor class A member 5	NM_001102499.1	F: AGGACCTACGCCTCAAGGAT R: GGGCCTCGATCACCTTTGAA	256	.
TIMP2	TIMP metallopeptidase inhibitor 2	NM_174472.4	F: GGGTCTCGCTGGACATTG R: TTGATGTTCTTCTCCGTGACC	256	(Ulbrich et al., 2011)
TIMP3	TIMP metallopeptidase inhibitor 3	NM_174473.4	F: CCTTTGGCACGATGGTCTACA R: CTCGGCCTGTCAGCAGGTA	156	(Sponchiado et al., 2017)
TRIM56	Tripartite motif containing 56	NM_001206574.1	F: TTCAGACCCCAAATCAGGAC R: TCTGGGCTCTGCTCTCTTTC	126	(Wang et al., 2021)
Housekeeping Transcripts					
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	NM_001034034.2	F: ACATCAAGTGGGGTGATGCT R: GGCATTGCTGACAATCTTGA	200	.
HPRT1	Hypoxanthine phosphoribosyltransferase 1	NM_001034035.2	F: GCCAGCCGGCTACGTTAT R: ATCCAACAGGTCGGCAAAGA	256	.
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta	NM_174814.2	F: TTGATCCCCAACGCTTCACA R: AGTTAAGGGCCAGACCCAGT	208	.

[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.
* Primers are reported in (Crites et al., 2022a).

7.3.6. Statistical Analysis

Data are presented as least square means (± SEM) with individual heifer as the experimental unit. Data were analyzed for normal distribution and homogeneity. When appropriate, qPCR data were natural log transformed given the samples were not distributed normally. The relative expression for each transcript identified in RNA-sequencing results were subjected to the Wald test using DESeq2 as described in section 9.3.3 RNA-Sequencing above.

To determine the effect of form of Se on concentrations of each mRNA transcript, data were analyzed using Student's T-Test with the PROC TTest procedure of SAS statistical software package (version 9.4; SAS Institute, Inc. Cary, NC), and each treatment group contained 6 heifers (ISe, n = 6; MIX, n = 6). Results were considered statistically significant at $P \leq 0.05$ or a tendency to differ at $0.05 < P \leq 0.10$.

7.4. Results

7.4.1. Cluster analyses

Principal component analysis (PCA) of all RNA-Seq data was performed to evaluate the relative relationships and variation among the individual heifers. The score plot (Figure 7.3A) demonstrates that principal component 1 (PC#1, x-axis) explained 25% of variance among the samples and principal component 2 (PC#2, y-axis) explained 12% of variance. Results indicate that there is slight separation between treatment groups with some similarities in the expression of DEGs.

Hierarchical clustering analysis of the top 1000 DEGs (Figure 7.3B) indicates a considerable separation between Se treatments, but there may be some overlap in transcriptomic profiles of the CAR.

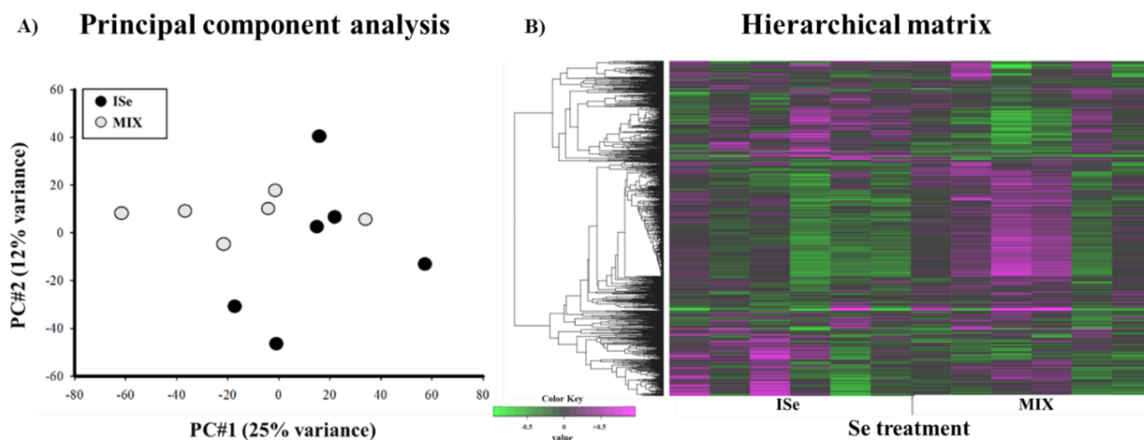

Figure 7.3. A) Score plot and B) hierarchical matrix of data derived from RNA-Seq analysis of each sample of caruncular (CAR) tissue from heifers supplemented with 3 mg Se/d in vitamin-mineral mixes as ISe (n = 6) or MIX (n = 6). A) Principal component 1 (PC#1) accounted for 25% of the variance and principal component 2 (PC#2) accounted for 12% of the variation among samples. B) Hierarchical cluster analysis was composed of the top 1000 differentially expressed transcripts using iDEP.96. Significance was determined by Wald test with significance declared at $P < 0.05$ for all.

Table 7.2. Top 10 most highly up- and down- regulated DEGs in the CAR of MIX (n = 6) versus ISe (n = 6) treated heifers.[1]

Gene ID	Gene Description	Fold Change	P-Value[2]
Up regulated in MIX			
CCDC152	Coiled-coil domain containing 152	4.03	0.0111
CD79B	CD79b molecule	3.75	0.0329
TM4SF5	Transmembrane 4 L six family member 5	3.39	0.0366
C22orf31	Chromosome 22 open reading frame 31	3.38	0.0152
SLC17A7	Solute carrier family 17 member 7	3.34	0.0338
CCL21	C-C motif chemokine ligand 21	2.93	0.0359
FAM180B	Family with sequence similarity 180 member B	2.88	0.0103
COL17A1	Collagen type XVII alpha 1 chain	2.35	0.0019
CCR9	C-C motif chemokine receptor 9	2.34	0.0184
OMG	Oligodendrocyte myelin glycoprotein	2.33	0.0429
Down regulated in MIX			
DPYSL4	Dihydropyrimidinase like 4	-4.07	0.0108
KLK5	Kallikrein related peptidase 5	-4.03	0.0004
GRM3	Glutamate metabotropic receptor 3	-3.70	0.0004
NIPAL4	NIPA like domain containing 4	-3.59	0.0020
NALCN	Sodium leak channel, non-selective	-3.26	0.0134
PTGER1	Prostaglandin E receptor 1	-3.16	0.0178
COL28A1	Collagen type XXVIII alpha 1 chain	-3.12	0.0105
SLC26A8	Solute carrier family 26 member 8	-3.12	0.0026
SLC34A3	Solute carrier family 34 member 3	-3.11	0.0237
DMKN	Dermokine	-2.93	< 0.0001

[1] Selenium was supplemented at 35 ppm as ISe (sodium selenite) or MIX (sodium selenite and SEL-PLEX).
[2] For statistical analysis, p-values were determined using the Wald test in DESeq2.

7.4.2. Differentially Expressed Genes

The Wald test of DESeq2 was used to identify changes in CAR between the ISe and MIX supplemented heifers. At $P < 0.05$, there was a total of 2029 DEGs with a total of 1038 transcripts upregulated, and 991 transcripts downregulated in MIX. The most differentiated genes that are increased and decreased according to fold-change in MIX compared to ISe are provided in Table 7.2.

7.4.3. Pathway and Gene Network Analysis

To determine global changes in DEGs in response to form of Se, bioinformatic analysis was performed using QIAGEN's Ingenuity Pathway Analysis (IPA, QIAGEN, Redwood, City, CA). Canonical pathway analysis indicated the top five canonical pathways (Table 7.3) based on p-values that were affected by the form of Se are ATM signaling ($P < 0.0001$), spliceosomal cycle ($P < 0.0001$), role of protein kinase R (PKR) in interferon induction and antiviral response ($P < 0.0001$), coordinated lysosomal expression and regulation (CLEAR) signaling pathway ($P < 0.0001$), and autophagy ($P < 0.0001$). Further, Figure 7.4 shows the top canonical pathways in CAR ranked by z-score. Interestingly, the most significantly affected pathways that were positively affected are spliceosomal cycle (z-score = 2.673), cold shock domain containing E1 (CSDE1) signaling pathway (z-score = 2.138), cell-cycle-control of chromosomal replication (z-score = 1.508), cell-cycle: G1/S checkpoint regulation (z-score = 1.414), and 3-phosphoinositide biosynthesis (z-score = 1.342). The most negatively affected pathways based on z-score are the role of PKR in interferon induction and antiviral response (z-score = -3.128), interferon signaling (z-score = -2.333), CD40 signaling (z-score = -

2.333), death receptor signaling (z-score = -2.183), and TNF receptor superfamily member 1A (TNFR1) signaling (z-score = -2.121).

The top upstream regulators identified using IPA at $P < 0.05$ were hepatocyte nuclear factor 4 alpha (HNF4A), estrogen receptor 1 (ESR1), KRAS proto-oncogene, GTPase (KRAS), transforming growth factor beta 1 (TGFB1), and Pyridostatin. Top 5 molecular and cellular functions with specific actions identified by IPA are indicated in Table 7.4. Only functions with a z-score of greater than or equal to the absolute value of 0.5 are reported.

Table 7.3. Top 5 IPA-identified canonical pathways of genes differentially expressed from CAR of heifers supplemented with 3 mg Se/d in vitamin-mineral mixes as ISe (n = 6) or MIX (n = 6).[1]

Canonical Pathway[2]	Gene Symbols	Ratio[3]	Z-Score	P-value
ATM Signaling	**Up:** ATR, CBX1, CBX3, CBX5, CCNB2, CDK2, HP1BP3, MDC1, PPP2R1B, RAD50, RAD51, RBBP8, RNF8, SMC2, SMC3, SMC1A, ZNF420 **Down:** BID, BRAT1, MAPK12, MAPK13, PPM1L, PPP2R1A, TP73, TRRAP	0.25 (25/100)	0.471	< 0.0001
Spliceosomal Cycle	**UP:** BCAS2, CDC5L, CTNNBL1, CWC15, DDX23, DHX38, ISY1-RAB43, MAGOHB, RBM8A, SLU7, SNRNP200, ZNF830 **Down:** PRPF19, SF3B3	0.29 (14/49)	2.673	< 0.0001
Role of PKR in Interferon Induction and Antiviral Response	**Up:** APAF1, DNAJC3, HMGB1, HSP90AA1, HSP90AB1, HSP90B1, HSPA4, METAP2, MSR1, REL **Down:** ATF3, BID, FADD, FOS, HSPA6, IRF1, IRF9, MAPK12, MAPK13, PDGFRB, PYCARD, RELA, STAT2, STAT3, TARBP2, TNFRSF1A	0.19 (26/136)	-3.128	< 0.0001
CLEAR Signaling Pathway	**Up:** ATP6V1C1, ATP6V1E1, BECN1, BMPR1B, HPS5, ITPR2, MAP4K3, NRBF2, PPP2R1B, RRAGB, VPS26A, YWHAE **Down:** ATP6V0A1, ATP6V0D1, BLOC1S3, BMP6, CRTC2, CTNS, CTSA, FLT1, GBA1, GLB1, KCNIP3, MAPK7, MAPK12, MAPK13, MLST8, PDGFRB, PML, PPM1L, PPP2R1A, PRKAG1, PRKCB, RAB7A, RPTOR, SEC13, SESN2, TCIRG1, TGFA, TGFBR3, TNFRSF1A, TRPM1, TSC2	0.15 (43/285)	-0.762	< 0.0001

Table 7.3. (Continued)

Autophagy	**Up:** ATG3, ATG10, ATR, BECN1, CALM1, CDKN1B, GNAI3, NRBF2, PIK3C2A, PPP2R1B, RB1CC1, SESN1, STX17, VPS41 **Down:** ATG2A, BMP6, FOS, IRS1, IRS2, MAPK12, MLST8, PI4K2A, PPM1L, PPP2R1A, PRKAG1, RAB7A, RAB7B, RIPK1, RPTOR, SLC7A5, TGFA, TNFRSF1A, TSC2, ULK1, WIPI2	0.16 35/216	0.845	< 0.0001

[1] Selenium was supplemented at 35 ppm as ISe (sodium selenite) or MIX (SEL-PLEX).
[2] Results from IPA were obtained based on log2 fold changes calculated using DESeq2.
[3] 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.

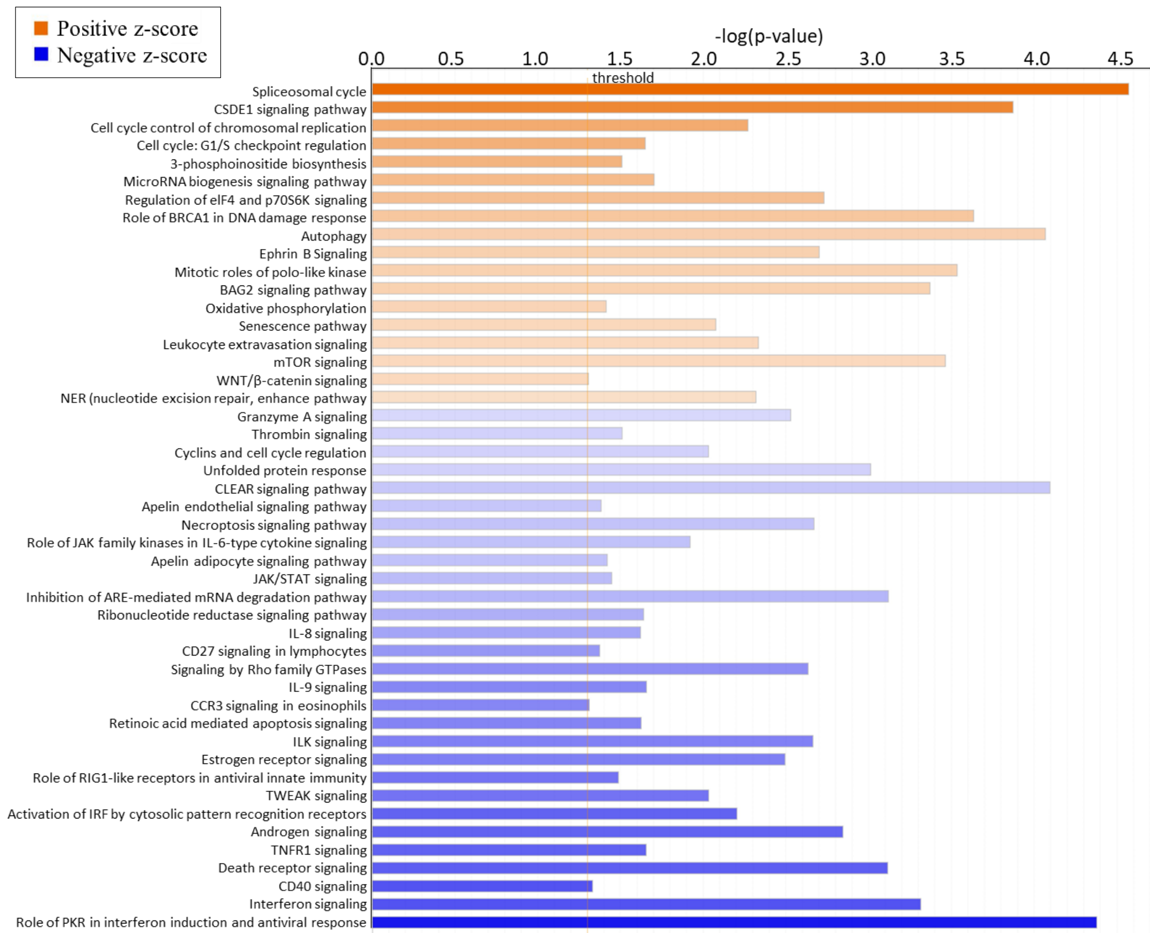

Figure 7.4. Top IPA-identified canonical pathways of genes differentially expressed from CAR of heifers supplemented with 3 mg Se/d in vitamin-mineral mixes as ISe (n = 6) or MIX (n = 6). Pathways are ranked by z-score with orange indicating a pathway that is predicted to be upregulated (positive z-score) and the blue indicates a pathway that is predicted to be down regulated (negative z-score).

Table 7.4. Top 5 IPA-identified molecular and cellular functions in CAR of heifers supplemented with 3 mg Se/d in vitamin-mineral mixes as ISe (n = 6) or MIX (n = 6).[1]

Molecular and Cellular Functions[2]	Z-score[3]	P-value[4]
Cell death and survival (732 molecules)		
Necrosis	-1.392	< 0.0001
Apoptosis	-2.252	< 0.0001
Cell death of tumor cell lines	-1.757	< 0.0001
Cell survival	1.029	< 0.0001
Apoptosis of tumor cell lines	-1.425	< 0.0001
Cell viability	1.112	< 0.0001
Cell viability of tumor cell lines	1.278	< 0.0001
Necrosis of epithelial tissue	-1.682	< 0.0001
Cell death of osteosarcoma cells	-1.671	< 0.0001
Cell death of bone cancer cell lines	-0.434	< 0.0001
Cell death of blood cells	-1.469	< 0.0001
Cell death of sarcoma cell lines	-0.943	< 0.0001
Colony survival of tumor cell lines	1.183	< 0.0001
Necrosis of tumor	-1.881	< 0.0001
Cell death of breast cancer cell lines	-2.267	< 0.0001
Apoptosis of peritoneal macrophages	-0.574	< 0.0001
Cellular response to therapeutics (149 molecules)		
Radiosensitivity of cells	-1.38	< 0.0001
Gene expression (462 molecules)		
Expression of RNA	-3.621	< 0.0001
Transcription of RNA	-2.971	< 0.0001
Transcription	-2.877	< 0.0001
Transcription of DNA	-2.485	< 0.0001
Activation of DNA endogenous promoter	-2.373	< 0.0001
Transactivation	1.115	< 0.0001
Transactivation of RNA	0.804	< 0.0001
Repression of RNA	0.646	< 0.0001
RNA post-translational modification (92 molecules)		
Processing of RNA	-1.066	< 0.0001
Processing of mRNA	-1.635	< 0.0001
Splicing of RNA	-1.339	< 0.0001
Splicing of mRNA	-1.682	< 0.0001
Cellular assembly and organization (505 molecules)		
Development of cytoplasm	-1.738	< 0.0001
Cohesion of sister chromatids	0.854	< 0.0001
Formation of centriole	0.921	< 0.0001
Remodeling of chromatin	0.900	< 0.0001

Table 7.4. (Continued)

Formation of cellular protrusions	-0.647	< 0.0001
Replication of centriole	0.921	< 0.0001

[1] Selenium was supplemented at 35 ppm as ISe (sodium selenite) or MIX (sodium selenite and SEL-PLEX).
[2] Results from IPA were obtained based on log2 fold changes calculated using DESeq2.
[3] Z-score is calculated using IPA and is used to infer activation state. Only functions reporting a z-score with an absolute value $\geq$ 0.5 are reported herein.
[4] P-values are determined using IPA.

7.4.4. Real-time PCR analysis of select mRNA transcripts

To validate the outcomes of the RNA-seq, real-time PCR analysis was conducted on select transcripts that are responsive to interferons or are relative to uterine function at MRP (Table 7.5). The results of qPCR were consistent with RNA-Seq analysis. Except for *Scara5* and *Timp2*, all targeted genes differed in both analyses at $P < 0.05$. Table 7.6 below lists relevant transcripts to the analysis herein, but these genes were not corroborated using qPCR.

Table 7.5. RNA-Seq corroboration using select gene interferon responsive transcripts at MRP from CAR endometrium of heifers supplemented with 3 mg Se/d in vitamin-mineral mixes as ISe (n=6) or MIX (n=6).[1]

Gene	Gene Name	RNA-Seq[2]			qPCR[2]			
		ISe	MIX	P-value	ISe	MIX	SEM	P-value
IFIT2	Interferon induced protein with tetratricopeptide repeats 2	1.00	0.71	0.01	1.07	0.66	0.15	0.08
IFIT3*	Interferon induced protein with tetratricopeptide repeats 3	1.00	0.78	0.02	1.08	0.65	0.13	0.04
IRF1*	Interferon regulatory factor 1	1.00	0.79	0.02	1.05	0.75	0.11	0.08
IRF9	Interferon regulatory factor 9	1.00	0.83	< 0.01	1.02	0.92	0.07	0.36
ISG15*	ISG15 ubiquitin like modifier	1.00	0.78	< 0.01	1.04	0.76	0.09	0.05
ISG20	Interferon stimulated exonuclease, transcript variant 1	1.00	0.74	< 0.01	1.07	0.82	0.13	0.13
MMP19	Matrix metallopeptidase 19	1.00	0.80	< 0.01	1.06	0.80	0.13	0.18
OAS2*	2'-5'-oligoadenylate synthetase 2	1.00	0.81	0.01	1.03	0.67	0.10	0.01
RSAD2*	Radical S-adenosyl methionine domain containing 2	1.00	0.75	< 0.01	1.05	0.57	0.14	0.01
SCARA5	Scavenger receptor class A member 5	1.00	0.87	0.09	1.03	0.65	0.08	0.01
TIMP2	TIMP metallopeptidase inhibitor 2	1.00	0.86	0.26	1.02	0.86	0.09	0.25
TIMP3	TIMP metallopeptidase inhibitor 3	1.00	0.83	< 0.01	1.04	0.92	0.12	0.53
TRIM56	Tripartite motif containing 56	1.00	0.83	0.02	1.03	0.71	0.08	0.02

[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 to treatment groups *ad libitum*.

[2] Data are expressed as a ratio of MIX relative to ISe, and P-values are associated with statistical significance between treatment groups for each respective test.
* qPCR results are reported in (Crites et al., 2022a), and P-values were obtained from one-way ANOVA using the PROC GLM procedure of SAS statistical software package (version 9.4; SAS Institute, Inc.) at n = 6 per treatment.

Table 7.6. Identification of selected interferon-stimulated genes and activated genes during peri-implantation period from CAR endometrium of heifers supplemented with 3 mg Se/d in vitamin-mineral mixes as ISe (n=6) or MIX (n=6).[1]

Gene	Gene Name	RNA-Seq[2]		
		ISe	MIX	P-value
IDO1	Indoleamine 2,3-dioxygenase 1	1.00	0.68	0.02
IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	1.00	0.83	0.04
IFIT2	Interferon induced protein with tetratricopeptide repeats 2	1.00	0.71	0.01
IFIT3	Interferon induced protein with tetratricopeptide repeats 3	1.00	0.78	0.02
IRF1	Interferon regulatory factor 1	1.00	0.79	0.02
IRF2	Interferon regulatory factor 2	1.00	0.98	0.63
IRF9	Interferon regulatory factor 3	1.00	0.83	< 0.01
ISG15	ISG15 ubiquitin like modifier	1.00	0.78	< 0.01
ISG20	Interferon stimulated exonuclease, transcript variant 1	1.00	0.74	< 0.01
MMP2	Matrix metallopeptidase 2	1.00	0.90	0.25
MMP14	Matrix metallopeptidase 14	1.00	0.85	0.09
MMP16	Matrix metallopeptidase 16	1.00	1.18	0.03
MMP19	Matrix metallopeptidase 19	1.00	0.8	< 0.01
OAS2*	2'-5'-oligoadenylate synthetase 2	1.00	0.81	0.01
RSAD1	Radical S-adenosyl methionine domain containing 1	1.00	0.83	< 0.01
RSAD2*	Radical S-adenosyl methionine domain containing 2	1.00	0.75	< 0.01
SCARA5	Scavenger receptor class A member 5	1.00	0.87	0.09
SCARB1	Scavenger receptor class B member 1	1.00	0.83	0.01
STAT1	Signal transducer and activator of transcription 1	1.00	0.92	0.16

Table 7.6 (Continued)

STAT2	Signal transducer and activator of transcription 2	1.00	0.84	0.02
TGFB1	Transforming growth factor beta 1	1.00	0.87	0.06
TIMP2	TIMP metallopeptidase inhibitor 2	1.00	0.86	0.26
TIMP3	TIMP metallopeptidase inhibitor 3	1.00	0.83	< 0.01
TRIM56	Tripartite motif containing 56	1.00	0.83	0.02

[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 to treatment groups *ad libitum*.
[2] Data are expressed as a ratio of MIX relative to ISe, and P-values are associated with statistical significance between treatment groups for each respective test.
* qPCR results are reported in (Crites et al., 2022a) and P-values were obtained from one-way ANOVA using the PROC GLM procedure of SAS statistical software package (version 9.4; SAS Institute, Inc.) at n = 6 per treatment.

7.5. Discussion

This study sought to augment previous findings in our lab that identified key changes in the abundance of mRNA encoding the P4 and IFNτ-induced DGAT2, OAS2, and MX1 in response to MIX-form Se diet compared to ISe, and these changes occurred synonymously with a longer conceptuses in MIX-supplemented heifers at MRP (Crites et al., 2022a). Respectively, we hypothesized that the maximal expression of IFNτ may be occurring earlier in MIX supplemented heifers, indicating a shift in the timing of MRP. This hypothesis of the shift to an earlier time is supported by the presence of significantly longer conceptuses in MIX-supplemented heifers on d 17 of gestation (Crites et al., 2022a). However, this remains speculative as the effects of form of Se on changes of mRNA transcripts in CAR had not yet been fully defined. Therefore, the objective of this study was to examine transcriptomic changes in the CAR tissue of the endometrium at MRP, testing the hypothesis that there would be changes in the endometrium indicating a shift in the timing of MRP and an advancement in CAR preparation for attachment and implantation of the conceptus. Results from the current transcriptomic analysis correspond with this hypothesis as evident by the significant changes in both interferon signaling which is affecting the transcription of interferon responsive genes, and by the noted changes in transcripts regulating cellular organization, survival, and death.

Prominently, the top two canonical pathways down regulated in MIX were associated with endometrial response to type I interferon signaling ("Role of PKR in interferon induction and antiviral response," and "Interferon signaling") and the trophoblast derived IFNτ is a type I interferon that signals by binding to the ubiquitously expressed type 1 interferon receptors (IFNAR1 and IFNAR2) on the endometrial luminal

and superficial glandular epithelium (Spencer et al., 2008; Walker et al., 2010). The primary function of this signal is to inhibit transcription of the ER, effectively preventing estrogen-induced increases in the synthesis of ER, PGR, and OTR, thus blocking production of luteolytic pulses of $PGF_{2\alpha}$. This mechanism also appears to involve the actions of IRF2 (Spencer and Bazer, 2002). Presently and reported in (Crites et al., 2022a), the presence of a more developed conceptus in MIX- versus ISe-treated heifers demonstrates successful signaling of MRP. However, the absence of Se-form effects on the abundance of mRNA encoding IFNAR1, IFNAR2, IRF2, ER, or OXTR suggests that there are mechanisms still to be elucidated that may be of significance. Additionally, as reported in (Crites et al., 2022a) qPCR analysis revealed a tendency for *Pgr* mRNA to be less abundant in MIX, which is consistent with the previously observed decrease in the expression of *Pgr* in response to continuous exposure of P4 in the endometrium (Spencer et al., 2004, 2008).

In conjunction with being antiluteolytic, IFNτ exerts immense effects in the endometrium during MRP by promoting the transcription of interferon stimulated genes (ISGs, (Bazer et al., 2008; Bazer et al., 2009)). To do so, IFNτ binds IFNAR1 and IFNAR2 to activate the JAK/STAT signal transduction pathway. STAT1 can form a homodimer that translocates into the nucleus to stimulate the transcription of specific genes by binding to interferon-gamma-activated sequence (GAS). Alternatively, STAT1 and STAT2 form a heterodimer and interact with IRF9 to form the transcriptionally active gene complex factor 3. This complex migrates into the nucleus to bind interferon stimulated response elements (ISRE) that facilitate transcription of ISGs (Figure 7.5) (Bazer et al., 2008; Walker et al., 2010; Forde et al., 2011b).

Results of the present transcriptomic analysis revealed significant MIX-form down regulation in the abundance of ISGs, particularly to those that are activated by ISRE. During RNA-sequencing analysis, we observed a significant MIX-induced decrease in *Stat2* and *Irf9* mRNA with no change in mRNA encoding STAT1. This suggests that down regulation of ISG is occurring via the interferon gene complex factor 3 protein complex that translocates into the nucleus to bind ISRE sequences in the promoter regions of ISGs (Walker et al., 2010). Additionally, STAT2 and IRF9 self regulate transciption of their own genes, thus further down regulating the type 1interferon JAK/STAT signaling tranduction pathway (Mesev et al., 2019).

Of the classical ISGs that are transcribed following activation of ISRE, mRNA transcripts for IFIT2, IFIT3, IRF9, ISG15, OAS2, and STAT2 were significantly down regulated in MIX-supplemented heifers (Figure 7.5). We further detected significant decreases in mRNAs encoding the interferon-stimulated genes RSAD2 and ISG20, and we have previously reported a MIX-Se down regulation of *Mx1* mRNA at this time (Crites et al., 2022a).

Results support the present hypothesis that the peak signal of IFNτ from the developing conceptus occurred earlier in MIX heifers as there was a stark decrease in the abundance of mRNA transcripts encoding IRF9. Notably, *Irf9* mRNA is typically more concentrated between days 14-18 compared to being significantly lower on days 25-40 of pregnancy in cattle, coincident with decreases in mRNA encoding IRF3, MX1α, MX1β, and MX2 in CAR at these two time points (Shirozu et al., 2016). Unfortunately, recovery of the uterine fluid at retrieval of these samples proved too inconsistent for subsequent quantification of concentrations of IFNτ protein in this experiment.

Additionally, for successful implantation to occur, there must be restructuring of the endometrium and evasion of the maternal immune system by the developing conceptus (Haeger et al., 2016; Ott, 2019). Functional analysis of the significantly affected mRNA transcripts in the present study have identified significant changes in the spliceosomal cycle, cell death and survival, cellular assembly and organization, and autophagy, suggesting significant structural and functional changes are occurring which indicate that the MIX-CAR is further developed and peak INFτ had occurred earlier in the establishment of pregnancy. The canonical pathway most predicted to be upregulated in MIX is the spliceosomal cycle. Most genes are transcribed as pre-mRNAs containing introns that must be spliced out to form mRNA for translation (Berget et al., 1977; Chow et al., 1977; Matera and Wang, 2014). Splicing of the pre-mRNA is facilitated by the enzymatic activity of the spliceosome, consisting of five ribonucleoprotein subunits (snRNPs) and various protein co-factors (Lerner et al., 1980; Will and Lührmann, 2011). This cycle is dependent on ATP (Will and Lührmann, 2011) and heavily regulated to ensure accurate splicing (Matera and Wang, 2014). Although the spliceosome cycle is significantly affected by form-of supplemental Se, the physiological relevance is unclear at this time.

Effective attachment and implantation also requires a transient evasion of the maternal immune system to allow the fetal allograft to closely interact with the maternal interface (Walker et al., 2010). The control of this mechanism appears to be modulated predominantly by the endometrium although it may also be stimulated by IFNτ and other signals from the developing conceptus (von Rango, 2008; Ott, 2019). This immunotolerant environment is the result of a careful balance between compositional

changes in the innate immune system consisting of granulocytes, monocytes, and dendritic cells, which may remain constitutively responsive to protect against pathogens (Walker et al., 2010), and the adaptive immune system which must be repressed as to not reject the invading conceptus (Walker et al., 2010; Ott, 2019).

Ligands from the conceptus as well as prostaglandins act on the endometrium that has been primed by P4 to effect changes in both the innate and adaptive immune responses. There is a significant increase in the abundance of the MHC II-expressing molecules, macrophages, and dendritic cells, as well as NK cells that migrate into the uterine epithelial, endothelial, and stomal cells in the presence of elevated levels of P4. Additionally, there is an increased abundance of molecules that promote immune tolerance, indoleamine 2,3-dioxygenase 1 (IDO1) and subsequently kynurenine, IL10, TGFB, and various cell surface receptors (CTLA4, PD-L1, and LAG3). Furthermore, there is increased expression of several ISG associated with immune tolerance in the uterus and circulating immune cells (Ott, 2019).

Of interest, IDO1 catalyzes the rate limiting step in the catabolism of tryptophan via the kynurenine pathway, although it is critical in immune tolerance and protecting the embryo from rejection (Groebner et al., 2011; Ott, 2019). The relative abundance of this transcript was significantly less in MIX versus ISe CAR samples. When comparing pregnancy to cyclic cows, IDO1 is significantly more abundant on day 17 of gestation (Groebner et al., 2011; Kamat et al., 2016), but then dramatically declines by day 20 (Kamat et al., 2016). This elevated expression of IDO1 is coupled with a decrease in the relative concentration of tryptophan: kynurenine (Groebner et al., 2011), and kynurenine can further affect immune tolerance (Vacca et al 2010). Relatedly, mRNA encoding

ISG20 and SCARA1 were significantly lower and mRNA encoding the transforming growth factor beta 1 (TGFB1) tended to be lower in the MIX supplemented heifers, which is indicative of a decreased antiviral response and an increased tolerance for cell invasion (Walker et al., 2010; Ott, 2019).

In combination, these findings suggest more advanced preparation of the developing conceptus to evade immune rejection by the maternal endometrium. This supports our hypothesis that there is a shift in the timing of peak IFNτ signaling (MRP), and advancement of the conceptus and endometrium to better support impending attachment, implantation, and placentation.

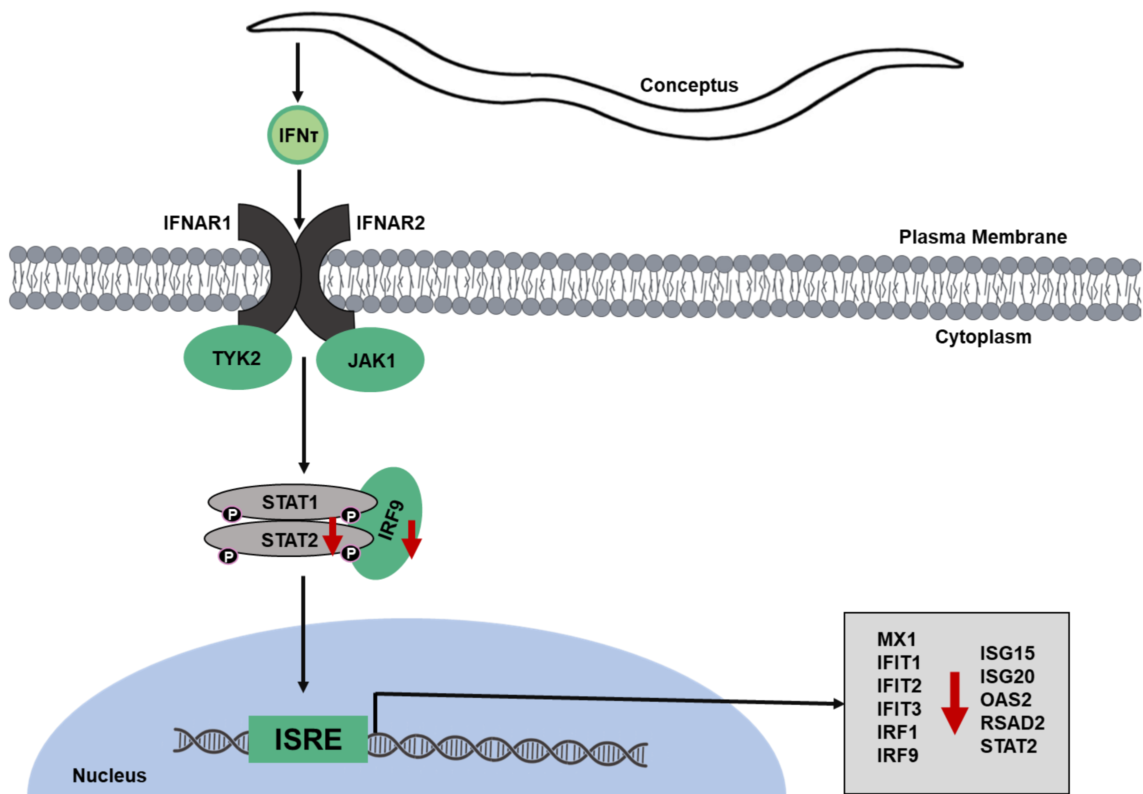

Figure 7.5. Mechanistic illustration of the downregulation of interferon-reponsive genes and signaling pathway. Red arrows demonstrate lower transcript abundance in MIX-Se supplemented heifers.

7.5.1. Conclusion

This research sought to expand upon previous findings in our lab that identified key changes in the abundance of mRNA encoding the P4 and IFNτ-induced DGAT2, OAS2, and MX1 in response to MIX-form Se diet compared to ISe which occurred synonymously with a longer conceptuses in MIX-supplemented heifers at MRP (Crites et al., 2022a). Therefore, the objective of this study was to examine transcriptomic changes in the CAR tissue of the endometrium at MRP, testing the hypothesis that there will be changes in the endometrium indicating a shift in the timing of MRP and an advancement in CAR preparation for implantation of the conceptus. Results from the current transcriptomic analysis correspond with this hypothesis as evident by the significant down regulation of interferon signaling through the JAK/STAT signal transduction pathway and downregulation of mRNA encoding the classical ISGs: IFIT1, IFIT2, IFIT3, IRF1, IRF9, ISG15, OAS2, and RSAD2. This presumed shift in the timing of MRP appears plausible given the additional findings of changes in the cellular organization and function, and immune evasian occurring in MIX supplemented heifers compared to those supplemented with ISe alone.

Chapter 8. Conclusions and implications

Selenium (Se) deficient soils that result in the growth of Se-deficient forages are widespread across the United States (Dargatz and Ross, 1996), and present challenges to grass-fed cattle producers due to the negative effects of low Se intake on immunity (Erskine et al., 1989), growth (Gleed et al., 1983), and fertility (McClure et al., 1986). Conventionally, this essential trace mineral is supplemented to cattle grazing these forages as an inorganic form (ISe, sodium selenite or sodium selenate), although organic (OSe)-forms (selenomethionine and selenocysteine) are available when cattle consume the forage itself (Ammerman and Miller, 1975) and OSe is more bioavailable than ISe (Khanam and Platel, 2016). Functionally, Se is incorporated into selenoproteins, some of which function as important antioxidants.

Additionally, cattle producers are challenged with a high percentage of early embryonic loss. Rate of fertilization to artificial insemination (AI) ranges between 90-100% given high fertility semen is used (Diskin et al., 2006; Diskin and Morris, 2008); however, it is estimated that only 50-60% of inseminated cows remain pregnant by day 30 of gestation (Bridges et al., 2013). This large proportion of early embryonic mortality primarily occurs between days 8 and 16 of gestation (Sreenan and Diskin, 1986).

Successful establishment and maintenance of pregnancy is dependent on progesterone (P4). During the early luteal phase, a relatively lower concentration of P4 can lead to lower conception rates (Mann and Lamming, 1999) whereas elevated concentrations of P4 have resulted in more advanced development of the conceptus (Carter et al., 2008) and increased production of IFNτ, the signal for maternal recognition of pregnancy (MRP) in cattle (Mann and Lamming, 2001). IFNτ from the developing

conceptus blocks the luteolytic $PGF_{2\alpha}$ from the endometrium, maintaining the production and secretion of P4 from the corpus luteum (CL). Progesterone stimulates the production and release of histotroph to nourish the developing post-hatched embryo, signals endometrial changes allowing for implantation of the conceptus, and is required for a successful pregnancy (Spencer et al., 2007).

Concerning the aforementioned challenges, we observed that feeding a MIX form of Se containing a 1:1 ratio of ISe:OSe forms, compared to ISe alone, resulted in a significant increase in the peripheral concentration of P4 on day 6 of the estrous cycle, with no concurrent change to the diameter of the CL (Cerny et al., 2016b), leading to the research reported herein which globally investigated the Se-form (ISe or MIX) induced mechanistic changes in the early luteal phase CL, as well as changes in the blood and endometrium during early gestation in cows via four experiments.

In Experiment 1 (Chapter 4) we sought to (1) confirm and expand upon our previous report that the form of Se provided to cows affects early luteal-phase concentrations of systemic P4, (2) determine the effects of the form of Se (ISe or MIX) on concentrations of P4 during gestation, and (3) determine the effects of the form of Se on concentrations of prolactin (PRL) during lactation. Angus-cross cattle were supplemented with either ISe or MIX and serum was collected on days 4, 7 and 10 of the early luteal phase, bimonthly during gestation, and monthly during lactation. We observed a MIX-induced increase ($p = 0.006$) in systemic concentrations of P4 on day 7 but not on days 4 or 10 of the estrous cycle, as well as MIX-induced increase ($p = 0.02$) in the systemic concentration of P4 at months 1, 3, 5, and 7 of gestation. In addition, we observed a MIX-induced decrease ($p < 0.05$) in systemic concentrations of PRL at

months 5 and 6 of lactation. The form of Se can be manipulated to affect circulating concentrations of early luteal phase and gestational concentrations of P4, and postpartum concentrations of PRL. These results confirm the previous results that MIX-supplementation increases early luteal phase P4, which is occurring concurrently with a time in which concentration of P4 can significantly affect pregnancy outcomes.

Subsequently, we sought to investigate the mechanistic changes in the CL that occur in response to form of dietary Se, and specifically why MIX versus ISe results in an elevated systemic concentration of P4 during the early luteal phase. Experiment 2 (Chapter 5) investigated the effect of form of Se on (1) the relative abundance of mRNA transcripts that encode selenoproteins and targeted steroidogenic enzymes in the CL, and (2) the ability of dissociated luteal cells to synthesize P4 and respond to key agonists *in vitro*. Nongravid Angus-cross cows were supplemented with ISe or MIX and the CL were retrieved on Day 7 post-estrus. Half of each CL was used for analysis of targeted mRNA transcripts and the remainder was dissociated for culture with select agonists. The expression of three selenoprotein transcripts and one selenoprotein P receptor was increased ($P < 0.05$), with an additional five transcripts tending to be increased ($P < 0.10$), in cows supplemented with MIX versus ISe. In cultures of luteal cells, hCG-induced increases in P4 ($P < 0.05$) were observed in CL obtained from ISe-supplemented cows. The abundance of steroidogenic transcripts in the CL was not affected by the form of Se; however, the abundance of mRNA encoding 2 key transcripts regulating cholesterol uptake (*Ldlr* and *Hsl*) was increased ($P < 0.05$) in MIX-supplemented cows. These results indicate the effects of form of Se on luteal production of P4 may to be the

result of changes in cholesterol availability rather than an upregulation of steroidogenesis in the CL.

In an additional experiment that is not reported herein, transcriptomic analysis of the CL confirmed that the MIX-induced increase in systemic production of P4 was at least partially due to an increase in cholesterol transport into the cells via LDLR, cleavage from lipid droplets via HSL, and upregulation of the *de novo* synthesis pathway in the early luteal CL (Crites et al., 2022b), which may result in changes in the early gestational endometrium and developing conceptus. We then expanded our research to investigate the effects of form of Se (ISe or MIX) on mRNA transcripts encoding IFNτ- and P4-induced proteins in the caruncular (CAR) and intercaruncular (ICAR) tissues of the uterine endometrium at maternal recognition of pregnancy (MRP) (Crites et al., 2022a). MIX-supplemented heifers had an increased abundance of mRNA encoding myostatin (MSTN) in ICAR, and a decrease in the relative abundance of mRNA encoding diacylglycerol o-acyltransferase 2 (DGAT2) in CAR which may affect the availability of glucose and triglycerides to the developing conceptus, and this occurred concurrently with significantly longer conceptuses at MRP in MIX-Se supplemented heifers (Crites et al., 2022a). Given these findings, we hypothesized that there may be changes in metabolic serological parameters, particularly cholesterol and lipoproteins, that are affecting the CL on d 7 as well on d 17 of gestation at maternal recognition of pregnancy (MRP).

Therefore, Experiment 3 (Chapter 6) aimed to investigate Se-form-specific changes in serum metabolic parameters at estrus, during the early luteal phase, and at MRP, with special consideration given for effects on systemic concentrations of

lipoproteins, glucose, cholesterol, and triglycerides. Commercial Angus-cross heifers (ISe vs MIX-supplementation) were estrous synchronized, artificially inseminated, and killed at MRP (d 17). Reproductive tracts were collected and only heifers with an intact conceptus were used for analysis. From each heifer, we collected whole blood and serum during experimentation for quantification of lipoproteins and metabolites. Heifers assigned to MIX versus ISe had a significantly lower concentration of total serum cholesterol ($P = 0.01$), and saliently, a decrease in both total and free LDL/VLDL protein fractions on d 7 ($P < 0.05$) and d 17 ($P < 0.05$) of gestation. Additionally, the form of Se significantly affected serum glucose ($P = 0.03$), aspartate aminotransferase (AST, $P < 0.01$), and beta hydroxybutyrate (BHBA, $P = 0.04$), with no effect observed on serum triglycerides ($P > 0.05$). These results indicate that Se-form may be altering the serum metabolic and lipid profiles of the heifer, with changes to whole animal physiology affecting fertility.

Our initial studies in the endometrium further suggested that there may be a form of Se-induced shift in the timing of MRP, with peak INFτ secretion occurring earlier in MIX compared to ISe supplemented heifers (Crites et al., 2022a). We further investigated the effects of the form of Se at MRP in heifers, and Experiment 4 (Chapter 7) was conducted to determine form of Se-induced transcriptomic changes in the CAR tissue of the endometrium at MRP. Angus-cross heifers were supplemented with ISe or MIX, as described earlier. Following estrous synchronization and artificial insemination, heifers were killed at MRP (d 17) and reproductive tracts were collected. Only heifers with a fully intact recovered conceptus were utilized for analyses. Transcriptomic analysis using RNA-sequencing was conducted using total mRNA from CAR samples. There was a

total of 2029 DEGs with a total of 1038 transcripts upregulated, and 991 transcripts downregulated in CAR samples from MIX versus ISe-heifers. Saliently, the interferon JAK/STAT pathway singing through the STAT1/2 heterodimer was significantly down regulated as were the following interferon-responsive genes IFIT1, IFIT2, IFIT3, IRF1, IRF9, ISG15, ISG20, OAS2, RSAD2, and STAT2. Considering the present results and our previous finding of significantly longer conceptus on d 17 of pregnancy, it appears that MIX-supplemented heifers are indeed experiencing earlier timing of peak INFτ signaling and MRP as indicated by the relatedly lower concentration of mRNA encoding the JAK/STAT signaling pathway and INFτ stimulated genes.

Globally, this dissertational research describes mechanistic interactions of dietary forms of Se (ISe or MIX) on the CL, in blood, and in the endometrium. Particularly, it first validated previous findings that MIX-Se supplementation increased early luteal phase P4 and then determined that this increase is not a direct result of increased expression of steroidogenic enzymes. Results from the research herein and corroborated via transcriptomic analysis (Crites et al., 2022b) indicate that this early luteal phase increase in P4 is at least partially due to increase in transport into the cells via LDLR, cleavage from lipid droplets via HSL, and upregulation of the *de novo* synthesis pathway in the early luteal CL. Translationally, the MIX-form of Se significantly affected serum metabolites at estrus, at d 7 of the estrus cycle, and at d 17 of pregnancy (MRP), with concurrent decreases in INFτ second messenger signaling and the expression of INFτ-stimulated genes observed in CAR samples from MIX heifers at MRP. When considered with previous results reporting a longer conceptus in MIX-Se heifers at this same time

point (Crites et al., 2022a), it appears that MIX heifers may indeed be experiencing peak INFτ signaling earlier in the establishment of their pregnancies.

Impactfully, MIX-form Se as a producer-friendly dietary supplement appears to increase fertility in cattle, stimulating early luteal phase increases in P4, altering the expression of endometrial transcripts at MRP, and increasing the length of the pre-implantation conceptus. The maternal fetal interaction that results in longer conceptuses at MRP remains to be fully understood, and potential Se-form induced changes at attachment and during placental development remain to be defined.

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Master of Science, Animal Physiology, West Virginia University, Davis College, Morgantown, WV. 2015-2018.

Bachelor of Science, Agriculture and Natural Resources, Berea College, Department of Agriculture and Natural Resources, Berea, Ky. 2011-2015.

Professional Experience

Graduate Research Assistant, Supervisor Dr. Phillip Bridges, University of Kentucky, Department of Animal and Food Sciences, Lexington, Ky. 2018-2023.

Visiting Lecturer, Supervisor Dr. Sarah Hall, Berea College, Department of Agriculture and Natural Resources, Berea, Ky. 2020.

Graduate Research Assistant, Supervisor Dr. Marlon Knights, West Virginia University, Department of Animal and Nutritional Sciences, Morgantown, WV. 2017-2018.

Graduate Teaching Assistant, Supervisor Dr. Marlon Knights, West Virginia University, Department of Animal and Nutritional Sciences, Morgantown, WV. 2015-2017.

Teaching Assistant/ Laboratory Assistant, Supervisor Dr. Quinn Baptiste, Berea College, Department of Agriculture and Natural Resources, Berea, Ky. 2014-2015.

Publications

Refereed Journal Articles

Crites BR, Carr SN, Anderson LH, Matthew JC, and Bridges PJ. 2022. 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. Journal of Animal Science. 100:1-15. DOI: 10.1093/jas/skac137

Crites BR, Carr SN, Matthews JC, and Bridges PJ. 2022. Form of dietary selenium affects mRNA encoding cholesterol biosynthesis and immune response elements in the early luteal phase bovine corpus luteum. Journal of Animal Science. 100:1-15. DOI: 10.1093/jas/skac135

Carr SN, Crites BR, Pate JL, Hughes CHK, Matthews JC, Bridges PJ. (2022) Form of supplemental selenium affects the expression of mRNA transcripts encoding selenoproteins, and proteins regulating cholesterol uptake, in the corpus luteum of grazing beef cows. Animals. 12:313. DOI: 10.3390/ani12030313

Carr SN, Jia Y, Crites BR, Hamilton CH, Burris WR, Edwards JL, Matthews JC, Bridges PJ. 2020. Form of supplemental selenium in vitamin-mineral premixes differentially affects early luteal and gestational concentrations of progesterone, and postpartum concentrations of prolactin in beef cows. Animals. 10:967. DOI: 10.3390/ani10060967

Abstracts

Carr SN, Crites BR, and Bridges PJ. (2023). Effects of form of selenium on serum metabolites and iodothyronine deiodinases during early gestation in beef heifers (Abstract). SSR Annual Meeting, Ottawa, Ontario, Canada, July 11-14, 2023.

Carr SN, Crites BR, Jia Y, Burris WR, Hamilton CH, Matthews, JC, and Bridges PJ. (2020). Form of supplemental selenium affects steroidogenesis by the early luteal phase corpus luteum in beef cattle (Abstract 2009). SSR Annual Meeting, e-poster, July 8-12, 2020.

Crites BR, Carr SN**,** Hamilton CH, Matthews JC, Burris WR, and Bridges PJ. (2020). Form of dietary selenium affects mRNA encoding cholesterol biosynthesis and immune response elements in the early luteal phase bovine corpus luteum (Abstract 2120). SSR Annual Meeting, e-poster, July 8-12, 2020.

Carr SN, Redhead AK, Nabers AN, Paul CD, Powell KJ, Adebiyi AK, and Knights M (2017). Effects of Pre-Breeding Nutritional Management, Weight Changes, and Age on Ewe Lamb Fertility (Abstract 670). ASAS-CSAS Annual Meeting and Trade Show, Baltimore, MD, USA, July8-12, 2017.

Carr SN and Baptiste QS (2015). Gross morphology, morphometric characteristics, and sequential changes in digesta fiber fractions of gastrointestinal tract segments from high postpartum piglet mortality extensively reared swine (Abstract 444). ASAS Midwest Section and ADSA Midwest Branch, Des Moines, IA, USA, March 16-18, 2015. Journal of Animal Science. 93(Suppl. 1): 199.

Professional Honors

Postdoctoral Fellowship ($225,000), Agriculture and Food Research Initiative (AFRI), National Institute of Food and Agriculture (NIFA), 2023.

Emeriti Fellowship, University of Kentucky, 2022

Richards Graduate Student Research Award, University of Kentucky, 2019-2022.

Graduate Student Congress Research Award, University of Kentucky, 2020.

Young Innovators Scholar, West Virginia University, 2017-2018.

Oliver Ruth Russel Fellowship, Berea College, 2015-2018.

Gilman International Scholar, Benjamin A. Gilman International Scholar Program, 2014.

International Scholar, American Institute for Foreign Study, 2014.