

## REPRODUCTION

# Evaluation of the molecular response of corpora lutea to manganese–amino acid complex supplementation in gilts

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## Abstract

Porcine pregnancy establishment and maintenance are dependent on the formation of functional corpora lutea (CL). Manganese (Mn) is critical for CL function as it is a cofactor for Mn superoxide dismutase and enzymes involved in cholesterol synthesis. Previously, we have shown that luteal Mn content increased and luteal progesterone ( $P_4$ ) concentration decreased in the CL of gilts fed diets supplemented with an Mn–amino acid complex (Availa-Mn; Zinpro Corporation) compared with controls fed Mn sulfate. Importantly, serum  $P_4$  increased from 0 (estrus onset) to 12 d post estrus (dpe), as expected, but  $P_4$  abundance in circulation was not affected by dietary Mn source ( $P = 0.15$ ). We hypothesized that a more bioavailable Mn source (which results in increased luteal Mn content) would alter the luteal proteome and abundance of mRNA associated with steroid biogenesis during the mid-luteal phase of the estrous cycle. Postpubertal gilts ( $n = 32$ ) were assigned to one of the four gestation diets. The control diet (CON) contained 20 ppm of supplemental Mn in the form of Mn sulfate. Three additional diets included 20 (TRT1), 40 (TRT2), or 60 (TRT3) ppm of supplemental Mn in the form of a Mn–amino acid complex instead of Mn sulfate. Dietary treatment began at estrus synchronization (approximately 20 d before estrus) and continued through 12 dpe when gilts were euthanized and tissues were collected. Protein and total RNA extracts from the CL were used for proteomic analysis via label-free liquid chromatography with tandem mass spectrometry to assess global protein abundance and quantitative real-time polymerase chain reaction (qRT-PCR) to assess specific mRNA abundance, respectively. Compared with CON, 188, 382, and 401 proteins were differentially abundant ( $P < 0.10$ ) in TRT1, TRT2, and TRT3, respectively. Gene Ontology enrichment software revealed that proteins involved in  $P_4$  signaling and cholesterol synthesis were downregulated in CL of gilts fed Mn–amino acid complex compared with controls. Quantitative RT-PCR showed that relative transcript abundance of genes encoding steroidogenic enzymes (CYP11A1 and StAR) in CL tissue was decreased in gilts from TRT2 compared with CON ( $P = 0.02$ ), but TRT1 and TRT3 were not affected ( $P \geq 0.30$ ). Collectively, these data support our hypothesis that a more bioavailable dietary Mn source may influence luteal function by altering the abundance of protein and mRNA involved in steroidogenesis.

**Key words:** cholesterol, corpus luteum, pig, progesterone, proteome, reproduction

## Abbreviations

BCA	bicinchoninic acid
CL	corpus luteum
C <sub>T</sub>	cycle threshold
dpe	days post estrus
FC	fold change
FDR	false discovery rate
glog	generalized logarithm
GO	gene ontology
LC-MS/MS	liquid-chromatography with tandem mass spectrometry
LDL	low density lipoprotein
LDLR	low-density lipoprotein receptor
NRC	National Research Council
P <sub>4</sub>	progesterone
PCR	polymerase chain reaction
PRTC	Peptide Retention Time Calibration
qRT-PCR	quantitative real-time polymerase chain reaction
ROS	reactive oxygen species

## Introduction

The corpus luteum (CL) is a temporary endocrine structure formed on the ovary from the ruptured follicle following ovulation. It is essential to the establishment and maintenance of pregnancy, required for implantation, placentation, and fetal-placental growth and development (Meidan, 2017). The CL is an extremely active gland that produces large amounts of progesterone (P<sub>4</sub>) and efficiently releases it into systemic circulation due to a dense capillary network (Wiltbank, 1994; Meidan, 2017). The intense vascularization of the CL allows each luteal cell to be in contact with several capillaries, giving the CL one of the highest rates of blood flow in the entire organism (Stocco et al., 2007). Regulation of luteal steroidogenesis can be categorized into three major events: luteinization, luteal regression, and pregnancy-induced rescue/maintenance (Christenson and Devoto, 2003).

The essential element manganese (Mn) is a redox-active metal that is utilized by almost all forms of life and is required for enzyme activation in nearly every cellular compartment (Culotta et al., 2005). Since Mn can be toxic, Mn transport to its target requires homeostatic factors to guide Mn through a designated trafficking pathway, leading to enzyme activation (Culotta et al., 2005). Manganese is also involved in skeletal development, energy metabolism, nervous system function, immune function, and reproductive hormone activity (Santamaria, 2008). It is also a cofactor for Mn superoxide dismutase (MnSOD) and enzymes involved in cholesterol synthesis such as mevalonate kinase (MVK) and farnesyl pyrophosphate synthase (Curran, 1954; Benedict et al., 1965; Goering, 2003). The mitochondrial antioxidant enzyme MnSOD is the first to act on superoxide anions generated by the electron transport chain by catalyzing the dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen (Fridavich, 1995; Holley et al., 2011; Kamiński et al., 2012). Induced Mn deficiency can cause a variety of detrimental effects, including impaired growth, poor bone formation, skeletal defects, reduced fertility, birth defects, and altered lipid and carbohydrate metabolism (Keen et al., 1999; Aschner and Aschner, 2005; Li and Yang, 2018).

Reactive oxygen species (ROS) and antioxidant enzymes are important factors involved in ovarian physiological metabolism. ROS are involved in follicular growth, oocyte maturation, and steroid hormone synthesis (Fujii et al., 2005; Wang et al., 2017). Preovulatory follicles are formed under the influence of follicle-stimulating hormone and once developed, ovulation

occurs following a surge of luteinizing hormone (Gougeon, 2010; Soede et al., 2011). Ovulation is an inflammatory process and ROS function as critical modulators during the catabolic reactions involved in follicular rupture. As such, inhibition of ROS can hinder ovulation (Shkolnik et al., 2011; Rizzo et al., 2012). During the mid-luteal phase of the estrous cycle, CL P<sub>4</sub> synthesis is maximized (Guthrie et al., 1972), resulting in ROS production, and overaccumulation of ROS may restrict P<sub>4</sub> synthesis (Carlson et al., 1995; Wang et al., 2017). A balance between antioxidant enzymes and ROS is necessary to protect oocytes from oxidative stress while also ensuring some ROS remain to aid in ovulation (Wang et al., 2017). After ovulation, SODs are very active in the CL. As an example, copper/zinc SOD (Cu/ZnSOD) activity is correlated with P<sub>4</sub> fluctuation in the luteal phase, whereas MnSOD activity is enhanced during CL regression to protect luteal cells from oxidative damage caused by inflammation (Behrman et al., 2001). Thus, Mn clearly contributes to biological processes necessary for proper CL function.

Previously, we have demonstrated that Mn–amino acid complex supplementation increased luteal Mn content and decreased calcium concentration compared with CL of gilts fed Mn sulfate (Studer et al., 2021). Interestingly, CL P<sub>4</sub> concentration was also altered, although P<sub>4</sub> in circulation was not different between gilts fed the more bioavailable Mn source compared with gilts fed Mn sulfate (Studer et al., 2021). Given that the CL is an incredibly essential and highly efficient structure, the objective of this study was to evaluate if luteal function could be influenced by trace mineral supplementation. We hypothesized that dietary fortification with a more bioavailable form of Mn (Availa-Mn; Zinpro Corporation) during the mid-luteal phase of the estrous cycle in gilts would alter the luteal proteome and transcript abundance of genes associated with steroid biogenesis. Understanding how trace mineral concentration and biological availability may affect reproductive tissues is essential to maximizing a sow's reproductive potential.

## Materials and Methods

### Animals and experimental design

All animal procedures were reviewed and approved by the Iowa State University (ISU) Institutional Animal Care and Use Committee. Thirty-two postpubertal crossbred gilts were individually housed at the ISU Swine Nutrition Farm and acclimated for a minimum of 3 d in their pens with individual access to feed and water in thermal neutral conditions (18 ± 0.5 °C).

Gilts were allocated into dietary treatments by body weight (154 ± 8 kg). Diets were formulated using corn, distillers dried grains with solubles, and soybean meal based on NRC (2012) gilt requirements for less than 90 d gestation, and Mn was included in a vitamin and mineral premix as previously described (Studer et al., 2021). Briefly, the control diet (CON) contained 20 ppm of added Mn in the form of Mn sulfate. Three additional diets included 20 (TRT1), 40 (TRT2), or 60 (TRT3) ppm of added Mn from a Mn–amino acid complex instead of Mn sulfate. Diets were mixed at the ISU Swine Nutrition Farm, and samples from each diet were submitted to Midwest Laboratories (Omaha, NE) for proximate and trace mineral analysis and have been previously reported (Studer et al., 2021). Gilts were limit fed 2.7 kg at 0800 hours daily throughout the trial.

Gilt estrous cycles were synchronized using 15.0 mg altrenogest (Matrix; Merck Animal Health) per day delivered

per os for 15 d. Administration of dietary treatments began at the onset of synchronization (approximately 20 d before estrus) and continued throughout the ensuing estrous cycle. Beginning 4 d post-altrenogest withdrawal, animals were observed for behavioral signs of standing estrus twice-daily using direct boar exposure. Characteristics of estrus such as a swollen vulva, reddening of the vulva, and vulva discharge were recorded. Animals were classified as being in estrus when they would stand in response to back pressure, and time of estrus detection was assigned as 0 d post estrus (dpe).

### Sample collection and processing

Gilts were humanely euthanized using captive bolt penetration followed by exsanguination at 12 dpe. Following euthanasia, ovaries were extracted, weighed, and the number of CL on each ovary was counted. CL on one ovary were measured by digital calipers, and the entire ovary was fixed in 4% paraformaldehyde for 24 h and then transferred to 70% ethanol for long-term storage. The remaining ovary had each CL excised, weighed, measured in three dimensions using digital calipers, flash-frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ .

### Protein extraction and proteomic analysis via liquid chromatography with tandem mass spectrometry

Excised CL from each animal were powdered using a mortar and pestle on dry ice. Approximately, 50 mg of powdered CL tissue per animal was weighed out into fresh sterile tubes and tissue lysis buffer (50 mM Tris HCl, 1 mM ethylenediaminetetraacetic acid [EDTA], pH 8.5) was added. Samples were homogenized by sonication and centrifuged at  $16,128 \times g$  at  $4^{\circ}\text{C}$  for 20 min. Supernatant was collected into fresh tubes and stored at  $-80^{\circ}\text{C}$  until further analysis. Protein concentration was determined using a bicinchoninic acid (BCA) assay (Pierce BCA Protein Assay Kit, ThermoFisher Scientific). Protein was diluted to  $1 \mu\text{g}/\mu\text{L}$  in nuclease-free water and submitted to the ISU Protein Facility for proteomic analysis via label-free liquid chromatography with tandem mass spectrometry (LC-MS/MS) to assess global protein abundance.

Crude protein extracts were digested in a solution of trypsin and Lys-C. After digestion, Pierce Peptide Retention Time Calibration (PRTC) Mixture (ThermoFisher Scientific) was added to each sample to serve as an internal control. High-performance liquid chromatography was performed using an EASY nLC-1200 System coupled to a Nanospray Flex Ion Source (ThermoFisher Scientific) using a pulled glass emitter  $75 \mu\text{m} \times 20 \text{ cm}$  (Agilent) packed with SB-C18 5 micron packing material (Agilent) and UChrom 3 micron material (nanoLCMS Solutions). Buffer A contained 0.1% formic acid in water and buffer B contained 0.1% formic acid in acetonitrile. After separation, the peptides were fragmented and analyzed by MS/MS using a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer with an higher-energy collisional dissociation fragmentation cell (ThermoFisher Scientific). The raw peptide data were analyzed in Proteome Discoverer 2.2 (ThermoFisher Scientific) and data were searched in Mascot against the *Sus scrofa* database and Sequest HT against the PRTC database.

Data from the Protein Facility were processed and normalized at the Genome Informatics Facility at ISU for conversion of raw data into biologically informative data. Proteins that were detected with low confidence were excluded from analysis. For each peptide, the signal intensity was divided by the arithmetic mean of the PRTC as the normalization factor before further analysis. MetaboAnalyst 4.0 (Chong et al., 2018)

was used for data analysis. Upon finding data integrity to be satisfactory (no peptide with more than 50% missing replicates and positive values for the area), missing value estimation was imputed using the singular value decomposition method. Non-informative values that were near constant throughout the experimental conditions were detected using the interquartile range estimation method and were deleted. Data transformation was performed based on generalized logarithm transformation (glog) to make individual features more comparable.

### RNA isolation and quantitative one-step real-time-polymerase chain reaction

Powdered CL tissue was homogenized in QIAzol Lysis Reagent by sonication, and total RNA was isolated using the miRNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted using the QuantiTect SYBR Green RT-PCR Kit (Qiagen) and measured on an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). Relative quantification of mRNA abundance was evaluated with the comparative cycle threshold ( $C_t$ ) method as previously described (Ashworth et al., 2010). The  $\Delta C_t$  value was determined by subtracting the target  $C_t$  of each sample from an internal control (GAPDH)  $C_t$  value. Calculation of  $\Delta\Delta C_t$  used the greatest sample  $\Delta C_t$  (the sample with the lowest expression) as an arbitrary constant to subtract from all other sample  $\Delta C_t$  values. Relative differences for each sample were calculated by applying the equation  $2^{-\Delta\Delta C_t}$  relying on the assumption that a single cycle difference during the exponential phase of amplification is equivalent to a 2-fold difference in abundance. Statistical analysis was performed using the normalized  $\Delta C_t$  values. Negative controls were included for each gene of interest to confirm the presence of the specific gene product desired, by excluding template or reverse transcriptase enzyme in the reaction. Furthermore, a melting curve analysis was conducted for each assay to confirm the production of a single product. Results are reported as relative transcript abundance normalized to GAPDH as an internal control. Primer sequences used for gene amplification are reported in Table 1.

### Statistical analysis

Effects of treatment (fixed effects) on gene expression were assessed as a completely randomized design in SAS 9.4 (Cary, NC) using a PROC MIXED analysis procedure. Gene expression data are represented as relative abundance and considered significant if  $P \leq 0.05$  or indicating a tendency if  $0.05 < P < 0.10$ . Comparisons between individual treatments are represented as differences of least squares means using the probability of differences function.

For protein data, the control and treatment samples were compared by t-test with the adjusted  $P$ -value false discovery rate (FDR) cutoff of 0.10. Fold change (FC) analysis with threshold of 2 was performed to compare the absolute value of change between control and treatment values. The threshold for inclusion of proteins in the pathway analysis was  $P < 0.10$  to allow for a comprehensive evaluation.

## Results

### Dietary Mn source alters the luteal proteome during diestrus

Proteomic analysis of luteal protein extracts via label-free LC/MS-MS identified an average of 1,771 proteins. Duplicate proteins and proteins with low mascot scores were removed



**Table 1.** Primer sequences used for quantitative real-time polymerase chain reaction

Gene <sup>1</sup>	Forward primer sequence, 5'–3'	Reverse primer sequence, 5'–3'	Annealing temperature, °C
GAPDH	TGGTGAAGGTCGGAGTGAAC	GAAGGGGTCATTGATGGCGA	57.0
SOD1	ATCAAGAGAGGCACGTTGGA	TCTGCCCAAGTCATCTGGTT	57.0
SOD2	TCAAGGAGAAGTTGACCGCT	AGGTAATACGCATGCTCCCA	57.0
BCL2	TTCTTTGAGTTCGGTGGGG	CCAGGAGAAATCAAATAGAGGC	57.0
FOXO3	AGCTGTGCACATCAGTCCAG	AGTGGCATTGGCAATGAGT	57.0
CYP11A1	TTCCAGAAGTATGGTCCCATTTA	TGAGCATGGGGACACTAGTGTGG	57.0
StAR	TTGGAAGAGACGGATGGAAG	CCCACATTCCTGCTATTGCT	51.8

<sup>1</sup>Glyceraldehyde 3-phosphate dehydrogenase, copper/zinc superoxide dismutase, manganese superoxide dismutase, B-cell lymphoma 2, forkhead box O3, cholesterol side-chain cleavage enzyme, and steroidogenic acute regulatory protein.

prior to analysis to identify differentially abundant proteins. In luteal protein extracts from gilts in TRT1, 188 proteins were identified that were differentially ( $P < 0.10$ ) abundant compared with luteal protein extracts from CON gilts. Of these differentially abundant proteins, 94 were increased and the remaining 94 were decreased compared with CL from CON gilts. In luteal protein extracts from gilts in TRT2, 382 proteins were identified to be differentially ( $P < 0.10$ ) abundant compared with luteal protein extracts from CON gilts, of which 182 were more abundant, while 200 were less abundant in TRT2 compared with CON. Similarly, 401 proteins were identified having altered abundance ( $P < 0.10$ ) in luteal protein extracts from gilts fed TRT3 compared with CON, of which 176 were increased and 225 were decreased in CL from TRT3 compared with CON. In total, and across all treatments compared with CON, 567 unique proteins were differentially abundant, of which 109 proteins were observed between each TRT1, TRT2, and TRT3 in comparison to CON (Figure 1). FC and P-value information for proteins in TRT1, TRT2, and TRT3 in comparison to CON is listed in Supplementary Table 1, and proteins altered due to source of Mn are represented by the comparison between CON (20 ppm Mn sulfate) and TRT1 (20 ppm Mn–amino acid complex). Proteins altered in TRT2 (40 ppm Mn–amino acid complex) and TRT3 (60 ppm Mn–amino acid complex) compared with CON (20 ppm Mn sulfate) cannot be explained as an effect of source or concentration of Mn since both source and concentration differ between CON and the treatment diets. Proteins altered due to concentration of Mn–amino acid complex (TRT2 and TRT3 compared with TRT1) are listed in Supplementary Table 2. Additionally, the 100 most abundant proteins in CL from CON gilts are listed in Supplementary Table 3.

Subsequently, Gene Ontology (GO) enrichment software was utilized to identify biological processes involving these proteins using an overrepresentation test (Ashburner et al., 2000; Gene Ontology Consortium, 2021). The cholesterol biosynthesis and steroid metabolism pathways were enriched in GO ( $P < 0.01$ ; FDR  $< 0.01$ ), as proteins involved in these pathways were differentially abundant in CL of gilts supplemented with Mn–amino acid complex compared with controls. The differentially abundant proteins represented in these pathways include low-density lipoprotein receptor (LDLR), squalene monooxygenase (SQLE), delta(24)-sterol reductase (DHCR24), neutral cholesterol ester hydrolase 1 isoform b (NCEH1), diphosphomevalonate decarboxylase (MVD), 7-dehydrocholesterol reductase (DHCR7), MVK, 3-hydroxy-3-methylglutaryl CoA reductase (HMGCR), 3-hydroxy-3-methylglutaryl CoA synthase (HMGCS1), sterol-4-alpha-carboxylate 3-dehydrogenase, decarboxylating (NSDHL), glucosylceramidase (GBA), ovarian and testicular apolipoprotein N (APON), EH domain-containing protein 1 (EHD1), LIM domain and actin-binding protein 1 (LIMA1), and scavenger receptor class

B member 1 (SCARB1). A summary of the cholesterol biosynthesis pathway and proteins affected by Mn–amino acid complex supplementation is shown in Figure 2. Additionally, membrane-associated P<sub>4</sub> receptor component 2 (PGRMC2), which is involved in P<sub>4</sub> signaling, was downregulated in Mn–amino acid complex supplemented treatments compared with CON. Proteins involved in cholesterol and steroid metabolism affected by Mn–amino acid complex supplementation are summarized in Table 2.

Multiple proteins involved in the oxidative stress response were also differentially abundant ( $P < 0.10$ ) in CL from Mn–amino acid complex supplemented gilts compared with CON including peroxiredoxin-6 (PRDX6), glutathione synthetase (GSS), lactoylglutathione lyase (GLO1), and mitochondrial aldehyde dehydrogenase 2 (ALDH2) along with other additional proteins involved in oxidative stress (Table 3). Proteins involved in angiogenesis (Table 4) were also differentially abundant compared with CON including platelet-derived growth factor receptor beta (PDGFRB) and vascular endothelial growth factor D (VEGFD).

Several proteins that utilize Mn as a cofactor (Table 5) were differentially abundant in CL of Mn–amino acid complex-supplemented gilts compared with CON ( $P < 0.10$ ). These enzymes include malic enzyme (ME) 2 (ME2), ME3, serine/threonine protein phosphatase (PPP1CA), acetyl-CoA carboxylase 1 (ACACA), GLO1, and hydroxymethylglutaryl-CoA lyase (HMGCL).

### Effect of Mn source and concentration on luteal gene expression

No effect of treatment was observed on relative transcript abundance of Cu/ZnSOD, MnSOD, or BCL2 ( $P > 0.30$ ) in total RNA from CL tissue. Relative abundance of forkhead box O3 (FOXO3) mRNA was affected ( $P = 0.06$ ) by dietary treatment (Figure 3), with increases observed in TRT1 compared with TRT2 ( $P = 0.01$ ) and TRT3 ( $P = 0.04$ ). Relative abundance of StAR mRNA was affected ( $P = 0.07$ ) by dietary treatment (Figure 4), with decreases observed in TRT2 compared with CON ( $P = 0.03$ ), TRT1 ( $P = 0.02$ ), and TRT3 ( $P = 0.09$ ). Relative abundance of cholesterol side chain cleavage enzyme (CYP11A1) mRNA did not demonstrate an overall treatment effect ( $P = 0.11$ ; Figure 5); however, a decrease in TRT2 was observed when directly compared with CON ( $P = 0.02$ ).

### Discussion

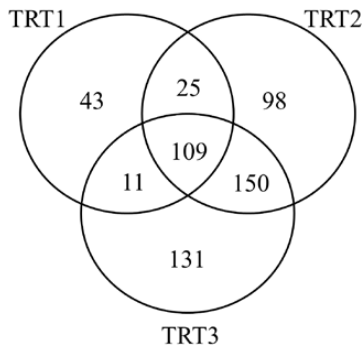
Manganese is an essential trace element required for a variety of metabolic processes including enzyme activation and reproductive hormone function (Santamaria, 2008). It is a cofactor for a wide range of metalloenzymes including proteins involved in cholesterol synthesis and the antioxidant enzyme MnSOD (Curran, 1954; Benedict et al., 1965; Goering, 2003). Cholesterol is required for P<sub>4</sub> synthesis, which is primarily produced by the CL. The CL is a highly efficient structure that produces large

amounts of  $P_4$  with peak secretion in swine occurring during the mid-luteal phase (approximately 12 dpe) of the estrous cycle (Stabenfeldt et al., 1969; Henricks et al., 1972; Anderson, 2009). Although Mn contributes to biological processes necessary for CL function, little information on the role of Mn in CL function exists. The purpose of this study was to understand if the luteal proteome is influenced by supplementation of trace minerals with different bioavailability. We hypothesized that dietary supplementation with Mn–amino acid complex would alter the luteal proteome and transcript abundance of genes associated with steroid biogenesis during the mid-luteal phase of the estrous cycle in gilts.

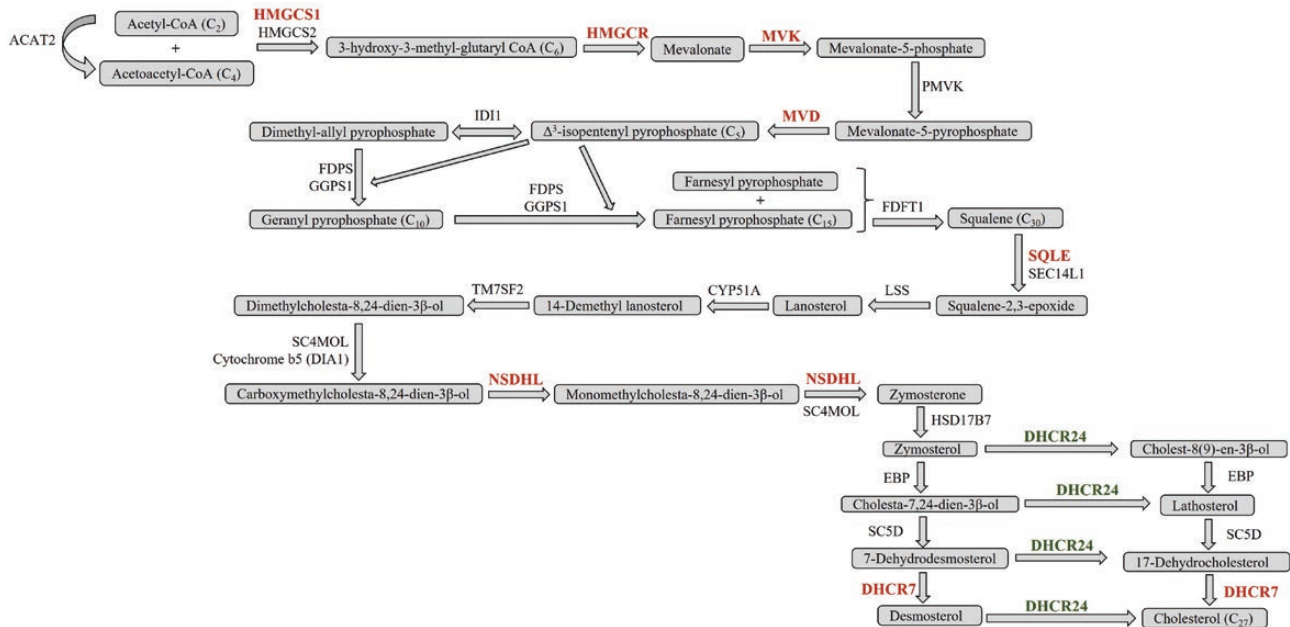
Previously, we demonstrated that dietary supplementation with Mn–amino acid complex increased luteal Mn concentration and decreased luteal  $P_4$  concentration at 12 dpe in gilts although

serum  $P_4$  concentration was not affected (Studer et al., 2021). Given that luteal  $P_4$  concentrations were decreased, it makes biological sense that proteins involved in  $P_4$  signaling as well as cholesterol synthesis and transport would also be downregulated in the CL. This postulation raises the question if the CL is capable of regulating serum  $P_4$  homeostasis by varying the levels of intracellular  $P_4$  production and storage, although the answer remains to be determined.

During the luteal phase,  $P_4$  is produced by both small and large luteal cells and synthesis begins with cholesterol transport into the inner mitochondrial matrix by STAR (Stocco and Clark, 1996). Cholesterol for luteal steroidogenesis can be obtained from multiple sources: de novo synthesis from acetate, circulating lipoproteins, and intracellular stores such as cholesteryl esters deposited in lipid droplets and cholesterol within the plasma membrane (Gwynne and Strauss, 1982). The major source of cholesterol for luteal cells is circulating high-density lipoproteins (HDL) and low-density lipoproteins (LDL; Grummer and Carroll, 1988; Wiltbank et al., 1990). LDLR is a main player in cholesterol uptake from circulation and was downregulated in CL from Mn–amino acid complex-supplemented gilts compared with CON (Luo et al., 2020). De novo cholesterol synthesis by luteal cells is uncommon since HMGCR (the rate-limiting enzyme of cholesterol biosynthesis) and other key cholesterol biosynthesis enzymes are scarce in luteal cells (Gwynne and Strauss, 1982; Christenson and Devoto, 2003). Interestingly, multiple proteins involved in the cholesterol biosynthesis pathway, including HMGCR and SQLE, were decreased in abundance in luteal tissue from Mn–amino acid complex-supplemented gilts compared with CL from CON gilts which would result in decreased cholesterol synthesis. This may suggest that luteal cells of CON gilts were utilizing de novo synthesis to obtain cholesterol at a higher rate than luteal cells from Mn–amino acid complex-supplemented gilts, which may also explain the reason CON gilts had greater concentrations of luteal  $P_4$ .



**Figure 1.** Venn diagram illustrating the overlap of differentially abundant proteins ( $P < 0.10$ ) in CL of gilts fed 20 (TRT1), 40 (TRT2), or 60 (TRT3) ppm Mn–amino acid complex compared with CL of gilts fed 20 ppm Mn sulfate (CON). Of the 109 differentially abundant proteins shared between Mn–amino acid complex treatments, 54 were more abundant in Mn–amino acid complex treatments, while 55 were more abundant in CON.



**Figure 2.** Proteins involved in the cholesterol biosynthesis pathway were differentially abundant ( $P < 0.10$ ) in CL of gilts fed 20 (TRT1), 40 (TRT2), or 60 (TRT3) ppm Mn–amino acid complex compared with CL of gilts fed 20 ppm Mn sulfate (CON). Proteins in colored bold text were differentially abundant in at least one Mn–amino acid complex treatment compared with CON. Proteins in red were less abundant, whereas proteins in green were more abundant in Mn–amino acid complex treatments compared with CON. Fold changes and  $P$ -values associated with these proteins are listed in Table 2.

**Table 2.** Proteins involved in cholesterol and steroid metabolism in corpora lutea of Mn–amino acid complex-supplemented gilts compared with CON<sup>1</sup>

UniProt ID <sup>2</sup>	Protein name	Gene name	Fold change <sup>3</sup>			P-Value <sup>4</sup>		
			TRT1	TRT2	TRT3	TRT1	TRT2	TRT3
I3LM80	Delta(24)-sterol reductase	DHCR24	1.24	1.28	1.46	0.02	0.01	0.01
F1RRB7	Acetyl-CoA acyltransferase 1	ACAA1	0.68	0.60	0.56	<0.01	<0.01	0.02
I3LB89	Low-density lipoprotein receptor	LDLR	0.85	0.63	0.57	0.07	<0.01	<0.01
A7L861	Squalene monooxygenase	SQLE	0.76	0.65	0.68	0.04	0.02	0.02
Q28956	17beta-estradiol dehydrogenase	HSD17B4	0.56	0.57	0.64	0.03	0.04	0.09
A0A287A310	Neutral cholesterol ester hydrolase 1	NCEH1	1.35	1.30	1.33	<0.01	0.02	0.01
A9LM01	Membrane-associated P4 receptor component 2	PGRMC2	0.73	0.59	0.51	<0.01	<0.01	<0.01
F2Z4Y1	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein eta	YWHAH	1.08	— <sup>5</sup>	0.90	0.09	—	0.07
F1SUB2	Sphingosine-1-phosphate lyase 1	SGPL1	—	0.59	0.51	—	0.03	0.01
A0A287AB49	Diphosphomevalonate decarboxylase	MVD	—	0.64	0.62	—	0.01	<0.01
I3LKI5	7-dehydrocholesterol reductase	DHCR7	—	0.83	0.80	—	0.02	0.02
Q8SQC1	Scavenger receptor class B member 1	SCARB1	—	0.67	0.63	—	0.06	0.02
F1RQR4	EH domain-containing protein 1	EHD1	—	0.63	—	—	0.09	—
F1RGS2	Glucosylceramidase	GBA	—	0.77	—	—	0.03	—
A0A287ASS9	Sterol-4-alpha-carboxylate 3-dehydrogenase, decarboxylating	NSDHL	—	—	0.82	—	—	0.05
Q68RU1	Ovarian and testicular apolipoprotein N	APON	—	—	1.60	—	—	0.06
F1RIS8	Mevalonate kinase	MVK	—	—	0.58	—	—	<0.01
F1SMG8	3-hydroxy-3-methylglutaryl CoA synthase	HMGCS1	—	—	0.80	—	—	0.08
F1S2I4	3-hydroxy-3-methylglutaryl CoA reductase	HMGCR	—	—	0.62	—	—	0.04
A0A287A2U9	LIM domain and actin-binding protein 1	LIMA1	—	—	0.74	—	—	0.09

<sup>1</sup>CON, diet contained 20 ppm of added Mn in the form of Mn sulphate; TRT1, diet included 20 ppm of added Mn from a Mn–amino acid complex instead of Mn sulphate; TRT2, diet included 40 ppm of added Mn from a Mn–amino acid complex instead of Mn sulphate; TRT3, diet included 60 ppm of added Mn from a Mn–amino acid complex instead of Mn sulphate.

<sup>2</sup>Universal Protein Resource.

<sup>3</sup>Fold change relative to control.

<sup>4</sup>Proteins are differentially abundant compared with control with significance level of  $P < 0.10$ .

<sup>5</sup>Dash (—) indicates that protein was not differentially abundant compared with control.

**Table 3.** Proteins involved in the oxidative stress response in corpora lutea of Mn–amino acid complex-supplemented gilts compared with CON<sup>1</sup>

UniProt ID <sup>2</sup>	Protein name	Gene name	Fold change <sup>3</sup>			P-Value <sup>4</sup>		
			TRT1	TRT2	TRT3	TRT1	TRT2	TRT3
F1S4X9	Glutathione synthetase	GSS	0.71	0.70	— <sup>5</sup>	0.02	0.05	—
F1SJP6	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6	NDUFA6	—	2.91	2.83	—	<0.01	0.01
A0A287A096	Eukaryotic translation initiation factor 2 subunit 1	EIF2S1	—	0.87	0.88	—	0.02	0.09
I3LDM7	Lactoylglutathione lyase	GLO1	—	1.38	1.43	—	<0.01	<0.01
Q9TSX9	Peroxiredoxin-6	PRDX6	—	0.81	—	—	0.01	—
A0A286ZND5	Peroxiredoxin-1	PRDX1	—	—	1.23	—	—	0.01
B2ZF47	Mitochondrial aldehyde dehydrogenase 2	ALDH2	—	—	0.70	—	—	0.08

<sup>1</sup>CON, diet contained 20 ppm of added Mn in the form of Mn sulphate; TRT1, diet included 20 ppm of added Mn from a Mn–amino acid complex instead of Mn sulphate; TRT2, diet included 40 ppm of added Mn from a Mn–amino acid complex instead of Mn sulphate; TRT3, diet included 60 ppm of added Mn from a Mn–amino acid complex instead of Mn sulphate.

<sup>2</sup>Universal Protein Resource.

<sup>3</sup>Fold change relative to control.

<sup>4</sup>Proteins are differentially abundant compared with CON with significance level of  $P < 0.10$ .

<sup>5</sup>Dash (—) indicates that protein was not differentially abundant compared with control.

Apolipoprotein N is related to the cholesterol transport regulatory protein apolipoprotein F (APOF) and has been previously identified in the HDL and LDL fractions of bovine ovarian follicular fluid as well as in the HDL fraction of bovine serum (O'Bryan et al., 2004). HDLs are involved in the regulation

and support of gonadal steroidogenesis, and the association between ApoN and HDL/LDL in follicular fluid suggests that it may have a role in transport of cholesterol or fatty acids, and ultimately in ovarian steroidogenesis (Andersen and Dietschy, 1978; Gwynne and Strauss, 1982; Gwynne and Mahaffee, 1986;

**Table 4.** Proteins involved in angiogenesis in corpora lutea of Mn–amino acid complex-supplemented gilts compared with CON<sup>1</sup>

UniProt <sup>2</sup> ID	Protein	Gene name	Fold change <sup>3</sup>			P-Value <sup>4</sup>		
			TRT1	TRT2	TRT3	TRT1	TRT2	TRT3
A0A287ADH9	Chloride intracellular channel protein	CLIC4	0.82	0.82	0.85	0.03	0.03	0.09
M3UZ63	Myeloid-derived growth factor	MYDGF	1.22	1.28	— <sup>5</sup>	0.04	0.07	—
F1RPH0	Phosphoglycerate kinase	PGK1	—	0.87	—	—	0.09	—
A0A286ZU54	Metadherin	MTDH	—	1.32	—	—	0.01	—
B2D1T2	Cathepsin H	CTSH	—	—	0.62	—	—	0.04
F1RM62	Heat shock protein family B (small) member 6	HSPB6	—	—	0.84	—	—	0.09
A0A287BNK4	Signal transducer and activator of transcription	STAT1	—	—	0.72	—	—	0.09
F1RL75	Platelet-derived growth factor receptor beta	PDGFRB	—	—	3.76	—	—	<0.01
F1SQU4	Vascular endothelial growth factor D	VEGFD	—	—	2.39	—	—	<0.01

<sup>1</sup>CON, diet contained 20 ppm of added Mn in the form of Mn sulphate; TRT1, diet included 20 ppm of added Mn from a Mn–amino acid complex instead of Mn sulphate; TRT2, diet included 40 ppm of added Mn from a Mn–amino acid complex instead of Mn sulphate; TRT3, diet included 60 ppm of added Mn from a Mn–amino acid complex instead of Mn sulphate.

<sup>2</sup>Universal Protein Resource.

<sup>3</sup>Fold change relative to control.

<sup>4</sup>Proteins are differentially abundant compared with CON with significance level of  $P < 0.10$ .

<sup>5</sup>Dash (—) indicates that protein was not differentially abundant compared with control.

**Table 5.** Proteins that utilize Mn as a cofactor in corpora lutea of Mn–amino acid complex-supplemented gilts compared with CON<sup>1</sup>

UniProt <sup>2</sup> ID	Protein	Gene name	Fold change <sup>3</sup>			P-Value <sup>4</sup>		
			TRT1	TRT2	TRT3	TRT1	TRT2	TRT3
F1RPN9	Malic enzyme 2	ME2	0.63	0.54	0.53	0.01	<0.01	0.01
A0A286ZL42	Serine/threonine-protein phosphatase	PPP1CA	1.28	1.27	— <sup>5</sup>	<0.01	<0.01	—
A0A287AU59	Acetyl-CoA carboxylase 1	ACACA	0.58	—	0.43	0.08	—	0.01
I3LDM7	Lactoylglutathione lyase	GLO1	—	1.38	1.43	—	<0.01	<0.01
FISNL7	Carnosine dipeptidase 2	CNDP2	—	0.71	0.56	—	0.01	<0.01
I3LTC6	Inositol-1-monophosphatase	IMPA1	—	0.51	—	—	<0.01	—
F1STS4	Malic enzyme 3	ME3	—	0.54	—	—	0.01	—
F1RKQ4	Triokinase/FMN cyclase	TKFC	—	0.74	—	—	0.09	—
A0A287ANI6	Hydroxymethylglutaryl-CoA lyase	HMGCL	—	—	1.30	—	—	0.08

<sup>1</sup>CON, diet contained 20 ppm of added Mn in the form of Mn sulphate; TRT1, diet included 20 ppm of added Mn from a Mn–amino acid complex instead of Mn sulphate; TRT2, diet included 40 ppm of added Mn from a Mn–amino acid complex instead of Mn sulphate; TRT3, diet included 60 ppm of added Mn from a Mn–amino acid complex instead of Mn sulphate.

<sup>2</sup>Universal Protein Resource.

<sup>3</sup>Fold change relative to control.

<sup>4</sup>Proteins are differentially abundant compared with CON with significance level  $P < 0.10$ .

<sup>5</sup>Dash (—) indicates that protein was not differentially abundant compared with control.

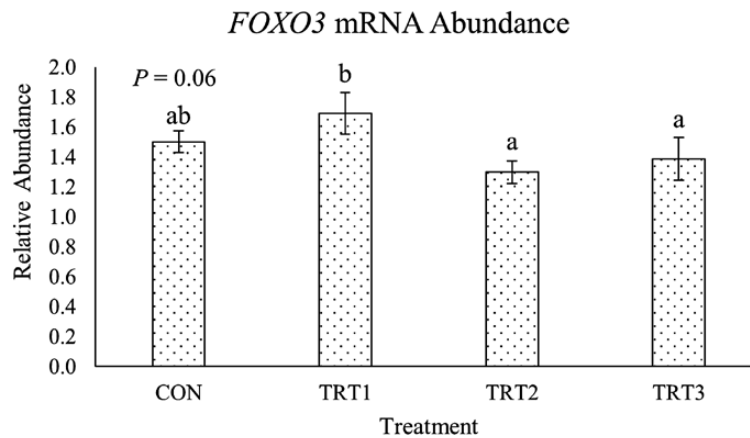
O'Bryan et al., 2004). The occurrence of increased ApoN abundance in luteal tissue of gilts supplemented with 60 ppm Mn–amino acid complex in concert with decreased luteal  $P_4$  concentrations (Studer et al., 2021) gives reason to speculate that increased ApoN is indicative of a cellular response to generate additional cholesterol available for increased luteal  $P_4$  production.

Interference with luteal cholesterol transport can be caused by oxidative stress, which is also linked to negative consequences such as apoptosis of luteal cells (Al-Gubory et al., 2012; Likso et al., 2019). Antioxidant enzymes PRDX6 and GSS, both downregulated in CL of Mn–amino acid complex-supplemented gilts, have previously been identified in luteinized follicles in swine and play a major role in removing ROS (Agca et al., 2006; Likso et al., 2019). Other proteins identified also involved in the anti-oxidative stress response include GLO1 and ALDH2. The protein GLO1, which was increased in CL of gilts supplemented with 40 and 60 ppm Mn–amino acid complex, alleviates ovarian oxidative stress by detoxifying carbonyl compounds resulting from lipid oxidation (Fujii et al., 2005;

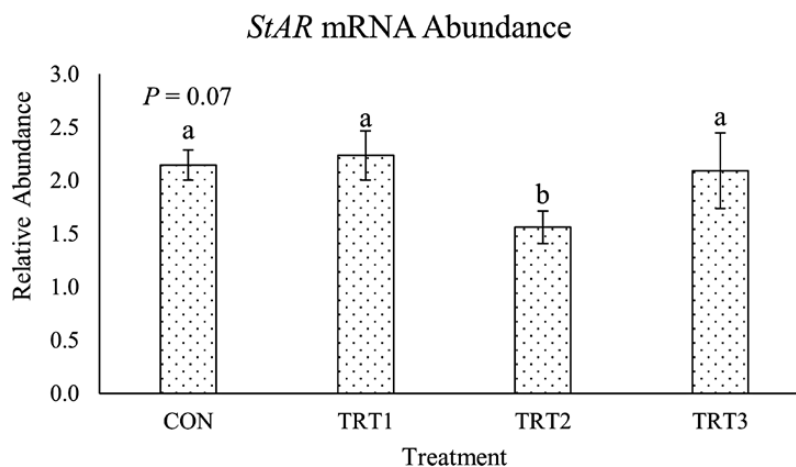
Brouwers et al., 2011). Given that redox homeostasis requires a balance between pro-oxidants and antioxidants, it makes sense that some proteins have increased abundance while others have decreased abundance.

Angiogenesis is the formation of a dense and intricate capillary network and is essential for proper CL function. This process allows hormone-producing luteal cells to obtain the oxygen, nutrients, and precursors needed for synthesis and release of  $P_4$ , necessary for the establishment and maintenance of pregnancy (Fraser and Wulff, 2003). This process requires a variety of factors including VEGFD, a regulator of lymphatic endothelial cells, and PDGFRB, responsible for activation of pericytes, which are a vital part of the microvasculature that form close contact with endothelial cells (Fraser and Wulff, 2003; Meidan, 2017). Both proteins were increased in CL of gilts supplemented with 60 ppm Mn–amino acid complex compared with CON suggesting that higher Mn could assist in capillary network formation in luteal cells. Although a dense capillary network would presumably aid luteal cells in





**Figure 3.** Relative abundance of FOXO3 mRNA was quantified via one-step quantitative real-time polymerase chain reaction from RNA extracted from luteal tissue of gilts fed 20 ppm Mn sulfate (CON), 20 ppm Mn–amino acid complex (TRT1), 40 ppm Mn amino–acid complex (TRT2), or 60 ppm Mn–amino acid complex (TRT3). Relative gene abundance was normalized to GAPDH as an internal control. There was an overall effect of dietary treatment on FOXO3 mRNA abundance ( $P = 0.06$ ), and TRT1 was increased compared with TRT2 ( $P = 0.01$ ) and TRT3 ( $P = 0.04$ ). Bars with differing superscripts denote significant differences between individual treatments ( $P < 0.05$ ).



**Figure 4.** Relative abundance of Star mRNA was quantified via one-step quantitative real-time polymerase chain reaction from RNA extracted from luteal tissue of gilts fed 20 ppm Mn sulfate (CON), 20 ppm Mn–amino acid complex (TRT1), 40 ppm Mn–amino acid complex (TRT2), or 60 ppm Mn–amino acid complex (TRT3). Relative gene abundance was normalized to GAPDH as an internal control. There was an overall effect of dietary treatment on Star mRNA abundance ( $P = 0.07$ ), and TRT2 was decreased compared with CON ( $P = 0.03$ ), TRT1 ( $P = 0.02$ ), and TRT3 ( $P = 0.09$ ). Bars with differing superscripts indicate significant differences ( $P < 0.05$ ) or a tendency for a difference ( $0.05 < P < 0.10$ ) between individual treatments.

obtaining necessary precursors for  $P_4$  synthesis, gilts in TRT3 had decreased luteal  $P_4$  compared with CON. While paradoxical and difficult to explain, it is possible that an attempt to form a denser capillary network in luteal tissue could be a paracrine feedback response to reduced local  $P_4$  to increase the availability of precursors for  $P_4$  synthesis.

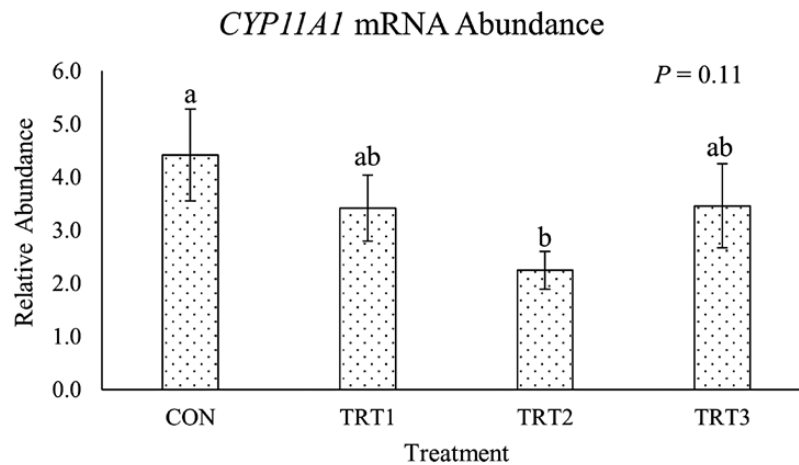
Supplementation with Mn–amino acid complex decreased abundance of ME2 and ME3, both of which utilize Mn as a cofactor. Three ME isoforms have been identified in mammalian cells: a cytosolic nicotinamide adenine dinucleotide phosphate (NADP)<sup>+</sup>-dependent isoform (ME1) and two mitochondrial NAD(P)<sup>+</sup>-dependent isoforms, ME2 and ME3 (Pongratz et al., 2007; Jiang et al., 2013; Dey et al., 2017). The mitochondrial MEs are oxidative decarboxylases that are essential for regeneration of nicotinamide adenine dinucleotide phosphate (reduced) (NADPH) and ROS homeostasis (Pongratz et al., 2007; Jiang et al., 2013; Dey et al., 2017). It is noteworthy that these mitochondrial ME isoforms were affected by

Mn–amino acid complex supplementation since Mn localizes to the mitochondria (Maynard and Cotzias, 1955).

Another protein that utilizes Mn as a cofactor and is also located within mitochondria is HMGCL, which had increased abundance in CL of gilts supplemented with 40 ppm Mn–amino acid complex. This enzyme catalyzes the cleavage of HMG-CoA to acetoacetic acid and acetyl-CoA inside the mitochondrial matrix (Ashmarina et al., 1996). Acetyl-CoA can then be used for de novo synthesis of cholesterol by luteal cells as a precursor for  $P_4$  (González-Fernández et al., 2008). It appears as though that supplementation with Mn–amino acid complex affects abundance of enzymes that utilize Mn as a cofactor differently, possibly depending on the function of each specific enzyme.

FOXO3 has been reported to protect cells from oxidative stress by stimulating transcription of MnSOD (Kops et al., 2002), although an increase in MnSOD transcript abundance was not observed herein. Relative transcript abundance of FOXO3 was not affected by dietary source of Mn, since no differences were





**Figure 5.** Relative abundance of CYP11A1 mRNA was quantified via one-step quantitative real-time polymerase chain reaction from RNA extracted from luteal tissue of gilts fed 20 ppm Mn sulfate (CON), 20 ppm Mn-amino acid complex (TRT1), 40 ppm Mn-amino acid complex (TRT2), or 60 ppm Mn-amino acid complex (TRT3). Relative gene abundance was normalized to GAPDH as an internal control. There was no overall effect of dietary treatment on mRNA abundance of CYP11A1 ( $P = 0.11$ ) but comparisons between individual treatments revealed decreased CYP11A1 mRNA abundance in TRT2 compared to CON ( $P = 0.02$ ). Bars with differing superscripts denote significant differences between individual treatments ( $P < 0.05$ ).

observed in Mn-amino acid complex treatments compared with CON. Dietary concentration of Mn-amino acid complex seemed to have an effect, since relative transcript abundance in TRT1 (20 ppm Mn-amino acid complex) was increased compared with TRT2 (40 ppm Mn-amino acid complex) and TRT3 (60 ppm Mn-amino acid complex).

The enzymes STAR and CYP11A1 are important components of the  $P_4$  biosynthesis pathway. Specifically, STAR functions as the rate-limiting step in steroid biosynthesis by mediating the transport of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane to enable conversion of cholesterol into pregnenolone, whereas CYP11A1 is responsible for catalyzing the conversion of cholesterol to pregnenolone (Miller and Auchus, 2011). The final step in  $P_4$  biosynthesis is conversion of pregnenolone into  $P_4$  by  $3\beta$ -hydroxysteroid dehydrogenase ( $3\beta$ HSD), although this enzyme was not analyzed in the current study (Christenson and Devoto, 2003). A study in cultured rat Leydig cells found that Mn exposure reduced StAR gene expression in a dose-dependent manner and that long periods of Mn exposure reduced the activity of CYP11A1 (Cheng et al., 2003). These findings partially corroborate results from our study, where relative abundance of StAR and CYP11A1 mRNA was lower in CL of gilts supplemented with 40 ppm of Mn-amino acid complex (TRT2) compared with gilts fed 20 ppm of Mn sulfate (CON). However, this was not detected across all Mn-amino acid complex treatments when compared with CON. Additionally, we also observed an apparent dose effect in regard to relative abundance of StAR and CYP11A1 mRNA, with CL of TRT2 fed gilts having less StAR mRNA abundance than CON, TRT1, and TRT3, and a lack of an effect of dietary Mn source, as TRT1, TRT3, and CON were not different from each other. Of further interest, CYP11A1, for which activity could presumably influence steroid hormone biosynthesis in the CL, was decreased in TRT2 compared with CON.

## Conclusions

Understanding the role of specific dietary components, particularly trace minerals with a presumably improved bioavailability, in the function of specific reproductive tissues is relatively unexplored. In this study, dietary supplementation

with a more bioavailable form of Mn altered relative abundance of protein and transcripts involved in luteal steroidogenesis. This was accompanied by an increase in luteal Mn content and a decrease in luteal  $P_4$  production (Studer et al., 2021). Collectively, these data demonstrate that CL function can be influenced by trace minerals in the diet and should be considered when optimizing nutritional strategies to maximize reproductive potential.

## Supplementary Data

Supplementary data are available at *Journal of Animal Science* online.

## Acknowledgments

We would like to acknowledge Trey Faaborg and the undergraduate students who assisted with the live animal phase of the project along with Kristen Olsen who formulated the diets. Additionally, we also acknowledge Joel Nott from the ISU Protein Facility as well as Maryam Sayadi and Andrew Severin from the Genome Informatics Facility for their assistance with the proteome work. This project was supported by Zinpro Corporation, Eden Prairie, MN.

## Conflict of interest statement

J.M.S., Z.E.K., B.M.G., A.F.K., L.H.B., and J.W.R. report no conflict of interest, and Z.J.R., W.P.S., M.E.W., and C.R. are or were employed by the Zinpro Corporation at the time of the study.

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