

Evaluation of dietary trace mineral supplementation in young horses challenged with intra-articular lipopolysaccharide¹

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ABSTRACT: Sixteen weanling Quarter Horses (255 ± 22 kg) were utilized in a 56-d trial to evaluate the effects of trace mineral (TM) source on intra-articular inflammation following a single acute inflammatory insult. Horses were stratified by age, sex, and BW and then randomly assigned to dietary treatment: concentrate formulated with Zn, Mn, Cu, and Co as inorganic sources (CON; *n* = 8) or complexed TMs (CTM; *n* = 8). Added TM were formulated at iso-levels across treatments and intakes met or exceeded NRC requirements. Horses were offered 1.75% BW (as-fed) of treatment concentrate and 0.75% BW (as-fed) coastal Bermudagrass hay. Growth measurements were collected on days 0, 28, and 56, and plasma was collected biweekly for determination of Mn, Cu, Zn, and Co concentrations. On day 42, carpal joints were randomly assigned to receive injections of 0.5 ng lipopolysaccharide (LPS) or sterile lactated Ringer's solution (LRS; contralateral control). Synovial fluid was collected at preinjection hours (PIH) 0, and 6, 12, 24, 168, and 336 h post-injection and analyzed for TM concentration, prostaglandin E₂ (PGE₂), carboxypeptide of type II collagen (CPII), collagenase cleavage neopeptide (C2C), and aggrecan chondroitin sulfate 846 epitope (CS846). Data were analyzed using the

MIXED procedure of SAS. Results showed a TM source × LPS × h effect for synovial fluid Co, Cu, and Se (*P* < 0.05); concentrations of TM peaked at hour 6 and decreased to preinjection values by hour 168 in both CON and CTM–LPS knees. A delayed peak was observed at hour 12 for CTM–LRS. Peak synovial fluid Cu and Se concentrations were higher in LPS knees, and Co was highest in CTM–LPS. A TM source × h interaction was observed for Zn (*P* < 0.05); concentrations peaked at hour 6 in CON vs. hour 12 for CTM. An LPS × h interaction was observed for Mn (*P* < 0.01); synovial concentration peaked at hour 6 in LPS knees compared with hour 24 in LRS. Synovial PGE₂, C2C, CPII, and CS846 concentrations were greater with LPS (*P* ≤ 0.01), and C2C was greater (*P* < 0.01) in CTM compared with CON. Concentrations of CPII and PGE₂ were unaffected by diet. A TM source × h × LPS interaction was observed for CS846 (*P* = 0.02). Concentrations of CS846 in CTM peaked at 12 h, whereas CON peaked at a lower concentration at 24 h (*P* < 0.05). Data indicate sufficient intake of a complexed TM source may support cartilage metabolism through increased aggrecan synthesis and type II collagen breakdown following an intra-articular LPS challenge in growing horses.

Key words: equine, inflammation, lipopolysaccharide, trace minerals

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INTRODUCTION

Homeostatic maintenance of articulating joints requires Cu, Mn, and Zn for turnover of collagen fibrils and to contribute to molecules within the extracellular matrix (Hostetler et al., 2003; Richards et al., 2010). The role of dietary trace minerals (TM) in equine articulating joints remains undetermined; however, information gained from other species indicates that metal amino acid-complexed TM provide a more biologically available source of TM (Osorio et al., 2012). In hens challenged with systemic lipopolysaccharide (LPS) and supplemented with Zn amino acid complex, serum interleukin-1 β (IL-1 β) concentration increased to 3 h post-induction, but at 12 h, concentrations were lower than hens receiving Zn sulfate (Cheng and Guo, 2004).

Increased bioavailability and utilization of complexed TM may allow for greater incorporation of TM into articular cartilage and a more rapid achievement in homeostasis following endotoxin injection. Utilizing an intra-articular LPS challenge to induce localized inflammation and cartilage turnover in young horses provides the ability to evaluate the effect of TM source on cartilage metabolism, joint inflammation, and synovial fluid TM concentrations post-induction (Leatherwood et al., 2016) through biomarkers relative to inflammation and cartilage turnover.

Synovial prostaglandin E₂ (PGE₂) concentration increases in response to intra-articular LPS. Resulting inflammation influences collagen metabolism and aggrecan synthesis by increasing catabolic collagenase cleavage neopeptide (C2C), anabolic carboxypropeptide of type II collagen (CPII), and chondroitin sulfate 846 epitope (CS846; de Grauw et al., 2009; Lucia et al., 2013). Therefore, objectives of this experiment were to compare effects of dietary TM source (organic vs. inorganic) on growth, joint inflammation, cartilage metabolism, and synovial fluid TM concentrations in response to an intra-articular LPS challenge in young horses.

MATERIALS AND METHODS

All care, handling, and procedures for experiment were approved by the Texas A&M University Institutional Animal Care and Use Committee.

Horses and Treatments

Sixteen weanling Quarter Horses (mean \pm SEM; initial BW of 255 \pm 22 kg BW; n = 9 colts; n = 7 fillies) were used in a complete randomized design. Prior to the initiation of dietary treatments, mares and foals were maintained on the same commercial concentrate that contained inorganic mineral sources (Producer's Cooperative Association, Bryan, TX). The concentrate was provided as a creep feed to foals beginning at 90 d of age. Foals were weaned at 150 \pm 11 d of age and maintained on the same concentrate until the initiation of the study (233 \pm 20 d of age).

Radiographs (lateral, flexed lateral, and cranial-caudal views) of both radial carpal joints were performed at the Texas A&M University Large Animal Hospital (College Station, TX) prior to initiation of the study. All horses considered to be radiologically normal by a licensed veterinarian were stratified by age, sex, BW, and BCS, and randomly assigned to dietary treatment. Treatment diets consisted of isocaloric, isonitrogenous pelleted concentrate formulated with either inorganic (CON; 100% inorganic CuSO₄, ZnSO₄, and MnSO₄ and CoCO₃; n = 8) or TM complexes (CTM; zinc methionine, manganese methionine, copper lysine, and cobalt glucoheptonate; n = 8). Added levels of Zn, Mn, Cu, and Co were formulated at iso-levels in both pelleted treatment diets, and daily mineral intakes met or exceeded 2007 NRC minimum recommended requirements. All personnel involved in performing the study were blinded to dietary treatment. Composited samples of concentrate and hay were analyzed by Equi-Analytical Laboratories (Ithaca, NY) commercial analysis (Table 1) for dry matter (method 930.15; AOAC, 2019), crude petrol (method 990.03; AOAC, 2019), crude fat (method

Table 1. Nutrient composition of concentrates and hay (DM basis) fed to weanling horses

	Concentrate		Forage ³
	CON ¹	CTM ²	
Dry matter, %	89.50	91.3	89.16
Nutrient, % DM			
CP	19.30	19.6	12.51
ADF	25.90	23.7	35.51
NDF	40.00	36.1	65.99
Fat	7.70	7.9	2.26
Ca	0.94	1.26	0.32
P	0.92	1.16	0.29
Trace minerals ⁴ , ppm			
Zn	197.90	184.7	17.9
Cu	51.90	57.9	5.8
Mn	216.00	226.9	192.2
Co	7.10	9.5	<1.0
Se ⁵	0.73	0.89	

¹Control: pelleted concentrate formulated with 100% inorganic mineral sources (CuSO₄, ZnSO₄, and MnSO₄ and CoCO₃), supplied for 56 d at 1.75% BW (as-fed), *n* = 8.

²Trace mineral complexes: 7 g of 4-Plex C (Zinpro Corporation, Eden Prairie, MN) replaced a portion of inorganic added trace mineral, supplied for 56 d at 1.75% BW (as-fed), *n* = 8.

³Coastal bermudagrass, *Cynodon dactylon*, supplied to both treatment groups at 0.75% BW.

⁴Total mineral content.

⁵Reported on a 100% dry basis.

2003.05; AOAC, 2019), acid detergent fiber (ANKOM Technology Method 5), neutral detergent fiber (ANKOM Technology Method 6), Ca, and P (inductively coupled plasma analysis [ICP]), and TM concentrations (Fe, Zn, Cu, Mn, Mo, Co, Se) were analyzed by Michigan State University DCPAH (Lansing, MI) with an ICP/mass spectrometer (ICP/MS).

Weanlings received their respective pelleted concentrate at 1.75% BW/d (as-fed) and 0.75% BW/d (as-fed) of coastal bermudagrass (*Cynodon dactylon*) hay divided evenly between 2 feedings at 0600 and 1800. Horses were fed individually and maintained in 3 × 3 m stalls with ad libitum access to water. Intakes and orts were obtained and measured daily. Every 7 d, BW was obtained utilizing a calibrated digital platform scale (Bastrop Scale Inc., Bastrop, TX) and diets adjusted accordingly. All horses were allowed 8 h of free exercise in a dry lot (58.5 × 79.2 m) daily.

Growth and Performance Characteristics

Body condition score, rump fat (RF), wither height (WH), hip height (HH), body length (BL), and heart girth circumference (HG) were taken

on days 0, 28, and 56 by the same 3 independent observers. Rump fat was measured at 5 cm lateral from the midline, halfway between the first coccygeal vertebrae and the ischium (Westervelt et al., 1976). An altitude stick was used to measure WH and HH. Body length and HG were taken using a soft measuring tape. Ultrasonic images were also captured of the *longissimus dorsi* muscle (LM) were captured by a certified technician (Designer Genes Technologies, Inc., Harrison, AR) to determine muscle area, back fat thickness (BFT), and intramuscular fat (IMF) deposition. The transducer was placed to obtain a cross-sectional image taken between the 13th and 14th as well as the 17th and 18th ribs. Subcutaneous fat thickness was measured at three-fourths the distance from the medial end of the LM; 4 independent images were collected laterally across the 17th and 18th ribs to estimate IMF within the LM. Four independent images were necessary to follow Annual Proficiency Testing and Certification standard format for data submission. Proper contact between transducer and horse was insured by fitting the transducer with a PIA contour pad (Animal Ultrasound Services, Ithaca, NY) designed to conform to the curvature of the horse's back. In addition, corn oil was applied to promote acoustical contact between animal and transducer (Perkins et al., 1992). An independent laboratory interpreted all images; personnel were blinded to treatment (Designer Genes Technologies, Inc.).

Incorporation into Plasma and Synovial Fluid

Plasma samples for TM analysis were collected every 14 d into a 6-mL trace element K₂ EDTA, 10.8 mg, additive tube (BD Vacutainer, Franklin Lakes, NJ) prior to the morning feeding. Samples were immediately placed on ice until centrifugation at 2,000 × *g* for 10 min at 4 °C within 1 h of collection. After centrifugation, plasma was aliquoted into 1.5-mL microcentrifuge tubes and stored at -80 °C until analysis. A certified veterinarian from the Texas A&M University Large Animal Clinic (Dr. C. E. Arnold) performed carpal arthrocentesis on both radial carpal joints on day 0. Horses were sedated using xylazine HCl, administered intravenously at recommended dosages. The carpal joint was aseptically aspirated using a location medial to the extensor carpi radialis tendon in the palpable depression between the radial carpal bone and the third carpal bone, to a depth of approximately 12.7 mm to avoid unnecessary contact with articular cartilage (McIlwraith and Trotter, 1996). Pooled synovial fluid between carpal joints

(1 to 4 mL) was transferred into sterile nonadditive tubes (BD Vacutainer) and was immediately placed on ice and stored at -80°C until laboratory analysis.

Intra-articular LPS Challenge

On day 42 of the study, all horses were subjected to an intra-articular LPS challenge. One radiocarpal joint was randomly selected within each horse for injection with LPS, whereas the other radiocarpal joint served as a contralateral control (injection of sterile lactated Ringer's solution; LRS). The use of an LRS joint was based on previous data in our laboratory that suggested repeated arthrocentesis influenced local inflammatory status in horses regardless of treatment (LRS or LPS) as evidenced by an alteration in circulating leukocytes, monocytes, or platelets (Hunt et al., 2018).

At PIH 0, the carpal joint was aseptically prepared for arthrocentesis and horses were sedated as previously described. Purified LPS derived from *Escherichia coli* O55:B5 (Sigma Aldrich, St. Louis, MO) was reconstituted and diluted in sterile lactated Ringer's solution; individual doses were 0.8 mL with a final concentration of 0.5 ng/mL. Dosage of LPS was based on previous work in our laboratory (Lucia et al., 2013; Leatherwood et al., 2016; Kahn et al., 2017). The LPS solution was inserted aseptically into the randomly selected carpal joint, and LRS joints were injected with 0.8 mL of sterile lactated Ringer's solution after the withdrawal of the PIH 0 sample. Synovial fluid samples (1 to 4 mL) were obtained at PIH 0 and 6, 12, 24, 168, and 336 h post-injection). All personnel were blinded to injection type.

All synovial samples were collected and transferred to sterile nonadditive tubes (BD Vacutainer Blood Serum Collection Tubes; Becton-Dickinson and Company, Franklin Lakes, NJ) and immediately placed on ice until aliquoted into 1.5-mL microcentrifuge tubes. Aliquots were stored at -80°C until later analysis of C2C, CPII, CS846, PGE_2 , and TM concentrations. All horses were monitored for signs of anaphylaxis over the initial 24 h post-injection. Rectal temperature (RT; $^{\circ}\text{C}$), heart rate (HR; beats/min), and respiratory rate (RR; breaths/min) were recorded prior to arthrocentesis at PIH 0 and at 6, 12, and 24 h post-injection. Carpal circumference (cm) was measured at the level of the accessory carpal bone with a soft tape measure that was performed by a single individual to maintain consistency.

Sample Analysis

Plasma and synovial fluid samples were sent to the Michigan State Diagnostics Laboratory (Lansing, MI) for TM analysis to establish TM composition. In brief, samples were diluted 20-fold with a solution containing 0.5% EDTA and Triton X-100, 1% ammonium hydroxide, 2% propanol, and 20 ppb of scandium, rhodium, indium, and bismuth as internal standards. The ICP/MS was tuned to yield a minimum of 7,500 cps sensitivity for 1 ppb yttrium (mass 89), less than 1.0% oxide level as determined by the 156/140 mass ratio and less than 2.0% double charged ions as determined by the 70/140 mass ratio (Wahlen et al., 2005). Elemental concentrations were calibrated using a 4-point linear curve of the analyte-internal standard response ratio. Standards were from Inorganic Ventures (Christiansburg, VA). In-house serum pools were used as controls.

Synovial fluid concentrations CPII, C2C, and CS846 were measured using commercially available ELISA kits (IBEX Pharmaceuticals Inc., Montreal, QC, Canada) previously validated in horses (de Grauw et al., 2009; Lucia et al., 2013). Synovial fluid samples were analyzed in duplicate, and standards were prepared according to manufacturer's recommendations with samples prepared at a 1:4 dilution for both CPII and C2C. Sample dilutions for CS846 ranged from 1:50 to 1:1,000 depending on time post-injection, to remain within detectable limits of the ELISA. Dilutions were made with calibrator diluents provided by the kit prior to beginning the assay. Mean detectable concentrations for CPII, C2C, and CS846 were 50, 10, and 20 ng/mL, respectively. Shifts in cartilage metabolism were evaluated by the ratio of CPII to C2C with individual ratios calculated for each horse.

Synovial fluid samples were analyzed in duplicate for concentrations of PGE_2 utilizing an enzyme-linked immunoassay (R&D Systems, Minneapolis, MN), previously validated in horses (Bertone et al., 2001; de Grauw et al., 2006; Lucia et al., 2013). Samples intended for PGE_2 analysis were diluted from 1:1 to 1:10 depending on time post-injection to remain within detectable limits of the ELISA; dilutions were prepared using the calibrator diluent provided by the kit with a mean detectable dose of PGE_2 of 39 pg/mL.

Final concentrations of all markers were read using a microplate reader (Synergy H1, Biotek, Winooski, VT) with optical density set at 450 nm. Intracoefficients of variation for CPII, C2C, and

PGE₂ were less than or equal to 15% and less than 20% for CS846.

Statistical Analysis

Intake data were analyzed by using the MIXED procedure of SAS v9.4 (SAS Inst. Inc., Cary, NC). The model contained fixed effects of TM source and time (d). Initial, final, and delta values for growth parameters were analyzed using the MIXED procedure of SAS v9.4 (SAS Inst. Inc., Cary, NC). The model contained a fixed effect of TM source and used a random variable of horse within treatment. Plasma mineral concentration utilized the same model with an additional fixed effect of time (d) and a TM source × d interaction.

In response to the LPS challenge, statistical analysis of synovial fluid biomarker and TM concentration were analyzed using PROC MIXED of SAS v9.4 (SAS Inst. Inc., Cary, NC). The model contained fixed effects of TM source, time (h), injection type (LPS), and their respective interactions. This model included a random effect of horse within treatment and a repeated variable (time). The covariate structure was utilized to specify a random effect for differences between animals within treatment, creating a correlation structure within animals that decreases with the increasing amount of time between measurements (Littell et al., 1998). The model for synovial fluid TM analysis also included hour 0 as a covariate. Post hoc comparison of TM source and individual time points was conducted using a paired *t*-test. Significant differences were declared as $P \leq 0.05$, and $P \leq 0.10$ was considered a trend toward significance. Plots of residual variation were used to evaluate normal

distributions for traits based on continuous variables. One biomarker (CPII) exhibited non-normal data; therefore, the data were log transformed for normalization. Outliers were identified using box plots of the residuals and removed if greater than 3 SD from the mean. Data are reported as mean ± SEM.

RESULTS

Target intakes of 1.75% BW (as-fed) from concentrate and 0.75% BW (as-fed) from hay were achieved as horses consumed their respective diets with no significant refusals across treatment groups over the 56-d trial. Weanling horses consumed energy and associated nutrients to meet or exceed recommended requirements (NRC, 2007). Total dry matter intake throughout the 56-d study did not differ between TM source ($P = 0.89$), with daily intakes of 5.74 and 5.71 ± 0.15 kg for CON and CTM, respectively. Individually, concentrate and forage intakes were not different between TM sources ($P = 0.54$ and $P = 0.47$, respectively). Similarly, TM source did not affect ($P = 0.78$) final BW (285.8 and 288.8 ± 5.57 kg for CON and CTM, respectively), with all horses gaining 32 ± 2 kg during the 56 d ($P = 0.90$), validating the diets were isocaloric.

No effect of TM source was observed in synovial biomarker concentrations during the pre-LPS dietary adaptation period; however, a TM source × d interaction was observed for CS846 ($P = 0.05$; Table 2). Synovial fluid CS846 concentration increased from days 0 to 42 in CTM horses, while remaining similar from day 0 to day 42 in CON horses. Conversely, the ratio of CPII to C2C increased in all horses from days 0 to 42 ($P = 0.04$),

Table 2. Cartilage biomarkers and inflammatory markers within the synovial fluid of weanling Quarter Horses before (day 0) and (day 42) 42 d of receiving a pelleted concentrate at 1.75% BW (as-fed) containing either inorganic (CON; $n = 8$) or organic (CTM; $n = 8$) dietary trace mineral supplementation

Synovial fluid variable ²	Dietary treatments ¹				SEM	<i>P</i> -value		
	CON		CTM			TM source	d	TM source × d
	Day 0	Day 42	Day 0	Day 42				
CPII, ng/mL	764.65	967.17	713.96	988.23	231.33	0.96	0.13	0.82
C2C, ng/mL	270.93	260.86	276.66	297.89	29.23	0.48	0.80	0.49
CPII:C2C	2.76	3.82	2.54	3.90	0.91	0.95	0.04	0.80
CS846, ng/mL	23,820 ^a	24,803 ^a	19,701 ^a	29,717 ^b	3,411	0.92	0.02	0.05
PGE ₂ , pg/mL	596.85	336.37	750.68	423.02	87.90	0.13	<0.01	0.64

¹Control (CON): pelleted concentrate formulated with 100% inorganic mineral sources (CuSO₄, ZnSO₄, and MnSO₄ and CoCO₃), supplied at 1.75% BW (as-fed), $n = 8$. Trace mineral complexes (CTM): 7 g of 4-Plex C (Zinpro Corporation, Eden Prairie, MN) replaced a portion of inorganic added trace mineral, supplied at 1.75% BW (as-fed), $n = 8$.

²CPII = carboxypropeptide of type II collagen; C2C = collagenase cleavage neopeptide; CS846 = chondroitin sulfate 846 epitope; PGE₂ = prostaglandin E₂.

^{ab}Within row, means with different letters differ ($P < 0.05$).

whereas PGE₂ decreased over time ($P < 0.01$), regardless of TM source (Table 2).

Growth Metrics

Growth measurements including WH, HH, HG, and BL were not affected ($P \geq 0.56$) by TM source (Table 3). Similarly, no differences between TM sources were observed for final IMF, BFT, or rump fat ($P > 0.10$). However, final BCS tended to be lower ($P = 0.06$) for horses fed CTM when compared with CON. Final LM area at both measured locations was not affected by TM source ($P \geq 0.60$). Overall change in area at the 17th and 18th rib was unaffected by TM source, but a positive change in area over the 56-d trial was observed in CON (+1.72 cm²) and CTM treatments (+1.97 cm²), indicating growth.

Plasma Mineral Concentration

No significant interaction or main effects of TM source and d were observed for Mn ($P \geq 0.18$) with average concentrations of 1.73 and 1.62 ± 0.09 ng/mL for CTM and CON, respectively. No interaction or effect of TM was observed for Cu ($P \geq 0.41$); however, an effect of day ($P < 0.01$) was present. Plasma Cu concentrations increased from days 0 to 42 ($P < 0.01$; 1.07 to 1.22 ± 0.05 µg/mL for days 0 and 42, respectively) and remained elevated through day 56. A TM source × d interaction was present for Zn ($P = 0.02$; Fig. 1). Concentrations decreased in CON from days 0 to 56 while increasing in CTM from day 14 to day 28 before declining to levels similar to CON by day 42. A significant TM source × d interaction was observed for Co concentrations ($P < 0.01$; Fig. 2). Cobalt concentrations increased from days 0 to 14 for all horses regardless of TM source; however, horses receiving CTM had greater Co plasma concentrations compared with the control CON, beginning at day 14 through day 56 of the study.

LPS Challenge

Clinical assessment During the LPS challenge, no TM source × h interactions were present for clinical parameters, including HR, RR, and RT ($P \geq 0.33$; data not shown). Average beats per minute were 48.4 and 50.9 ± 21.6 ($P \geq 0.22$), for CON and CTM horses, respectively. Respiration rate was affected by hour ($P < 0.01$), with an increase from PIH 0 (20 ± 2 breaths/min) to hour 6 (24 ± 2 breaths/min), before decreasing to below PIH 0 levels at

Table 3. Growth parameters and composition of weanling Quarter Horses before (day 0) and after 56 d of receiving a pelleted concentrate at 1.75% BW (as-fed) containing either inorganic (CON; $n = 8$) or organic (CTM; $n = 8$) dietary trace mineral supplementation

Variable	Dietary treatments		SEM	P-value
	CON ¹	CTM ²		
BW, kg				
Day 0	256.4	253.7	8.0	0.82
Day 56	288.8	285.8	7.6	0.78
Wither height, cm				
Day 0	128.19	127.56	0.97	0.65
Day 56	131.68	131.60	0.67	0.93
Hip height, cm				
Day 0	134.54	134.38	1.12	0.92
Day 56	137.80	138.19	0.99	0.78
Heart girth, cm				
Day 0	143.25	141.67	1.97	0.56
Day 56	149.52	148.13	1.82	0.60
Body length, cm				
Day 0	135.21	134.94	1.39	0.89
Day 56	144.74	145.31	1.35	0.77
Body condition score				
Day 0	5.65	5.52	0.16	0.58
Day 56	6.55	6.08	0.16	0.06
Rump fat ³				
Day 0	0.14	0.15	0.006	0.79
Day 56	0.16	0.16	0.005	0.86
Intramuscular fat, %				
Day 0	3.62	3.65	0.17	0.91
Day 56	3.64	3.48	0.17	0.52
Back fat thickness, 13th and 14th rib				
Day 0	0.17	0.15	0.02	0.42
Day 56	0.16	0.17	0.01	0.57
Back fat thickness, 17th and 18th rib				
Day 0	0.14	0.14	0.01	0.74
Day 56	0.14	0.14	0.01	0.81
LM area, cm ² , 13th and 14th rib				
Day 0	10.57	10.27	0.45	0.65
Day 56	11.75	12.00	0.59	0.77
LM area, cm ² , 17th and 18th rib				
Day 0	12.01	11.63	0.52	0.61
Day 56	13.73	13.60	0.28	0.60

¹Control: pelleted concentrate formulated with 100% inorganic mineral sources (CuSO₄, ZnSO₄, and MnSO₄ and CoCO₃), supplied for 56 d at 1.75% BW (as-fed), $n = 8$.

²Trace mineral complexes: 7 g of 4-Plex C (Zinpro Corporation, Eden Prairie, MN) replaced a portion of inorganic added trace mineral, supplied for 56 d at 1.75% BW (as-fed), $n = 8$.

³Rump fat thickness measured by ultrasound.

hour 12 (16 ± 2 breaths/min) and hour 24 (15 ± 2 breaths/min). An effect of h was also observed for RT ($P = 0.01$). Rectal temperature increased from PIH 0 (38.0 ± 0.1 °C) to 38.4 ± 0.1 °C at hour 6

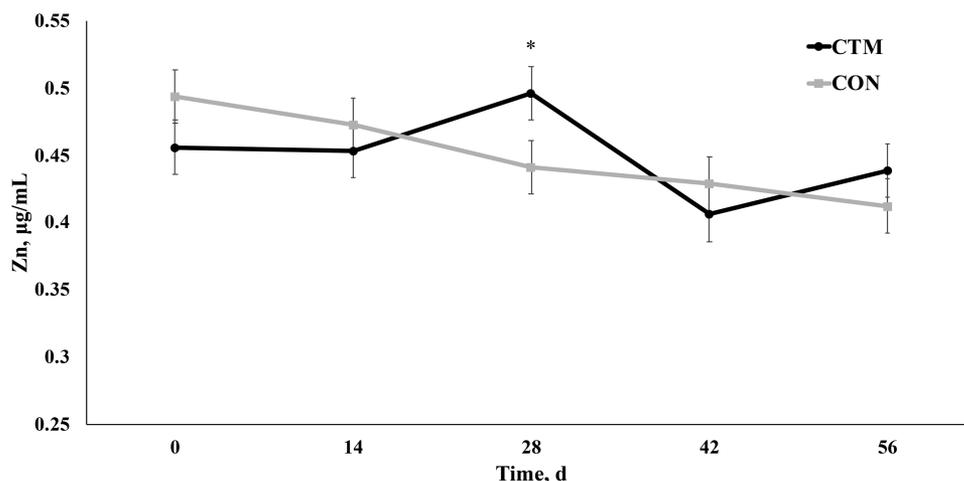


Figure 1. Mean plasma Zn concentrations over 56-d trial in weanling horses receiving a pelleted concentrate at 1.75% BW (as-fed) containing either 100% inorganic ZnSO₄, CuSO₄, and MnSO₄ and CoCO₃ (CON; *n* = 8) or trace mineral complexes zinc methionine, manganese methionine, copper lysine, and cobalt glucoheptonate (CTM; *n* = 8). Added levels of Zn, Mn, Cu, and Co were at iso-levels in both pelleted treatment diets, and daily mineral intakes met or exceeded NRC minimum requirements. Trace mineral source × d interaction (*P* < 0.05). *CTM greater than CON at day 28 (*P* < 0.05).

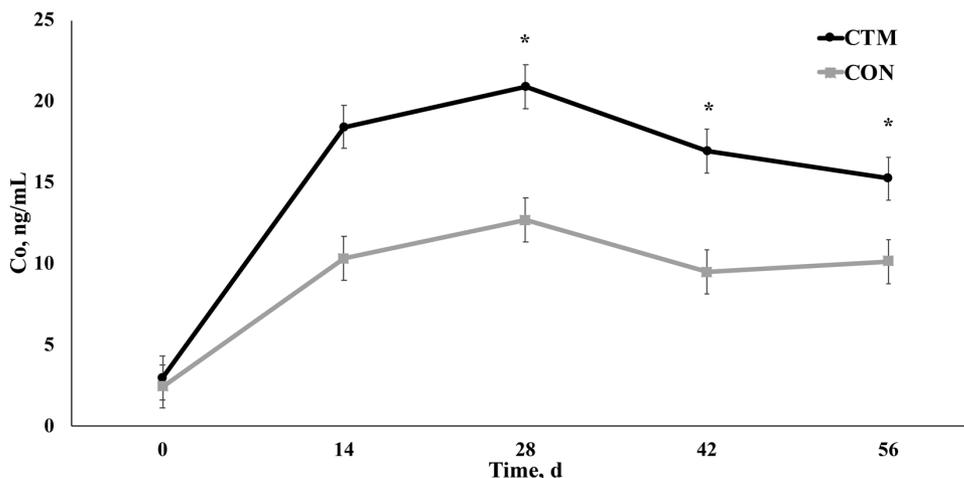


Figure 2. Mean plasma Co concentrations over 56-d trial in weanling horses receiving a pelleted concentrate fed at 1.75% BW (as-fed) containing either 100% inorganic ZnSO₄, CuSO₄, and MnSO₄ and CoCO₃ (CON; *n* = 8) or trace mineral complexes Zn methionine, Mn methionine, Cu lysine, and Co glucoheptonate (CTM; *n* = 8). Added levels of Zn, Mn, Cu, and Co were at iso-levels in both pelleted treatment diets, and daily mineral intakes met or exceeded NRC minimum requirements. Trace mineral source × d interaction (*P* < 0.01). *CTM higher than CON (*P* < 0.01).

before returning to baseline by hour 24 (38.0 ± 0.1 °C). All values remained within normal physiological limits throughout the 24-h period. An LPS × h interaction was observed for carpal circumference (*P* < 0.01), as the LPS injected knees increased to hour 12 and remained elevated to hour 24, whereas the LRS knee increased in circumference to hour 12 and returned to baseline by hour 24. Carpal circumference increased over time (*P* < 0.01; data not shown), but was not affected by TM source.

Synovial fluid mineral concentration A TM source × LPS × h interaction was observed for Co (*P* < 0.01; Fig. 3). Cobalt concentration at hour 6 was the greatest in CTM-LPS (*P* < 0.01). In

addition, at hour 12, Co was greater in CTM-CON than CON-LPS and CON-LRS (*P* < 0.01).

A TM source × LPS × h effect was observed for Cu (*P* < 0.01; Fig. 4). Concentrations of Cu increased in both LPS and CON-LRS to peak concentrations by hour 6 then decreased to hour 168, whereas concentration of Cu was lowest at hour 6 in CTM-LRS (*P* = 0.01). Concentrations of Cu in CTM-LRS increased from hours 6 to 12 and then declined to hour 168. Peak concentration for CTM-LRS was observed at hour 12, and the Cu concentration was similar to other knees at that time point. No differences were observed at hour 168 or 336. Trace mineral source × LPS × h effect was also observed for Se (*P* = 0.02; Fig. 5), with

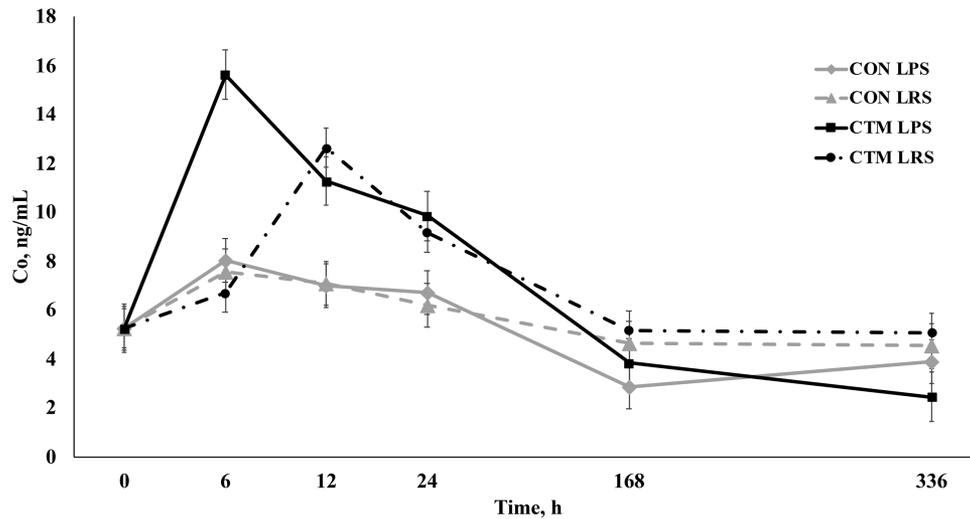


Figure 3. Mean synovial fluid cobalt concentrations following a 0.5-ng intra-articular lipopolysaccharide (LPS; derived from *Escherichia coli* O55:B5) injection at 0 to 336 h post-injection or lactated Ringer's solution (LRS; contralateral control). Dietary treatments consisted of pelleted concentrates containing either 100% inorganic ZnSO₄, CuSO₄, and MnSO₄ and CoCO₃ (CON; *n* = 8) or trace mineral complexes zinc methionine, manganese methionine, copper lysine, and cobalt glucoheptonate (CTM; *n* = 8). Added levels of Zn, Mn, Cu, and Co were at iso-levels in both pelleted treatment diets, and daily mineral intakes met or exceeded NRC minimum requirements. Cobalt concentrations: trace mineral source × LPS × h interaction (*P* < 0.01). ^{abc}Denotes differences within each time point between trace mineral source and LPS (*P* < 0.05).

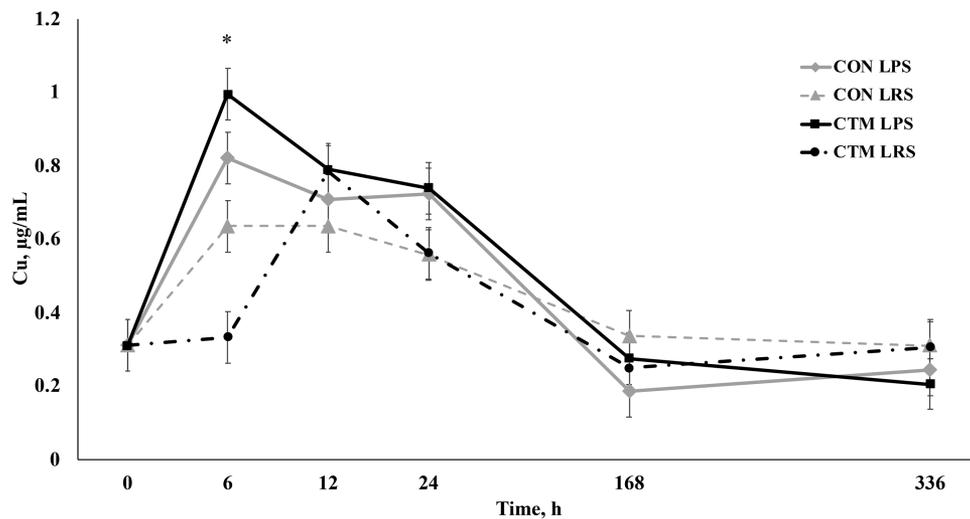


Figure 4. Mean synovial fluid copper concentrations following a 0.5-ng intra-articular lipopolysaccharide (LPS; derived from *Escherichia coli* O55:B5) injection at 0 to 336 h post-injection or lactated Ringer's solution (LRS; contralateral control). Dietary treatments consisted of pelleted concentrates containing either 100% inorganic ZnSO₄, CuSO₄, and MnSO₄ and CoCO₃ (CON; *n* = 8) or trace mineral complexes zinc methionine, manganese methionine, copper lysine, and cobalt glucoheptonate (CTM; *n* = 8). Added levels of Zn, Mn, Cu, and Co were at iso-levels in both pelleted treatment diets, and daily mineral intakes met or exceeded NRC minimum requirements. Copper concentrations: trace mineral source × LPS × h interaction (*P* = 0.01). ^{abc}Differences within each time point between trace mineral source and LPS (*P* < 0.05).

concentrations following the same basic pattern as Cu. Concentrations of Se were lowest in CTM–LRS at hour 6 vs. other knees (*P* < 0.01) and then increased to similar concentrations at hour 12 to those in other knees. Concentrations of Se then declined in all knees to hour 168, and maintained similar concentrations to hour 336. No TM source × LPS × h interaction was present for Zn (*P* = 0.21; Fig. 6), although concentrations followed the same general pattern as Cu and Se. A TM source × h interaction was observed for Zn (*P* = 0.02). Zinc increased in concentration, peaking at hour 6 in

CON and hour 12 for CTM before decreasing to hour 168. At hours 12, 24, and 168, CON had a lower concentration of Zn than CTM (*P* < 0.01). An LPS × h interaction (*P* < 0.01) was observed for Zn; concentrations were higher in LPS knees at hours 6 and 24 (*P* ≤ 0.01) and tended to be higher at hour 12 than CON (*P* = 0.06).

No TM source × h × LPS interaction (*P* = 0.74; Fig. 7) was observed for Mn; however, an h × LPS interaction was present (*P* < 0.01). Manganese concentration increased in both LPS knees to hour 6 and then decreased steadily to baseline levels by

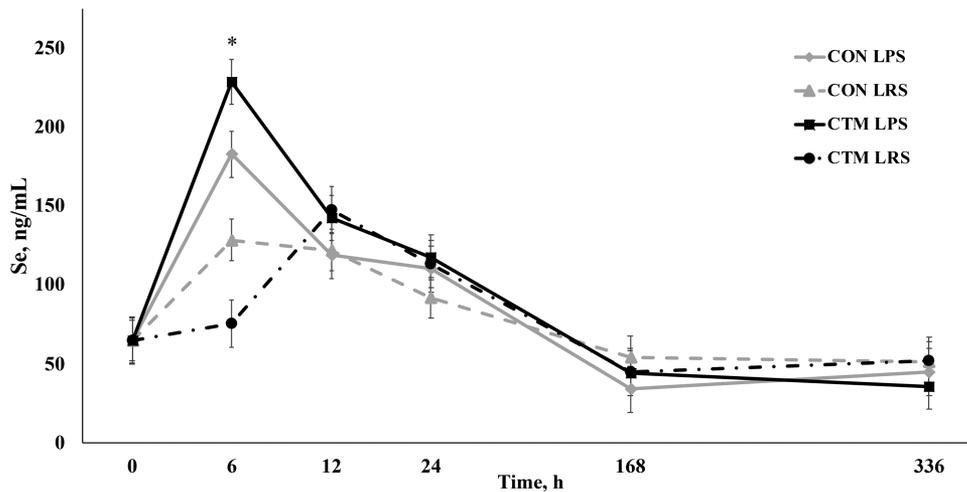


Figure 5. Mean synovial fluid selenium concentrations following a 0.5-ng intra-articular lipopolysaccharide (LPS; derived from *Escherichia coli* O55:B5) injection at 0 to 336 h post-injection or lactated Ringer's solution (LRS; contralateral control). Dietary treatments consisted of pelleted concentrates containing either 100% inorganic $ZnSO_4$, $CuSO_4$, and $MnSO_4$ and $CoCO_3$ (CON; $n = 8$) or trace mineral complexes zinc methionine, manganese methionine, copper lysine, and cobalt glucoheptonate (CTM; $n = 8$). Added levels of Zn, Mn, Cu, and Co were at iso-levels in both pelleted treatment diets, and daily mineral intakes met or exceeded NRC minimum requirements. Selenium concentrations: trace mineral source \times LPS \times h interaction ($P = 0.02$). ^{abc}Differences within each time point between trace mineral source and LPS ($P < 0.05$).

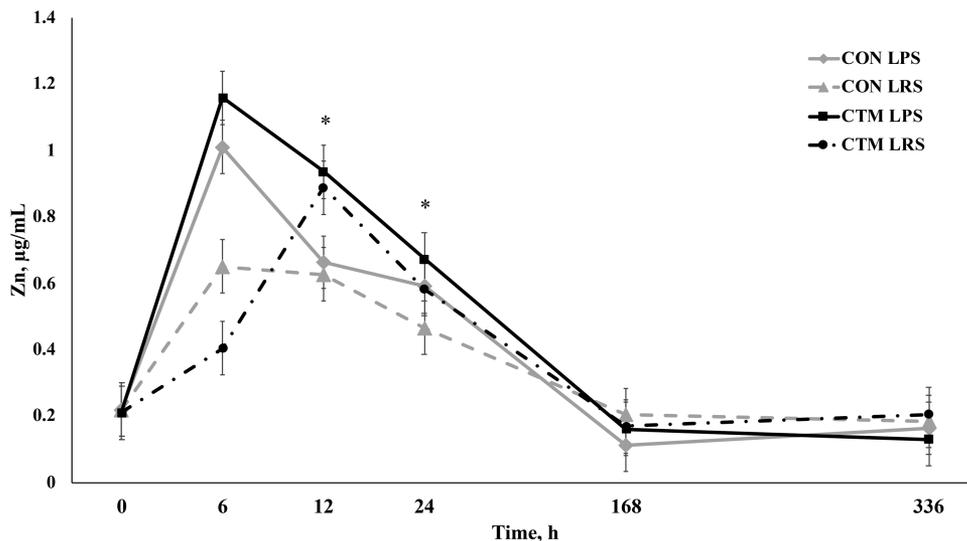


Figure 6. Mean synovial fluid zinc concentrations following a 0.5-ng intra-articular lipopolysaccharide (LPS; derived from *Escherichia coli* O55:B5) injection at 0 to 336 h post-injection or lactated Ringer's solution (LRS; contralateral control). Dietary treatments consisted of pelleted concentrates containing either 100% inorganic $ZnSO_4$, $CuSO_4$, and $MnSO_4$ and $CoCO_3$ (CON; $n = 8$) or trace mineral complexes zinc methionine, manganese methionine, copper lysine, and cobalt glucoheptonate (CTM; $n = 8$). Added levels of Zn, Mn, Cu, and Co were at iso-levels in both pelleted treatment diets, and daily mineral intakes met or exceeded NRC minimum requirements. Zinc concentrations: trace mineral source \times LPS \times h ($P = 0.21$), trace mineral source \times h ($P < 0.05$), h \times LPS ($P < 0.01$). *Differences within each time point between trace mineral source ($P < 0.05$). ^aDifferences within each time point between injection type (LPS).

hour 168. LRS knees displayed an increase from hour 12 to 24 resulting in higher Mn concentration in LRS compared with LPS knees at hour 24 ($P < 0.01$). Concentration of Mn in LRS knees then decreased to baseline levels by hour 168.

Synovial inflammation An LPS \times h interaction ($P \leq 0.01$; Fig. 8) was observed for synovial PGE_2 . Concentration of PGE_2 was greater in the LPS injected knee when compared with LRS at hours 6, 12, and 24 ($P < 0.01$). Synovial PGE_2 was not affected by TM source ($P = 0.13$).

Cartilage markers An LPS \times h interaction was present for synovial C2C ($P < 0.01$; Fig. 9). Concentrations of C2C in LPS knees were greater than concentrations in LRS knees at hours 6, 12, 24, and 168 ($P \leq 0.04$). A tendency for an interaction of TM source \times LPS ($P = 0.09$) was observed with concentrations of C2C being greater in CTM-LPS compared with CON-LPS ($P < 0.01$). An LPS \times h interaction ($P \leq 0.01$; Fig. 10) was observed for anabolic CPII with LPS injection resulting in greater

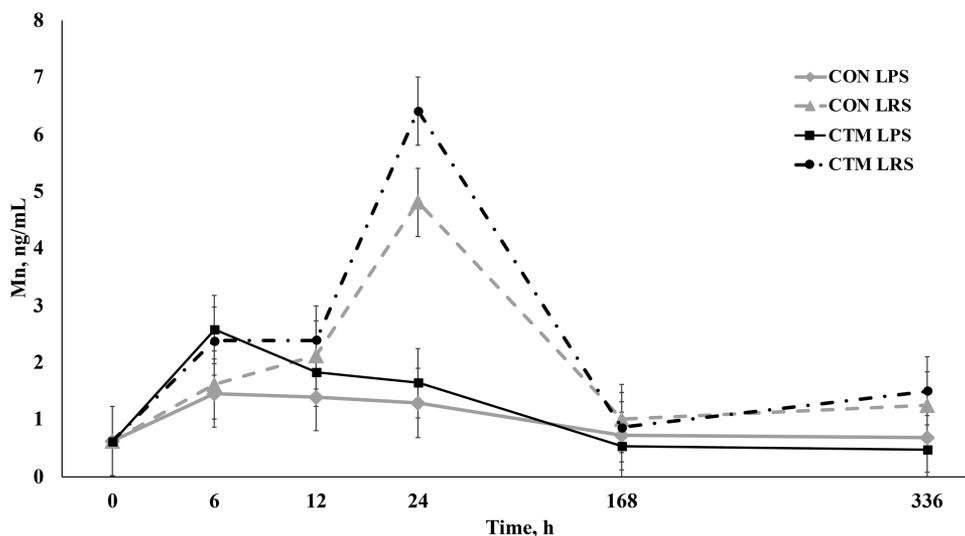


Figure 7. Mean synovial fluid manganese concentrations following a 0.5-ng intra-articular lipopolysaccharide (LPS; derived from *Escherichia coli* O55:B5) injection at 0 to 336 h post-injection or lactated Ringer's solution (LRS; contralateral control). Dietary treatments consisted of pelleted concentrates containing either 100% inorganic ZnSO₄, CuSO₄, and MnSO₄ and CoCO₃ (CON; *n* = 8) or trace mineral complexes zinc methionine, manganese methionine, copper lysine, and cobalt glucoheptonate (CTM; *n* = 8). Added levels of Zn, Mn, Cu, and Co were at iso-levels in both pelleted treatment diets, and daily mineral intakes met or exceeded NRC minimum requirements. Manganese concentrations: trace mineral source × h × LPS interaction (*P* = 0.74), LPS × h interaction (*P* < 0.01), trace mineral source (*P* = 0.35). *Differences between LRS knees and LPS knees (*P* < 0.01).

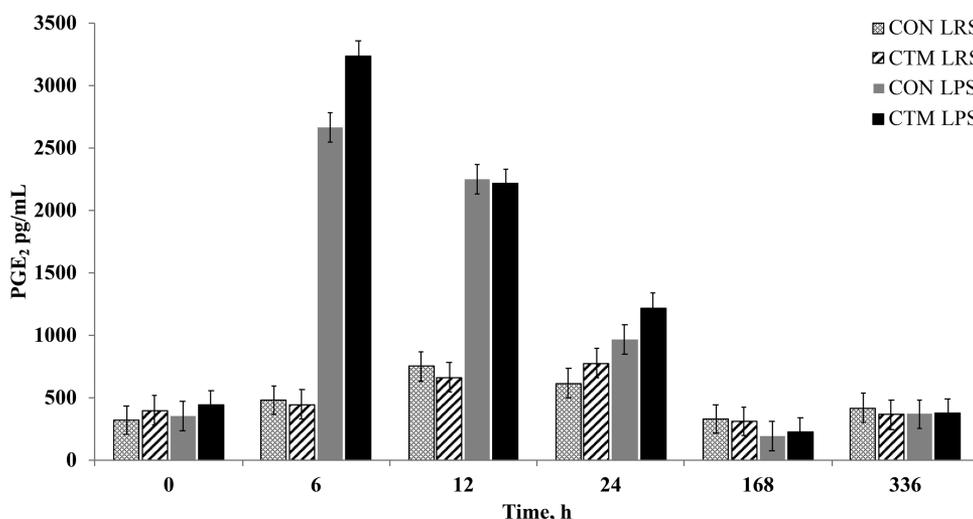


Figure 8. Mean synovial prostaglandin E₂ (PGE₂) concentrations following a 0.5-ng intra-articular lipopolysaccharide (LPS; derived from *Escherichia coli* O55:B5) injection at 0 to 336 h post-injection or lactated Ringer's solution (LRS; contralateral control). Dietary treatments consisted of pelleted concentrates containing either 100% inorganic ZnSO₄, CuSO₄, and MnSO₄ and CoCO₃ (CON; *n* = 8) or trace mineral complexes zinc methionine, manganese methionine, copper lysine, and cobalt glucoheptonate (CTM; *n* = 8). Added levels of Zn, Mn, Cu, and Co were at iso-levels in both pelleted treatment diets, and daily mineral intakes met or exceeded NRC minimum requirements. Trace mineral source × LPS × h (*P* = 0.48), LPS × h interaction (*P* < 0.01), trace mineral source (*P* = 0.13).

CPII concentrations at 6, 12, and 168 h (*P* ≤ 0.01) compared with LRS. The CPII to C2C ratio was not significantly affected by TM source (*P* = 0.57), LPS (*P* = 0.11), or hour (*P* = 0.19; data not shown).

A significant interaction of TM source × LPS × h (*P* = 0.02; Fig. 11) was observed for synovial CS846 concentrations. Prior to injection of LPS (PIH 0) there were no differences in CS846 levels; however, 6 h after LPS injection, CS846 was higher

in LPS knees than LRS knees with no difference between dietary mineral sources. At hour 12, knees injected with LPS in CTM horses had the greatest concentration of CS846 (*P* < 0.01) followed by LPS knees in CON horses; LRS knees remained near baseline. By hour 24, LPS knees in CON horses had higher CS846 concentrations than CTM horses and LRS knees had increased above baseline but remained lower than LPS. By hour 168, no differences were detected.

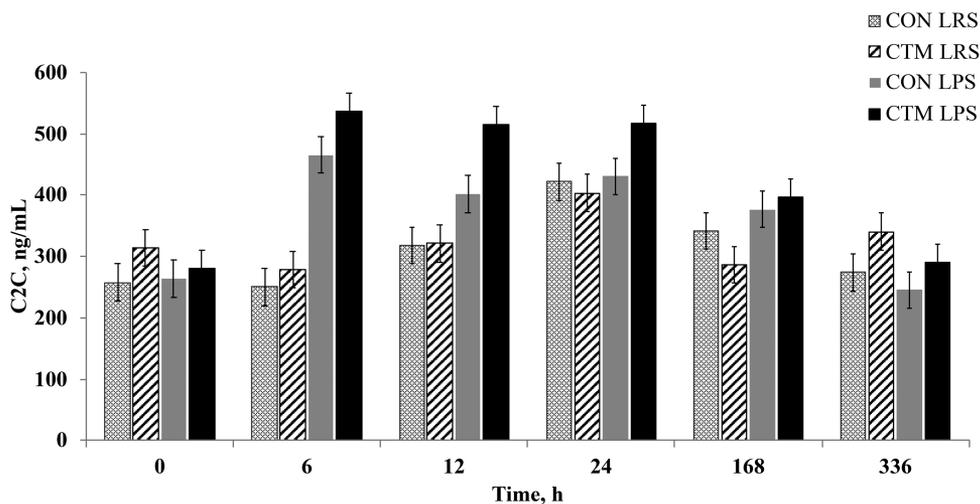


Figure 9. Mean synovial collagenase cleavage neopeptide (C2C) concentrations following a 0.5-ng intra-articular lipopolysaccharide (LPS; derived from *Escherichia coli* O55:B5) injection at 0 to 336 h post-injection or lactated Ringer's solution (LRS; contralateral control). Dietary treatments consisted of pelleted concentrates containing either 100% inorganic ZnSO₄, CuSO₄, and MnSO₄ and CoCO₃ (CON; *n* = 8) or trace mineral complexes zinc methionine, manganese methionine, copper lysine, and cobalt glucoheptonate (CTM; *n* = 8). Added levels of Zn, Mn, Cu, and Co were at iso-levels in both pelleted treatment diets, and daily mineral intakes met or exceeded NRC minimum requirements. Trace mineral source × LPS × h (*P* = 0.35), LPS × h (*P* < 0.01), trace mineral source × LPS (*P* = 0.09).

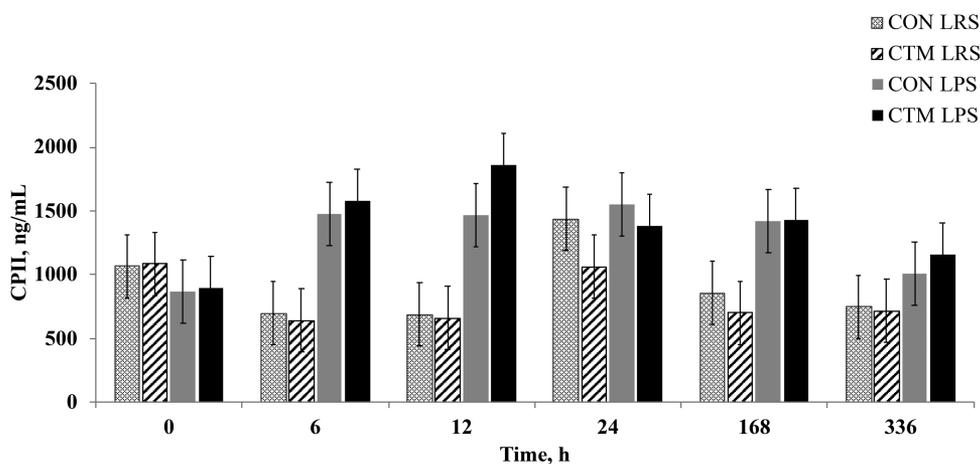


Figure 10. Mean synovial carboxypeptide of type II collagen (CPII) concentrations following a 0.5-ng intra-articular lipopolysaccharide (LPS; derived from *Escherichia coli* O55:B5) injection at 0 to 336 h post-injection or lactated Ringer's solution (LRS; contralateral control). Dietary treatments consisted of pelleted concentrates containing either 100% inorganic ZnSO₄, CuSO₄, and MnSO₄ and CoCO₃ (CON; *n* = 8) or trace mineral complexes zinc methionine, manganese methionine, copper lysine, and cobalt glucoheptonate (CTM; *n* = 8). Added levels of Zn, Mn, Cu, and Co were at iso-levels in both pelleted treatment diets, and daily mineral intakes met or exceeded NRC minimum requirements. Trace mineral source × LPS × h (*P* = 0.99), LPS × h (*P* < 0.01), trace mineral source (*P* = 0.82).

DISCUSSION

The present study examined the effect of complexed Zn, Cu, Mn, and Co source on growth and intra-articular inflammation in growing horses. Our study did not detect a positive effect of CTM on growth of yearling horses, which is in contrast with the prior reports that complexed TM increased growth in beef cattle and calves (Osorio et al., 2012; Genther-Schroeder et al., 2016a, b). Osorio et al. (2012) reported that wither height was increased in calves fed complexed TMs (Zn, Mn, Cu, and Co) vs. inorganic sources at weaning. In addition, the addition of dietary Zn amino acid

complex has been shown to increase long bone length and width in embryos and chicks (Favero et al., 2013). Limited data exist observing the effect of complexed TMs on equine growth; however, differences between TM proteinates and inorganic sources were evaluated in yearling horses fed for 112 d (Ott and Johnson, 2001). No effect of source on BW, WH, HG, or BL gains was reported; however, HH gain was greater for horses receiving proteinates than the inorganic supplemented horses. In the current 56-d study, all horses, regardless of diet, increased in BW, HH, WH, BL, and HG circumference.

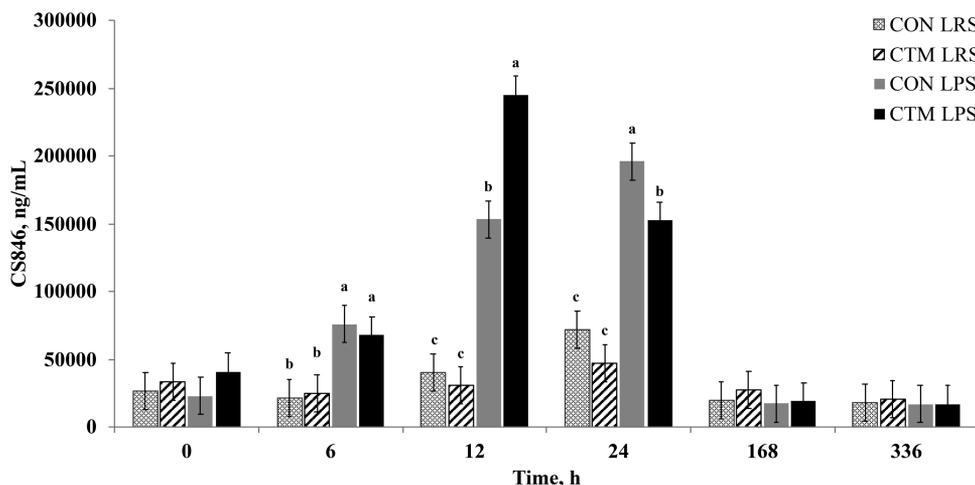


Figure 11. Mean synovial chondroitin sulfate epitope 846 (CS846) concentrations following a 0.5-ng intra-articular lipopolysaccharide (LPS; derived from *Escherichia coli* O55:B5) injection at 0 to 336 h post-injection or lactated Ringer's solution (LRS; contralateral control). Dietary treatments consisted of pelleted concentrates containing either 100% inorganic ZnSO₄, CuSO₄, and MnSO₄ and CoCO₃ (CON; *n* = 8) or trace mineral complexes zinc methionine, manganese methionine, copper lysine, and cobalt glucoheptonate (CTM; *n* = 8). Added levels of Zn, Mn, Cu, and Co were at iso-levels in both pelleted treatment diets, and daily mineral intakes met or exceeded NRC minimum requirements. Significant interactions: TM source × LPS × h interaction (*P* < 0.02). ^{abc}Differences in concentration among TM sources at specific time points post-intra-articular injection (*P* < 0.05).

In previous studies exposing young horses to intra-articular LPS, clinical responses for HR, RR, and RT showed no signs of systemic illness (de Grauw et al., 2009; Lucia et al., 2013). In the present study, clinical measures also remained within normal physiological ranges for horses of this age and demonstrated the inflammatory response remained localized. Joint circumference increased regardless of TM source or intra-articular treatment (LPS vs. LRS) above baseline values at 6 h, increased at 12 h, and began decreasing at 24 h; however, values did not return to baseline by 336 h. The lack of differences between joints receiving LPS vs. LRS further validates the inclusion of an LRS sham-injected knee in the LPS model to account for effects of repeated arthrocentesis. Data collected in the present study agree with previous literature, indicating 0.5 ng LPS causes acute synovitis resulting in minor carpal circumference increases with minimal physiological changes of HR, RR, and RT (Lucia et al., 2013; Kahn et al., 2017).

Synovial fluid TM concentrations in response to an inflammatory insult have not previously been reported in horses. Due to roles for various TMs as enzyme activators and cofactors, changes in concentrations have potential to affect other biochemical indicators. In the present study, all TMs measured varied over time, likely a result of inflammation associated with both LPS and repeated arthrocentesis. Trace minerals, Zn, Cu, Mn, and Se, were directly influenced by LPS injection, increasing in the synovial fluid post-injection. Concentrations of Cu, Zn, and Se responded similarly as concentrations

of each mineral increased to 6 h before returning to baseline or below baseline levels by 168 h post-injection in all knees except CTM–LRS, which showed a delayed increase at 12 h.

In human osteoarthritic patients, similar increases in synovial fluid Cu and positive correlations between synovial fluid Zn and Cu have been reported in response to inflammation (Yazar et al., 2005). The increases in concentrations of Zn, Cu, and Se in response to LPS could be due to their role in combating free radical formation. The presence of reactive oxygen species is damaging to the extracellular matrix (ECM) both structurally and functionally (Henrotin et al., 2003). Together, Se-containing glutathione peroxidase and superoxide dismutase (SOD) are the major antioxidant defense systems against oxygen free radicals. Three isoforms of SOD exist in mammals: cytoplasmic Cu/Zn SOD, mitochondrial Mn SOD, and extracellular Cu/Zn SOD (Fukai and Ushio-Fukai, 2011). Decreased SOD activity is exhibited in humans and mice with osteoarthritis (Regan et al., 2005). The increase in Cu could potentially be due to an increase in the Cu-containing acute phase protein, ceruloplasmin, which appears to exert antioxidant effects in human knee-joint synovial fluid (Blake et al., 1981).

In contrast to Zn, Cu, and Se, synovial fluid concentration of Mn was minimally responsive to LPS. Specifically, manganese concentrations of 6.41 and 4.81 ng/mL were exhibited in the CTM and CON–LRS knees, respectively, at 24 h. These values are approximately 7- to 10-fold higher than

values at hour 0, whereas Mn values in LPS knees were only 2 to 2.5 times greater (CTM and CON, respectively) than hour 0. The decrease in Mn in the LPS knees could be attributed to increased chondroitin sulfate synthesis, supported by the increase in CS846 in response to the LPS.

The 3-way interactions observed between diet, h, and LPS for Cu and Se indicate dietary source affected degree of response between knees. The peak concentrations were highest in the CTM–LPS knee for Cu and Se, $0.99 \pm 0.07 \mu\text{g/mL}$ and $228.62 \pm 14.2 \text{ ng/mL}$, respectively; although these concentrations were not high enough to differ from CON–LPS. At 6 h, the CTM–LRS knee had lower Cu and Se concentrations ($0.33 \pm 0.07 \mu\text{g/mL}$ and $75.6 \pm 13 \text{ ng/mL}$) than both LPS knees and CON–LRS. Concentrations of Cu and Se in CTM–LRS peaked at 12 h ($0.78 \pm 0.07 \mu\text{g/mL}$ and $147.21 \pm 13 \text{ ng/mL}$) that were similar levels to CTM–LPS 12 h. The elevated response to inflammation from either LPS or the delayed response to repeated arthrocentesis could be due to the form of Cu supplied in the diet or from the interactions of dietary from of minerals supplied with Se. Cobalt was the only mineral directly affected by diet; horses fed CTM had greater mean concentration of Co in the synovial fluid, 8.18 ± 0.5 vs. $5.86 \pm 0.5 \mu\text{g/mL}$. Therefore, Co glucoheptonate appears to be more bioavailable than inorganic Co. The 3-way interaction observed in relation to Co concentrations also conveys that horses fed CTM had a greater Co response to LPS and repeated arthrocentesis than CON horses, exhibiting higher peak concentrations in the synovial fluid for both LPS (hour 6) and LRS (hour 12) knees than CON horses. The exact role of Co in the joint has yet to be elucidated.

This study further demonstrates the importance of the use of controls, as regardless of injection type differed over time. Overall, these data indicate that acute joint inflammation altered synovial fluid concentrations of TM and that dietary source impacted the resulting degree of response. Currently, limited research exists relating TM concentrations and relative time course in equine joints under controlled inflammation; thus, data reported here are a preliminary exploration and do not fully explain the potential role of complex changes of TMs in joint inflammation. However, understanding how CTM affect the physiological response of TM concentrations and the biological roles of TMs within the joint further advances our knowledge of the role of TMs in joint health. These data ultimately provide a foundation for the development of further in-depth studies evaluating specific

mechanisms regarding interaction networks of TM under inflammatory conditions within the joint. Furthermore, research identifying the source of increased TM in equine synovial fluid would provide improved understanding of how nutritional mineral status and cartilage health could result in mineral extraction from articular cartilage.

Articular cartilage integrity is heavily dependent on the balance of metabolic activities (Mueller and Tuan, 2011). The presence of inflammation causes altered cartilage metabolism by decreasing anabolic and increasing catabolic activities (McIlwraith and Trotter, 1996). Although designed to promote healing, chronic inflammation can lead to articular degradation (Palmer and Bertone, 1994). Levels of cartilage biomarkers measured in the synovial fluid may be influenced by local inflammatory status. A useful indicator of inflammation and as a marker for the progression of joint disease is PGE_2 (Bertone et al., 2001). In the present study, PGE_2 concentrations were higher in LPS injected joints ($1210.98 \pm 37.43 \text{ pg/mL}$) than in LRS joints ($491.11 \pm 36.21 \text{ pg/mL}$). Concentrations of PGE_2 peaked at hour 6 in the LPS knee for both TM sources; however, horses receiving CTM had a more pronounced response ($3,239 \pm 133.86 \text{ pg/mL}$) vs. CON ($2,664 \pm 133.91 \text{ pg/mL}$). Similar inflammatory responses have been reported in an acute LPS model using chickens. When fed Zn amino acid complex, hens had a greater cytokine production ($\text{IL-}\beta$) 3 h post-challenge before reducing to a lower concentration than other diets at 12 h (Cheng and Guo, 2004). A similar increase at 6 h was reported in yearling and mature horses undergoing an intra-articular LPS challenge (de Grauw et al., 2009; Leatherwood et al., 2016). Although reported peak values for both studies were higher than peak concentration noted in the present study, this could be due to differences in age or to differences in total dietary TM fortification.

Aggrecan molecules are an essential component of the ECM and, due to their highly negative charge, are responsible for providing the joint with compressive strength (Frisbie et al., 2008). A key glycosaminoglycan of aggrecan is chondroitin sulfate; therefore, the impact of both diet and LPS on aggrecan molecule synthesis was measured through the CS846 epitope. A response of aggrecan synthesis was exhibited in the presence of LPS with highest concentrations at 12 h for CTM and 24 h for CON horses. de Grauw et al. (2009) also reported a short-lived inflammation-induced enhancement of CS846 synthesis, with concentrations highest at 24 h in mature horses. Interestingly, the CTM

horses had greater concentrations than their CON counterparts ($245,290 \pm 15,663$ and $196,008 \pm 13,653$ ng/mL, respectively) in the present study. In contrast, concentrations in CTM horses started to decrease by hour 24; however, concentrations returned to baseline by hour 168 for both TM sources. Multiple enzymes involved in the synthesis of chondroitin sulfate require Mn for synthesis (Leach, 1971); therefore, a potential explanation for the more rapid increase in CTM horses is that the complexed Mn may be more readily available for enzyme utilization.

Inflammation can lead to articular cartilage degradation, a key feature in the development of joint disease. The primary component of articular cartilage is type II collagen; its breakdown is highly involved in the development and progression of joint disease (McIlwraith and Trotter, 1996). The destruction of cartilage results in an accumulation of breakdown products in synovial fluid. The analysis of these fragments can help elucidate the degree of cartilage turnover and potentially highlight metabolic changes (Garvican et al., 2010). The breakdown of type II collagen has been measured using C2C (de Grauw et al., 2009). The unwinding or cleavage of collagens by collagenases exposes normally hidden epitopes, and these fragments are increased with joint inflammation measured in rabbits, dogs, and horses (Matyas et al., 2004; Lucia et al., 2013). Multiple studies have reported peak concentrations of C2C at 24 h post-LPS injection (de Grauw et al., 2009; Lucia et al., 2013; Leatherwood et al., 2016; Kahn et al., 2017). In the present study, LPS increased C2C concentrations, although peak concentrations were exhibited 6 h post-injection for both CTM and CON (536 ± 29.85 and 465 ± 29.85 ng/mL, respectively).

Concentrations of C2C were higher in joints of horses receiving CTM (373.51 ± 9.28 ng/mL) compared with CON (337.36 ± 9.28 ng/mL) horses. The breakdown and turnover of cartilage collagen is largely mediated by matrix metalloproteinases (MMPs), a family of degradative enzymes that require a metal ion for activation (Garvican et al., 2010). Synthesis of MMPs is regulated by cytokine and growth factor production (Milner et al., 2006). As an established model of inflammation, an increased cytokine production can be expected when challenged with LPS. Activation of most MMP requires Zn; therefore, readily available Zn may be allowing for the upregulation of MMPs and the inflammatory response, resulting in increased concentrations of C2C. Concentrations of C2C for

CTM horses remained higher at 12 and 24 h before decreasing to baseline at 336 h (290 ± 29.85 ng/mL). However, at 336 h, the highest C2C concentrations were observed in CTM-CON knees, (340.43 ± 29.85 ng/mL) further exhibiting the need for a sham control due to results from repeated arthrocentesis.

The rate of recent collagen synthesis can be measured using CPII (de Grauw et al., 2009). This molecule is proteolytically cleaved from the pro-collagen strand during fibril formation and has a half-life of 16 h in synovial fluid (Garvican et al., 2010). It has also been shown to increase in arthritic joints and in osteochondrosis in horses (Frisbie et al., 2008). In addition, CPII concentrations have been shown to increase in response to intra-articular LPS injection in both growing and mature horses, with variation in concentration between studies (de Grauw et al., 2009; Lucia et al., 2013; Leatherwood et al., 2016; Kahn et al., 2017). Results from the present study are consistent with previous work (de Grauw et al., 2009; Lucia et al., 2013; Leatherwood et al., 2016; Kahn et al., 2017) in that LPS caused an increase in CPII concentrations, regardless of diet, with highest concentrations at 12 h (1663.09 ± 175.45 ng/mL) when compared with CON knees that peaked later at 24 h (1248.87 ± 175.45 ng/mL).

Potential dilution effects of biomarkers could be a confounding factor in synovial fluid biomarker analysis; thus, evaluation of ratios looking at the anabolic to catabolic processes in the joint may prevent biases (de Grauw et al., 2011; Te Moller and van Weeren, 2017). It also allows for observation of metabolic shifts. Previous data have shown a shift toward synthesis in response to inflammation. This shift allows for damage within the cartilage framework to repair; however, replacement of damaged matrix may not return the joint to its original state or function (Garvican et al., 2010). In the present study, the ratio of CPII to C2C was analyzed, and even though an increase in C2C was observed in CTM horses, the ratio of type II collagen synthesis to degradation was unchanged. The intra-articular LPS tended to increase the ratio due to minimal increases in anabolic processes, likely a result of damage caused by inflammatory mediators.

In conclusion, the intra-articular LPS challenge was sufficient in inducing inflammation, cartilage turnover, and aggrecan synthesis, thus allowing for the determination of dietary impact on these synovial fluid biomarkers. Compared with inorganic mineral sources, these data suggest that supplemental intake of a complexed TM source may support ECM turnover in response to an LPS

challenge as evidenced by an increase in type II collagen degradation and a more rapid rise in aggrecan synthesis. Additional research is needed to fully understand the impact of TMs and their physiological role within the joint and the ability of TM supplementation to delay the onset of joint disease in young horses.

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