Forms of selenium in vitamin-mineral mixes differentially affect serum prolactin concentration and hepatic glutamine synthetase activity of steers grazing endophyte-infected tall fescue

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ABSTRACT: The goal of this study was to test the hypothesis that sodium selenite (ISe), SEL-PLEX (OSe), vs. an 1:1 blend (MIX) of ISe and OSe in a basal vitamin-mineral (VM) mix would differentially affect metabolic parameters and performance of growing steers grazing toxic endophyte-infected tall fescue mixed forage (E+) pasture. Predominately-Angus steers (BW = 183 ± 34 kg) were randomly selected from herds of fall-calving cows grazing E+ pasture and consuming VM mixes that contained 35 ppm Se as ISe, OSe, and MIX forms. Steers were weaned, depleted of Se for 98 d, and subjected to summer-long common grazing of an E+ pasture (0.51 ppm total ergovaline per ergovalinine; 10.1 ha). Steers were assigned ($n = 8$ per treatment) to the same Se-form treatments upon which they were raised. Selenium treatments were administered by daily top-dressing 85 g of VM mix onto 0.23 kg soyhulls, using in-pasture Calan gates. The PROC MIXED procedure of SAS was used to assess effect of Se-form treatments on whole blood Se (ng/mL) and serum prolactin (ng/mL) at day 0, 22, 43, 64, and 86, and caudal arterial area (mm²) at day -7, 43, and 86. The effect of Se treatment on ADG (day 86), and liver glutamine synthetase

(GS) mRNA, protein, and activity (nmol/mg wet tissue/min) were assessed using the PROC GLM procedure of SAS. Fisher's protected LSD procedure was used to separate treatment means. Whole blood Se increased ($P < 0.01$) for all treatments from day 0 to 22 and then did not change ($P \geq 0.17$), and was greater ($P \leq 0.04$) for MIX and OSe steers. Serum prolactin decreased ($P < 0.01$) over time and was greater ($P < 0.05$) for MIX and OSe steers. Liver GS mRNA content was 66% and 59% greater ($P < 0.05$) in MIX and OSe steers, respectively, than ISe steers. Liver GS protein content in MIX steers was 94% more ($P < 0.01$) than ISe steers. Moreover, MIX and OSe steers had 99% and 55% more ($P \leq 0.01$) liver GS activity, respectively, than ISe steers. ADG was not affected ($P = 0.36$) by Se treatments. We conclude that consumption of 3 mg Se/d as OSe or MIX forms of Se in VM mixes increased 1) whole blood Se content, an indicator of greater whole-body Se assimilation; 2) serum prolactin, the reduction of which is a hallmark of fescue toxicosis; and 3) hepatic GS activity, indicating greater hepatic assimilation of acinar ammonia. However, 4) these positive effects on metabolic parameters were not accompanied by increased growth performance.

Key words: fescue toxicosis, glutamine synthetase, prolactin, selenium supplementation, steer

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J. Anim. Sci. 2018.96:715–727

doi: 10.1093/jas/skx068

This is publication No. 17-07-115 of the Kentucky Agricultural Experiment Station and is published with the approval of the Director. This work is supported by a United States Department of Agriculture-Agricultural Research Service Cooperative Agreement (JCM) and by the National Institute of Food and Agriculture, U.S. Department of Agriculture, Hatch project No. 1010352.

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Received October 13, 2017.

Accepted December 19, 2017.

INTRODUCTION

It is well established that cattle consuming tall fescue (*Lolium arundinaceum*) infected with a symbiont endophyte (*Epichloë coenophiala*) develop a syndrome of negatively altered physiological systems, collectively known as fescue toxicosis (Strickland et al., 2011). In addition to the classical decrease in prolactin, recent studies have identified that the hepatic metabolism of specific AA is altered in fescue toxicosis cattle (Brown et al., 2009; Liao et al., 2015), including a putative decrease in glutamine synthetase (GS) expression (Matthews and Bridges, 2014).

Another challenge to endophyte-infected tall fescue-based beef cattle operations is that the soils often are Se poor, necessitating the need to provide supplemental Se (Dargatz and Ross, 1996). Inorganic Se (ISe, sodium selenite) is the most common Se supplemented in cattle diets, whereas organic forms of Se (OSe), derived from specially cultivated *Saccharomyces cerevisiae*, also are available and approved for use in beef cattle diets. Supplementation (3 mg/d) with an 1:1 blend of ISe:OSe (MIX) or OSe vs. ISe results in greater amounts of Se in blood and liver of cattle (Brennan et al., 2011). Moreover, MIX stimulates expression of genes involved in hepatic selenoprotein synthesis and glutamate/glutamine metabolism relative to ISe or OSe (Matthews et al., 2014). Serendipitously, some of the genes upregulated by MIX were found downregulated in the liver (Liao et al., 2015) and pituitary (Li et al., 2017) of steers grazing high vs. low endophyte-infected forages.

The goal of this study was to test the general hypotheses that the form (ISe, OSe, and MIX) of supplemental Se in vitamin-mineral (VM) mixes consumed by growing beef steers grazing endophyte-infected tall fescue forage would ameliorate negative physiological parameters associated with fescue toxicosis, including depressed serum prolactin and hepatic GS.

MATERIALS AND METHODS

All experimental procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Animals

Twenty-four suckling, predominantly Angus, beef steers (BW: 182.6 ± 33.9 kg, age: 165.5 ± 14.2 day) were randomly selected ($n = 8$) from herds of fall-calving cows grazing toxic endophyte-infected tall fescue mixed forage pastures

and consuming VM mixes that contained 35 mg/kg Se as ISe (sodium selenite, Prince Se Concentrate; Prince Agri Products, Inc., Quincy, IL), OSe (SELPLEX, Alltech Inc., Nicholasville, KY), or an 1:1 blend of ISe:OSe (MIX) forms.

Steers were removed from their dams; housed in feedlot pens of 4; fed (ad libitum access) a hay, soyhull, and DDGS-based “adaptation” diet; and had ad libitum access to a basal free-choice, VM mix (see below) that lacked Se for 40 d. Then, over a 39-d period (in consecutive groups of six, two steers from each treatment group, 2 wk/group), steers were moved to an in-pasture Calan gate facility and trained to eat the adaptation diet from individual in-pasture Calan gates. During this 39-d period, steers continued to have ad libitum access to the Se-free basal VM mix. Next, over a 19-d period, steers were transitioned from the adaptation diet to a common mixed-forage pasture that lacked endophyte-infected tall fescue and trained to consume the Se-free basal VM mix from individual Calan gates by top-dressing 85 g of the basal VM mix onto 227 g soyhulls. In summary, over a 98-d period (March 11 to June 17, 2015) steers were weaned, commonly depleted of Se, and trained to consume VM mix from in-pasture Calan gates. During this pretrial Se-depletion period, the ADG of steers was 0.82 ± 0.18 kg/d.

Steers then began (day 0) an 86-d (June 17 to September 9, 2015) period in which they commonly grazed a predominately endophyte-infected tall fescue-mixed pasture (10.1 ha) and individually consumed their respective Se-form-specific VM mix treatments, through the use of in-pasture Calan gates. During this 86-d period, two ISe steers were removed from the trial due to a bad hoof and failure to consume their mineral treatment. Thus, the final number of experimental observations was as follows: ISe = 6, and OSe and MIX = 8.

The composition (analyzed by Dairy One Cooperative, Inc., Ithaca, NY) of the basal VM mix was 11.62% Ca; 4.88% P; 1.05% S; 1.95% Mg; 0.88% K; 9.95% Na; 1,321 mg/kg Fe; 3,388 mg/kg Zn; 1,761 mg/kg Cu; 5,262 mg/kg Mn; 0.98 mg/kg Mo; 19.07 mg/kg Co; 550,000 IU/kg vitamin A; and 550 IU/kg vitamin E. The Se-specific mixes contained the basal mix plus 35 mg/kg Se as ISe, OSe, or MIX. As noted above, to ensure individual consumption of 3 mg Se/d/steer, 85 g of the specific VM mix (35 mg/kg Se) was top-dressed onto 227 g of soyhulls in each individual Calan gate feeder. The consumption of the mixture of soyhulls/VM mix was monitored daily, and all steers consumed all of the mixture every day. Thus, every steer consumed 3 mg of supplemental Se/d.

On day 0 and 86, shrunk BW (steers were denied access to water and forage for 12 h) was determined to calculate ADG. After day 86 shrunk BW determination, steers were then returned to pasture and, within their Se treatment, randomly assigned to 1 of 8 slaughter d (from day 93 to 119) and maintained on their Se treatments until their slaughter.

Pasture Sampling and Analysis

Pasture samples were collected on day 0, 22, 43, 64, 86, and 115 for determination of ergot alkaloid (ergovaline and ergovalinine) concentrations as previously described (Brown et al., 2009). Samples were stored on ice during transportation to the laboratory and then frozen and stored at -20°C . Analysis of ergot alkaloids was performed by a high-performance liquid chromatography fluorescence procedure (Carter et al., 2010). Proximate analysis and mineral content were determined (<http://dairyone.com/wp-content/uploads/2014/02/Forage-Lab-Analytical-Procedures-Listing-Alphabetical-July-2015.pdf>) by Dairy One Cooperative, Inc.

Blood Collection

Jugular vein blood samples were collected by venipuncture on day 0, 22, 43, 64, and 86. For the preparation of whole blood, 8 mL of blood was collected in sodium heparin-containing blood collection tubes (Becton Dickinson, Franklin Lakes, NJ). For serum, 16 mL of blood was collected in serum blood collection tubes (Becton Dickinson) without an anticoagulant. Serum was recovered by centrifugation at $3,000 \times g$ for 10 min at 4°C and stored at -80°C . Whole blood Se concentrations were analyzed by Michigan State University Diagnostic Center for Population and Animal Health (DCPAH) using an Agilent 7900 inductively coupled plasma-mass spectrometer as described previously (Wahlen et al., 2005). Serum prolactin concentrations were quantified by the laboratory of Dennis Hallford (New Mexico State University), using a double antibody RIA as described previously (Spoon and Hallford, 1989). The interassay CV was 9.5%, and the intra-assay CV was 6.6%.

Slaughter and Tissue Collection

Steers were slaughtered over a 26-d period, from day 93 to 119 (September 17 to October 13, 2015) of the study. Specifically, one OSe and one MIX steer were killed on the first two slaughter d. On the remaining six slaughter d, one steer

each from ISe, OSe, and MIX treatment groups were killed. On any given slaughter day, steers were transported from the University of Kentucky Research and Education Center at Princeton, KY, to the University of Kentucky Meat Laboratory at Lexington, KY, and allowed to rest 1 to 2 h before slaughter and had ad libitum access to water. Body weight was determined, and steers were stunned by captive-bolt gun followed by exsanguination. Liver samples were collected from the mid-lower right lobe and used fresh for determination of GS activity. For real-time RT-PCR analysis, liver samples were placed in foil packs, snap-frozen in liquid nitrogen, and stored at -80°C . *Longissimus dorsi* (LM) tissue was collected from between the 12th and 13th rib from the left side of the carcass and used fresh for determination of GS activity.

Analysis of GS Activity in Hepatic and LM Tissue Homogenates

After collection, fresh liver and LM muscle tissue were put on ice and immediately transferred to the laboratory. About 0.4 g of tissue was homogenized (30,000 rpm for two 15 s cycles) in five volumes of ice-cold extraction buffer (pH 7.9), containing 50 mM Tris and 2 mM EDTA, using a PowerGen 124 homogenizer (Thermo Fisher Scientific, Waltham, MA). The homogenate was centrifuged at $2,000 \times g$ and 4°C for 10 min. The derived supernatant was assayed for GS (EC 6.3.1.2) activity using a radiochemical method modified from James et al. (1998). A 50 μL aliquot of the supernatant was mixed with 200 μL of reaction medium which consisted of 0.25 μCi L-[1- ^{14}C] glutamate (ARC, Saint Louis, MO; specific activity: 50 to 60 mCi/mmol), 25 mM unlabeled glutamate, 25 mM MgCl_2 , 25 mM NH_4Cl , 18.75 mM ATP, 12.5 mM phosphocreatine, 4 units creatine kinase, and 62.5 mM imidazole-HCl buffer, at pH 7.6. The mixture was incubated at 37°C for 20 min, and the reaction was then terminated by adding 1 mL of ice-cold 20 mM imidazole-HCl buffer, at pH 7.5. A sample of the incubate (1 mL) was loaded into a 4 mL prefilled (formate resin) ion-exchange column (Bio-Rad, Hercules, CA) previously rinsed by distilled water. The column was washed with 4 mL of distilled water, and the effluent was collected. A sample of effluent (1 mL) was mixed with 4 mL of a ScintiSafe Plus 50% cocktail (Thermo Fisher Scientific), and the radioactivity was measured in a liquid scintillation analyzer TRI-CARB 2900TR (PerkinElmer, Downers Grove, IL). Assays were conducted in triplicate. Positive control contained

2.5 units of GS from *Escherichia coli* (Sigma, Saint Louis, MO) instead of supernatant from liver or LM muscle tissue. Negative control contained neither supernatant nor GS. GS activity is expressed as nmol/min/mg wet mass. The intra- and interassay CV were 8.5% and 8.8%, respectively.

Caudal Artery Area Measurement

Ultrasound measures of caudal artery area were performed on day -7, 43, and 86 of the experiment as previously described (Aiken et al., 2007). Briefly, caudal artery at the fourth coccygeal vertebra was scanned by an Aloka 3500 Ultrasound Unit (Aloka Inc., Wallingford, CT) with a UST-5542 (13 MHz) linear array transducer set to a depth of 2 cm. Three cross-sectional color Doppler flow scans were taken to determine the mean artery lumen area (mm²).

RNA Extraction and Analysis

For each animal, total RNA was extracted from 300 mg of frozen liver tissue using TRIzol Reagent (Invitrogen Corporation, Carlsbad, CA) following the manufacturer's instructions. The purity and concentration of total RNA samples were analyzed using a NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). All samples had an average concentration of 1.05 µg/µL and a high purity with 260/280 absorbance ratios of 1.98 to 2.03 and 260/230 absorbance ratios ranging from 1.89 to 2.18. The integrity of total RNA was examined by gel electrophoresis using an Agilent 2100 Bioanalyzer System (Agilent Technologies, Santa Clara, CA) at the University of Kentucky Microarray Core Facility. Visualization of gel images and electropherograms showed that all RNA samples were of high quality with RNA integrity numbers (RIN) being greater than 8.7 and 28S/18S rRNA absorbance ratios greater than 2.

Real-Time RT-PCR Analysis

The quantification of relative mRNA for genes of interest was performed using standard procedures in our laboratory, as previously described (Cerny et al., 2016). Briefly, 1 µg of each steer's liver RNA was reversely transcribed to cDNA using the SuperScript III 1st Strand Synthesis System (Invitrogen). Real-time RT-PCR were performed using an Eppendorf Mastercycler ep realplex2 system (Eppendorf, Hamburg, Germany) with iQ SYBR Green Supermix (Bio-Rad). A total volume of 25 µL was used in each real-time RT-PCR

reaction containing 5 µL of cDNA, 1 µL of a 10 µM stock of each primer (forward and reverse), 12.5 µL of 2× SYBR Green PCR Master Mix, and 5.5 µL of nuclease-free water. The relative amount of each transcript was calculated using the 2^{-ΔΔCT} method (Livak and Schmittgen, 2001). Primer sets (Supplementary Table S1) for genes of interest were designed and obtained from NCBI Primer-BLAST tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) against RefSeq sequence (accessed February to April 2016).

All real-time RT-PCR cDNA products were validated by DNA sequencing verification and were 97% to 100% identical with their reference mRNA sequences (Supplementary Table S1). Briefly, the PCR-amplified cDNA products were electrophoresed in a 1.2% agarose (UltraPure Agarose, Invitrogen) slab gel. A single cDNA band at the desired size was identified under a UV light, excised from the gel, purified using the PureLink Quick Gel Extraction Kit (Invitrogen), then sequenced by Eurofins Genomics (Eurofins MWG Operon LLC, Louisville, KY). The resulting sequences (Supplementary Table S1) were compared with the NCBI RefSeq mRNA sequences used as templates for primer pair set design. Three constitutively expressed genes (GAPDH, PLOD1, and PCK2) were used, and their CT values were not affected (*P* > 0.43) by the Se-form treatment. Thus, the relative mRNA expression was normalized to the geometric means of three constitutively expressed genes. For the RT-PCR analysis, *n* = 6, 8, and 8 samples were used for ISe, OSe, and MIX treatments, respectively. RT-PCR reactions were performed in triplicate.

Immunoblot Analysis

All immunoblot and densitometric analyses for the relative expression of targeted proteins were performed using a standard protocol of our lab as previously described (Miles et al., 2015). Approximately 0.2 g of liver was homogenized on ice for 30 s (setting 11, Polytron Model PT10/35, Kinematic Inc., Lucerne, Switzerland) in 7.5 mL of 4 °C sample extraction buffer solution (0.25 mM sucrose, 10 mM HEPES-KOH pH 7.5, 1 mM EDTA, and 50 µL of protease inhibitor [Sigma]). Protein was quantified by a modified Lowry assay, using bovine serum albumin as a standard (Kilberg, 1989). Proteins were separated using 12% SDS-PAGE and electrotransferred onto a 0.45 µm nitrocellulose membrane (Bio-Rad) and then stained with Fast-Green (Fisher, Pittsburgh, PA). The relative amount of stained protein per

lane per sample was determined by densitometric analysis and recorded as arbitrary units (Brown et al., 2009).

The relative content of GS in liver homogenate was evaluated using an antibody as previously described (Miles et al., 2015). Briefly, blots were hybridized with 1.25 µg of IgG anti-sheep polyclonal antibody (BD Biosciences, San Jose, CA) per milliliter of blocking solution (5% nonfat dry milk [wt/vol], 10 mM Tris-Cl [pH 7.5], 100 mM NaCl, 0.1% Tween 20 [vol/vol]) for 1 h at 37 °C with gentle rocking. Protein-primary antibody binding reactions were visualized with a chemiluminescence kit (Pierce, Rockford, IL) after hybridization of primary antibody with horseradish peroxidase-conjugated goat anti-mouse IgG (BD Biosciences; GS, 1:5000).

Densitometric analysis of immunoreactive products was performed as described previously (Miles et al., 2015). After exposure of auto-radiographic film (Amersham, Arlington Heights, IL), digital images of all observed immunoreactive species were recorded and quantified as described (Dehnes et al., 1998) using the Bio-Rad Versadoc imaging system and the Quantity One Program (Version 4.2.3, Bio-Rad). A single immunoreaction product (GS, 42.5 kDa) was assessed for treatment effect by densitometric analysis. The linearity of antibody-ligand immunoreactions and densitometry were validated using immunoblots containing protein gradients (data not shown). Data were collected as arbitrary densitometric units and then were corrected for unequal loading and/or transfer of proteins by normalization to densitometric values of Fast-Green-stained proteins common to all immunoblot lanes/samples. The range of Fast-Green normalization for GS was 6.9% to 8.2%. For all results, densitometric values were normalized to ISe steers by obtaining an average ISe steer densitometric value and dividing all results by this value.

Statistical Analysis

Data are presented as least square means ($\pm$ SEM). Steers were the experimental units. The effect of Se supplementation on whole blood Se, serum prolactin, and caudal artery area was evaluated by ANOVA, using the PROC MIXED procedure of SAS (v 9.4, SAS Inst. Inc., Cary, NC). The statistical model included Se supplementation, time, and their interaction as fixed effects. class variables were Se supplementation and steer, with steer included in the random statement. The Kenward-Roger adjustment was used to calculate the denominator of df (Kenward and Roger, 1997). Within Se

supplement treatment, nonorthogonal polynomial contrasts (linear, quadratic, cubic, and quartic) were used to characterize the effect of Se treatments over time on whole blood Se and serum prolactin concentration, using the PROC IML procedure of SAS to generate coefficients for unequally spaced contrasts. After slaughter, the effect of Se supplementation on hepatic GS activity, and the relative abundance of hepatic GS mRNA and protein was assessed by ANOVA, using the PROC GLM procedure of SAS. Fisher's protected LSD procedure was used to separate treatment means of the time-course data.

RESULTS

Nutrient and Ergot Alkaloid Profiles of Forages

The composited means of proximate, mineral, and alkaloid analysis of pasture samples are presented in Table 1. The pasture samples from throughout the grazing periods had a CP of 16.3%, Se of 0.07 µg/g DM, and total ergot alkaloids of 0.51 µg/g.

Whole Blood Se Concentration

The concentration of Se in whole blood was affected by Se supplementation treatment ($P = 0.01$), time ($P < 0.01$), and their interaction ($P < 0.01$) (Figure 1A). Specifically, OSe steers and MIX steers had 11% ($P < 0.01$) and 7.5% ($P = 0.04$) more whole blood Se than ISe steers, respectively, and did not differ from each other ($P = 0.30$). Across Se supplementation treatments, the whole blood Se concentration increased ($P < 0.01$) 49% from day 0 to 22 and then did not change ($P \geq 0.17$) throughout day 86 (Figure 1A). Within Se treatments, non-orthogonal polynomial contrast analysis showed that the concentrations of whole blood Se essentially increased in a quadratic manner ($P < 0.01$) over time for all three Se treatments.

Serum Prolactin Concentration

The concentration of prolactin in serum was affected ($P < 0.05$) by Se treatment (Figure 1B). Across periods, OSe and MIX steers had 59% ($P < 0.03$) and 52% ($P < 0.05$) more serum prolactin than ISe steers, respectively. A time effect was found ($P < 0.01$) in serum prolactin concentration, but there was no ($P = 0.12$) Se treatment by time interaction. Across Se treatments, serum prolactin concentration decreased ($P < 0.01$) from

Table 1. Proximate, mineral, and alkaloid analysis of composited pasture samples (DM basis)¹

Item	Value
Proximate analysis, %	
DM	28.05
TDN	59.42
CP	16.26
Adjusted CP	15.84
ADF	37.58
aNDF	60.08
Crude fat	2.62
Lignin	6.46
Starch	0.89
Mineral analysis	
Ash, %	10.32
Calcium, %	0.6
Phosphorus, %	0.25
Magnesium, %	0.23
Potassium, %	2.08
Sodium, %	0.01
Sulfur, %	0.28
Iron, ppm	438.67
Zinc, ppm	28.5
Copper, ppm	6.17
Manganese, ppm	63.5
Molybdenum, ppm	1.68
Selenium, ppm	0.07
Ergot alkaloid analysis, µg/g	
Ergovaline	0.34
Ergovalinine	0.17
Total ergot alkaloids	0.51

¹Values are the average of samples collected on day 0, 22, 43, 64, 86, and 115, and presented on a DM basis. Samples were obtained systematically from approximately 30 sites from a common pasture, using a knife to cut the forage at approximately 2 cm above soil level.

day 0 to 22, plateaued ($P > 0.25$) from day 22 to 64, and then decreased ($P < 0.01$) again from day 64 to 86. Within Se treatments, nonorthogonal polynomial contrasts analysis revealed that serum prolactin essentially decreased in a linear ($P < 0.01$) manner for OSe and ISe steers. In contrast, serum prolactin essentially decreased in a cubic manner ($P < 0.01$) for MIX steers, apparently reflecting a decrease from day 0 to 22 and then from day 64 to 86.

GS Activity and Expression

At slaughter, Se treatment affected ($P < 0.01$) hepatic GS activity of steers (Figure 2), where MIX steers had 99% ($P < 0.01$) and 29% ($P = 0.03$) more activity than ISe and OSe steers, respectively, and OSe steers had 55% more ($P = 0.01$) than ISe steers. In contrast, Se treatment did not affect ($P = 0.21$) GS activity in LM tissue (Figure 2).

Figure 1. Whole blood selenium (Se) (A) and serum prolactin (B) concentrations in growing beef steers grazing endophyte-infected tall fescue and supplemented with 3 mg Se/d in VM mixes as sodium selenite (ISe), SEL-PLEX (OSe), or an 1:1 blend of ISe and OSe (MIX). Data are least squares means ($n = 6$ for ISe, $n = 8$ for OSe and MIX) $\pm$ SE. (A) Selenium form ($P = 0.01$), time ($P < 0.01$), and Se form by time interaction ($P < 0.01$) effects. Nonorthogonal polynomial contrasts: ISe, linear ($P < 0.01$), quadratic ($P < 0.01$), cubic ($P < 0.01$), and quartic ($P = 0.03$) effects; OSe, linear ($P < 0.01$), quadratic ($P < 0.01$), and cubic ($P = 0.03$) effects; MIX steers, linear ($P < 0.01$), quadratic ($P < 0.01$), and quartic ($P = 0.04$) effects. (B) Selenium form ($P < 0.05$) and time ($P < 0.01$) effects. Nonorthogonal polynomial contrasts: ISe, linear ($P < 0.01$) effect; OSe, linear ($P < 0.01$) effects; MIX, linear ($P < 0.01$) and cubic ($P < 0.01$) effects.

The relative expression of hepatic GS mRNA differed ($P = 0.02$) among Se treatment steers (Table 2). MIX steers had 66% more ($P < 0.01$) hepatic GS mRNA expression than did ISe steers, and there was 59% more ($P < 0.05$) hepatic GS mRNA in OSe than in ISe steers. However, hepatic GS mRNA content did not differ ($P = 0.75$) between OSe and MIX steers. Similarly, the relative hepatic GS protein content was affected ($P = 0.02$) by Se treatments (Table 2; Figure 3). Specifically, MIX steers had 94% more ($P < 0.01$) hepatic GS protein than ISe steers. However, the hepatic GS protein content of OSe steers did not differ ($P > 0.05$) with that of both ISe and MIX steers.

Caudal Artery Area

Selenium form did not affect ($P = 0.65$) caudal artery area, nor ($P = 0.89$) was there a Se form

× time interaction (Figure 4). However, there was a time effect ($P = 0.03$). Specifically, across Se treatments, the caudal artery area at day 43 ($6.19 \pm 1.3 \text{ mm}^2$) was 41% greater ($P = 0.03$) than day -7 ($4.4 \pm 0.69 \text{ mm}^2$) and 40% greater ($P = 0.02$) than day 86 ($4.41 \pm 0.77 \text{ mm}^2$), whereas the caudal area did not differ ($P = 0.98$) between day -7 and 86.

Animal Performance

Final BW were 293.6, 293.5, and 272.0 kg, respectively, for ISe, OSe, and MIX steers, and did not differ ($P = 0.34$). Average daily gain was 0.43, 0.53, and 0.47 kg/day for ISe, OSe, and MIX steers, respectively, and was not affected by Se treatment ($P = 0.36$).

Figure 2. GS activity in liver and *Longissimus dorsi* (Muscle) homogenates of growing beef steers grazing endophyte-infected tall fescue and supplemented with 3 mg Se/d in VM mixes as either sodium selenite (ISe), SEL-PLEX (OSe), or an 1:1 blend of ISe and OSe (MIX). Data are least squares means ($n = 6$ for ISe, $n = 8$ for OSe and MIX) $\pm$ SE. Selenium form effect on liver GS activity ($P < 0.01$). Means with different letters differ ($P < 0.05$).

DISCUSSION

Animal Model

The goal of this study was to test the general hypotheses that the form of Se (ISe, OSe, and MIX) in VM mixes would affect the expression of important physiological parameters in growing beef steers commonly grazing an endophyte-infected tall fescue pasture. Steers had ad libitum access to their respective supplemental Se treatments as suckling calves before selection into the trial. During a pre-trial Se-depletion period (98 d), steers received the basal VM mix that lacked any form of Se. From initiation of the trial to slaughter, steers individually consumed 85 g/d of a basal VM mix that contained 35 ppm Se supplied as ISe, OSe, or MIX forms. Thus, steers consumed 3 mg Se/d of their respective Se from initiation of the grazing period until their slaughter (up to 113 d) by use of Calan gates. The 3 mg Se/animal daily supplementation is the legal maximal limit for free-choice VM mixes allowed by the Food and Drug Administration and is considered adequate to achieve the NRC (NRC, 1996) recommendation of 0.1 mg/kg of diet. We chose to use a Se-adequate model to avoid confounding effects of Se deficiency and to legitimately compare the effect of Se form on experimental parameters.

Steers commonly grazed a single 10.1 ha predominantly tall fescue-mixed pasture, which contained 0.51 ppm total ergot alkaloids (Table 1). In a previous study by our group (Brown et al., 2009), a similar level of ergot alkaloid challenge (0.52 ppm) induced fescue toxicosis in beef steers grazing endophyte-infected tall fescue and consuming ad libitum amounts of a similar VM mix that contained the ISe form of Se. Importantly, these steers had similar concentrations of serum prolactin and levels of ADG, as did the ISe steers of this study. The rapid increase then stabilization of Se assimilation

Table 2. Comparison of glutamine synthetase mRNA and protein content in liver homogenates of growing beef steers grazing endophyte-infected tall fescue and supplemented with 3 mg Se/d in VM mixes as either sodium selenite (ISe), SEL-PLEX (OSe), or an 1:1 blend of ISe and OSe (MIX)¹

	Treatment ²			SEM ³	P value
	ISe	OSe	MIX		
Glutamine synthetase mRNA ⁴	1.03 ^a	1.64 ^b	1.71 ^b	0.15	0.02
Protein ⁵	2,098 ^a	3,041 ^{ab}	3,632 ^b	331	0.02

¹Values are least squares means ($n = 6$ for ISe, $n = 8$ for OSe and MIX) and pooled SEM.

²Means within a row that lack a common letter differ ($P < 0.05$).

³Most conservative error of the mean.

⁴Values are relative level of mRNA expression.

⁵Values (arbitrary densitometric units) were determined by densitometric evaluation of Western blot data (Figure 3).

Figure 3. Western blot analysis of glutamine synthetase protein (42.5 kDa) content in liver homogenates (20 µg/lane) of growing beef steers grazing endophyte-infected tall fescue and supplemented with 3 mg Se/d in VM mixes as sodium selenite (ISe), SEL-PLEX (OSe), or an 1:1 blend of ISe and OSe (MIX).

Figure 4. Caudal artery area in growing beef steers grazing endophyte-infected tall fescue and supplemented with 3 mg Se/d in VM mixes as sodium selenite (ISe), SEL-PLEX (OSe), or an 1:1 blend of ISe and OSe (MIX). Data are least squares means ($n = 6$ for ISe, $n = 8$ for OSe and MIX) $\pm$ SE. Time ($P = 0.03$) effect.

in the whole blood of all three Se treatment groups is consistent with our previous Se depletion/repletion regimens with growing cattle (Brennan et al., 2011; Liao et al., 2011). Thus, the animal model of this study was successfully established to properly evaluate the potential effect of different Se-form treatments on physiological parameters of growing beef steers subjected to summer-long grazing of toxic endophyte-infected tall fescue pasture.

OSe and MIX Forms of Se Supplementation Resulted in Greater Whole Blood Se Than ISe

Selenium status is commonly determined by measuring the concentration of Se in blood or tissues, or the activity of glutathione peroxidase in blood. Whole blood Se concentrations are well correlated with Se intake (Patterson et al., 2013). Although there is no clear agreement in the literature as to what concentrations of whole blood Se are considered to be deficient or adequate, studies have reported that cattle have adequate levels of Se when whole blood Se is greater than 100 ng/

mL (Gerloff, 1992) or between 81 to 160 ng/mL (Dargatz and Ross, 1996). In this study, across-periods, mean whole blood Se concentration ranged from 154 to 231 $\pm$ 5.4, 181 to 252 $\pm$ 4.7, and 153 to 257 $\pm$ 4.7 ng/mL in ISe, OSe, and MIX steers, respectively, indicating that all steers were Se adequate. Importantly, MIX and OSe steers had greater whole blood Se concentrations than did ISe steers. This finding is in agreement with previous studies, where greater whole blood Se was found in grazing cows supplemented with OSe at 3.3 mg/day·animal (Pehrson et al., 1999) or had ad libitum access to Se-containing mineral mixes (Gunter et al., 2013; Patterson et al., 2013). Of note, supplementation (3 mg/day) of slow maturing beef heifers with the MIX form of Se for 224 days resulted in similar Se concentrations in whole blood, red blood cells, serum, and liver compared with that supplemented with OSe (Brennan et al., 2011). Consistently, in this study with growing grazing steers, the whole blood Se concentration of MIX steers also was essentially the same as for OSe steers. This finding suggests that the MIX form of Se (1:1 equal blend

of ISe and OSe) was assimilated as efficiently as was the 100% OSe.

Concentrations of Serum Prolactin Were Greater in MIX and OSe Steers

Reduced serum prolactin concentrations are a hallmark of fescue toxicosis (Goetsch et al., 1987; Davenport et al., 1993). One mechanism by which ergot alkaloids exert their inhibitory effect on serum prolactin is to bind dopamine receptor D₂ (Larson et al., 1994), resulting in suppression of prolactin synthesis and release by lactotrophs within the anterior pituitary (Schillo et al., 1988; Li et al., 2017). In this study, OSe and MIX steers had greater serum prolactin concentrations than ISe steers throughout the grazing period. A priori, this novel finding suggests that supplemental OSe interacts in some manner with the prolactin synthetic cascade or enzymes involved in the degradation of circulating prolactin.

With regard to the first possibility, it may be reasonable to speculate that potential links exist between Se and prolactin given the critical roles of Se and selenoproteins in neurotransmission and brain function (Solovyev, 2015). More specifically, consumption (1 to 9 µg/g DM) of ISe (selenite), but not OSe resulted in increased dopamine concentrations in murine striatum (Tsunoda et al., 2000). As noted above, one of the key roles of dopamine is to negatively regulate prolactin secretion by binding and stimulating the dopamine receptor D₂-mediated inhibition of prolactin synthesis (Fitzgerald and Dinan, 2008). The greater serum prolactin concentrations of MIX and OSe vs. ISe steers in this study appears consistent with the understanding that selenite supplementation increases dopamine content, accompanied by decreased prolactin synthesis in mouse brain models. Thus, it may be reasonable to speculate that the serum prolactin difference in this study may be due to the different dopamine concentrations induced by different form of Se. However, more research is needed about the expression and function of key enzyme/signaling proteins involved in lactotroph dopamine–prolactin interactions (such as dopamine receptor D₂ (Beaulieu and Gainetdinov, 2011), prolactin receptor (Bernard et al., 2015), and Jak-Stat cascades (Rawlings et al., 2004)) to support this speculation.

As discussed early, consumption of endophyte-infected tall fescue suppresses serum prolactin. Unsurprisingly, in this study, the serum prolactin concentration decreased (86%) from day 0 to its nadir on day 86, regardless of the Se-form

treatments of the steers. More specifically, from day 0 to 22, the serum prolactin concentration of all steers quickly dropped 43%, accounting for approximately half of the total reduction. The rapid decrease of serum prolactin concentrations after consumption of forages containing endophyte-infected tall fescue has been reported in steers (Aldrich et al., 1993; Jackson et al., 2015). The serum prolactin depression from day 0 to 84 observed in this study was approximately 86% (from 73.4 to 10.1 ng/mL). This magnitude of the reduction is in agreement with reported reductions of serum prolactin concentrations of 80% (Aldrich et al., 1993), 81% (Schillo et al., 1988), and 90% (Brown et al., 2009) in cattle consuming endophyte-infected vs. endophyte-free tall fescue.

This summer-long grazing study was conducted during June to September of 2015. Because reduction of photoperiod decreases concentrations of circulating prolactin in many species, including cattle (Dahl et al., 2000), it should be noted that the decrease in daylight also may have contributed to the depression of serum prolactin in steers from all three Se treatments.

Hepatic GS Activity, mRNA, and Protein Expression Are Greater in MIX Than ISe Steers

Part of the general hypotheses of this trial was that the form of supplemental Se would affect GS expression. This hypothesis was derived from a preliminary understanding (Matthews and Bridges, 2014) that the MIX form of Se increases the content of GS mRNA in steer liver, whereas the consumption of endophyte-infected tall fescue mixed forage decreases hepatic GS mRNA content. Thus, an important finding from this study was that hepatic GS activity was strongly affected (MIX > OSe > ISe) by the form of Se in the VM mix (Figure 2). In contrast, LM GS activity was not affected by Se-form treatment. Hepatic GS expression is restricted to pericentral hepatocytes (Haussinger, 1990). As such, GS is involved in scavenging ammonia that escapes periportal hepatocyte detoxification (urea cycle) by synthesizing glutamine from sinusoidal ammonia and glutamate (Wagenaar et al., 1994). Given the marked increase in hepatic GS activity by MIX and OSe steers, it is reasonable to expect that they possessed an increased N recycling capacity.

Although the regulatory mechanisms of GS have not been clearly defined, several studies indicate that GS activity is at least partially under transcriptional regulation (Gaunitz et al., 2001). More specifically, regulatory elements have been reported

in the first intron (Fahrner et al., 1993) and in 5' upstream regions (Gaunitz et al., 2001) of the GS gene. These regions contain binding sites for various transcription factors and have a functional role in the organ-specific expression patterns of GS, and in the restricted pericentral expression of GS within the liver (Lie-Venema et al., 1995). Moreover, it has been reported that posttranscriptional regulations such as alternative splicing also may contribute to the tissue-specific expression of GS (Shin et al., 2003). Although GS isolated from brain and other tissues of the same species have identical sequences, tissue-specific differences in substrate feedback regulation exist (Tate et al., 1972). Therefore, post-translational modifications may exist (Eisenberg et al., 2000).

To gain insight into potential transcription, translational, or both, processes accounting for Se-form differences in hepatic GS activity, the relative mRNA, and protein abundance of GS was determined (Table 2). In agreement with the 99% greater hepatic GS activity, MIX steers had a 66% and 94% more hepatic GS mRNA and protein, respectively, than ISe steers. Although statistically not different from ISe steers, the 50% quantitatively greater GS protein content of OSe vs. ISe steers is consistent with the 59% greater mRNA content and 55% greater GS activity. Collectively, these data suggest that the MIX and OSe forms of Se affected at least the transcriptional regulation of hepatic GS.

Little is known about the potential for Se or selenoproteins to interact with *GLUL* (gene for GS) or GS. However, it has been reported that long-term supranutritional supplementation of sodium selenite to diabetic mice (vs. no supplementation) results in increased GS mRNA and protein (Wang et al., 2014). Although the specific mechanism(s) is unclear, our current finding in steer liver, coupled with this limited report in mouse liver, suggests that hepatic GS expression and activity may be sensitive to the form of Se and, possibly, the amount of supplemental Se.

Caudal Artery Area and Animal Performance Were Not Affected by Se Treatment

Caudal artery area. Consumption of ergot alkaloids by cattle is known to constrict the caudal artery (Aiken et al., 2007), right ruminal artery and vein (Foote et al., 2011), and mesenteric artery and vein (Jia et al., 2015). Ergot alkaloid-induced vasoconstriction is thought to be a contributor to several symptoms of fescue toxicosis (Klotz

and Nicol, 2016). Therefore, the caudal artery luminal area was determined in this study as a parameter to assess potential Se-form treatment differences. However, the caudal artery area was not affected by Se treatment. The range of the caudal artery areas was 4.40 to 6.19 mm², which was higher (thus, less restricted) than the 2.0 to 3.8 mm² (Aiken et al., 2007) and 1.2 to 5.0 mm² (Aiken et al., 2009) reported for heifers (345 to 375 kg) consuming endophyte-infected tall fescue seed plus chopped alfalfa hay/concentrate diets. The difference in caudal artery areas in this study employing grazing steers and the earlier studies using confined heifers may be due to the difference of ergot alkaloids load. That is, in this study, the pasture contained 0.51 ppm ergovaline and ergovalinine, whereas endophyte-infected seed containing 1.17 (Aiken et al., 2009) to 1.21 ppm (Aiken et al., 2007) of ergovaline and ergovalinine were consumed by confined heifers. Another possible explanation for the relatively higher caudal artery area in this study may be an inherent difference between heifers and steers in artery size and luminal diameter. For example, intravascular ultrasound analysis has revealed that left main and proximal left anterior descending coronary arterial areas were smaller in women than in men (Sheifer et al., 2000).

Growth performance. As noted above, the amount of ADG achieved by the ISe steers (0.43 kg/d) is almost identical to that achieved (0.40) by beef steers consuming endophyte-infected tall fescue pastures (0.52 ppm ergovaline/ergovalinine) and that had ad libitum access to ISe-containing (35 ppm) VM mix over a 85-d summer-long grazing period (Brown et al., 2009). In mammals, prolactin affects various physiologic parameters besides lactation, including osmoregulation, immunoregulation, and growth and development (Bole-Feysot et al., 1998). Moreover, prolactin has a somatogenic effect on postnatal body growth and BW of male rats (Perez-Villamil et al., 1992). However, the ADG by ISe steers did not differ from that by MIX or OSe steers over the 86-d grazing period. The form of Se (ISe, OSe, MIX) also does not affect the ADG of slow-growing beef heifers supplemented with 3 mg Se/day (Brennan et al., 2011). Similarly, no significant improvement on animal growth performances (ADG, ADI, or gain: feed ratio) has been reported by studies comparing the effects of OSe and ISe supplementation (Lawler et al., 2004; Brennan et al., 2011).

In summary, predominately Angus steers subjected to summer-long grazing of endophyte-infected

pasture and supplemented (3 mg/d) with MIX or OSe forms of Se had greater whole blood Se, serum prolactin concentrations, and greater hepatic GS activity than ISe-supplemented steers. Despite these effects, no difference in growth performance was found.

SUPPLEMENTARY DATA

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

Conflict of interest statement. None declared.

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