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Effect of inorganic or organic copper fed without or with added sulfur and molybdenum on the performance, indicators of copper status, and hepatic mRNA in dairy cows

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ABSTRACT

The effect of inorganic (INORG) or organic (ORG) Cu, fed without (−) or with (+) additional S and Mo on Cu status and performance was examined using 56 early lactation dairy cows in a 2 × 2 factorial study design. Supplementary Cu was added as either $CuSO_4$ or Bioplex Cu (Alltech Inc., Nicholasville, KY) to provide an additional 10 mg of Cu/kg of dry matter (DM), with S added at 1.5 g/kg of DM and Mo at 6.8 mg/kg of DM to reduce Cu bioavailability. The basal ration was composed of corn and grass silages (2:1 respectively, DM basis) and straight feeds. Cows commenced the study at wk 7 of lactation and remained on treatment for 16 wk. An interaction existed between Cu source and added S and Mo on DM intake, with cows offered INORG− Cu having an increased intake compared with those offered INORG+ or ORG− Cu. Milk yield averaged 35.4 kg/d, and was 5% higher with milk fat content 6% lower in cows fed INORG compared with ORG Cu, but milk fat yield, energy-corrected milk yield, and milk protein content did not differ between treatments. A trend existed for cows to have a higher body weight gain when offered ORG compared with INORG Cu. Cows fed diets containing INORG Cu had a higher milk concentration of C17:0 and C18:3n-3 compared with those fed diets containing ORG Cu. Cows fed added S and Mo had a lower milk concentration of C17:0 and C18:0 compared with those that were not supplemented. No effect was observed of dietary treatment on plasma Cu concentration, which averaged 13.1 μmol/L, except during wk 12 when cows receiving added S and Mo had a lower concentration. No effect was observed of Cu source on mean plasma Mo concentrations, but during wk 16 cows offered INORG Cu had a higher concentration than those offered ORG Cu. Hepatic Cu levels decreased by approximately 0.9 mg/kg of DM per day when fed additional S and Mo, but no effect of Cu source was observed. A trend existed for hepatic ATPase, Cu++ transporting, beta polypeptide (ATP7B) to be upregulated in cows when fed S and Mo along with ORG but not INORG Cu. In conclusion, the inclusion of an ORG compared with an INORG source of Cu reduced milk yield but increased milk fat concentration and body weight gain, with no effect on energy-corrected milk yield. Little effect was observed of dietary Cu supply on plasma mineral concentration, liver mRNA abundance, or milk fatty acid profile, whereas the addition of S and Mo reduced hepatic Cu concentrations.

Key words: copper, dairy cow, liver, milk production

INTRODUCTION

Interest in Cu in ruminant diets centers around its role as an essential trace element required within numerous key enzymes, including cytochrome c oxidase, tyrosinase, lysyl oxidase, and ceruloplasmin (Suttle, 2010). As a consequence, a deficiency of Cu is related to an impairment of growth, reproduction, connective tissue development, and pigmentation (McDowell, 1985). A deficiency in Cu can be regarded as being either primary, due to a lack of Cu in the diet, or secondary, whereby an interaction exists between Cu and antagonists that reduce its absorption or function (Phillippo et al., 1987). The most widely researched antagonists include S, Mo, and Fe, and it is generally regarded that secondary deficiency is more common and economically important (Suttle, 2010). A 3-way interaction exists between Cu, S, and Mo that reduces the absorption of Cu, with sulfide forming within the rumen from dietary sulfate, which then reacts with molybdate to form insoluble Cu thiomolybdates (Suttle, 1991; Gould and Kendall, 2011). Interest in this interaction has been revived recently with the suggestion that Mo toxicity occurs where, under conditions of high Mo (>8 mg/kg of DM) and low Cu:Mo ratios, thiomolybdates are absorbed into the blood stream and may have inhibitory effects on critical metalloenzymes, although Mo toxicity is a controversial subject (Telfer et al., 2004).

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It has been suggested that supplying minerals in an organic rather than inorganic form may increase their bioavailability, increasing absorption in the gastrointestinal tract (Spears, 1996) and altering physiological function (Cope et al., 2009). A form of dietary Cu that avoids the interaction with S and Mo in the rumen, yet is absorbed in the small intestine, is a desirable characteristic of a supplementary source. The effects of supplying Cu in an organic form on ruminant performance and health are, however, conflicting. For example, Chase et al. (2000) reported no benefit to Cu-lysine on intake, milk performance, or SCC in dairy cows, although a trend was observed for plasma Cu levels to be increased on the organic Cu treatment. Similarly, Du et al. (1996) reported no effect of Cu-proteinate or $CuSO_4$ on animal performance. Studies that have reported a benefit to supplying Cu in an organic form are often confounded by supplementation with a combination of minerals (Nocek et al., 2006; Rabiee et al., 2010). It was suggested by Ward et al. (1996) that any benefits to supplementing Cu in an organic form would be more apparent in situations of high dietary concentrations of antagonists to Cu absorption. The objectives of the current study were to determine the effect of supplementing dietary Cu in an inorganic or organic (proteinate) form, either without or with dietary Cu antagonists (S and Mo) on the Cu status, performance, milk FA content, and hepatic mRNA expression in early lactation dairy cows.

MATERIALS AND METHODS

Animals, Management, and Treatments

All the procedures involving animals were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986. Based on recordings taken in the week before allocation, 56 dairy cows (16 primiparous and 40 multiparous) were stratified according to parity (primiparous or multiparous), DIM (35 d; SD: 12.8), milk yield (36.9 kg/d; SD: 7.71), and BW (627 kg; SD: 69.0) and randomly allocated to 1 of 4 dietary treatments. Cows remained on study for 16 wk.

All cows received a TMR based on corn and grass silages (2:1 DM basis) and straight feeds, formulated according to Thomas (2004; Table 1). The basal diet was predicted to supply 6.99 and 1.67 mg/kg of DM of Cu and Mo, respectively, and 1.68 g of S/kg of DM. Supplementary Cu was added as either $CuSO_4 \cdot 5H_2O$ or Bioplex Cu (Alltech Inc., Nicholasville, KY) to provide an additional 10 mg of Cu/kg of DM and be approximately 50% in excess of NRC (2001) requirements. Bioplex Cu is a Cu proteinate and contains 10% Cu. Within each of the supplementary Cu sources, a Cu antagonist mix was added that contained ammonium sulfate (TG Tennants, West Bromwich, UK) and sodium molybdate (Acros Organics, Geel, Belgium) to supply an additional 1.5 g of S/kg of DM and 6.8 mg of Mo/kg of DM. To balance for the increased rumen-degradable N supplied by the ammonium sulfate, animals not receiving additional S were fed an equivalent amount of N as feed-grade urea. This resulted in 4 dietary treatments: 10 mg/kg of DM of supplementary Cu as $CuSO_4$ (inorganic, **INORG**), no added S or Mo (**INORG−**); 10 mg/kg of DM of supplementary Cu as $CuSO_4$, with added S and Mo (**INORG+**); 10 mg/kg of DM of supplementary organic (**ORG**) Cu, no added S or Mo (**ORG−**); and 10 mg/kg of DM of supplementary ORG Cu, with added S and Mo (**ORG+**).

All dietary ingredients were mixed daily and fed as a TMR using a forage mixer calibrated to ±1 kg and fed through Insentec roughage intake feeders (RIC feeders; Insentec BV, Marknesse, the Netherlands) fitted with an automatic animal identification and forage weighing system calibrated to ±0.1 kg (Sinclair et al., 2005). Fresh feed was offered at 1.05 of ad libitum intake with refusals collected twice weekly. The cows were housed in the same portion of a building containing freestalls fitted with foam mattresses. The passageways were scraped every 4 h by automatic scrapers, and the stalls bedded twice weekly with chopped, limed paper. All cows had continual access to water.

Experimental Routine

Cows were milked twice daily at approximately 0600 and 1600 h, with yield recorded at each milking and subsamples taken fortnightly at 2 consecutive milkings for subsequent analysis of fat, protein, and lactose. Energy-corrected milk yield (kg) was calculated as follows: (0.0384 × g/kg of milk fat + 0.0223 × g/kg of milk CP + 0.0199 × g/kg of milk lactose − 0.108) × kg of milk/3.08 (AFRC, 1993). During the first and final week of the study, additional milk samples were collected and the fat extracted by the method of Feng et al. (2004) and stored at −20°C before the subsequent determination of milk FA content. The cows were weighed and condition scored (Lowman et al., 1976) after an evening milking during the week before allocation, and then every 2 wk. Forage samples were collected weekly and split into 2; one sample was oven dried and the ratio of corn to grass silage was adjusted to achieve the desired ratio of 2:1 (DM basis). The other sample was frozen at −20°C and bulked before analysis. Weekly samples of caustic-treated wheat were also taken, oven dried, and the dietary inclusion rate adjusted. Samples of the TMR were collected weekly immediately following feed-out, and stored at −20°C. Blood samples were

Table 1. Chemical composition of diets that contained inorganic (INORG) or organic (ORG) Cu fed without (−) or with (+) added S and Mo[1]

	Diet			
Item	INORG−	INORG+	ORG−	ORG+
Ingredient, g/kg of DM				
Grass silage	202	201	202	201
Maize silage	405	402	405	402
Caustic-treated wheat	84	84	84	84
Sugar beet pulp	70	70	70	70
Rapeseed meal	79	79	79	79
Soybean meal	88	88	88	88
Sunflower meal	20	20	20	20
Rouxminate liquid feed[2]	33	33	33	33
Protected fat[3]	13	13	13	13
Feed-grade urea	4	—	4	—
Ammonium sulfate[4]	—	8	—	8
Minerals and vitamins[5]	4	4	4	4
Chemical analysis				
DM, g/kg	407	400	403	398
CP, g/kg of DM	168	168	171	169
NDF, g/kg of DM	396	387	388	397
Ash, g/kg of DM	78	81	78	81
Ca, g/kg of DM	6.5	6.4	6.2	7.1
P, g/kg of DM	4.2	4.2	4.2	4.5
Mg, g/kg of DM	2.6	2.8	2.5	2.8
Cu, mg/kg of DM	16.4	17.7	16.4	17.3
Mo, mg/kg of DM	1.21	8.81	1.23	8.85
Fe, mg/kg of DM	271	277	259	286
Zn, mg/kg of DM	52.7	58.9	52.4	60.4
S, g/kg of DM	2.35	3.48	2.29	3.50

[1]Dietary treatments INORG− and INORG+ also included 10 mg of Cu/kg of DM as $CuSO_4$ and ORG− and ORG+ included 10 mg of Cu/kg of DM as Bioplex Cu. Diets INORG+ and ORG+ also received 6.8 mg of Mo/kg of DM as sodium molybdate dehydrate.

[2]KW Feeds (Leeds, UK). A liquid feed produced from a blend of co-products from the sugar and wheat-processing industries.

[3]Calcium salts of long-chain palm FA (Nutrion Internacional SL, Madrid, Spain).

[4]TG Tennants (West Bromwich Essex, UK).

[5]Mineral/vitamin premix (Rumenco Ltd., Staffordshire, UK). Major minerals (g/kg): Ca, 240; P, 80; and Mg, 120; trace minerals (mg/kg): Cu, 0; Zn, 7,000; Mn, 2,000; I, 400; Co, 80; and Se, 50; vitamins (mg/kg): retinol, 105; cholecalciferol, 1.75; and all *rac* α-tocopherol acetate, 5,000.

collected from the jugular vein at 1000 h during wk 0, 1, 2, 4, 8, 12, and 16 of the study into Vacutainers (Becton Dickinson Vacutainer Systems, Plymouth, UK) containing EDTA (for samples used to determine whole blood hematology), silica (for samples used to determine ceruloplasmin), or lithium heparin (for samples used to determine superoxide dismutase activity) and sodium heparin (for samples used to determine mineral concentrations). After centrifuging at 1,000 × *g* for 15 min at 4°C the plasma was removed and stored at −20°C. Silica tubes were refrigerated for 24 h before centrifuging and subsequent removal of serum, which was stored at −20°C before analysis. Samples collected into EDTA tubes were analyzed directly for hematology, and a subsample of whole blood stored at −20°C. Liver biopsy samples were obtained from all animals during wk 0 and 16 of the study by insertion of a needle through the 11th intercostal space using the procedure described by Davies and Jebbett (1981). The sample was immediately split into 2; approximately 500 mg was stored at −80°C, whereas approximately 300 mg was immersed in 1 mL of RNAlater (Ambion Inc., Austin, TX), stored at 4°C for 24 h, the RNAlater was removed after centrifugation at 13,000 × *g* for 10 min at room temperature, and the sample was stored at −80°C

Chemical Analysis

Weekly TMR samples were bulked within month and analyzed according to AOAC International (2000) methods for DM (934.01) and CP (988.05), whereas NDF was determined according to Van Soest et al. (1991). Neutral detergent fiber determination was conducted without sodium sulfite, with α-amylase, and corrected for ash. Metabolizable energy content of the forages was determined by near-infrared reflectance spectroscopy at AFBI Hillsborough (Hillsborough, Co. Down,

UK). Dietary Cu, Fe, Zn, Mn, and Mo were extracted using the DigiPREP digestion system (QMX Laboratories Ltd., Essex, UK) and analyzed by inductively coupled plasma-mass spectrometry (**ICP-MS**; Thermo Fisher Scientific Inc., Hemel Hempstead, UK) following dilution in 2% HNO_3, 1% methanol, and 0.1% Triton X-100 (Sigma-Aldrich, Dorset, UK), as described by Cope et al. (2009). Milk samples were analyzed using a MilkoScan Minor (Foss UK Ltd., Warrington, UK) calibrated by the methods of AOAC International (2000). Fatty acid methyl esters were prepared by base-catalyzed transmethylation according to Christie (1982), with modifications by Chouinard et al. (1999), as described by Lock et al. (2006). Fatty acid methyl esters were quantified using a gas chromatograph (GC system 6890+ with flame ionization detector; Agilent Inc., Wilmington, DE) equipped with a CP-SIL 88 fused-silica capillary column [100 m × 0.25 mm (i.d.) with 0.2-μm film thickness; Varian Inc., Walnut Creek, CA], with hydrogen as the carrier gas and a programmed temperature sequence; further details and conditions have been described previously (Lock et al., 2006). Fatty acid identification and recoveries were determined using pure methyl ester standards (Nu-Chek Prep, Elysian, MN; Natural ASA, Hovdebygda, Norway) for the C18 unsaturated FA. Additionally, a FA methyl ester mix (47885-U; Supelco, Gillingham, UK) was used for the identification of other FA (C8–C20). Lipids in feed were extracted and the FA were methylated and quantified according to Sukhija and Palmquist (1988) with modifications by Loor et al. (2004) using nonadecanoic acid as an internal standard. Gas chromatography analysis and column conditions were the same as that for milk FA analysis. Whole blood samples were analyzed for white blood cells, red blood cells, and hemoglobin using a Vet Animal Blood Counter (Woodley Equipment Company Ltd., Bolton, UK). Serum samples were analyzed for ceruloplasmin according to Henry et al. (1974) and whole blood samples for superoxide dismutase (**SOD**; Randox Laboratories, Crumlin, Co. Antrim, UK; kit catalog no. SD 125) using a Cobas-Mira Plus autoanalyzer (ABX Diagnostics, Bedfordshire, UK). Additionally, plasma samples were analyzed for Cu, Fe, Zn, Mn, and Mo by ICP-MS by diluting 1:20 in 0.5% HNO_3 (Cope et al., 2009). Liver samples were analyzed for Cu, Mo, Zn, Mn, and Fe by ICP-MS after digestion overnight at 60°C in concentrated nitric acid. Samples were made up to 50 mL in a DigiPREP tube (QMX Laboratories Ltd.) and diluted (1:20) in 2% HNO_3, 1% methanol, and 0.1% Triton X-100 and analyzed by ICP-MS.

Hepatic total RNA was extracted using the SV Total RNA Isolation kit that included a 15-min DNase treatment (Promega Corp., Madison, WI). The concentration and purity of the isolated RNA samples was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). All samples had an absorbance at 260 nm/absorbance at 280 nm ($A_{260}/_{280}$) ratio of between 1.8 and 2.0. Primer sequences for bovine copper transporter 1 (**CTR1**); hepatic ATPase, Cu++ transporting, beta polypeptide (**ATP7B**); cytochrome c oxidase 17 (**COX17**); copper chaperone for superoxide dismutase (CCS); and GAPDH (internal reference: accession numbers NM_001100381, XM_596258, BC103398, NM_001046187, and BC102589, respectively) were as published by Han et al. (2009), and those for ceruloplasmin and antioxidant protein 1 homolog (**ATOX1**) were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to generate an amplicon size of approximately 100 bp as for the other primers. For ceruloplasmin, the accession number, forward primer, and reverse primers were XM_002685007.2, AGACAGCAACTGCGTGACCCG, and GCCCAATGAGTCCTGAGGCGA, and for ATOX1 were NM_001130758.1, AGAGCCGGTGGAGGCGTAGTC, and CGGAGAACTCGTGCTTCGGCAT, respectively. Primer alignment specificity was checked using the BLAST search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All oligonucleotides were commercially synthesized as highly purified salt-free products (MWG-Biotech AG, London, UK).

Reactions were optimized using a temperature gradient and the presence of a single product was confirmed by electrophoresis on a 2% (wt/vol) agarose gel. Diluted total RNA (20 ng/μL) was used as a template for reverse-transcription quantitative PCR (**RT-qPCR**) amplification carried out using the iScript One-Step RT-PCR Kit With SYBR Green kit (Bio-Rad Laboratories Inc., Hercules, CA) in a reaction volume of 20 μL. For each assay, a master mix was prepared containing 2× reaction buffer [containing a 0.4 m*M* concentration of each deoxyribonucleotide triphosphate (dNTP) magnesium ions, iTaq DNA polymerase, 20 n*M* fluorescein, SYBR Green I dye, and stabilizers; Bio-Rad Laboratories Inc.], 300 n*M* final concentration of each primer, iScript RNase H^+ reverse transcriptase, and nuclease-free water. For all unknown samples measured, 20-μL reactions were prepared in clear 96-well plates containing the prepared master mix plus 100 ng of total RNA. External standards were run on the same plate in 20-μL reaction volumes. To minimize variation, all samples included in each analysis were prepared under the same conditions and were analyzed on a single plate in triplicate. The CTR1, ATP7B, COX17, CCS, ATOX1, and ceruloplasmin mRNA expression levels were measured on samples collected at wk 0 and 16 of the study and were reported relative to GAPDH. Complementary DNA synthesis was carried out as the

first step in the reaction through incubation at 50°C for 10 min using iScript RNase H^+ reverse transcriptase as included with the kit. A 5-min step at 95°C was included for deactivation of the enzyme. The RT-qPCR reactions were performed using 95°C for 30 s, and at 60°C for 5 s for 40 cycles (Bio-Rad iCycler; Bio-Rad Laboratories Inc.). Following RT-qPCR, amplified cDNA were melted (melting curve) to ensure the quality of amplification. For melting curve analysis, RT-qPCR products were incubated for 10 s at each step, with an increase in temperature of 0.5°C from 55 to 95°C in each cycle. Gene expression levels were calculated using the Pfaffl method (Pfaffl, 2001).

Statistical Analysis

Performance, blood hematology, plasma minerals, and biochemistry were analyzed by repeated-measures ANOVA as a 2 × 2 factorial design. Treatment degrees of freedom were split into main effects of Cu source (INORG Cu vs. ORG Cu), antagonist [without antagonist (−) vs. with antagonist (+)] and their interaction and was analyzed as follows:

$$Y_{ijkl} = \mu + B_i + F_j + A_k + T_l + FA_{jk} + FT_{jl} + AT_{kl} + FAT_{jkl} + \varepsilon_{ijkl},$$

where Y_{ijkl} = dependent variable; μ = overall mean; B_i = fixed effect of blocks; F_j = effect of form (j = INORG or ORG); A_k = effect of S and Mo (k = − or +); T_l = effect of time; FA_{jk} = interactions between form and antagonist; FT_{jl} = interaction between form and time; AT_{kl} = interaction between time and antagonist; FAT_{jkl} = interaction between form, antagonist and time; and ε_{ijkl} = residual error.

Hepatic mineral concentration, milk FA, and hepatic mRNA were analyzed by ANOVA in a 2 × 2 factorial design as follows:

$$Y_{ijk} = \mu + B_i + F_j + A_k + FA_{jk} + \varepsilon_{ijk},$$

where Y_{ijk} = dependent variable, μ = overall mean, B_i = fixed effect of blocks, F_j = effect of form (j = INORG or ORG), A_k = effect of S and Mo (k = − or +), FA_{jk} = interactions between form and antagonist, and ε_{ijk} = residual error. For hepatic mineral concentrations, the concentration during wk 0 was used as a covariate to determine the final and rate of mineral deposition or mobilization. All statistical analyses were conducted using GenStat version 14.1 (VSN Int. Ltd., Oxford, UK) and are presented as means with standard error of the mean; $P < 0.05$ was used as the significant threshold and a trend was considered at $P < 0.1$.

RESULTS

Diet Analysis, Intake, and Animal Performance

All 4 diets had a similar DM, CP, NDF, and ash content, with mean values of 402, 169, 392, and 80 g/kg of DM, respectively (Table 1). Similarly, the content of Cu was comparable between all 4 diets, with a mean value of 16.9 mg/kg of DM. In contrast, the concentration of S and Mo was higher in INORG+ and ORG+ (mean values of 3.49 g of S/kg of DM and 8.83 mg of Mo/kg of DM) than INORG− and ORG− (mean values of 2.32 g of S/kg of DM and 1.22 mg of Mo/kg of DM). An interaction ($P = 0.025$) was observed between Cu source and antagonist on DMI, with cows offered INORG− having an increased DMI compared with those offered INORG+ or ORG− (Table 2). Milk yield was 5% higher and milk fat content 6% lower ($P < 0.05$) in cows fed INORG compared with ORG Cu, but milk fat yield and ECM did not differ between treatments ($P > 0.05$). No effect ($P > 0.05$) was observed of treatment on milk protein content or yield, or lactose content, but lactose yield was lower ($P < 0.01$) in cows offered ORG Cu compared with INORG Cu. A trend ($P = 0.06$) existed for cows receiving ORG Cu to have a greater BW gain over the experimental period than those fed INORG Cu.

Milk FA

No effect ($P > 0.05$) was observed of dietary treatment on milk FA concentrations of 8:0, 10:0, 12:0, 14:0, 14:1, 15:0, 16:0, 16:1, 18:1, 18:2, or 20:0 (Table 3). Cows fed INORG Cu had a higher ($P < 0.05$) concentration of C17:0 and 18:3n-3 ($P < 0.01$) compared with those fed ORG Cu. Supplementation with S and Mo (+) resulted in a lower ($P < 0.05$) milk concentration of 17:0 and 18:0 compared with animals that were unsupplemented (−), but no effect ($P > 0.05$) was observed of dietary treatment on the content of SFA, MUFA, or PUFA.

Plasma Mineral Profile, Cu-Mediated Enzymes, and Hematology Profile

No effect ($P > 0.05$) was observed of Cu source on plasma Cu concentration during wk 1, 2, 4, 8, or 16 or the mean concentration (Figure 1 and Table 4). During wk 12, however, cows receiving added S and Mo had a lower ($P < 0.05$) plasma Cu concentration than those that were not supplemented, with mean values of 11.4 and 12.9 and μmol/L for + and −, respectively. No effect ($P > 0.05$) was observed of Cu source on mean plasma Mo concentrations, but during wk 16 cows offered INORG Cu had a higher ($P < 0.05$)

Table 2. Effect of dietary inorganic (INORG) or organic (ORG) Cu fed without (−) or with (+) added S and Mo on the performance of early lactation dairy cows

	Diet					Significance,[1] *P*-value		
Item	INORG−	INORG+	ORG−	ORG+	SEM	Cu	Ant	Int
Intake, kg of DM/d	22.6	20.8	21.0	21.4	0.54	0.27	0.13	0.025
Milk yield, kg/d	36.1	36.5	34.9	34.2	0.92	0.044	0.86	0.63
ECM, kg/d	33.2	33.8	32.2	33.0	1.06	0.36	0.53	0.92
Fat, g/kg	35.8	35.5	36.9	38.7	1.03	0.042	0.48	0.34
Protein, g/kg	30.7	30.5	31.8	30.9	0.48	0.11	0.28	0.56
Lactose, g/kg	45.8	45.7	45.7	45.8	0.40	0.92	0.96	0.88
Fat yield, kg/d	1.29	1.28	1.23	1.31	0.043	0.69	0.41	0.31
Protein yield, kg/d	1.10	1.11	1.06	1.06	0.035	0.16	0.89	0.88
Lactose yield, kg/d	1.65	1.71	1.53	1.57	0.045	0.005	0.28	0.81
BW, kg	634	636	647	634	5.1	0.27	0.31	0.14
BW change, kg/d	0.16	0.08	0.35	0.20	0.055	0.056	0.15	0.65
Condition score	2.61	2.55	2.64	2.57	0.040	0.52	0.13	0.97
Condition score change	0.43	0.23	0.39	0.37	0.052	0.52	0.18	0.23

[1]Cu = main effect of copper; Ant = main effect of antagonists; Int = interaction between copper and antagonists.

concentration than those offered ORG Cu, with mean values of 0.25 and 0.21 μmol/L, respectively. Following additional dietary S and Mo (treatments INORG+ and ORG+), plasma Mo concentrations approximately doubled within 1 wk ($P < 0.001$), and remained high for the duration of the study (Figure 2). No effect ($P > 0.05$) existed of dietary treatment on plasma Fe or Zn concentration. Also, no effect ($P > 0.05$) was observed of Cu source or added S and Mo on whole-blood SOD, serum ceruloplasmin, or weekly ceruloplasmin:Cu ratio (Figure 3). Similarly, no effect ($P > 0.05$) was observed of Cu source on red blood cell count, white blood cell count, or blood hemoglobin concentration.

Hepatic Mineral Concentration and mRNA

The mean hepatic concentration of Cu, Zn, Mo, Fe, and Mn at the beginning of the study was 382, 86, 2.75, 192, and 9.9 mg/kg of DM, respectively, with no difference ($P > 0.05$) between treatments. Hepatic Cu

Table 3. Effect of dietary inorganic (INORG) or organic (ORG) Cu fed without (−) or with (+) added S and Mo on the FA composition [g/100 g of FA methyl esters (FAME)] of milk fat in early lactation dairy cows[1]

	Diet					Significance,[2] *P*-value		
FA, g/100 g of FAME	INORG−	INORG+	ORG−	ORG+	SEM	Cu	Ant	Int
8:0	0.92	0.80	0.94	0.92	0.028	0.27	0.19	0.54
10:0	2.55	2.37	2.59	2.53	0.093	0.37	0.21	0.57
12:0	3.21	2.99	3.28	3.21	0.117	0.30	0.20	0.52
14:0	11.3	10.9	11.3	11.0	0.322	0.84	0.25	0.82
cis-9 14:1	0.23	0.22	0.22	0.21	0.008	0.29	0.22	0.91
15:0	1.49	1.48	1.52	1.54	0.074	0.59	0.96	0.89
16:0	36.7	36.5	36.5	38.0	0.70	0.40	0.36	0.23
cis-9 16:1	2.02	2.05	2.13	2.21	0.105	0.22	0.59	0.81
17:0	0.54	0.52	0.53	0.48	0.014	0.047	0.024	0.45
18:0	10.0	9.32	9.60	9.04	0.296	0.25	0.041	0.85
trans-9 18:1	1.74	1.90	1.76	1.75	0.061	0.34	0.27	0.18
cis-9 18:1	20.9	22.6	20.2	20.8	0.88	0.52	0.47	0.52
cis-9,*cis*-12 18:2	1.68	1.73	1.70	1.67	0.054	0.43	0.80	0.60
cis-9,*trans*-11 18:2[3]	0.55	0.52	0.55	0.52	0.042	0.99	0.56	0.98
trans-10,*cis*-12 18:2	0.10	0.10	0.12	0.11	0.008	0.49	0.62	0.43
cis-9,*cis*-12,*cis*-15 18:3	0.33	0.32	0.30	0.27	0.013	0.003	0.25	0.60
20:0	0.13	0.13	0.14	0.12	0.007	0.68	0.10	0.38
ΣSFA	66.9	65.1	66.4	66.9	1.01	0.60	0.51	0.26
ΣMUFA	24.9	26.9	25.3	24.9	0.91	0.49	0.39	0.20
ΣPUFA	2.67	2.68	2.63	2.55	0.066	0.23	0.64	0.44

[1]Wk 0 values were used as a covariate.

[2]Cu = main effect of copper; Ant = main effect of antagonists; Int = interaction between copper and antagonists.

[3]Milk fat contains several additional isomers of conjugated linoleic acid (*cis*/*trans, cis*/*cis* and *trans*/*trans*) and these co-elute under the analytical conditions used in the present study.

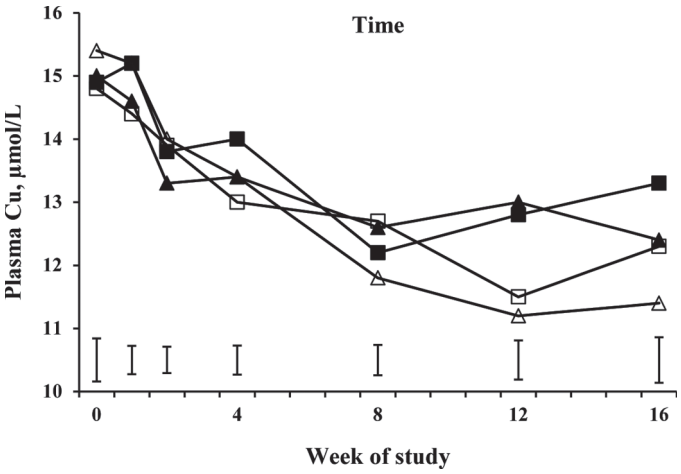

Figure 1. Plasma Cu concentration in early lactation dairy cows fed diets supplemented with inorganic Cu fed without (■) or with (□) added S and Mo, or organic Cu fed without (▲) or with (Δ) added S and Mo. Error bars indicate SEM. Statistically significant ($P < 0.05$) effects of copper source, antagonist, time, copper source × antagonist, copper × time, antagonist × time, and copper × antagonist × time are given in the figure.

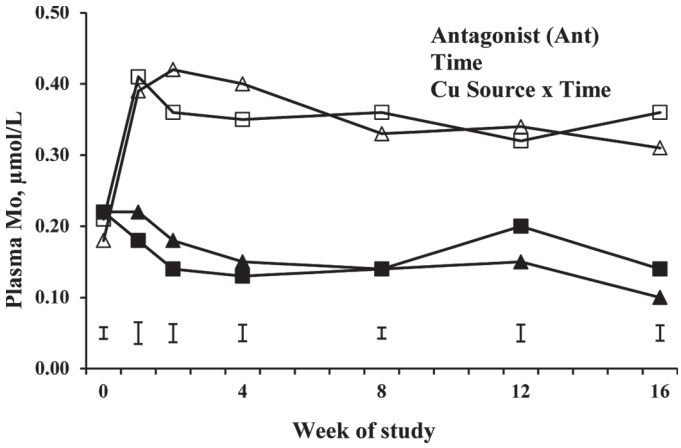

Figure 2. Plasma Mo concentration in early lactation dairy cows fed diets supplemented with inorganic Cu fed without (■) or with (□) added S and Mo, or organic Cu fed without (▲) or with (Δ) added S and Mo. Error bars indicate SEM. Statistically significant ($P < 0.05$) effects of copper source, antagonist, time, copper source × antagonist, copper × time, antagonist × time, and copper × antagonist × time are given in the figure.

concentrations at wk 16 were lower ($P < 0.001$) and decreased to a greater extent ($P < 0.001$) in cows when fed added S and Mo, with a mean reduction of approximately 0.9 mg/kg of DM per day, compared with an increase of 0.13 mg/kg of DM per for animals not receiving added S and Mo (Table 5). No effect ($P > 0.05$) was observed of dietary form of Cu on the rate of change or final hepatic Cu concentration. Similarly, no effect ($P > 0.05$) was observed of dietary treatment on the final or change in hepatic concentrations of Zn, Fe, or Mn. In contrast, the final hepatic concentration of Mo was higher ($P < 0.05$) in cows receiving added S and Mo, but no effect ($P > 0.05$) of dietary Cu source was observed.

A trend ($P = 0.06$) occurred for ATP7B to be upregulated in cows fed S and Mo along with ORG Cu compared with the other treatments, although relative abundance decreased over the study period (Figure 4). No effect ($P > 0.05$) was observed of dietary Cu source or the inclusion of Cu antagonists on hepatic mRNA expression of ATOX1, ceruloplasmin, CTR1, or COX17.

DISCUSSION

Intake and Performance

The majority of studies that have investigated the effect of Cu on dairy cow intake have examined the

Table 4. Effect of dietary inorganic (INORG) or organic (ORG) Cu fed without (−) or with (+) added S and Mo on mean indicators of blood Cu status over the study period of early lactation dairy cows[1]

	Diet					Significance,[3] *P*-value		
Item[2]	INORG−	INORG+	ORG−	ORG+	SEM	Cu	Ant	Int
Cu, µmol/L	13.5	12.9	13.2	12.8	0.33	0.53	0.14	0.78
Mo, µmol/L	0.16	0.36	0.16	0.36	0.012	0.60	<0.001	0.91
Fe, µmol/L	27.5	26.5	29.2	27.3	1.04	0.24	0.19	0.66
Zn, µmol/L	10.4	10.2	10.4	10.6	0.28	0.54	0.98	0.53
Ceruloplasmin, mg/dL	20.2	18.8	18.9	19.8	1.12	0.88	0.79	0.30
Ceruloplasmin:Cu	1.53	1.49	1.43	1.53	0.06	0.65	0.62	0.25
Superoxide dismutase, U/g of Hb	2,670	2,511	2,532	2,557	70.1	0.51	0.31	0.19
WBC, 10^3/mm^3	7.79	8.39	7.97	7.45	0.31	0.21	0.91	0.075
Hb, g/dL	10.17	9.97	9.89	9.62	0.20	0.13	0.24	0.86
RBC, 10^6/mm^3	6.18	6.13	5.95	5.84	0.12	0.060	0.53	0.80

[1]Wk 0 values were used as a covariate.

[2]Hb = hemoglobin; WBC = white blood cells; RBC = red blood cells.

[3]Cu = main effect of copper; Ant = main effect of antagonists; Int = interaction between copper and antagonists.

Table 5. Effect of dietary inorganic (INORG) or organic (ORG) Cu fed without (−) or with (+) added S and Mo on the hepatic mineral concentrations of early lactation dairy cows[1]

	Diet					Significance,[2] *P*-value		
Item	INORG−	INORG+	ORG−	ORG+	SEM	Cu	Ant	Int
Final Cu, mg/kg of DM	419	280	375	285	22.1	0.65	<0.001	0.26
Cu change, mg/kg of DM per day	0.33	−0.91	−0.07	−0.87	0.197	0.65	<0.001	0.26
Final Zn, mg/kg of DM	87.9	88.4	81.8	93.6	7.71	0.84	0.46	0.46
Zn change, μg/kg of DM per day	20.8	25.6	−33.5	72.4	68.88	0.84	0.46	0.46
Final Mo, mg/kg of DM	2.93	3.10	2.89	3.09	0.08	0.79	0.035	0.84
Mo change, μg/kg of DM per day	1.61	3.09	1.25	3.05	0.76	0.79	0.035	0.84
Final Fe, mg/kg of DM	164	172	168	166	11.6	0.93	0.78	0.67
Fe change, μg/kg of DM per day	−237	−178	−245	−232	107.0	0.77	0.73	0.83
Final Mn, mg/kg of DM	8.22	8.41	8.18	8.34	0.395	0.89	0.67	0.96
Mn change, μg/kg of DM per day	−15.1	−13.4	−15.5	−14.1	3.53	0.89	0.67	0.97

[1]Wk 0 value used as a covariate where appropriate.

[2]Cu = main effect of copper; Ant = main effect of antagonists; Int = interaction between copper and antagonists.

influence of level rather than form of supplementation. For example, Engle et al. (2001) reported no effect of increasing supplementary Cu in the form of sulfate from 0 to 40 mg/kg of DM on intake or performance, whereas Chase et al. (2000) reported no effect of level (0, 15, or 30 mg of Cu/kg of DM) or antagonist (Fe) on intake in dairy cows. Similarly, the level of Cu inclusion had no effect on DMI in the study of Brzóska and Sala (2001) or Sol Morales et al. (2000). In contrast, beef steers supplemented with Cu (10 mg/kg of DM) as sulfate or oxide had a decreased intake compared with unsupplemented animals (Wittenberg and Boila, 1988) or those receiving a Cu injection, possibly as a consequence of interactions in the rumen. In the current study, feeding 16.9 mg/kg of DM resulted in an interaction between dietary Cu form and S and Mo on DMI, with intake decreasing in response to antagonists in cows when fed the INORG but not ORG source. Previous studies that have used the same Cu-proteinate (Hart et al., 2011) have reported no effect on DMI. Similarly, no difference existed in intake of growing heifers fed sources of Cu-proteinate or sulfate in the study of Ward et al. (1996). Hart et al. (2011) fed diets that were low in S and Mo (approximately 2.3 and 1.5 mg/kg of DM, respectively), and it is possible that the differences in intake in the current study may be attributed to interactions between Cu source and antagonists in the rumen.

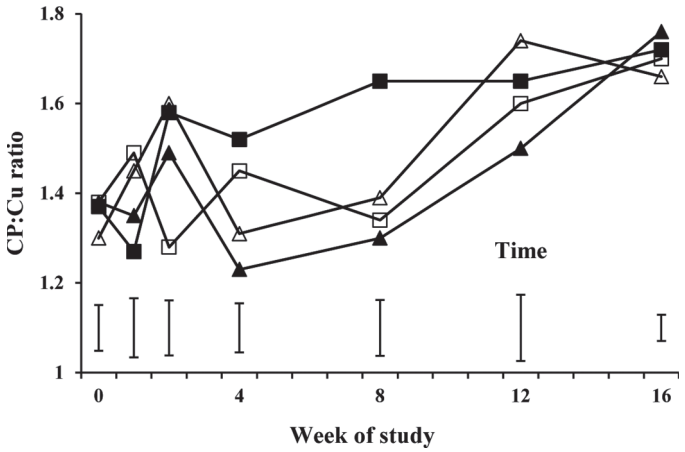

Figure 3. Serum ceruloplasmin to plasma Cu ratio in early lactation dairy cows fed diets supplemented with inorganic Cu fed without (■) or with (□) added S and Mo, or organic Cu fed without (▲) or with (Δ) added S and Mo. Error bars indicate SEM. Statistically significant ($P < 0.05$) effects of copper source, antagonist, time, copper source × antagonist, copper × time, antagonist × time, and copper × antagonist × time are given in the figure.

Milk yield was 1.7 kg/d lower in animals receiving ORG compared with INORG Cu, but no effect on ECM yield was observed, primarily due to a corresponding increase in fat content in cows receiving Cu in an ORG form. A decrease in milk fat content from 42 to 40 g/kg has been reported when dietary Cu level was increased from 14 to 60 mg of Cu/kg of DM in the study of Brzóska and Sala (2001). Dietary levels used in the current study were considerably lower than that used by Brzóska and Sala (2001), but the similar effect on milk fat concentration when Cu was supplied in an ORG form may indicate a greater availability of Cu. Similarly, Formigoni et al. (2011) reported an increase in milk fat content of 1.7 g/kg in early lactation dairy cows receiving a mixture of organic trace elements that included Cu, but no effect occurred on milk yield. In a meta-analysis of the effects of combined organic minerals across 20 studies, Rabiee et al. (2010) found a net increase in milk yield attributable to organic minerals of 0.93 kg/d and milk fat yield of 0.04 kg/d. Performance changes in such studies should, however, be interpreted with caution, as it is not possible to determine the effect of an individual element, or associative effects between elements. Additionally, there

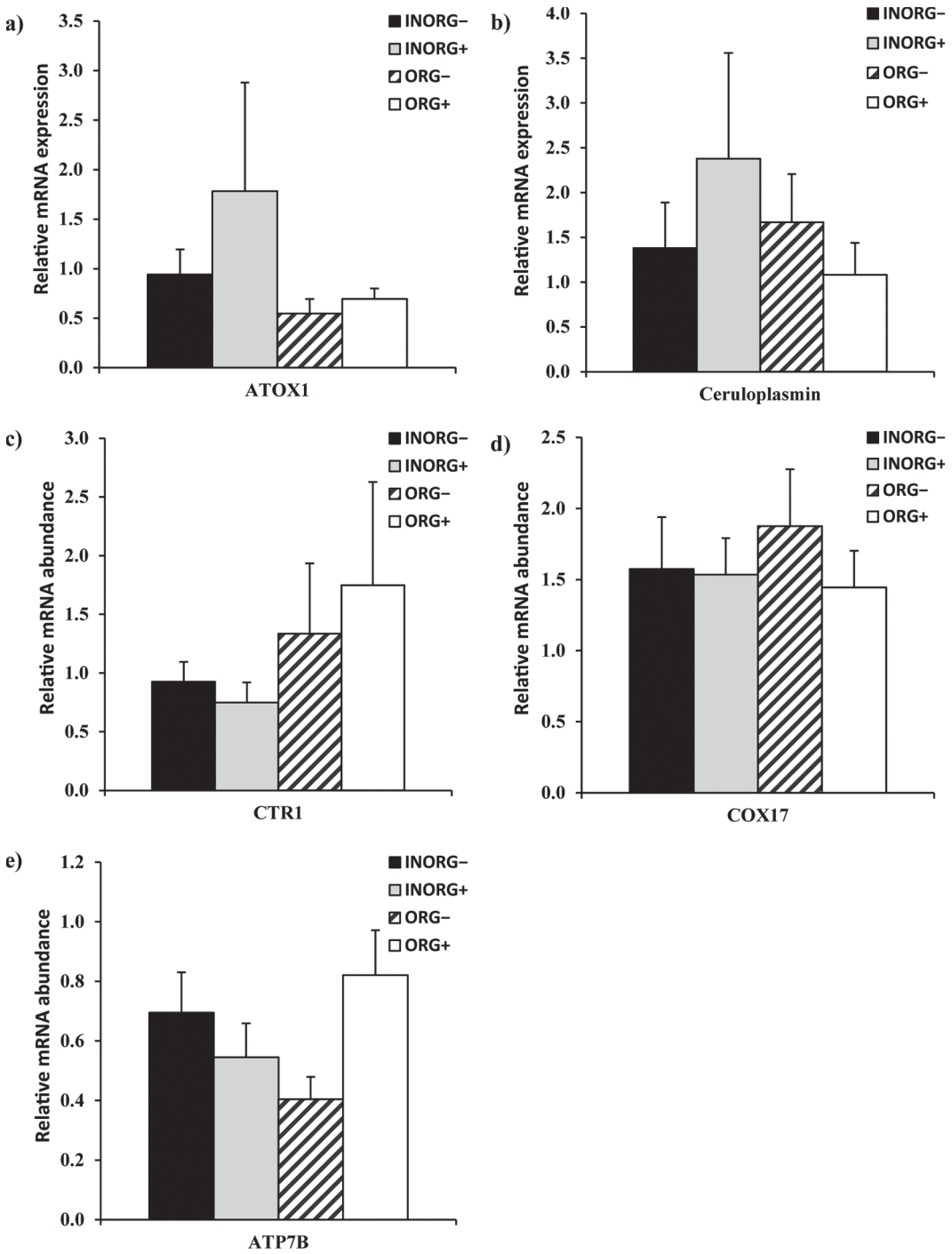

Figure 4. Hepatic mRNA relative expression of (a) antioxidant protein 1 homolog (ATOX1), (b) ceruloplasmin, (c) copper transporter 1 (CTR1), (d) cytochrome c oxidase 17 (COX17), and (e) ATPase, Cu++ transporting, beta polypeptide (ATP7B) in early lactation dairy cows fed diets supplemented with inorganic Cu fed without (INORG−) or with (INORG+) added S and Mo, or organic Cu fed without (ORG−) or with (ORG+) added S and Mo. Values are expressed relative to GAPDH and are presented as the ratio of the value obtained at wk 16 to 0 of the study. Error bars indicate SE. Statistically significant ($P < 0.05$) effects of copper source, antagonist, and copper source × antagonist are given in the figure.

is the added complication of variations in response between studies being due to different organic forms of Cu. Evidence exists that Cu-lysine, for example, will rapidly disintegrate within the rumen, with the Cu being present as carbonate and phosphate complexes (Attaelmannan and Reid, 1996), although the integrity

of other forms of organically complexed Cu in the rumen is less clear.

Milk FA Profile

Dietary Cu has been suggested to influence milk FA, either through altering mammary metabolism or more probably inhibition of ruminal biohydrogenation of dietary PUFA (Engle et al., 2001). A decrease in ruminal *trans* 18:1 was reported in steers (Engle et al., 2000) and milk *trans* C18:1 content was decreased in cows that received 10 or 40 mg of additional Cu/kg of DM in the study of Engle et al. (2001), an effect that was attributed to an interference with the reduction process during biohydrogenation in the rumen, resulting in a diversion in the flow of hydrogen. However, no effect was observed of Cu form on any of the milk *trans* FA identified in the current study, and it can be suggested that form of Cu supplementation has a minor, if any, effect on ruminal biohydrogenation. In the current study, however, a small but significant decrease was observed in 17:0 when Cu was fed in an ORG form. Although some studies have reported alterations in synthesis of odd-chain FA in the mammary gland, changes in milk fat content are mainly affected by the incorporation of ruminal bacterial lipids, (see review of Vlaeminck et al., 2006). In particular, amylolytic bacteria appear to have a higher content of odd-chain FA, and it is, therefore, possible that supplying Cu in an ORG form altered the microbial population. Engle and Spears (2004) also reported that the level of Cu supplementation affected the 17:0 concentration in muscle in finishing steers, with a decrease following supplementation with a Cu bolus. Increasing the dietary concentration of Cu sulfate was associated with a decrease in milk PUFA and MUFA concentrations in the study of Engle et al. (2001), although the effect was small and occurred only when high dietary levels (40 mg of Cu/kg of DM) were included. In the current study, milk PUFA content was unaffected by dietary Cu source, despite the decrease in milk 18:3n-3 observed in cows when fed the ORG source. Few studies have investigated the direct effects of additional S and Mo on milk FA profile, with those that have been conducted often confounding Cu level and the inclusion of antagonists (e.g., Sol Morales et al., 2000). In the current study, the effects of the antagonists were small, with a decrease in 17:0 and 18:0 concentrations.

Plasma Mineral Status

Plasma Cu has been suggested to be an insensitive measure of Cu status, as during Cu depletion or repletion, levels are maintained by alterations in hepatic concentration (Laven and Livesey, 2005). Indeed, in the current study, no effect was observed of dietary Cu source or the presence of added S and Mo at concentrations that were anticipated to reduce the bioavailability of Cu on plasma Cu concentrations. Plasma Cu concentrations were observed to decline across all treatments as lactation progressed, but were always well above the 9 μmol/L generally regarded as adequate (Laven and Livesey, 2005). Ward et al. (1996) also reported no difference in plasma Cu concentrations in beef heifers when supplemented with $CuSO_4$, $CuCO_3$, or Cu-proteinate without added S and Mo. When S and Mo were included at 1.5 and 5 mg/kg of DM, respectively, the Cu-proteinate treatment maintained plasma concentrations at a higher level, although initial liver Cu concentrations were low at 36 mg/kg of DM (Ward et al., 1996).

As a consequence of the unresponsiveness of plasma Cu to dietary changes, except in situations where animals have low liver Cu reserves, other indicators of Cu status including Cu-containing enzymes such as SOD_1 and CP have been investigated, along with CP:Cu ratio. In the current study, however, no effect was observed of Cu source or added S and Mo on any of the indicators of Cu status. The ratio of CP:Cu has also been suggested as an indicator of Mo toxicity rather than Cu deficiency (Telfer et al., 2004), whereby thiomolybdates formed in the rumen in the absence of adequate quantities of Cu are absorbed into the blood stream and may have inhibitory effects on critical metalloenzymes (Mason, 1986; Williams et al., 2001; Gould and Kendall, 2011). In the current study, a rapid doubling in plasma Mo concentration occurred following the addition of dietary S and Mo, but there was no effect on the CP:Cu ratio, which was in excess of the 1.5 threshold regarded as indicative of toxicity (Telfer et al., 2004). The dietary ratio of Cu:Mo was reduced from 13.4 to 2.0:1 following the addition of Mo, but this value was still in excess of the 1:1 ratio that has been suggested as being necessary before thiomolybdates are absorbed (Suttle, 2010) and may explain the lack of response in plasma CP:Cu ratio in the current study. The organically complexed Cu may also have been expected to have reduced the ruminal availability of Cu to bind with thiomolybdates in the rumen and, therefore, increase the likelihood of thiomolybdates being absorbed, but no difference in any of the indicators of Cu status was observed.

Hepatic Mineral Concentration and mRNA

The liver is the main storage organ for Cu and a strong correlation exists between liver Cu content and dietary levels, although it has been suggested that hepatic concentrations may not necessarily reflect Cu

status across other tissues (Suttle, 2010). Hepatic Cu concentrations at the end of the study averaged 397 and 283 mg/kg of DM in cows receiving no or additional S and Mo, respectively. These values are well in excess of the 6 to 19 mg/kg of DM considered to be marginal, and close to the upper limit of 508 mg/kg of DM suggested by Livesey et al. (2002). Grace et al. (2010) reported high and variable liver Cu concentrations in commercial dairy cows when samples were collected at slaughter or biopsy, with values ranging from approximately 150 to 600 mg/kg of DM. When dairy cows were supplemented with Cu at 10 mg/kg of DMI as Cu sulfate, liver concentrations increased by 1.9 mg/kg of DM per day (Engle et al., 2001). In the current study, a similar level of Cu supplementation resulted in a positive but smaller net increase of 0.33 mg of Cu/kg of DM per day. This lower rate of increase may partly be attributed to the lower dietary Cu concentration in the current study than that of Engle et al. (2001; 16.9 vs. 18.9 mg/kg of DM, respectively). The inclusion of additional S and Mo in the present study resulted in a substantial decrease in liver Cu concentrations of 0.89 mg/kg of DM per day. At this rate of depletion, cows would have been expected to have exhibited clinical signs of deficiency after a further 300 d on treatment and would justify a higher Cu inclusion rate, although these levels of antagonists are rarely encountered under commercial feeding conditions (NRC, 2001).

No effect was observed of form of dietary Cu supplementation on the rate of change in liver Cu concentrations in the current study, either when included without or with added S and Mo. Previous studies have demonstrated an increase in the bioavailability of Cu when supplied in an organic form in monogastrics, but the evidence in ruminants is more limited. For example, Du et al. (1996) found no difference between Cu sulfate or proteinate on hepatic Cu concentrations in lactating dairy cows following supplementation at 5 or 80 mg/kg of DM. Similarly, no effect was reported when either form of Cu was fed to dairy heifers that had previously been fed a Cu-depleted ration (Du et al., 1996). In contrast, bioavailability of Cu glycinate increased compared with Cu sulfate in steers, particularly when they were supplemented with S and Mo (Hansen et al., 2008). Similarly, Ward et al. (1996) reported no difference in final liver Cu concentrations in beef heifers when fed Cu sulfate or proteinate, although following the addition of S and Mo, there was a benefit to the proteinate. Considerable variation, therefore, appears to exist between organic sources in their bioavailability relative to inorganic sources, with any benefit that may be present generally being more apparent when antagonists are fed and (or) the animals have a low Cu status.

Absorbed molybdate is normally stored in tissues as molybdoprotein in the liver, kidney, and adrenal gland where it has been shown to be bound in mitochondrial membranes to sulfite oxidase and to aldehyde oxidase and xanthine dehydrogenase in the cytosol (Johnson, 1997). Previous studies have demonstrated that when provided with additional S, Mo retention increases (Grace and Suttle, 1979). However, only a small effect of additional S and Mo on liver Mo concentration was observed in the current study, despite a doubling in plasma Mo concentrations. We suggest, therefore, that the liver is not a major depot of Mo in dairy cows, or that in the current study the absorbed Mo was unavailable for uptake by the liver. Liver concentrations of Fe, Mn, and Zn were also unaffected by dietary treatment, and the liver is generally accepted as not being a major store for these minerals (Suttle, 2010).

In the current study, hepatic ATP7B expression tended to be upregulated in cows when fed additional S and Mo in combination with the organic form of Cu. ATP7B exports Cu out of the liver and into bile (Suttle, 2010), and the current finding is consistent with the observed reduction in liver Cu concentration in animals receiving ORG+. It is puzzling, however, that the same effect was not observed in cows when supplemented with the INORG source of Cu, despite a similar rate of hepatic Cu depletion. This may suggest that the reduction in hepatic Cu in cows fed the INORG form was due to a reduction in tissue supply rather than rate of mobilization. Pooling of the initial liver ATP7B mRNA expression with liver Cu concentration revealed a negative correlation (−0.126) that was not significant ($P = 0.39$). In contrast, Han et al. (2009) reported a negative but strong correlation between ATP7B mRNA expression and liver Cu levels in young beef bulls. Reasons for the difference between the 2 studies are unclear, but may be related to factors including basal diet, dietary Cu levels, and physiological state. Additionally, Han et al. (2009) found very low levels of ATP7B mRNA expression in animals with high liver Cu concentrations, and the comparatively high initial values recorded in the current study may have affected the results.

CONCLUSIONS

The inclusion of an ORG compared with INORG form of Cu decreased milk yield but increased milk fat concentration, with ECM yield being similar in cows receiving either Cu source. Additional dietary S and Mo reduced DMI in cows fed the INORG but not ORG Cu, and reduced liver Cu levels by approximately 0.9 mg/kg of DM per day. Little effect was observed of dietary Cu supply on plasma mineral concentration or liver

mRNA abundance, except ATP7B, which tended to be increased in cows when fed the ORG but not INORG Cu when the diet was supplemented with S and Mo. The form of Cu and the addition of S and Mo had only minor effects on the milk FA profile. In the absence of high levels of antagonists, a dietary concentration of 17 mg of Cu/ kg of DM was more than sufficient to meet Cu requirements, as indicated by the positive liver Cu balance.

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