Added dietary sulfur and molybdenum has a greater influence on hepatic copper concentration, intake, and performance in Holstein-Friesian dairy cows offered a grass silage- rather than corn silage-based diet

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ABSTRACT

To test the hypothesis that the metabolism of Cu in dairy cows is affected by basal forage and added S and Mo, 56 dairy cows that were 35 (standard error $\pm$ 2.2) days postcalving and yielding 38.9 kg of milk/d (standard error $\pm$ 0.91) were offered 1 of 4 diets in a 2×2 factorial design for a 14-wk period. The 4 diets contained approximately 20 mg of Cu/kg of dry matter (DM), and had a corn silage-to-grass silage ratio of 0.75:0.25 (C) or 0.25:0.75 (G) and were either unsupplemented (–) or supplemented (+) with an additional 2 g of S/kg of DM and 6.5 mg of Mo/kg of DM. We found an interaction between forage source and added S and Mo on DM intake, with cows offered G+ having a 2.1 kg of DM lower intake than those offered G–, but no effect on the corn silage-based diets. Mean milk yield was 38.9 kg/d and we observed an interaction between basal forage and added S and Mo, with yield being decreased in cows offered G+ but increased on C+. No effect of dietary treatment on milk composition or live weight was noted, but body condition was lower in cows fed added S and Mo irrespective of forage source. We found an interaction between forage source and added S and Mo on milk somatic cell count, which was higher in cows offered G+ compared with G–, but not in cows fed the corn silage-based diets, although all values were low (mean values of 1.72, 1.50, 1.39, and 1.67 log₁₀/mL for C–, C+, G–, and G+, respectively). Mean plasma Cu, Fe, and Mn concentrations were 13.8, 41.3, and 0.25 μ mol/L, respectively, and were not affected by dietary treatment, whereas plasma Mo was 0.2 μ mol/L higher in cows receiving added S and Mo. The addition of dietary S and Mo decreased liver Cu balance over

the study period in cows fed either basal forage, but the decrease was considerably greater in cows receiving the grass silage-based diet. Similarly, hepatic Fe decreased more in cows receiving G than C when S and Mo were included in the diet. We concluded that added S and Mo reduces hepatic Cu reserves irrespective of basal forage source, but this decrease is considerably more pronounced in cows receiving grass silage- than corn silage-based rations and is associated with a decrease in intake and milk performance and an increase in milk somatic cell count.

Key words: copper, corn silage, dairy cow, grass silage, liver

INTRODUCTION

It has long been recognized that Cu is an important trace element for normal health and performance in dairy cattle, principally due to its requirement in approximately 300 different proteins with functions ranging from efficient iron metabolism, hair pigmentation, antioxidants, release of hormones, and synthesis of connective tissue (Suttle, 2010). As a consequence, Cu-responsive disorders result in production and economic losses due to effects on fertility, performance, and health (NRC, 2005). Clinical signs in dairy cows can be caused by a dietary deficiency of Cu, but often are related to interactions with dietary antagonists such as S and Mo, Fe, and Zn that inhibit Cu absorption or metabolism (Suttle, 2010), with S and Mo receiving the most research attention. It has been proposed that dietary sulfates present in feed or water are reduced in the rumen to sulfides, which then react with molybdate to form thiomolybdates (Dick et al., 1975). Gould and Kendall (2011) discussed that thiomolybdates may be present in the rumen as di-, tri-, or tetrathiomolybdates, with trimolybdate predominant at a ruminal pH of 6.5, whereas tetrathiomolybdate is most prevalent at lower pH values. Thiomolybdates form insoluble complexes with Cu, rendering it unabsorbable (Suttle, 1991), resulting in Cu-responsive disorders. At high

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Mo intakes (e.g., >8 mg of Mo/kg of DM) and very low Cu:Mo ratios (less than 1:1), thiomolybdates may also leave the rumen and be absorbed (Suttle, 2010), subsequently binding to Cu-containing enzymes such as ceruloplasmin (**Cp**), impairing their function (Gould and Kendall, 2011).

It is recognized that the degree of thiomolybdate formation in the rumen can also be affected by the basal forage and method of preservation (Suttle, 1974, 1983, 2010), although our understanding of the mechanism remains poor. For example, in grass hays, the inhibitory effect of Mo on Cu absorption is less than that of S, whereas in fresh grass Cu absorption is greatly affected by small additions of S and Mo, with semipurified diets being intermediate (Suttle, 1983). A large body of literature exists comparing the effect of grass silage with corn silage on dairy cow intake and performance (e.g., Phipps et al., 1995; Hart et al., 2015), and, in general, replacing grass silage with corn silage results in an increase in DMI, milk yield, and milk protein content. However, little information is available on the relative effects of either of these forages on Cu metabolism in Holstein-Friesian dairy cows, despite their importance in contemporary dairy cow rations. A lack of understanding of the influence of S and Mo on Cu metabolism in dairy cows fed different forages may be contributing to the unnecessary oversupplementation of Cu. Indeed, recent surveys of commercial trace element feeding rates in the United States and United Kingdom (e.g., Castillo et al., 2013; Sinclair and Atkins, 2015) have reported that dietary Cu is frequently fed at levels well above that recommended by national feed standards, such as ARC (1980) or NRC (2001). Feeding Cu above nutritional requirements can result in chronic Cu poisoning, whereby a gradual increase in hepatic Cu concentrations occurs, ultimately leading to rupture of lysosomes, hepatic necrosis, hemoglobinuria, methemoglobinemia, and rapid death (Bidewell et al., 2000). The objectives of our current study were to determine the effect of level of inclusion of corn silage and grass silage fed either without or with added sulfur and molybdenum on indicators of copper status, performance, and health in Holstein-Friesian dairy cows.

MATERIALS AND METHODS

Animals, Management, and Treatments

The procedures involving animals were conducted in accordance with the UK Animals (Scientific Procedures) Act (UK Parliament, 2012), and were approved by the Harper Adams Animal Welfare and Ethical Review Board. Fifty-six Holstein-Friesian dairy cows

(8 primiparous and 48 multiparous) that were 35 (SE ± 2.2) d postcalving and yielding 38.9 kg/d (SE ± 0.91) of milk were used. From calving until wk 5 of lactation the cows were group housed and fed a diet containing 95 g/kg of DM of grass silage, 90 g/kg of DM of alfalfa silage; 324 g/kg of DM of corn silage; 20 g/kg of DM of chopped wheat straw; 100 g/kg of DM of urea-treated wheat; 80 g/kg of DM of soy hulls; 50 g/kg of DM of molasses; 66 g/kg of DM of soybean meal; 64 g/kg of DM of rapeseed meal; 64 g/kg of DM of distillers grains; 18 g/kg of DM of palm kernel meal; 14 g/kg of DM of protected fat; and 15 g/kg of DM of minerals and vitamins. Based on recordings taken in wk 4 of lactation the animals were blocked and allocated to 1 of 4 dietary treatments according to lactation number (primiparous or multiparous), calving date, milk yield, milk composition, BCS (using a 1–5 scoring system on a quarter point scale; Lowman et al., 1976), and live weight. Cows remained on study for 14 wk.

Based on the mineral analysis of the forages (Table 1) and NRC (2001) values for the other feeds, 4 diets were formulated to contain approximately 20 mg of Cu/kg of DM and a corn silage-to-grass silage ratio of 0.75:0.25 (**C**) or 0.25:0.75 (**G**; DM basis; Table 2). To evaluate the effects of dietary antagonists on Cu metabolism, the diets were either unsupplemented (–) or supplemented (+) with additional S and Mo, to result in a total dietary concentration of approximately 3.5 g of S/kg of DM or 7.5 mg of Mo/kg of DM [an increase of approximately 2 g of S/kg of DM (+160%) and 6.5 mg of Mo/kg of DM (+500%)]. Therefore, we used 4 dietary treatments: C– [0.75:0.25 corn silage:grass silage (DM basis), no additional antagonists]; C+ (0.75:0.25 corn silage:grass silage, with additional S and Mo); G– (0.25:0.75 corn silage:grass silage, no additional antagonists); and G+ (0.25:0.75 corn silage:grass silage, with additional S and Mo). Additional Cu was supplied as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, sulfur as ammonium sulfate (TG Tennants, West Bromwich, United Kingdom), and molybdenum as sodium molybdate (Acros Organics, Geel, Belgium). Feed-grade urea was added to G– and C– to provide an equivalent amount of rumen-degradable N as supplied by the ammonium sulfate. The diets were supplemented with other feed ingredients to support a milk production of approximately 38 kg/d according to Thomas (2004; Table 2). All dietary ingredients were mixed and fed as a TMR using a forage mixer calibrated to ± 1 kg, and fed through roughage intake feeders (Hokofarm Group BV, Insentec, 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 daily at 105% of ad libitum intake with refusals collected twice

Table 1. Chemical composition of corn and grass silage

Item	Corn silage	Grass silage
DM, g/kg	341	256
CP, g/kg of DM	75	157
Ash, g/kg of DM	46	
Ammonia-N, g/kg of total N	9.03	8.39
pH	3.6	3.9
ME, MJ/kg of DM	10.8	11.2
Water-soluble carbohydrate, g/kg of DM	26.2	68.8
NDF, g/kg of DM	449	439
ADF, g/kg of DM	229	246
VFA, g/kg of DM		
Lactic	62.1	105.3
Acetic	16.1	22.6
Propionic	0.92	1.06
Butyric	<0.6	<0.6
Ethanol, g/kg of DM	1.84	28.1
Minerals		
Ca, g/kg of DM	2.3	7.3
P, g/kg of DM	2.3	3.3
Mg, g/kg of DM	1.5	1.7
S, g/kg of DM	0.9	3.1
Cu, mg/kg of DM	4.7	8.0
Mo, mg/kg of DM	0.59	1.43
Fe, mg/kg of DM	65.0	159.4
Zn, mg/kg of DM	23.6	37.8
Mn, mg/kg of DM	15.6	34.8

weekly on a Tuesday and Friday. The cows were housed in the same portion of a freestall building containing freestalls fitted with foam mattresses. The passageways were scraped using automatic scrapers and the stalls bedded twice weekly with sawdust. All cows had continual access to fresh bore-hole water, which contained concentrations of S, Fe, Cu, and Mo of 19.3 mg/L, 6.5, 2.9, and 0.5 µg/L, respectively.

Experimental Routine

Cows were milked twice daily, at approximately 0530 and 1530 h, with yield recorded at each milking and samples taken fortnightly at consecutive morning and afternoon milkings for subsequent composition and SCC analysis. The cows were weighed and BCS was recorded after the evening milking in the week before allocation, and then fortnightly afterward. Forage samples were collected weekly; half the sample was oven-dried at 70°C to constant weight, and the amount of corn silage to grass silage was adjusted to achieve the desired ratio. The other sample was frozen and bulked for subsequent analysis. Samples of each of the 4 diets were collected immediately following feeding once per week and stored at −20°C before subsequent analysis. During wk 0, 1, 2, 4, 8, and 14 of the study, blood samples were collected at 1000 h via jugular venipuncture into vacutainers (Becton Dickinson Vacutainer Systems, Plymouth, United Kingdom) containing silica (for samples used

to determine Cp), lithium heparin [for samples used to determine superoxide dismutase (SOD) activity], or sodium heparin (for samples used to determine mineral concentrations and metabolites). During wk 0 and 14 of the study, liver biopsy samples were collected from all cows through the 11th intercostal space, as described by Davies and Jebbitt (1981), and stored at −80°C before subsequent analysis.

Chemical Analysis

Weekly forage and TMR samples were bulked within month and analyzed according to AOAC International (2012) for DM (method 934.01), CP (method 990.03), and starch (method 920.40). In addition, forage samples were analyzed for pH, ammonia-N, water-soluble carbohydrates (MAFF, 1986), and VFA based on the method of Jones and Kay (1976). The analysis of NDF and ADF were conducted according to Van Soest et al. (1991) with the use of a heat-stable α-amylase (Sigma, Gillingham, UK), and expressed exclusive of residual ash. The ME content of the forages was determined by near infrared reflectance spectroscopy (Eurofins Laboratories, Wolverhampton, UK) using a system approved by United Kingdom advisory services (Offer et al., 1996). Forage and TMR minerals were extracted using the DigiPREP digestion system (Qmx Laboratories, Essex, UK), and analyzed as described by Cope et al. (2009) by inductively coupled plasma-MS (Thermo Fisher Scientific Inc., Hemel Hempstead, UK). Serum samples were analyzed for Cp according to Henry et al. (1974), and plasma samples for SOD (Randox Laboratories, Crumlin, UK; kit catalog no. SD 125), BHB, and urea (Randox Laboratories; kit catalog no. RB 1007 and UR221, respectively) using a Cobas Miras Plus autoanalyser (ABX Diagnostics, Bedfordshire, UK). Plasma and liver samples were analyzed for Cu, Fe, Mn, and Mo by inductively coupled plasma-MS as described by Sinclair et al. (2013). Milk samples were analyzed using a Milkoscan Minor (FOSS, Warrington, UK) calibrated by the methods of AOAC International (2012), and SCC was determined by Eurofins Laboratories.

Statistical Analysis

Performance, plasma minerals, and metabolites were analyzed by repeated measures ANOVA as a 2 × 2 factorial design. Milk SCC was transformed to log₁₀ before analysis. Treatment degrees of freedom were split into main effects of forage source (corn vs. grass silage), antagonist [without (−) vs. with (+)] and their interaction and analyzed as

Table 2. Diet composition and chemical analysis of diets high in corn silage (C) or grass silage (G) fed without (–) or with (+) added S and Mo¹

Item	C–	C+	G–	G+
Ingredient, g/kg of DM				
Grass silage	133	134	398	399
Corn silage	400	401	133	134
Urea-treated wheat	111	111	167	167
Soy hulls	89	89	89	89
Rapeseed meal	58	58	31	31
Soybean meal	96	96	31	31
Distillers dark grains with solubles	58	58	31	31
Sopralin ²	—	—	58	58
Molasses	33	33	33	33
Protected fat	13	13	20	20
Urea	2	—	2	—
Minerals/vitamins ³	7	7	7	7
Total	1,000	1,000	1,000	1,000
Chemical analysis				
DM, g/kg	404	421	364	368
Ash, g/kg of DM	71	71	92	93
CP, g/kg of DM	181	185	193	194
NDF, g/kg of DM	407	403	381	387
ADF, g/kg of DM	222	224	228	224
Ca, g/kg of DM	5.40	5.45	7.84	7.49
P, g/kg of DM	3.57	3.82	3.96	3.69
Mg, g/kg of DM	2.72	2.84	2.92	2.79
S, g/kg of DM	1.20	3.15	1.32	3.45
Cu, mg/kg of DM	19.9	19.5	20.7	20.5
Mo, mg/kg of DM	1.17	7.94	1.48	7.70
Fe, mg/kg of DM	183	226	287	252
Zn, mg/kg of DM	49.2	46.3	51.8	48.8
Mn, mg/kg of DM	61	68	70	60

¹C+ and G+ diets also received additional ammonium sulfate and sodium molybdate dihydrate. SEM for differences between dietary concentrations (n = 8 per treatment) for S and Mo was 0.11 and 0.29, respectively.

²Formaldehyde-treated soybean meal, Frank Wright Trouw, Ashbourne, United Kingdom.

³Mineral/vitamin premix (Rumenco, Staffordshire, UK). Major minerals (g/kg): Ca, 240; P, 80; 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.

$$Y_{ijkl} = \mu + B_i + F_j + A_k + T_l + F.A_{jk} + F.T_{jl} \\ + A.T_{kl} + F.A.T_{jkl} + \epsilon_{ijkl},$$

where Y_{ijkl} = dependent variable; μ = overall mean; B_i = fixed effect of blocks; F_j = effect of forage (j = corn or grass silage); A_k = effect of S and Mo (k = –, +); T_l = effect of time; $F.A_{jk}$ = interactions between forage and antagonist; $F.T_{jl}$ = interaction between forage and time; $A.T_{kl}$ = interaction between antagonist and time; $F.A.T_{jkl}$ = interaction between forage, antagonist, and time; and ϵ_{ijkl} = residual error.

Hepatic mineral concentration was analyzed by ANOVA as a 2×2 factorial design as

$$Y_{ijk} = \mu + B_i + F_j + A_k + F.A_{jk} + \epsilon_{ijk},$$

where Y_{ijk} = dependent variable; μ = overall mean; B_i = fixed effect of blocks; F_j = effect of forage (j = corn or grass silage); A_k = effect of S and Mo (k = –, +); $F.A_{jk}$ = interaction between forage and antagonist; and ϵ_{ijk} =

residual error. For hepatic mineral concentrations, the concentration during wk 0 was used where appropriate as a covariate to determine the final and rate of mineral deposition or mobilization. All statistical analysis was conducted using Genstat version 17.1 (VSN Int. Ltd., Oxford, UK) and is presented as means with standard error of the mean; $P < 0.05$ was used as the significant threshold and a trend was considered when $P < 0.1$.

RESULTS

Diet Analysis, Intake, and Animal Performance

Compared with the corn silage, the grass silage contained 85 g/kg less DM and was 82 g/kg of DM higher in CP and 0.4 MJ/kg of DM higher in ME (Table 1). The 2 forages had a similar fiber content, but the grass silage was 43.2 g/kg of DM higher in lactic acid than the corn silage. Compared with the corn silage, the mean content of Ca, P, Mg, and S was 5.0, 1.0, 0.2, and 2.2 g/kg of DM higher, respectively, and Cu, Mo,

Table 3. Intake and performance of early-lactation dairy cows fed diets high in corn silage (C) or grass (G) silage without (–) or with (+) added S and Mo

Item	Diet				SEM	<i>P</i> -value ¹		
	C–	C+	G–	G+		F	A	Int
Intake								
DM, kg/d	23.5	24.0	22.6	20.5	0.48	<0.001	0.111	0.012
Cu, mg/d	467	466	467	418	9.6	0.022	0.007	0.015
Mo, mg/d	27.4	190.2	33.5	157.5	2.69	<0.001	<0.001	<0.001
S, g/d	28.1	74.9	29.9	70.4	1.25	0.302	<0.001	0.013
Fe, g/d	4.30	5.42	6.43	5.17	0.121	<0.001	0.564	<0.001
Milk yield, kg/d	38.1	40.6	38.9	37.9	0.77	0.225	0.373	0.034
Fat, g/kg	37.8	36.6	38.2	37.4	1.37	0.656	0.475	0.889
Protein, g/kg	32.5	32.6	31.6	32.5	0.80	0.173	0.901	0.646
Lactose, g/kg	46.5	46.4	46.7	46.3	0.32	0.975	0.328	0.680
Fat yield, kg/d	1.43	1.43	1.47	1.39	0.059	0.944	0.484	0.468
Protein yield, kg/d	1.23	1.30	1.22	1.21	0.024	0.049	0.242	0.142
Lactose yield, kg/d	1.77	1.93	1.80	1.73	0.063	0.185	0.434	0.060
Live weight, kg	651	653	646	639	7.9	0.237	0.818	0.587
Live weight change, kg/d	0.43	0.30	0.20	0.25	0.131	0.309	0.738	0.518
BCS	2.49	2.35	2.49	2.31	0.047	0.803	0.001	0.744
BCS change	0.35	0.13	0.27	0.09	0.081	0.470	0.019	0.801
Milk SCC, log ₁₀ /mL	1.72	1.50	1.39	1.67	0.086	0.381	0.714	0.017

¹F = main effect of forage source, A = main effect of antagonists, Int = interaction between forage and antagonists.

Fe, and Zn were 3.3, 0.84, 94, and 14.2 mg/kg of DM higher, respectively, in the grass silage.

The DM content of the corn based diets (C– and C+) was 47 g/kg higher than the grass silage-based diets (G– and G+), whereas CP was on average 11 g/kg of DM higher in the grass than the corn silage-based diets (Table 2). The content of NDF was higher in the corn than the grass silage-based diets, but ADF concentration was similar across all 4 diets, averaging 225 g/kg of DM. All 4 diets had a similar P and Mg concentration, but the grass silage-based diets (G– and G+) contained approximately 2 g/kg of DM more Ca. The mean concentration of Cu was 20 mg/kg of DM, and the 2 diets with added antagonists (C+ and G+) had concentrations of S and Mo of 3.3 g/kg of DM and 7.8 mg/kg of DM, respectively, which were close ($P > 0.05$) to the predicted values of 3.5 g/kg of DM and 7.5 mg/kg of DM, respectively. In contrast, the 2 diets with no added S and Mo (C– and G–) had low concentrations of S and Mo, at 1.3 g/kg of DM and 1.3 mg/kg of DM, respectively, that were also close ($P > 0.05$) to predicted.

Cows offered the corn silage-based diets had a daily DMI that was 2.2 kg/d higher ($P < 0.001$) than those offered the grass silage-based diets (Table 3), an effect that was evident from wk 1 of the study (Figure 1). We noted an interaction ($P < 0.05$) between forage source and Cu antagonists, where adding S and Mo reduced DMI by 2.1 kg/d in cows fed the grass silage- but not the corn silage-based diet. We also found an interaction between forage source and antagonist on Cu intake, which was lowest ($P < 0.05$) in cows fed G+ compared

with the other 3 treatments. An interaction ($P < 0.05$) between forage source and Cu antagonists on milk yield was observed, with yield decreasing with the addition of S and Mo in cows fed the grass silage-based diet but increasing in those offered the corn silage-based diet. In contrast, we found no effect ($P > 0.05$) of dietary treatment on milk fat, protein, or lactose content or daily fat yield, but we found that daily milk protein yield was 0.05 kg/d higher ($P < 0.05$) in cows fed the corn silage-based diet. We found no effect ($P > 0.05$) of dietary treatment on live weight or daily live weight change, but an effect of antagonist on BCS and BCS change ($P < 0.05$), with cows fed added S and Mo (C+ and G+) having a lower score and gaining less BCS over the study period than those not supplemented with S and Mo (C– and G–; Figure 2). We observed an interaction ($P < 0.05$) between forage source and Cu antagonists on milk SCC count, with the addition of S and Mo increasing SCC in cows fed the grass silage- but not the corn silage-based diet.

Plasma Mineral Profile, Cu-Mediated Enzymes, and Metabolites

We found no effect ($P > 0.05$) of dietary treatment on plasma Cu concentration, with a mean value of 13.7 $\mu\text{mol/L}$ (Table 4). An effect of time on plasma Cu was noted, with the concentration increasing in the first week of the study and then fluctuating in subsequent weeks (Figure 3). We also found an effect ($P < 0.001$) of dietary treatment on mean plasma Mo concentrations, which were higher in cows fed added S and Mo, but

Figure 1. Weekly DMI in early-lactation dairy cows fed diets high in corn silage without (■) or with (□) added S and Mo, or diets high in grass silage without (▲) or with (△) added S and Mo. Pooled SEM = 0.72. Forage, $P < 0.001$; forage $\times$ antagonist, $P = 0.012$; time, $P < 0.001$; forage $\times$ time, $P = 0.003$.

observed no effect ($P > 0.05$) of basal forage. We noted no effect ($P > 0.05$) of dietary treatment on plasma Fe or Mn concentrations. Serum Cp concentrations were higher ($P < 0.01$) in cows fed the grass silage-based

diets or with added S and Mo ($P < 0.05$). In contrast, we found no effect of dietary treatment on blood Cp-to-Cu ratio, although a trend ($P < 0.1$) was noted for a lower ratio in cows fed the corn silage-based diets

Figure 2. Fortnightly BCS in early-lactation dairy cows fed diets high in corn silage without (■) or with (□) added S and Mo, or diets high in grass silage without (▲) or with (△) added S and Mo. Pooled SEM = 0.067. Antagonist, $P < 0.001$; time, $P < 0.001$; time $\times$ antagonist, $P = 0.077$.

Table 4. Plasma mineral concentration and metabolites and serum ceruloplasmin in early-lactation dairy cows fed diets high in corn silage (C) or grass silage (G) without (–) or with (+) added S and Mo¹

Item	Diet				SEM	<i>P</i> -value ²		
	C–	C+	G–	G+		F	A	Int
Plasma Cu, µmol/L	13.3	13.7	14.3	13.7	0.51	0.340	0.889	0.332
Plasma Mo, µmol/L	0.33	0.50	0.27	0.50	0.029	0.271	<0.001	0.375
Plasma Fe, µmol/L	43.2	40.5	40.7	40.9	1.61	0.519	0.446	0.384
Plasma Mn, µmol/L	0.25	0.24	0.27	0.25	0.010	0.124	0.239	0.740
Ceruloplasmin, mg/dL	17.9	15.9	20.3	18.1	0.79	0.006	0.010	0.909
Ceruloplasmin:Cu	1.37	1.22	1.41	1.36	0.057	0.096	0.090	0.377
SOD, ³ U/g of Hb	2,960	2,841	2,954	2,915	89.8	0.710	0.387	0.657
BHB, mmol/L	0.42	0.38	0.44	0.48	0.048	0.210	0.963	0.406
BUN, mmol/L	5.22	5.44	5.70	5.39	0.189	0.265	0.802	0.172

¹Blood samples were collected during wk 0, 1, 2, 4, 8 and 14 of the study.

²F = main effect of forage source, A = main effect of antagonists, Int = interaction between forage and antagonists. We noted time × treatment effect on plasma Mo ($P < 0.05$), which increased with time in animals receiving C+ and G+ compared with C– and G–.

³SOD = superoxide dismutase.

or in animals receiving added S and Mo. We observed no effect ($P > 0.05$) of dietary treatment on plasma SOD, BHB, or BUN concentrations, with mean values of 2918 U/g of Hb, 0.43 mmol/L, and 5.44 mmol/L, respectively.

Hepatic Mineral Concentration

We found no difference between dietary treatments ($P > 0.05$) in initial hepatic Cu concentration, which averaged 443 mg/kg of DM (Table 5). We did find an effect of forage source on final Cu concentration, which

was higher ($P < 0.05$) in cows fed the corn silage- compared with the grass silage-based diets. An effect of Cu antagonists on final hepatic Cu concentration was also noted, which was 142 mg/kg of DM lower ($P < 0.01$) in cows fed added S and Mo. We observed a trend ($P < 0.1$) for an interaction between forage source and Cu antagonists on the rate of change in hepatic Cu concentration, with a decrease of 61 mg/kg of DM over the 14-wk study period in cows fed added S and Mo in combination with grass silage (G+), but an increase of 11 mg/kg of DM in cows offered the corn silage-based diet (C+).

Figure 3. Plasma Cu concentrations in early-lactation dairy cows fed diets high in corn silage without (■) or with (□) added S and Mo, or diets high in grass silage without (▲) or with (△) added S and Mo. Pooled SEM = 0.87. Time, $P < 0.001$.

Table 5. Liver mineral concentrations in early-lactation dairy cows fed diets high in corn silage (C) or grass silage (G) without (–) or with (+) added S and Mo

Item	Diet				SEM	<i>P</i> -value ¹		
	C–	C+	G–	G+		F	A	Int
Initial Cu, mg/kg of DM	522	426	407	418	47.0	0.201	0.372	0.262
Final Cu, mg/kg of DM	587	437	490	357	41.0	0.038	0.002	0.837
Cu change, mg/kg of DM per day	0.66	0.11	0.84	–0.62	0.253	0.275	0.001	0.078
Initial Mo, mg/kg of DM	3.90	3.50	3.39	4.12	0.356	0.878	0.636	0.120
Final Mo, mg/kg of DM	3.92	4.19	3.79	4.71	0.221	0.377	0.011	0.149
Mo change, µg/kg of DM per day	0.20	6.94	4.08	6.02	4.622	0.750	0.356	0.600
Initial Fe, mg/kg of DM	378	313	288	295	36.6	0.150	0.422	0.334
Final Fe, mg/kg of DM	411	319	352	253	31.8	0.057	0.005	0.908
Fe change, µg/kg of DM per day	336	61	653	–429	222.4	0.690	0.005	0.079
Initial Mn, mg/kg of DM	10.20	9.60	9.15	10.41	0.565	0.839	0.560	0.109
Final Mn, mg/kg of DM	10.18	10.38	9.96	9.84	0.305	0.223	0.895	0.610
Mn change, µg/kg of DM per day	–0.20	7.96	8.26	–5.82	5.704	0.641	0.605	0.060

¹F = main effect of forage source, A = main effect of antagonists, Int = interaction between forage and antagonists.

We found no difference between treatments in initial hepatic Mo concentration ($P > 0.05$), whereas final Mo concentration was higher ($P < 0.05$) in cows fed added S and Mo (C+ and G+). Initial hepatic Fe concentration did not differ between treatments ($P > 0.05$), whereas final concentration was lower ($P < 0.01$) in cows fed added S and Mo, and a trend ($P < 0.1$) for final hepatic Fe concentration to be higher was found in cows offered the corn silage- compared with the grass silage-based diet. The addition of S and Mo resulted in a net decrease in hepatic Fe concentration over the study period of 19 mg/kg of DM compared with an increase in cows that were not supplemented with S and Mo of 50 mg/kg of DM, although most of this difference could be attributed to cows fed the grass silage-based ration with added S and Mo (G+) decreasing in hepatic Fe concentration ($P < 0.1$) compared with an increase in cows fed any of the other dietary treatments. Finally, we found no effect ($P < 0.05$) of dietary treatment on hepatic Mn concentrations, although cows fed the grass silage with Cu antagonists (G+) tended ($P < 0.1$) to decrease by the greatest amount.

DISCUSSION

Intake and Performance

Our study is the first to determine Cu status and metabolism in high-yielding dairy cows when fed corn silage- or grass silage-based rations at different S and Mo concentrations. Corn silage is generally regarded as having a lower Cu concentration than grass silage (NRC, 2001), but we supplemented the diets to ensure that levels were similar across all treatments, averaging 20.0 mg of Cu/kg of DM. The dietary level of 20 mg of Cu/kg of DM was lower than the mean value of 27.9

mg/kg of DM per day that was reported in the diet of early-lactation cows in the United Kingdom (Sinclair and Atkins, 2015), but similar to the 18 mg/kg of DM reported on 39 Californian dairy units by Castillo et al. (2013). Additionally, we added S and Mo at a rate to ensure that the supplemented diets (C+ and G+) had similar concentrations, which would be expected to substantially reduce Cu absorption and subsequent metabolism. Differences in dietary S and Mo concentration between diets within the same level of antagonist was small ($P > 0.05$), and therefore the main effect was the difference between the unsupplemented and supplemented diets. Using the equations of Suttle and McLauchlin (1976), we predicted that the C– and G– diets would result in an apparent absorption coefficient of Cu of approximately 0.054, whereas the C+ and G+ diets would be two-thirds lower at approximately 0.018. As a consequence, we predicted that animals receiving C– or G– had a similar Cu supply but were over supplied by approximately 220 mg of Cu/d, whereas those receiving C+ or G+ were undersupplied by approximately 200 mg of Cu/d. However, the use of the current equations did not predict any interaction between forage source and antagonist on Cu status or performance.

Similar to other studies that have investigated the effect of replacing grass silage with corn silage (Phipps et al., 1995; Hart et al., 2015), we found that DMI was increased at the higher corn inclusion rate, although it is accepted that the change in forage composition from the prestudy diet was greater for cows on G than C diets. However, we also found an interaction between forage inclusion level and Cu antagonists on intake, with added S and Mo having little effect in cows fed the corn silage-based diet but reduced intake by 2.1 kg of DM/d in those receiving the grass silage-based diet. Our diets were supplemented with both S and Mo, and it is

therefore not possible to determine the effects of each element independently. Some authors have reported a decrease in DMI in cattle when dietary S exceeded 2 g/kg of DM (Spears et al., 2011), although others have reported little effect of dietary S concentration up to 6 g/kg of DM (Richter et al., 2012). Under acidic ruminal conditions, most of the S would be present as H_2S , which may be eructated and absorbed by the lungs or absorbed across the rumen epithelium (Bray and Till, 1975; Drewnoski et al., 2012). High circulating concentrations of H_2S can have neurological effects, including polioencephalomalacia, which is associated with a reduced intake (Gould, 1998). The large role that ruminal pH plays in the form of sulfide present in the rumen has been suggested as a possible explanation for the differences observed in sulfur tolerance between concentrate and roughage fed cattle (Drewnoski et al., 2012), and could explain the reduced DMI of cows offered G+ in our study. However, we did not monitor ruminal H_2S or pH levels, and the influence of level of inclusion of corn and grass silage on ruminal pH is difficult to predict, as it is dependent on several factors including initial forage pH, buffering capacity of the diet, forage particle length, and supplementary feed level, composition, and degree of processing (Krause and Oetzel, 2006).

Molybdenum interacts with S in the rumen, resulting in the formation of various isomers of thiomolybdate, a reaction that is reversible and pH-dependent, with the formation of tetra-thiomolybdate being favored at lower ruminal pH values (Gould and Kendall, 2011). Indeed, the dietary addition of Mo has been proposed as a potential sink for H_2S in the rumen (Kessler et al., 2012), potentially reducing the negative effects of excess dietary S on intake, although this approach has not been supported by recent studies with beef animals (Kessler et al., 2012). An alternative hypothesis for the effect of added S and Mo on intake may be related to the absorption of tetra-thiomolybdates, as these can have a direct effect on Cu containing enzymes such as peptidylglycine α -amidating monooxygenase, which exerts an influence on the appetite-regulating hormones cholecystokinin and gastrin (Suttle, 2010), although studies in this area in ruminants are scarce. Ruminal absorption of tetra-thiomolybdates is increased at lower ruminal pH values, and it is possible that differences in the ruminal pH in cows fed the different forages affected uptake. The conditions under which thiomolybdates are absorbed is, however, a controversial subject area; Suttle (2010) proposed that absorption was unlikely unless dietary Cu:Mo ratios were below 1:1, well below the 2.5:1 in our C+ and G+ diets. It is also possible that the added Mo resulted in molybdenosis, however, no characteristic signs such as scouring were noted and dietary values were well below that reported in other

studies that have also reported no signs (Raisbeck et al., 2006).

Studies that have fed varying levels of Cu to dairy cows in the absence of high levels of dietary antagonists have reported little effect on DMI (see review of Sinclair and Mackenzie, 2013); therefore, it appears unlikely that a lower tissue supply of Cu per se was responsible for the differences in DMI reported here. It is of interest that the inclusion of S and Mo reduced BCS in the cows in our study, irrespective of basal forage level. This effect may be attributed to different mechanisms for each of the forage treatments, as milk yield was higher in cows fed C+ compared with C-, whereas intake was lower in cows fed G+ compared with G-.

The interaction between basal forage source and Cu antagonists on milk SCC in our study is difficult to explain, although all values were low. The role of Cu on milk SCC has been demonstrated in dairy cattle in some but not all studies. For example, increasing dietary Cu concentration from a suboptimal level of 6.5 to 26.5 mg/kg of DM was shown to reduce the peak increase in milk SCC following a challenge with *Escherichia coli*, which was attributed to a greater ability of neutrophils to kill invading bacteria, although the duration of the infection was unaffected (Scaletti et al., 2003). In contrast, dietary Cu concentration was not shown to have an effect on milk SCC concentration following a challenge with *E. coli* in the studies of Scaletti and Harmon (2012), or when different levels of dietary Cu were fed (Chase et al., 2000). In our study, cows receiving G+ were in negative Cu balance, as evidenced by the depletion of hepatic Cu reserves, whereas all other treatments were in positive balance. It is therefore possible that this lower Cu status contributed to the increased milk SCC, although other indicators of Cu status, such as plasma Cu and plasma Cp:Cu, were unaffected by dietary treatment. The lower DMI we observed in cows receiving G+ may also have contributed to a greater metabolic stress and indirectly increased milk SCC.

Plasma Mineral Profile, Cu-Mediated Enzymes, and Metabolites

We found that plasma Cu concentrations were unaffected by dietary treatment, with all values being above the 9 mmol/L considered to be adequate (Laven and Livesey, 2005). Our finding is consistent with others that have supplemented Cu at different levels (Chase et al., 2000), with different levels of dietary S and Mo (Sinclair et al., 2013), or with different dietary sources of Cu (Scaletti and Harmon, 2012; Sinclair et al., 2013). In a meta-analysis of the relationship between dietary concentration of Cu, S, and Mo and plasma Cu in grow-

ing cattle, Dias et al. (2013) concluded that any prediction equation would be limited, and that it is only when animals have either very low or high hepatic Cu reserves that plasma values can be usefully employed as an indicator of Cu status (Laven and Livesey, 2005). The plasma Cp:Cu ratios reported in our study were generally low and unaffected by dietary treatment. Similarly, we found that plasma SOD, a Cu-containing enzyme involved in the defense against free radicals (Suttle, 2010), was unaffected by dietary treatment. Our findings therefore support Suttle (2010), who suggested that the dietary ratio of Cu to Mo needed to be close to 1:1 before a risk of thiomolybdates causing a systemic impairment of Cu-containing enzymes occurs.

Hepatic Mineral Concentration

One of the first biochemical changes observed under Cu deprivation is a decrease in hepatic concentration (Suttle, 2010), as the liver is generally regarded as the principal storage organ for Cu (Laven and Livesey, 2005). In our study, initial hepatic Cu levels were high and variable at 443 ± 29.2 (SE) mg/kg of DM, although most (68%) animals were below the upper limit of 510 mg/kg of DM suggested to pose a risk of toxicity (Livesey et al., 2002). The initial mean hepatic Cu concentration that we found was also lower than that reported by Kendall et al. (2015), where almost 40% of cull dairy cows in the United Kingdom were reported to have a concentration above 500 mg of Cu/kg of DM. As we anticipated, a significant reduction in hepatic Cu concentration occurred following the addition of dietary S and Mo, but the greater reduction in cows fed a grass silage- compared with a corn silage-based diet was unexpected, although the difference failed to reach full statistical significance. Suttle (2013) discussed changes in hepatic Cu concentration that are an exponential function of initial hepatic Cu concentration, most probably due to a greater rate of biliary excretion at higher liver concentrations. Therefore, we $\log_e$ -transformed and reanalyzed the initial and final hepatic Cu concentrations to more accurately determine the influence of diet on hepatic Cu reserves. Similar to the untransformed data, we found no difference ($P > 0.1$) between treatments in initial liver Cu concentration, but we did find an interaction ($P < 0.05$) between forage source and Cu antagonist on daily liver Cu balance ($\log_e \text{ final} - \log_e \text{ initial}$), confirming that high dietary concentrations of S and Mo have a greater effect on Cu metabolism in cows receiving a grass silage- than a corn silage-based diet.

The influence of forage source on the absorption of Cu is well demonstrated in sheep (e.g., Suttle, 1983, 2010), and in the absence of high Mo concentrations,

the absorption coefficient of Cu was reported to be 0.014 in grazed grass, 0.049 in grass silage, 0.073 in hay, and 0.128 in leafy brassicas. Our study, however, is the first to report a substantial difference in Cu status in dairy cows fed corn silage- or grass silage-based rations, but only when S and Mo concentrations were high. Dietary Fe may interact with added S reducing hepatic Cu concentration (Suttle, 2010). However, the low dietary concentration of Fe in all of our diets compared with that reported for typical dairy cow rations in the United Kingdom (Sinclair and Atkins, 2015) or California (Castillo et al., 2013), in combination with the similarity in dietary Fe and S concentration between C+ and G+, does not support Fe as having a major influence. Consideration should also be given to the lower DMI of cows receiving G+, which resulted in a lower Cu intake of 49 mg/d than G-. Nevertheless, at the rate of decline in hepatic Cu concentration in cows receiving G+, concentrations would reduce and eventually approach the 25 mg of Cu/kg of DM threshold considered to deficient (Laven and Livesey, 2005). In contrast, in cows fed C- or G-, feeding 20 mg of Cu/kg of DM would result in a rapid increase in hepatic Cu concentration, whereas those receiving C+ would be relatively unchanged. Given such large differences in Cu status when fed the same dietary level, we recommend that forage source as well as dietary S and Mo concentration should be taken into account when supplementing dairy cows with Cu.

Similar to our previous study (Sinclair et al., 2013), liver Mo concentrations were little affected by dietary treatment, despite a 6.5 mg/kg of DM difference in dietary concentration between (-) and (+) treatments, and we can conclude that the liver does not appear to be either a major store or a sensitive indicator of Mo status. Ferritin is the main storage form of Fe in the body and is particularly concentrated in the liver, where concentrations of between 100 to 1000 mg of Fe/kg of DM are considered to be normal in cattle (Suttle, 2010). Hepatic Fe concentrations at the beginning and end of our study were within this range, but, similar to Cu, hepatic Fe concentrations were negatively affected by the addition of S and Mo, particularly in the grass silage-based diet. In contrast, Phillipppo et al. (1987) reported in growing calves fed a barley straw-based diet that an additional 5 mg of Mo/kg of DM increased liver Fe concentrations, which was associated with a decrease in plasma Fe concentrations.

CONCLUSIONS

Addition of S and Mo had no effect on DMI or milk yield in cows fed a corn silage-based ration, but DMI and milk yield were reduced and milk SCC increased

when a grass silage-based diet was fed. In the absence of additional S and Mo, a diet containing 20 mg of Cu/kg of DM, whether based on grass or corn silage, supplies well in excess of requirements, as evidenced by the net increase in hepatic Cu concentration. In contrast, in the presence of high levels of S and Mo, feeding 20 mg of Cu/kg of DM will result in a rapid depletion of hepatic Cu concentrations in cows fed grass silage-, but not corn silage-based diets. Within the limits of this study we also found little effect of added Cu antagonists on plasma Cu or indicators of plasma Cu enzyme activity, even at the high levels of S and Mo, and concluded that use of these parameters to predict Cu status is limited. Reasons for the differences in Cu metabolism in cows when fed grass or corn silage based rations is unclear and require further investigation, but our results highlight the importance of taking account of forage source when formulating diets for dairy cows, particularly when dietary S and Mo levels are high.

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