Dietary starch concentration alters reticular pH, hepatic copper concentration, and performance in lactating Holstein-Friesian dairy cows receiving added dietary sulfur and molybdenum

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ABSTRACT

To test the hypothesis that Cu metabolism in dairy cows is affected by dietary starch concentration and additional sulfur S and Mo, 60 Holstein-Friesian dairy cows that were [mean $\pm$ standard error (SE)] 33 ± 2.5 days postcalving and yielding 41 ± 0.9 kg of milk/d were fed 1 of 4 diets in a 2×2 factorial design experiment over a 14-wk period. The 4 diets had a Cu concentration of approximately 15 mg/kg of dry matter (DM), a grass silage-to-corn silage ratio of 1:1, a dietary starch concentration of either 150 g/kg of DM (low starch, LS) or 220 g/kg of DM (high starch, HS), and were either unsupplemented (–) or supplemented (+) with an additional 0.8 g of S/kg of DM and 4.4 mg of Mo/kg of DM. We found an effect of dietary starch concentration on mean reticular pH, which was 0.15 pH units lower in cows fed the high starch diets. The addition of S and Mo decreased intake by 1.8 kg of DM/d, an effect that was evident beginning in wk 1 of the study. Mean milk and fat yields were 37.0 and 1.51 kg/d, respectively, and were not affected by dietary treatment. We found an effect of dietary starch concentration on milk protein concentration, protein yield, and urea nitrogen, which were increased by 2.8 g/kg, 0.09 kg/d, and 2.1 mg/dL, respectively, in cows fed the high starch diets. We found no effect of dietary treatment on either cow live weight or body condition. Mean plasma Cu, Fe, and Zn concentrations were 15.3, 42.1, and 14.4 μ mol/L, respectively, and were not affected by dietary treatment. In contrast, we found an interaction between dietary starch concentration and Cu antagonists on plasma Mo, where feeding additional S and Mo increased plasma Mo to a greater extent when cows were offered the high versus low starch diet. We also found that increasing dietary starch concentration increased serum ceruloplasmin activity, but serum

haptoglobin concentration was not affected by dietary treatment. The addition of S and Mo decreased hepatic Cu concentration, whereas in cows fed the higher dietary starch concentration, hepatic Cu concentration was increased over the period of our study. We concluded that increasing dietary starch concentration decreases rumen pH and increases milk protein yield and hepatic Cu concentration, whereas feeding additional S and Mo decreases intake and hepatic Cu concentration. **Key words:** copper, dairy cow, dietary starch, rumen pH

INTRODUCTION

Copper is an essential trace element, and its dietary supply has consequences for dairy cow health and performance (Suttle, 2010). Copper-responsive disorders have been related to impairments of growth, reproduction, and red blood cell formation (McDowell, 1985; Suttle et al., 1987). Although a dietary deficiency of Cu is possible, these disorders are more commonly related to interactions with dietary antagonists such as S, Mo, Fe, and Zn, which inhibit Cu absorption (Suttle, 2010; Gould and Kendall, 2011). It has been proposed that dietary sulfates in feed and water are reduced to sulfides within the rumen (Bradley et al., 2011) and then react sequentially with molybdate in a stepwise manner to form mono-, di-, tri-, and tetrathiomolybdates (Dick et al., 1975; Gould and Kendall, 2011), with each thought to have differing consequences for Cu absorption (Suttle, 1991). Clarke and Laurie (1980) reported that thiomolybdate formation was highly pH dependent, with a greater proportion of tetrathiomolybdates formed at lower pH values. It is also proposed that other dietary factors such as basal forage and preservation method may affect Cu status (Suttle, 1983; 2010), although our understanding of the underlying mechanisms remains poor. For example, Sinclair et al. (2017) reported a greater decrease in hepatic Cu concentration when cows were fed additional S and Mo in a grass silage-based versus a corn silage-based diet, and it was

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suggested that this may have been due to the potential effect of rumen pH on S metabolism and thiomolybdate formation.

It is well established that the fermentable carbohydrate concentration of the diet can influence rumen pH (Humer et al., 2018). Corn silage is higher in starch than other forages such as grass or alfalfa silage (Hassanat et al., 2013; Tayyab et al., 2019), and its inclusion is often associated with a decrease in rumen pH (Firkins, 1997; Tayyab et al., 2019). There is also a large body of evidence regarding the effect of dietary starch source and concentration on rumen pH, as well as milk, fat, and protein yields (Gómez et al., 2016). There is, however, little information on the effect of dietary starch concentration on Cu metabolism in lactating Holstein-Friesian dairy cows, despite reported links between dietary starch, rumen pH, and thiomolybdate formation (Gould and Kendall, 2011; Tayyab et al., 2019). This lack of understanding may be contributing to the over-supplementation of Cu in dairy cow rations (Suttle, 2016). Surveys in both the United States and United Kingdom (Castillo et al., 2013; Sinclair and Atkins, 2015) have reported Cu supplementation on farm to be in excess of the nutritional guidelines proposed by NRC (2001) and ARC (1980). Feeding Cu above the animal's requirement can result in clinical Cu poisoning, a condition whereby high hepatic Cu concentrations lead to lysosome rupture, hepatic necrosis, and eventual death (Bidewell et al., 2000). Kendall et al. (2015) reported that 38% of Holstein-Friesian dairy cows within the United Kingdom have hepatic Cu concentrations at slaughter above the 508 mg of Cu/kg of DM threshold generally regarded to pose a risk of clinical Cu toxicity. The objectives of the current study were to determine the effect of dietary starch concentration when fed with or without additional S and Mo on rumen pH, performance, and indicators of Cu status in lactating Holstein-Friesian dairy cows.

MATERIALS AND METHODS

Animals, Treatments, Housing, and Management

All procedures involving animals were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 (Amended Regulations; UK Parliament, 2012), and received local ethical approval. Sixty Holstein-Friesian dairy cows (48 multiparous and 12 primiparous) that were (mean $\pm$ SE) 33 ± 2.5 d postcalving, with a live weight of 659 ± 17.7 kg and yielding 41 ± 0.9 kg/d were used. Based upon recordings taken in the week before allocation, cows were blocked and allocated to 1 of 4 dietary treatments according to calving date, parity (multiparous or primiparous), milk

yield, and BCS (5-point scale with 0.25 increments; Ferguson et al., 1994). Cows remained on study for a total of 98 d.

Four isoenergetic and isonitrogenous diets were formulated to contain a grass:corn silage ratio of 1:1 (DM basis), 15 mg of Cu/kg of DM, and a dietary starch concentration of 150 (low starch, **LS**) or 220 (high starch, **HS**) g/kg of DM (Table 1). The starch concentration of the treatment diets was obtained by partially replacing wheat with a combination of soy hulls and molassed sugar beet feed, with further manipulation of the dietary ingredients to maintain the same metabolizable protein-rumen energy limited (**MPE**) supply of 104 g/kg of DM and metabolizable protein-rumen nitrogen limited (**MPN**) supply of 116 (**LS**) or 114 (**HS**) g/kg of DM, according to Thomas (2004). To determine the effects of Cu antagonists, the diets were either unsupplemented (–) or supplemented (+) with added S and Mo. Additional Cu was supplied as copper sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), sulfur as flowers of sulfur, and molybdenum as sodium molybdate (Rumenco, Staffordshire, UK). The antagonist-supplemented diets were formulated to contain a total dietary concentration of approximately 3.0 g of S/kg of DM and 5.5 mg of Mo/kg of DM, an increase of approximately 0.8 g of S/kg of DM and 4.4 mg of Mo/kg of DM, respectively, compared with the unsupplemented diets. The 4 dietary treatments were **LS**– (150 g/kg of DM dietary starch, no additional antagonists), **LS**+ (150 g/kg of DM dietary starch, with additional S and Mo), **HS**– (220 g/kg of DM dietary starch, no additional antagonists), and **HS**+ (220 g/kg of DM dietary starch, with additional S and Mo). All 4 diets were formulated to support a milk yield of approximately 38 kg/d according to Thomas (2004). Dietary ingredients were mixed using a forage mixer calibrated to ± 0.1 kg, with the resulting TMR being fed through roughage intake feeders (Hokofarm, Marknesse, the Netherlands), fitted with an automatic weighing and animal identification system calibrated to ± 0.1 kg (Sinclair et al., 2007). Fresh feed was offered at 0900 h daily at 105% of the previous recorded intake, and refusals were collected 3 times per week on Monday, Wednesday, and Friday. Cows were housed in freestalls fitted with mattresses in the same area of an open span building. Stalls were bedded 3 times a week with a sawdust-lime mix, and passageways were scraped by automatic scrapers. All cows had continual access to fresh water.

Experimental Routine

Cows were milked twice daily through a 40-point internal rotary parlor at approximately 0600 and 1600 h. The milk yield of each cow was recorded at each milk-

Table 1. Diet composition and chemical analysis of low- (LS) and high-starch (HS) rations fed either without (–) or with (+) added S and Mo

Item	LS–	LS+	HS–	HS+
Ingredient, g/kg of DM				
Grass silage	264	264	265	265
Corn silage	264	264	265	265
Rolled wheat	13	13	212	212
SoyPass ¹	42	42	—	—
Soy hulls	157	157	59	59
Molassed sugar beet feed	114	114	—	—
Soybean meal	59	59	86	86
Rapeseed meal	29	28	43	43
Wheat distillers dark grains	29	29	43	43
Palm kernel meal	8	8	12	12
Molasses	3	3	3	3
Limestone	—	1	—	1
Salt	1	1	1	1
Protected fat	8	8	2	2
Minerals and vitamins ^{2,3}	9 ²	9 ³	9 ²	9 ³
Total	1,000	1,000	1,000	1,000
Chemical analysis				
DM, g/kg	430	426	429	423
CP, g/kg of DM	166	164	167	166
Ash, g/kg of DM	78	79	68	70
OM, g/kg of DM	922	921	932	930
NDF, g/kg of DM	435	441	376	374
ADF, g/kg of DM	273	267	229	223
Starch, g/kg of DM	149	148	224	225
Ether extract, g/kg of DM	22	23	24	22
ME, MJ/kg of DM	12.0	12.0	12.1	12.1
Ca, g/kg of DM	6.77	5.26	6.79	5.31
P, g/kg of DM	4.03	4.04	4.14	4.20
Mg, g/kg of DM	2.28	2.20	2.25	2.17
S, g/kg of DM	2.25	3.04	2.20	2.96
Cu, mg/kg of DM	15.1	14.9	14.8	14.6
Mo, mg/kg of DM	1.1	5.7	1.1	5.5
Zn, mg/kg of DM	70.6	71.4	73.0	68.1
Fe, mg/kg of DM	248	242	252	234
Mn, mg/kg of DM	62.4	65.9	66.4	59.0

¹Xylose-treated soybean meal (KW Alternative Feeds, Staffordshire, UK).

²LS– and HS– mineral/vitamin premix (Rumenco, Staffordshire, UK). Major minerals (g/kg): Ca 180, P 53, Mg 75, Na 75, and S 0; Trace minerals (mg/kg): Cu 825, Zn 4,500, Mn 1,500, I 300, Co 30, Se 26, and Mo 0; vitamins (IU/kg) were retinol 225,000, cholecalciferol 75,000, and all-*rac* α -tocopherol acetate 3,000.

³LS+ and HS+ mineral/vitamin premix (Rumenco). Major minerals (g/kg): Ca 148, P 53, Mg 75, Na 75, and S 90; trace minerals (mg/kg): Cu 825, Zn 4,500, Mn 1,500, I 300, Co 30, Se 26, and Mo 488; vitamins (IU/kg) were retinol 225,000, cholecalciferol 75,000, and all-*rac* α -tocopherol acetate 3,000.

ing, with samples taken every other week at consecutive morning and afternoon milkings for analysis of composition and SCC. Cows were weighed and scored for body condition before the afternoon milking in the week before allocation, and every 2 wk thereafter. Reticular pH boluses (eCow Ltd., Devon, UK) that recorded reticular pH at 15-min intervals (96 data points/d) were administered to 24 cows (6 per treatment) in the week before commencing the study. Forage samples were collected weekly, dried to a constant weight, and the corn to grass silage adjusted to the desired ratio. Fresh samples of the 4 diets were collected immediately after feeding on a weekly basis and stored at -20°C before subsequent analysis. Blood samples were collected by jugular venipuncture at 1100 h during wk 0, 1, 2, 4, 6,

10, and 14 of the study into Vacutainers (Becton Dickinson Vacutainer Systems, Plymouth, UK) containing lithium heparin [to determine plasma urea N (**PUN**) and BHB], silica gel [to determine serum haptoglobin (**Hp**) and ceruloplasmin (**Cp**) activity], fluoride/oxalate (to determine plasma glucose), tripotassium EDTA [to determine superoxide dismutase (**SOD**) activity], and trace element Vacutainers containing dipotassium EDTA (to determine plasma minerals; guaranteed maximum copper level of $5.0\text{ }\mu\text{g/L}$; catalog no. 36381). Liver biopsy samples were collected during wk 0 and 14 of the study via the 11th intercostal space, as described by Davies and Jebbitt (1981), immediately snap frozen in liquid nitrogen, and stored at -80°C before subsequent analysis.

Chemical Analysis

Weekly diet samples were bulked monthly and analyzed according to AOAC International (2012) for DM [method 934.01; intra-assay coefficient of variation (CV) of 1.5%], CP (method 990.03; intra-assay CV of 1.6%), ether extract (method 2003.05; intra-assay CV of 5.3%), and ash (method 942.05; intra-assay CV of 0.8%). Samples were also analyzed for starch (intra-assay CV of 3.3%) according to ISO 6493 (ISO, 2000) at Sciantic Analytical (Stockbridge Technology Centre, North Yorkshire, UK), and the ME content of the diets was estimated from their composition. The NDF and ADF contents of the diets were determined according to Van Soest et al. (1991); heat-stable α -amylase was used for NDF determination (Sigma Aldrich, Poole, UK; intra-assay CV of 1.2% for NDF and 2.7% for ADF). Dietary minerals were extracted using a DigiPREP digestion system (QMX Laboratories, Essex, UK), and analyzed by inductively coupled plasma-mass spectrometry (ICP-MS; Nexion 2000; Perkin Elmer, Beaconsfield, UK) as described by Sinclair and Atkins (2015), with the use of Ga as an internal standard. Accuracy of dietary mineral analysis was checked by extraction and reference to certified European Union (EU) reference samples of hay (BCR-129) and dairy concentrate (BCR-185). Plasma samples were analyzed for PUN, BHB, and glucose (Randox Laboratories, Antrim, UK; kit catalog nos. UR221, RB1007, GL1611; intra-assay CV of 2.6, 4.0, and 0.9% respectively). Serum samples were analyzed for Cp activity according to Henry et al. (1974; intra-assay CV of 0.9%), and Hp (Tridelta Development Ltd., Kildare, Ireland; kit catalog no. HP801; intra-assay CV of 0.8%). Whole-blood samples were analyzed for SOD activity (Randox Laboratories; kit catalog no. SD 125; intra-assay CV of 3.3%). The analysis of all plasma, serum, and whole-blood samples was conducted using a Cobas Miras Plus auto-analyzer (ABX Diagnostics, Bedfordshire, UK). Liver samples were dried at 60°C to a constant mass, digested overnight at 60°C in concentrated nitric acid, and made up to 50 mL in a DigiPREP tube (QMX Laboratories). Plasma and liver samples were analyzed for Cu, Fe, Zn, and Mo by ICP-MS following a 1:50 dilution in 0.5% HNO₃, 1% HPLC-grade methanol, and 0.05% Triton X-100, with the use of Ga as an internal standard. Accuracy of plasma and liver mineral analysis was checked by reference to ClinCheck certified lyophilized plasma control sample 2 (product no. 8885, RECIPE Chemicals and Instruments GmbH, Munich, Germany), and a certified EU reference bovine liver sample (BCR-185). Milk samples were analyzed for fat, protein, lactose, MUN and SCC by Eurofins Laboratories (Wolverhampton, UK).

Calculations and Statistical Analysis

The percentage of time that daily reticular pH was below the threshold values of pH 5.8, 6.0, 6.2, and 6.5 was calculated by assuming that the change in reticular pH between 15-min time points was linear.

Continuous performance, rumen pH, and blood parameters were analyzed as a 2 × 2 factorial design using a repeated-measures ANOVA. The treatment degrees of freedom were split into main effects of dietary starch concentration (low versus high), antagonist [with (+) versus without (−)], and their interaction, and analyzed as follows:

$$Y_{ijkl} = \mu + B_i + S_j + A_k + T_l + S \cdot A_{jk} + S \cdot T_{jl} \\ + A \cdot T_{kl} + S \cdot A \cdot T_{jkl} + \epsilon_{ijkl},$$

where Y_{ijkl} = dependent variable; μ = overall mean; B_i = fixed effect of blocks; S_j = effect of starch concentration (j = low or high); A_k = effect of S and Mo (k = with or without); T_l = effect of time (time of day or week of study, depending on the variable); $S \cdot A_{jk}$ = interaction between dietary starch concentration and antagonists; $S \cdot T_{jl}$ = interaction between dietary starch concentration and time; $A \cdot T_{kl}$ = interaction between dietary antagonists and time; $S \cdot A \cdot T_{jkl}$ = interaction between starch concentration, antagonists, and time; and ϵ_{ijkl} = residual error.

Noncontinuous performance parameters including hepatic mineral concentration were analyzed as a 2 × 2 factorial design ANOVA, as follows:

$$Y_{ijk} = \mu + B_i + S_j + A_k + S \cdot A_{jk} + \epsilon_{ijk},$$

where Y_{ijk} = dependent variable; μ = overall mean; B_i = fixed effect of blocks; S_j = effect of starch concentration (j = low or high); A_k = effect of S and Mo (k = with or without); $S \cdot A_{jk}$ = interaction between starch concentration and antagonists; and ϵ_{ijk} = residual error. Milk SCC was log₁₀ transformed before analysis, and live weight change was determined by linear regression of live weight measurements every 2 wk. All statistical analysis was conducted using Genstat version 18 (VSN International Ltd., Oxford, UK), and means are presented with their associated standard error of the mean.

RESULTS

Dietary Analysis, Reticular pH, Intake, and Animal Performance

We observed all 4 diets to have similar contents of DM, CP, and ether extract, with mean values of 427 g/

Figure 1. Reticular pH of early lactation dairy cows fed low-starch diets without ($\blacktriangle$) or with ($\triangle$) added S and Mo or high-starch diets fed without ($\blacksquare$) or with ($\square$) added S and Mo. Pooled SEM = 0.067. Starch, $P = 0.022$; antagonist, $P = 0.755$; starch $\times$ antagonist, $P = 0.487$; time, $P < 0.001$; time $\times$ starch, $P = 0.256$; time $\times$ antagonist, $P = 0.601$; time $\times$ starch $\times$ antagonist, $P = 0.305$. Fresh feed was offered daily at 0900 h.

kg, 166 g/kg of DM, and 23 g/kg of DM respectively (Table 1). Mean dietary NDF and ADF values were 63 and 44 g/kg of DM higher, respectively, in the low- (LS- and LS+) compared with the high- (HS- and HS+) starch diets, whereas the mean dietary starch content of the high-starch diets was 76 g/kg of DM higher than the low-starch diets, with mean concentrations of 149 and 225 g/kg of DM, respectively. All 4 diets had similar concentrations of Mg and P, with mean values of 2.23 and 4.10 g/kg of DM, respectively. We also observed similar dietary Cu concentrations across all 4 diets, with a mean value of 14.9 mg/kg of DM. In contrast, diets containing additional antagonists (LS+ and HS+) had S and Mo concentrations that were 0.78 g/kg of DM and 4.5 mg/kg of DM higher, respectively, than that of the unsupplemented diets (LS- and HS-).

We observed reticular pH to be at its highest immediately before feeding, with a decline in cows fed any of the treatments, thereafter reaching a nadir at approximately 1800 h ($P < 0.001$; Figure 1). We also noted an effect of dietary starch concentration on mean reticular pH, which was 0.15 pH units lower ($P = 0.022$) in cows fed the high- versus low-starch diets (pH 6.23 vs. 6.38 for HS and LS, respectively; Table 2). Cows fed the high-starch diets had a minimum daily reticular pH that was 0.20 pH units lower ($P = 0.008$; pH 5.81 versus 6.01 for HS and LS, respectively), and spent a greater percentage of time below pH 5.8 ($P = 0.048$; 90 vs. 1 min/d) and pH 6.0 ($P = 0.017$; 307 vs. 65

min/d) compared with those fed the low-starch diets. In contrast, we did not observe an effect of additional S and Mo on reticular pH.

Cows offered diets containing additional S and Mo had a daily DMI that was 1.8 kg of DM/d lower ($P < 0.001$) than those fed unsupplemented diets (Table 3), an effect that was evident from wk 1 of the study (Figure 2). We also noted that the addition of S and Mo decreased DMI to a greater extent when cows were fed high- versus low-starch diets (-2.7 vs. -0.9 kg/d for HS and LS, respectively; $P = 0.057$). In contrast, we did not find an effect of dietary treatment on milk yield, with a mean value of 37.0 kg/d. Cows fed the high-starch diets had milk protein content, protein yield, and MUN that were 2.8 g/kg ($P < 0.001$), 0.09 kg/d ($P = 0.004$), and 2.1 mg/dL ($P = 0.034$) higher than those offered the low-starch diets; however, there was no effect of dietary treatment on either the content or yield of milk fat or lactose. We also found no effect of dietary treatment on BW, daily BW change, BCS, or BCS change.

Hepatic Mineral Concentration

We found that cows offered the high-starch diets had hepatic Cu concentrations at the end of the study that were 57 mg/kg of DM higher ($P = 0.018$) than those fed the low-starch diets (Table 4). We also observed an effect of Cu antagonists on final hepatic Cu concentra-

Table 2. Daily reticular pH of dairy cows fed low- (LS) or high-starch (HS) diets without (–) or with (+) added S and Mo

Item	Diet				SEM	<i>P</i> -value ¹		
	LS–	LS+	HS–	HS+		S	A	Int
Daily minimum pH	5.97	6.05	5.86	5.76	0.062	0.008	0.856	0.168
Daily maximum pH	6.72	6.77	6.63	6.67	0.068	0.199	0.513	0.914
Mean pH	6.35	6.41	6.24	6.22	0.056	0.022	0.755	0.487
% time <5.8 pH ²	0.09	0.00	3.50	9.04	2.889	0.048	0.404	0.316
% time <6.0 pH	4.96	4.01	19.00	23.57	6.013	0.017	0.769	0.655
% time <6.2 pH	24.0	22.9	43.5	40.9	8.49	0.050	0.828	0.932
% time <6.5 pH	73.9	60.7	78.5	78.6	7.00	0.137	0.370	0.365

¹S = main effect of dietary starch level, A = main effect of dietary antagonists. Int = interaction between dietary starch level and antagonists.

²Data not normally distributed.

tion, which was 108 mg of Cu/kg of DM lower ($P < 0.001$) in cows fed additional S and Mo compared with those offered the unsupplemented diets.

We found no difference between treatments in initial hepatic Mo concentration, but final Mo concentrations were 0.26 mg/kg of DM higher ($P = 0.008$) in cows offered diets containing additional S and Mo. We also observed that cows fed the high-starch diets had 0.19 mg/kg of DM lower ($P = 0.049$) final hepatic Mo concentration than those fed the low-starch diets. In contrast, we found no effect of dietary treatment on hepatic Fe or Zn concentration, although cows fed diets containing additional S and Mo had a 0.21 mg/kg of DM/d higher net gain in hepatic Zn ($P = 0.076$).

Plasma Mineral Profile, Blood Metabolites, and Cu-Mediated Enzymes

We did not find an effect of dietary treatment on plasma Cu concentration, with a mean of 15.3 $\mu\text{mol/L}$ (Table 5). In contrast, we found an effect ($P < 0.001$) of time on plasma Cu concentration, which increased

between wk 0 and 2 of the study, before decreasing until wk 4, and fluctuating thereafter (Figure 3a). We also found an increase ($P = 0.004$) in mean plasma Mo concentration when cows were offered the high- versus low-starch diets, and when cows were fed additional S and Mo compared with unsupplemented diets ($P < 0.001$). An interaction ($P = 0.037$) between dietary starch concentration and Cu antagonists on mean plasma Mo concentration was also noted, where the addition of S and Mo increased plasma Mo concentration to a greater extent compared with unsupplemented cows when offered the high- versus low-starch diets (+0.57 vs. +0.42 $\mu\text{mol/L}$ for HS and LS, respectively). An effect of time ($P < 0.001$; Figure 3b) on plasma Mo was also noted, with the concentration increasing between wk 0 and 4 in our study, but remaining relatively stable thereafter. In contrast, we found no effect of dietary treatment on plasma Zn or Fe concentration, with mean values of 14.4 and 42.1 $\mu\text{mol/L}$, respectively. Serum Cp activity was higher ($P = 0.001$) in cows fed our high-starch diets, but there was no effect of additional S and Mo. We also found an effect of dietary starch on PUN, which was 1.81 mg/dL higher ($P = 0.003$) in cows fed the high- versus low-starch diets. Finally, we found no effect of dietary treatment on plasma glucose, BHB, or serum Hp concentrations, with mean values of 3.47 mmol/L, 0.55 mmol/L, and 0.31 mg/mL, respectively.

DISCUSSION

Performance and Intake

Our study is the first to determine Cu status and metabolism in early lactation, high-yielding dairy cows fed different dietary concentrations of starch. The mean dietary Cu concentration of 14.9 mg/kg of DM used in our study was higher than the NRC (2001) recommendations of approximately 11 mg/kg of DM, but lower than the 27.9 mg/kg of DM reported in the diet of early

Figure 2. Weekly DMI in early lactation dairy cows fed low-starch diets without (■) or with (△) added S and Mo or high-starch diets fed without (●) or with (□) added S and Mo. Pooled SEM = 0.64. Antagonist, $P < 0.001$; time, $P < 0.001$.

Table 3. Intake and performance in dairy cows fed low- (LS) or high-starch (HS) diets without (–) or with (+) added S and Mo

Item	Diet				SEM	P-value ¹		
	LS–	LS+	HS–	HS+		S	A	Int
DMI, kg/d	21.7	20.8	22.5	19.8	0.46	0.845	<0.001	0.057
Milk yield, kg/d	38.2	36.7	37.1	35.8	0.98	0.330	0.159	0.926
Fat, g/kg	39.5	42.0	40.8	41.2	1.22	0.794	0.241	0.400
Protein, g/kg	29.9	31.4	33.8	33.0	0.56	<0.001	0.549	0.048
Lactose, g/kg	46.7	46.3	46.2	45.9	0.30	0.150	0.276	0.950
Fat yield, kg/d	1.52	1.51	1.51	1.49	0.048	0.756	0.774	0.799
Protein yield, kg/d	1.14	1.14	1.27	1.18	0.027	0.004	0.077	0.100
Lactose yield, kg/d	1.78	1.70	1.75	1.64	0.047	0.324	0.060	0.823
MUN, mg/dL	22.2	23.6	24.5	25.4	0.98	0.034	0.237	0.780
Milk SCC, log ₁₀ /mL	1.66	1.39	1.43	1.58	0.076	0.799	0.421	0.010
BW, kg	654	654	654	649	17.5	0.877	0.881	0.905
BW change, ² kg/d	0.10	0.23	0.24	0.36	0.083	0.130	0.149	0.987
BCS	2.57	2.65	2.67	2.65	0.070	0.087	0.619	0.794
BCS change ²	–0.15	–0.08	–0.05	–0.01	0.058	0.130	0.347	0.859

¹S = main effect of starch level, A = main effect of antagonists, Int = interaction between starch level and antagonists.

²From wk 0 to 14.

lactation dairy cows in the United Kingdom (Sinclair and Atkins, 2015). It was, however, similar to the mean dietary concentration of 18 mg/kg of DM reported by Castillo et al. (2013) on 39 Californian dairy farms. We added dietary S and Mo (LS+ and HS+) at a level that was predicted to reduce Cu absorption and substantially alter Cu metabolism. Using the equations of Suttle and McLauchlin (1976), we predicted that the apparent Cu absorption coefficient of the LS+ and HS+ diets would be 0.025, approximately 40% lower than the 0.044 predicted for the LS– and HS– diets. The use of these equations, however, did not predict any effect of dietary starch concentration or reticular pH on apparent Cu availability or indicators of Cu status.

One of the primary challenges in dairy cow nutrition is the provision of a diet with sufficient energy density to minimize the negative energy balance during early

lactation without compromising the ruminal ecosystem (Zebeli et al., 2008; Humer et al., 2018). When this is attempted by feeding high-starch concentrates at the expense of more fibrous forages (Allen, 1997; Zebeli et al., 2012), intermittent drops in ruminal pH may cause SARA (Dirksen, 1985; Humer et al., 2018), a condition associated with decreased intake, milk yield, and altered milk composition (NRC, 2001). Zebeli et al. (2008) conducted a meta-analysis examining the effects of diet on ruminal pH and concluded that ruminal pH, when measured in the ventral sac, should not drop below pH 5.8 for longer than 5.24 h/d to minimize the risk of SARA occurrence. Falk et al. (2016) reported that, in lactating Holstein dairy cows, the mean daily pH when measured in the reticulum was 0.2 pH units higher than the corresponding values in the ventral sac of the rumen. As the boluses used in our study mea-

Table 4. Hepatic mineral concentrations in dairy cows fed low- (LS) or high-starch (HS) diets without (–) or with (+) added S and Mo

Item ¹	Diet				SEM	P-value ²		
	LS–	LS+	HS–	HS+		S	A	Int
Final Cu, ³ mg/kg of DM	522	387	551	471	24.2	0.018	<0.001	0.262
Cu change, ³ mg/kg of DM/d	0.72	–0.65	1.02	0.20	0.247	0.019	<0.001	0.262
Initial Fe, mg/kg of DM	278	241	291	307	21.1	0.071	0.632	0.222
Final Fe, mg/kg of DM	285	255	271	273	34.6	0.972	0.686	0.648
Fe change, µg/kg of DM/d	78	143	–207	–352	300.5	0.204	0.895	0.729
Final Mo, ³ mg/kg of DM	3.76	4.03	3.58	3.83	0.090	0.049	0.008	0.869
Mo change, ³ µg/kg of DM/d	1.54	4.32	–0.22	2.25	0.917	0.049	0.008	0.867
Initial Zn, mg/kg of DM	99.5	85.1	78.1	78.9	9.13	0.142	0.462	0.410
Final Zn, mg/kg of DM	85.7	95.2	75.6	93.4	7.34	0.426	0.073	0.578
Zn change, mg/kg of DM/d	–0.14	0.10	–0.03	0.15	0.114	0.487	0.076	0.757

¹Hepatic DM (g/kg) at wk 14: LS– = 291, LS+ = 288, HS– = 287, and HS+ = 286.

²S = main effect of starch level, A = main effect of antagonists, Int = interaction between starch level and antagonists.

³Week 0 values used as a covariate.

Table 5. Mean plasma mineral concentrations, blood metabolites, and ceruloplasmin in dairy cows fed low- (LS) or high-starch (HS) diets without (–) or with (+) added S and Mo

Item ¹	Diet				SEM	P-value ²		
	LS–	LS+	HS–	HS+		S	A	Int
Plasma Cu, $\mu\text{mol/L}$	15.5	15.6	14.6	15.6	0.56	0.387	0.324	0.454
Plasma Mo, $\mu\text{mol/L}$	0.22	0.64	0.32	0.89	0.035	0.004	<0.001	0.037
Plasma Fe, $\mu\text{mol/L}$	40.8	42.0	42.0	43.5	1.37	0.341	0.323	0.895
Plasma Zn, $\mu\text{mol/L}$	14.6	14.2	14.1	14.7	0.39	0.959	0.708	0.229
SOD, U/g of Hb ³	2,377	2,444	2,519	2,347	95.7	0.823	0.502	0.214
Ceruloplasmin, mg/L	18.5	17.5	21.2	22.6	1.11	0.001	0.872	0.288
BHB, mmol/L	0.60	0.53	0.49	0.58	0.048	0.511	0.796	0.122
Glucose, mmol/L	3.41	3.43	3.62	3.43	0.067	0.133	0.234	0.113
PUN, mg/dL	10.36	10.24	12.11	12.10	0.565	0.003	0.914	0.924
Haptoglobin, mg/mL	0.37	0.25	0.24	0.38	0.082	0.951	0.888	0.118

¹Blood samples were collected during wk 0, 1, 2, 4, 6, 10 and 14 of the study. Plasma mineral concentrations were analyzed for all weeks sampled. Superoxide dismutase (SOD) was analyzed during wk 0, 2, 6, and 14. Serum ceruloplasmin and plasma glucose were analyzed during wk 0, 1, 2, 6, 10, and 14. Plasma urea nitrogen (PUN) and BHB were analyzed during wk 0, 2, 6, 10, and 14. Haptoglobin was analyzed during wk 0, 1, 6, and 14.

²S = main effect of starch level, A = main effect of antagonists, Int = interaction between starch level and antagonists.

³Hemoglobin.

sured reticular pH, the corresponding ventral sac values for cows fed the high-starch diets dropped below pH 6.0 for only 5.11 h/d, less than the 5.24 h/d suggested by

Figure 3. Plasma concentrations of copper (a) and molybdenum (b) in early lactation dairy cows fed low-starch diets without (▲) or with (△) added S and Mo or high-starch diets fed without (■) or with (□) added S and Mo. For plasma copper; pooled SEM = 0.75. Time $P < 0.001$; time $\times$ starch $\times$ antagonist, $P = 0.003$. For plasma molybdenum; pooled SEM = 0.051. Time, $P < 0.001$; time $\times$ starch, $P < 0.001$; time $\times$ antagonist, $P = 0.006$; time $\times$ starch $\times$ antagonist, $P < 0.001$.

Zebeli et al. (2008). Additionally, there was little effect of increased dietary starch concentration on cow performance or milk fat content, and it is therefore unlikely that our cows fed the high-starch diets were experiencing SARA (NRC, 2001; Zebeli et al., 2008). We found that increasing dietary starch concentration caused a similar reduction in reticular pH (pH 6.38 vs. 6.23 for LS and HS) to that observed by Tayyab et al. (2019), when grass silage was substituted for corn silage in the diet (pH 6.42 vs. 6.34 for grass and corn silage, respectively) of lactating Holstein-Friesian dairy cows. Under commercial conditions, it should be considered that changes in ruminal pH are difficult to predict because of a variety of factors, including feed composition, level of processing, diet buffering capacity, and forage chop length (Krause and Oetzel, 2006; Beauchemin, 2018).

Similar to our findings, several other studies have reported a decrease in DMI as a result of feeding additional S and Mo. For example, Sinclair et al. (2017) reported a reduced intake of 2.1 kg of DM/d in lactating Holstein-Friesian dairy cows when a grass silage-based diet was supplemented with 2 g of S/kg of DM and 6.5 mg of Mo/kg of DM. Given that our diets were supplemented with both S and Mo, it is not possible to determine the effects of each element in isolation, but the potential of S to reduce intake has been extensively studied in beef cattle fed high S concentrations as a result of the dietary inclusion of ethanol co-products (Drewnoski et al., 2014). Evidence from these studies is conflicting, however. For example, Spears et al. (2011) reported a decreased intake when dietary S concentrations exceeded 2 g of S/kg of DM, but Richter et al. (2012) observed no effect of dietary S on DMI at concentrations as high as 6 g of S/kg of DM. In the rumen,

sulfur-reducing bacteria convert inorganic sulfates and S-containing amino acids into hydrogen sulfide (H_2S ; Bradley et al., 2011), which may migrate to the gas cap of the rumen or disassociate to form bisulfide in the liquid fraction (Schoonmaker and Beitz, 2012). This disassociation is thought to be pH-dependent, with a greater proportion of bisulfide in the ruminal fluid at higher pH values (Schoonmaker and Beitz, 2012). For example, at a pH of 7.0 and a pK_a of 7.04, approximately 50% of H_2S will disassociate to form bisulfide compared with a 5% disassociation rate at a pH of 5.5 (Drewnoski et al., 2014). It has been suggested that increased ruminal H_2S decreases DMI by decreasing rumen motility and increasing feedstuff retention time (Uwituze et al., 2011), or by causing the animal discomfort (Drewnoski et al., 2014). Increased ruminal H_2S may therefore explain the decreased intake in cows fed additional S and Mo in our study (Spears et al., 2011), an effect that was potentially amplified in cows fed the high-starch diets due to a lower reticular pH (Schoonmaker and Beitz, 2012). Indeed, it is hypothesized that ruminal pH is the major reason for differences in sulfur tolerance between forage- and concentrate-fed cattle (Drewnoski et al., 2012). Additional sulfur was supplied as elemental S in our study, whereas others have often used an inorganic salt (Spears et al., 2011; Sinclair et al., 2017). The consequences of this substitution are likely to be minimal, however, as bacteria have been shown to readily utilize elemental S as a substrate (Barton and Fauque, 2009).

Similar to other studies that have investigated the effect of dietary starch concentration on milk protein production (Carmo et al., 2015; Hills et al., 2015), we found an increase in both milk protein concentration and yield as dietary starch concentration increased. Increasing dietary starch concentration increases fermentable energy supply to the rumen microbes, resulting in increased microbial protein synthesis (Oba and Allen, 1999). Diets that maximize microbial protein production tend to have an increased digestibility and an improved amino acid profile (O'Connor et al., 1993), with the potential to increase milk protein yield (Carmo et al., 2015), although microbial production was not measured in our study.

Hepatic Mineral Concentration

The liver is generally regarded as the primary organ of Cu storage, and one of the first changes to occur under Cu deprivation is a decrease in hepatic Cu concentration (Suttle, 2010). We found that the final hepatic Cu concentrations of cows fed our LS- or HS- diets were above the upper threshold of 508 mg/kg of DM thought to pose a risk of clinical Cu toxicity (Livesey et al., 2002). Current prediction equations do not consider

the potential effects of dietary starch concentration or rumen pH on Cu absorption (Suttle and McLauchlin, 1976), and subsequently did not predict the increased hepatic Cu retention of cows fed our high-starch diets (Suttle and McLauchlin, 1976; Suttle, 2010). Our study is not the first to report an effect of diet on apparent Cu availability, with Sinclair et al. (2017) also reporting an increase in hepatic Cu retention when cows were fed additional S and Mo in a corn compared with a grass silage-based diet. Although Sinclair et al. (2017) did not measure rumen pH, others such as Tayyab et al. (2019) have reported a lower pH in cows fed corn silage- versus grass silage-based diets. These differences in rumen pH may explain the increased hepatic Cu retention of cows fed the high-starch diets in our study or the corn silage-based diets by Sinclair et al. (2017), either by altering the extent of formation or the speciation of thiomolybdates in the rumen.

Dietary sulfides combine with molybdate in the rumen to form thiomolybdates, which form a complex with Cu preventing its absorption (Suttle, 1991); as a consequence, our cows fed additional S and Mo had lower final hepatic Cu concentrations. During thiomolybdate formation, molybdate reacts with bisulfide in a stepwise, reversible, and pH-dependent manner, commencing with the formation of monothiomolybdate (TM_1) to di- (TM_2), tri- (TM_3), and tetrathiomolybdate (TM_4) sequentially (Gould and Kendall, 2011), with each form proposed to have differing effects on Cu absorption and metabolism (Suttle, 1991). Trithiomolybdate and TM_4 are thought to irreversibly bind Cu to high-molecular-weight proteins, rendering it unavailable for absorption in the small intestine (Suttle and Field, 1983), whereas it is proposed that TM_1 is broken down by the acidity of the abomasum, freeing any complexed Cu for absorption (Price et al., 1987; Suttle, 1991). Clarke and Laurie (1980) identified increased rates of TM_4 formation in vitro at lower ruminal pH values; in our study, cows fed the high-starch diets had a lower rumen pH and might have been expected to have had a lower final hepatic concentration than those fed the low-starch diets, but this was not the case. However, a lower ruminal pH also reduces the availability of bisulfide for thiomolybdate formation in vivo (Clarke and Laurie, 1980). The lower reticular pH of cows fed our high-starch diets may therefore have reduced the bisulfide available for thiomolybdate formation (Schoonmaker and Beitz, 2012), with a decreased quantity of thiomolybdate resulting in an increase in both apparent Cu availability and final hepatic Cu concentration (Suttle, 1991; Gould and Kendall, 2011). In cows fed our LS- or HS- diets, feeding 15 mg of Cu/kg of DM resulted in a rapid increase in hepatic Cu concentration, whereas those receiving HS+ experienced a small gain. In con-

trast, cows fed LS+ experienced a rapid decline in hepatic Cu concentration, which would eventually reach the 19 mg of Cu/kg of DM considered the threshold for deficiency (Laven and Livesey, 2005), if fed for a further 566 d. Given that feeding the same concentration of dietary Cu resulted in such large differences in hepatic Cu status, dietary starch concentration and rumen pH, as well as dietary S and Mo concentrations, should be taken into consideration when calculating appropriate Cu supplementation rates for dairy cows.

Similar to other studies that have investigated the effects of feeding additional S and Mo to lactating dairy cows (Sinclair et al., 2013, 2017), we found a higher final hepatic Mo concentration in cows fed the antagonist-supplemented diets (LS+ and HS+). This is not surprising, because absorbed molybdate is normally stored in tissues such as the kidneys and liver as molybdoprotein, where it binds to various enzymes of the mitochondria and cytosol (Johnston, 1997). It was surprising, however, that we found increased final hepatic Mo concentrations in cows fed the low-starch diets (Suttle, 1991; Johnston, 1997), which is difficult to explain. There is evidence that when TM_3 is administered intravenously, it can be stored in the liver (Wang et al., 1987), and it may be possible that an increased quantity of ruminal TM_3 in cows fed our low-starch diets resulted in hepatic accumulation of Mo-bound thiomolybdate (Clarke and Laurie, 1980; Wang et al., 1987). In contrast, we found that both hepatic Fe and Zn were unaffected by dietary treatment, with the liver generally not regarded as a major storage organ for either of these minerals (Suttle, 2010).

Plasma Mineral Profile, Cu-Mediated Enzymes, and Blood Metabolites

We found no effect of dietary treatment on plasma Cu concentrations, which were all well above the 9 $\mu\text{mol/L}$ threshold considered to denote adequacy (Laven and Livesey, 2005). These findings are consistent with those of other studies that have either fed additional S and Mo (Sinclair et al., 2013) or varied dietary Cu concentration (Engle et al., 2001). Dias et al. (2013) conducted a meta-analysis examining the relationship between plasma Cu and dietary Cu, S, and Mo concentrations, and concluded that plasma concentration is a poor indicator of Cu status, and the parameter may only be useful when animals experience very high or low hepatic Cu concentrations (Dias et al., 2013). In contrast to other studies that have used heparin as an anticoagulant for the determination of plasma Cu (Engle et al., 2001; Sinclair et al., 2017), we used Vacutainers containing EDTA according to the manufacturer's stated lower risk of trace element contamination. Evidence in cattle

is scarce, but Cu chelation by EDTA in human blood is unlikely to affect plasma Cu concentration compared with heparin, particularly when determined by ICP-MS (Frank et al., 2001). Similar to other studies that have supplemented lactating dairy cow diets with S and Mo (Sinclair et al., 2013, 2017), we found increased plasma Mo concentrations in cows fed these Cu antagonists. It is surprising, however, that we found an interaction between dietary starch and Cu antagonists on plasma Mo, where additional S and Mo increased plasma Mo concentration to a greater extent when cows were fed the high- compared with the low-starch diets. This interaction is difficult to explain but may have resulted from decreased ruminal thiomolybdate formation in cows fed our high-starch diets (Clarke and Laurie, 1980; Schoonmaker and Beitz, 2012), which resulted in a greater quantity of Mo that was free for absorption into the bloodstream (Suttle, 1991; Turnlund and Friberg, 2007).

Evidence regarding the effects of Cu antagonists on Cp activity is conflicting, with some studies reporting decreased activity along with a lower plasma Cu concentration following S and Mo supplementation (Ward et al., 1993), although most support our current finding that there was no effect of additional S and Mo on Cp activity (Sinclair et al., 2013, 2017). The potential of thiomolybdates to inhibit Cp activity has only been documented in vitro and at pharmacological dose rates in vivo (Kelleher and Mason, 1986; Lannon and Mason, 1986). It is surprising that we found an effect of dietary starch on Cp activity, which was higher in cows fed high-starch diets. This is not, however, the only report of dietary factors influencing Cp activity with, for example, Sinclair et al. (2017) reporting an increase in Cp activity when corn silage was replaced with grass silage in the diet of lactating Holstein-Friesian cows. Ceruloplasmin can act as a minor acute phase protein during the inflammatory response that follows infection or trauma (Matsuda et al., 1974; Kaya et al., 2016), and Suttle (1994) reported the potential of both vaccines and infections to induce Cp synthesis in hypocupremic animals. It may be possible that a decreased reticular pH triggered an acute phase response and induced Cp synthesis in cows fed our high-starch diets (Cannizzo et al., 2012; Kaya et al., 2016). If this were indeed the case, an increase in the major acute phase protein haptoglobin, combined with a decrease in plasma Zn (Plaizier et al., 2008) and an increase in plasma Cu concentration, may have been expected (Sattar et al., 1997), but none of these occurred in our study. There is a second explanation relating to the potentially increased quantity of ruminal thiomolybdates in cows fed our low-starch diets (Clarke and Laurie, 1980; Schoonmaker and Beitz, 2012), which if absorbed

into the bloodstream could inhibit Cp synthesis and activity (Kelleher and Mason, 1986; Lannon and Mason, 1986), although this effect would have been expected to have been greater in cows fed LS+ than LS−, which we did not observe. The absorption of thiomolybdates is, however, a controversial subject area, and Suttle (2010) proposed that absorption is unlikely unless the Cu:Mo ratio is less than 1:1, well below the mean value of 2.6:1 in our LS+ and HS+ diets. Suttle (2010) noted that there can be a wide range in this ratio, resulting from a variety of factors such as feed composition and dietary S concentration.

CONCLUSIONS

We found that increasing dietary starch concentration decreased mean reticular pH but increased milk protein concentration and yield, with milk fat yield being unaffected. The addition of S and Mo decreased DMI but increased plasma Mo concentration. Feeding lactating Holstein-Friesian dairy cows Cu at a dietary concentration of 15 mg of Cu/kg of DM in the absence of additional S and Mo resulted in hepatic Cu accumulation, demonstrating that this dietary concentration is more than sufficient to meet the cow's requirements. In contrast, feeding 15 mg of Cu/kg of DM in a diet with high S and Mo concentrations will result in hepatic Cu depletion. Importantly, cows fed a high- versus a lower-starch diet had an increased hepatic Cu concentration when fed without or with added antagonists. Reasons for these differences in Cu absorption and metabolism as a result of dietary starch concentration or reticular pH are unclear and require further investigation, but highlight the need to take these factors into account when calculating appropriate Cu supplementation levels for lactating dairy cows.

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