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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Interpretive Summary

Dietary starch concentration alters copper metabolism in lactating Holstein-Friesian dairy cows receiving added dietary sulfur and molybdenum.

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Copper is a trace element that is essential for dairy cow health and performance. The absorption of copper has been shown to vary according to a variety of dietary factors, although the mechanisms are not well understood. The current study found that higher dietary starch concentrations that resulted in a lower rumen pH increased copper absorption. This information allows dairy farmers to more accurately provide the correct amount of dietary copper.

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 33 (standard error ± 2.5) days post calving and yielding 41 (standard error ± 0.9) kg of milk/d were fed 1 of 4 diets in a 2 x 2 factorial design experiment over a 14-week 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 (**LS**) or 220 g/kg of DM (**HS**), and were either unsupplemented (-) or supplemented (+) with an additional 0.8 g of S/ kg 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 DM/d, an effect that was evident from week 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 in cows fed the high starch diets. There was 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 µmol/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 our high compared to our low starch diets. 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 an impairment of growth, reproduction and red blood cell formation (McDowell, 1985; Suttle et al., 1987). Whilst 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 (Gould and Kendall, 2011; Dick et al., 1975), 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- compared to a corn silage-based diet, and it was 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., 2018), and its inclusion is often associated with a decrease in rumen pH (Firkins, 1997; Tayyab et al., 2018). 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 yield (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., 2018). This lack of understanding may be contributing to an over-supplementation of Cu in dairy cow rations (Suttle, 2016). Surveys in both the United Kingdom and United States (Sinclair and Atkins, 2015; Castillo et al., 2013), 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 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, 2012), and received local ethical approval. Sixty Holstein-Friesian dairy cows (48 multiparous and 12 primiparous) that were 33 (SE ± 2.5) days post calving, with a live weight of 659 (SE ± 17.7) kg and yielding 41 (SE ± 0.9) kg were used. Based upon recordings taken in the week prior to 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 days.

Four isoenergetic and isonitrogenous diets were formulated to contain a grass:corn silage ratio of 1:1 (DM basis), 15 mg Cu/kg of DM, and a dietary starch concentration of 150 (**LS**) or 220 g/kg of DM (**HS**; 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 g/kg of DM (HS), according to Thomas (2004). In order 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 ($CuSO_4.5H_2O$), sulfur as flowers of sulfur, and molybdenum as sodium molybdate (Rumenco, Staffordshire, United Kingdom). 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 S/kg of DM and 4.4 mg Mo/kg of DM respectively compared to the unsupplemented diets. The 4 dietary treatments were therefore: 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]; 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, 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 collected three times per week on a Monday, Wednesday and Friday. Cows were housed in free stalls fitted with mattresses in the same area of an open span building. Stalls were bedded three times per 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 parlour at approximately 0600 and 1600 h. The milk yield of each cow was recorded at each milking, with samples taken on a fortnightly basis at consecutive morning and afternoon milkings for analysis of composition and SCC. Cows were weighed and body condition scored before afternoon milking in the week prior to allocation, and on a fortnightly basis thereafter. Reticular pH boluses (eCow Ltd, Devon, United Kingdom) that recorded reticular pH at 15-minute intervals (96 data points/d) were administered to twenty-four cows (six per treatment) in the week prior to commencing the study. Forage samples were collected on a weekly basis, dried to a constant weight, and the corn to grass silage adjusted to the desired ratio. Fresh samples of the four diets were collected immediately post-feeding on a weekly basis and stored at -20^{o}C prior to subsequent analysis. Blood samples were collected by jugular venepuncture at 1100 h during weeks 0, 1, 2, 4, 6, 10, and 14 of the study into vacutainers (Becton Dickinson Vacutainer Systems, Plymouth, United Kingdom) 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 ethylenediaminetetraacetic acid [**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 µg/L; catalogue no. 36381). Liver biopsy samples were collected during weeks 0 and 14 of the study via the 11th intercostal space as described by Davies and Jebbett (1981), immediately snap frozen in liquid nitrogen, and stored at -80^{o}C prior to subsequent analysis.

Chemical Analysis

Weekly diet samples were bulked on a monthly basis and analyzed according to AOAC (2012) for DM (934.01; intra-assay CV of 1.5%), crude protein (990.03; intra-assay CV of 1.6%), ether extract (2003.05; intra-assay CV of 5.3%), and ash (942.05; intra-assay CV of 0.8%). Samples were also analyzed for starch (intra-assay CV of 3.3%) according to ISO 6493 (2000) at Sciantic Analytical (Stockbridge Technology Centre, North Yorkshire, United Kingdom), and the ME content of the diets was estimated from their composition. The NDF and ADF content of the diets were determined according to Van Soest et al. (1991); heat-stable α-amylase was used for NDF determination (Sigma Aldrich, Dorset, United Kingdom; intra-assay CV of 1.2 % for NDF and 2.7% for ADF respectively). Dietary minerals were extracted using a DigiPREP digestion system (QMX Laboratories, Essex, United Kingdom), and analyzed by inductively coupled plasma-mass spectrometry (ICP-MS; Nexion 2000; Perkin Elmer, Beaconsfield, United Kingdom) 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 EU reference samples of hay (BCR-129) and dairy concentrate (BCR-185). Plasma samples were analyzed for PUN, BHB, and glucose (Randox Laboratories, Antrim, United Kingdom; kit catalogue no. 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, Republic of Ireland; kit catalogue no. HP801; intra-assay CV of 0.8%). Whole blood samples were analyzed for SOD activity (Randox Laboratories, Antrim, United Kingdom; kit catalogue 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, United Kingdom). Liver samples were dried at 60^{o}C to a constant mass, digested overnight at 60^{o}C in concentrated nitric acid, and made up to 50 mL in a DigiPREP tube (QMX Laboratories, Essex, United Kingdom). Plasma and liver samples were analyzed for Cu, Fe, Zn and Mo by ICP-MS following a 1:50 dilution in 0.5% HNO_3, 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 lyophilised plasma control sample two (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, United Kingdom).

Calculations and Statistical Analysis

The percentage of time 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-minute time points was linear.

Continuous performance, rumen pH and blood parameters were analyzed as a 2 x 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:

197 $$Y_{ijkl} = \mu + B_i + S_j + A_k + T_l + S.A_{jk} + S.T_{jl} + A.T_{Kl} + S.A.T_{jkl} + \varepsilon_{ijkl}$$

198

199 where Y_{ijk} = dependent variable; μ = overall mean; B_i = fixed effect of blocks; S_j = effect of
200 starch level (j = low or high); A_k = effect of S and Mo (k = with or without); T_l= effect of time
201 $S.A_{jk}$ = interaction between dietary starch concentration and antagonists; $A.T_{kl}$ = interactions
202 between dietary concentration and time; $S.A.T_{jkl}$ = interaction between starch concentration,
203 antagonists, and time, and ε_{ijkl} = residual error.

204 Non-continuous performance parameters including hepatic mineral concentration were
205 analyzed as a 2 x 2 factorial design ANOVA as:

206 $$Y_{ijk} = \mu + B_i + S_j + A_k + S.A_{jk} + \varepsilon_{ijk}$$

207 where Y_{ijk} = dependent variable; μ = overall mean; B_i = fixed effect of blocks; C_j = effect of
208 starch concentration (j = low or high); A_k = effect of S and Mo (k = with or without); $S.A_{jk}$ =
209 interaction between starch concentration and antagonist, and ε_{ijk} = residual error. Milk SCC
210 was $\log_{10}$ transformed prior to analysis, and live weight change was determined by linear
211 regression of fortnightly live weight measurements. All statistical analysis was conducted using
212 Genstat version 18 (VSN International, Ltd, Oxford, United Kingdom), and means are
213 presented with their associated standard error of the mean.

214

215

216 **RESULTS**

217 ***Dietary Analysis, Reticular pH, Intake, and Animal Performance***

218 We observed all four diets to have a similar content of DM, CP and ether extract, with
219 mean values of 427 g/kg, 166, and 23 g/kg of DM respectively (Table 1). Mean dietary NDF
220 and ADF values were 63, and 49 g/kg of DM higher respectively in the low (LS- and LS+)
221 compared to 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 g/kg of DM and 225 g/kg of DM respectively. All four 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 a similar dietary Cu concentration across all four 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 the unsupplemented diets (LS- and HS-).

We observed reticular pH to be at its highest immediately prior to 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 compared to the low starch diets (pH 6.23 versus pH 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 pH 6.01 for HS and LS respectively), and spent a greater percentage of time below pH 5.8 ($P = 0.048$; 90 versus 1 min/d) and pH 6.0 ($P = 0.017$; 307 versus 65 min/d) compared to 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 DM/d lower ($P < 0.001$) than those fed unsupplemented diets (Table 3), an effect that was evident from week 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 compared to low starch diets (-2.7 kg/d versus -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 a 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 liver 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 concentration, which was 108 mg Cu/kg of DM lower ($P < 0.001$) in cows fed additional S and Mo compared to those offered our 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 a 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 our 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 µmol/ L (Table 5). In contrast, we found an effect ($P < 0.001$) of time on plasma Cu concentration, which increased between week 0 and week 2 of the study, before decreasing until week 4, and fluctuating thereafter (Figure 3a). We also found an increase ($P = 0.004$) on mean plasma Mo concentration when cows were offered the high compared to the low starch diets, and when cows were fed additional S and Mo compared to 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 to unsupplemented cows when offered the high compared to the low starch diets (+0.61 µmol/ L versus +0.43 µmol/ L for HS and LS respectively). An effect of time (P < 0.001) on plasma Mo was also noted, with the concentration increasing between week 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 µmol/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 compared to the low starch diets. Finally, we found no effect of dietary treatment on either plasma glucose, BHB, or serum Hp concentrations, with mean values of 3.47, 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 above 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 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

297 coefficient of the LS+ and HS+ diets would be 0.025, approximately 40% lower than the 0.044
298 predicted for the LS- and HS- diets. The use of these equations however did not predict any
299 effect of dietary starch concentration or reticular pH on apparent Cu availability or indicators
300 of Cu status.

301 One of the primary challenges in dairy cow nutrition is the provision of a diet with
302 sufficient energy density to minimise the negative energy balance during early lactation
303 without compromising the ruminal ecosystem (Humer et al., 2018; Zebeli et al., 2008). When
304 this is attempted by feeding high starch concentrates at the expense of more fibrous forages
305 (Allen, 1997; Zebeli et al., 2012), intermittent drops in ruminal pH may cause subacute ruminal
306 acidosis (**SARA**; Dirksen, 1985; Humer et al., 2018), a condition associated with decreased
307 intake, milk yield, and altered milk composition (NRC, 2001). Zebeli et al. (2008) conducted
308 a meta-analysis examining the effects of diet on ruminal pH, and concluded that ruminal pH
309 when measured in the ventral sac should not drop below pH 5.8 for longer than 5.24 h/d in
310 order to minimise the risk of SARA occurrence. Falk et al. (2016) reported that in lactating
311 Holstein dairy cows the mean daily pH when measured in the reticulum was 0.2 pH units higher
312 than the corresponding values in the ventral sac of the rumen. As the boluses used in our study
313 measured reticular pH, then the corresponding ventral sac values for cows fed the high starch
314 diets dropped below pH 6.0 for only 5.11 h/d, less than the 5.24 h/d suggested by Zebeli et al.
315 (2008). Additionally, there was little effect of increased dietary starch concentration on cow
316 performance or milk fat content, and it is therefore unlikely that our cows fed the high starch
317 diets were not experiencing SARA (NRC, 2001; Zebeli et al, 2008). We found that increasing
318 dietary starch concentration caused a similar reduction in reticular pH (pH 6.38 versus 6.23 for
319 LS and HS) to that observed by Tayyab et al. (2018), when grass silage was substituted for
320 corn silage in the diet (pH 6.42 versus 6.34 for grass and corn silage respectively) of lactating
321 Holstein-Friesian dairy cows. Under commercial conditions, it should however be taken into

consideration that changes in ruminal pH are difficult to predict due to a variety of factors including feed composition, level of processing, diet buffering capacity, and forage chop length (Beauchemin, 2018; Krause and Oetzel, 2006).

Similar to our findings, several other studies have also 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 DM/d in lactating Holstein-Friesian dairy cows when a grass silage-based diet was supplemented with 2 g S/kg of DM and 6.5 mg 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 however conflicting. For example, Spears et al. (2011) reported a decreased intake when dietary S concentrations exceeded 2 g S/kg of DM, but Richter et al. (2012) observed no effect of dietary S on DM intake at concentrations as high as 6 g 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 (**HS^-**) in the liquid fraction (Schoonmaker and Beitz, 2012). This disassociation is thought to be pH-dependent, with a greater proportion of HS^- in the ruminal fluid at higher pH values (Schoonmaker and Beitz, 2012). For example, at a pH of 7.0 and a pKa of 7.04 approximately 50% of H_2S will disassociate to form HS^- compared to 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 which was potentially amplified in cows fed the high starch diets due to a lower reticular pH

(Schoonmaker and Beitz, 2012). It is indeed hypothesised that ruminal pH in 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 (Sinclair et al., 2017; Spears et al., 2011). The consequences of this substitution are however likely to be minimal, as bacteria have been shown to readily utilise 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. It has been shown that increasing dietary starch concentration increases fermentable metabolisable energy supply to the rumen microbes resulting in increased microbial protein synthesis (Oba and Allen, 2003). Diets which maximise 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 suggested to pose a risk of clinical Cu toxicity (Livesey et al., 2002). Current prediction equations do not take into consideration the potential effects of dietary starch concentration or rumen pH on Cu absorption (Suttle and McLauchlin, 1976), and subsequently did not predicted the increased hepatic Cu retention of cows fed our high starch diets (Suttle, 2010; Suttle and McLauchlin, 1976). Our study is not however, 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 to a grass silage-based diet. Although Sinclair et al. (2017) did not measure rumen pH, others such as Tayyab et al. (2018) have reported a lower pH in cows fed corn compared to grass silage-based diets. These differences in rumen pH may explain the increased hepatic Cu retention of cows 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 that form a complex with Cu preventing its absorption (Suttle, 1991), and as a consequence our cows fed additional S and Mo had lower final hepatic Cu concentrations. During thiomolybdate formation, molybdate reacts with HS^- in a stepwise, reversible, and pH-dependent manner, commencing with the sequential formation of monothiomolybdate (**TM_1**) through 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 in the rumen 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, and in our study cows fed the high starch diets had a lower rumen pH and may therefore 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 HS^- 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 HS^- 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 Cu/kg of DM resulted in a rapid increase in hepatic Cu concentration, whereas those receiving HS+ experienced a small gain. In contrast, cows fed LS+ experienced a rapid decline in hepatic Cu concentration, which would eventually reach the 19 mg Cu/ kg DM considered to be the threshold for deficiency (Laven and Livesey, 2005), if fed for a further 566 days. Given that feeding the same concentration of dietary Cu results in such large differences in hepatic Cu status, dietary starch concentration and rumen pH, as well as dietary S and Mo concentration 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, as 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 however surprising that we found increased final hepatic Mo concentrations in cows fed the low starch diets (Johnston, 1997; Suttle, 1991), which is difficult to explain. There is however evidence that when TM_3 is administered intravenously, it can be stored in the liver (Wang et al., 1987), and it may therefore be possible that an increased quantity of ruminal TM_3 in cows fed our low starch diets resulted in the 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 µmol/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 it has been demonstrated that Cu chelation by EDTA in human blood is unlikely to affect plasma Cu concentration compared to 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 however surprising 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 to 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 a 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 our 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 which 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 hypocupraemic animals. It may be possible therefore, 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 TM in cows fed our low starch diets (Clarke and Laurie, 1980; Schoonmaker and Beitz, 2012), which if absorbed into the bloodstream has the potential to 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, which was well below the mean value of 2.6:1 in our LS+ and HS+ diets. It was however

noted by Suttle (2010) 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, and increased milk protein concentration and yield, with milk fat yield being unaffected. We also found that the addition of S and Mo decreased DMI, but increased plasma Mo concentration. Feeding lactating Holstein-Friesian dairy cows Cu a dietary concentration of 15 mg 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 Cu/kg of DM in a diet with high S and Mo concentrations will result in hepatic Cu depletion. Importantly, cows fed a high compared to 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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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
Soy pass[1]	42	42	--	--
Soy hulls	157	157	59	59
Molassed sugar beet feed	114	114	--	--
Soy bean meal	59	59	86	86
Rapeseed meal	29	28	43	43
Wheat distiller's 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
Mins/vits[2/3]	9[2]	9[3]	9[2]	9[3]
Total	1000	1000	1000	1000
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
Organic matter, 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

[1] Xylose-treated soya bean meal, KW Alternative Feeds, Staffordshire, United Kingdom).

[2] LS- and HS- mineral/vitamin premix (Rumenco, Staffordshire, UK). Major minerals (g/kg): Ca 180, P 53, Mg 75, Na 75, 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.

[3]LS+ and HS+ mineral/vitamin premix (Rumenco, Staffordshire, UK). Major minerals (g/kg): Ca 148, P 53, Mg 75, Na 75, 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.

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

	Diet					P-value[1]		
Item	LS-	LS+	HS-	HS+	SEM	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

[1] S = main effect of dietary starch level, A = main effect of dietary antagonists. Int = interaction between dietary starch level and antagonists.
[2] Data not normally distributed.

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

	Diet					P-value[1]		
Item	LS-	LS+	HS-	HS+	SEM	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

[1] S= main effect of starch level, A = main effect of antagonists, Int = interaction between starch level and antagonists; [2]Wks 0-14.

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

	Diet					P-value[1]		
Item[2]	LS-	LS+	HS-	HS+	SEM	S	A	Int
Final Cu, mg/kg of DM[2,3]	522	387	551	471	24.2	0.018	<0.001	0.262
Cu change, mg/kg of DM/d[3]	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

[1] S= main effect of starch level, A = main effect of antagonists, Int = interaction between starch level and antagonists.
[2]Hepatic DM (g/kg): LS- = 291, LS+ = 288, HS- = 287, and HS+ = 286.
[3] Week 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

	Diet					P-value[1]		
Item[2]	LS-	LS+	HS-	HS+	SEM	S	A	Int
Plasma Cu, µmol/L	15.5	15.6	14.6	15.6	0.56	0.387	0.324	0.454
Plasma Mo, µmol/L	0.22	0.64	0.32	0.89	0.035	0.004	<0.001	0.037
Plasma Fe, µmol/L	40.8	42.0	42.0	43.5	1.37	0.341	0.323	0.895
Plasma Zn, µmol/L	14.6	14.2	14.1	14.7	0.39	0.959	0.708	0.229
SOD[3] U/ g of Hb[2]	2377	2444	2519	2347	95.7	0.823	0.502	0.214
Ceruloplasmin, mg/ dL	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[4], 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

[1] S= main effect of starch level, A = main effect of antagonists, Int = interaction between starch level and antagonists.
[2] Blood samples were collected during weeks 0, 1, 2, 4, 6, 10 and 14 of the study. Plasma mineral concentrations were analyzed for all weeks sampled. Superoxide dismutase was analyzed during week 0, 2, 6, and 14. Serum ceruloplasmin and plasma glucose were analyzed during week 0, 1, 2, 6, 10, and 14. Plasma urea nitrogen and BHB were analyzed during week 0, 2, 6, 10, and 14. Haptoglobin was analyzed during week 0, 1, 6, and 14.
[3] SOD = superoxide dismutase.
[4] PUN = plasma urea nitrogen.

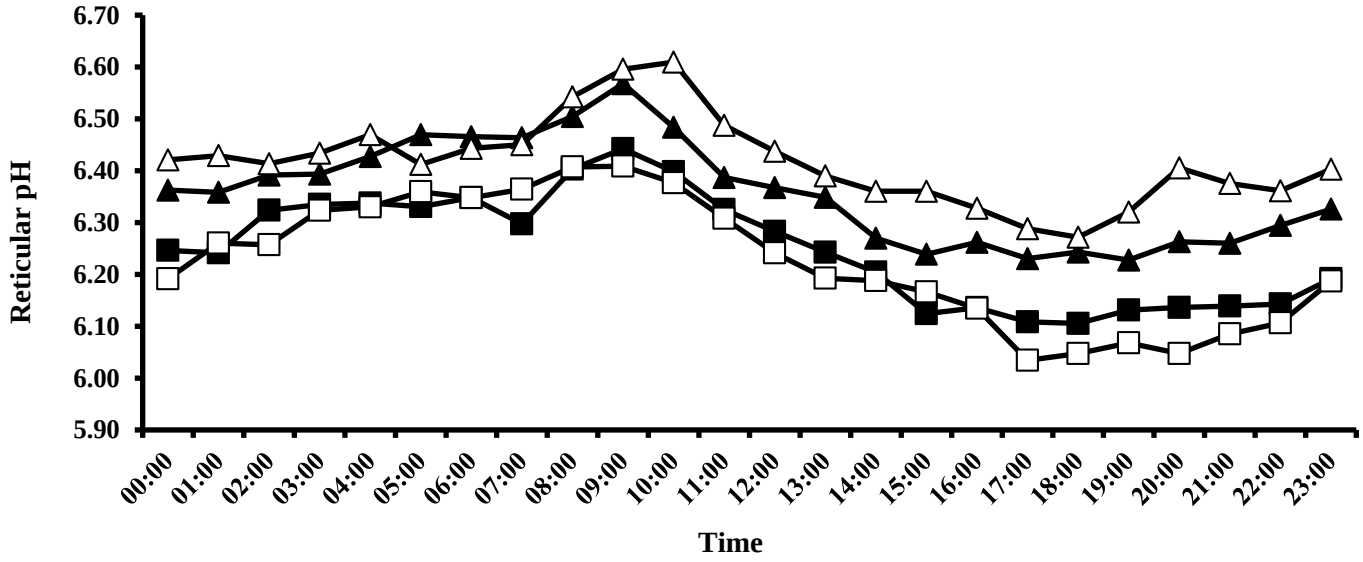

Figure 1. Reticular pH of early lactation dairy cows fed low starch diets without (▲) or with (△) added S and Mo, or high starch diets fed without (■) or with (□) added S and Mo. Pooled SEM = 0.067. Starch, P = 0.022; antagonist, P = 0.755; starch x antagonist, P = 0.487; time, P < 0.001; time x starch, P = 0.256; time x antagonist, P = 0.601; time x starch x antagonist, P = 0.305. Fresh feed was offered daily at 0900 h.

Figure 1: Manuscript ID JDS.2020-18453

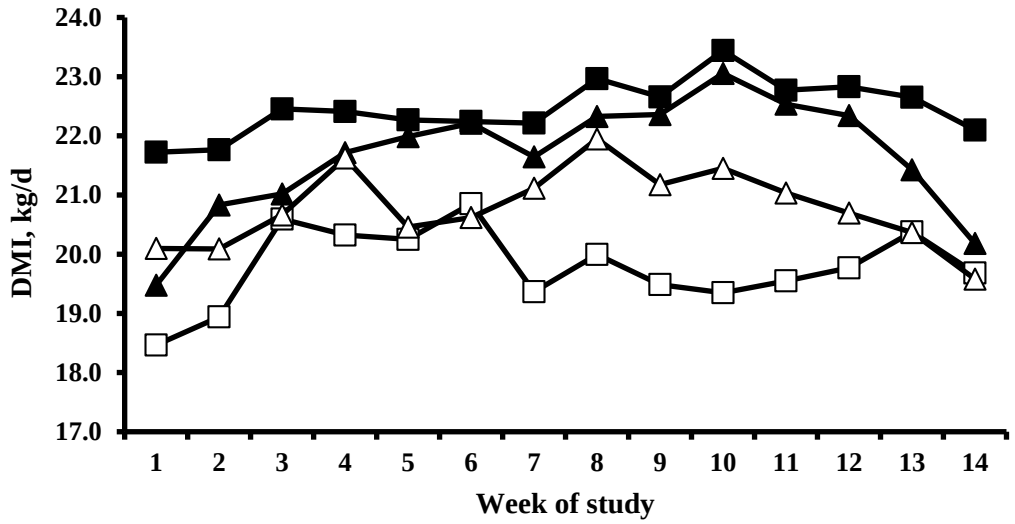

Figure 2. Weekly dry matter intake (DMI) in early lactation dairy cows fed low starch diets without (▲) or with (△) added S and Mo, or high starch diets fed without (■) or with (□) added S and Mo. Pooled SEM = 0.64. Antagonist, $P < 0.001$; time, $P < 0.001$.

Figure 2: Manuscript ID JDS.2020-18453

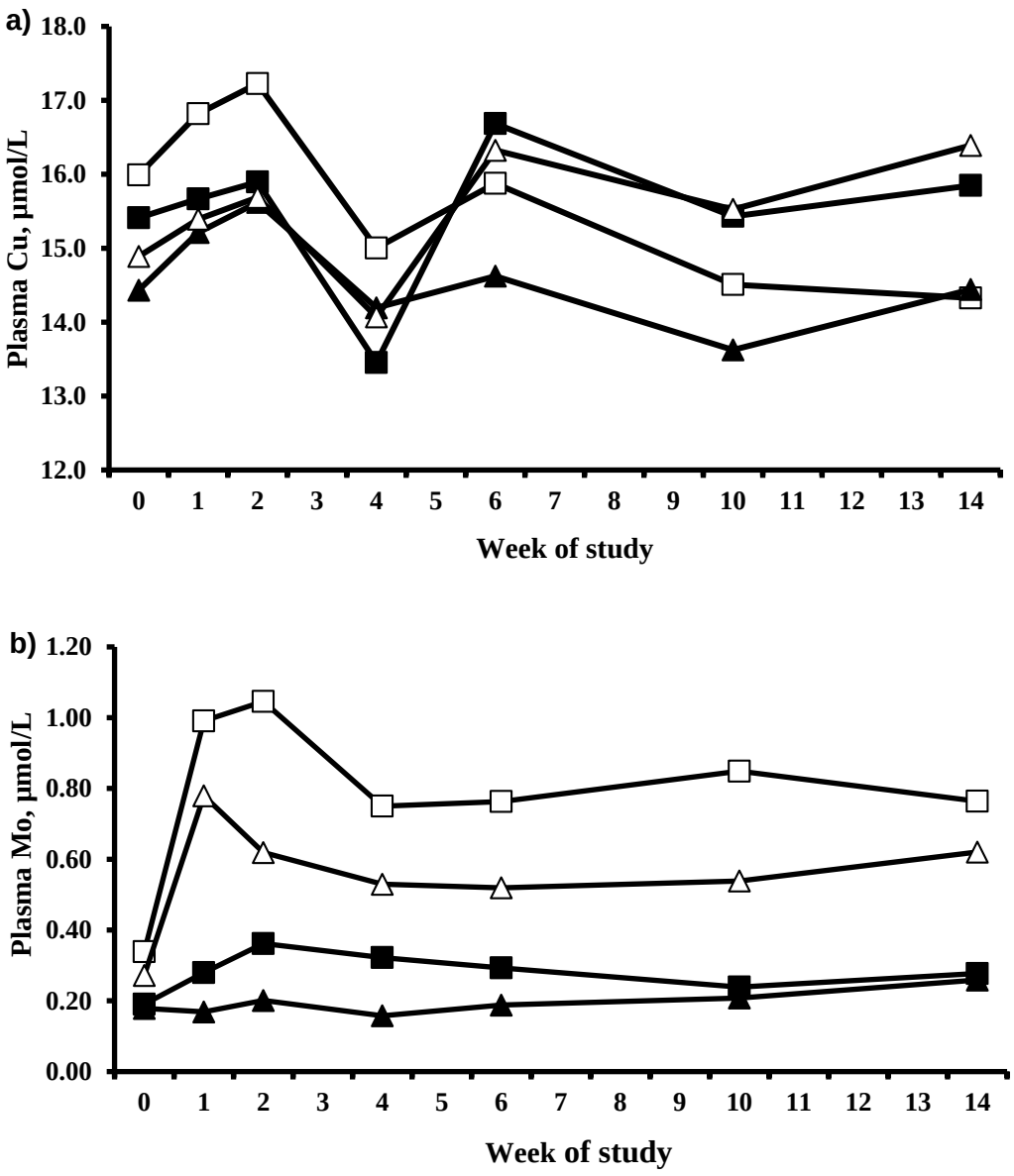

Figure 3. Plasma copper (a) and molybdenum (b) concentrations of early lactation dairy cows fed low starch diets without (▲) or with (△) added S and Mo, or high starch diets fed without (■) or with (□) added S and Mo. For plasma copper; pooled SEM = 0.75. Time < 0.001; time x starch x antagonist, P =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

Figure 3: Manuscript ID JDS.2020-18453