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

by Sinclair, L.A., Johnson, D., Wilson, S. and Mackenzie, A.M.

Sinclair LA[1], Johnson D[2], Wilson S[2], Mackenzie AM[2].

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INTERPRETIVE SUMMARY

Added dietary sulfur and molybdenum has a greater influence on hepatic copper concentration, intake and performance in Holstein-Friesian dairy cows offered a grass silage than a corn silage based diet *By Sinclair*. The objectives of our study were to determine the effect of different forages on the copper status and milk performance in dairy cows when fed without or with antagonists to copper absorption. We found that, only in the high inclusion grass silage based diet did the addition of dietary sulphur and molybdenum reduce intake and milk yield and increase somatic cell count. Liver copper concentration also declined more rapidly in cows offered a grass silage diet with added sulfur and molybdenum, but blood copper levels were unaffected. We advise that the basal forage should be taken into account when supplementing copper, particularly if sulfur and molybdenum levels are high.

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

L. A. Sinclair*[1], D. Johnson*,†, S. Wilson* and A. M. Mackenzie*

*Department of Animal Production, Welfare and Veterinary Sciences, Harper Adams University, Edgmond, Newport, Shropshire, UK, TF10 8NB

†**Current address:** Faculty of Health and Social Sciences, Leeds Beckett University, Leeds, UK, LS1 3HE

[1] Corresponding author: lsinclair@harper-adams.ac.uk

Key words: copper, dairy cow, forage, liver,

ABSTRACT

To test the hypothesis that the metabolism of Cu in dairy cows is affected by basal forage and added S and Mo, 56 dairy cows that were 35 (SE +/- 2.2) days post calving and yielding 38.9 kg milk/d (SE +/- 0.91) were offered one of four diets in a 2 x 2 factorial design for a 14 wk period. The four diets contained approximately 20 mg Cu/kg DM, and had a corn silage to grass silage ratio of 0.75:0.25 (C) or 0.25:0.75 (G) and were either unsupplemented (-) or supplemented (+) with an additional 2g S/kg DM and 6.5 mg Mo/kg DM. There was an interaction between forage source and added S and Mo on DM intake, with cows offered G+ having a 2.1 kg DM lower intake than those offered G-, but there was no effect on the corn silage based diets. Mean milk yield was 38.9 kg/d, and there was an interaction between basal forage and added S and Mo, with yield being decreased in cows offered G+, but increased on C+. There was no effect of dietary treatment on milk composition or live weight, but body condition was lower in cows fed added S and Mo irrespective of forage source. There was an interaction between forage source and added S and Mo on milk somatic cell count, which was higher in cows offered G+ compared to G-, but not in cows fed the corn silage based diets, although all values were low (mean values of 1.75, 1.50, 1.39 and 1.67 $\log_{10}$/mL for C-, C+, G- and G+ respectively). Mean plasma Cu, Fe and Mn concentrations were 13.8, 41.3 and 0.25 µmol/L respectively and were not affected by dietary treatment, whereas plasma Mo was 0.2 µmol/L higher in cows receiving added S and Mo. The addition of dietary S and Mo decreased liver Cu balance over the study period in cows fed either basal forage, but the decrease was considerably greater in cows receiving the grass silage based diet. Similarly, hepatic Fe decreased more in cows receiving G than C when S and Mo were included in the diet. It is concluded that added S and Mo reduces hepatic Cu reserves irrespective of basal forage source, but this decrease is considerably more pronounced in cows receiving grass silage than corn silage based rations, and is associated with a decrease in intake, milk performance and increase in milk somatic cell count.

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

INTRODUCTION

It has long been recognized that Cu is an important trace element for normal health and performance in dairy cattle, principally due to its requirement in approximately 300 different proteins with functions ranging from efficient iron metabolism, hair pigmentation, antioxidants, release of hormones and synthesis of connective tissue (Suttle, 2010). As a consequence, Cu responsive disorders result in production and economic losses due to effects on fertility, performance and health (NRC, 2005). Clinical signs in dairy cows can be caused by a dietary deficiency of Cu, but are often are related to interactions with dietary antagonists such as S and Mo, Fe and Zn that inhibit Cu absorption and/or metabolism (Suttle, 2010), with S and Mo receiving the most research attention. It has been proposed that dietary sulfates present in feed or water are reduced in the rumen to sulfides which then react with molybdate to form thiomolybdates (Dick et al., 1975). Gould and Kendall (2011) discussed that thiomolybdates may be present in the rumen as di, tri or tetrathiomolybdates, with trimolybdate predominant at a ruminal pH of 6.5, whereas tetrathiomolybdate is most prevalent at lower pH values. Thiomolybdates form insoluble complexes with Cu rendering it unabsorbable (Suttle, 1991), resulting in Cu responsive disorders. At high Mo intakes (e.g. >8 mg Mo/kg DM) and very low Cu:Mo ratios (less than 1:1) thiomolybdates may also leave the rumen and be absorbed (Suttle 2010), subsequently binding to Cu containing enzymes such as caeruloplasmin (**Cp**), impairing their function (Gould and Kendall, 2011).

It is recognized that the degree of thiomolybdate formation in the rumen can also be affected by the basal forage and method of preservation (Suttle 1974; Suttle 1983; Suttle

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

MATERIALS AND METHODS

Animals, Management and Treatments. The procedures involving animals were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986, and were approved by the Harper Adams Animal Welfare and Ethical Review Board. Fifty-six Holstein-Friesian dairy cows (8 primiparous and 48 multiparous) that were 35 (SE +/- 2.2) days post calving and yielding 38.9 kg/d (SE +/- 0.91) of milk were used. From calving until wk 5 of lactation the cows were group housed and fed a diet containing (g/kg DM) grass silage 95, alfalfa silage 90; corn silage 324; chopped wheat straw 20; urea treated wheat 100; soy hulls 80; molasses 50; soybean meal 66; rapeseed meal 64; distillers grains 64; palm kernel meal 18; protected fat 14; minerals and vitamins 15. Based on recordings taken in wk 4 of lactation the animals were blocked and allocated to one of four dietary treatments according to lactation number (prima or multi), calving date, milk yield, milk composition, BCS (using a 1-5 scoring system on a quarter point scale; Lowman et al., 1976) and live weight. Cows remained on study for 14 wks.

Based on the mineral analysis of the forages (Table 1) and NRC (2001) values for the other feeds, fours diets were formulated to contain approximately 20 mg Cu/kg DM and a corn silage to grass silage ratio of 0.75:0.25 (C) or 0.25:0.75 (G: DM basis; Table 2). To evaluate the effects of dietary antagonists on Cu metabolism, the diets were either unsupplemented (-) or supplemented (+) with additional S and Mo, to result in a total dietary concentration of approximately 3.5 g S/kg DM or 7.5 mg Mo/kg DM (an increase of approximately 2 g S/kg DM (+160%) and 6.5 mg Mo/kg DM (+ 500%). There were therefore 4 dietary treatments: C- (0.75 corn silage:0.25 grass silage (DM basis), no additional antagonists); C+ (0.75 corn silage:0.25 grass silage, with additional S and Mo); G- (0.25 corn silage:0.75 grass silage, no additional antagonists) and G+ (0.25 corn silage:0.75 grass silage, with additional S and Mo). Additional Cu was supplied as $CuSO_4.5H_2O$, sulfur as ammonium sulfate (TG Tennants, West Bromwich, UK) and molybdenum as sodium molybdate (Acros Organics, Geel, Belgium). Feed grade urea was added to G- and C- to provide an equivalent amount of rumen degradable N as supplied by the ammonium sulphate. The diets were supplemented with other feed ingredients to support a milk production of approximately 38 kg/d according to Thomas (2004; Table 2). All dietary ingredients were mixed and fed as a TMR using a forage mixer calibrated to ± 1 kg, and fed through Insentec roughage intake feeders fitted with an automatic animal identification and forage weighing system calibrated to ± 0.1 kg (Sinclair et al., 2005). Fresh feed was offered daily at 1.05 of *ad libitum* intake with refusals collected twice weekly on a Tuesday and Friday. The cows were housed in the same portion of a free stall building containing Super Comfort free stalls fitted with foam mattresses. The passageways were scraped using automatic scrapers and the stalls bedded twice weekly with sawdust. All cows had continual access to fresh bore-hole water which contained a concentration of S, Fe, Cu and Mo of 19.3 mg/L, 6.5, 2.9 and 0.5 μg/L respectively.

Experimental routine. Cows were milked twice daily at approximately 0530 h and 1530 h, with yield recorded at each milking and samples taken fortnightly at consecutive am and pm milkings for subsequent composition and somatic cell count (SCC) analysis. The cows were weighed and BCS recorded after the evening milking in the wk prior to allocation and then fortnightly. Forage samples were collected weekly: half the sample was oven dried at 70°C to constant weight, and the amount of corn silage to grass silage adjusted to achieve the desired ratio. The other sample was frozen and bulked for subsequent analysis. Samples of each of the four diets were collected immediately following feeding once per wk and stored at -20ºC prior to subsequent analysis. During wks 0, 1, 2, 4, 8 and 14 of the study blood samples were collected at 1000 h via jugular venipuncture into vacutainers (Becton Dickinson Vacutainer Systems, Plymouth, UK) containing, silica (for samples used to determine Cp), or lithium heparin (for samples used to determine superoxide dismutase (SOD) activity) and sodium heparin (for samples used to determine mineral concentrations and metabolites). During wk 0 and 14 of the study liver biopsy samples were collected from all cows through the 11th intercostal space as described by Davies and Jebbett (1981), and stored at -80°C prior to subsequent analysis.

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

Statistical analysis. Performance, plasma minerals and metabolites were analyzed by repeated measures ANOVA as a 2 x 2 factorial design. Milk SCC was transformed to $\log_{10}$ prior to analysis. Treatment degrees of freedom were split into main effects of forage source (corn versus grass silage), antagonist (Ant; without; (-) versus with; (+)) and their interaction (Int) and analyzed as:

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

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

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

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

Where Y_{ijk} = dependent variable; μ = overall mean; B_i = fixed effect of blocks; F_j = effect of forage (j = corn or grass silage); A_k = effect of S and Mo (k = -, +); $F.A_{jk}$ = interactions between forage and antagonist; and ε_{ijk} = residual error. For hepatic mineral concentrations the concentration during wk 0 was used where appropriate as a covariate to determine the final and rate of mineral deposition or mobilization. All statistical analysis was conducted using Genstat version 17.1 (VSN Int. Ltd., Oxford, UK) and is presented as means with standard error of the mean (SEM); $P < 0.05$ was used as the significant threshold and a trend was considered when $P < 0.1$.

RESULTS

Diet Analysis, Intake and Animal Performance. Compared to the corn silage, the grass silage contained 85 g/kg less DM, and was 82 g/kg DM higher in CP and 0.4 MJ/kg DM higher in ME (Table 1). The two forages had a similar fiber content, but the grass silage was 43.2 g/kg DM higher in lactic acid than the corn silage. Compared to the corn silage, the mean content of Ca, P, Mg and S was 5.0, 1.0, 0.2 and 2.2 g/kg DM higher respectively, and Cu, Mo, Fe and Zn 3.3, 0.84, 94 and 14.2 mg/kg DM higher respectively in the grass silage.

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

Cows offered the corn silage based diets had a daily DM intake that was 2.2 kg/d higher ($P < 0.001$) than those offered the grass silage based diets (Table 3), an effect that was evident from wk 1 of the study (Fig 1). There was an interaction ($P < 0.05$) between forage source and Cu antagonists; adding S and Mo reduced DM intake by 2.1 kg/d in cows fed the grass silage but not the corn silage based diet. We also found an interaction between forage source and antagonist on Cu intake, which was lowest ($P < 0.05$) in cows fed G+ compared to the other 3 treatments. There was an interaction ($P < 0.05$) between forage source and Cu antagonists on milk yield, with yield decreasing with the addition of S and Mo in cows fed the grass silage based diet, but increasing in those offered the corn silage based diet. In contrast, there was no effect ($P > 0.05$) of dietary treatment on milk fat, protein or lactose content or daily fat yield, but we found that daily milk protein yield was 0.05 kg/d higher (*P*

< 0.05) in cows fed the corn silage based diet. We found no effect ($P > 0.05$) of dietary treatment on live weight or daily live weight change, but there was an effect of antagonist on BCS and BCS change ($P < 0.05$), with cows fed added S and Mo (C+ and G+) having a lower score and gained less BCS over the study period than those not supplemented with S and Mo (C- and G-; Fig 2). There was an interaction ($P < 0.05$) between forage source and Cu antagonists on milk SCC count, with the addition of S and Mo increasing SCC in cows fed the grass but not the corn silage based diet.

Plasma Mineral Profile, Cu Mediated Enzymes and Metabolites. We found no effect ($P > 0.05$) of dietary treatment on plasma Cu concentration, with a mean value of 13.7 µmol/L (Table 4). There was an effect of time on plasma Cu, with the concentration increasing in the first wk of the study, and then fluctuating in subsequent wks (Fig 3). We also found an effect ($P < 0.001$) of dietary treatment on mean plasma Mo concentrations, which were higher in cows fed added S and Mo, but there was no effect ($P > 0.05$) of basal forage. There was no effect ($P > 0.05$) of dietary treatment on plasma Fe or Mn concentrations. Serum Cp concentrations were higher ($P < 0.01$) in cows fed the grass silage based diets or with added S and Mo ($P < 0.05$). In contrast, we found no effect of dietary treatment on blood Cp:Cu ratio, although there was a trend ($P < 0.1$) for a lower ratio in cows fed the corn silage based diets, or in animals receiving added S and Mo. There was no effect ($P > 0.05$) of dietary treatment on plasma SOD, BHBA or BUN concentrations, with mean values of 2918 U/gHb, 0.43 mmol/L and 5.44 mmol/L respectively.

Hepatic Mineral Concentration. There was no difference between dietary treatments ($P > 0.05$) in initial hepatic Cu concentration, which averaged 443 mg/kg DM (Table 5). We did find an effect of forage source on final Cu concentration, which was higher ($P < 0.05$) in cows fed the corn compared to the grass silage based diets. There was also an effect of Cu antagonists on final hepatic Cu concentration, which was 142 mg/kg DM lower ($P < 0.01$) in cows fed added S and Mo. There was a trend ($P < 0.1$) for an interaction between forage source and Cu antagonists on the rate of change in hepatic Cu concentration, with a decrease of 61 mg/kg DM over the 14 wk study period in cows fed added S and Mo in combination with grass silage (G+), but an increase of 11 mg/kg DM in cows offered the corn silage based diet (C+).

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

DISCUSSION

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

20 mg Cu /kg DM was lower than the mean value of 27.9 mg/kg DM/d that was reported in the diet of early lactation cows in the UK (Sinclair and Atkins, 2013), but similar to the 18 mg/kg DM reported on 39 Californian dairy units by Castillo et al., (2013). Additionally, we added S and Mo at a rate to ensure that the supplemented diets (C+ and G+) had similar concentrations which would be expected to substantially reduce Cu absorption and subsequent metabolism. Differences in dietary S and Mo concentration between diets within the same level of antagonist was small ($P > 0.05$), and therefore the main effect was the difference between the unsupplemented and supplemented diets. Using the equations of Suttle and McLauchlan (1976), we predicted that the C- and G- diets would result in an apparent digestibility co-efficient of Cu of approximately 0.054, whereas the C+ and G+ diets would be two-thirds lower at approximately 0.018. As a consequence, we predicted that animals receiving C- or G- had a similar Cu supply but were over supplied by approximately 220 mg Cu/d whereas those receiving C+ or G+ were undersupplied by approximately 200 mg Cu/d. However, the use of the current equations did not predict any interaction between forage source and antagonist on Cu status or performance.

Similar to other studies that have investigated the effect of replacing grass silage with corn silage (Phipps et al., 1995; Hart et al., 2015), we found that DM intake was increased at the higher corn inclusion rate, although it is accepted that the change in forage composition from the pre-study diet was greater for cows on G than C diets. However, we also found an interaction between forage inclusion level and Cu antagonists on intake, with added S and Mo having little effect in cows fed the corn silage based diet, but reduced intake by 2.1 kg DM/d in those receiving the grass silage based diet. Our diets were supplemented with both S and Mo, and it is therefore not possible to determine the effects of each element independently. Some authors have reported a decrease in DMI in cattle when dietary S exceeded 2 g/kg DM (Spears et al., 2011), although others have reported little effect of dietary S concentration up to 6 g/kg DM (Richter et al., 2012). Under acidic ruminal conditions most of the S would be present as H_2S, which may be eructated and absorbed by the lungs or absorbed across the rumen epithelium (Bray and Till, 1975; Drewnoski et al., 2012). High circulating concentrations of H_2S can have neurological effects including polioenchalomalacia that is associated with a reduced intake (Gould, 1998). The large role that ruminal pH plays in the form of sulphide present in the rumen has been suggested as a possible explanation for the differences observed in sulfur tolerance between concentrate and roughage fed cattle (Drewnoski et al., 2012), and could explain the reduced DMI of cows offered G+ in our study. However, we did not monitor ruminal H_2S or pH levels, and the influence of level of inclusion of corn and grass silage on ruminal pH is difficult to predict as it is dependent on a number of factors including initial forage pH, buffering capacity of the diet, forage particle length, and supplementary feed level, composition, and degree of processing (Krause and Oetzel, 2006).

Molybdenum interacts with S in the rumen resulting in the formation of various isomers of thiomolybdate, a reaction which is reversible and pH dependent, with the formation of tetra-thiomolybdate being favored at lower ruminal pH values (Gould and Kendall, 2011). Indeed, the dietary addition of Mo has been proposed as a potential sink for H_2S in the rumen (Kessler et al., 2012), potentially reducing the negative effects of excess dietary S on intake, although this approach has not been supported by recent studies with beef animals (Kessler et al., 2012). An alternative hypothesis for the effect of added S and Mo on intake may be related to the absorption of tetra-thiomolybdates as these can have a direct effect on Cu containing enzymes such as peptidylglycine α-amidating monooxygenase which exerts an influence on the appetite-regulating hormones cholecystokinin and gastrin (Suttle, 2010), although studies in this area in ruminants are scarce. Ruminal absorption of tetra-thiomolybdates is increased at lower ruminal pH values, and it is possible that differences in the ruminal pH in cows fed the different forages affected uptake. The conditions under which thiomoybdates are absorbed is, however, a controversial subject area, and it was proposed by Suttle et al., (2010) that absorption was unlikely unless dietary Cu:Mo rations were below 1:1, well below the 2.5:1 in our C+ and G+ diets. It is also possible that the added Mo resulted in molybdenosis, however, no characteristic signs such as scouring were noted and dietary values were well below that reported in other studies that have also reported no signs (Raisbeck et al. 2006).

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

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

Plasma Mineral Profile, Cu Mediated Enzymes and Metabolites. We found that plasma Cu concentrations were unaffected by dietary treatment, with all values being above the 9 mmol/L considered to be adequate (Laven and Livesey, 2005). Our finding is consistent with others that have supplemented Cu at different levels (Chase et al., 2000), with different levels of dietary S and Mo (Sinclair et al., 2013), or with different dietary sources of Cu (Scaletti and Harmon, 2012; Sinclair et al., 2013). In a meta-analysis of the relationship between dietary concentration of Cu, S and Mo and plasma Cu in growing cattle, Dias et al., (2013) concluded that any prediction equation would be limited, and that it is only when animals have either very low or high hepatic Cu reserves that plasma values can be usefully employed as an indicator of Cu status (Laven and Livesey, 2005). The plasma Cu:Cp ratios reported in our study were generally low, and unaffected by dietary treatment. Similarly, we found that plasma SOD, a Cu containing enzyme involved in the defense against free radicals (Suttle, 2010), was unaffected by dietary treatment. Our findings therefore support Suttle (2010) who suggested that the dietary ratio of Cu:Mo needed to be close to 1:1 before there is a risk of thiomolybdates causing a systemic impairment of Cu containing enzymes.

Hepatic Mineral Concentration. One of the first biochemical changes observed under Cu deprivation is a decrease in hepatic concentration (Suttle, 2010), as the liver is generally regarded as the principal storage organ for Cu (Laven and Livesey, 2005). In our study initial hepatic Cu levels were high and variable at 443 ± 29.2 (SE) mg/kg DM, although most (68%)

animals were below the upper limit of 510 mg/kg DM suggested to pose a risk of toxicity (Livesey et al., 2002). The initial mean hepatic Cu concentration that we found was also lower than that reported by Kendall et al., (2015), where almost 40% of cull dairy cows in the UK were reported to have a concentration above 500 mg Cu/kg DM. As we anticipated, there was a significant reduction in hepatic Cu concentration following the addition of dietary S and Mo, but the greater reduction in cows fed a grass silage compared to the corn silage based diet was unexpected, although the difference failed to reach full statistical significance. Suttle (2013) discussed that changes in hepatic Cu concentration are an exponential function of initial hepatic Cu concentration, most probably due to a greater rate of biliary excretion at higher liver concentrations. We therefore $\log_e$ transformed and re-analyzed the initial and final hepatic Cu concentrations to more accurately determine the influence of diet on hepatic Cu reserves. Similar to the untransformed data, we found no difference ($P > 0.1$) between treatments in initial liver Cu concentration, but we did now find an interaction ($P < 0.05$) between forage source and Cu antagonist on daily liver Cu balance ($\log_e$ final – $\log_e$ initial), confirming that high dietary concentrations of S and Mo have a greater effect on Cu metabolism in cows receiving a grass silage than a corn silage based diet.

The influence of forage source on the absorption of Cu is well demonstrated in sheep (e.g. Suttle 1983; Suttle 2010), and in the absence of high Mo concentrations, the absorption coefficient of Cu was reported to be 0.014 in grazed grass, 0.049 in grass silage, 0.073 in hay and 0.128 in leafy brassicas. This is however, the first study to report a substantial difference in Cu status in dairy cows fed corn or grass silage based rations, but only when S and Mo concentrations were high. Dietary Fe may interact with added S reducing hepatic Cu concentration (Suttle, 2010). However, the low dietary concentration of Fe in all of our diets compared to that reported for typical dairy cow rations in the UK (Sinclair and Atkins, 2013) or California (Castillo et al., 2013), in combination with the similarity in dietary Fe and S concentration between C+ and G+, does not support Fe as having a major influence in our study. Consideration should also be given to the lower DM intake of cows receiving G+ which resulted in a lower Cu intake of 49 mg/d than G-. Nevertheless, at the rate of decline in hepatic Cu concentration in cows receiving G+, concentrations would reduce and eventually approach the 25 mg Cu/kg DM threshold considered to deficient (Laven and Livesey, 2005). In contrast, in cows fed C- or G-, feeding 20 mg Cu/kg DM would result in a rapid increase in hepatic Cu concentration, whereas those receiving C+ would be relatively unchanged. Given such large differences in Cu status when fed the same dietary level, we recommend that forage source as well as dietary S and Mo concentration should be taken into account when supplementing dairy cows with Cu.

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

CONCLUSIONS

We found that the addition of S and Mo had no effect on DM intake or milk yield in cows fed a corn silage based ration, but were reduced and milk SCC increased when a grass silage based diet was fed. In the absence of additional S and Mo, a diet containing 20 mg Cu/kg DM whether based on grass or corn silage, contains well in excess of requirements as evidenced by the net increase in hepatic Cu concentration. In contrast, in the presence of high levels of S and Mo, feeding 20 mg Cu/kg DM will result in a rapid depletion of hepatic Cu concentrations in cows fed grass silage, but not corn silage based diets. Within the limits of this study we also found that there was little effect of added Cu antagonists on plasma Cu or indicators of plasma Cu enzyme activity, even at the high levels of S and Mo, and suggest that use of these parameters to predict Cu status is limited. Reasons for the differences in Cu metabolism in cows when fed grass or corn silage based rations is unclear and require further investigation, but our results highlight the importance of taking account of forage source when formulating diets for dairy cows, particularly when dietary S and Mo levels are high.

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Table 1. Chemical composition of corn and grass silage

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

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

	C-	C+	G-	G+
Ingredient, g/kg DM				
Grass silage	133	134	398	399
Corn silage	400	401	133	134
Urea-treated wheat	111	111	167	167
Soy hulls	89	89	89	89
Rapeseed meal	58	58	31	31
Soybean meal	96	96	31	31
Distillers dark grains with solubles	58	58	31	31
Sopralin[1]	---	---	58	58
Molasses	33	33	33	33
Protected fat	13	13	20	20
Urea	2	---	2	---
Mins/vits[2]	7	7	7	7
Total	1000	1000	1000	1000
Chemical analysis				
DM, g/kg	404	421	364	368
Ash, g/kg DM	71	71	92	93
CP, g/kg DM	181	185	193	194
NDF, g/kg DM	407	403	381	387
ADF, g/kg DM	222	224	228	224
Ca, g/kg DM	5.40	5.45	7.84	7.49
P, g/kg DM	3.57	3.82	3.96	3.69
Mg, g/kg DM	2.72	2.84	2.92	2.79
S, g/kg DM	1.20	3.15	1.32	3.45
Cu, mg/kg DM	19.9	19.5	20.7	20.5
Mo, mg/kg DM	1.17	7.94	1.48	7.70
Fe, mg/kg DM	183	226	287	252
Zn, mg/kg DM	49.2	46.3	51.8	48.8
Mn, mg/kg DM	61	68	70	60

[1] Formaldehyde treated soybean meal, Frank Wright Trouw, Ashbourne, UK [2] Mineral/vitamin premix (Rumenco, Staffordshire, UK). Major minerals (g/kg): Ca 240, P 80, Mg 120; Trace minerals (mg/kg): Cu 0, Zn 7,000, Mn 2,000, I 400, Co 80, and Se 50; vitamins (mg/kg) were: retinol 105, cholecalciferol 1.75, and all *rac* α-tocopherol acetate 5,000. [3] SEM for differences between dietary concentrations (n = 8 per treatment) for S and Mo was 0.11 and 0.29 respectively.

C+ and G+ diets also received additional ammonium sulfate and sodium molybdate dihydrate.

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

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

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

Table 4. Plasma mineral concentration and metabolites and serum caeruloplasmin in early lactation dairy cows fed diets high in corn silage (C) or grass silage (G) fed without (-) or with (+) added S and Mo. Blood samples were collected during wks 0, 1, 2, 4, 8 and 14 of the study.

	Diets					Significance, *P*-value[1]		
	C-	C+	G-	G+	SEM	F	A	Int
Plasma Cu, µmol/L	13.3	13.7	14.3	13.7	0.51	0.340	0.889	0.332
Plasma Mo, µmol/L	0.33	0.50	0.27	0.50	0.029	0.271	<0.001	0.375
Plasma Fe, µmol/L	43.2	40.5	40.7	40.9	1.61	0.519	0.446	0.384
Plasma Mn, µmol/L	0.25	0.24	0.27	0.25	0.010	0.124	0.239	0.740
Caeruloplasmin, mg/dL	17.9	15.9	20.3	18.1	0.79	0.006	0.010	0.909
Caeruloplasmin:Cu	1.37	1.22	1.41	1.36	0.057	0.096	0.090	0.377
SOD[2] U/gHb	2960	2841	2954	2915	89.8	0.710	0.387	0.657
BHBA, mmol/L	0.42	0.38	0.44	0.48	0.048	0.210	0.963	0.406
BUN, mmol/L	5.22	5.44	5.70	5.39	0.189	0.265	0.802	0.172

[1] F= main effect of forage source, A = main effect of antagonists, Int = interaction between forage and antagonists. There was a time x treatment effect on plasma Mo ($P < 0.05$), which increased with time in animals receiving C+ and G+ compared to C- and G-

[2] Superoxide dismutase

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

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

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

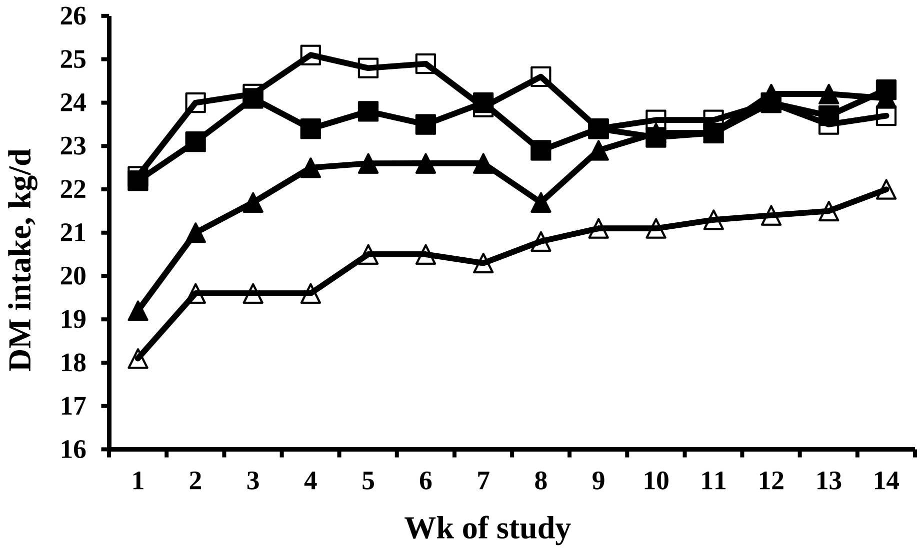

Figure 1. Weekly DM intake in early lactation dairy cows fed diets high in corn silage and fed without (■) or with (□) added S and Mo, or diets high in grass silage fed without (▲) or with (△) added S and Mo. Pooled SEM = 0.72. Forage, *P* < 0.001; Forage x Ant, *P* = 0.012; Time, *P* < 0.001; Forage x time, *P* =0.003.

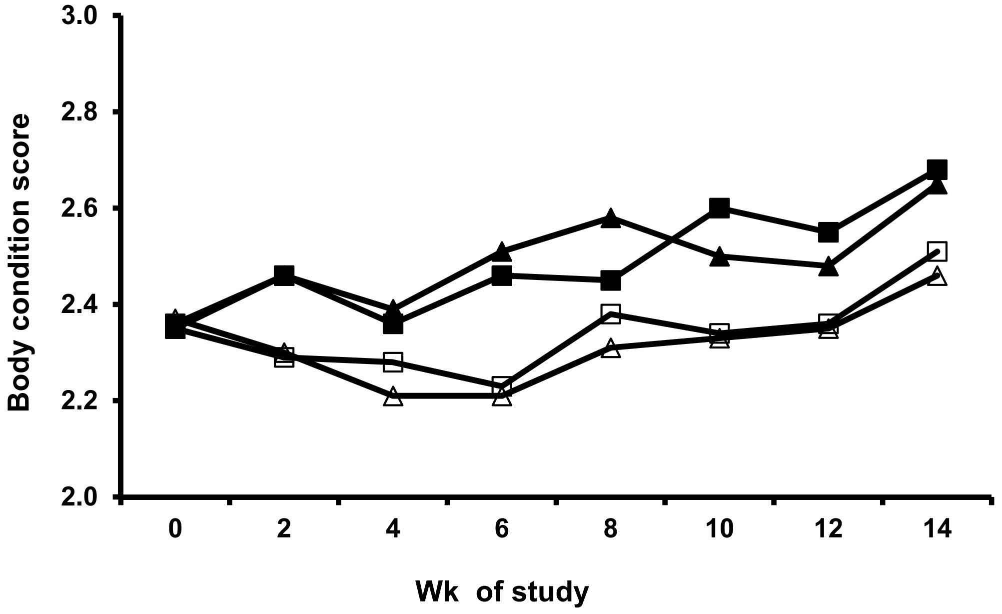

Figure 2. Fortnightly BCS in early lactation dairy cows fed diets high in corn silage and fed without (■) or with (□) added S and Mo, or diets high in grass silage fed without (▲) or with (△) added S and Mo. Pooled SEM = 0.067. Ant, *P* < 0.001; Time, *P* < 0.001; Time x ant, *P* =0.077.

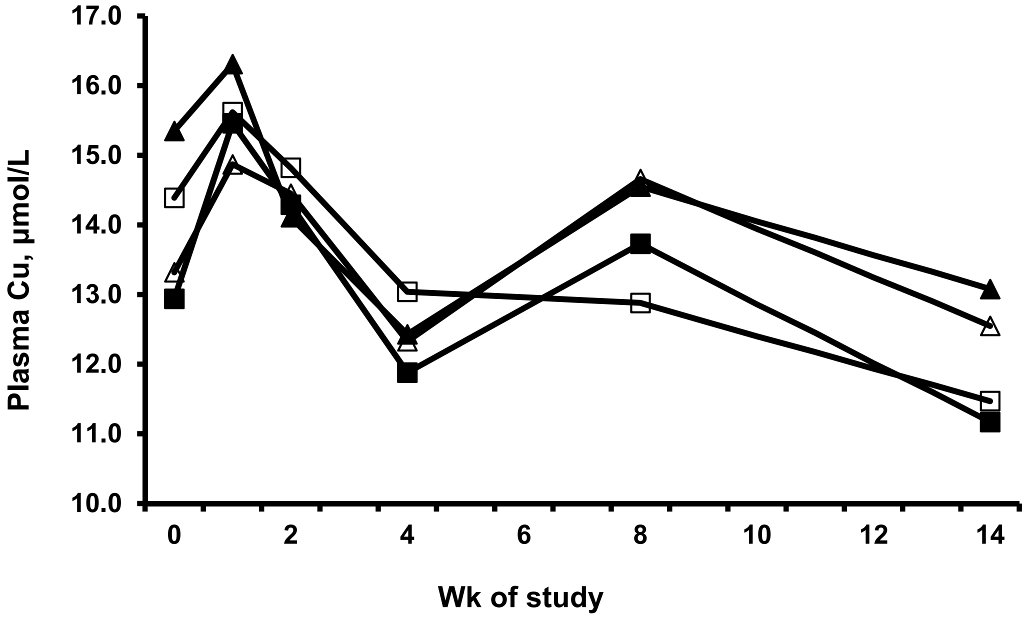

Figure 3. Plasma Cu concentrations in early lactation dairy cows fed diets high in corn silage and fed without (■) or with (□) added S and Mo, or diets high in grass silage fed without (▲) or with (△) added S and Mo. Pooled SEM = 0.87. Time, *P* < 0.001.