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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.

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

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

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ABSTRACT

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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 log₁₀/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

74 silage based rations, and is associated with a decrease in intake, milk performance and increase
75 in milk somatic cell count.

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

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INTRODUCTION

79 It has long been recognized that Cu is an important trace element for normal health and
80 performance in dairy cattle, principally due to its requirement in approximately 300 different
81 proteins with functions ranging from efficient iron metabolism, hair pigmentation,
82 antioxidants, release of hormones and synthesis of connective tissue (Suttle, 2010). As a
83 consequence, Cu responsive disorders result in production and economic losses due to effects
84 on fertility, performance and health (NRC, 2005). Clinical signs in dairy cows can be caused
85 by a dietary deficiency of Cu, but are often are related to interactions with dietary antagonists
86 such as S and Mo, Fe and Zn that inhibit Cu absorption and/or metabolism (Suttle, 2010), with
87 S and Mo receiving the most research attention. It has been proposed that dietary sulfates
88 present in feed or water are reduced in the rumen to sulfides which then react with molybdate
89 to form thiomolybdates (Dick et al., 1975). Gould and Kendall (2011) discussed that
90 thiomolybdates may be present in the rumen as di, tri or tetrathiomolybdates, with trimolybdate
91 predominant at a ruminal pH of 6.5, whereas tetrathiomolybdate is most prevalent at lower pH
92 values. Thiomolybdates form insoluble complexes with Cu rendering it unabsorbable (Suttle,
93 1991), resulting in Cu responsive disorders. At high Mo intakes (e.g. >8 mg Mo/kg DM) and
94 very low Cu:Mo ratios (less than 1:1) thiomolybdates may also leave the rumen and be
95 absorbed (Suttle 2010), subsequently binding to Cu containing enzymes such as
96 caeruloplasmin (**Cp**), impairing their function (Gould and Kendall, 2011).

97 It is recognized that the degree of thiomolybdate formation in the rumen can also be
98 affected by the basal forage and method of preservation (Suttle 1974; Suttle 1983; Suttle 2010),

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

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MATERIALS AND METHODS

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Animals, Management and Treatments. The procedures involving animals were

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conducted in accordance with the UK Animals (Scientific Procedures) Act 1986, and were

124 approved by the Harper Adams Animal Welfare and Ethical Review Board. Fifty-six Holstein-
125 Friesian dairy cows (8 primiparous and 48 multiparous) that were 35 (SE +/- 2.2) days post
126 calving and yielding 38.9 kg/d (SE +/- 0.91) of milk were used. From calving until wk 5 of
127 lactation the cows were group housed and fed a diet containing (g/kg DM) grass silage 95,
128 alfalfa silage 90; corn silage 324; chopped wheat straw 20; urea treated wheat 100; soy hulls
129 80; molasses 50; soybean meal 66; rapeseed meal 64; distillers grains 64; palm kernel meal 18;
130 protected fat 14; minerals and vitamins 15. Based on recordings taken in wk 4 of lactation the
131 animals were blocked and allocated to one of four dietary treatments according to lactation
132 number (prima or multi), calving date, milk yield, milk composition, BCS (using a 1-5 scoring
133 system on a quarter point scale; Lowman et al., 1976) and live weight. Cows remained on
134 study for 14 wks.

135 Based on the mineral analysis of the forages (Table 1) and NRC (2001) values for the
136 other feeds, four diets were formulated to contain approximately 20 mg Cu/kg DM and a corn
137 silage to grass silage ratio of 0.75:0.25 (C) or 0.25:0.75 (G: DM basis; Table 2). To evaluate
138 the effects of dietary antagonists on Cu metabolism, the diets were either unsupplemented (-)
139 or supplemented (+) with additional S and Mo, to result in a total dietary concentration of
140 approximately 3.5 g S/kg DM or 7.5 mg Mo/kg DM (an increase of approximately 2 g S/kg
141 DM (+160%) and 6.5 mg Mo/kg DM (+ 500%). There were therefore 4 dietary treatments: C-
142 (0.75 corn silage:0.25 grass silage (DM basis), no additional antagonists); C+ (0.75 corn
143 silage:0.25 grass silage, with additional S and Mo); G- (0.25 corn silage:0.75 grass silage, no
144 additional antagonists) and G+ (0.25 corn silage:0.75 grass silage, with additional S and Mo).
145 Additional Cu was supplied as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, sulfur as ammonium sulfate (TG Tennants, West
146 Bromwich, UK) and molybdenum as sodium molybdate (Acros Organics, Geel, Belgium).
147 Feed grade urea was added to G- and C- to provide an equivalent amount of rumen degradable
148 N as supplied by the ammonium sulphate. The diets were supplemented with other feed

149 ingredients to support a milk production of approximately 38 kg/d according to Thomas (2004;
150 Table 2). All dietary ingredients were mixed and fed as a TMR using a forage mixer calibrated
151 to ± 1 kg, and fed through Insentec roughage intake feeders fitted with an automatic animal
152 identification and forage weighing system calibrated to ± 0.1 kg (Sinclair et al., 2005). Fresh
153 feed was offered daily at 1.05 of *ad libitum* intake with refusals collected twice weekly on a
154 Tuesday and Friday. The cows were housed in the same portion of a free stall building
155 containing Super Comfort free stalls fitted with foam mattresses. The passageways were
156 scraped using automatic scrapers and the stalls bedded twice weekly with sawdust. All cows
157 had continual access to fresh bore-hole water which contained a concentration of S, Fe, Cu and
158 Mo of 19.3 mg/L, 6.5, 2.9 and 0.5 $\mu\text{g/L}$ respectively.

159 ***Experimental routine.*** Cows were milked twice daily at approximately 0530 h and
160 1530 h, with yield recorded at each milking and samples taken fortnightly at consecutive am
161 and pm milkings for subsequent composition and somatic cell count (SCC) analysis. The cows
162 were weighed and BCS recorded after the evening milking in the wk prior to allocation and
163 then fortnightly. Forage samples were collected weekly: half the sample was oven dried at 70°C
164 to constant weight, and the amount of corn silage to grass silage adjusted to achieve the desired
165 ratio. The other sample was frozen and bulked for subsequent analysis. Samples of each of the
166 four diets were collected immediately following feeding once per wk and stored at -20°C prior
167 to subsequent analysis. During wks 0, 1, 2, 4, 8 and 14 of the study blood samples were
168 collected at 1000 h via jugular venipuncture into vacutainers (Becton Dickinson Vacutainer
169 Systems, Plymouth, UK) containing, silica (for samples used to determine Cp), or lithium
170 heparin (for samples used to determine superoxide dismutase (SOD) activity) and sodium
171 heparin (for samples used to determine mineral concentrations and metabolites). During wk 0
172 and 14 of the study liver biopsy samples were collected from all cows through the 11th

173 intercostal space as described by Davies and Jebbett (1981), and stored at -80°C prior to
174 subsequent analysis.

175 **Chemical analysis.** Weekly forage and TMR samples were bulked within month and
176 analyzed according to AOAC (2012) for DM (934.01), CP (990.03) and starch (920.40). In
177 addition, forage samples were analyzed for pH, ammonia-N, water soluble carbohydrates
178 (MAFF, 1986), and VFA based on the method of Jones and Kay (1976). The analysis of NDF
179 and ADF were conducted according to Van Soest et al. (1991) with the use of a heat-stable α -
180 amylase (Sigma, Gillingham, UK), and expressed exclusive of residual ash. The ME content
181 of the forages was determined by near infra-red reflectance spectroscopy (Eurofins
182 Laboratories, Wolverhampton, UK) using a system approved by the UK advisory services
183 (Offer et al., 1996). Forage and TMR minerals were extracted using the DigiPREP digestion
184 system (Qmx Laboratories, Essex, UK), and analyzed as described by Cope et al. (2009) by
185 inductively coupled plasma-mass spectrometry (ICP-MS; Thermo Fisher Scientific Inc.,
186 Hemel Hempstead, UK). Serum samples were analyzed for Cp according to Henry et al. (1974)
187 and plasma samples for superoxide dismutase (**SOD**; Randox Laboratories, kit catalogue no.
188 SD 125), BHBA and urea (Randox Laboratories, County Antrim, UK; kit catalogue no. RB
189 1007, and UR221 respectively) using a Cobas Miras Plus autoanalyser (ABX Diagnostics,
190 Bedfordshire, UK). Plasma and liver samples were analyzed for Cu, Fe, Mn and Mo by ICP-
191 MS as described by Sinclair et al., (2013). Milk samples were analyzed using a Milkoscan
192 Minor (FOSS, Warrington, UK) calibrated by the methods of AOAC (2012), and SCC was
193 determined by Eurofins Laboratories (Wolverhampton, UK).

194 **Statistical analysis.** Performance, plasma minerals and metabolites were analyzed by
195 repeated measures ANOVA as a 2 x 2 factorial design. Milk SCC was transformed to \log_{10}
196 prior to analysis. Treatment degrees of freedom were split into main effects of forage source

197 (corn versus grass silage), antagonist (Ant; without; (-) versus with; (+)) and their interaction
198 (Int) and analyzed as:

$$199 \quad Y_{ijkl} = \mu + B_i + F_j + A_k + T_l + F.A_{jk} + F.T_{jl} + A.T_{kl} + F.A.T_{jkl} + \varepsilon_{ijkl}$$

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

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

$$206 \quad Y_{ijk} = \mu + B_i + F_j + A_k + F.A_{jk} + \varepsilon_{ijk}$$

207 Where Y_{ijk} = dependent variable; μ = overall mean; B_i = fixed effect of blocks; F_j = effect of
208 forage (j = corn or grass silage); A_k = effect of S and Mo (k = -, +); $F.A_{jk}$ = interactions between
209 forage and antagonist; and ε_{ijk} = residual error. For hepatic mineral concentrations the
210 concentration during wk 0 was used where appropriate as a covariate to determine the final and
211 rate of mineral deposition or mobilization. All statistical analysis was conducted using Genstat
212 version 17.1 (VSN Int. Ltd., Oxford, UK) and is presented as means with standard error of the
213 mean (SEM); $P < 0.05$ was used as the significant threshold and a trend was considered when
214 $P < 0.1$.

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RESULTS

217 ***Diet Analysis, Intake and Animal Performance.*** Compared to the corn silage, the grass
218 silage contained 85 g/kg less DM, and was 82 g/kg DM higher in CP and 0.4 MJ/kg DM higher
219 in ME (Table 1). The two forages had a similar fiber content, but the grass silage was 43.2 g/kg
220 DM higher in lactic acid than the corn silage. Compared to the corn silage, the mean content

221 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
222 Zn 3.3, 0.84, 94 and 14.2 mg/kg DM higher respectively in the grass silage.

223 The DM content of the corn based diets (C- and C+) was 47 g/kg higher than the grass
224 silage based diets (G- and G+), whereas CP was on average 11 g/kg DM higher in the grass
225 than the corn silage based diets (Table 2). The content of NDF was higher in the corn than the
226 grass silage based diets, but ADF concentration was similar across all four diets, averaging 225
227 g/kg DM. All four diets had a similar P and Mg concentration, but the grass silage based diets
228 (G- and G+) contained approximately 2 g/kg DM more Ca. The mean concentration of Cu was
229 20 mg/kg DM, and the two diets with added antagonists (C+ and G+) had concentrations of S
230 and Mo of 3.3 g/kg DM and 7.8 mg/kg DM respectively, which were close ($P > 0.05$) to the
231 predicted values of 3.5 g/kg DM and 7.5 mg/kg DM respectively. In contrast, the two diets
232 with no added S and Mo (Corn- and Grass-) had low concentrations of S and Mo at 1.3 g/kg
233 DM and 1.3 mg/kg DM respectively that were also close ($P > 0.05$) to predicted.

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

246 live weight change, but there was an effect of antagonist on BCS and BCS change ($P < 0.05$),
247 with cows fed added S and Mo (C+ and G+) having a lower score and gained less BCS over
248 the study period than those not supplemented with S and Mo (C- and G-; Fig 2). There was an
249 interaction ($P < 0.05$) between forage source and Cu antagonists on milk SCC count, with the
250 addition of S and Mo increasing SCC in cows fed the grass but not the corn silage based diet.

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

264 ***Hepatic Mineral Concentration.*** There was no difference between dietary treatments
265 ($P > 0.05$) in initial hepatic Cu concentration, which averaged 443 mg/kg DM (Table 5). We
266 did find an effect of forage source on final Cu concentration, which was higher ($P < 0.05$) in
267 cows fed the corn compared to the grass silage based diets. There was also an effect of Cu
268 antagonists on final hepatic Cu concentration, which was 142 mg/kg DM lower ($P < 0.01$) in
269 cows fed added S and Mo. There was a trend ($P < 0.1$) for an interaction between forage source
270 and Cu antagonists on the rate of change in hepatic Cu concentration, with a decrease of 61

271 mg/kg DM over the 14 wk study period in cows fed added S and Mo in combination with grass
272 silage (G+), but an increase of 11 mg/kg DM in cows offered the corn silage based diet (C+).

273 We found no difference between treatments in initial hepatic Mo concentration ($P >$
274 0.05), whereas final Mo concentration was higher ($P < 0.05$) in cows fed added S and Mo (C+
275 and G+). Initial hepatic Fe concentration did not differ between treatments ($P > 0.05$), whereas
276 final concentration was lower ($P < 0.01$) in cows fed added S and Mo, and there was a trend
277 ($P < 0.1$) for final hepatic Fe concentration to be higher in cows offered the corn compared to
278 the grass silage based diet. The addition of S and Mo resulted in a net decrease in hepatic Fe
279 concentration over the study period of 19 mg/kg DM compared to an increase in cows that
280 were not supplemented with S and Mo of 50 mg/kg DM, although most of this difference could
281 be attributed to cows fed the grass silage based ration with added S and Mo (G+) decreasing in
282 hepatic Fe concentration ($P < 0.1$) compared to an increase in cows fed any of the other dietary
283 treatments. Finally, we found no effect ($P < 0.05$) of dietary treatment on hepatic Mn
284 concentrations, although cows fed the grass silage with Cu antagonists (G+) tended ($P < 0.1$)
285 to decrease by the greatest amount.

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DISCUSSION

288 ***Intake and Performance.*** Our study is the first to determine Cu status and metabolism
289 in high yielding dairy cows when fed corn or grass silage based rations at different S and Mo
290 concentrations. Corn silage is generally regarded as having a lower Cu concentration than grass
291 silage (NRC, 2001), but we supplemented the diets to ensure that levels were similar across all
292 treatments, averaging 20.0 mg Cu/kg DM. The dietary level of 20 mg Cu /kg DM was lower
293 than the mean value of 27.9 mg/kg DM/d that was reported in the diet of early lactation cows
294 in the UK (Sinclair and Atkins, 2013), but similar to the 18 mg/kg DM reported on 39
295 Californian dairy units by Castillo et al., (2013). Additionally, we added S and Mo at a rate to

296 ensure that the supplemented diets (C+ and G+) had similar concentrations which would be
297 expected to substantially reduce Cu absorption and subsequent metabolism. Differences in
298 dietary S and Mo concentration between diets within the same level of antagonist was small (P
299 > 0.05), and therefore the main effect was the difference between the unsupplemented and
300 supplemented diets. Using the equations of Suttle and McLauchlan (1976), we predicted that
301 the C- and G- diets would result in an apparent digestibility co-efficient of Cu of approximately
302 0.054, whereas the C+ and G+ diets would be two-thirds lower at approximately 0.018. As a
303 consequence, we predicted that animals receiving C- or G- had a similar Cu supply but were
304 over supplied by approximately 220 mg Cu/d whereas those receiving C+ or G+ were
305 undersupplied by approximately 200 mg Cu/d. However, the use of the current equations did
306 not predict any interaction between forage source and antagonist on Cu status or performance.

307 Similar to other studies that have investigated the effect of replacing grass silage with
308 corn silage (Phipps et al., 1995; Hart et al., 2015), we found that DM intake was increased at
309 the higher corn inclusion rate, although it is accepted that the change in forage composition
310 from the pre-study diet was greater for cows on G than C diets. However, we also found an
311 interaction between forage inclusion level and Cu antagonists on intake, with added S and Mo
312 having little effect in cows fed the corn silage based diet, but reduced intake by 2.1 kg DM/d
313 in those receiving the grass silage based diet. Our diets were supplemented with both S and
314 Mo, and it is therefore not possible to determine the effects of each element independently.
315 Some authors have reported a decrease in DMI in cattle when dietary S exceeded 2 g/kg DM
316 (Spears et al., 2011), although others have reported little effect of dietary S concentration up to
317 6 g/kg DM (Richter et al., 2012). Under acidic ruminal conditions most of the S would be
318 present as H₂S, which may be eructated and absorbed by the lungs or absorbed across the rumen
319 epithelium (Bray and Till, 1975; Drewnoski et al., 2012). High circulating concentrations of
320 H₂S can have neurological effects including polioenchalomalacia that is associated with a

321 reduced intake (Gould, 1998). The large role that ruminal pH plays in the form of sulphide
322 present in the rumen has been suggested as a possible explanation for the differences observed
323 in sulfur tolerance between concentrate and roughage fed cattle (Drewnoski et al., 2012), and
324 could explain the reduced DMI of cows offered G+ in our study. However, we did not monitor
325 ruminal H₂S or pH levels, and the influence of level of inclusion of corn and grass silage on
326 ruminal pH is difficult to predict as it is dependent on a number of factors including initial
327 forage pH, buffering capacity of the diet, forage particle length, and supplementary feed level,
328 composition, and degree of processing (Krause and Oetzel, 2006).

329 Molybdenum interacts with S in the rumen resulting in the formation of various
330 isomers of thiomolybdate, a reaction which is reversible and pH dependent, with the
331 formation of tetra-thiomolybdate being favored at lower ruminal pH values (Gould and
332 Kendall, 2011). Indeed, the dietary addition of Mo has been proposed as a potential sink for
333 H₂S in the rumen (Kessler et al., 2012), potentially reducing the negative effects of excess
334 dietary S on intake, although this approach has not been supported by recent studies with beef
335 animals (Kessler et al., 2012). An alternative hypothesis for the effect of added S and Mo on
336 intake may be related to the absorption of tetra-thiomolybdates as these can have a direct
337 effect on Cu containing enzymes such as peptidylglycine α -amidating monooxygenase which
338 exerts an influence on the appetite-regulating hormones cholecystokinin and gastrin (Suttle,
339 2010), although studies in this area in ruminants are scarce. Ruminal absorption of tetra-
340 thiomolybdates is increased at lower ruminal pH values, and it is possible that differences in
341 the ruminal pH in cows fed the different forages affected uptake. The conditions under which
342 thiomoybdates are absorbed is, however, a controversial subject area, and it was proposed by
343 Suttle et al., (2010) that absorption was unlikely unless dietary Cu:Mo ratios were below
344 1:1, well below the 2.5:1 in our C+ and G+ diets. It is also possible that the added Mo
345 resulted in molybdenosis, however, no characteristic signs such as scouring were noted and

346 dietary values were well below that reported in other studies that have also reported no signs
347 (Raisbeck et al. 2006).

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

356 The interaction between basal forage source and Cu antagonists on milk SCC in our
357 study is difficult to explain, although all values were low. The role of Cu on milk SCC has been
358 demonstrated in dairy cattle in some but not all studies. For example, increasing dietary Cu
359 concentration from a sub-optimal level of 6.5 mg/kg DM to 26.5 mg/kg DM was shown to
360 reduce the peak increase in milk SCC following a challenge with *E. Coli* which was attributed
361 to a greater ability of neutrophils to kill invading bacteria, although the duration of the infection
362 was unaffected (Scaletti et al., 2003). In contrast, dietary Cu concentration was not shown to
363 have an effect on milk SCC concentration following a challenge with *E. Coli* in the studies of
364 Scaletti and Harmon (2012), or when different levels of dietary Cu were fed (Chase et al.,
365 2000). In our study, cows receiving G+ were in negative Cu balance as evidenced by the
366 depletion of hepatic Cu reserves, whereas all other treatments were in positive balance. It is
367 therefore possible that this lower Cu status contributed to the increased milk SCC, although
368 other indicators of Cu status such as plasma Cu and plasma Cu:Cp were unaffected by dietary
369 treatment. The lower DM intake that we observed in cows receiving G+ may also have
370 contributed to a greater metabolic stress and indirectly increased milk SCC.

371

372 ***Plasma Mineral Profile, Cu Mediated Enzymes and Metabolites.*** We found that plasma Cu
373 concentrations were unaffected by dietary treatment, with all values being above the 9 mmol/L
374 considered to be adequate (Laven and Livesey, 2005). Our finding is consistent with others that
375 have supplemented Cu at different levels (Chase et al., 2000), with different levels of dietary S
376 and Mo (Sinclair et al., 2013), or with different dietary sources of Cu (Scaletti and Harmon,
377 2012; Sinclair et al., 2013). In a meta-analysis of the relationship between dietary concentration
378 of Cu, S and Mo and plasma Cu in growing cattle, Dias et al., (2013) concluded that any
379 prediction equation would be limited, and that it is only when animals have either very low or
380 high hepatic Cu reserves that plasma values can be usefully employed as an indicator of Cu
381 status (Laven and Livesey, 2005). The plasma Cu:Cp ratios reported in our study were
382 generally low, and unaffected by dietary treatment. Similarly, we found that plasma SOD, a Cu
383 containing enzyme involved in the defense against free radicals (Suttle, 2010), was unaffected
384 by dietary treatment. Our findings therefore support Suttle (2010) who suggested that the
385 dietary ratio of Cu:Mo needed to be close to 1:1 before there is a risk of thiomolybdates causing
386 a systemic impairment of Cu containing enzymes.

387 ***Hepatic Mineral Concentration.*** One of the first biochemical changes observed under
388 Cu deprivation is a decrease in hepatic concentration (Suttle, 2010), as the liver is generally
389 regarded as the principal storage organ for Cu (Laven and Livesey, 2005). In our study initial
390 hepatic Cu levels were high and variable at 443 ± 29.2 (SE) mg/kg DM, although most (68%)
391 animals were below the upper limit of 510 mg/kg DM suggested to pose a risk of toxicity
392 (Livesey et al., 2002). The initial mean hepatic Cu concentration that we found was also lower
393 than that reported by Kendall et al., (2015), where almost 40% of cull dairy cows in the UK
394 were reported to have a concentration above 500 mg Cu/kg DM. As we anticipated, there was
395 a significant reduction in hepatic Cu concentration following the addition of dietary S and Mo,

396 but the greater reduction in cows fed a grass silage compared to the corn silage based diet was
397 unexpected, although the difference failed to reach full statistical significance. Suttle (2013)
398 discussed that changes in hepatic Cu concentration are an exponential function of initial hepatic
399 Cu concentration, most probably due to a greater rate of biliary excretion at higher liver
400 concentrations. We therefore \log_e transformed and re-analyzed the initial and final hepatic Cu
401 concentrations to more accurately determine the influence of diet on hepatic Cu reserves.
402 Similar to the untransformed data, we found no difference ($P > 0.1$) between treatments in
403 initial liver Cu concentration, but we did now find an interaction ($P < 0.05$) between forage
404 source and Cu antagonist on daily liver Cu balance (\log_e final – \log_e initial), confirming that
405 high dietary concentrations of S and Mo have a greater effect on Cu metabolism in cows
406 receiving a grass silage than a corn silage based diet.

407 The influence of forage source on the absorption of Cu is well demonstrated in sheep
408 (e.g. Suttle 1983; Suttle 2010), and in the absence of high Mo concentrations, the absorption
409 coefficient of Cu was reported to be 0.014 in grazed grass, 0.049 in grass silage, 0.073 in hay
410 and 0.128 in leafy brassicas. This is however, the first study to report a substantial difference
411 in Cu status in dairy cows fed corn or grass silage based rations, but only when S and Mo
412 concentrations were high. Dietary Fe may interact with added S reducing hepatic Cu
413 concentration (Suttle, 2010). However, the low dietary concentration of Fe in all of our diets
414 compared to that reported for typical dairy cow rations in the UK (Sinclair and Atkins, 2013)
415 or California (Castillo et al., 2013), in combination with the similarity in dietary Fe and S
416 concentration between C+ and G+, does not support Fe as having a major influence in our
417 study. Consideration should also be given to the lower DM intake of cows receiving G+ which
418 resulted in a lower Cu intake of 49 mg/d than G-. Nevertheless, at the rate of decline in hepatic
419 Cu concentration in cows receiving G+, concentrations would reduce and eventually approach
420 the 25 mg Cu/kg DM threshold considered to deficient (Laven and Livesey, 2005). In contrast,

421 in cows fed C- or G-, feeding 20 mg Cu/kg DM would result in a rapid increase in hepatic Cu
422 concentration, whereas those receiving C+ would be relatively unchanged. Given such large
423 differences in Cu status when fed the same dietary level, we recommend that forage source as
424 well as dietary S and Mo concentration should be taken into account when supplementing dairy
425 cows with Cu.

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

437

438 **CONCLUSIONS**

439 We found that the addition of S and Mo had no effect on DM intake or milk yield in
440 cows fed a corn silage based ration, but were reduced and milk SCC increased when a grass
441 silage based diet was fed. In the absence of additional S and Mo, a diet containing 20 mg Cu/kg
442 DM whether based on grass or corn silage, contains well in excess of requirements as evidenced
443 by the net increase in hepatic Cu concentration. In contrast, in the presence of high levels of S
444 and Mo, feeding 20 mg Cu/kg DM will result in a rapid depletion of hepatic Cu concentrations
445 in cows fed grass silage, but not corn silage based diets. Within the limits of this study we also

446 found that there was little effect of added Cu antagonists on plasma Cu or indicators of plasma
447 Cu enzyme activity, even at the high levels of S and Mo, and suggest that use of these
448 parameters to predict Cu status is limited. Reasons for the differences in Cu metabolism in
449 cows when fed grass or corn silage based rations is unclear and require further investigation,
450 but our results highlight the importance of taking account of forage source when formulating
451 diets for dairy cows, particularly when dietary S and Mo levels are high.

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453

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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

¹Formaldehyde treated soybean meal, Frank Wright Trouw, Ashbourne, UK ²Mineral/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. ³SEM 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				SEM	Significance, <i>P</i> -value ¹		
	C-	C+	G-	G+		F	A	Int
Intake								
DM, kg/d	23.5	24.0	22.6	20.5	0.48	<0.001	0.111	0.012
Cu, mg/d	467	466	467	418	9.6	0.022	0.007	0.015
Mo, mg/d	27.4	190.2	33.5	157.5	2.69	<0.001	<0.001	<0.001
S, g/d	28.1	74.9	29.9	70.4	1.25	0.302	<0.001	0.013
Fe, g/d	4.30	5.42	6.43	5.17	0.121	<0.001	0.564	<0.001
Milk yield, kg/d	38.1	40.6	38.9	37.9	0.77	0.225	0.373	0.034
Fat, g/kg	37.8	36.6	38.2	37.4	1.37	0.656	0.475	0.889
Protein, g/kg	32.5	32.6	31.6	32.5	0.80	0.173	0.901	0.646
Lactose, g/kg	46.5	46.4	46.7	46.3	0.32	0.975	0.328	0.680
Fat yield, kg/d	1.43	1.43	1.47	1.39	0.059	0.944	0.484	0.468
Protein yield, kg/d	1.23	1.30	1.22	1.21	0.024	0.049	0.242	0.142
Lactose yield, kg/d	1.77	1.93	1.80	1.73	0.063	0.185	0.434	0.060
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

¹F= 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				SEM	Significance, <i>P</i> -value ¹		
	C-	C+	G-	G+		F	A	Int
Plasma Cu, $\mu\text{mol/L}$	13.3	13.7	14.3	13.7	0.51	0.340	0.889	0.332
Plasma Mo, $\mu\text{mol/L}$	0.33	0.50	0.27	0.50	0.029	0.271	<0.001	0.375
Plasma Fe, $\mu\text{mol/L}$	43.2	40.5	40.7	40.9	1.61	0.519	0.446	0.384
Plasma Mn, $\mu\text{mol/L}$	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

¹F= 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-

²Superoxide 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				SEM	Significance, <i>P</i> -value ¹		
	C-	C+	G-	G+		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

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

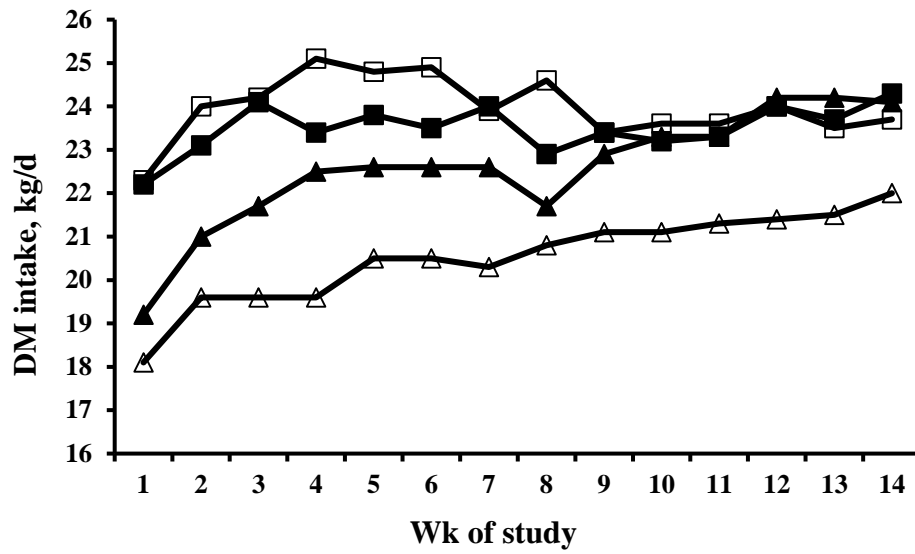


Figure 1. Weekly DM intake in early lactation dairy cows fed diets high in corn silage and fed without (■) or with (□) added S and Mo, or diets high in grass silage fed without (▲) or with (△) added S and Mo. Pooled SEM = 0.72. Forage, $P < 0.001$; Forage x Ant, $P = 0.012$; Time, $P < 0.001$; Forage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

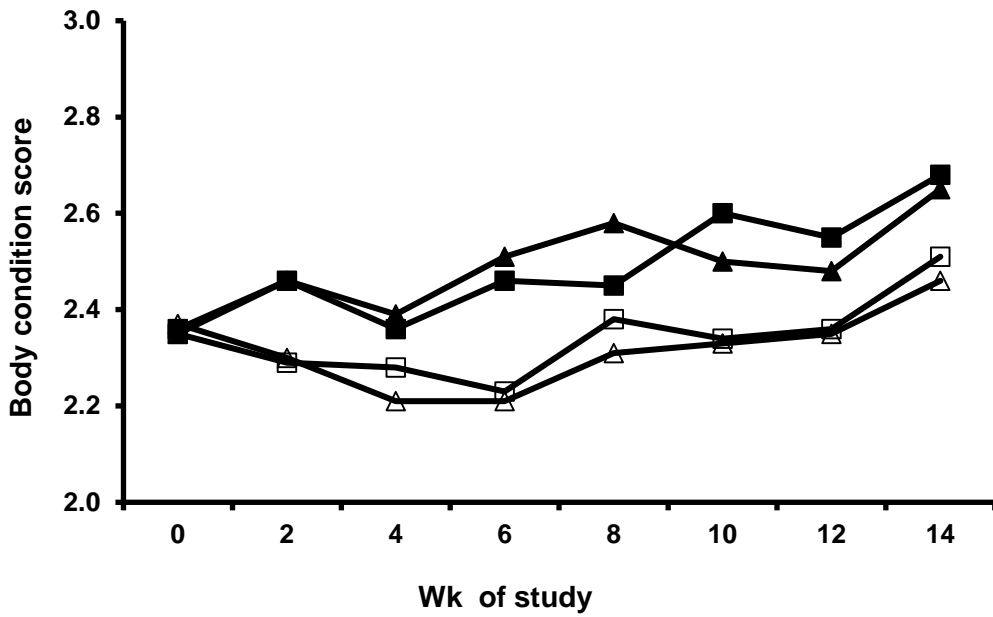


Figure 2. Fortnightly BCS in early lactation dairy cows fed diets high in corn silage and fed without (■) or with (□) added S and Mo, or diets high in grass silage fed without (▲) or with (△) added S and Mo. Pooled SEM = 0.067. Ant, $P < 0.001$; Time, $P < 0.001$; Time x ant, $P = 0.077$.

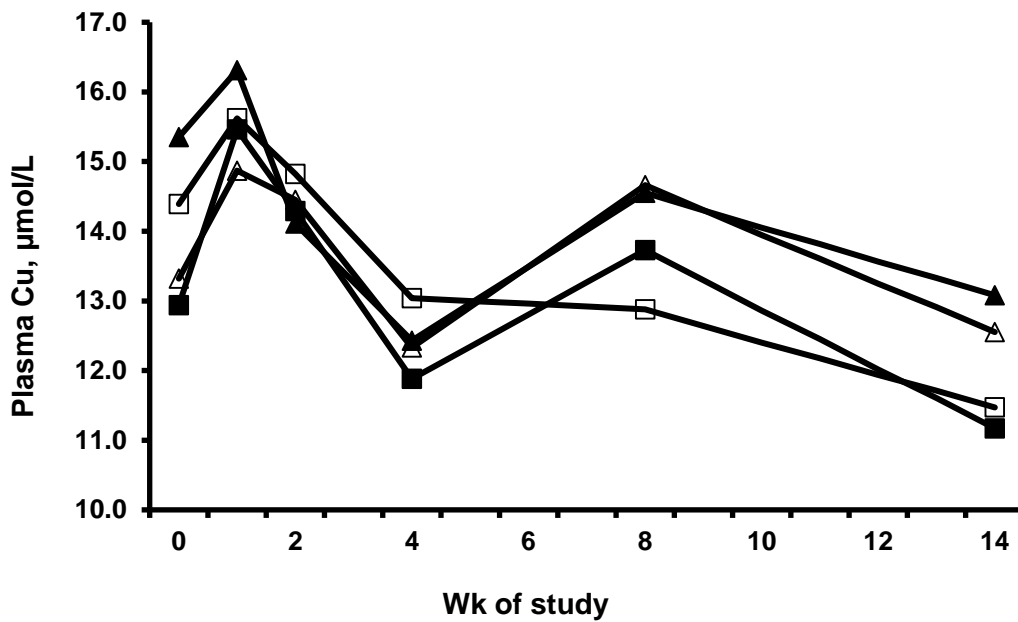


Figure 3. Plasma Cu concentrations in early lactation dairy cows fed diets high in corn silage and fed without (■) or with (□) added S and Mo, or diets high in grass silage fed without (▲) or with (△) added S and Mo. Pooled SEM = 0.87. Time, $P < 0.001$.