Evaluation of liver and fur copper concentrations in dairy cows

- in relation to dietary intake of copper and molybdenum

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Utvärdering av lever- och pälskopparkoncentrationer hos mjölkkor – i relation till utfodring av koppar och molybden

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Sammanfattning

Koppar (Cu) har en avgörande roll i flera viktiga metaboliska funktioner och är en essentiell mineral för idisslare. Kopparbrist kan resultera i ett flertal problem som påverkar djurets hälsa och produktion, men även överutfodring av Cu kan resultera i allvarliga kliniska symptom på grund av ackumulering av Cu i levern. Utöver detta är kopparmetabolismen hos idisslare mer komplex än hos enkelmagade djur. I våmmen sker interaktioner som minskar tillgängligheten av Cu. Den huvudsakliga antagonisten är mineralen molybden (Mo) som tillsammans med svavel (S) binder till Cu och skapar olösliga komplex. Det finns ett starkt samband mellan Mo i jorden och Mo i växter. I områden med höga halter av Mo i jorden resulterar detta även i foder med höga koncentrationer av Mo. Detta kompenseras ofta med extra utfodring av Cu, vilket gör det viktigt med övervakning av kopparstatus i besättningarna. Den huvudsakliga metoden för att övervaka kopparstatus hos besättningar idag är genom leverprover, antingen biopsier eller prover tagna i samband med slakt. Syftet med den här masteruppsatsen var att utvärdera kopparstatus i relation till intag av Cu och Mo i foder hos mjölkkobesättningar runt Falköping i Sverige där områden med höga koncentrationer av Mo i jorden finns. Det var även meningen att utvärdera om prover på päls är en lämplig metod för att övervaka kopparstatus, då provtagning av lever är komplext och kan vara ekonomiskt kostsamt.

Tio mjölkgårdar valdes ut baserat på koncentration av Mo i deras egenodlade grovfoder. Fem gårdar med molybdenkoncentration $\leq$ 1.2 mg/kg TS (LOW) och fem gårdar med molybdenkoncentration $\geq$ 5 mg/kg TS (HIGH). På varje gård togs pälsprover från fem slumpmässigt utvalda lakterande kor och leverprover togs från de upp till sex första korna som skickades till slakt efter gårdsbesöket. Alla prover skickades till ett laboratorium för analys av kopparkoncentration. Utöver det så mättes bröstomfånget på alla djur som provtogs för päls och en uppskattning av dagligt intag av alla foderkomponenter samt foderanalyser gjordes för att kunna göra en så kallad endagars utfodringskontroll, en kontroll som utvärderar mjölkkornas näringstillförsel. Resultaten analyserades sedan statistisk för korrelationer mellan relevanta variabler och variationer inom och mellan besättningar.

Resultaten visade att en majoritet av leverproverna hade kopparkoncentrationer under toxiska nivåer och ingen visade nivåer som skulle indikera kopparbrist. Det tyder på att tillskottsutfodringen av Cu var tillräcklig. Förhållandet mellan Cu och Mo (Cu:Mo) varierade dock kraftigt mellan 1.4-31.8:1 och Mo hade en tydligt minskande effekt på kopparkoncentrationen i både lever och päls. Intag av Cu i foder visade ingen effekt på kopparkoncentrationen i levern och på kopparkoncentrationen i päls hade intag av Cu i fodret en negativ effekt. Leverproverna visade stor variation i kopparinnehåll inom besättning medan det var en liten variation inom besättning för kopparinnehållet i päls. Det tyder på att om fortsatta studier kan bekräfta att kopparkoncentrationen i päls har ett samband med kopparstatus, kan prover från ett fåtal individer representera en hel besättning.

Nyckelord: idisslare, koppar, kopparstatus, lever, mjölkko, molybden, päls

Abstract

With its' vital roll in several important metabolic functions, copper (Cu) is an essential mineral for ruminants. Deficiency can cause a variety of problems affecting the animals' health and production, but excess of Cu can also result in severe clinical symptoms because of its' accumulation in the liver. In addition to this, Cu metabolism is particularly complex in ruminants compared to monogastric animals. In the rumen interactions that decrease Cu availability occurs. The main antagonist is the mineral molybdenum (Mo) which toghether with sulphur (S) bind to Cu and form insoluble precipitates. There is a strong correlation between Mo in soil and Mo in crops. In geochemical areas high of Mo, this results in feed with high concentrations of Mo which need to be compensated with extra supplementat of dietary Cu, making monitoring of Cu status important. The main method for monitoring of Cu status today is liver samples, either biopsies or samples taken at slaughter. The aim of this master thesis was therefore to evaluate Cu status in relation to dietary intake of Cu and Mo of dairy herds located around Falköping, Sweden, where geochemical areas with high Mo in soil exists. And also to evaluate if fur samples is an adequate method to monitor Cu status, since taking liver samples are complex and can be costly.

Ten dairy farms where selected based on Mo concentration in homegrown roughage. Five farms with Mo concentration ≤ 1.2 mg/kg DM (LOW) and five farms with Mo concentrations ≥ 5 mg/kg DM (HIGH). On all farms, five random lactating animals were sampled for fur, however liver samples were taken from the first up to six animals sent to slaughter after farm visits. All samples were sent to a laboratory and analysed for Cu concentration. In addition, chest circumference was measured on the fur sampled animals and an estimation of daily feed intake including all feed components and feed analyses were made in order to do an one-day feeding control, a control to evaluate nutrional supply to a group of lactating dairy cows. The results were then analysed for correlations between relevant variables and variations within and between herds.

The results showed that majority of the liver samples had Cu below toxic levels and no one showed levels that indicated deficiency which suggest that supplement of Cu was sufficient. However the Cu:Mo ratio varied from 1.4-31.8:1 and there was a clear decreasing effect of dietary Mo on both Cu content in liver and fur. Dietary Cu showed no effect on liver Cu concentration and on fur Cu concentration dietary Cu had a decreasing effect. Liver samples showed a large variation within herd but there was a small variation in fur Cu content within herd. This indicate that if further studies can confirm fur Cu contents' correlation to Cu status, fur samples from only a few individuals can represent the whole herd.

Keywords: copper, copper status, dairy cow, fur, liver, molybdenum, ruminant

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Abbreviations

B-SFMS	Biota Sector Field Mass Spectrometry
Ca	Calcium
Co	Cobolt
Cu	Copper
CV	Covariance
DM	Dry Matter
DMT1	Divalent Metal Transporter 1
Fe	Iron
HIGH	Farms with >5 mg Mo/kg DM in their homegrown roughage
I	Iodine
ICP-AES	Inductively Coupled Plasma Atomic Emission Spectroscopy
ICP-SFMS	Inductively Coupled Plasma Sector Field Mass Spectrometry
ID	Identity number
K	Potassium
LOW	Farms with <1,2 mg Mo/kg DM in their homegrown roughage
Mg	Magnesium
Mn	Manganese
Mo	Molybdenum
Na	Sodium
NIR	Near Infrared Reflectance
P	Phosphorus
S	Sulfur
SD	Standard Deviation
Se	Selenium
SLU	Swedish University of Agricultural Sciences
SOC	Soil Organic Content
Zn	Zinc

1. Introduction

Copper (Cu) has a vital role in several enzyme systems, it is necessary for haemoglobin formation and for normal pigmentation of hair, among others. The metabolic functions of Cu makes it an essential mineral and deficiency of Cu can cause a variety of problems. Most deficiency symptoms in ruminants are observed among sheep but appear in cattle as well (López-Alonso & Miranda 2020). Symptoms include anemia, insufficient growth, infertility, depigmentation of fur and gastrointestinal disturbances, among others (McDonald et al. 2011). However, if Cu is overfed, it will accumulate in the liver (McDonald et al. 2011) because ruminants have a limited capacity to excrete redundant Cu (López-Alonso & Miranda 2020). Slowly this will cause tissue damage without clinical symptoms, making Cu a sort of cumulative poison. Cumulated Cu can then be released spontaneously or by stressors, causing destruction of red blood cells (haemolysis) (McDonald et al. 2011). In ruminants, interactions occur in the rumen between molybdenum (Mo), sulfur (S) and Cu, making Cu metabolism in ruminants particularly complex. In the rumen S and Mo form thiomolybdate that binds to Cu, making it insoluble. This interaction make ruminants particularily susceptible for Cu deficiency (López-Alonso & Miranda 2020). There is a correlation between Mo in soil and Mo in plant (Lätt 2019; Eriksson 2010). Additionaly, in some areas, for example around the high plateus in Falköping, Västra Götaland region in Sweden, Mo concentrations in soil are documented at high levels (Eriksson et al. 2017; Appendix 3). Recommendations for Cu:Mo ratio varies. Some says that ratios below 3:1 increases the risk of deficiency (Underwood & Suttle 1999) and others that ratios more than 6:1 are ideal (Puls 1994), with no studies conducted in Sweden as far as we know. In Falköping, feed advisors use a recommendation of Cu:Mo ratio at 4:1 (personal communication T. Lundborg, Växa). One sign of high intake of Mo is stiffed gait, and Mo is also thought to interfere with estrogen metabolism, causing impairment in fertility of heifers (Suttle 1991). A study on Danish farms showed toxic levels of Cu in liver with moderate dietary levels of Cu, although they had no information of dietary intake of Mo (Krüger & Kristensen 2020). Supplementation recommendations of Cu in Norfor (2011) were revised based on slightly decreased US recommendations from 2001 (National Research Council, 2001) to 2021 (NASEM 2021). From this and with information of the geochemistry in Falköping areas, evaluation of Cu status of dairy cows around Falköping was therefore of interest, to see if dietary Cu supplementation is adequate. The aim of this study was therefore to examine how supplementation of Cu in relation to high Mo concentrations in feed affect storage and secretion of Cu in dairy cattle around Falköping.

1.1 Objectives of this study

Objectives of this study was to examine effects of dietary Cu in relation to dietary Mo on dairy cows from farms with high or low molybdenum in homegrown feed around Falköping, and to answer the questions:

- How does Cu supplementation in relation to dietary levels of Mo affect levels of Cu in the liver, are correlation possible to observe?
- Is it possible to observe a correlation between concentration of Cu stored in fur and Cu and Mo concentrations in feed and is Cu concentration in fur an adequate method to monitor Cu status in a herd?
- Is the supplementation of Cu adequate on the farms in this study?

2. Litterature Brief

2.1 From soil to feed

Many trace elements have a significant correlation between concentration in the soil and concentration of the same element in the plant. For several elements there is a decreasing concentration in the crop when soil pH increase. Although, this is not the case for Mo which occurs as anions. The uptake of Mo in crops will therefore be highest at neutral pH in contrast to other elements that occurs as cations (Eriksson et al. 2017). Concentrations of trace elements in the soil are also affected by provision of the element from air pollution and manure, other input means and partly by abduction through crops and leaching. Cu and Mo concentration in crops (wheat, barley and oats) are positively correlated to Cu and Mo concentrations in soil. Also zinc (Zn) concentration in crops has a positive correlation to Zn concentration in soil, although not as strong as Cu and Mo. A majority of the S in the soil is found in the organic matter (Eriksson 2010). In both Norway and Sweden Cu concentration in soil has shown to correlate positively with clay content (Govasmark et al. 2005; Eriksson et al. 2017). In Sweden Zn concentration also showed to correlate with clay content, further both Cu and Zn concentration showed a weak correlation to soil organic carbon (SOC) content (Eriksson et al. 2017). Concentration of Mo was on the other hand independent of both clay and SOC content (Eriksson et al. 2017). In first cut herbage in Norway, Cu concentration was also positively correlated to organic matter content in the soil and negatively correlated to herbage yield. In contrast, in second cut herbage Cu content was positively correlated to herbage yield (Govasmark et al. 2005). Both Cu and Mo concentration in herbage was higher in the second cut which could be due to higher percentage of clover, earlier harvesting could also affect Cu concentration to rise. Soil pH was positively correlated to Mo in herbage and the factor that contributes most to variation of Mo concentration in herbage. In the first cut, Mo in herbage was positively correlated with soil organic matter and negatively with dry matter (DM) yield. Most samples in their study had a lower Cu:Mo ratio in the second cut which probably is because Mo increases a bit more than Cu (Govasmark et al. 2005). Suttle (1991) meant that to improve animal health one of the most successful applications of geochemistry is analysis of soils for Mo.

When farmers convert from conventional to organic farming the soil pH tend to rise which favours Mo concentration in the soils (Govasmark et al. 2005). In Västra Götaland some soils are influenced by accumulation of alum shale originating from plateau mountains, causing an increase of Mo in the soil (Eriksson et al. 2017).

Regionally, low crop concentrations of essential trace elements are probably more important for intensive animal production than for the human diet because of our geographically diverse origin of food. In areas of Sweden, mineral supplementation occur in intensive animal production irrespectively of the trace mineral concentration in homegrown feed (Eriksson et al. 2017).

2.2 Copper Metabolism

The process of Cu absorption is similar across animal species. Dietary Cu needs to be in its' most reactive state (Cu^{+}) to be absorbed. Therefore Cu present as Cu^{2+} needs to be reduced into Cu^{+}. The Cu specific transporter (Ctr1) is present in the intestinal microvillies and responsible for 70% of Cu uptake into the enterocyte. This leaves remaining Cu to be attached by the non-specific transporter divalent metal transporter 1 (DMT1). The main competitors with Cu for the non-specific transporter DMT1 is iron (Fe) and Zn (Clarkson et al. 2020; López-Alonso & Miranda 2020). Inside the enterocyte Cu chaperone proteins bind Cu for transport to other specific proteins or enzymes for incorporation. Copper in excess of cellular requirements enter the secretory pathway to bind to metallothionein in the golgi (Clarkson et al. 2020; López-Alonso & Miranda 2020). When bound to metallothionein Cu is stored in the lysosomes, and the cell is then protected from free Cu (López-Alonso & Miranda 2020). When metallothionein reaches saturation capacity in the lysosome, Cu will efflux the enterocyte into the blood circulation (Clarkson et al. 2020).

When leaving the enterocyte Cu is bound to albumin, an abundant plasma protein which accounts for 15-20% of Cu transport. Transcuprein, a specific Cu carrier protein, carry 10-30% of total transported Cu from the small intestine to the blood. Albumin and transcuprein transport Cu through the systemic circulation from the intestines to the liver. The mechanism when Cu arrives at the membrane of the hepatocyte is similar as when entering the enterocytes, Cu is reduced and transported by Ctr1. In the liver dietary Cu from the portal circulation is bound to newly synthesised, predominant Cu transporter caeruloplasmin. In ruminants 88% of total plasma Cu is present bound to caeruloplasmin which distributes Cu to other tissues (Clarkson et al. 2020).

By regulating secretion of excess Cu into the bile, the liver is the major regulator of Cu. The main difference between enterocytes and hepatocytes is the uniqe chaperone ATP7B in the secretory pathway of the Golgi (Clarkson et al. 2020). ATP7B directs Cu to be incorporated to caeruloplasmin which then enters the circulation to distribute Cu to other tissues. When caeruloplasmin-bound Cu from peripheral tissues returns to the liver, the whole molecule is absorbed to be destructed and effluxed through the biliary route (Clarkson et al. 2020).

2.2.1 Limitations in ruminant copper metabolism

Ruminants unique digestive system affects their Cu metabolism. In the rumen an interaction between Cu, Mo and S occur (Suttle 1991). This interaction results in a much lower absorption of Cu in the intestinal tract, although the range varies greatly depending on the relative presence of the antagonists Mo and S. When there is high concentreations of these Cu antagonists, Cu availability is very low (López-Alonso & Miranda 2020). In the rumen S och Mo react together and form thiomolybdates, which strongly bind Cu to form Cu-thiomolybdates. These precipitates are insoluble and will therefore greatly decrease Cu availability in the intestine (Clarkson et al. 2020). Inorganic and organic S has similar effects in the S-Mo-Cu antagonism, as both are degraded to S^{2-} (Suttle 1975). If there is no available Cu, the thiomolybdates will be absorbed either through the rumen wall or the small intestine (López-Alonso & Miranda 2020), into the systematic circulation and can pass through the cell membrane. Inside the cell they can disrupt Cu transport by binding Cu bound to chaperones, transporters and enzymes. The thiomolybdates have no effect on other trace metals such as Fe, Zn or Cd although they have similar properties as Cu (Clarkson et al. 2020). Further, thiomolybdates can deplete body reserves of Cu in the liver by enhancing Cu excretion through increased biliary Cu concentration (Suttle 1991; Gooneratne et al. 1994). This could be explained by thiomolybdates removing Cu from the lysosomes and cytosol of hepatocytes (Suttle 1991).

Most non-ruminant species can tolerate high levels of dietary Cu, because a predominant part of the Cu is bound to metallothionein (López-Alonso & Miranda 2020). Cattle on the other hand, have a lower capacity to store Cu bound to metallothionein compared to single-stomached animals. Metallothionein acts as a storage buffer that protects the cell from free Cu (López-Alonso & Miranda 2020). Cattle and sheep have less than half the amount of metallothionein as other non-ruminants like pigs and dogs (Clarkson et al. 2020). If there is an influx of Cu that exceeds the metallothionein and lysosomal capacity, unbound Cu will appear in the cytosol and cause severe cell damage by entering the nucleus (Clarkson et al. 2020). This can cause major changes in liver structure and function, and limit the ability to make new red blood cells to replace destroyed ones (haemolytic crisis) (López-

Alonso & Miranda 2020). There seems to be a breed difference in Cu-tolerance where Holstein and Angus are more Cu-tolerant than Jersey, Charolais and Simmental (Clarkson et al. 2020).

The microbes in the rumen metabolize both organic and inorganic S and thus producing sulphide. By forming insoluble Cu-sulphide, S can reduce Cu bioavailability further. Iron can also affect Cu absorption negatively. With S and Cu, Fe reacts and form non-absorbable Fe-Cu-S complex (López-Alonso & Miranda 2020).

2.2.2 Distribution of copper in the liver

50-60% of the body Cu content is found in the liver (Miranda et al. 2010) and the distribution of Cu varies between sites in the liver (Puls 1994). In beef calves of Holstein Friesian and Galician Blonds, concentration of Cu in the liver was highest in the left lobe, followed by the processus papillaris and lowest in the quadrate and caudate lobes (Miranda et al. 2010). This in contrast to an other study where concentration of Cu was highest in the caudate lobe (Braselton et al. 1997). Possible explanations could be factors such as age, diet, sex or genetic factors and liver Cu concentrations were generally high in the study by Braselton et al. (1997). A difference in total hepatic Cu levels between the breeds Holstein Friesian (175 mg/kg in caudate lobe) and Galician Blonds (140 mg/kg in caudate lobe) could be observed at all different sample sites, but no difference in distribution of Cu in the liver was observed between breeds (Miranda et al. 2010).

In cattle, most Cu is found in the caudate lobe, where distribution is 39% in mitochondria and lysosomes (large-granule fraction). 30% is found in the cytosol, 21% in the nucleus and only 10% is found in the microsomal fraction (endoplasmatic reticulum, golgi apparatus and ribosomes). In most other mammalian species Cu is mainly found in the cytosol and only 20% is found in the large-granule fraction. When total Cu concentration in the liver increases, the proportion of Cu in the large-granule fraction decrease and the nuclear fraction accumulate Cu faster (López-Alonso et al. 2005).

2.3 Dietary mineral recommendations

Cu is an essential mineral that functions in the activity of several enzymes (López-Alonso & Miranda 2020; Suttle 2022). Absence of Cu can cause a variety of deficiency symptoms with first symptom as change in fur structure, dullness in fur colour and loss of fur. Other symptoms of deficiency include anemia, anoxia (Suttle 2022), insufficient growth, infertility, and gastrointestinal disturbances among others (McDonald et al. 2011), making it important with a sufficient availability of dietary Cu. With the effect of Mo on Cu metabolism stated before (Suttle 1991; Clarkson et al. 2020; López-Alonso & Miranda 2020), supplementation of Mo is unnecessary (NASEM 2021) but maximum recommended levels exist (INRA 2019; Puls 1994). In table 1 a compilation of mineral recommendations for lactating cows can be found.

Table 1. Dietary mineral recommended and maximum tolerable level for copper (Cu), molybdenum (Mo), and sulphur (S), in g or mg/kg dry matter (DM) and Cu:Mo ratio for lactating cows of dairy breeds.

Reference	INRA 2019	NASEM 2021	Suttle 2022	Puls 1994
Cu mg/kg DM	10	8-10[1]	>6.0	10-25
Max. Cu mg/kg DM	40	20-40	50-300[2]	80-100[3] 200-800[4,5]
Mo mg/kg DM	*	*	*	*
Max. Mo mg/kg DM	5	*	*	>10
S g/kg DM	2	2	0,8-1,5[1]	2.1-3.6
Max. S g/kg DM	3.0 high conc 5.0 forage	5.0	3.0-4.0	4, 33[5] (>5, 270[6])
Cu:Mo	*	*	>2:1/30-60:1[2]	>6:1

** No recommendation; [1] Depending on breed, milk yield, body weight, gestation stage and growth; [2]values below rule out cronic copper poisoning values over strongly suggest cronic copper poisoning; [3] Maximum safe tolerable level; [4] Acute toxic dose; [5] mg/kg body weight; [6]total in feed ration/day; ()=fatal dose*

2.4 Monitoring copper status

2.4.1 Liver samples

Concentration of Cu in liver samples reflect dietary intake of Cu (Grace et al. 2010) and Cu status is reflected best in liver tissue (Combs et al. 1982; INRA 2019). Liver biopsies can be taken at any time and are possible to follow up (Grace et al. 2010) which makes it the best sampling method for liver tissues (Puls 1994). However, a study from New Zeeland suggested that also liver samples taken at slaughter houses could be used to categorise Cu status in a herd (Grace et al. 2010). In the study by Grace et al. (2010), they compared variation of Cu concentration in liver samples between 10 farms with samples from 12 cows on each farm (both biopsies and samples taken at slaughter). There was a large variation within herd (covariance (CV) 50%) but there was no major difference in Cu concentration between biopsy and slaughter samples (Grace et al. 2010). Samples from slaughter houses are also suggested by EFSA as a way to monitor redundant Cu accumulation in cattle (EFSA FEEDAP 2016). At a dietary intake of Cu at 10.3 ± 0.1 mg/kg DM, Mo 1.0 ± 0.1 mg/kg DM and S 2.16 ± 0.07 g/kg DM in lactating cows mean concentration of Cu in the caudate liver lobes were 388 mg/kg DM (Buckley 1991). Puls (1994) set the limits of 0.5-10 mg Cu/kg in liver as deficient, 5.0-25 mg/kg as marginal, 25-199 mg/kg as adequate, 200-550 mg/kg as high and 250-800 mg/kg as toxic. López-Alonso & Miranda (2020) mean that 150 mg Cu/kg is genereally accepted as an indication of toxic levels of Cu in the liver. In NASEM (2021) the indicational limits for Cu concentration in the liver are <10 mg/kg DM for deficiency, <35 mg/kg DM suboptimal, and 300-350 mg/kg DM toxicity. From INRA (2019) the values for Cu concentration in the liver are limited to 3.7-11 mg/kg DM as deficiency and 230-580 mg/kg DM as excess. Suttle (2022) has a marginal range of 6.4-19.2 mg/kg DM for Cu concentrations in the liver. Values within that marginal range indicate an increased probability for hypocuprosis and values over do not. With the number of individuals within the marginal range increasing in a herd, the likelihood of the herd to benefit from Cu supplementation also increses. Suttle (2022) also gives a marginal range of 406-1014 mg Cu/kg DM in the liver where values below rule out cronic copper poisoning and values over strongly suggest cronic copper poisoning. In studies on liver DM and liver lipids it is shown that liver DM is around 30% (Rosendo & McDowell 2003; Tajik et al. 2012). A summary of Cu concentrations in liver samples where litterature that refers in mg/kg DM are calculated to mg/kg with liver DM set at 30% can be found in table 2.

In a herd of approximately 250 Jersey cows, sudden death of six cows in connection to dry off evoke attention (Johnston et al. 2014). At examination of the carcasses high liver Cu concentrations was found, which led to examination of ten random living cows from the herd by liver biopsies. High liver concentrations (mean 2620 µmol/kg) were found and 0.5 g of ammonium molybdate and 1 g sodium sulfate/cow/day was administered by drenching, for five days. This gave an average reduction in liver Cu by 37.3% in these cows (Johnston et al. 2014). In addition McCaughern et al. (2020) found that when increasing dietary S from 2.2 g/kg DM to 3.0 g /kg DM and dietary Mo from 1.1 mg/kg DM to 5.5 mg/kg DM in a diet with 15 mg Cu/kg DM, in Holstein-Friesan dairy cows, liver Cu concentration decreased. When giving the cows extra S and Mo they also observed a decrease in DM intake of 1.8 kg DM/day (McCaughern et al. 2020).

2.4.2 Fur samples

Growing fur is metabolically active and could reflect concentration of minerals in the hair follicle when growth occurred (Combs et al. 1982). During growth of hair keratin is formed in the millimetres closest under the skin. When this keratin structure is established the hair is metabolically inactive (Schwertl et al. 2003). The growth rate of fur differs depending on body location. Tail hair has the highest growth rate and growth rate on hair from the hip and shoulder areas on cows are similar to each other (Burnett et al. 2014). For tail switch hair collected with scissors from Limousin, Aberdeen Angus, Simmental or crossbreed of these cattle Schwertl et al. (2003) calculated the growth rate to be 0.6-1.06 mm/d. Burnett et al. (2014) calculated it to be 0.51 ± 0.05 mm/d on scissor cut tail switch hair from Holstein cattle. For the hip and shoulder areas the calculated growth rates are 0.04 ± 0.05 and 0.03 ± 0.05 mm/d respectively (Burnett et al. 2014).

Analysing mineral status is usually a costly and complex issue which makes fur with its characteristics an interesting alternative to more invasive sampling methods (Combs 1987). There will be a stubble left when cutting fur and not plucking (Schwertl et al. 2003) and interpretive limits for fur samples are that samples need to be clean and growth need to be recent (Suttle 2022). Advantages on the other hand, include easy and non invasive sampling and also easy storage without quality decline (Kellaway et al. 1978; Combs et al. 1982).

There is no correlation between color of cattle fur and Cu content in the fur (Cunningham & Hogan 1958) in contrast with for example selenium (Se) where a correlation have been observed with fur color of Holstein cows (Christodoulopoulos et al. 2003). Cu in fur on the other hand, have a correlation with Cu in liver (Combs 1987) and in a study on mule deer a correlation between Cu serum and Cu in fur could be observed (Roug et al. 2015). The correlations were strongest during growth of winter coat or closely after (Roug et al. 2015) which agrees with the hair follicles being active during growth (Combs et al. 1982). Unfortunatley correlations in their study were not sufficient to suggest fur samples as a reliable source for monitoring Cu status (Roug et al. 2015). To get a result as reliable as possible it is suitable to collect fur samples in relation to its' growth periods (Combs et al. 1982). Cu content in fur is markedly affected by dietary intake of Mo and S. By adding Mo and S in feed, Cu content of fur decreases (Cunningham & Hogan 1958; Kellaway et al. 1978). Fur Cu content decreased from 6.7 mg/kg to 1.4 mg/kg when going from a diet with 4.1 mg Cu/kg DM, 1.1 g S/kg DM and 0.3 mg Mo/kg DM to a diet with 3.7 mg Cu/kg DM, 3.8 g S/kg DM and 2.7 mg Mo/kg DM. When going from the first mentioned diet of Cu, S and Mo to a diet with 4.0 mg Cu/kg DM, 7.0 g S/kg DM and 5.7 mg Mo/kg DM, Cu content in fur decreased from 6.4 to 1.0 mg/kg DM. When injected with Cu glycinate, fur levels of Cu increased for 14-28 days but after that showed evidence of a new decline (Kellaway et al. 1978). Kellaway et al. (1978) suggested fur samples as a reasonable tool for diagnosis of sub-clinical Cu deficiency after their observings that the levels of Cu in hair was reduced in response to reduced Cu availability. Cunningham & Hogan (1958) could also observe that the decrease in Cu concentration caused by dietary Mo and S was prevented if Cu was added at the same time. In the study by Kellaway et al. (1978) they also took liver biopsies that showed the same trend of decreasing Cu concentration in relation to the diets with higher concentration of Mo and S. In addition they observed a correlation between liver Cu content and fur Cu content, and suggest that when Cu levels in fur is below 4 mg/kg then Cu levels in liver is below 20 mg/kg which is a minimum level to avoid clinical symptoms of Cu deficiency (Kellaway et al. 1978). Cunningham & Hogan 1958 observed an average of Cu content in fur on cattle on pasture to be 9 mg/kg. From Puls (1994) the limits of Cu content in fur is deficient at 1.0-6.7 mg/kg DM, marginal at 4.3-8.3 mg/kg DM and adequate at 6.7-32 mg/kg DM. However, Puls (1994) states that Cu levels in fur only reflects dietary intake when Cu liver levels are deficient. From INRA (2019) limits on Cu concentration in fur are <4.0-8.0 mg/kg as deficiency, and >30 mg/kg as excess. For Cu concentration in fur Suttle (2022) has a marginal band of 4.0-8.0 mg/kg with the same prerequsisites as for Cu liver concentrations. Together with Cu concentrations limits of liver samples a compilation of Cu concentration limits for fur can be found in table 2.

Table 2. Compilation of cut off points for Cu concentration in liver in mg/kgand fur tissue in mg/kg or mg/kg dry matter (DM).

	Deficient	Adequate	Toxic[1]/excess [2]	Reference
Liver				
	0,5-10	25-199	200[7] 250-800[1]	Puls 1994
	<3[4]	<11[4,5]	90-105[1,4]	NASEM 2021
	1-3[4]		69-174[2,4]	INRA 2019
	2-6[4]	122-304[3,4]		Suttle 2022
	< 20			Kellaway et al. 1978
Fur				
	1.0-6.7[6]	6.7-32[6]		Puls 1994
	<4.0-8.0		>30[2]	INRA 2019
	4.0-8.0	>8.0		Suttle 2022
	< 4			Kellaway et al. 1978

[1] *Reference use the term toxic.;* [2] *Reference use the term excess.;* [3] *Values below rule out cronic copper poisoning values over strongly suggest cronic copper poisoning.;* [4] *Calculated from dry matter (DM) that the litterature originally states with a liver DM set at 30%* (Rosendo & McDowell 2003; Tajik et al. 2012) *and rounded to integer.;* [5] *Suboptimal;* [6] *Expressed in DM.;* [7]*High but not toxic*

3. Material and methods

To study the effect of Cu and Mo intake on Cu concentrations in fur and liver, ten dairy farms in Skaraborg were selected based on earlier knowledge from a cattle feed advisor on molybdenum concentrations in homegrown roughage. Five farms were selected for low Mo concentrations in their homegrown roughage ≤ 1.2 mg/kg DM (LOW) and five farms for high Mo concentrations ≥ 5 mg/kg DM (HIGH). In table 3 information about production system for each farm can be found and what group they belonged to. For the organic farms the herd size, which here only includes lactating cows, was 50-150. For conventional farms it differed between 110-320. On each farm five lactating cows were sampled for fur and at the slaughter house liver samples were collected from up to six other lactating cows. A majority of the cows in this study was of Swedish Holstein breed, but other breeds included was Swedish Red, Swedish Polled Cattle, Swedish Ayshire, Swedish Jersey and crosses of those breeds. Feed analyses including 14 mineralswere obtained on all feedstuffs for lactating cows on every farm.

Table 3. Information on farms in the present study

Farm	Production system	Molybdenum
1	Conventional	LOW
2	Conventional	LOW
3	Conventional	LOW
4	Conventional	LOW
5	Conventional	LOW
6	Conventional	HIGH
7	Organic	HIGH
8	Organic	HIGH
9	Conventional	HIGH
10	Organic	HIGH

LOW = ≤ 1 mg/kg DM in roughage HIGH=≥ 5 mg/kg DM in roughage

In prior to farm visits ten lactating cows with a maximum limit of 250 days in milk were randomly selected on each farm. This, to ensure that they were in lactation at the day of farm visit. For the selection, lists of all lactating cows on each farm were provided by Växa Sverige. From these lists lactating cows of 1-250 days in milk were selected with the Microsoft Excel function =RANDBETWEEN. Ten animals were selected to ensure that five animals were available for sampling at the day of the farm visit, in case of an animal being culled, dried off or sold before sampling day. Five of the selected animals were sampled for fur. The lactation number for those cows are shown in table 4. Chest circumference was measured to estimate body weight in kg. The equation used for weight estimation was 239.2-(4.4*cm)+(0.031*cm^2) (Pönniäinen 1989), for Swedish red, Swedish Jersey, Swedish Ayshire or crosses with these breeds. For Holstein and crosses including Holstein the equation 167.5–(3.7682*cm)+(0.0295*cm^2) (Coburn 2000) was used. A mean weight of these five animals was then calculated. During sampling, the animals were fixed in feeding fronts or with a rope behind them in a cubicle. Samples were taken at the end of January 2023.

Table 4. Cross table showing how many cows in each laction (from 1-6) that were sampled for fur on each farm.

Lactation No.	**1**	**2**	**3**	**4**	**5**	**6**
Farm						
1	3	1	0	0	0	1
2	0	2	2	1	0	0
3	1	3	0	1	0	0
4	1	1	1	2	0	0
5	2	1	1	1	0	0
6	1	1	2	0	1	0
7	2	0	3	0	0	0
8	1	2	1	0	0	1
9	1	0	3	0	0	1
10	3	1	1	0	0	0

For practical reasons, personell at slaughter houses were asked to take liver samples from the first five animals sent to slaughter after the farm visit. Therefore, they are not from the same animals as the ones sampled and measured on farm. The lactation number for the sampled cows are shown in table 5. The samples were taken at three different slaughter houses during February to April 2023.

3.1 Feed

Several farms had analyses on their roughage, but if not or if extended mineral analysis including Mo were missing, new samples were taken. Roughage was sampled with a silage drill either from a bunker silo or wrapped big bale. On all farms, grain was sent for analysis of nutrient and mineral concentrations. Grain samples were taken either from a rolled mix or directly from silos. Samples were sent to Eurofins (Kristianstad, Sweden) and roughage was analysed with near infrared reflectance (NIR) for full nutrional composition and the minerals phosphorus (P), calcium (Ca), manganese (Mn), magnesium (Mg), Cu, Zn, Fe, potassium (K), sodium (Na), S, Se, iodine (I), Mo and cobolt (Co) were analysed with inductively coupled plasma atomic emission spectroscopy (ICP-AES). Grain samples were analysed with chemical methods for nutrional composition and with

ICP-AES for minerals. Composition of compound feed and mineral feed were provided by the feed manufacturers.

All roughages was analyzed for DM the same day as the farm visit, or stored in freezer and analysed within two days. Approximately 100 g of sample was dried at 90°C for one hour and the remaining weight was assumed to be the DM. Samples were taken with a silage drill exept on two of the farms. On one farm, the farmer mixed silage so a sample could be collected manually. On the other farm, using the drill was not possible and samples were obtained by hand, picking roughage from different locations in the bunker silo.

A one-day feeding control was performed. The farmers weighed or estimated all roughages and concentrates that were offered to the lactating dairy cows during a day and also weighed or estimated the refusals. The number of lactating cows and the daily milk production of the group was registered. This information and the calculated mean weight of the cows was used in the programme Typfoder (Växa Sverige; NorFor Plan 1.24.0.690, FST version 2.09, FRC version 2.13, OFC version 1.41) to evaluate the daily diet to the cows. No manual correction between primiparous and multiparous cows was done for feed intake. For dry cows and pregnant heifers a fill value balance at 100% was aspired. Because our DM results and the results on DM from the laboratory differed greatly on several farms, we decided to use the results from the laboratory on all farm calculations because it affected the reults on fill balance in Typfoder greatly. The main focus in this study was on dietary supply of Cu and Mo and the estimation of the animals' Cu requirement.

3.2 Fur

Fur was sampled from the left dorsal side of the cow, from the caudal side of the shoulder blade back to the hip joint. On all farms except one, the area of sampling was brushed to remove excess dirt and environmental contaminants. To minimise hygiene risks, the farms' own electronic clippers with a blade variety of 3-10 mm were used to the greatest extent. If clippers were not available or if cows were too short haired, a brought electronic clipper with a blade of 1.9 mm was used. Samples were frozen at -19°C for 1-9 days and then brought to Swedish University of Agricultural Sciences (SLU), Ultuna. To minimise contamination further, samples were rinsed in warm water twice and then soaked in acetone for at least 12 minutes. Acetone was poured out and the fur was spread out on a white paper and allowed to dry over night. When dry, the fur was collected in 50 ml test tubes. Any remaining hair was collected in plastic zip lock bags and saved. All samples were stored in -20°C pending analysis. The fur samples were marked with a serial number and sent to ALS Global (Luleå, Sweden) for analysis of copper concentration. The analysis was done by determination of metals in biota according to SS-EN ISO 17294-2:2016, US EPA Method 200.8:1994, biota sector field mass spectrometry (B-SFMS). Prior to analysis the sample was digested according to B-VKSPEC (ALS Global). There was a measurement uncertainty of 14% meaning all sample reults are ±14%.

3.3 Liver

Liver samples were taken in connection with slaughter by personell at the slaughter house. Three different slaughter houses were used by the farms in this study. Before slaughter the farmers provided us information regarding which cows where going to be slaughtered and what day. This information was then passed to the contact person at the slaughter house so they could be prepared. At the transport day, a notification that samples was going to be taken were made on the information paper from that farm, and which cows it applied to. The driver was also verbally informed about this to pass the information forward to personell at slaughter house arrival. Employees on these slaughter houses were asked to take liver samples, approximately 5 x 5 cm from the caudate lobe (Gerspach et al. 2017) in concern of the variation of copper distribution in the liver (Puls 1988). The samples were marked with farm SE-number and cow's identity number (ID) and stored in a freezer at the slaughterhouse until all samples were obtained. Samples were then picked up by an employee from Växa Sverige and brought to SLU, Ultuna. On SLU the samples were marked with a serial number and sent to ALS Global (Luleå, Sweden) for analysis of copper by B-SFMS. There was a measurement uncertainty of 14% meaning that all samples results are ±14%.

No samples could be obtained from farm 6, but farm 6 had participated in an earlier study where liver samples were obtained in August 2022. The analytical results from that study was obtained from SVA (Uppsala, Sweden) who also used ALS Global (Luleå, Sweden) and according to information received, the samples were digested according to SE-POP-0128 in prior to analysis. Thereafter determination of Cu concentration in the liver was analysed by inductively coupled plasma sector field mass spectrometry (ICP-SFMF) according to SS-EN ISO 17294-2:2016 and US EPA method 200.8:1994. The location of sampling in the liver in that study was unknown.

Table 5. Cross table showing how many cows in each laction (from 1-7) that were sampled for liver on each farm.

Lactation No.	1	2	3	4	5	6/7
Farm						
1	1	0	0	3	1	0
2	1	2	0	0	0	0
3	1	3	0	0	0	1
4	3	0	1	1	0	0
5	3	1	0	0	0	0
6	2	0	0	1	0	0
7	0	1	1	0	0	0
8	0	2	1	1	1	0
9	2	0	1	1	1	1
10	1	0	0	0	0	0

3.4 Statistical methods

Univariate linear regression models where used for statistical analyses on correlations between relevant variables. This was done in the statistical programme R (4.2.3). The basic formula code used in R;

lm(formula = ***Y*** *~* ***X****)*

where Y is the outcome variable and X is the predictor variable.

Analysis of to what extent distribution of variation in samples could be explained by herd inhesion was done in the statistical programme SAS (9.4), using a mixed model with one random factor. The basic formula code used in SAS;

Proc mixed;class ***Herd****;model* ***Y****=;random herd;run;*

Where the random factor was Herd and the outcome variable is Y.

To visualize simple correlations and associations, Microsoft Excel was used to make scatter plots. The excel function =TTEST (t-test) was used to investigate if there was a significant difference between groups of LOW farms and HIGH farms. For mean values the function =AVERAGE was used and the function =STDEV.S to calculate standard deviations (SD).

Level of significance was in all models set to $p<0.05$ and tendency at $p<0.1$.

4. Results

4.1 One-day feeding control

Farms in this study was chosen based on information on Mo concentration in their homegrown roughage from earlier years. When analysing the roughage used in this study some of the HIGH farms did not reach >5 mg Mo/kg DM in their homegrown roughage and some had bought roughage that decreased the total concentration of Mo in the roughage they fed to their cows (Table 6). In addition to this, the roughage of one LOW farm had more than 1.2 mg Mo/kg DM which was the limit set in this study (Table 6).

Table 6. Molybdenum (Mo) /kg dry matter (DM) of farm used roughage, if a farm used more than one batch of roughage a mean of those are presented. Total Mo/kg DM showing the mean Mo/kg DM when all feed components fed every day are included.

Farm	1	2	3	4	5	6	7	8	9	10
Roughage Mo/kg DM	0.7	0.37	1.25	0.9	0.8	4.8	9.4	8.0	2.7	7.4
Total Mo/kg DM	0.67	0.38	0.83	0.76	0.53	3.93	7.67	6.16	2.66	6.93

Feed intake and mineral content in the diets are presented for each farm in table 6 and on average for LOW and HIGH farms with p-values in Table 7. Dietary Mo was higher in HIGH farms than LOW (Table 8). Three of five farms had Mo intake over 5 mg/kg DM per day and the Cu:Mo ratio on all farms varied from 1.4:1 to 32:1 (Table 7). The HIGH farms had a higher percentage intake of roughage (Table 8) which was expected since three out of five farms was organic and all organic farms could be found in the HIGH group (Table 3). Positive correlations were found between Mo concentration in feed and organic farms ($p<0.001$; figure 1). For S concentration in feed and total S fed per day there was a significant negative correlation to organic farms ($p<0.001$ and <0.001 respectively; figure 2). In addition farms with higher dietary Mo generally had lower dietary S ($p<0.001$). A slightly higher intake of Cu in mg/kg DM was seen in the HIGH group ($p=0.05$) which was also expected because of extra supplementation of Cu in relation to higher Mo intake.

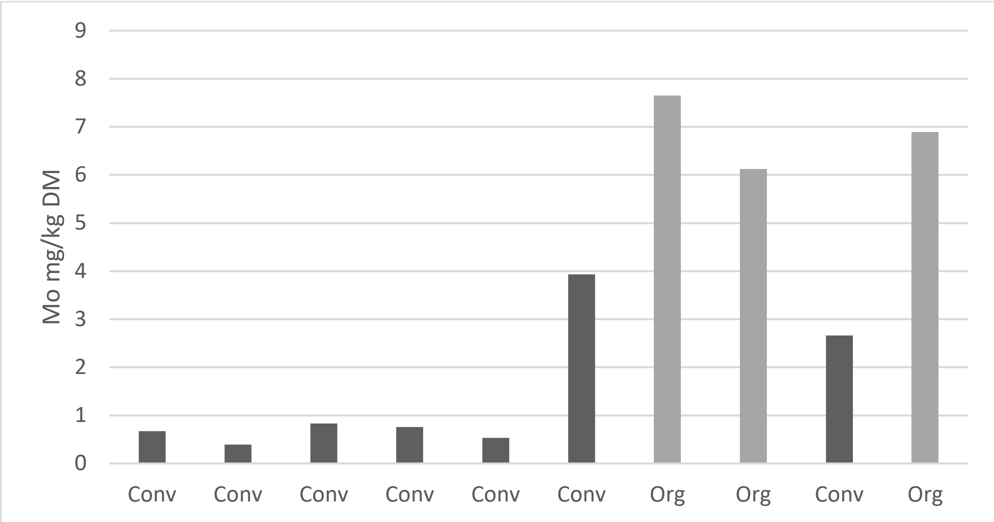

Figure 1. Molybdenum (Mo) concentration in total diet on herd level with explanations if the farm was organic (Org) or conventional (Conv). From the left, farms in order 1-10 with farm 1-5 with molybdenum (Mo) concentrations <1.2 mg/kg DM in their homegrown roughage (LOW) and farm 6-10 with Mo concentrations above >5 mg/kg DM in their homegrown roughage (HIGH). $p<0.001$.

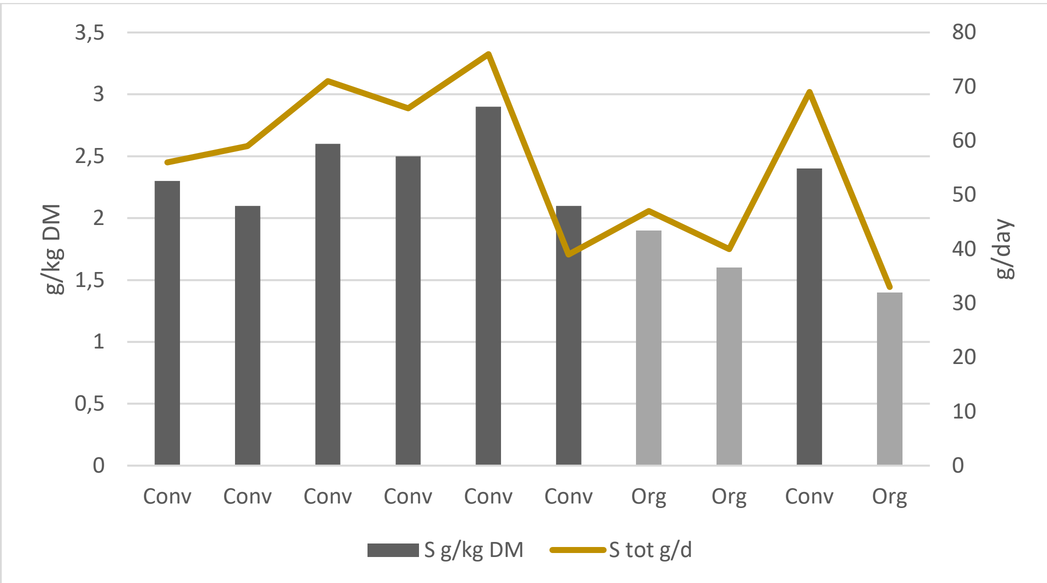

Figure 2. Dietary sulphur (S) both concentration in feed (g/kg dry matter (DM)) on herd level and total ration per day (tot g/d) and explanations if the farm was organic (Org) or conventional (Conv). From the left, farms in order 1-10 with farm 1-5 with molybdenum (Mo) concentrations <1.2 mg/kg DM in their homegrown roughage (LOW) and farm 6-10 with Mo concentrations above >5 mg/kg DM in their homegrown roughage (HIGH). $p<0.001$.

Table 7. Results from one-day feeding control on daily feed intake, intake and concentrations of copper (Cu), molybdenum (Mo), sulphur (S), iron (Fe) and zinc (Zn) ,and Cu:Mo ratio in each herd.

Farm	1	2	3	4	5	6	7	8	9	10
Feed intake, kg DM/day	24.6	27.5	27.1	26.6	26.2	18.4	24.7	25.2	28.1	24.1

Roughage %	54.7	59	58.8	48.1	50.4	61	63.4	55.7	61.2	72.2
Roughage kg DM	13.5	16.2	15.9	12.8	13.2	11.2	15.7	14	17.2	17.4
Concentrate kg DM	11.2	11.3	11.2	13.8	13	7.2	9.1	11.1	10.9	6.7
Cu mg/kg DM	13.2	12.4	16.4	9.8	11.3	15.7	10.9	18.5	16.5	9.8
Cu mg/day	325	340	444	261	296	289	270	465	463	238
Mo mg/kg DM	0.67	0.39	0.83	0.76	0.53	3.93	7.65	6.12	2.66	6.89
Mo mg/day	16	10	22	20	14	72	189	154	75	166
Cu:Mo	20	32	20	12.9	21	4	1.4	3	6.2	1.4
S g/kg DM	2.3	2.1	2.6	2.5	2.9	2.1	1.9	1.6	2.4	1.4
S g/day	56	59	71	66	76	39	47	40	69	33
Fe mg/kg DM	94.9	113.6	170.7	236.8	196	201.1	155.9	238.4	462.2	157.7
Fe mg/day	2336	3126	4621	6310	5129	3698	3856	6002	13003	3807
Zn mg/kg DM	64.1	55.7	85.1	60.5	50	77.7	48.5	45.8	77.7	40
Zn mg/day	1578	1532	2304	1613	1308	1428	1200	1154	2187	965

Table 8. Mean ($\bar{x}$) values on herd levels for farms with molybdenum (Mo) concentrations <1.2 mg/kg DM in their homegrown roughage (LOW) and farms with Mo concentrations above >5 mg/kg DM in their homegrown roughage (HIGH), standard deviations (SD) and p-values (p) for daily feed intake, the intake and concentration of copper (Cu), Mo, sulphur (S), iron (Fe) and zinc (Zn) and Cu:Mo ratio.

Farm	$\bar{x}$ LOW	SD	$\bar{x}$ HIGH	SD	p
Feed intake, kg DM/day	26.4	1.12	24.1	3.54	0.001
Roughage %	54.2	4.90	62.7	6.02	<0.001
Roughage kg DM/day	14.3	1.60	15.1	2,57	0.36
Concentrate kg DM/day	12.1	1.22	9	2.03	<0.001
Cu mg/kg DM	12.6	2.47	14.28	3.75	0.05
Cu mg/day	333	68.9	345	110	0.62
Mo mg/kg DM	0.6	0.18	5.45	2.09	<0.001
Mo mg/day	16	4.77	131	54.6	<0.001
Cu:Mo	21	6.81	3.2	2.01	<0.001
S g/kg DM	2.5	0.30	1.88	0.40	<0.001
S g/day	65	8.26	45.6	13.99	<0.001
Fe mg/kg DM	162	58.46	243	127	0.003
Fe mg/day	4304	1586	6073	3991	0.03
Zn mg/kg DM	63.1	13.4	57.94	18.3	0.22
Zn mg/day	1667	375	1386	477	0.015

Mo concentration increased with roughage proportion (p<0.001) which also total Mo fed per day did (p<0.001; figure 3). In contrast, both Mo concentration and total Mo fed per day decreased with total daily ration of concentrate (p<0.001 for both parameters). On organic farms both Mo concentration and total Mo fed per day was significantly higher (p<0.001 for both). Roughage proportion was also significantly higher on organic farms (p<0.001). The total daily ration of concentrate was lower on organic farms (p<0.001).

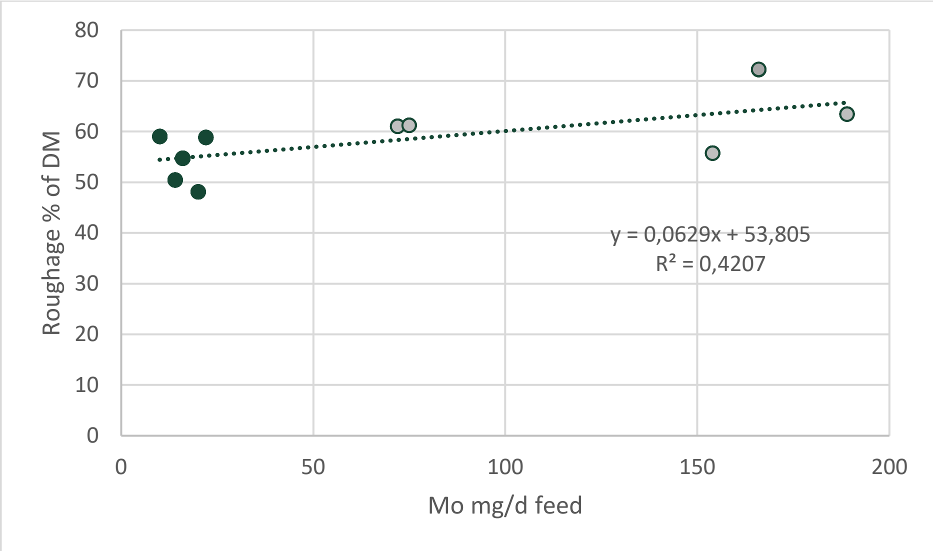

Figure 3. Correlation between total dietary molybdenum (Mo) feed per day and roughage share on herd level. Darker dots are farms with molybdenum (Mo) concentrations below 1.2 mg/kg dry matter (DM) in their homegrown roughage (LOW) and brighter plots are farms with Mo concentrations above 5 mg/kg DM in their homegrown roughage (HIGH). p<0.001.

4.2 Fur samples

In table 9 below, the results on Cu concentration in fur samples (mean values) from live cows are presented. In appendix 1 results on individual cows can be found. There was a significant difference between the groups of LOW and HIGH farms ($p<0.001$). Fur Cu was lower on organic farms than on conventional farms ($p=0.002$).

Table 9. Mean ($\bar{x}$) Copper (Cu) concentration in fur samples expressed as mg/kg and standard deviations (SD) for each farm and for groups of farms with molybdenum (Mo) concentrations below 1.2 mg/kg dry matter (DM) in their homegrown roughage (LOW) and farms with Mo concentrations above 5 mg/kg DM in their homegrown roughage (HIGH).

Farm	$\bar{x}$	SD
1	8.05	0.92
2	13.8	1.62
3	8.69	0.84
4	12.0	1.43
5	10.1	0.84
LOW	10.5	2.43
6	9.51	0.56
7	8.60	0.86
8	7.56	0.78
9	7.95	0.26
10	7.75	1.68
HIGH	8.27	1.14

Fur Cu concentration was negatively correlated to Mo concentration in feed ($p<0.001$) and total Mo fed per day ($p<0.001$) which are shown in figure 4 and 5. There was also a negative correlation between fur Cu concentrations and Cu concentration in feed ($p=0.011$). Between fur Cu concentration and total Cu fed per day there was a tendency to negative correlation ($p=0.059$). Positive correlations were found between fur Cu concentration and total S fed per day and S concentration in feed ($p= 0.028$ and $p=0.038$ respectivley). Fur Cu concentrations was lower on organic farms ($p=0.002$).

Neither dietary Fe or Zn showed relation to fur Cu concentrations ($p=0.118$ for Fe concentration, $p=0.191$ for total Fe fed per day, $p=0.935$ for Zn concentration and $p=0.774$ for total Zn fed per day). Milk yield and lactation number was not related to fur Cu concentrations ($p= 0.474$ and $p=0.193$ respectivley).

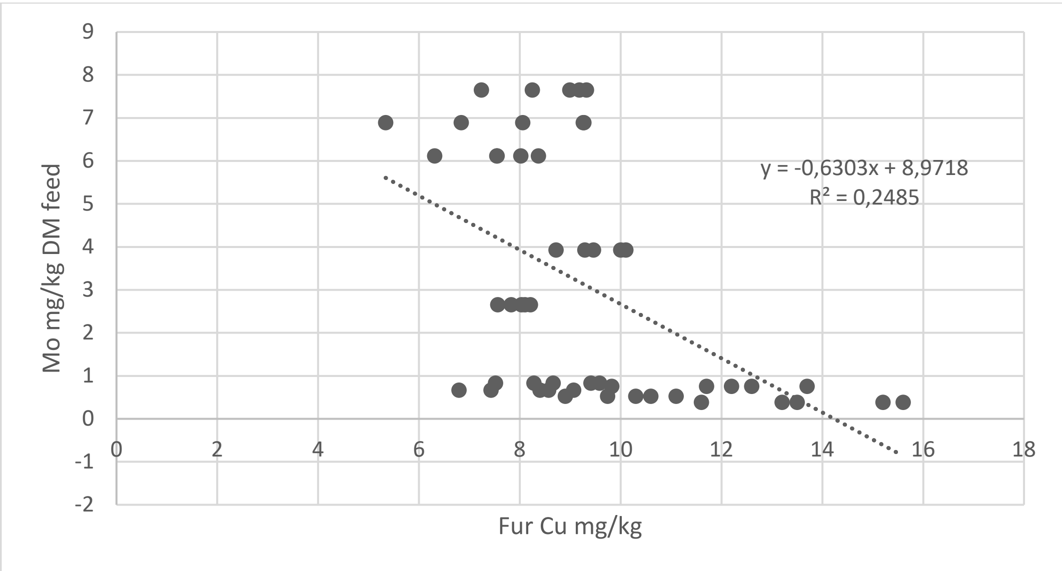

Figure 4. Scatterplot of relationship between molybdenum (Mo) concentration in dry matter (DM) on herd level and copper (Cu) concentration in individual fur samples (Fur Cu). $p<0.001$.

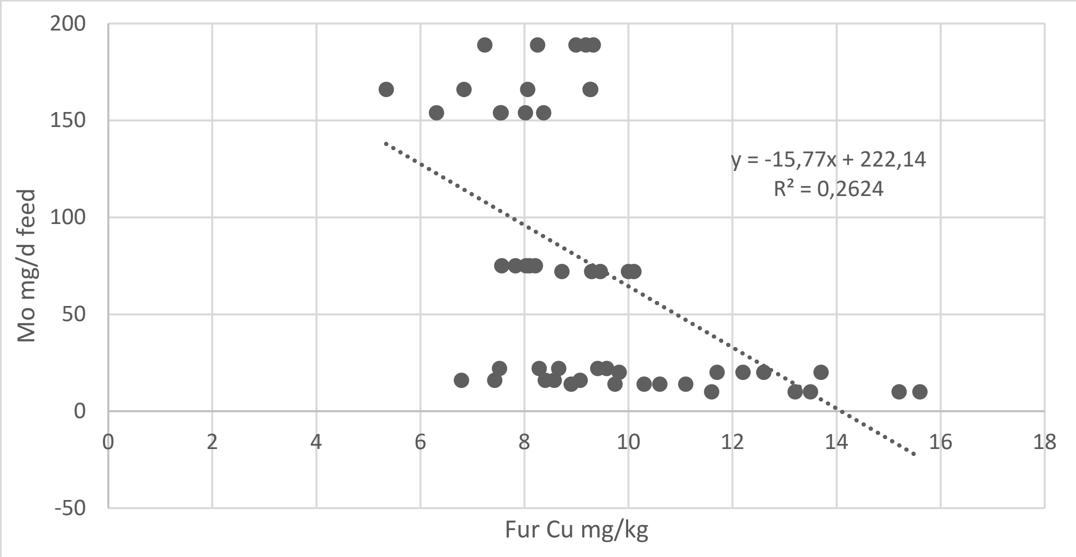

Figure 5. Scatterplot of relationship between total dietary intake of molybdenum (Mo) on herd level and copper (Cu) concentration in individual fur samples (Fur Cu). $p<0.001$.

The CV for fur Cu concentration showed that 78% of the variation in fur Cu concentration can be explained by origin of farm and is showed in figure 6 below.

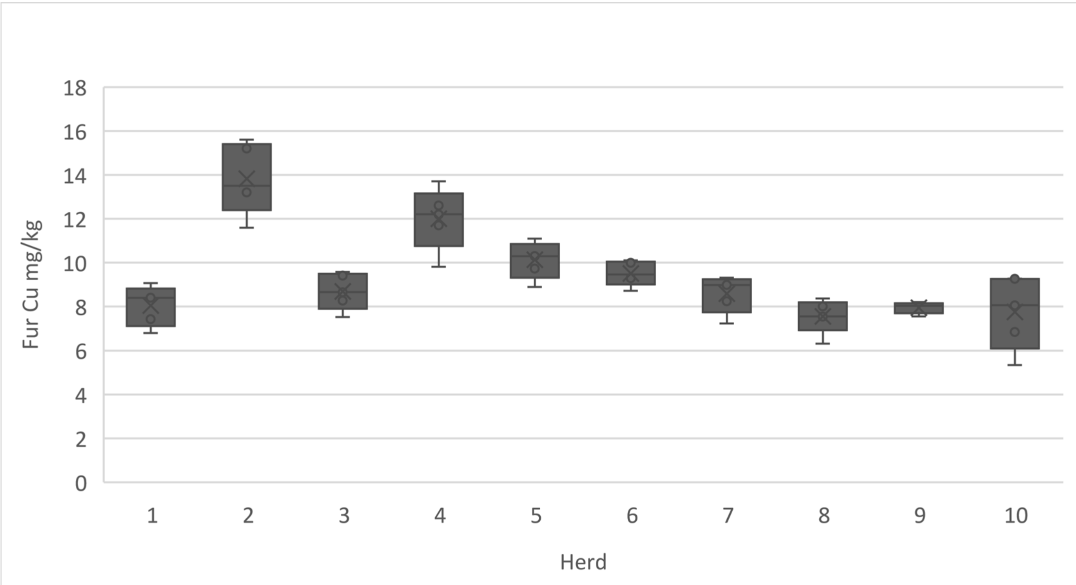

Figure 6. Boxplot of distribution of copper (Cu) concentration in fur samples within herd. Covariance 78%.

4.3 Liver samples

Because of limitations in time, differences in herd sizes and difficulties in communication, the time from sampling on farm until day of slaughter and sampling of liver differed greatly between farms from two weeks to two and a half months. Sampling date ranged from February to the end of April. This also resulted in only one sample from one farm, two samples from one farm, three samples from two farms, four samples from one farm and from one farm six samples were obtained (see appendix 2). In table 10 below, the results on Cu concentration in liver samples from cows are presented. In appendix 2 results on individual cows can be found. Liver Cu tended to be lower in the HIGH group compared to the LOW (p=0.096). No difference in liver Cu concentration between organic and conventional farms was observed (p=0.137).

Table 10. Mean ($\bar{x}$) Copper (Cu) concentration in liver samples expressed as mg/kg wet weight and standard deviations (SD) for each herd and for groups of farms with molybdenum (Mo) concentrations below 1.2 mg/kg dry matter (DM) in their homegrown roughage (LOW) and farms with Mo concentrations above 5 mg/kg DM in their homegrown roughage (HIGH).

Farm	$\bar{x}$ Cu	SD
1	248	68.58
2	190	27.43
3	156	30.33
4	112	36.74
5	98.8	52.78
LOW	161	71.04
6	135	43.49
7	77.7	51.41
8	133	9.76
9	137	31.25
10	132	0.00
HIGH	128	33.11

In figure 7 and 8 it is shown that liver Cu concentration has a negative correlation to total daily intake of Mo mg/day (p=0.048) and to Fe concentration in feed (p=0.046). Liver Cu concentrations only showed a tendency to decrease with total daily ration of Fe in mg/day (p=0.075). Concentration of Cu in liver samples increased with age (p=0.032) which is shown in figure 9. No correlations were found between liver Cu concentrations and dietary intake of Cu in mg/day (p=0.603) or mg/kg DM (p=0.523). Neither did dietary S in g/kg DM nor g/day show any correlation to liver Cu concentrations (p=0.772 and p=0.697, respectively). Total Zn fed per day in mg/kg or Zn concentration in mg/kg DM did not correlated to liver Cu concentrations either (p=0.385 and p=0.274 respectively).

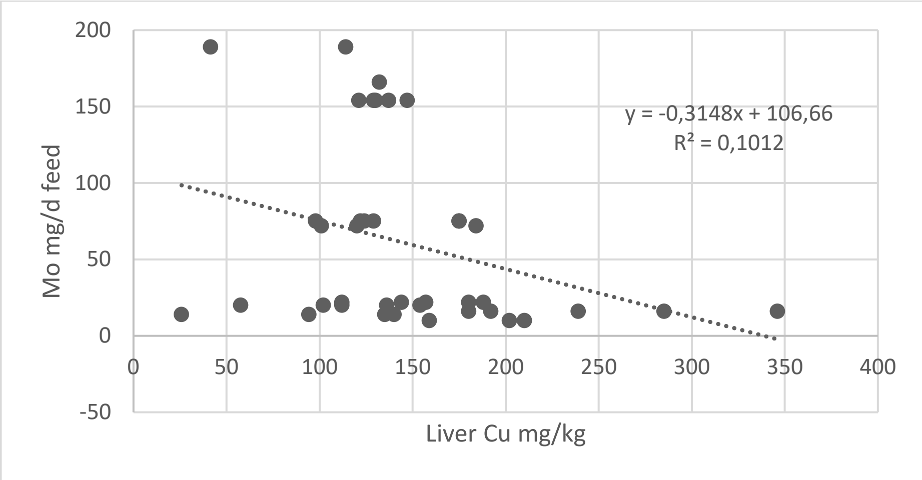

Figure 7. Scatterplot of relationship between total dietary intake of molybdenum (Mo) on herd level and copper (Cu) concentration in individual liver samples (Liver Cu). p=0.048.

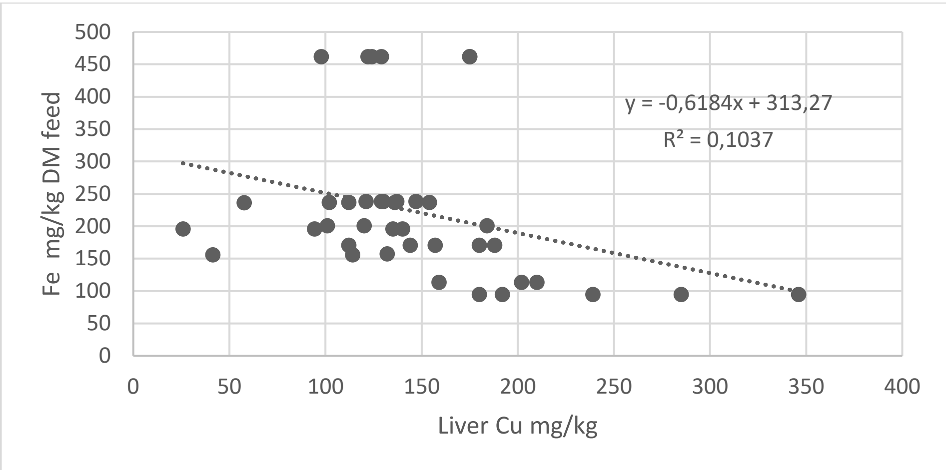

Figure 8. Scatterplot of relationship between iron (Fe) concentration in dry matter (DM) on herd level and copper (Cu) concentration in individual liver samples (Liver Cu). p=0.046.

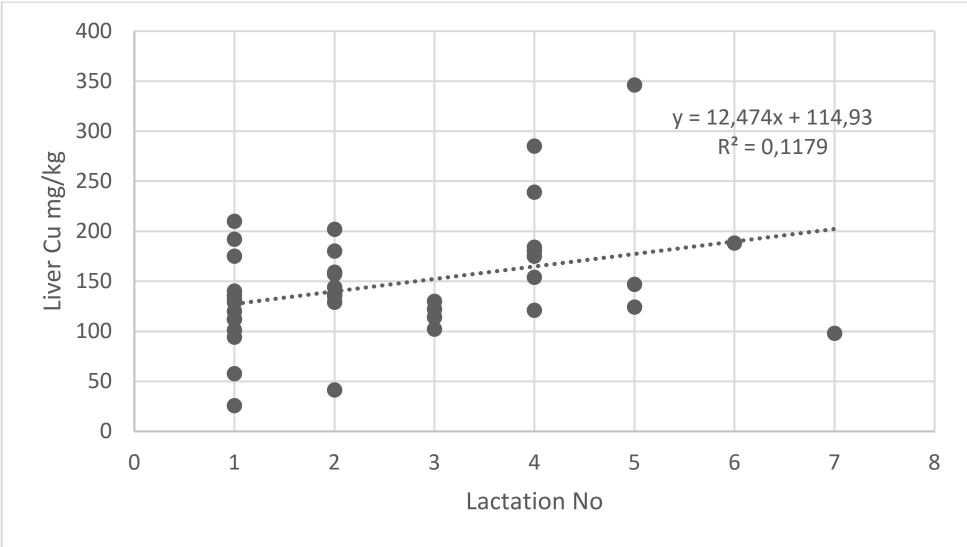

Figure 9. Scatterplot of relationship between copper (Cu) concentration in individual liver samples (Liver Cu) and lactation number (No). p=0.032.

The CV for liver Cu concentration showed that 54% of the variation in liver Cu concentration can be explained by origin of farm and is showed in figure 10 below.

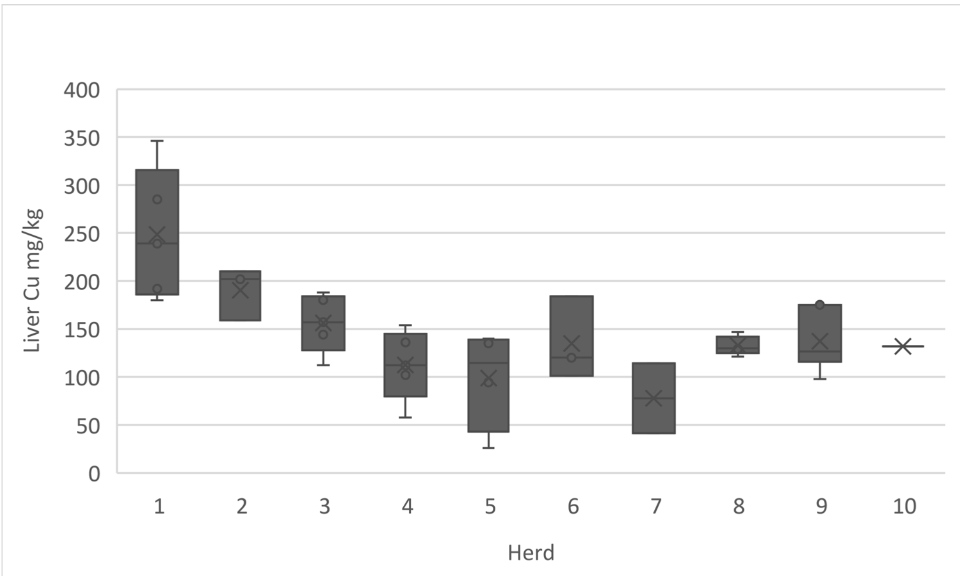

Figure 10. Boxplot of distribution of copper (Cu) concentration in liver samples within herd. Covariance 54%.

There were no correlation between fur Cu concentration and liver Cu concentration (p=0.897). This is shown in figure 11 where the data points are very scattered.

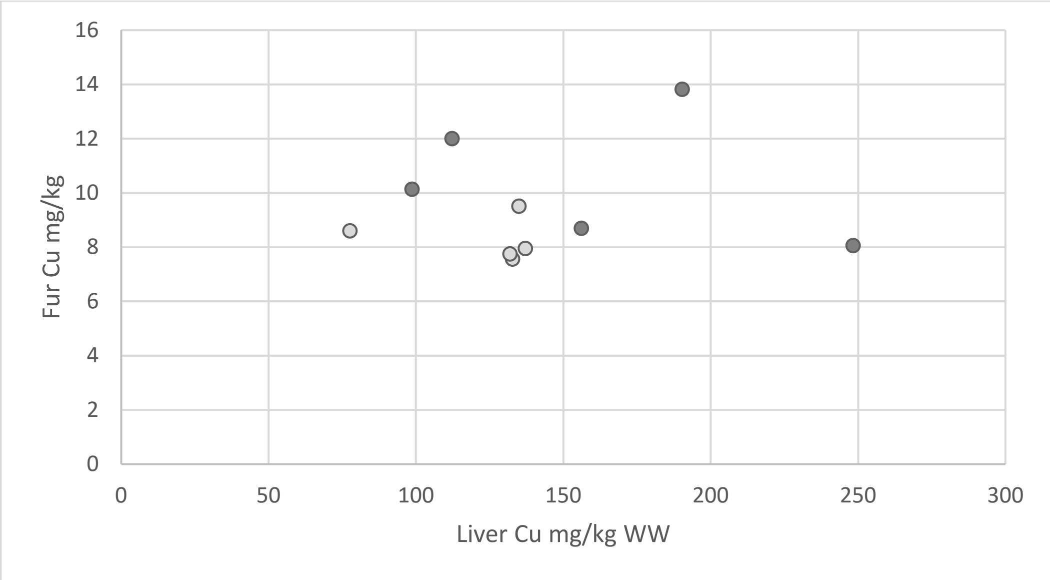

Figure 11. Scatter plot of mean fur copper (Cu) concentrations and mean liver Cu concentrations for each herd. Both expressed in mg/kg. Darker dots are farms with molybdenum (Mo) concentrations below 1.2 mg/kg dry matter (DM) in their homegrown roughage (LOW) and brighter plots are farms with Mo concentrations above 5 mg/kg DM in their homegrown roughage (HIGH). $p=0.897$.

5. Discussion

Several farmers and their feed advisors in the area of Falköping were aware of the Mo levels in their feed. If high, they supplemented with extra dietary Cu to compensate the inhibitory effect Mo has on Cu metabolism (Suttle 1991; Clarkson et al. 2020; López-Alonso & Miranda 2020). This study was as far as we knew the first to evaluate Cu status on a number of farms in this region. As in the study by Grace et al. (2010) there was a large variation of Cu concentration in liver samples within herd (CV 54%) compared with their CV on 50%. In this study farm 10 could be considered an outlier because it only had one liver sample taken and farm 1 being an outlier with a larger variation within herd than the other farms (figure 10) on Cu concentration in liver samples.

The results showed that two out of five LOW farms were in risk of Cu toxicity (figure 12). Two of the five HIGH farms had low Cu:Mo ratios (Suttle 2022; Puls 1988). The expectation that the HIGH farms would have a lot of extra Cu supplementation in relation to dietary Mo (at least Cu:Mo 4:1) was not the case (table 6). With the significantly lower Cu:Mo ratios on the HIGH farms, this could explain why the HIGH farms showed a tendency on lower liver Cu concentrations than the LOW farms, yet no farm showed deficient levels of Cu in liver samples (figure 12). This study showed that liver Cu decreased with increased intake of dietary Mo which is in agreement with previous findings of Johnston et al. (2014) and McCaughern et al. (2020) that addition of Mo reduce liver Cu. Even though litterature say that Cu content in liver samples reflect dietary intake of Cu (Grace et al. 2010) and that liver is the best tissue in reflecting Cu status (Combs et al. 1982; INRA 2019), results on Cu concentration in liver showed no correlation to dietary Cu in this study.

The period from one day feeding controls and liver sampling at the slaughter houses varied greatly from two weeks to two months. Even though differences in feeding could occur during this period the LOW and HIGH farms will probably continue to have similar Mo levels in feed as long as they use home grown roughage and grains. In addition to this, the cows probably got a a diet with similar Cu and Mo levels for several months before the one day feeding control.

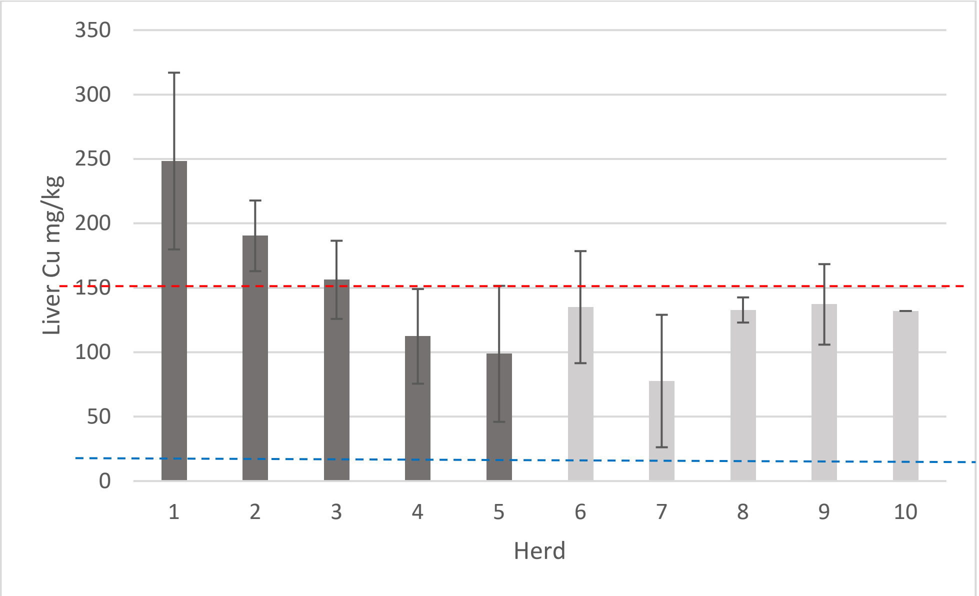

Figure 12. Mean values (with ±14% measurement uncertainty) of liver copper concentrations in mg/kg for each herd (1 to 10) expressed in mg/kg and standard deviations. Darker bars are farms with molybdenum (Mo) concentrations below 1.2 mg/kg dry matter (DM) in their homegrown roughage (LOW) and brighter bars are farms with Mo concentrations above 5 mg/kg DM in their homegrown roughage (HIGH). Values over the top red line are excess or toxic *(López-Alonso & Miranda 2020;NASEM 2021;INRA 2019)* and values below the lower blue line are deficient *(Kellaway et al. 1978; Suttle 2022;INRA 2019)*.

Both Kellaway et al. (1978) and Cunningham & Hogan (1958) showed in their studies that dietary Mo decreased Cu concentration in fur samples which agrees with the findings in this study. Cunningham & Hogan (1958) showed that this effect was prevented if dietary Cu was added to the ration. In addition, Kellaway et al. (1978) saw that if Cu glycinate was injected fur Cu concentration increased. In this study Cu concentration in fur showed a decrase in relation to dietary Cu intake in mg/kg DM and a tendency to decrease in relation to Cu intake in mg/day. This could be because the HIGH groups had significantly lower Cu:Mo (table 7) ratios that overruled the high dietary intake of Cu in the LOW group (table 7). On the other hand the LOW group had significantly higher levels of S (table 7) which has showed to affect Cu concentrations in fur earlier (Cunningham & Hogan 1958; Kellaway et al. 1978) even if it did not in this study where total S fed (g/day) and S concentration (g/kg DM) correlated positively with Cu concentration in fur.

The variation of fur Cu concentration within herd was lower (CV 78%) than it was for liver Cu concentrations (CV 54%). In contrast to the liver Cu concentrations (figure 12) the literature suggest that several farms in this study were on the limit of Cu deficiency according to the fur samples, and no farms were over 30 mg Cu/kg and at risk of excess (INRA 2019) in their fur samples (figure 13). When cutting fur and not plucking there will be a stubble left (Schwertl et al. 2003). It could therefore be a risk of missing Cu concentrations in this stubble, if Cu concentration in fur varies depending on location in the hair strand like it does in the liver (Puls 1988). The fur is metabolically active during growth when keratin is formed (Schwertl et al. 2003) and the mineral concentration in fur could reflect the concentration of minerals in the hair follicle during growth (Combs et al. 1982). This together with the information that fur grows at different rates at different parts of the body (Schwertl et al. 2003; Burnett et al. 2014) could be an argument to investigate Cu concentration in fur depending on sampling site. In this study it should however not be a limitation because samples where taken somewhere between the shoulder and hip joint, an area where the growth rate is similar (Burnett et al. 2014). A limitation could be that depending on fur length and how recent they had been cut, could show different recent Cu concentrations in the fur.

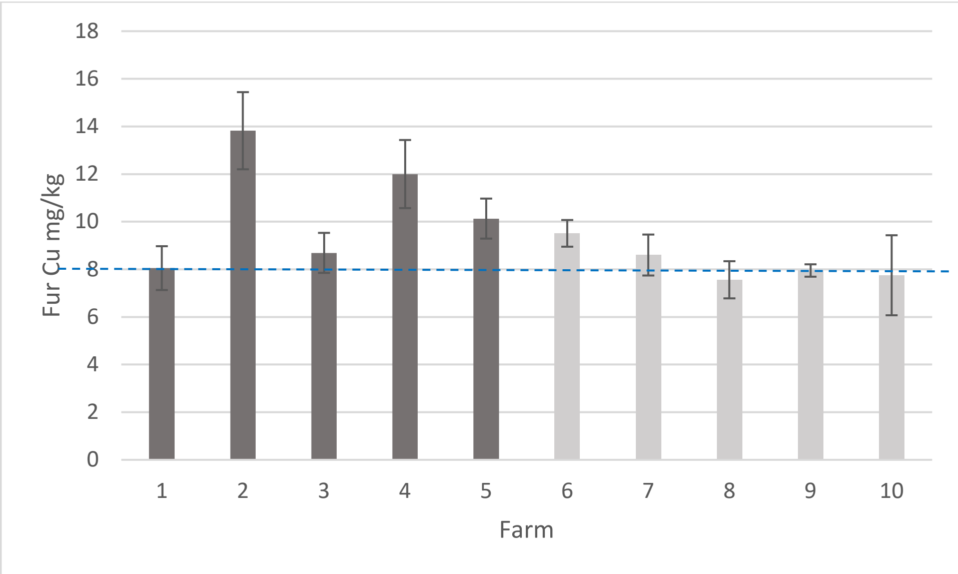

Figure 13. Mean values (with ±14% measurement uncertainty) of fur copper concentrations in mg/kg for each herd and standard deviations. Darker bars are farms with molybdenum (Mo) concentrations below 1.2 mg/kg dry matter (DM) in their homegrown roughage (LOW) and brighter bars are farms with Mo concentrations above 5 mg/kg DM in their homegrown roughage (HIGH). Values below the blue line are considered deficient *(Kellaway et al. 1978; Suttle 2022;INRA 2019)*. No values were above 30 mg/kg that is considered as Cu fed in excess.

Copper status is reflected best in liver tissue (Combs et al. 1982;INRA 2019) but Cu concentration in fur has been suggested as a diagnostic tool for sub-clinical Cu deficiency (Kellaway et al. 1978). This is because liver Cu concentrations and fur Cu concentrations correlate (Kellaway et al. 1978; Combs 1987). However, in the present study there was no correlation between fur Cu and liver Cu. Liver- and fur samples were not taken from the same individuals and there was probably several different reasons why just these animals were sent to slaughter. Reasons that perhaps could have affected feed intake and therefore Cu intake, which is unknown due to feed results on herd level and not individual level. All fur samples were far from excess levels in Cu but both herd 1 and 3 have liver samples that show excess or toxic levels and fur samples that indicate deficiency. There is also a 14% measurement uncertainty on the laboratory results, but even if multiplying by 1.14, fur samples will not reach toxic levels yet this uncertainty can also mean that true values are lower than shown.

In intensive animal production in areas of Sweden, mineral supplementation occur irrespectively of knowledge on mineral concentrations in homegrown feed (Eriksson et al. 2017). The reason that farmers and advisors are so aware of the Mo concentrations in their homegrown feed is the routine of nutrient analysis. Nutrient analysis of homegrown feed is probably more common than analysis of soil for Mo that Suttle (1991) mentioned as important for animal health. Although, general mappings done by Eriksson et al. (2017) can show risk areas where analysis of the four minerals Se, I, Mo and Co that generally are not included in the base package of analyse is extra important. The feed advisors in the area of Falköping strive for a Cu:Mo ratio of 4:1, but in this study three out of five HIGH farms were below that limit. Additionally all the LOW farms were far beyond that limit which could be considered unessecary because of several liver samples with high Cu concentrations (figure 12). It is however worth notifying that one-day feeding controls in this study were practiced by ten different people and that human variation in estimating amounts could have affected the results on mineral concentrations in the herd specific diets. The laboratory results on DM differed from our results on DM. It is difficult to measure DM on farm because of its' sensitivity to temperature, but DM can also differ greatly from different roughage lots. This would probably not have affected the results on Cu to a greater extent because Cu was mainly supplied by mineral feed and concentrates, yet it could have had an effect on the Mo results since Mo mainly originated from homegrown roughages.

Mo concentration in the daily diet increased with a higher roughage proportion and decresed with a higher daily ration of bought concentrate. The same trend also applies for total intake of Mo per day. The organic farms had larger roughage proportions and lower concentrate proportions. The organic farms in the present study were all in the HIGH group (table 3). They also had the highest Mo concentration in their daily diet to the cows (table 6) which could explain the positive correlations between Mo concentration in feed and organic farms. However the results also agrees with that soil pH tend to rise when converting from conventional to organic farming (Govasmark et al. 2005) which favour Mo concentration in soil (Eriksson et al. 2017).

An unexpected positive correlation was found between Cu concentration in fur and both S concentration in feed and total intake of S per day. In earlier studies adding dietary S has decreased Cu concentrations in fur (Kellaway et al. 1978). In their study S concentration was 3.8 mg/kg DM (Kellaway et al. 1978) which was much higher rations of S than in the herds of the present study (table 6). Iron and Zn are the main competitors with Cu for DMT1 (Clarkson et al. 2020; López-Alonso & Miranda 2020) and Fe has the possibility to interact in non-absorbable complex Fe-Cu-S (López-Alonso & Miranda 2020). Because of this correlations between fur Cu concentrations and dietary Fe and Zn was investigated, however no correlations could be found. Neither milk yield or lactation number was correlated to fur Cu concentrations, which was interesting to investigate since factors as age, diet or genetic factors can affect liver Cu concentrations (Miranda et al. 2010).

As mentioned before Fe has two ways to interact in cattle Cu metabolism (Clarkson et al. 2020; López-Alonso & Miranda 2020), so that liver Cu concentration decreased with increasing Fe concentration (mg/kg DM) in feed in this study was no suprise. In contrast Zn, which only competes with Cu for DMT1 (Clarkson et al. 2020; López-Alonso & Miranda 2020) did not show any correlation to liver Cu concentrations. That liver Cu concentrations can be affected by age (Miranda et al. 2010) and that the liver accumulate excess Cu over time (McDonald et al. 2011) agrees with our results that Cu concentration in liver increased with age.

Combs (1987) stated that mineral status is complex and costly to analyse which is one of the reasons why fur is an interesting option. In this study and in a collaborative study we got the same price per sample for the Cu analyses no matter of origin (liver, fur, feaces, urine and milk). Liver samples however, entailed extra fees at the slaughter houses for taking out samples. If biopsies would have been taken that would also have generated an extra veterinary fee. This extra cost does not occur with fur samples that is possible for the farmer to obtain by themselves. That sampling fur is non-invasive and easy to store is also in its advantage (Kellaway et al. 1978; Combs et al. 1982).

6. Conclusion

There was a decreasing effect of dietary Mo intake on both Cu content in liver and fur. In this study no correlation between liver Cu concentration and intake of dietary Cu could be shown, yet other studies have shown that and liver samples are generally recommended as a tool to predict Cu status in a herd. That no correlation on this could be shown, can however be explained by the aim of finding farms with high or low dietary Mo intake in this study and that the high Mo concentrations might have overruled the correlation to dietary Cu. Fur Cu concentration decreased with increased dietary intake of Cu which was also unexpected and could be explained by the same reason as for liver Cu concentrations. There was however a small variation within herd on fur Cu concentration and if further studies continue to show that fur samples can be an adequate method to monitor Cu status in a herd, samples from only a few individuals could reflect the herd status. A majority of the liver samples in this study had liver Cu concentrations below toxic levels and no one showed deficiency. This could indicate that the nutritional recommendations in this area are sufficient. Though, feed advisors implies that their strive is a Cu:Mo ratio at 4:1 which agrees with the litterature that suggest 3-4:1 even if other antagonists should also be of consideration. If the advisors want to continue to strive for that, a review of the routines might be beneficial since the Cu:Mo ratio in study varied from 1.4 to 32:1.

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Popular science summary

Koppar är vad man kallar en essentiell mineral för idisslare, vilket innebär att den är livsnödvändig och måste tillföras med fodret. Att tillskottsutfodra koppar är dock utmanande då även överutfodring kan resultera i allvarliga kliniska symptom och försämrad produktion. Det på grund av att koppar lagras i levern och kan frisättas av stressfaktorer som foder- eller stallbyte. Utöver detta så är kopparmetabolismen hos idisslare ytterligare kompex då upptaget av koppar påverkas av andra tillgängliga mineraler.

De huvudsakliga mineralerna som hämmar upptaget av koppar är molybden och svavel. I våmmen sker en reaktion där molybden och svavel binder till varandra för att sedan binda till sig koppar. Det gör kopparen blir otillgänglig för idisslaren att ta upp när den når tunntarmen.

Det finns ett starkt samband mellan molybden i jorden och molybden i växter vilket resulterar i att områden med höga koncentrationer av molybden i jorden även får höga koncentrationer av molybden i fodret. Ett högt intag av molybden i fodret kompenseras ofta med extra utfodring av koppar, vilket ökar vikten av att kontrollera kopparstatusen i dessa besättningar med tanke på koppars negativa effekter både vid brist och överskott. Idag kontrolleras kopparstatus huvudsakligen genom leverbiopsier eller leverprover tagna vid slakt. Syftet med den här masteruppsatsen var att utvärdera kopparstatus i relation till koppar och molybden i foder hos mjölkkobesättningar runt Falköping i Västra Götaland, ett område där flera lantbrukare har jordar med höga koncentrationer av molybden till följd av ackumulering av alunskiffer från platåbergen i området. I och med att provtagning av lever kan vara komplext och ekonomiskt kostsamt utvärderades även om prover på päls är en lämplig metod för att kontrollera kopparstatus.

Totalt 10 gårdar och hårprov från 50 slumpmässigt valda mjölkkor inkluderades i studien. Av praktiska skäl togs leverprover från de upp till sex första djuren som skickades till slakt på varje gård och det var därför inte samma djur som provtogs för päls. Proverna blev analyserade för kopparinnehåll och foderstaterna på

varje gård utvärderades med fokus på dess innehåll av molybden, svavel samt koppar. Studien utfördes under vårterminen 2023.

Resultaten visade att en majoritet av leverproverna hade kopparkoncentrationer inom referensintervallet, vilket tyder på att tillskottsutfodringen av koppar var tillräcklig utan att orsaka risk för förgiftning. Det var dock en kraftig variation mellan gårdar i förhållandet mellan koppar och molybden (Cu:Mo) i fodret, där mängden koppar i jämförelse med mängden molybden varierade från 1.4-31.8, och molybden i fodret hade en tydligt minskande effekt på kopparkoncentrationen i både lever- och pälsprover. Av kopparintaget via fodret kunde ingen effekt ses på kopparkoncentrationen i leverprover medans koppar i fodret hade en minskande effekt på kopparkoncentrationen i pälsprover. I pälsproverna var det liten variation inom besättningarna vilket tyder på att om fortsatta studier kan bekräfta att kopparkoncentrationen i päls har ett samband med kopparstatus hos kor så kan prover från ett fåtal individer representera en hel besättning.

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To start with, I would like to thank Nötkreatursstiftelsen Skaraborg who made this possible by funding this study. A huge thank you to the farmers for welcoming us to their farms with warmth and patiently received questions by text and phone calls. Some who also deserves a huge thank you are the participating slaughter houses, KLS (Dalsjöfors) slakteri, Skövde slakteri and HK Scan slakteri for receiving my emails at the last minute and still managing to solve everything I asked for! All my amazing classmates no one namned no one forgotten, who supported me through all the ups and downs. And last but not least I want to thank both my supervisors at SLU and Växa Sverigefor all the support and patience with me during this whole project.

Appendix 1

Fur Samples with Cu concentrations expressed in mg/kg for individual cows and mean ($\bar{x}$) Cu concentration and standard deviations (SD) for each farm and mean ($\bar{x}$) Cu concentration and standard deviations (SD) for groups of LOW and HIGH farms.

Farm	Cow 1	Cow 2	Cow 3	Cow 4	Cow 5	$\bar{x}$	SD
1	8.4	9.07	8.57	7.43	6.79	8.05	0.92
2	13.2	15.2	15.6	11.6	13.5	13.82	1.62
3	8.28	9.58	9.41	8.66	7.52	8.69	0.84
4	13.7	12.2	12.6	11.7	9.82	12.00	1.43
5	11.1	8.9	9.74	10.6	10.3	10.13	0.84
LOW						10.54	2.43
6	9.29	10.1	8.72	10	9.46	9.51	0.56
7	9.18	7.24	8.25	8.99	9.32	8.60	0.86
8	7.54	8.37	6.31	8.02	7.55	7.56	0.78
9	8.21	7.83	8.1	7.56	8.03	7.95	0.26
10	8.06	9.27	6.84	9.26	5.34	7.75	1.68
HIGH						8.27	1.14

Appendix 2

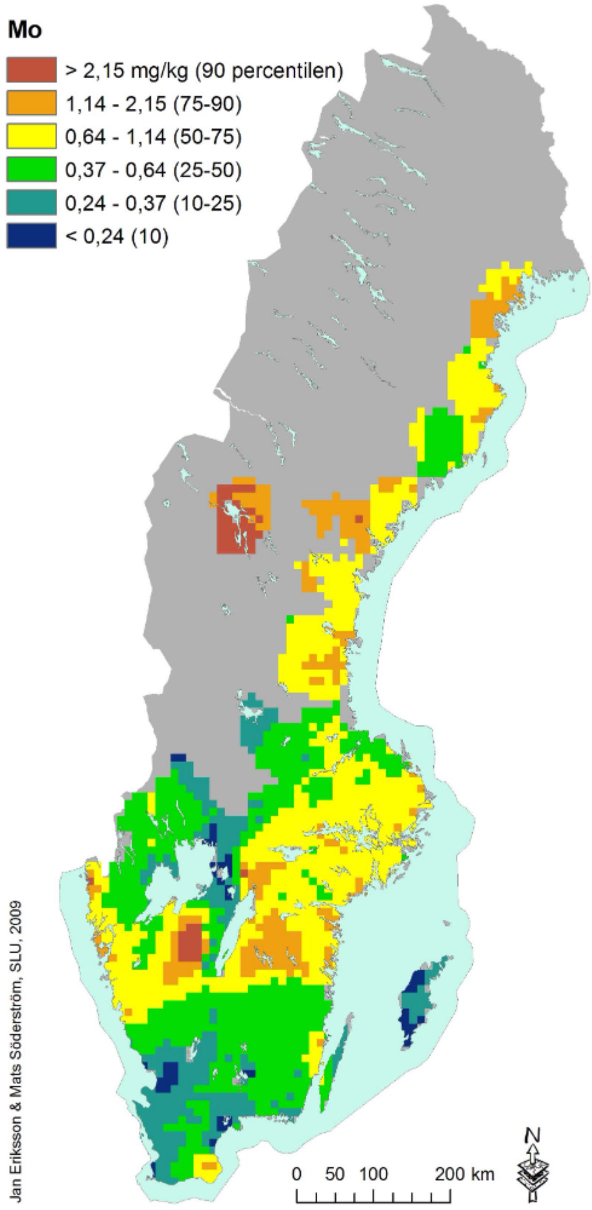
Liver Samples with Cu concentrations expressed in mg/kg for individual cows and mean ($\bar{x}$) Cu concentration and standard deviations (SD) for each farm and mean ($\bar{x}$) Cu concentration and standard deviations (SD) for groups of LOW and HIGH farms.

Farm	Cow 6	Cow 7	Cow 8	Cow 9	Cow 10	Cow11	$\bar{x}$	SD
1	192	239	285	180	346	-	248.40	68.58
2	210	202	159	-	-	-	190.33	27.43
3	188	144	180	157	112	-	156.20	30.33
4	136	102	154	112	57.6	-	112.32	36.74
5	94.2	25.8	140	135	-	-	98.75	52.78
LOW							161.39	71.04
6	101	184	120	-	-	-	135.00	43.49
7	114	41,3	-	-	-	-	77.65	51.41
8	130	121	129	137	147	-	132.80	9.76
9	97.8	175	175	124	129	122	137.13	31.25
10	132	-	-	-	-	-	132.00	0.00
HIGH							128.18	33.11

- No sample obtained

Appendix 3

A map of molybdenum (Mo) concentrations in soil in Sweden. From Swedish University of Agricultural Sciences, Kartor. https://www.slu.se/institutioner/mark-miljo/miljoanalys/akermarksinventeringen/kartor/ [2023-05-25].



Molybdenhalter (7M HNO_3) i matjorden. Data från omdrev 1 och 2 sammanslagna. Antal värden 4 641.

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