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Seasonal and individual variation in hepatic copper concentrations in a flock of Norwegian Dala sheep

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ABSTRACT

This study focused on a flock of Dala sheep with recurrent cases of chronic copper (Cu) poisoning. The seasonal variation in hepatic Cu concentration was followed in individual sheep with repeated liver biopsies, four times per year, in two consecutive years. Thirty-six ewes were included, yielding a total of 279 biopsies. Cu concentrations were measured by atomic absorption spectroscopy. Hepatic Cu concentrations remained almost stable from December to March, fell substantially from March to June, and rose sharply during the summer pasture period from June to October. There were large individual differences in hepatic Cu levels. These differences remained stable through the two years. Treatment with ammonium tetrathiomolybdate (3 × 3.4 mg per kg bodyweight (bw) s.c.) in June had only weak and inconsistent effect on hepatic Cu levels in October. The results may partly explain why chronic Cu poisoning in sheep in Norway predominantly occur in the autumn and winter months.

1. Introduction

Chronic copper poisoning in sheep is well known in Norway (Nordstoga, 1962). In much of the world, outbreaks of this disease are most commonly seen in housed lambs or milk sheep, when they are fed concentrates with an improper mineral balance (Greene and Huston, 1999; Underwood and Suttle, 1999). In Norway, chronic Cu poisoning is an endemic, low-frequency, spontaneous disease, encountered under normal grazing conditions. It is primarily seen in inland districts, during the autumn and early winter months, in sheep that have been on mountain or woodland pasture in the summer. The condition is seen mostly in ewes above one year of age (Nordstoga, 1962; Sivertsen et al., 1995; Søli, 1980). Previous research has indicated that a main background for these disease cases is a severe hepatic copper accumulation in a high percentage of sheep in some Norwegian inland districts (Frøslie, 1980), which seems to be related to low molybdenum concentrations and high copper/molybdenum ratios in local grass (Frøslie and Norheim, 1983) and mountain pasture plants (Garmo et al., 1986).

From the 60s to the 90s, cases of chronic copper poisoning was fairly common, and led to much concern in the sheep industry (Frøslie, 1980; Sivertsen et al., 1995). Since then, the prevalence seems to have subsided, though cases are still diagnosed by the Norwegian Veterinary Institute almost every year (Bernhoft, 2013). The reasons for this apparent change in prevalence is not fully understood.

A recent nation-wide survey of hepatic trace element concentrations in slaughtered Norwegian sheep and lambs did however confirm that serious hepatic Cu accumulation in the autumn is still widespread in sheep in Norway (Sivertsen et al., 2009). Hepatic Cu concentrations up to 690 μg/g wet weight (ww) were observed, and 13% of sampled livers from adult sheep had Cu concentrations

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above 150 μg/g ww. This led us to reconsider the previously unpublished results from a study of the seasonal variation of hepatic Cu concentrations performed in the 1990's, and prepare it for publication.

Though most clinical cases of chronic Cu poisoning in Norway have always occurred sporadically, the prevalence might in these years be considerable in some flocks (Sivertsen and Wie, 1996), and one of these flocks was used in the investigation.

Several important questions concerning Cu accumulation and poisoning in Norwegian sheep have remained unanswered by previous research. Among these are the reasons for the seasonal occurrence of the disease, and the individual factors that determine which animals within the flock that will be clinically affected. One of the limitations of previous studies is that they have all been based on samples collected at slaughter, and in Norway the great majority of sheep are slaughtered in a few autumn months, from September till November. In an attempt to improve the knowledge on the seasonal variation in hepatic Cu accumulation, we used repeated liver biopsies to track hepatic Cu concentrations in a number of sheep from the selected flock; through two consecutive years. We also tested the effect of three s.c. injections with ammonium tetrathiomolybdate (Humphries et al., 1988) in June on the hepatic Cu concentration in October, and of similar injections in November upon the hepatic concentrations two weeks later. Tetrathiomolybdate (TTM) is an effective chelator of Cu ions, and parenteral TTM injections have been used successfully to reduce hepatic Cu levels in flock outbreaks of chronic Cu poisoning (Gooneratne et al., 1981; Humphries et al., 1988). In order to evaluate the possible effect of the mineral content of the winter feed, we analyzed Cu, zinc (Zn), molybdenum (Mo) and sulfur (S) concentrations in the hay and silage fed to the flock in the indoor season.

Permission to conduct the liver biopsy study was granted by the National Animal Research Authority in Norway. Some preliminary results from the first year of the study were presented at two symposia in Norway in the nineties (Sivertsen et al., 1995; Sivertsen and Wie, 1996).

2. Materials and methods

2.1. Animals, feeding and pasture

The flock used in this study consisted of about 60 winter-fed ewes, of the Norwegian Dala breed. In four out of six years before the study was started, the owner had lost ewes from copper poisoning. A total of 7 ewes were lost during the worst year. All the lost ewes were adult, all died in the autumn, and all had shown the typical signs of copper-induced hemolytic crisis and hemoglobinuric nephrosis (Moeller, 2004; Søli, 1980). Mean hepatic copper concentrations in clinically normal ewes from the flock slaughtered in autumn had in these years varied from 257 to 465 μg/g ww (Sivertsen et al., 1995).

The flock was from a small mountain farm in Namdalen, an inland area in the county of Nord-Trøndelag, at 64° 40′ latitude and 450 m above sea level. As in most mountain areas in Norway, the surface varies between bare rock and thin deposits of glacial origin. Soils are predominantly podzols, with patches of swamp soils in between (Låg, 1983). Climatically, the area lies mainly within a low alpine vegetation zone (Moen, 1999). From the middle of October to the middle of June the sheep were kept indoors in an unheated barn and mainly fed silage and hay grown on the farm. Concentrates were used in small amounts, mainly in the months before and after lambing. The compound concentrates and the mineral supplements used in the flock were without added copper, but contained standard recommended amounts of other trace elements (Zinc, cobalt, selenium, iodine, manganese). From the start of July till the middle of October the flock was grazing in the surrounding mountain area. No changes due to this study were made in the normal management of the farm, nor with the owner's recruitment and slaughter policy.

The study was performed in the years 1994–96. It was begun in late November. Ten adult ewes aged 1½–7 years and 10 ewe lambs born in the spring were selected for inclusion. Liver biopsies were taken at the start in late November, and in March, June, October and December the following year. As three of the original animals selected were slaughtered in the spring, three new ones were recruited.

In December of the second year, 10 adult ewes (age 1½–7 years) and 10 new ewe lambs were again selected. Seven of the adult ewes had also been sampled the first year, while 3 were new. The second year samples were taken in December, and in March, June and October in the following season. Altogether 36 animals were sampled. Two of the animals we managed to sample only once. As they would give no information on temporal change, the results from these animals are not included in the tables, figures and main statistical calculations. Due to logistical problems, the liver samples from four of the ewes in October the last year had to be collected at the abattoir, after slaughter. An overview of the sampling, treatment and fate of each of the 34 animals included in the results are shown in Table 1.

Twenty-nine samples of the silage and hay fed to the sheep in the winter season were collected at intervals both winters (Tables 1 and 3).

2.2. Ammonium tetrathiomolybdate treatment

In each of the seasons studied, half of the animals included in the study were treated with ammonium tetrathiomolybdate (TTM) in June, to see if this affected the levels in October (Table 1). In assigning the animals to the treatment and non-treatment groups, efforts were made to obtain an even distribution between groups. The distribution of adult and one year old ewes as well as the hepatic Cu levels recorded in March were taken into account.

In the first autumn, half of the animals that had been sampled in October were given a new TTM treatment in November, ending two weeks before the sampling of new liver biopsies in December. None of these animals were included in the second year of the study (Table 1). This November TTM treatment did therefore not influence the results reported in Table 2.

The treatments comprised three s.c. injections of 3.4 mg TTM per kg bw, with 48 hours' intervals (Humphries et al., 1988). The TTM had been bought as crystalline ammonium tetrathiomolybdate from Rowett Research Services, Aberdeen, UK, and kept dry and cool till it was used. Immediately before usage it was dissolved in sterile 0.9% saline solution to a concentration of 40 mg/ml. The dissolved batch was stored in a refrigerator between treatments and discarded after the third treatment.

2.3. Liver biopsy method

The biopsies were taken in the barn, using a modified version of the percutaneous approach described by Harvey et al. (1984) and Humann et al. (1999). No sedation was necessary. An area above the 10th and 11th costae on the right side of the animal was clipped and shaved. To select the most suitable place to insert the biopsy needle, and the appropriate depth from the skin surface, a portable ultrasound scanner (Aloka Echo Camera SSD-500 with 7.5 MHz scanner head, Aloka Co ltd, Tokyo, Japan) was used. Usually a point in the 11th intercostal space was chosen, about a third of the length from the top of the palpable costa. In some sheep, especially when pregnant, a point slightly further down in the 10th intercostal space was found to be more suitable. After disinfection with 2% iodine tincture, and local anesthesia with 5 ml of 20 mg/ml Lidocaine, a small incision was made in the skin. The biopsies were taken with a sterile disposable biopsy needle with depth markings (Bard® Biopty-cut 14 G × 160 mm) mounted on an automatic biopsy pistol (Bard® Biopty, Bard biopsy systems, Tempe, Arizona). In late November the first year, only one biopsy was taken from each animal. At the subsequent sampling dates, we attempted to take two biopsies each time. After the last biopsy, the incision wound was closed with a wound clip. Immediately after excision, the biopsy was dropped into an isolated box filled with liquid nitrogen. Thereafter, each of the frozen biopsies was put into a closed and pre-marked plastic vial (Nalgene Cryoware 1.2 ml, Nalge (UK) Ltd., Rotherwas, UK). The vials were

Table 1
Overview of ewes included in the experiment: year of birth, TTM treatments, liver biopsies taken, and fate of the animal. Periods of indoor feeding and pasture for the flock are designated at bottom, together with time of collection of hay and silage samples. M P: Mountain pasture. Bs in italics indicate samples collected at the abattoir.

Exp. year:		1st year													2nd year										
Month:		N	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O
Ewe	Born	Biopsies collected (B), TTM treatment (T), and fate of animal*																							
1	88	B				B		S	–				–		–			–			–				–
2	89	B				B			B	T			B		B			B			B				*B*
3	90	B				B			B				B	T	B[1]	E		–			–			–	
4	92	B				B			B	T			B		B			B			B	T			*B*
5	92	B				B			B	T			B	S	–			–			–				–
6	92	B				B		S	–				–		–			–			–				–
7	92	B				U			B				B	T	B[1]	E		–			–			–	
8	93	B				B			B				B		B			B			B	T			B
9	93	B				B			B	T			B	T	B[1]	E		–			–			–	
10	93	B				U			B				B	T	B[1]	E		–			–			–	
11	94	B				B			B	T			B	T	B[1]	E		–			–			–	
12	94	B				B			B	T			B		B			B			B	T			B
13	94	B				B			B				B	S	–			–			–				–
14	94	B				B			B				U	S	–			–			–				–
15	94	B				B			B	T			B	S	–			–			–				–
16	94	B				B			B				B	T	B[1]	E		–			–			–	
17	94	B				B			B				B	T	B[1]	E		–			–			–	
18	94	B				B			B	T			B	S	–			–			–				–
19	94	B				B			B				B		B			B			B	T		S	–
20	90								B				U		B			B			B				B
21	93								B	T			B		B			B			B				B
22	92														B			B			B				B
23	93														B			B			B	T			B
24	94														B			B			B				*B*
25	95														B			B			B	S			–
26	95														B			B			B			S	–
27	95														B			B			B	T			B
28	95														B			B			B	T			B
29	95														B			B			B	S			–
30	95														B			B			B				B
31	95														B			B			B	T			B
32	95														B			B			U				B
33	95														B			B			B				*B*
34	95														B			B			B	S			–
Feeding		Indoor feeding								M P			Indoor feeding									M P			
Feed smpl.						X		X					X		X	X		X							

* S: Slaughtered or lost. U: Unavailable, or unsuccessful sampling. E: Not included in 2nd year.
[1] Biopsy only for evaluation of November TTM treatment. Not included in 2nd year figures.

Table 2
Copper concentrations in repeated liver biopsies from a sheep flock with high prevalence of copper poisoning. Means, standard deviations (in italics) and ranges (in brackets). All values in μg/g ww (wet weight).

	Sampling dates:			
1st year:	Nov. 30th (n = 19)	March 4th (n = 17)	June 5th (n = 18)	Oct. 2nd
				Untreated (n = 8)	TTM-treated (n = 9)
Adult ewes	248, *68* (149–359)	218, *88* (101–383)	103, *57* (34–223)	256, *97* (154–405)	221, *61* (156–336)
1 year old ewes*	147, *41* (89–221)	127, *28* (81–176)	56, *21* (23–89)	227, *64* (157–306)	165, *6* (155–171)

	Sampling dates:			
2nd year:	Dec. 9th (n = 20)	March 1st (n = 20)	June 7th (n = 19)	Oct. 5th
				Untreated (n = 8)	TTM-treated (n = 7)
Adult ewes	187, *97* (66–374)	169, *107* (48–352)	99, *83* (16–296)	193, *60* (122–288)	248, *165* (81–494)
1 year old ewes*	120, *30* (87–186)	127, *33* (94–198)	96, *9* (47–173)	186, *65* (132–277)	205, *88* (135–330)

* Recruited as ewe lambs; born in May.

transported to the laboratory floating in liquid nitrogen, and were stored at −70°. The vials were kept unopened until the frozen biopsy rods were weighed for analysis.

Altogether 279 biopsies were collected successfully. At one occasion acceptable biopsies were not achieved from two of the selected animals. In 3 other cases, only one of the intended two biopsies was acquired. Once, one of the animals was found dead the next morning. The autopsy showed hemorrhage to the abdominal cavity, apparently from the small biopsy wound in the liver. On two other occasions, a sampled ewe was reluctant to eat the first hour after sampling. No other signs of disease or discomfort were noted.

2.4. Chemical analysis

The biopsies were weighed at −40° on a low-temperature laboratory scale (Cahn scale USA Model C27, Cahn Instruments, Serritos, California installed in an Electrolux freezer, Electrolux AB, Stockholm, Sweden). The mean weight of the biopsies was 20.6 mg ww (frozen), with a standard deviation (s.d.) of 4.6 mg. Wet oxidative digestion of the samples was carried out in a mixture of concentrated nitric (65% HNO_3, p.a., Merck) and perchloric acid (70% $HClO_4$, p.a., Merck). The ratio nitric acid: perchloric acid was 3:1; total volume added was 16 ml. Automatic heat digestion was done overnight, using a Tecator 1012 Controller connected to a 1016 Digester (Tecator AB, Höganäs, Sweden). The heating program went up to 220 °C. The samples were diluted to 25 ml with ionchanged water before analysis. The copper content in the biopsies were analyzed by atomic absorption spectroscopy (AAS) with flame atomization, using a Varian SpectrAA-600 (Varian Techtron Pty Ltd., Mulgrave, Australia).

The forage samples were dried in a drying cabinet at 85 °C overnight (Termaks TS 4115, Termaks, Bergen, Norway) and homogenized in a common kitchen homogenizer. Cu and Zn concentrations were measured by flame atomization AAS after wet oxidative digestion, using the same analytical equipment as for the liver biopsies. The oxidative digestion was done in a mixture of 4 ml concentrated nitric acid (65–70% HNO_3, suprapure, Merck), 2 ml water, and 1 ml hydrogen peroxide (30% H_2O_2, suprapure, Merck); using a microwave oven (Ethos Plus, Milestone s.r.l., Sorisole, Italy). The samples were diluted to 25 ml with ionchanged water before analysis. Mo concentrations were measured by electrothermal AAS (ETAAS), using a Varian AA-300 spectrophotometer with a GTA-96 graphite tube atomizer (Varian Techtron Pty Ltd., Mulgrave, Australia).

A quality control system included the use of certified control material based on liver matrix for the copper, zinc and molybdenum analyses. (Bovine liver 1577b, Standard Reference Material, NIST, Canada and BCR no.185 Bovine Liver, Standard Reference Material, Commission of the European Community, Community Bureau of Reference). Additionally, a reference material based on a matrix of hay (V-10, IAEA, Austria) served as a control for Mo.

The detection limits were 0.2 μg/g for Cu (flame-AAS), 0.3 μg/g for Zn (flame-AAS) and 0.2 μg/g for Mo (ETAAS).

The reliability of the biopsy method was tested by analyzing both test biopsies and standard 2 g samples from 18 livers collected at the abattoirs, both from the flock studied and from other flocks.

S concentrations in the forage samples were measured at Norwegian University of Life Sciences, Ås. The analysis was done by an internal method (Selmer-Olsen, 1986), based on oxidation of all sulfur compounds to sulfate, precipitation with barium and turbidimetric determination, using a Technicon® AA model 1 autoanalyzer (Technicon corp., USA).

2.5. Calculations and statistical evaluations

In two sets of parallel biopsies, one of the parallels was excluded as an outlier, because of extreme difference to the other parallel biopsy and to other samples from the same animal. In the other 126 sets of parallel biopsies, the mean value was used as hepatic Cu concentration in further calculations and presentation of results. In the remaining 23 cases, the available single biopsy result was used.

For the statistical evaluation of changes from month to month and differences between age groups, a generalized linear mixed model was used, with date of biopsy and age group as fixed effect variables, and with correction for clustering using individual as a random variable (Dohoo et al., 2009). The STATA statistical software (STATA 12.0, Stata Corporation, College Station, Texas, USA) was used for all statistical calculations. Anscombe residual graphs were used to check the validity of the model. Each year of the study was treated separately.

To test the effect of the TTM treatments, the change in hepatic Cu concentrations from the biopsy taken before treatment to the next biopsy sampling was calculated, and the groups compared with Student's *t*-test. In all statistical evaluations, the limit of significance was set at $p = 0.05$.

3. Results

3.1. Liver biopsy method and variation between biopsies

The mean Cu concentration in all biopsies (the two outliers excluded) was 151 μg/g ww (s.d. 86 μg/g). In the 126 sets of parallel biopsies taken out from the same animal at the same occasion, mean difference between the Cu concentrations measured in the two parallels was 21 μg/g ww (s.d. 24 μg/g), or 16% (s.d. 19%) of the mean value for each pair.

The test of the reliability of the biopsy method performed on abattoir-collected livers showed good correspondence between the analyses of the test biopsies and the standard 2 g samples from the same livers. The mean relative ratio between measured concentrations in the test biopsy and in the standard sample was 1.04, and the pairwise correlation coefficient between the results of the two methods was 0.95 ($p < 0.0001$, $n = 18$).

3.2. Seasonal and individual variation

Means, s.d.'s and ranges of copper concentrations in liver biopsies from the one year old ewes and the older ewes are shown for both years in Table 2. Ewe no. 20, with only one successful sampling the first year (Table 1), is not included in the results for that year.

From November/December to March, the mean hepatic Cu concentrations in the different groups of animals remained stable or fell somewhat, but not more than 30 μg/g ww. From March to June, however, the hepatic Cu levels fell in all animals except one. The fall was substantial in all groups. From June to October the hepatic Cu concentrations rose sharply again, reaching similar or (especially for the one year old ewes) higher levels than in the previous autumn. Statistically, the hepatic Cu concentrations in June were significantly lower than the concentrations in the previous November/December ($p < 0.001$ both years). The moderate fall from November/December to March was significant the first year ($p \sim 0.01$), but not the second year. The moderate rise from November/December to the following October was significant the second year ($p \sim 0.02$), but not the first.

Both years, the mean hepatic Cu concentrations were lower in the one year old ewes than in the adult ones. Seen over the whole year, the difference between the age groups was statistically significant the first year ($p \sim 0.004$), but not the second, but the difference decreased gradually through the year (Table 2). From 1½ years and upward, we found no correlation between age and hepatic Cu levels.

Differences in Cu concentrations between individual sheep tended to remain stable (Fig. 1). As mentioned, the Cu concentrations were followed through both years in seven of the ewes (Table 1). The individual time/Cu concentration graphs for these animals are shown in Fig. 2.

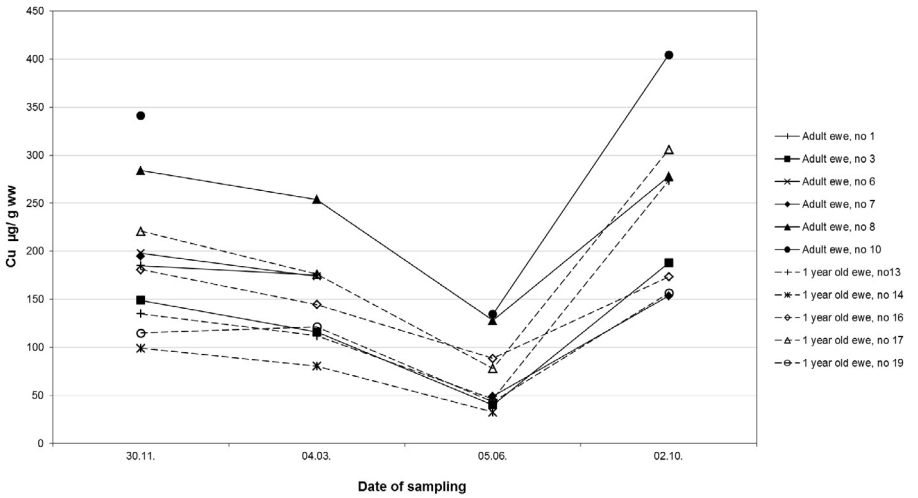

Fig. 1. Copper concentrations (µg/g wet weight) in repeated liver biopsies from untreated sheep, in the first year of the study.

3.3. Effect of treatment with ammonium tetrathiomolybdate

In the first year of the study, the hepatic Cu concentrations in the animals treated with TTM in June rose in average by 108 µg/g till October, compared to166 µg/g in the untreated group. In the second year, the mean rise in hepatic Cu from June till October in those treated with TTM was 112 µg/g, compared to116 µg/g in the untreated group. Statistically, the difference between the groups was just below significance ($p = 0.054$) the first year, and far from significance the second year.

In the animals treated with TTM in November the second year (Table 1), the mean hepatic Cu concentrations in December had fallen to 178 µg/g, compared to 229 µg/g in October. The mean Cu level in the untreated group was practically unchanged (225 µg/g in December vs 223 µg/g in October). The difference between these groups was statistically significant ($p \sim 0.03$).

3.4. Forage samples

The results of the chemical analyses of silage and hay samples are shown in Table 2. Twenty-three out of 29 samples had a Cu/Mo ratio above 20. The mean S concentration in the silage samples was significantly higher than in the hay samples ($p \sim 0.006$), otherwise there were no significant differences between silage and hay. The mean Mo concentration was lower (0.16 vs 0.49 mg/kg DM, $p \sim 0.03$) and the mean Cu/Mo relation higher (78 vs 20, $p \sim 0.001$) in the roughage samples from the second season than in those from the first. There were no significant differences between the years for the other parameters.

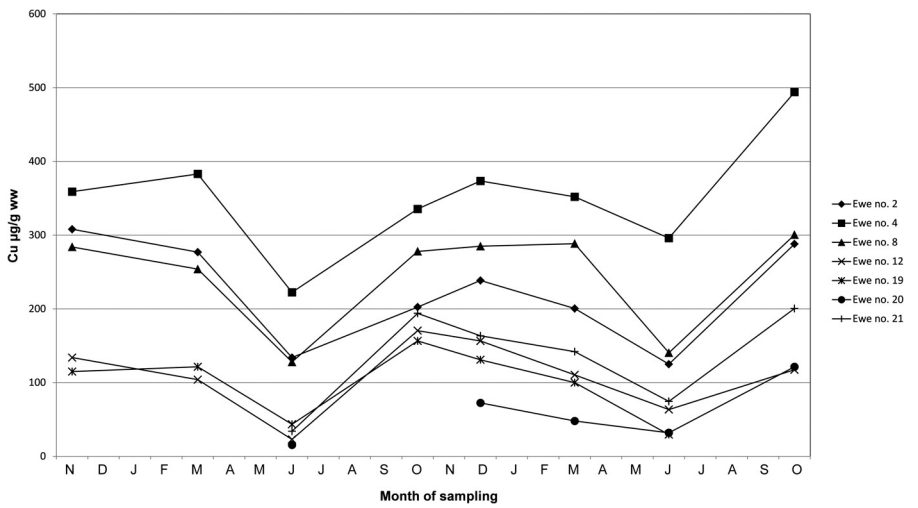

Fig. 2. Copper concentrations (µg/g wet weight) in repeated liver biopsies from seven different ewes in the flock; followed through two years. The samples from ewes no. 2 and no. 4 in October the last year were taken at slaughter.

4. Discussion

4.1. Methodological considerations

Liver biopsies have been used for many years in the study of copper metabolism in sheep (Bickhardt et al., 1997; Dick, 1944). However, the present study put several demands both on the biopsy technique and on the handling, storage and transport of the biopsies. First, the study was done in a small, privately owned flock with copper poisoning problems. This demanded that the biopsy technique had to be simple, safe and acceptable to the owner. We therefore decided for the least invasive technique available, with standard biopsy equipment used in human medicine (Harvey et al., 1984; Humann et al., 1999), and used ultrasound scanning to improve the safety and efficiency.

A disadvantage of this technique is that the biopsies acquired are small, with an average weight of about 20 mg ww, and rapidly lose weight by evaporation. Therefore, the biopsies must either be allowed to dry, with subsequent Cu analysis on a dry matter basis; or they must be frozen immediately and effectively kept from changing weight until analysis. Before the study, we tested both possibilities, and found that drying the biopsies gave considerably less reliable results than the fast-freezing and storage technique described in materials and methods. The analysis of test biopsies taken out from abattoir-collected livers showed acceptable correspondence to standard samples. Most probably, the biopsies taken out *in vivo* contain more blood than both the test biopsies and standard samples from livers collected at slaughter. Therefore, there is a possibility that our biopsy results underestimate the Cu concentrations somewhat, compared to values found in abattoir-collected livers. However, this does not affect our conclusions about seasonal and individual variation in hepatic Cu concentrations.

The variation between parallel biopsies taken out from the same animal at the same occasion was larger than the variation usually seen in repeated analyses of standard samples from an abattoir-collected liver. As the parallel biopsies were taken at almost the same place anatomically, large-scale variation in Cu distribution in the liver must have been of minor importance. Most probably, the main source of the variation between the parallel biopsies was small-scale differences in the amount of hepatic cells compared to blood, intracellular fluid and connective tissue from biopsy to biopsy. Variations in the loss of water in the freezing and storage process may also have played a part.

4.2. Seasonal variation in hepatic Cu concentrations and relation to clinical Cu poisoning

The first remarkable finding in this study was a characteristic seasonal variation in the hepatic Cu concentrations in the sheep studied (Table 2). This pattern was evident not only in average values, but also for each individual sheep, as illustrated by Figs. 1 and 2. It was only weakly and inconsistently influenced by treatment of some of the animals by subcutaneous TTM in June and did not change substantially from the first to the second year of the study (Fig. 2).

The relationship between chronic hepatic Cu accumulation and the outbreak of clinical disease in individual animals is not straightforward. Experimentally, typical chronic Cu poisoning may be induced reproducibly in 2–3 months, by oral Cu dosing (Søli and Frøslie, 1977). Under practical conditions, however, a large number of sheep may be heavily affected by hepatic Cu overload, without showing any signs of disease (Frøslie, 1980).

Even in the flock used in this investigation, where clinical chronic Cu poisoning was a problem of serious concern, two of the six years before the study started had shown no clinical cases, although the mean hepatic Cu levels in the ewes slaughtered at autumn had consistently been above 250 μg/g ww (Sivertsen et al., 1995). It is therefore obvious that additional stress factors are important for the induction of clinical disease (Moeller, 2004; Nordstoga, 1962). The sensitivity to these stress factors may vary. It has been shown that Cu sensitive breeds differ from Cu tolerant breeds not only in their tendency to accumulate Cu, but also in their sensitivity to the effects of this accumulation (Haywood et al., 2005). On the other hand, hepatic Cu accumulation is generally acknowledged as a prerequisite for the development of clinical disease. The critical level is by different authors set between 150 and 250 μg/g ww (Moeller, 2004; Radostits et al., 2007). In comparison between three affected flocks we found indications that the flock morbidity over time was related to the mean hepatic Cu concentrations in adult sheep from the flock at slaughter (Sivertsen and Wie, 1996).

The focus of the present study was not to elucidate the factors leading from hepatic Cu overload to clinical toxicosis in individual sheep, but to clarify if there was seasonal variations in hepatic Cu concentrations that might contribute to the understanding of the epidemiology. As it turned out, only one clinical case was seen in the flock during the investigation. As in previous years, it affected an adult ewe in the autumn, but this ewe had not been selected for the biopsy study. However, the seasonal pattern in hepatic Cu concentrations we found is in accordance with the seasonal occurrence of clinical cases of copper-induced hemolytic crisis observed in the flock in all the years; before and during the study.

In the ewes studied, the hepatic Cu levels fell only moderately from November/December till March. If this was representative for the situation in the flock over time, one might have expected some of the cases in this flock to occur also in midwinter. The dominating occurrence of clinical cases in the autumn may be result of the combined effect of high hepatic Cu levels and stress factors related to return from pasture, change of feed, etc. (Nordstoga, 1962). On the other hand, the lack of clinical cases in the otherwise stressful time around lambing in May is readily explained by our results.

To the extent that the findings in this flock are representative, they may also explain the general experience that copper poisoning is an autumn and winter disease in Norway (Nordstoga, 1962; Sivertsen and Wie, 1996). A limited, additional study of biopsies before and after summer pasture was done in Buskerud county in the southern inland in 1996 (Sivertsen and Thomassen, unpublished), comprising 10 ewes from three flocks with known sporadic

Table 3
Copper, molybdenum, zinc and sulfur content in silage and hay fed to the flock in the two winter seasons when the biopsy study was done. Means, standard deviations (in italics) and ranges (in brackets). All values per kg DM (dry matter).

	Cu (mg/kg DM)	Mo (mg/kg DM)	Cu/Mo	Zn (mg/kg DM)	S (g/kg DM)
Silage (n = 13)	8.7, *5.1* (5.0–22)	0.27, *0.50* (0.05–1.9)	65, *37* (12–137)	32, *14* (16–68)	2.6, *1.0* (1.7–5.0)
Hay (n = 16)	7.1, *2.8* (3.0–12)	0.23, *0.21* (0.05–0.9)	59, *53* (10–163)	34, *19* (15–80)	1.7, *0.4* (1.3–2.7)

occurrence of copper poisoning. The mean hepatic levels were lower than in Namdalen, but the seasonal trend was the same: Mean hepatic Cu concentrations increased in all 10 ewes over the summer pasture period, from a mean level of 58 μg/g ww in late May to 114 μg/g ww in late September.

4.3. Relation to trace elements in the feed and other factors

Cu/Mo ratios above 20 in the feed are known to induce hepatic Cu accumulation in sheep and represent a risk for clinical Cu poisoning (Frøslie and Norheim, 1983; Hogan et al., 1968; Osweiler et al., 1985). Most of the winter feed of this flock in the relevant years had levels well above this limit (Table 3). As previously found in studies of grass and pasture plants in Norway (Frøslie and Norheim, 1983; Garmo et al., 1986), the Cu/Mo ratios in our forage samples were more strongly linked to the variation in Mo concentrations (correlation coefficient of log values −0.88) than to the variation in Cu concentrations (correlation coefficient of log values 0.40). Other samples of winter forage from the same farm through several years preceding the biopsy study had shown similar levels of Cu, Mo and Cu/Mo ratios (Sivertsen and Wie, 1996). These results are consistent with the generally high hepatic Cu levels and recurring cases of clinical Cu poisoning cases in the flock. However, in spite of the high Cu/Mo ratios in the forage, the hepatic Cu levels in the flock tended to be stable or fall slightly in the winter period from October to March.

The observed fall in hepatic Cu from March to June may have several reasons. Feeding factors cannot be excluded. As in other sheep flocks, the amount of concentrates used was higher in the spring than during the winter. As mentioned, the concentrates and mineral mixtures used in the flock were without copper supplement, and contained standard recommended amounts of Zn, but no added iron. Cu and Mo concentrations in samples of concentrates were not measured, but Norwegian grain and concentrates are generally somewhat higher in Mo and lower in Cu/Mo ratios (Frøslie et al., 1983) than the roughage of this flock.

One of the factors that may have contributed to the observed spring fall in hepatic Cu is transfer of Cu to the lambs in utero (Gooneratne et al., 1989). Cu is important for the fetus, and studies in several species have shown that there is an active transfer of Cu across the placenta (McArdle, 1995). Some Cu may also have been transferred by lactation, especially in colostrum (Davis and Mertz, 1987). With three exceptions, all the animals sampled in June had been pregnant in the spring, 97% of them lambing between the 8th of May and the 3rd of June. The three ewes that were found to be empty were all one year old ewes in the second year of the study. One of these was the only one showing a rise in hepatic Cu level from March to June. In the two others the levels fell moderately. Seen as a group, these three one year old ewes showed small variation in hepatic Cu levels through the year; the mean concentrations being 123 μg/g (range 94–142 μg/g) in December, 139 μg/g (range 104–152) in March and 122 μg/g (range 69–173) in June. Being without lambs, they were all slaughtered before the summer season.

The strong rise in hepatic Cu during summer pasture may indicate that the plants grazed by the sheep at pasture had an even higher Cu/Mo ratio than the winter ration. The mountain pasture of this flock covers a large area, and the botanical variation is substantial. A systematic study of mineral contents of pasture plants in the whole area was outside the scope of the study. In a large study of pasture plants in another inland mountain district in Norway, Garmo et al. (1986) found higher average Cu/Mo ratios in most mountain pasture plants than Frøslie and Norheim (1983) had found in harvested grass samples. A recent, countrywide study of trace elements in natural sheep pasture plants in Norway did largely confirm these results (Sivertsen et al., 2009). One of our previous studies based on liver samples collected from abattoirs in the autumn, did also show significantly higher hepatic Cu levels in lambs from mountain pasture than in lambs that had grazed lowland or cultured pastures (Sivertsen and Plassen, 2004).

4.4. Individual variation in hepatic Cu levels

The second striking observation in this study was that the large individual differences in hepatic Cu concentrations remained generally stable over time. Parallel seasonal variations in hepatic Cu were found in all the sheep, but at different levels (Fig. 1). This was a consistent finding, and for the sheep that were followed through both years, the hepatic Cu curves of the second year were nearly identical to the first (Fig. 2). It has been known for many years that hepatic Cu concentrations at slaughter can vary considerably within the same flock (Frøslie, 1980; Nordstoga, 1962; Sivertsen and Plassen, 2004). However, to the knowledge of the authors, the stable character of these differences over time has not been reported before.

Accidental variations in additional stress factors and individual differences in sensitivity to the stress of Cu overload (Haywood et al., 2005) may be most decisive for the development of clinical disease in only a fraction of sheep

with hepatic Cu overload. Our observation of stable individual differences in hepatic Cu concentrations over time does however indicate that not all animals may be at risk, even in flocks with consistent problems.

The large and stable individual differences in hepatic Cu levels found in this study is remarkable, considering that all these animals were of the same breed, belonged to the same small flock, were fed the same feed all the winter, and grazed the same mountain pastures in the summer. Some of these individual differences may be genetically based. Differences in hepatic Cu accumulation between sheep of different breeds are well documented (Wiener et al., 1978). These differences are also found between the progeny of rams and ewes of different breeds (Littledike and Young, 1993).

4.5. Problems of prophylaxis

With the considerable number of clinical cases of Cu poisoning experienced in this flock, the need for prophylactic measures was obvious. However, the fact that the strongest hepatic Cu accumulation occur in the summer pasture period complicates most practical methods of prophylaxis. In these areas, the sheep graze over large mountain areas through the summer. The use of Mo-containing lick stones or other kinds of additional feeding are not easily practicable. A previous attempt to use Mo-containing rumen tablets did not show significant effect (Sivertsen and Wie, 1996).

As a part of this study, we therefore decided to test if a set of subcutaneous TTM injections in June would influence the hepatic Cu levels in October. As shown, the injections led only to a small reduction in the rise of hepatic Cu in the pasture period of the first year, and had no effect in the second year. However, the additional test of the same TTM procedure in November the first year did show a discernible effect on the hepatic Cu levels two weeks later. These results confirm the effect of TTM on the short-term hepatic Cu stores previously found by Gooneratne et al. (1981, 1989) and Humphries et al. (1988), but do also show that such injections have little prophylactic effect on the long-term Cu accumulation problems on Norwegian mountain pasture. Findings of severe side effects after use of TTM treatment in some flocks (Haywood et al., 2004) have also made mass treatments with TTM as a prophylactic measure less relevant.

After this study was done, the prevalence of Cu poisoning in the affected flock did gradually subside. At present, clinical cases have not been observed in the flock for many years. As mentioned, it seems to have been a general trend toward reduced prevalence of this disease, also on a national level. The particular change of prevalence in this flock is however also consistent with practical experience in other affected districts in previous years. Cases have occurred repeatedly in a flock for a number of years, and then the problem have apparently disappeared, without obvious changes in the feeding or pasturing conditions. Whether these national and local variations in prevalence of the disease are results of genetic drift, ecological changes or other factors, is still not clarified.

5. Conclusions

The study did reveal new information on the seasonal and individual variations in hepatic Cu concentrations in a flock with chronic Cu poisoning problems. Hepatic Cu concentrations in ewes were almost stable through the winter, fell substantially in the spring, and rose sharply again during the mountain pasture period. Treatment with ammonium tetrathiomolybdate before sending to mountain pasture had only weak and inconsistent effect on hepatic Cu levels in the autumn. The findings may explain the previously known seasonal pattern of spontaneous chronic Cu poisoning in sheep in Norway. However, they also emphasize that efficient prophylaxis against this disease in affected flocks under Norwegian pasturing systems is a difficult task.

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